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Speckle echocardiographic left atrial strain as predictor of atrial fibrillation recurrence in hypertensive patients

Istvan Gyalai-Korpos, Minodora Andor, Mirela Cleopatra Tomescu, Aurora Bordejevic, Iosif Marincu

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Abstract

Background: Echocardiographic assessed left atrial (LA) strain parameters are associated with atrial fibrillation (AF). Our aim was to determine if changes in left atrial peak systolic longitudinal strain (LAS) after electric conversion (CV) of atrial fibrillation to sinus rhythm is associated with AF recurrence in hypertensive patients.

Methods and results: 25 hypertensive patients with persistent AF and 31 age-matched hypertensive patients with no AF were included in the study. LAS was measured before and 24 hours after cardioversion (CV), using 2D speckle tracking. Follow-up period was 6 months. We divided AF patients after CV in patients who maintained sinus rhythm (MSR) and patients with recurrent atrial fibrillation (RAF). Pre-CV mean LAS was lower in patients with AF as compared to control group (16.9±2.2 vs 34.7± 2.9, $p<0.0001$). Immediately after CV, we noted a significantly increase in the mean LAS (19.0±2.3 vs 16.9±2.2, $p<0.01$), but without reaching the values of the control group. The changes in LAS after cardioversion were significantly higher in the patients who maintained sinus rhythm ($p<0.04$) than in those who experienced recurrent AF ($p=0.35$).

Conclusions: LAS differed between hypertensive patients with and without AF. Baseline LAS was not associated with AF recurrence. However, a smaller change in LAS after CV was associated with recurrent arrhythmia.

Key words: Atrial fibrillation, Arterial hypertension, Left atrial strain

Introduction

Atrial fibrillation (AF) is one of the most common arrhythmia and the population with AF is increasing [1,2]. Left atrium (LA) has a major role in the occurrence of atrial fibrillation, by pathological remodeling, including fibrosis and myofiber disarray [3]. Increased diameter of the left atrium (LA), assessed by echocardiography, is associated with an increased risk of recurrent AF [4]. Left atrial strain, strain rate and stiffness, assessed echocardiographic by tissue Doppler Imaging (TDI) and 2D speckle tracking imaging (2DSI) can be used as surrogate markers of atrial structural and functional remodeling [5-7]. 2DSI measures local atrial myocardial deformation, and, compared to TDI, has the advantage of being angle independent and not affected by cardiac translation [8]. According to some studies, LA strain parameters could be independent risk predictors for AF recurrence [9].

The aim of this study was to determine if LA peak systolic longitudinal strain (LAS) changes after CV in hypertensive patients with persistent AF are associated with the maintenance of sinus rhythm.

Methods

Patient population

This study was approved the Timisoara "V. Babes" University of Medicine and Pharmacy. Between January of 2011 and January of 2013, 59 hypertensive patients were enrolled. 28 hypertensive patients had persistent, but less than three months duration, AF. They underwent electrical cardioversion (CV) for AF and were enrolled in the AF group , if the CV was successful . 31 subjects were age-matched hypertensive patients with no history of AF. These were enrolled in the control group. All patients signed a written-informed consent prior to enrollment. Exclusion criteria were: significant (moderate or severe) valvular lesions, history of myocardial infarction, presence of intracardiac thrombus, paced atrial or ventricular rhythm, history of cardiac surgery, atrial septal

defect, pregnancy, age less than 18. Three AF patients were excluded from the study because they failed cardioversion. All patients were examined by standard 2-Dimensional transthoracic echocardiography (TTE). In AF patients, TTE was performed prior to CV and repeated within 24 hours post-CV. CV for AF was performed respecting ESC guidelines - with direct-current, synchronized electric shock . The patients were fasting and with intravenous sedation [10, 11].

Echocardiography

Standard TTE was performed using a Vivid 5S (GE Healthcare) echocardiograph and a 1.7/3.4 MHz transducer. Machine settings were adjusted by the echographer to optimize endocardial and myocardial gray scale definition. The images were acquired during end-expiratory apnea. We stored loops of 4 cardiac cycles. The offline analyze was done using Echo PAC, GE Healthcare, software package.

LA diameter (LAD) was measured at end-systole in parasternal long-axis view. The maximum LA volume was calculated using the biplane method of disks, at end-ventricular systole, in apical 4 and apical 2-chamber views [10,11]. In all patients , we measured left ventricular end-systolic and end-diastolic volumes, as well as left ventricular ejection fraction (LVEF). Mitral flow velocity (E) was measured from apical 4 chamber view, by pulsed-wave Doppler, with the sample volume placed in diastole between the tips of the mitral leaflets. Peak flow velocities were averaged from measurements of five consecutive cardiac cycles. TDI was used to measure early diastolic velocity (E´) of the mitral annulus. Ventricular filling pressures were estimated using the E/E' ratio [10,11].

Speckle tracking imaging

LA peak systolic longitudinal strain (LAS) was calculated using offline 2DSI technique. At peak systole, corresponding with the closure of the aortic valve, a line was drawn manually along the LA endocardium. The software generated automatically additional lines near the atrial epicardium. Manual adjustments were made if the tracking of the LA endocardium was not satisfactory. LAS was measured dividing the LA wall into 6 segments, in both 4 and 2 chamber views. LAS was measured for all segments from the annulus to the roof of LA (Figure 1) and was then averaged. Approximately 85% of the individual segments had adequate speckle tracking. The rest of 15% were excluded from the analysis because of inadequate speckle tracking.

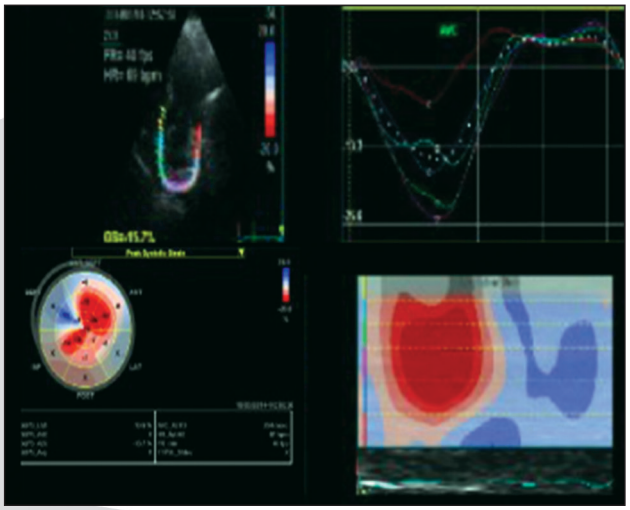

Figure 1. Speckle tracking of the left atrium in a hypertensive patient with recurrent atrial fibrillation

Statistical analysis

Statistical analysis was performed using MedCalc statistical software for Windows, version 12.7.7.0. The differences between study groups were compared using Chi square tests for categorical variables and Student's t test for continuous variables. Ordinal variables are presented as mean ± 1 SD.

Results

Clinical characteristics

The general clinical characteristics of the enrolled hypertensive patients are presented in Table 1. Compared to the control group, subjects in AF group had a higher incidence of obesity, dyslipidemia, coronary artery disease and systolic heart failure.

Comparison of echocardiographic and strain parameters

The echocardiographic measurements and strain parameters in hypertensive patients with and without AF are presented in Table 2. Pre-cardioversion mean LAS was significantly lower in subjects with AF when compared to the control group (16.9 ± 2.2 vs 34.7 ± 2.9, p<0.0001) (Figure 2). Mean post-cardioversion LAS was also significant lower (19.0 ±

2.3 vs 34.7 ± 2.9, p <0.0001), in patients with AF when compared to controls (Figure 2). We noted a significant increase in mean LAS after CV (16.9 ± 2.2 vs 19.0 ± 2.3, p<0.01) (Figure 3).

Table 1. Baseline clinical characteristics of patients with atrial fibrillation (AF) as compared to the control group

	AF (25) n (%)	Controls (31) n (%)	P value
Mean age (years)	62.5 ± 1.7	61.7 ± 2.4	0.12
Male sex	15 (60)	19 (61.2)	0.85
BMI (Kg/m2)	29.5 ± 2.3	27.8 ± 1.6	<0.01
Comorbid conditions:			
CAD	8 (32)	2 (6)	<0.03
Systolic HF	8 (32)	1 (3)	<0.01
CVA	1 (4)	0	0.91
DM	5 (21)	3 (9.6)	0.4
Dyslipidemia	14 (56)	5 (15)	<0.01
Medications:			
Statins	15 (60)	6 (19)	<0.01
Beta blockers	27 (65.8)	9 (21.9)	<0.01
ACE-i/ARB	15 (60)	18 (58)	0.90
CCB	10 (40)	10 (33)	0.79
Digoxin	3 (12)	0	0.16
Diuretics	13 (52)	15 (48)	0.97
ASA	3 (10)	4 (14)	0.96
Clopidogrel	4 (15)	5 (16)	0.78
Acenocumarol	25 (100)	0	<0.0001
Amiodarone	8 (32)	0	<0.01
Sotalol	3 (12)	0	0.16

BMI-Body mass index, CAD-Coronary artery disease, CVA- Cerebrovascular accident, HF- heart failure, ACE-i/ARB – Angiotensinogen converting enzyme inhibitors/Angiotensin receptor blockers, CCB – Calcium channel blockers, ASA –Aspirin

Table 2. Echocardiographic and strain variables of hypertensive patients with atrial fibrillation (AF) as compared to the control group

Echo variables	AF (n=25)			Controls (n=31)	P value
LVIDd, mm	51.4 ± 2.6			49.9 ± 2.3	0.02
LVIDs, mm	37.6 ± 3.6			35.5 ± 2.8	<0.02
IVSTd, mm	10 ± 0.6			9.5 ± 0.5	<0.01
PWTd, mm	10 ± 0.6			9.4 ± 0.6	<0.02
LVEF (%)	54.0 ± 3.6			56.1 ± 2.4	<0.02
LAD, mm	43.7 ± 3.8			39.4 ± 3.1	<0.0001
LAVI, ml/M2	32.5 ± 4.0			27.4 ± 3.6	<0.0001
E/E’	12.7 ± 1.3			7.9 ± 1.5	<0.0001
	Pre-CV	Post-CV	P value		
2CLAS, %	17.7±2,4	19.5± 2.1	<0.01	32.7± 4,1	<0.0001
4CLAS, %	16.4 ± 2.1	18.6± 2.7	<0.01	33.1±2.5	<0.0001
LAS, %	16.9±2.2	19.0±2.3	<0.01	34.7± 2.9	<0.0001

LVIDd – Left ventricular internal diameter at end-diastole, LVIDs – Left ventricular internal diameter at end-systole, STd – Interventricular septum thickness at end-diastole, PWTd – Left ventricular posterior wall thickness at end-diastole, LVEF – Left ventricular ejection fraction, LAD – Left atrial diameter, LAVI – Left atrial volume index, E = early filling velocity of transmitral Doppler flow, E’ = Average early diastolic velocity of medial and lateral mitral annulus, 2CLAS -2 chamber average peak systolic strain, 4CLAS -4 chamber average peak systolic strain, LAS – Mean peak systolic strain of 2C and 4C,

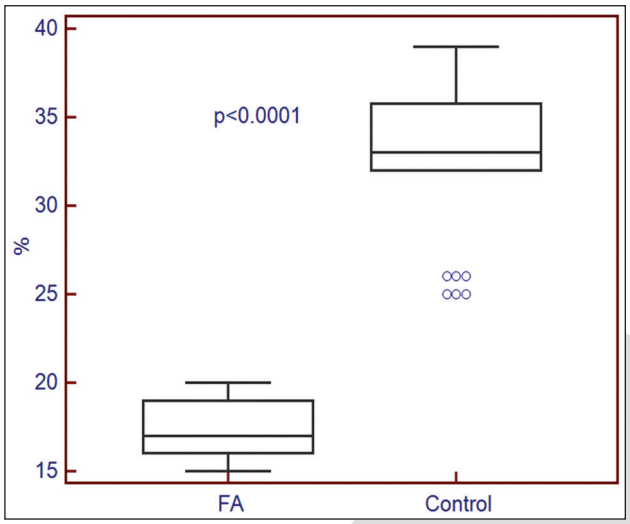

Figure 2. Comparison of mean LAS at baseline between FA and control group patients

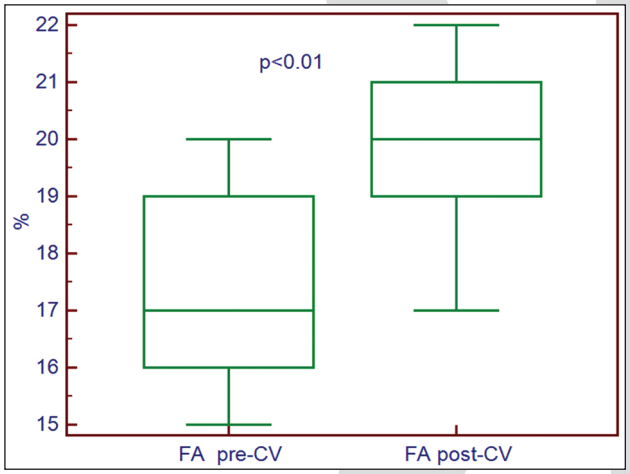

Figure 3. Comparison of mean LAS in patients with AF pre- and post- cardioversion (CV)

Clinical characteristics of the maintained sinus rhythm (MSR) and AF recurrence (AFR) subgroups

Eighteen (72%) of participants undergoing CV for persistent AF remained in normal sinus rhythm throughout the 6-month follow-up period, whereas 7 (28%) experienced at least 1 recurrence of AF. There was no significant difference in age, sex, body mass index (BMI), or medications, especially anti-arrhythmic drugs between both the groups (Table 3). Coronary artery disease had a significant higher prevalence in patients with AF recurrence (71% vs 16%, p=0.02). All AF hypertensive patients continued anticoagulation with Acenocumarol after CV. None of the study subjects experienced stroke or transient ischemic attack during the 6 month follow up.

Comparison of echocardiographic and strain parameters in the MSR and AFR group

The echocardiographic variables and strain parameters of MSR and AFR groups are recorded in Table 4.

The AFR subgroup patients, compared to MSR subgroup, had significant higher values for left ventricular internal diameter and end-diastole (52.5±3,6 mm vs 48.6±2.6mm, p<0.01), for left atrial diameter (49.7±3.4mm vs 44.5±3.8 mm, p<0.01) as well as for E/E´ratio (13.5±2.8 vs 9.8±2.5, p<0.01). Also, post-conversion mean heart rate was higher in patients with recurrent atrial fibrillation compared to those who maintained sinus rhythmus

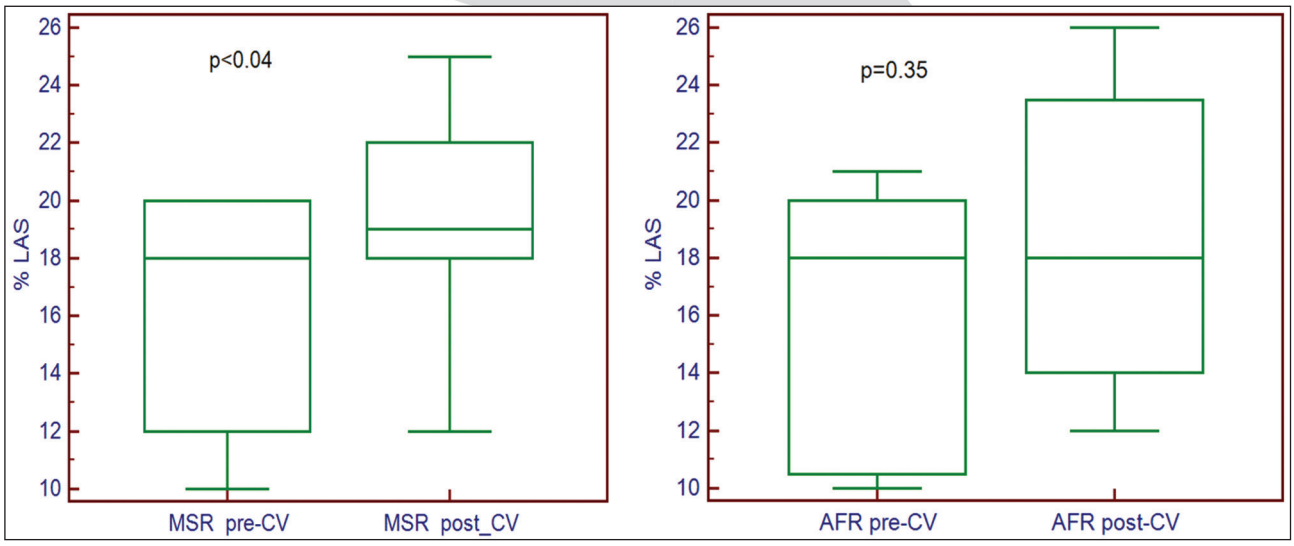

Figure 4. Post-CV mean LAS as compared to pre-CV mean LAS in the maintained sinus rhythm group (MSR) and in the atrial fibrillation recurrence group (AFR)

Table 3. Baseline clinical characteristics of hypertensive patients in the AF group who maintained sinus rhythmus (MSR) compared to those with atrial fibrillation recurrence (AFR)

	MSR (18) n (%)	AFR (7) n (%)	P value
Mean age (years)	61 ± 1.7	62 ± 2.1	0.22
Male Sex	12 (66)	3 (43)	0.54
BMI (Kg/m2)	29 ± 3.0	30 ± 1.6	0.41
Comorbid conditions:			
CAD	3 (16)	5 (71)	0.02
Systolic HF	4 (22)	4 (57)	0.22
CVA	0 (0)	1 (14)	0.63
DM	2 (11)	3 (43)	0.21
Dyslipidemia	12 (66)	2 (29)	0.22
Medications:			
Statins	10 (55)	5 (71)	0.78
Beta blockers	12 (66)	5 (71)	0.81
ACE-i/ARB	12 (66)	3 (43)	0.54
CCB	8 (44)	2 (29)	0.81
Digoxin	0 (0)	0 (0)	
Diuretics	12 (88)	1 (14)	0.05
ASA	3 (16)	0 (0)	0.28
Clopidogrel	3 (16)	1 (14)	0.62
Acenocumarol	18 (100)	7 (100)	
Amiodarone	13 (72)	2 (29)	0.12
Sotalol	2 (7.4)	3 (21.4)	0.32
Propaphenone	5 (28)	2 (29)	0.65

BMI-Body mass index, CAD-Coronary artery disease, CVA- Cerebrovascular accident, HF- heart failure, ACE-i/ARB – Angiotensinogen converting enzyme inhibitors/Angiotensin receptor blockers, CCB – Calcium channel blockers, ASA –Aspirin

Table 4 Echocardiographic and strain variables of patients with maintained sinus rhythm (MSR) group as compared to the atrial fibrillation recurrence (AFR) group

Echo variables	MSR (n=18)			AFR (n=7)		P value
LVIDd, mm	48.6 ± 2.6			52.5 ± 3.6		<0.01
LVIDs, mm	36.8 ± 4.6			37.2 ± 4.8		0.84
STd, mm	10.2 ± 0.8			10.3 ± 0.8		0.78
PWTd, mm	10.1 ± 0.8			10.5 ± 0.9		0.28
LVEF (%)	53.8 ± 5.4			50.5 ± 5.7		0.18
LAD, mm	44.5 ± 3.8			49.7 ± 3.4		<0.01
Pre-CV LAVI, ml/M2	35.5 ±3.2			36.9 ± 4.6		0.39
Post-CV LAVI, ml/M2	33.5 ± 5.4			34.2 ± 5.1		0.77
E/E'	9.8 ± 2.5			13.5 ± 2.8		<0.01
	Pre-CV	Post-CV	P value	Pre-CV	Post-CV	P value
HR	84.0 ±12	63.0± 7	<0.0001	87.9 ± 10	68.7± 9	<0.0001
LAVI, ml/M2	34.7± 5.3	33.8±3.5	0.55	36.5± 4.8	35.2± 2.8	0.54
2CLAS, %	15.6 ± 5.3	19.9± 4,4	<0.02	15.4± 6.2	18.2± 7.3	0.45
4CLAS, %	16.8 ± 3.5	18.9 ±2.1	<0.04	16.2± 3.8	18.7± 3.1	0.17
LAS, %	16.5±3.9	19.2± 3,3	<0.04	15.8± 4.9	18.5 ± 5.5	0.35

LVIDd – Left ventricular internal diameter at end-diastole, LVIDs – Left ventricular internal diameter at end-systole, STd – Interventricular septum thickness at end-diastole, PWTd – Left ventricular posterior wall thickness at end-diastole, LVEF – Left ventricular ejection fraction, LAD – Left atrial diameter, LAVI – Left atrial volume index, E = early filling velocity of transmitral Doppler flow, E' = Average early diastolic velocity of medial and lateral mitral annulus, HR – Heart rate, 2CLAS 2 chamber average peak systolic strain, 4CLAS -4 chamber average peak systolic strain, LAS – Mean peak systolic strain of 2C and 4C

(68.7±9 vs 63,0±7, p<0.0001). There were no significant differences between mean pre-CV LAS in MSR and AFR subgroups (16.5 ± 3.9% vs 15.8 ± 4.9%, p=0.71), nor between mean post-CV LAS (19.2 ± 3.3% vs 18.5 ± 5.5%, p=0.69). But we noticed that in MSR subgroup, there was a significant increase in mean LAS post-CV as compared to pre-CV (19.2 ± 3.3% vs 16.5 ± 3.9%, p<0.03), while in the AFR subgroup the increase was not significant LAS (18.5 ± 5.5 vs 15.8 ± 4.9, p=0.35 (Figure 4).

Discussion

In this clinical study involving 56 participants, we found that mean LAS was lower in subjects with AF as compared to those in normal sinus rhythm and no history of AF. We also observed that, although mean LAS improved immediately after CV in subjects with AF, it remained lower than in the control group. Compared with individuals who experienced a recurrence of AF over the follow-up period, those who remained in normal sinus rhythm were more likely to experience a significant improvement in the LAS after CV.

Atrial myocardial deformation properties and AF Normal LA function includes three components: reservoir function, the LA storing the blood that comes from the pulmonary veins; conduit function, the LA passing the blood from the pulmonary veins to the left ventricle in early diastole; booster function, as LA contraction increases the end diastolic ventricular fiber stretch [8]. Atrial structural remodeling during AF includes fibrosis, hypertrophy, myofiber disarray and apoptosis [12]. Some studies have shown that atrial strain parameters correlate significantly with the underlying fibrosis and have been validated against MRI, as markers of regional atrial myocardial deformation [13]. But, these echocardiographic strain parameters are influenced by loading conditions and by rhythm irregularity, not only by fibrosis [14].

Interesting studies suggest that LA strain parameters may be used as independent predictors of atrial fibrillation recurrence after cardioversion or catheter ablation [15].

Our study aimed to explore if left atrial strain (LAS) is influenced by atrial fibrillation and if may predict the recurrence of atrial fibrillation, in hypertensive patients.

We found that LAS was significantly lower in subjects with AF as compared to hypertensive controls. This finding is similar to the findings of other studies [16]. Post-conversion to sinus rhythmus, LAS increased significantly, but did not achieve the values found in the controls. The same results were found in a 3-year follow-up study [17]. In contrast to this work, we measured LAS within 24 hours after CV. The improvement in LA after restoration of sinus rhythmus was previously explained by 'reverse atrial remodeling [17]. The immediate increase in LAS after cardioversion is probably explained by the restoration of atrial contraction. The fact that LAS measured 14 hours after CV did not improve to the levels seen among the control hypertensive patients might be explained by "myocardial atrial stunning" or by persistent LA structural abnormalities.

Decreased left atrial strain assesses the impairment in atrial compliance, and has been reported to be a predictor of atrial fibrillation recurrence after conversion to sinus rhythm in patients with persistent AF [18]. However, we did not find differences between basal LAS in patients who maintained sinus rhythm and in patients with AF recurrence. We found that the post-convertion change in LAS was significant associated with maintenance of sinus rhythm. This finding is concordant with the results of another study, who also showed that LAS continued to increase in subjects with MSR during a three month follow up, in contrast to subjects with AFR [6]. Due to the low rates of post-conversion recurrent AF, we consider that further larger studies, with a longer follow-up period, are needed in order to have the appropriate statistical power to clarify this issue.

Some recent studies suggest that LAS could be a reliable predictor of stroke risk and cardiovascular outcomes in patients with AF [19,20]. While baseline atrial strain was not associated with the recurrence of AF, the post-CV improvement in LAS could be a predictor of sinus rhythm maintenance.

Conclusion

We observed that LAS differed between hypertensive patients with and without AF, being lower in AF patients, both pre- and after conversion to sinus rhythm. Our results suggest that a failure to improve LAS after CV may be a predictor of recurrent

arrhythmia. Further larger and long term follow up studies are required to establish the prognostic role of this non-invasive surrogate markers of left atrial filling pressure, in hypertensive patients with persistent atrial fibrillation converted to sinus rhythm.

Acknowledgement

All authors contributed equally to this manuscript.

References

1. *Cho GY, Jo SH, Kim MK, Kim HS, Park WJ, et al. Left atrial dyssynchrony assessed by strain imaging in predicting future development of atrial fibrillation in patients with heart failure. Int J Cardiol 2009; 134(3): 336–341.*

2. *Thomas L, McKay T, Byth K, Marwick TH. Abnormalities of left atrial function after CV: an atrial strain rate study. Heart 2007; 93(1): 89–95.*

3. *McManus DD, Shaikh AY, Abhishek F, Vasan RS. Atrial fibrillation and heart failure parallels: lessons for atrial fibrillation prevention. Crit Pathw Cardiol 2011; 10(1): 46–51.*

4. *Vianna-Pinton R, Moreno CA, Baxter CM, Lee KS, Tsang TS, Appleton CP. Two-dimensional speckle-tracking echocardiography of the left atrium: feasibility and regional contraction and relaxation differences in normal subjects. J Am Soc Echocardiogr 2009; 22(3): 299–305.*

5. *Leung DY, Boyd A, Ng AA, Chi C, Thomas L. Echocardiographic evaluation of left atrial size and function: current understanding, pathophysiologic correlates, and prognostic implications. Am Heart J 2008; 156(6): 1056–1064.*

6. *Inaba Y, Yuda S, Kobayashi N, Hashimoto A, Uno K, et al. Strain rate imaging for noninvasive functional quantification of the left atrium: comparative studies in controls and patients with atrial fibrillation. J Am Soc Echocardiogr 2005; 18(7): 729–736.*

7. *Zhang Q, Yip GW, Yu CM. Approaching regional left atrial function by tissue Doppler velocity and strain imaging. Europace 2008; 10(Suppl 3): iii62–iii69.*

8. *Mirza M, Caracciolo G, Khan U, Mori N, Saha SK, et al. Left atrial reservoir function predicts atrial fibrillation recurrence after catheter ablation: a two-dimensional speckle strain study. J Interv Card Electrophysiol 2011; 31(3): 197–206.*

9. *Hammerstingl C, Schwekendiek M, Momcilovic D, Schueler R, Sinning JM, et al. Left atrial deformation imaging with ultrasound based two-dimensional speckle-tracking predicts the rate of recurrence of paroxysmal and persistent atrial fibrillation after successful ablation procedures. J Cardiovasc Electrophysiol 2011, doi: 10.1111/ j.1540-8167. 2011.02177.x. Epub ahead of print.*

10. *Fuster V, Ryden LE, Asinger RW, Cannom DS, Crijns HJ, et al. ACC/AHA/ ESC guidelines for the management of patients with atrial fibrillation: executive summary: a report of the American College of Cardiology/ American Heart Association Task Force on Practice Guidelines and the European Society of Cardiology Committee for Practice Guidelines and Policy Conferences (Committee to Develop Guidelines for the Management of Patients With Atrial Fibrillation). Circulation 2001; 104: 2118–2150.*

11. *Lang RM, Bierig M, Devereux RB, Flachskampf FA, Foster E, et al. Chamber Quantification Writing Group; American Society of Echocardiography's Guideline sand Standards Committee; European Association of Echocardiography: Recommendations for chamber quantification: a report from the American Society of Echocardiography'sGuidelinesand Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology. J Am Soc Echocardiogr 2005; 18(12): 1440–1463.*

12. *Hwang HJ, Choi EY, Rhee SJ, Joung B, Lee BH, et al. Left atrial strain as predictor of successful outcomes in catheter ablation for atrial fibrillation: a two-dimensional myocardial imaging study. J Interv Card Electrophysiol 2009; 26(2): 127–132.*

13. *Kuppahally SS, Akoum N, Burgon NS, Badger TJ, Kholmovski EG, et al. Left atrial strain and strain rate in patients with paroxysmal and persistent atrial fibrillation: relationship to left atrial structural remodeling detected by delayed-enhancement MRI. Circ Cardiovasc Imaging 2010; 3(3): 231–239.*

14. *Di Salvo G, Caso P, Lo Piccolo R, Fusco A, Martiniello AR, et al. Atrial myocardial deformation properties predict maintenance of sinus rhythm after external CV of recent-onset lone atrial fibrillation: a color Doppler myocardial imaging and transthoracic and transesophageal echocardiographic study. Circulation 2005; 112(3): 387–395.*

15. *Machino-Ohtsuka T, Seo Y, Tada H, Ishizu T, Machino T, et al. Left atrial stiffness relates to left ventricular diastolic dysfunction and recurrence after pulmonary vein isolation for atrial fibrillation. J Cardiovasc Electrophysiol 2011; 22(9): 999–1006.*

16. *Hammerstingl C, Schwekendiek M, Momcilovic D, Schueler R, Sinning JM, et al. Left atrial deformation imaging with ultrasound based two-dimensional speckle-tracking predicts the rate of recurrence of paroxysmal and persistent atrial fibrillation after successful ablation procedures. J Cardiovasc Electrophysiol 2011, doi:10.1111/ j.1540-8167.2011.02177.x. Epub ahead of print.*

17. *Thomas L, Boyd A, Thomas SP, Schiller NB, Ross DL. Atrial structural remodeling and restoration of atrial contraction after linear ablation for atrial fibrillation. Eur Heart J 2003; 24(21): 1942–1951.*

18. *Schneider C, Malisius R, Krause K, Lampe F, Bahlmann E, et al. Strain rate imaging for functional quantification of the left atrium: atrial deformation predicts the maintenance of sinus rhythm after catheter ablation of atrial fibrillation. Eur Heart J 2008; 29(11): 1397–1409.*

19. *Saha SK, Anderson PL, Caracciolo G, Kiotsekoglou A, Wilansky S, et al. Global left atrial strain correlates with CHADS2 risk score in patients with atrial fibrillation. J Am Soc Echocardiogr 2011; 24(5): 506–512.*

20. *Azemi T, Rabdiya VM, Ayirala SR, McCullough LD, Silverman DI. Left atrial strain is reduced in patients with atrial fibrillation, stroke or TIA, and low risk CHADS(2) scores. J Am Soc Echocardiogr 2012, doi:pii:S0894-7317(12) 00715-8. 10.1016/j.echo.2012.09.004. Epub ahead of print.*

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Prevalence of Depression among Male Prisoners at an Urban Jail in Pakistan

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Abstract

Introduction: In Pakistan, no study on mental health status of male prisoners has been done so far. The purpose of this cross-sectional study was to determine the prevalence of depression, anxiety and stress in male Pakistani prisoners at an urban jail.

Methods: This study was conducted on 100 male prisoners at District Jail Lahore, Pakistan. Basic demographic characteristics, past medical history, and history of substance abuse, child abuse and child labor were obtained. Presence of depression was assessed by a validated Urdu translation of the Beck Depression Inventory (BDI). Mean BDI scores for categories of binary variables were compared with each other by applying independent samples t-test.

Results: The total prevalence of depression as per the BDI was 85%. 30% of prisoners had mild depression, 20% had moderate depression and 35% had severe depression. Prisoners with a history of substance abuse, childhood sexual abuse, chronic medical diseases and child labor were found to have significantly higher BDI scores.

Conclusion: Pakistani male prisoners have a markedly high prevalence of depression leading to significant psychiatric morbidity. We suggest here, provision of psychological support along with depression management and rehabilitation therapies such as musical therapy among others as essential to reduce mental health problems in prison inmates.

Key Words: Depression, Beck Depression Inventory, Prisoners, Cross-sectional Study, Mental Health

Introduction

The high prevalence of psychiatric disorders among prisoners compared to the general population has long been recognized. The issue was beginning to be highlighted by many authors as early as the first half of the 19th century and has been a topic of active research since the 1980s[1-4]. The magnitude of the problem can be further appreciated by considering the sheer number of people in prison worldwide. By 2011, there were more than 10.1 million people in prisons around the world[5]. Around half of the prisoners worldwide are in the United States, Russia and China, with the United States having the highest incarceration rate in the world. Human Rights Commission of Pakistan reported that by the end of 2012 there were 75,444 detainees in Pakistan's prisons in total, against a total authorized capacity of 44,578[6].

Fazel[7, 8] did two large-scale systematic reviews of severe mental illness in prisoners worldwide separated by a decade. The first one done in 2002 showed that 10% of men and 12% of women had major depressive disorder. The second one done in 2012 analyzed data on 33,588 prisoners in 24 countries and reported similar prevalence of major depressive disorder: 10.2% in men and 14.1% in women. The prevalence of major depressive disorder in prisoners of low-income countries was 22.5%, which is more than double the prevalence of 10.0% in high-income countries. As most of the available literature is from the West, there is an urgent need to conduct studies on this topic in third world countries.

The mental health of prison inmates has been a much overlooked issue in Pakistan. A study done by Khan et al.[9] describes the incidence of depression in female prison inmates in Peshawar, Pakistan. This study concluded that 59.4% of the inmates suffered from depression. A study done on psychiatric morbidity among female inmates of District Jail Adyala, Rawalpindi, reported that affective disorders were found in 23.43%, of which 19.5% were depressive illnesses[10]. A small scale study by Zadeh[11] reported a number of de-

pressive symptoms among female prisoners in a jail in Karachi, with insomnia, aggression and subjective feeling of stress being the most common (19%, 17% and 16% respectively). All of these studies included only female prisoners. While some studies have reported a higher prevalence of psychiatric disorders in female prisoners[12], others have not found a substantial difference[7, 8]. Ours is the first study in Pakistan on the prevalence of depression in male prisoners.

Turney considered the association between incarceration and major depression showing that current and recent incarceration was substantially associated with risk of major depression, due not only to the direct psychological implications of confinement, but also difficulties related to economic and social reintegration during and following incarceration[13]. It is of paramount importance that administrative authorities be made aware of the disastrous implications of the poor mental health of prisoners, and the negative impact of not providing appropriate physical and mental health facilities to inmates, which may well cripple their social and mental faculties. The overall prevalence of anxiety and depressive disorders in the Pakistani population is reported to be 34%[14].

This study was undertaken in a jail in Lahore to measure the prevalence of depression in male prisoners and to relate it to variables such as income, history of previous police encounters, history of drug abuse and child abuse. Variables like drug abuse, unemployment and homelessness have been found to be positively associated with poor mental health in the jail population[15].

Methods

The present study is a cross-sectional survey of the prevalence of depression among prisoners, conducted at District Jail Lahore in December 2010. One hundred male inmates were interviewed. Before conducting interviews, informed consent was taken from the participants and they were assured about the confidentiality of their personal details. Basic demographic characteristics like age, gender, education, occupation and marital status were obtained from prisoners. Information was collected about birth order, total number of siblings and monthly family income. Past medical history included questions about presence of chronic medical conditions such as hypertension, diabetes, psychiatric illnesses and a history of admission to mental health institutions. Participants were further queried about a history of substance abuse, child abuse and child labor. Presence of depression was assessed by a validated Urdu translation of the Beck Depression Inventory (BDI)[16]. BDI is a widely used 21-item self-report questionnaire for measuring the severity of depression, originally published in 1961[17]. Every question has four possible answers, ranging in intensity from 0 to 3. The total score for all items is added up, and the following cut-offs are used to assess the severity of depressive symptoms: a score of 0–9 indicates minimal depression, 10–18 indicates mild depression, 19–29 indicates moderate depression, and a score above 30 indicates severe depression. SPSS version 20 was used for data analysis. Prevalence of Depression among the whole sample was calculated. Mean BDI scores for categories of binary variables were compared with each other by applying independent samples t-test. P value of less than 0.05 was considered significant.

Results

At the time of data collection, the prisoners were not taking any medication and were not being evaluated by a mental health specialist. The mean age of the subjects was 28.8± 12.2. The total prevalence of depression in this inmate population, as per the BDI scale, was 85%. Of the total, 30% had mild depression, 20% had moderate depression and 35% had severe depression. The prisoners were divided into those aged 25 years of age and below, and those 26 years and above. The average BDI score in inmates below 25 years was 27 and the score for those above 26 years was 21. The difference was found to be significant (P=0.03). Inmates educated from grade 1-5 had a mean BDI score of 28, while the inmates who had received education beyond grade 6 had a mean BDI score of 17, with the difference between the groups being statistically significant (P<0.01). Another significant variable that was analyzed was a history of childhood sexual abuse; those with a history of abuse had a mean BDI score of 48 and those without a history of abuse had a score of 20(P<0.01).

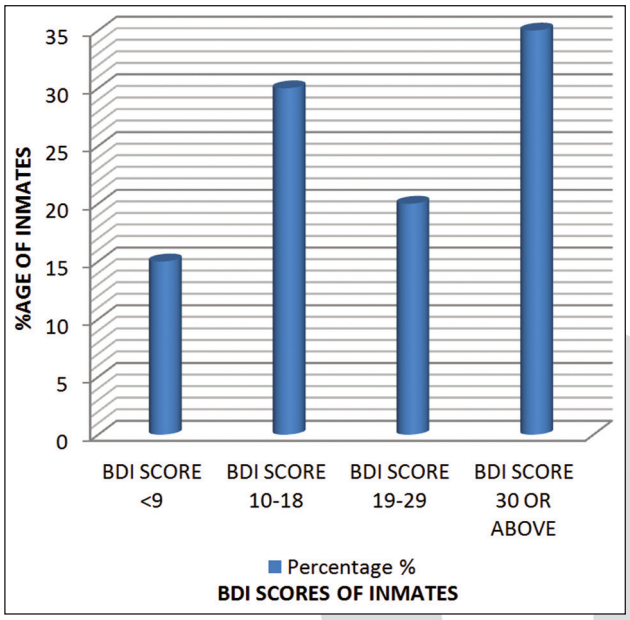

Figure 1. Graph showing the Percentage of Inmates and their BDI Scores

The presence of a history of substance abuse also was significant (P<0.01) with the mean BDI score being 29 in those with a positive history and 14 without history of substance abuse. The presence of chronic medical illness (Chronic Obstructive Pulmonary Disease, Hypertension, Tuberculosis, Congestive Heart Failure, Chronic Liver Disease, Chronic Renal Failure, Diabetes Mellitus) also increased the mean BDI score from 21 to 36 (P<0.01). Inmates with a history of previous court cases had a mean BDI score of 35, and those without had a score of 18 (P<0.01). The presence of a history of child labor (before 16 years of age) was also seen to have an effect on mean BDI score, raising it from 18 to 29 (P<0.01).The mean BDI scores of inmates are presented in Figure 1. Table 1 shows a comparison of mean BDI scores of inmates against predictor variables.

Discussion

The jail system in Pakistan is in a state of extreme neglect. It has never received much consideration from the government or the health care department. There is extreme overcrowding (total capacity around 45,000 with total number of inmates at any one time, over 75,000), forcing groups of inmates to sleep together on the floor. In seven jails in Punjab the number of prisoners is more than 200 percent higher than the sanctioned capacity. In 11 facilities, it is more than 100% higher, and in one particular location (District Jail Multan), it is nearly 350% higher[6]. As a result, the health of prisoners, both mental and physical, is highly neglected.

Our study helps to shed light on the level of depression in the prisoners of Pakistani jails. The total prevalence of depression in our study is 85%,

Table 1. Comparison of mean bdi scores against predictor variables of inmates in an urban jail

No.	Variables		BDI Scores (Mean +/- SD)	P-Value
1.	History of childhood sexual abuse	Present	47.67 +/- 2.582	<0.01
		Absent	19.94 +/- 9.112	
2.	History of substance abuse	Present	28.50 +/- 12.204	<0.01
		Absent	13.83 +/- 8.457	
3.	History of chronic disease eg. CLD,COPD,CHD,CRF,TB,HTN,DM	Present	35.50 +/- 16.819	<0.01
		Absent	21.25 +/- 10.216	
5.	Previous court cases	Present	34.71 +/- 12.470	<0.01
		Absent	18.38 +/- 9.283	
6.	History of labor at age <16 years	Present	29.09 +/- 14.321	<0.01
		Absent	18.00 +/- 7.906	
7.	History of living alone	Living alone	22.00 +/- 0.000	0.111
		Not living alone	24.21 +/- 13.387	
8.	Upbringing (Both v/s Single parents)	Both parents	24.21 +/- 13.387	0.111
		Single parent	22.00 +/- 0.000	
9.	Parents (married v/s Separated)	Married	20.63 +/- 8.037	-
		Separated	-	

which is a very high figure. The over-all prevalence of depression in men in Fazel's systematic review[8] was 10.2% and in prisoners of low income countries it was 22.5%.To take some examples from third world countries, a study from Nigeria[18] reported depression in 23% of prisoners; a study from Iran[19] reported 29%,; Brazil[20] 17.6%-18.8%; and India[21] 18%. Two prior studies from Pakistan on female prisoners have reported the prevalence to be prevalence to be 59.4%[9] and 19.5%[10]. What explains the excessively high prevalence of depression in our study? One factor might be the degree of depression that a study uses as a cut-off. For example, if we exclude the category of mild depression from our findings, the prevalence in our study falls to approximately 55%; still a very high figure but almost the same as reported by Khan et al.[9]. Andersen et al.[22] reported that the incidence of psychiatric disorders in prisoners is related to the level of stress. The difference in prevalence rates cited may very well be a function of the stress and the hardship prisoners have to bear in each particular prison. The District Jail Lahore in which our study was conducted has approximately 3000 prisoners while the authorized capacity is 1050 i.e. it is holding 200% more than capacity. This degree of over-crowding would be a constant source of stress for the prisoners. Other factors such as the behavior of the prison guards and the degree of harassment may also be contributing to the level of stress. Given that this is the only study on male prisoners in Pakistan, it is unknown whether this high prevalence will be found in male prisoners at other facilities as well.

Inmates with a lower level of education were found to have higher mean BDI scores in this prison population, while those with a higher level of education were seen to have lesser mean BDI scores. This is consistent with the literature, which has reported the protective effect of a higher level of education against depression[23]. Inmates with a history of chronic medical conditions were also found to be more depressed. Depression commonly co-occurs with medical illnesses and chronic pain, which can contribute to the somatic symptoms of depression, complicates diagnosis and treatment, worsens patient prognosis, and hinders recovery[24]. The occurrence of emotional, sexual and physical abuse is one of the most important factors for the development of depression[25] and depressive patients with a history of childhood adversity have more severe and chronic forms of depression with higher suicide rates[26]. Consistent with this, inmates with a previous history of childhood sexual abuse were found to have a higher mean BDI score in our study. Inmates with a prior history of substance abuse were also found to score higher on the BDI. The relationship between prior substance abuse and depression in prison inmates has also been reported previously[27]. Inmates with a history of child labor below the age of 16 were observed to have a higher mean BDI score. Studies have shown that childhood abuse, trauma and neglect put one at a significantly higher risk for mood disorders as an adult[28, 29].

Musical therapy was found useful in reducing mental health problems in prisoners in a study conducted in Bergen in 2008[30]. In Pakistan, such measures should be adopted to improve mental health status in prisoners and to reduce risk of suicide.

Limitations

One limitation of our study is that it was a single center study and given the varying conditions prisoners face at different prisons, it is difficult to be certain whether the results of this study can be generalized to other prisons as well. We restricted our study to male prisoners. Hence, we are not in a position to determine if there was any gender-based difference in the prevalence of depression at that particular prison. It was a cross-sectional study and therefore offers no information on the duration and course of the depressive symptoms in the prisoners. We did not keep a record of the duration of imprisonment for each participant, which can be a significant factor in the prevalence of depression in prison populations[31]. Studies carried out in future should address these limitations to improve existing literature on this topic in Pakistan.

Conclusion

Pakistani male prisoners have a markedly high prevalence of depression leading to significant psychiatric morbidity. This suggests the need for immediate interventions on the part of the government, prison administration and health depar-

tment. This is essential both to preserve discipline inside prisons as well as to help reintegrate inmates back into society following their release. We strongly recommend essential measures to reduce depression and stress in prisoners and rehabilitate them by different management programs.

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References

1. *Coid J. How many psychiatric patients in prison? Br J Psychiatry. Jul 1984; 145: 78-86.*
2. *Smith R. The mental health of prisoners. I--How many mentally abnormal prisoners? Br Med J (Clin Res Ed). Jan 28 1984; 288(6413): 308-310.*
3. *Smith R. The mental health of prisoners. II--The fate of the mentally abnormal in prison. Br Med J (Clin Res Ed). Feb 4 1984; 288(6414): 386-388.*
4. *Bluglass R. Mentally disordered prisoners: reports but no improvements. Br Med J (Clin Res Ed). Jun 25 1988; 296(6639): 1757-1758.*
5. *Walmsley R. World prison population list . 9 ed. London: Kings College London, International Centre for Prison Studies.; 2011.*
6. *State of Human Rights in 2012. Lahore: Human Rights Commission of Pakistan; 2013.*
7. *Fazel S, Danesh J. Serious mental disorder in 23000 prisoners: a systematic review of 62 surveys. Lancet. Feb 16 2002; 359(9306): 545-550.*
8. *Fazel S, Seewald K. Severe mental illness in 33,588 prisoners worldwide: systematic review and meta-regression analysis. Br J Psychiatry. May 2012; 200(5): 364-373.*
9. *Khan TM, Hussain H, Khan G, Khan A, Badshah A, Sarwar R. Incidence of Depression among Incarcerated Woman in Central Prison, Peshawar, Pakistan. Eur J Gen Med. 2012; 9(1): 33-38.*
10. *Bilal F, Saeed K. Psychiatric morbidity among the female inmates of district jail Adyala, Rawalpindi, Pakistan. Rawal Medical Journal. 2011; 36(2): 129-132.*
11. *Zadeh Z. Mental Health Issues of Women Prisoners in Karachi Pakistan. International Journal of Humanities and Social Science. 2012; 2(14): 310-318.*
12. *Binswanger IA, Merrill JO, Krueger PM, White MC, Booth RE, Elmore JG. Gender differences in chronic medical, psychiatric, and substance-dependence disorders among jail inmates. Am J Public Health. Mar 2010; 100(3): 476-482.*
13. *Turney K, Wildeman C, Schnittker J. As fathers and felons explaining the effects of current and recent incarceration on major depression. J Health Soc Behav. 2012; 53(4): 465-481.*
14. *Mirza I, Jenkins R. Risk factors, prevalence, and treatment of anxiety and depressive disorders in Pakistan: systematic review. BMJ. Apr 3 2004; 328(7443): 794.*
15. *Singleton N, Gatward R, Meltzer H, Britain G. Psychiatric morbidity among prisoners in England and Wales: Stationery Office London; 1998.*
16. *Sultan S. Psychological Aspects of Infertility [PhD Thesis]. Multan, Bahauddin Zakariya University, Multan; 2009.*
17. *Beck AT, Ward CH, Mendelson M, Mock J, Erbaugh J. An inventory for measuring depression. Arch Gen Psychiatry. Jun 1961; 4: 561-571.*
18. *Agbahowe S, Ohaeri J, Ogunlesi A, Osahon R. Prevalence of psychiatric morbidity among convicted inmates in a Nigerian prison community. East African medical journal. 1998; 75(1): 19-26.*
19. *Assadi SM, Noroozian M, Pakravannejad M, et al. Psychiatric morbidity among sentenced prisoners: prevalence study in Iran. Br J Psychiatry. Feb 2006; 188: 159-164.*
20. *Pondé MP, Freire AC, Mendonça MS. The Prevalence of Mental Disorders in Prisoners in the City of Salvador, Bahia, Brazil*. Journal of forensic sciences. 2011; 56(3): 679-682.*
21. *Goyal SK, Singh P, Gargi PD, Goyal S, Garg A. Psychiatric morbidity in prisoners. Indian J Psychiatry. Jul 2011; 53(3): 253-257.*
22. *Andersen HS, Sestoft D, Lillebæk T, Gabrielsen G, Hemmingsen R, Kramp P. A longitudinal study of prisoners on remand: psychiatric prevalence, incidence and psychopathology in solitary vs. non-solitary confinement. Acta Psychiatrica Scandinavica. 2000; 102(1): 19-25.*

23. *Bjelland I, Krokstad S, Mykletun A, Dahl AA, Tell GS, Tambs K. Does a higher educational level protect against anxiety and depression? The HUNT study. Social science & medicine. 2008; 66(6): 1334-1345.*

24. *Manning JS, Jackson WC. Depression, pain, and comorbid medical conditions. J Clin Psychiatry. Feb 2013; 74(2): e03.*

25. *De Venter M, Demyttenaere K, Bruffaerts R. The relationship between adverse childhood experiences and mental health in adulthood. A systematic literature review. Tijdschr Psychiatr. 2013; 55(4): 259-268.*

26. *Kim SW, Kang HJ, Kim SY, et al. Impact of childhood adversity on the course and suicidality of depressive disorders: the CRESCEND study. Depress Anxiety. Oct 2013; 30(10): 965-974.*

27. *Peters RH, Strozier AL, Murrin MR, Kearns WD. Treatment of substance-abusing jail inmates. Examination of gender differences. J Subst Abuse Treat. Jul-Aug 1997; 14(4): 339-349.*

28. *Heim C, Nemeroff CB. The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. Biol Psychiatry. Jun 15 2001; 49(12): 1023-1039.*

29. *Horwitz AV, Widom CS, McLaughlin J, White HR. The impact of childhood abuse and neglect on adult mental health: a prospective study. J Health Soc Behav. Jun 2001; 42(2): 184-201.*

30. *Gold C, Assmus J, Hjornevik K, et al. Music Therapy for Prisoners: Pilot Randomised Controlled Trial and Implications for Evaluating Psychosocial Interventions. Int J Offender Ther Comp Criminol. Aug 27 2013.*

31. *Hassan L, Birmingham L, Harty MA, et al. Prospective cohort study of mental health during imprisonment. Br J Psychiatry. Jan 2011; 198(1): 37-42.*

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Influence of rosuvastatin therapy on biochemical markers, functional and structural parameters of endothelial dysfunction in patients with essential hypertension

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Abstract

Background: Increased levels of asymmetric dimethyl arginine (ADMA) in hypertensive patients promote endothelial dysfunction and atherosclerosis, while statins improve endothelial function.

Aim: This study is aiming to investigate the influence of rosuvastatin therapy on plasma ADMA levels, flow-mediated forearm vasodilatation (FMD) and carotid IMT in patients with essential hypertension.

Material and method: The prospective present study was conducted during a period of 18 months and included a number of 63 patients with essential hypertension, without hypercholesterolemia. All patients received conventional antihypertensive therapy. Group I patients, with endothelial dysfunction and subclinical atherosclerosis received 20 rosuvastatin for 6 months. Group II patients, with endothelial dysfunction but normal IMT received 10 mg rosuvastatin for 6 months. Group III patients, with no endothelial dysfunction, received no statin therapy. Statistical analyses was performed using MedCalc 12.3.0.0 statistical software for Windows. Significant differences were assumed to be at $p< 0.05$.

Results: At 6 months, group I patients had a significant decrease in ADMA levels (from 0.96±0.07 to 0.73±0.06, $p<0.0001$), a significant decrease in IMT (from 1.15±0.1 to 1.02±0.11, $p<0.01$) and increase in FMD (from 6,53±0.76, to 7.55±0.81, $p<0.01$). In group II, ADMA levels decreased from 0.67±0.07 to 0.58±0,08 ($p<0.01$), IMT decreased from 0.82±0.07 to 0.80±0.10 ($p>0.05$), FMD increased from 8,48±0.79 to 9.10±0.71 ($p<0.01$). In the control group, the changes were not significant.

Conclusions: 6 months rosuvastatin therapy improved all biochemical, functional and structural parameters of endothelial dysfunction. The clinical benefits were dose dependent.

Key words: hypertension, asymmetric dimethyl arginine, flow mediated vasodilatation, intima-media thickness

1. Introduction

Hypertension is one of the major risk factors in cardiovascular disease, it accelerates the development of atherosclerosis and it affects 12% to 15% of the adult population. Ever since 1980, Furchgott [1,2], has stated that the acetylcholine induced vascular relaxation is critically linked to an intact endothelium. Loss of vascular endothelium integrity in various heart diseases is associated with nitric oxide (NO) production. Physiologically, NO (Nitric Oxide) is synthesized within the endothelium, based on a reaction between the amino acid (L-arginine) and the corresponding nitric oxide synthase enzyme (NOS) [3].

Endothelial dysfunction is the earliest marker for vascular endothelial damage. Endothelial dysfunction in hypertensive patients is a major factor for cardiovascular complications. Primary prevention of cardiovascular disease, makes is a main target, to identify endothelial dysfunction by means of non-invasive clinical and laboratory methods.

Classical noninvasive assessment methods of endothelial dysfunction include tests such as: Doppler echocardiography (ultrasound flow mediated vasodilatation - ex. brachial artery ultrasound), circulating markers of endothelial dysfunction (asymmetric dimethylarginine) and vascular inflammation (high-sensitivity C-reactive protein and soluble cellular adhesion molecules) [4].

Carotid intima-media thickness (IMT) is an important marker in subclinical vascular lesion

assessment and an independent predictor of future cardiovascular events in the general population[5]. An increased carotid IMT is considered by some authors a marker of subclinical atherosclerosis[6], while other studies show that and blood pressure plays and important role in increasing the IMT [7].

Statins are known to improve endothelial function in hypercholesterolemic patients, having anti-inflammatory effects, one of the most efficient being rosuvastatin [8,9].

The main study objective was to evaluate whether rosuvastatin treatment improves endothelial dysfunction in patients with essential hypertension and no hypercholesterolemia.

2. Material and methods

2.1. Study design and patients

This is a prospective study that was conducted during a period of 6 months and included hypertensive patients referred to a specialized Cardiology Clinic of the Internal Medicine Department. The inclusion criteria in the study were: age ≥ 18 years, diagnosis of mild/moderate essential hypertension (systolic blood pressure (BP) of 140-179 mmHg, diastolic BP of 90-119 mmHg), normal plasmatic cholesterol levels (<200 mg/dL), no cholesterol-lowering therapy six months prior to inclusion. We excluded the subjects presenting coronary artery disease, chronic heart failure, diabetes mellitus, chronic kidney or hepatic disease, stroke, bronchial asthma, peripheral arterial circulatory failure, and acute or chronic inflammatory states. All patients received conventional therapy for hypertension, such as beta blockers (nebivolol), diuretics and/or calcium antagonists.

Prior to being admitted in the study, all patients completed an informed consent form to participate in the study and to allow the retrospective evaluation of clinical data. The informed consent form was approved by the local ethical committee. The patients` participation in the study was completely voluntary, and they were able to withdraw it at any point.

We conducted a background check on the patients` medical history; in all patients, we performed physical examination, laboratory determination, electrocardiogram (ECG), echocardiography, vascular echocardiography evaluating flow mediated vasodilatation (FMD) at brachial artery and carotid intima-media thickness (IMT).

Blood pressure determinations were done after 30 minutes of rest, in supine position at the right brachial artery and expressed as the mean value of 3 measurements over a period of 30 minutes.

Laboratory determinations were performed in a fasting state and in a temperature-controlled room. The venous blood samples were collected after 15 minutes of rest, after an overnight fast (>12 hours since the last intake of food), immediately placed on ice and were drawn for glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides. All these analyses were measured by routine laboratory methods, inside the hospital. Total cholesterol ≥ 200 mg/dL and triglyceride levels ≥ 150 mg/dL were considered as dyslipidemia**.**

Plasma levels of high-sensitivity C-reactive protein (hs-CRP) were measured within the Laboratory Bioclinica SA, Timisoara through a highly sensitive quantitative immunoturbidimetric method, using a CRP Ultra kit, distributed by Abboth Diagnostics. The lowest detection limit for ultrasensitive method was 0. 01 mg / dL.

Plasma ADMA levels were measured in Laboratory Dr. Linbach Heidelberg by a simple and fast LC–MS–MS analytical method with fluorescence detection. Fluorescence was detected after solid phase extraction (SPE) with carboxylic acid (CBA) cartridges online o-phtaldialdehyde (OPA) derivation, and separation on a phenyl column (250 x 4.6 mm, Macherey-Nagel, Düren, Germany). For our laboratory, the reference value for ADMA: range of 0. 3 – 0. 8 μmol/L, for both sexes and all age groups over 18 years of age.

Determination of forearm flow mediated vasodilatation (FMD) was done using a VIVID S5 (GE Healthcare) equipment, according to the international guidelines [10]. The diameter of brachial artery was measured with a 7.5 MHz transducer. Measurements were obtained at 09: 00 AM — at baseline, after at least 20 min of resting in the supine position in a quiet room and 12 h without caffeine, smoking, food or any vasoactive drugs**.** Longitudinal scans of the brachial artery were obtained approximately 5cm proximal of the antecubital fossa. The transmit focus zone was set at the depth of the anterior brachial artery wall. A

view of a 5-cm longitudinal section of the brachial artery was recorded on S-VHS for 30 s at baseline, before and during peak (1 min) reactive hyperemia (induced by deflation of a blood pressure cuff previously inflated to 50 mm Hg above the subject's systolic blood pressure point). Endothelium-dependent, flow-mediated dilation was calculated due to percent change in diameter, 1 min after the cuff release and relative to the baseline diameter before cuff release.

Carotid intima-media thickness (IMT) of the common carotid artery was determined as an index of atherosclerosis, at baseline in both carotid arteries, as agreed in the Mannheim consensus [11], with a GE medical system VIVID S5, high-resolution ultrasonography system, equipped with a 9 MHz linear array transducer (figure 1). The carotid arterial scanning was performed by a certified sonographer. The subjects were examined in a supine position. The intima-media thickness was calculated online by built-in software of the ultrasound system. The mean IMT was calculated for each of the four measurements sites in each patient.

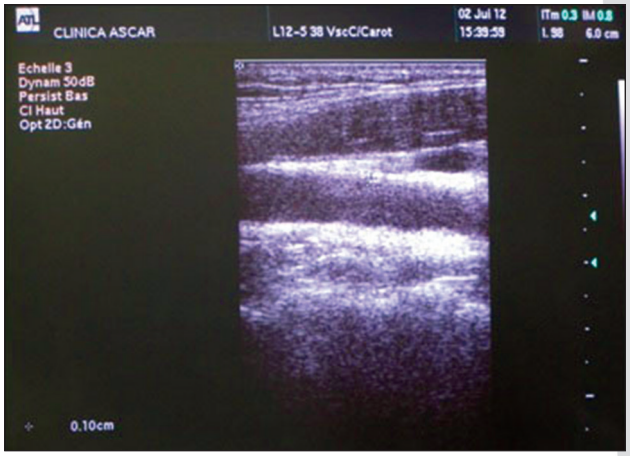

Figure 1. Ultrasound measurement technique of the carotid intima-media thickness (IMT)

All procedures and laboratory measurements were done at baseline and after 3 and 6 months.

According to the results of the investigations, the patients were distributed into three groups. Group I (n=23 patients) had endothelial dysfunction (FMD ≤10%) with subclinical carotid atherosclerosis (IMT≥0.9 mm). They received 20 mg rosuvastatin daily. Group II (n=25 patients) had endothelial dysfunction (FMD<10%) with normal carotid IMT (<0.9 mm). They received treatment with 10 mg rosuvastatin. Group III (n=15 patients) represented the control group (FMD>10%, IMT<0.9). They received no rosuvastatin therapy.

All procedures and laboratory measurements were done at baseline and after 6 months.

2.2. Statistical analysis

Continuous variables were evaluated using t – test. Pearson correlation coefficient (r) test was used to calculate the correlation between the analyzed variables. The value of r closer to 1 was considered to indicate a stronger correlation of the parameters. Simple linear regression analysis was performed to assess the relationship between the ADMA plasma levels, FMD and IMT in hypertensive patients. The independent relationship between these biological, functional and structural parameters of endothelial dysfunction were also tested by backward multiple regression analysis. Parametric data are reported as mean±1 standard deviation (SD). Significant differences were assumed to be at $p<0.05$. Statistical analysis was performed using MedCalc 12.3.0.0 statistical software for Windows.

3. Results

A total of 63 patients were enrolled. Sex distribution in the group was 30 (47.61 %) men and 33 (52.38 %) women. Mean age was 55±11, with limits between 35 -69 years.

Baseline characteristics of the patients are presented in table 1.

There were no significant differences between the groups with respect to all demographic and blood pressure parameters (p>0. 05). There were also no significant statistical differences regarding their mean plasma levels of LDL-cholesterol, HDL-cholesterol, triglycerides and glucose (p>0. 05).

According to the criteria of distribution, group I patients had significant higher carotid IMT comparative to group II and III patients (p<0.0001). % FMD was significant lower in group I and II patients versus group III patients (p<0.0001).

Mean plasma ADMA levels were significantly higher in group I (0. 96±0.07 μmol/L) in comparison with group II (0.67±0. 11 μmol/L; p<0.0001) and group III (0. 54±0. 06; p<0.0001). The difference between the mean plasma ADMA levels

Table 1. Baseline characteristics of the patients

Patients characteristics	Group I n=23	Group II n=25	Group III n=15	P value I vs. II	P value I vs. III	P value II vs. III
Male sex	10 (43 %)	11 (46 %)	9 (6 0%)	>0.05	>0.05	>0.05
Age years	53.45±12	51.27±13	52.74±10	>0.05	>0.05	>0.05
Heart rate (beats/min)	74.6±8.8	75,8±9.7	73.9±10.2	>0.05	>0.05	>0.05
Systolic pressure (mmHg)	160. 74±10.54	158.81±9.43	157.66±9.12	>0.05	>0.05	>0.05
Diastolic pressure (mmHg)	99.00±7.21	96.32±5.27	95.46±5.97	>0.05	>0.05	>0.05
Total cholesterol (mg/dL)	188±6.5	190±7.6	188±13.1	>0.05	>0.05	>0.05
LDL (mg/dL)	117±13	115±12	116±8	>0.05	>0.05	>0.05
HDL (mg/dL)	39±4	41±6	40±8	>0.05	>0.05	>0.05
Triglycerides (mg/dL)	138±8	137±10	139±7	>0.05	>0.05	>0.05
Glycaemia (mg/dL)	98±4	97±5	99±3	>0.05	>0.05	>0.05
Smokers %	15 (63 %)	13 (57 %)	9 (60%)	>0.05	>0.05	<0.05
hs-CRP (mg/L)	342±47	303±37	201±52	0.0061	<0.0001	<0.0001
Plasma ADMA (μmol/L)	0.96±0.07	0.67±0.07	0.54±0.07	<0.0001	<0.0001	<0.0001
Flow-mediated vasodilatation (%)	6.53±0.76	8.48±0.79	14.67±1.17	<0.0001	<0.0001	<0.0001
Intima-media thickness (IMT) (mm)	1.15±0.09	0.82±0.07	0.73±0.08	<0.0001	<0.0001	>0.05

Data are presented as number (%) for categorical variables and as mean±1 standard deviation for continuous variables. hs-CRP: high-sensitive C reactive protein, ADMA: asymmetric dimethyl arginine, BP: blood pressure, SD: standard deviation.

of group II and III patients was also significant (p=0.0002).

In group I patients, with endothelial dysfunction and subclinical carotid atherosclerosis, % FMD was significant correlated with ADMA levels (r=-0.83, p<0.0001) and IMT (r=-0.7, p=0.0002).

At multiple regression analysis, independent variables associated with % FMD were again IMT (p<0.001) and ADMA levels (P<0.01).

In group II, although the ADMA levels were still normal in the presence of demonstrated impaired flow-mediated vasodilatation, we found significant correlations between % FMD and ADMA levels (r=-0.93, p<0.001), systolic BP (r=0.87, p<0.0001), hs-CRP (r=0.79, p<0.0001), diastolic BP (r=0.77, p<0.0001), cholesterol level (r=0.71, p=0.0001), smoking (r=0.46, p=0.02) and age (r=0.67, p=0.0002). At backward multiple regression analysis, only one variable – ADMA, was independent associated with FMD (p<0.001).

In the non-interventional group III, %FMD correlated negative with ADMA levels (r=-0.55, p=0.03), cholesterol levels (r=-0.9, p<0.0001), hs-CRP (r=-0.96, p<0.0001), systolic BP (r=-0.95, p<0.0001), diastolic BP (r=-0.94, p<0.0001) and smoking (r=-0.7, p<0.003). The multivariable regression analysis found that independent variables associated with FMD were ADMA levels (p<0.01), hs-CRP (p<0.01) and systolic BP (p<0,001).

As we see, there is a significant negative and independent correlation between ADMA levels and %FMD, in all the three groups of hypertensive patients. In group I there was a significant positive correlation between ADMA levels and IMT. The association between FMD, ADMA levels and IMT in the three patient groups, at baseline, as outlined by a linear regression model, is presented in figure 2. All procedures and laboratory measurements were repeated after 6 months. Variations of carotid IMT were used as indicator of progression or regression of atherosclerosis, while those of % FMD as indicator of endothelial dysfunction evolution (table 2).

Analyzing the evolution of antioxidant ADMA levels in the three groups, we note that at 6 months a significant reduction occurred under rosuvastatin therapy (p<0.001) (table2). This reduction was observed in all hypertensive patients treated with rosuvastatin, but in group I, with high lipoprotein levels ADMA at baseline, the decrease was extremely significant (p<0.0001). A slight increase, not statistical significant, occurred in group III patients, without endothelial dysfunction and without

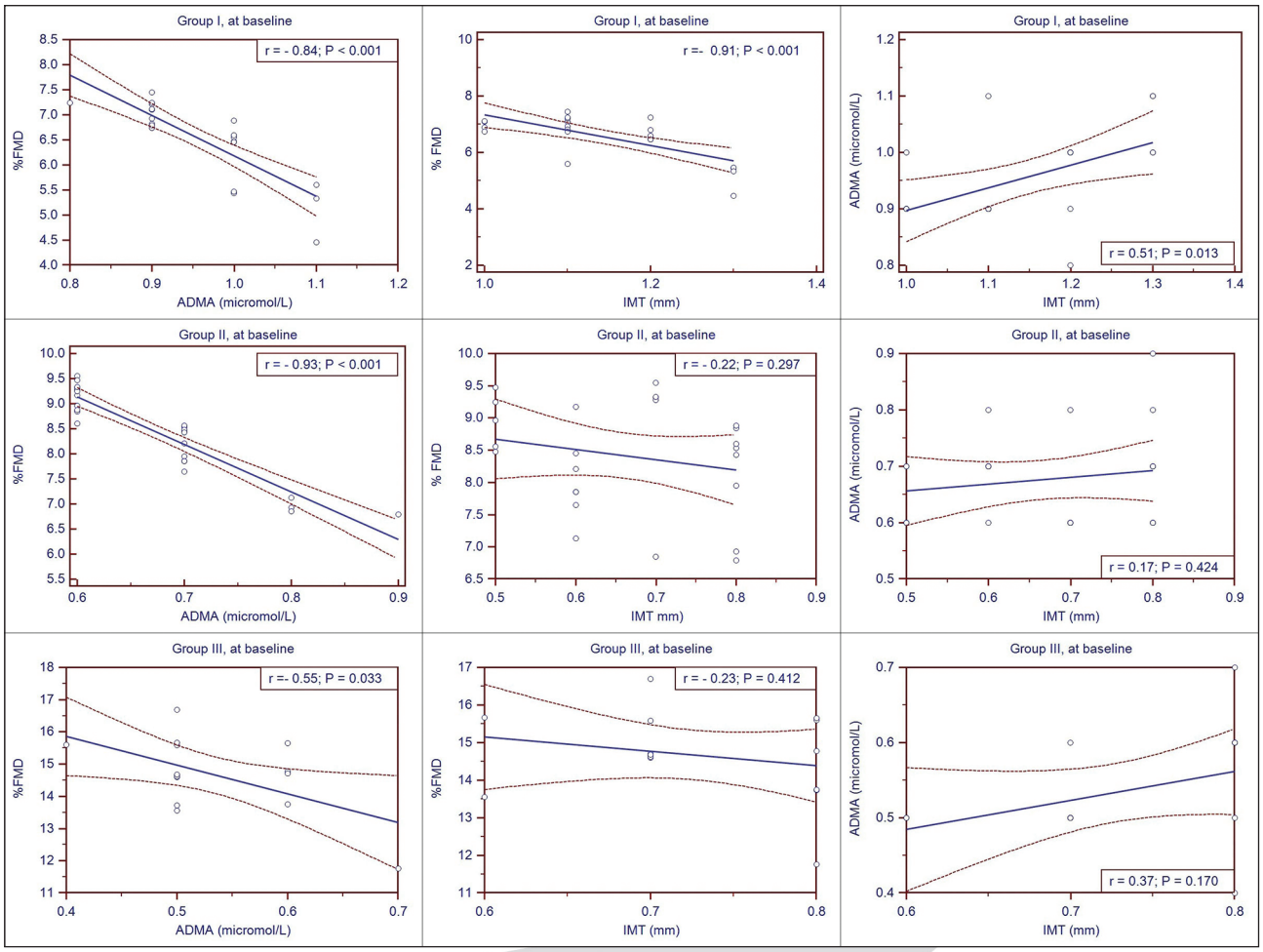

Figure 2. Association between plasma ADMA levels, FMD, carotid IMT, in the three groups of hypertensive patients, at baseline. %FMD: percent flow mediated forearm vasodilatation, IMT: carotid intima-media thickness, ADMA: asymmetric dimethyl arginine.

statin therapy. The dynamics of these results is shown in figure 3.

Concerning the changes at 6 months in endothelium dependent brachial flow mediated vasodilatation (table 2), we note a highly significant increase in group I ($p<0.001$), in patients treated with 20 mg rosuvastatin. In group II patients, treated with 10 mg rosuvastatin, the increase of % FMD was also significant ($p<0.01$). In the control group III, without statin therapy, there was a slight increase, but not significant ($p>0.05$). All these dynamic aspects of FMD values are represented in figure 4.

Table 2. Variations of FMD, carotid IMT and plasma ADMA levels

Values recorded at 6 months	Group I n=23 20 mg rosuvastatin	Group II n=25 10 mg rosuvastatin	Group III n=15 No rosuvastatin	p value I vs II	p value I vs III	p value II vs III
Plasma ADMA (μmol/L)	0.73±0.06	0.58±0.08	0.56±0.08	<0.0001	<0.0001	>0.05
P value 6 months vs baseline	P<0.0001	P<0.001	p>0.05			
FMD (%)	7.55±0.81	9.10±0.71	14.53±1.21	<0.0001	<0.0001	<0.0001
P value 6 months vs baseline	p<0.001	p<0.01	p>0.05			
Carotid IMT (mm)	1.02±0.11	0.80±0.10	0.79±0.11	<0.0001	<0.0001	>0.05
P value 6 months vs baseline	p<0.001	p>0.05	p>0.05			

Data are presented as mean±standard deviation. %FMD: percent flow mediated forearm vasodilatation, IMT: carotid intima-media thickness, ADMA: asymmetric dimethyl arginine

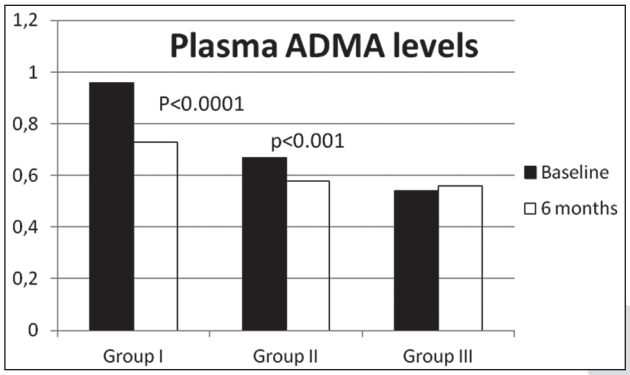

Figure 3. Dynamics of ADMA values (µmol/L) in hypertensive patients treated with rosuvastatin 20 mg (group I) or 10 mg (group II), versus no rosuvastatin therapy (group III). ADMA: Asymmetric dimethyl arginine

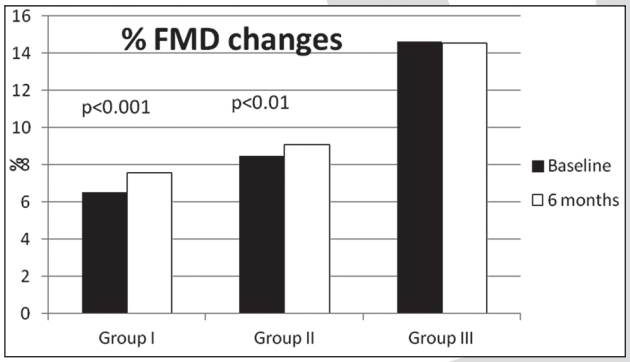

Figure 4. Dynamics of FMD values in hypertensive patients treated with rosuvastatin 20 mg (group I) or 10 mg (group II), versus no rosuvastatin therapy (group III). FMD: flow mediated vasodilatation

Regarding the changes of carotid IMT (table II), we note that in group I, with documented subclinical atherosclerosis, IMT decreased statistical significant (p<0.001) after 6 months treatment with rosuvastatin 20 mg daily. In group II (10 mg rosuvastatin therapy), IMT decreased not statistical significant (p>0.05), while in group III patients (no rosuvastatin), there was a slight increase in IMT (p>0.05). The dynamics of IMT is presented in figure 5.

The dose of 20 mg rosuvastatin administered daily for 6 months was highly efficient in improving endothelium dependent vasodilatation and in reducing carotid IMT (p<0.001) in group I hypertensive patients. At 6 months, the association between the biochemical, functional and structural parameters of endothelial dysfunction remained significant, but less strong than at baseline (% FMD with ADMA: r=-0.51, p<0.02; % FMD with IMT: r=0.51, p<0.02, ADMA with IMT: r=-0.44, p<0,04) (figure 6).

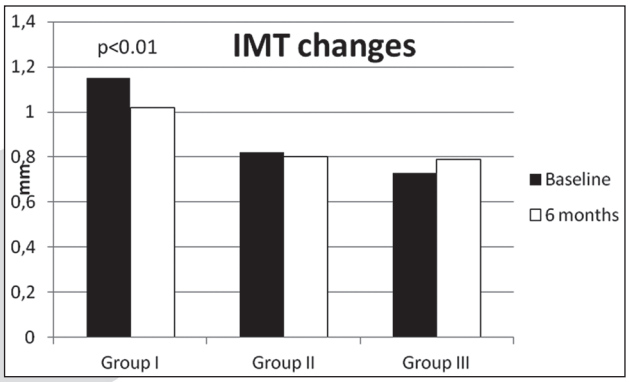

Figure 5. Dynamics of FMD values in hypertensive patients treated with rosuvastatin 20 mg (group I) or 10 mg (group II), versus no rosuvastatin therapy (group III). IMT: carotid intima-media thickness

In group II patients, 10 mg rosuvastatin administered daily for 6 months improved significant % FMD (p<0.01), but did not influence significantly carotid IMT. Plasma ADMA levels decreased significantly (p<0.0001) and correlated negative with % FMD (r=-0.51, p<0.02) and positive with carotid IMT(r=0.54, p<0.006) There was a strong positive association between %FMD and IMT (r=0.80, p<0.001).

In group III (control group), the changes in the evaluated parameters were not significant and the associations between %FMD, ADMA levels and IMT were not statistical significant (figure 6).

4. Discussion

Asymmetric dimethyl arginine (ADMA), an analogue of L-arginine, is a product of metabolism that is present in blood in normal conditions. Elevated ADMA levels induce endothelial dysfunction by inhibiting NO synthesis, and thereby favor atherosclerosis [12]. ADMA levels are increased in different conditions- essential hypertension, hypercholesterolemia, atherosclerosis, chronic heart failure, diabetes mellitus and chronic renal failure [13-14]. A number of studies have reported ADMA as a new and powerful risk marker of cardiovascular disease. Increased levels of ADMA in

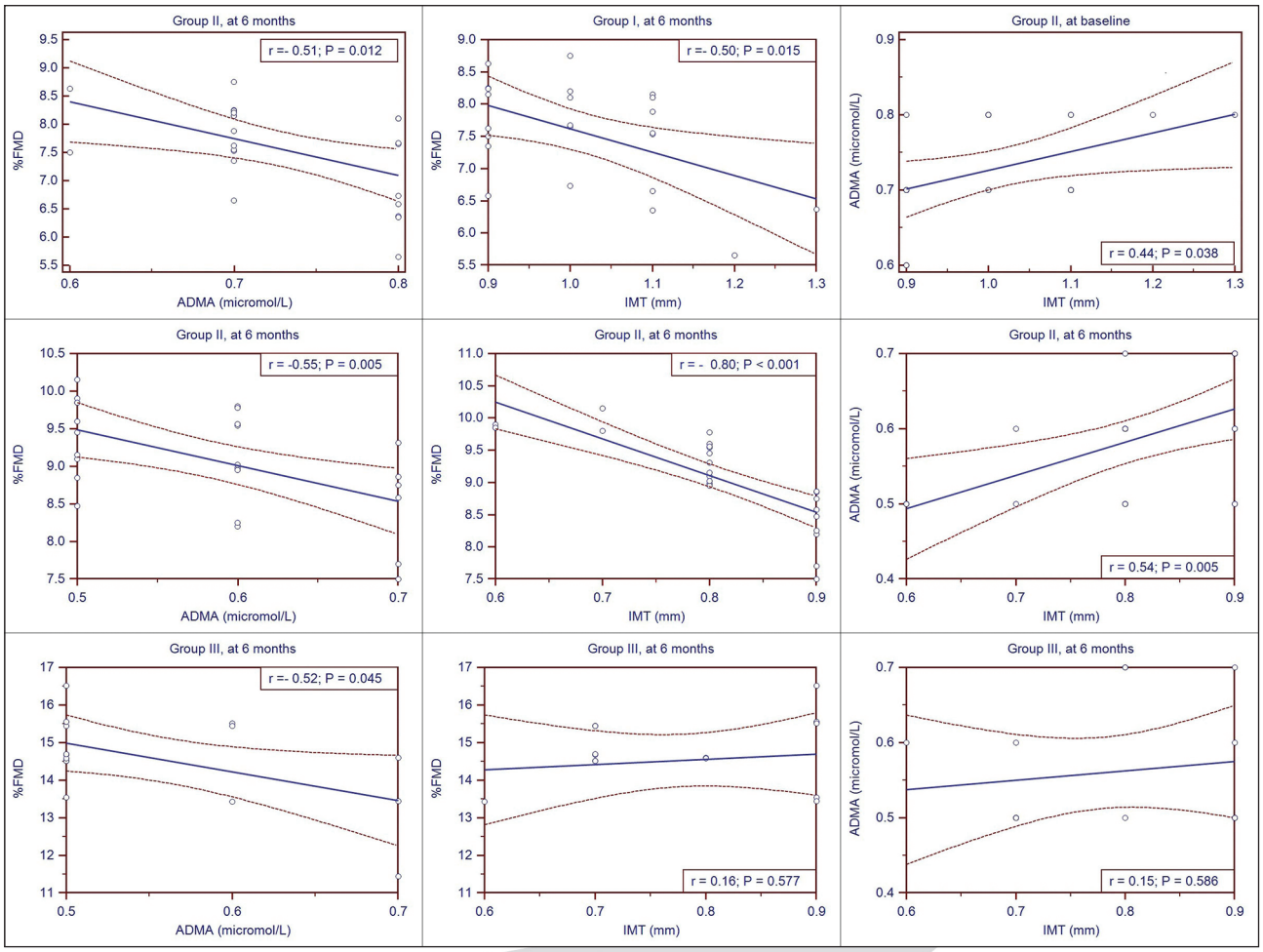

Figure 6. Association between ADMA levels and IMT, respective FMD, in the three groups of hypertensive patients, at six months. %FMD: percent flow mediated forearm vasodilatation, IMT: carotid intima-media thickness, ADMA: asymmetric dimethyl arginine.

patients with coronary artery disease are a strong predictor for cardiovascular events and for general and cardiovascular mortality, the predictive power overcoming traditional cardiovascular risk factors [15]. Interventions such as treatment with L-arginine have been shown to improve endothelium-mediated vasodilatation in people with high ADMA levels [16]. One study has stated that rosuvastatin therapy lowers plasma ADMA levels in patients with hypercholesterolemia [17].

The relationship between ADMA and essential hypertension is a rather new field of research. During recent studies, repeated measurements of ADMA plasma concentrations have been found to be higher in hypertensive patients, compared with normotensive healthy subjects, suggesting a systemic damage to the NO production levels [18-21].

In our study, hypertensive patients without hypercholesterolemia were included. Those with carotid IMT ≥ 0.9 mm, brachial FMD ≤ 10% and high plasma ADMA levels (≥0.8 μmol/L) were included in group I and received conventional antihypertensive therapy and 20 mg rosuvastatin daily. Group II patients had endothelial dysfunction (FMD≤10%) with normal carotid IMT (<0.9 mm) and normal plasma ADMA (<0.8 μmol/L). They received conventional antihypertensive therapy and treatment with 10 mg rosuvastatin daily. Group III patients represented the control group (FMD>10%, IMT<0.9 and plasma ADMA <0.8 μmol/L). They received only conventional antihypertensive therapy (no statin therapy).

The ADMA plasma levels were repeated at 6 months after treatment initiation, because a number of studies [22,23] reported that after a shorter time (1-3 months), only statistically not significant changes occur, for example after treatment with simvastatin 80 mg and 40 mg.

In groups I and II, with endothelial dysfunction demonstrated by reduced % FMD, rosuvastatin therapy for 6 months induced a highly statistically significant reduction in plasma ADMA levels: from 0.96±0.07 to 0, 73±0.06 ($p < 0.0001$) in group I and from 0.67±0.07 to 0.58±0.08 ($p<0.001$) in group II. Interestingly, the reduction of ADMA levels under rosuvastatin therapy occurred in all patients with endothelial dysfunction, even if they had normal ADMA values at baseline (group II). This is an interesting finding, not noted in previous studies. The difference in ADMA levels between groups I and II remained statistical significant at 6 months ($p<0.0001$)

In group III, with no endothelial dysfunction at baseline and no rosuvastatin therapy, there was a slight increase in ADMA values after 6 months (from 0.54±0.07 to 0.56±0.08, $p>0.05$). Even so, absolute plasma ADMA levels remained with in normal range. At 6 months, the difference in ADMA levels remained highly significant compared to those in group I ($p<0.0001$), but became not significant when compared to those in group II ($p>0.05$).

Endothelium-dependent vasodilation, assessed by brachial FMD, was significantly improved after rosuvastatin therapy for 6 months. The greatest improvement was observed in group I, at a dose of 20 mg, from 6.53±0.76 at baseline to 7.55±0.81 ($p<0.001$). In group II, at a dose of 10 mg rosuvastatin, the improvement was from 8.48±0.79 to 9.10±0.71 ($p<0.01$). In the control group, FMD increased not significant ($p>0.05$). At the end of the study, the differences between the three groups regarding %FMD remained highly statistical significant ($p<0.0001$). There are no data in the literature on the minimum time interval after which influence of statins on changes in endothelial function can be highlighted. In our study, the statin induced improvement in endothelium dependent vasodilatation was significant after six months and was dose-dependent.

Structural changes induced by statin therapy were assessed by echocardiographic measurements of carotid IMT, at baseline and after six months. A statistically significant decrease occurred only in group I, from 1.15±0, 10 to 1.02±0.11 ($p<0.001$), at a dose of 20 mg rosuvastatin. In group II, with normal IMT at baseline at 10 mg rosuvastatin, IMT decreased not significant ($p>0.05$). In group III there was a not significant increase, without overcoming normal values. These results suggest that high doses (20 mg) rosuvastatin act on subclinical atherosclerotic lesions and induce their regression, the effect being significant at six months. This result is concordant with the outcomes of other studies that reported a dose-dependent decrease of IMT under rosuvastatin therapy [24,25]. Following the dynamics of ADMA, FMD and IMT changes under rosuvastatin therapy, we observed that FMD and ADMA changes were dose independent, while IMT changed only at the dose of 20 mg.

At baseline, ADMA levels had a significant negative correlation with FMD in all three groups of hypertensive patients (group I: $r=-0.97$, $p<0.001$; group II: $r=-0.93$, $p<0.001$, group III: $r=-0.63$, $p<0.01$). We found a significant positive correlation between ADMA and IMT in group I ($r=0.92$, $p<0.001$) and in group II ($r=0.82$, $p<0.001$). There was no significant correlation between ADMA and IMT in group III ($r=0.23$, $p>0.05$). The strongest correlation between ADMA and FMD, respectively IMT was found in group I patients, with subclinical carotid atherosclerosis and endothelial dysfunction. These data suggest the possibility to use ADMA levels as a surrogate marker of these structural and functional conditions.

At 6 months evaluation, the strongest correlations between biochemical (ADMA), functional (FMD) and structural parameters (IMT) were found in group I, were we also noticed the best response to high dose rosuvastatin therapy for 6 months. Analyzing the negative correlation between ADMA and FMD, we notice that it is less strong than at baseline, but still significant in all three groups: group I ($r=-0.76$, $p<0.001$), group II ($r=-0.56$, $p<0.01$), group III ($r=-0.52$, $p=0.045$). These data are in concordance with literature [26].

The positive ADMA-IMT correlation remains significant, but less strong in group I ($r=0.62$, $p=0.002$) and in in group II ($r=0$, 57, $p=0.003$), in accordance with the literature [27]. This correlation remains not significant in the control group III ($p>0.05$). The significant reduction of IMT in group I indicates that high dose (20mg) rosuvastatin therapy may induce the regression of subclinical atherosclerotic lesions in hypertensive patients with high plasmatic ADMA levels.

Regarding the negative correlation FMD with IMT after 6 months of treatment, it remains significant

the first two groups and not significant in the control group, the data being in accordance with the literature [27]. This correlation was stronger in group II ($r=-0.77$, $p<0.01$) than in group I ($r=-0.71$, $p<0.01$). The progressive increase in FMD and decrease in IMT in hypertensive patients with endothelial dysfunction reflect functional and structural changes in the arterial wall under rosuvastatin therapy. In group III, the direction of correlation between FMD and IMT changed at 6 months, becoming positive, although it remained not statistical significant. This change of direction indicates a worsening of endothelial function, even in the absence of detectable functional or structural abnormalities of the arterial wall. All these aspects observed in our study demonstrate the benefits of rosuvastatin treatment in hypertensive patients with documented endothelial dysfunction. Rosuvastatin treatment had benefits in improving endothelial function in group I, this fact being demonstrated by the decrease in ADMA levels and the increase in FMD. In group II patients, rosuvastatin therapy prevented the progression of endothelial dysfunction, this fact being demonstrated by the decrease of the already normal ADMA levels and by the increase in FMD. The doses of 20 mg rosuvastatin were efficient in inducing the regression of subclinical atherosclerotic lesions. All these benefits observed in groups I and II are independent of the dose level and regardless of the severity of the functional or structural changes of the arterial wall. The intensity of the benefits is dose dependent.

These results suggest that rosuvastatin administration is efficient anytime in the treatment of hypertension, having a beneficial effect on the functional and structural condition of the endothelium. Rosuvastatin therapy prevented the occurrence and worsening of endothelial dysfunction. It also slowed the progression and promoted the regression of subclinical atherosclerotic lesions.

ADMA appears as a possible marker of endothelium functional status, its pathological changes are early and strong correlated with the degree of endothelial dysfunction, while dynamics can be used as a predictive marker for the evolution of subclinical atherosclerotic lesions in hypertensive patients.

Rosuvastatin treatment in patients with essential hypertension seems to improve all biochemical, structural and functional parameters of endothelial dysfunction. Our study demonstrates that a 6 months treatment with rosuvastatin significantly improves FMD, this improvement being significantly associated with a reduction in ADMA and IMT values. The clinical benefits are positive correlated with the doses of rosuvastatin used, being greater at the dose of 20mg vs 10 mg. These results suggest that rosuvastatin treatment in hypertensive patients can improve endothelial function and slow down the progression of subclinical atherosclerotic lesion. The dynamics of plasma ADMA correlates significantly with the functional and structural changes of the endothelium, suggesting its usefulness as a surrogate marker for these parameters.

Learning points

Endothelial dysfunction increases cardiovascular risk in patients with essential arterial hypertension.

Increased ADMA levels correlate strongly with impaired endothelium dependent flow-mediated vasodilatation and with the presence of subclinical atherosclerotic lesions. In hypertensive patients without hypercholesterolemia, Rosuvastatin therapy at 20 mg daily for 6 months reduces biochemical, functional and structural markers of endothelial dysfunction, while at 10 mg daily for 6 months it influences significantly ADMA levels and %FMD.

References

1. *Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980; 288: 373–6.*
2. *Furchgott RF. The role of the endothelium in the responses of vascular smooth muscle to drugs. Annu Rev Pharmacol Toxicol 1984; 24: 175–97.*
3. *Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci USA 1987; 84: 9265–9.*
4. *Andor M, Tomescu M. Endothelial dysfunction – Methods of assessment and pharmacological approach in cardiovascular disease. Timisoara Medical Journal 2005; 55: 58-63.*
5. *O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson SK Jr. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. N Engl J Med 1999; 340: 14–22.*

6. *Mancini GB, Dahlof B, Diez J. Surrogate markers for cardiovascular disease: structural markers. Circulation 2004; 109: IV22–IV30.*
7. *Lakka TA, Salonen R, Kaplan GA, Salonen JT. Blood pressure and the progression of carotid atherosclerosis in middle-aged men. Hypertension 1999; 34: 51–56.*
8. *Martinez-Gonzalez J, Badimon L. Influence of statin use on endothelial function: from bench to clinics. Curr Pharm Des 2007; 13: 1771-86.*
9. *Jones PH, Davidson MH, Stein EA, et al; STELLAR Study Group. Comparison of the efficacy and safety of rosuvastatin versus atorvastatin, simvastatin, and pravastatin across doses (STELLAR Trial). Am J Cardiol 2003; 92: 152-60.*
10. *Corretti MC, Anderson TJ, Benjamin EJ et al. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the international brachial artery reactivity task force. JACC 2002; 39: 257-65.*
11. *Touboul PJ, Hennerici MG, Meairs S, Adams H, Amarenco P, Bornstein N et al. Mannheim Carotid Intima-Media Thickness Consensus (2004 – 2006). An Update on Behalf of the Advisory Board of the 3rd and 4th Watching the Risk Symposium 13th and 15th European Stroke Conferences, Mannheim, Germany, 2004, and Brussels, Belgium, 2006. Cerebrovasc Dis 2006; 23: 75-80.*
12. *Anderssohn M, Schwedhelm E, Lue neburg N. Asymmetric dimethylarginine as a mediator of vascular dysfunction and a marker of cardiovascular disease and mortality: an intriguing interaction with diabetes mellitus. Diab Vasc Dis Res 2010; 7(2): 105–18.*
13. *Matsuguma K, Ueda S, Yamagishi S, Matsumoto Y, Kaneyuki U, Shibata R et al. Molecular mechanism for elevation of asymmetric dimethylarginine and its role for hypertension in chronic kidney disease. J Am Soc Nephrol 2006; 17: 2176–83.*
14. *Arnett DK et al. Hypertension and subclinical carotid artery atherosclerosis in blacks and whites: The Atherosclerosis Risk in Communities Study. Arch Intern Med 1996; 17: 1983–9.*
15. *Sibal L, Agarwal SA, D'Home P, Boger RH. The Role of Asymmetric Dimethyl arginine (ADMA) in endothelial dysfunction and cardiovascular disease. Curr Cardiol Revue 2010; 6(2): 82-90.*
16. *Wang H, Liu J. Plasma asymmetric dimethylarginine and L-arginine levels in Chinese patients with essentialhypertension without coronary artery disease. J Cardiovasc Dis Res. 2011 Jul; 2(3): 177-80.*
17. *Lu TM, Ding YA, Leu HB, Yin WH, Sheu WH, Chu KM. Effect of rosuvastatin on plasma levels of asymmetric dimethylarginine in patients with hypercholesterolemia. Am J Cardiol 2004; 94: 157-61.*
18. *Achan V, Broadhead M, Malaki M, Whitley G, Leiper J, MacAllister R, Vallance P. Asymmetric dimethylarginine causes hypertension and cardiac dysfunction in humans and is actively metabolized by dimethylarginine dimethylaminohydrolase. Arterioscler Thromb Vasc Biol 2003 Aug 1; 23(8): 1455-9.*
19. *Palloshi A, Fragasso G, Piatti P. Effect of oral L-arginine on blood pressure and symptoms and endothelial function in patients with systemic hypertension, positive exercise test, and normal coronary arteries. Am J Cardiol 2004; 93: 930–5.*
20. *Surdacki A, Nowicki M, Sandmann J. Reduced urinary excretion of nitric oxide metabolites and increased plasma levels of asymmetrical dimethylarginine in men with essential hypertension. J Cardiovasc Pharmacol 1999; 33: 652–8.*
21. *Calver A, Collier J, Leone A, Moncada S, Vallance P. Effect of local intra-arterial asymmetric dimethylarginine (ADMA) on the forearm arteriolar bed of healthy volunteers. J Hum Hypertens 1993; 7: 193–4.*
22. *Vladimirova-Kitova LG, Deneva-Koycheva TI. Asymmetric dimethylarginine--a determinant of the effect of the high dose Simvastatin. Clin Biochem. 2010 Jul; 43(10-11): 843-50*
23. *Vladimirova-Kitova LG, Deneva-Koycheva TI. The effect of simvastatin on asymmetric dimethylarginine and flow-mediated vasodilation after optimizing the LDL level: a randomized, placebo-controlled study. Vascul Pharmacol. 2012 Mar-Apr; 56(3-4): 122-30*
24. *Riccioni G, Bazzano LA, Bucciarelli T, Mancini B, di Ilio E, D'Orazio N. Rosuvastatin reduces intima-media thickness in hypercholesterolemic subjects with asymptomatic carotid artery disease. The Asymptomatic Carotid Atherosclerotic Disease in Manfredonia (ACADIM) Study. Expert Opin Pharmacother. 2008 Oct; 9(14): 2403-8.*
25. *Rossi R, Nuzzo AAU,Olaru AI, Origliani G, Modena MG. Endothelial function affects early carotid atherosclerosis. J Hypertens 2011; 29(6): 1136–44.*
26. *Feldstein CA. Statins in hypertension: are they a new class of antihypertensive agents? Am J Ther 2010 May-Jun; 17(3): 255-62.*
27. *Tsiara S, Elisaf M, Mikhailidis DP. Early vascular benefits of statin therapy. Curr Med Res Opin 2003; 19: 540-56.*

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Retrospective evaluation of patterns of hepatotoxicity and grading in patients with primary breast cancer on Doxorubicin-Cyclophosphamide protocol (AC-protocol)

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Abstract

Background & Objective: Doxorubicin-cyclophosphamide (AC) is used in the treatment of breast cancer as first line therapy. A retrospective study was conducted to determine the hepatotoxicity and grading of toxic effects on the basis of increase in the levels of hepatic enzymes by using this protocol in different age groups.

Methods: Total 75 patients (n=75) on AC protocol with breast cancer were included in this study. The patient records were retrospectively evaluated. The age, gender, body surface area, doses and disease status were recorded by reviewing their charts. Hepatotoxicity was diagnosed on the basis of abnormal level of liver enzymes and graded according to common terminology criteria for adverse events (CTCAE) in patients suffering from breast cancer in order of increasing adversity from grade-1 to grade-4.

Results: Out of 75 patients, 49 (65%) had value of liver enzymes within range. While remaining 26 patients (35%) had different grades of hepatotoxicity. According to the CTCAE out of 26 patients, the value of liver enzymes raised and graded as grade-1 toxicity in 22 patients (85%), grade-2 toxicity in 3 patients (11%) and garde-3 hepatotoxicity in only one patient (3%).

After applying ANOVA through SPSS version 21, results of the comparison between the baseline pretreatment values with the post-therapy values show highly significant increase ($p < 0.05$) in the levels of alanine transaminase (ALT), aspartate transaminase (AST) and gamma glutamyl transferase (GGT) in patients received AC protocol.

Conclusion: It is concluded that Doxorubicin-Cyclophosphamide (AC) protocol equally causes hepatotoxicity in the patients of all age groups with breast cancer. Therefore, AC affects the liver functions significantly as a result of increase in hepatic enzymes.

Key words: Breast cancer, AC Protocol, Hepatic enzymes, Hepatotoxicity

Introduction

Cancer is a fatal disease that results into more deaths than heart diseases (1). Cancer affects every human being regardless of the age, gender and socioeconomic status (2, 3). Global cancer prevalence is recorded to be 28.8 million from 2004 to 2008 (4). In the United States of America (USA), 1.6 million new cases of cancer are registered annually and according to a report, about 0.57 million patients died of cancer in the year 2012 (5). However in Europe, 3.45 million new cases of cancer are registered each year while 1.75 million died as a result of cancer in the year 2012 (6). Lung, colorectal, female breast and male prostate are the four main sites of cancer (7). Incidence of breast cancer is higher in patients who do not annually undergo screening examination through mammography (8). Several carcinogens cause cancer, which may be as minor as irritant scratch, to as complex as viruses and chemicals (9). Any transformation occurring in the genetic makeup of the cells may lead to cancer development of different intensities and stages (10, 11). Cancers are being treated stereoscopically by using surgical, radiological or chemotherapeutic methods with good or bad results (12). There are several anticancer drugs which are extensively used either alone or in combination (13). These drugs beside producing therapeutic effects exert deleterious side-effects (14) and bringing structural and physiological changes in

vital organs of the body(15) as liver, kidney, brain and blood (16). Risk of death also increases in patients being treated with anticancer drugs (17). For the sake of patient safety, it is very important to monitor therapy either by checking plasma concentration of drugs through therapeutic drug monitoring (TDM) (18) or by avoiding serious adverse effects by monitoring laboratory values (19).

Liver is involved in the regulation of vital functions including synthesis, biotransformation and clearance of xinobiotics (20). Majority of anticancer drugs are metabolized through cytochrome-P450 subfamily CYP3A4 (21, 22). Most of the drug-induced injuries are attributed to liver because of their direct involvement in metabolism especially xenobiotics. In xenobiotic-induced hepatic damage, the hepatocytes are often disrupted by the activity of cytochrome P-450 enzymes (23). Liver injury may be either drug related or idiosyncratic (24, 25). Chemotherapeutic agents also cause hypersensitivity reactions or direct hepatic toxicity in combination therapy as well as in monotherapy. It has been reported that permanent disability or death of patients can occur through idiosyncratic drug-induced liver injury (IDILI) (26). Glutathione S-transferase (GST) depletion is associated directly with the chemotherapeutic drug induced liver injuries (27). One of the reasons for drug withdrawal from the market is drug-induced liver injury (DILI) (28, 29). Drug-induced liver injury can be minimized or avoided by monitoring liver function tests (LFTs) on regular basis. As a result of liver injury the plasma concentration of cyctotoxic drugs having narrow therapeutic window increases that in turn increases the potential for the side-effects and adverse drug reactions (30). Therefore, it is important to monitor hepatic functions during antineoplastic chemotherapy (31).

Doxorubicin is an anthracycline antibiotic obtained from *Streptococcus peucetius* (32). It is extensively used as a part of therapeutic regimen in the treatment of various malignancies as lymphomas, sarcomas and leukemias (33, 34). It is quite frequently a part of therapeutic protocols like 5-flurouracil, doxorubicin and cyclophosphamide (FAC), doxorubicin, bleomycin, vinblastine, decarbazine (ABVD), doxorubicin and cyclophosphamide (AC) and cyclophosphamide, doxorubicin and 5-flurouracil (CAF) (13). Doxorubicin causes subclinical hepatotoxicity (35-37) and also has been observed to be hepatotoxic as a part of combination therapy in cancer patients (38). For instance doxorubicin is given as combination therapy with cyclophosphamide (AC- protocol) in the treatment of breast cancer (39). This protocol (AC) is considered to be preferred first line therapy in breast cancer (40). Doxorubicin and cyclophosphamide also have been reported to cause grade-2 to grade-3 hepatotoxicity when used in combination with docetexal combination chemotherapy protocol (41). Cyclophosphamide alone has been reported to cause hepatotoxicity at very low dose (42) and it undergoes redox cycling depleting glutathione (GSH) reserves in tissues leading to the generation of highly reactive species (free-radicals) and ultimately the hydrogen peroxide, which is a proposed mechanism of oxidative stress leading to hepatotoxicity (43, 44). Cyclophosphamide is administered intravenously and being cleared by hepatic metabolism and biliary excretion. Doxorubicin being metabolized through liver has direct adverse effects including hepatitis, cholestatic jaundice, steatosis and elevation of liver enzymes. Liver toxicity is due to mitochondrial oxidative stress and formation of peroxynitrite (45).

Materials and Methods

This study was conducted retrospectively at the specialized cancer research centre in Lahore, Pakistan after the approval from the ethical committees of the hospital and the University College of Pharmacy, University of the Punjab, Lahore, Pakistan. The patients included in this study were newly diagnosed with primary breast cancer having normal baseline hepatic functions and being treated with AC-protocol. Total sample size of study population was 75 patients (n=75). The patients were further categorized into three groups on the basis of age (20-35 years, 36-50 years and 51-65 years).

Baseline liver function tests (LFTs) were assessed before initiating drug therapy. The LFT values were checked every 21 days after each cycle (cycle 1, cycle 2, cycle 3 and cycle 4). The data was recorded on a data collection form, further SPSS (version 21) statistical software package for data entry and analysis was used. Statistical test ANOVA was applied for comparing the changes in LFTs from the baseline (pre-treatment) with the post-cycle laboratory values.

Results

Total 75 patients were categorized according to the age into three groups as group-1(20-35 years), group-2 (36-50 years) and group-3(51-65 years) and the distribution number of total patients according to age group was 18, 40 and 17 respectively

Table 1. Mean Baseline and Post Therapy Values of Liver Enzymes in different age groups

Laboratory Parameters	Age Group (Years)	Baseline Value U/L	Cycle 1 U/L	Cycle 2 U/L	Cycle 3 U/L	Cycle 4 U/L
Alanine transaminase (ALT)	20-35	20.72	25.83	26.86	28.89	32.80
	36-50	20.67	25.65	26.60	28.57	32.45
	51-65	20.33	25.58	26.23	27.12	30.45
Aspartate transaminase (AST)	20-35	20.72	22.82	25.31	27.13	30.41
	36-50	20.69	22.49	24.99	26.96	30.20
	51-65	20.67	22.55	24.33	25.43	28.30
Gamma glutamyl tranferase (GGT)	20-35	20.68	28.41	29.68	30.17	35.87
	36-50	20.99	28.91	30.28	30.35	36.93
	51-65	20.67	28.35	28.42	29.77	36.22

Table 2. Comparison of mean baseline and post-therapy value in total population

Laboratory Parameter (U/L)	Alanine transaminase (ALT) (U/L)	Aspartate transaminase (AST) (U/L)	Gamma glutamyl tranferase (GGT) (U/L)
Baseline Value	20.67	20.69	20.99
Post-therapy value	32.45	30.2	36.93
S.D	±21.57	±15.23	±30.75
M_4-M_0	11.78 U/L	9.51 U/L	15.94 U/L
p-value*	< 0.001**	< 0.001**	< 0.001**

** Comparison of mean baseline and post-therapy value* ***highly significant*

Table 3. Multiple Comparison of different groups using ANOVA

Multiple Comparisons		ALT		AST		GGT	
Cycles (I)	Cycles (J)	Mean Difference (I-J)	Sig.	Mean Difference (I-J)	Sig.	Mean Difference (I-J)	Sig.
Baseline	Cycle1	-4.98667	.281	-1.80000	.864	-7.92000	.230
	Cycle2	-5.93333	.133	-4.29333	.134	-9.29333	.106
	Cycle3	-7.90667*	.016	-6.26667*	.006	-9.36000	.102
	Cycle4	-11.78667*	.000	-9.50667*	.000	-15.94667*	.000
Cycle1	**Baseline**	**4.98667**	**.281**	**1.80000**	**.864**	**7.92000**	**.230**
	Cycle2	-.94667	.996	-2.49333	.654	-1.37333	.996
	Cycle3	-2.92000	.777	-4.46667	.108	-1.44000	.996
	Cycle4	-6.80000	.057	-7.70667*	.000	-8.02667	.218
Cycle2	**Baseline**	**5.93333**	**.133**	**4.29333**	**.134**	**9.29333**	**.106**
	Cycle1	.94667	.996	2.49333	.654	1.37333	.996
	Cycle3	-1.97333	.936	-1.97333	.819	-.06667	1.000
	Cycle4	-5.85333	.142	-5.21333*	.038	-6.65333	.405
Cycle3	**Baseline**	**7.90667***	**.016**	**6.26667***	**.006**	**9.36000**	**.102**
	Cycle1	2.92000	.777	4.46667	.108	1.44000	.996
	Cycle2	1.97333	.936	1.97333	.819	.06667	1.000
	Cycle4	-3.88000	.540	-3.24000	.395	-6.58667	.416
Cycle4	**Baseline**	**11.78667***	**.000**	**9.50667***	**.000**	**15.94667***	**.000**
	Cycle1	6.80000	.057	7.70667*	.000	8.02667	.218
	Cycle2	5.85333	.142	5.21333*	.038	6.65333	.405
	Cycle3	3.88000	.540	3.24000	.395	6.58667	.416

**The mean difference is significant at the 0.05 level.*

(n=75). Parameters as ALT, AST and GGT were included. After recording laboratory values of these parameters prior to treatment and at the end of each cycle, mean of baseline values after each cycle up to maximum cycle-4 were recorded Table 1.

Changes in Alanine transaminase (ALT) levels

Baseline value of ALT was normal in all patients before the start of therapy. After completion of cycle 4, value of ALT remained within range in 56 (75%) patients. The value of ALT according to the CTCAE showed grade-1 toxicity in 17 (22%) patients and grade-2 toxicity in 2 (3%) patients. In patients of age group 20-35 years, 13 (72%) patient values remained within normal range. Only 1 (6%) patient showed grade-2 toxicity and 4 (22%) patients showed grade-1 toxicity. In age group of 36-50 years, 29 (72%) patients remained within normal range. Only 1(3%) patient showed grade-2 toxicity and 10 (25%) patients with grade-1 toxicity. In elderly patients of age group 51-65 years, 17 (85%) patients were within normal range and grade-1 toxicity had been seen in 3 (15%) patients.

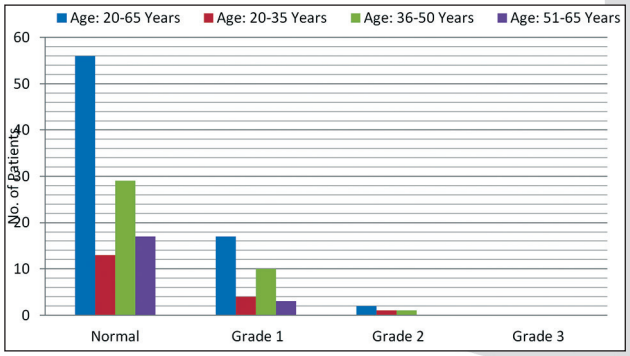

Figure 1. Hepatotoxicity grading in patients of different age groups on the basis of ALT value

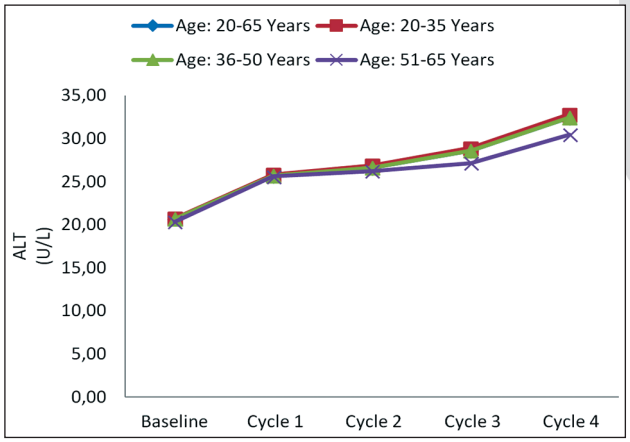

Figure 2. Trend graph of ALT during AC therapy in patients of different age groups.

Mean baseline value of ALT in patients receiving AC protocol

The mean baseline value of ALT in patients receiving AC protocol before starting therapy was 23 U/L and the value increased significantly ($p < 0.05$) after completion of cycle 4. The average values of all patients were 25.65 U/L, 26.60 U/L, 2, 28.57 U/L and 32.45 U/L after cycle 1, cycle 2, cycle 3 and cycle 4 respectively. In different age groups, same trend was observed and there was no significant difference in the average baseline values and post therapy values of ALT between different age groups.

Changes in Aspartate Transaminase (AST) Levels

Baseline value of AST was also within normal range in all patients. After completion of therapy, value of AST remained within range in 62 (83%) patients. The value of AST according to the CTCAE showed grade-1 toxicity in 13 (17%) patients. In patients of age group 20-35 years, 15 (83%) patients remained within normal range. Only 3 (17%) patients showed grade-1 toxicity. In age group of 36-50 years, 37 (92%) patients remained within normal range. Only 3 (8%) patients showed grade-1 toxicity. In elderly patients of age group 51-65 years, 10 (59%) patients were within normal range and grade-1 toxicity had been seen in 7 (41%) patients.

Average baseline value of AST before starting therapy was 20.69 U/L. The values of AST, 22.49 U/L, 24.99 U/L, 26.96 U/L and 30.20 U/L after cycle 1, cycle 2, cycle 3 and cycle 4 were observed respectively. In different age groups, same trend was seen and there was no significant difference in the average baseline values and post therapy values of AST of different age groups.

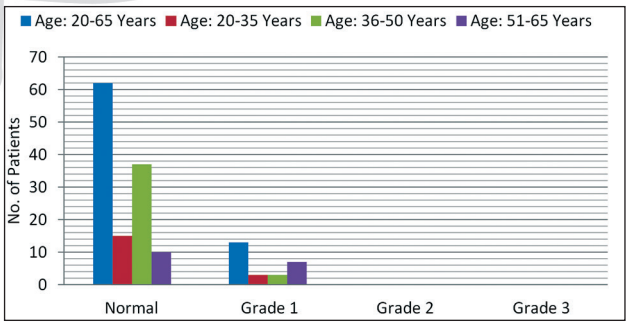

Figure 3. Hepatotoxicity grading in patients of different age groups on the basis of AST value

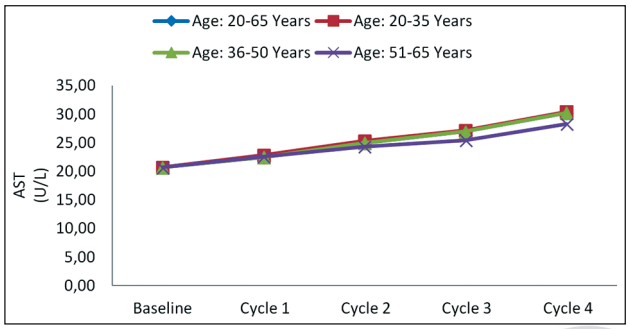

Figure 4. Trend graph of AST during AC therapy in patients of different age groups

Changes in Gamma Glutamyl Transferase (GGT)

After completion of therapy, value of GGT remained within normal range in 52 (69%) patients. The value of GGT according to the CTCAE showed grade-1 toxicity in 20 (27%) patients, grade-2 toxicity in 2 (3%) patients and 1(1%) patient with grade-3 toxicity. In patients of age group 20-35 years, 15 (83%) patients remained within normal range. Only 3 (17%) patients show grade-1toxicity. In age group of 36-50 years, 28 (70%) patients remain within normal range. A total of 11 (27%) patient showed grade-1 toxicity and 1 (3%) patient graded toxicity of grade 3. In elderly patients of age group 51-65 years, 9 (53%) patients were within normal range and grade-1 toxicity had been seen in 6 (35%) patients and grade-2 toxicity in 2 (12%) patients.

The average baseline value of GGT before starting therapy was 20.99 U/L and the value increased after completion of therapy. The values were 28.91 U/L, 30.28 U/L, 30.35 U/L and 36.93 U/L after cycles 1, 2, 3 and 4 respectively. The values increased in the same trend in all age groups during course of therapy.

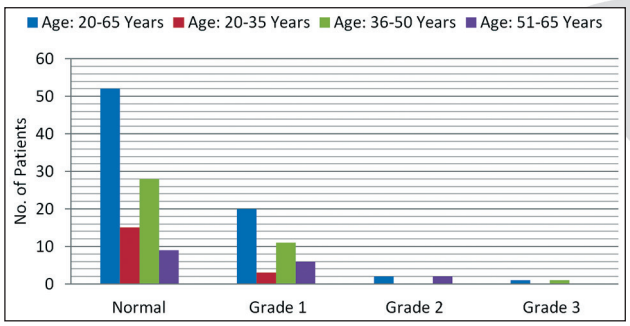

Figure 5. Hepatotoxicity grading in patients of different age groups on the basis of GGT value

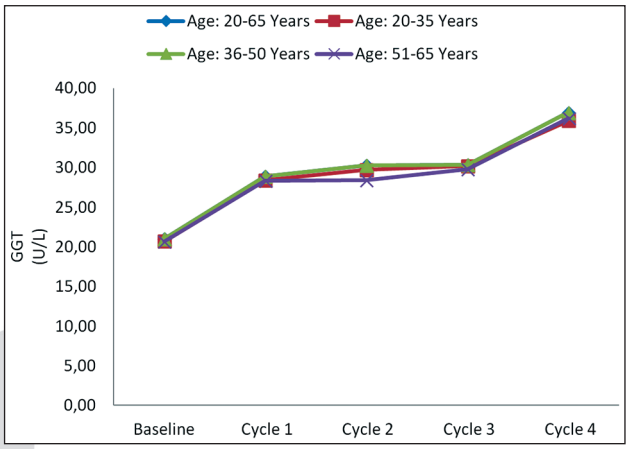

Figure 6. Trend graph of GGT during AC therapy in patients of different age groups

Discussion

Anticancer drugs are used under strict supervision because of their narrow therapeutic window, high toxicity profile, significance of drug interactions and finally to prevent recurrence of painful disease condition (46). Anticancer drugs affect every tissue of the body because the drug is unable to target the site of tumor (47). The drugs especially affect vital organs like brain, heart, kidney or liver and to those which undergo rapid cell division (30, 48). Therefore, careful monitoring is required to ensure desired outcomes. Liver enzymes are the most important parameter to evaluate liver functions (49) and the increased level of liver enzymes are the indicator of some sort of injury to hepatocytes (50, 51). A very limited data is available on hepatotoxicity induced by combination chemotherapy in patients with breast cancer. Mild hepatotoxicity had been reported in 75% patients on the basis of abnormal AST and ALT level during the course of docetaxel, doxorubicin, and cyclophosphamide therapy (41). In this study, after analyzing the data using SPSS statistical tool by applying paired ANOVA between baseline values (pretreatment values) and post treatment values after cycle 4, showed that there was highly significant increase ($p < 0.05$) in the levels of ALT, AST and GGT in patients receiving AC protocol. The baseline values of all patients included in this study were normal (pre-treatment). After completion of therapy, overall results shown the value of liver enzymes was within range in 49 (65%) patients and in remaining 26 (35%) patients with different grades of hepatotoxicity. According to the CTCAE out of these 26 patients, on the basis

of value of liver enzymes was categorized as grade-1 toxicity in 22 (85%) patients, grade-2 toxicity in 3(11%) patients and grade-3 hepatotoxicity in 1(3%) patient only. In patients of age group 20-35 years, 15 (87%) patients were within normal range and the remaining all 3 (17%) patients had shown grade-1 toxicity. In age group of 36-50 years, value of liver enzymes was within range in 27 (67%) patients and in remaining 13 (33%) patients shown different grades of hepatotoxicity after completion of therapy. According to the CTCAE, out of these 13 patients, the value of liver enzymes showed grade-1 toxicity in 11 (84%) patients, grade-2 toxicity in 1 (8%) patient and while grade-3 hepatotoxicity in 1 (8%) patient. In elderly patients of age group 51-65 years, 17 (85%) patients were within normal range and remaining 8 (47%) patients had shown different grades of hepatotoxicity. Of these 7 (78%) showed grade-1 toxicity and grade-2 toxicity had been observed in 3(22%) patients.

It demonstrated that when the offending agent was discontinued the elevated liver enzyme condition reverted back to normal assuring the involvement of drug in liver toxicity. Once the symptoms of drug-induced injury appear, discontinuation of the offending agent is the only measure practiced (52). The genetic background of each patient should be addressed by pharmacogenomic approaches (53). The only way to avoid drug-mediated liver injuries is screening methods for identification of enzymatic deficiencies that alter the metabolism and by modifying pharmacogenomic approaches before drug being used in patients (54). Doxorubicin induced hepatotoxicity, like cardiotoxicity (55) may be prevented by screening methods that can identify aberrant gene polymorphism or enzyme deficiency before a patient uses anthracycline oxidant drug (56). The patients should be given some additional supplements prophylactically like vitamins E and C which reduce the oxidative stress induced by doxorubicin (57).

Conclusion

Doxorubicin-Cyclophosphamide (AC) protocol equally causes hepatotoxicity in all age groups of patients with breast cancer. Therefore, it is concluded that AC affects the liver functions by significantly increasing the hepatic enzymes.

References

1. *Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al. Cancer statistics, 2008. CA: a cancer journal for clinicians. 2008; 58(2): 71-96.*
2. *Jemal A, Center MM, Ward E, Thun MJ. Cancer occurrence. Cancer Epidemiology: Springer; 2009; 3-29.*
3. *Babaei M, Mousavi S, Malek M, Tosi G, Masoumeh Z, Danaei N, et al. Cancer occurrence in Semnan Province, Iran: results of a population-based cancer registry. Asian Pac J Cancer Prev. 2005; 6(2): 159-64.*
4. *Bray F, Ren JS, Masuyer E, Ferlay J. Global estimates of cancer prevalence for 27 sites in the adult population in 2008. International Journal of Cancer. 2013; 132(5): 1133-45.*
5. *Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA: a cancer journal for clinicians. 2012; 62(1): 10-29.*
6. *Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh J, Comber H, et al. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. European Journal of Cancer. 2013.*
7. *Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA: a cancer journal for clinicians. 2013; 63(1): 11-30.*
8. *DeSantis C, Siegel R, Bandi P, Jemal A. Breast cancer statistics, 2011. CA: a cancer journal for clinicians. 2011; 61(6): 408-18.*
9. *Pohanish RP. Sittig's handbook of toxic and hazardous chemicals and carcinogens: Access Online via Elsevier; 2008.*
10. *Imai K, Yamamoto H. Carcinogenesis and microsatellite instability: the interrelationship between genetics and epigenetics. Carcinogenesis. 2008; 29(4): 673-80.*
11. *Berenblum I. The mechanism of carcinogenesis. A study of the significance of cocarcinogenic action and related phenomena. Cancer research. 1941; 1(10): 807-14.*
12. *Papadakis MA, McPhee SJ, Rabow MW. Current medical diagnosis & treatment 2013: McGraw-Hill Medical; 2013.*
13. *Wells BG, DiPiro JT, Schwinghammer TL, Hamilton CW. Pharmacotherapy handbook: McGraw-Hill, Medical Pub. Division; 2006.*
14. *Committee JF, Britain RPSoG. British national formulary (bnf): Pharmaceutical Press; 2012.*

15. *Harvey RA, Champe R. Lippincott's illustrated reviews pharmacology: Philadelphia: Lippincott-Raven, 2007.*

16. *Hashmi FK, Hussain K, Islam M, Ali M, Tipu MK, Khan MT, et al. Cisplatin-induced nephrotoxicity in different regimens of cancer chemotherapy. HealthMed. 2012; 6(6).*

17. *Swerdlow AJ, Higgins CD, Smith P, Cunningham D, Hancock BW, Horwich A, et al. Myocardial infarction mortality risk after treatment for Hodgkin disease: a collaborative British cohort study. Journal of the National Cancer Institute. 2007; 99(3): 206-14.*

18. *Galpin AJ, Evans WE. Therapeutic drug monitoring in cancer management. Clinical chemistry. 1993; 39(11): 2419-30.*

19. *Steinherz LJ, Graham T, Hurwitz R, Sondheimer HM, Schwartz RG, Shaffer EM, et al. Guidelines for cardiac monitoring of children during and after anthracycline therapy: report of the Cardiology Committee of the Childrens Cancer Study Group. Pediatrics. 1992; 89(5): 942-9.*

20. *Pavek P, Dvorak Z. Xenobiotic-induced transcriptional regulation of xenobiotic metabolizing enzymes of the cytochrome P450 superfamily in human extrahepatic tissues. Current drug metabolism. 2008; 9(2): 129-43.*

21. *Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacology & therapeutics. 2013.*

22. *Kivisto KT, Kroemer HK, Eichelbaum M. The role of human cytochrome P450 enzymes in the metabolism of anticancer agents: implications for drug interactions. British journal of clinical pharmacology. 1995; 40(6): 523-30.*

23. *Sturgill MG, Lambert GH. Xenobiotic-induced hepatotoxicity: mechanisms of liver injury and methods of monitoring hepatic function. Clinical chemistry. 1997; 43(8): 1512-26.*

24. *Maurel M, Rosenbaum J. Closing the gap on drug-induced liver injury. Hepatology. 2012; 56(2): 781-3.*

25. *Holt MP, Ju C. Mechanisms of drug-induced liver injury. The AAPS journal. 2006; 8(1): E48-E54.*

26. *Lucena MI, Andrade RJ, Kaplowitz N, García-Cortes M, Fernández MC, Romero-Gomez M, et al. Phenotypic characterization of idiosyncratic drug-induced liver injury: The influence of age and sex. Hepatology. 2009; 49(6): 2001-9.*

27. *Bailey WJ, Holder D, Patel H, Devlin P, Gonzalez RJ, Hamilton V, et al. A Performance Evaluation of Three Drug-Induced Liver Injury Biomarkers in the Rat: Alpha-Glutathione S-Transferase, Arginase 1, and 4-Hydroxyphenyl-Pyruvate Dioxygenase. Toxicological Sciences. 2012; 130(2): 229-44.*

28. *Lee WM. Drug-induced hepatotoxicity. New England Journal of Medicine. 2003; 349(5): 474-85.*

29. *Wysowski DK, Swartz L. Adverse drug event surveillance and drug withdrawals in the United States, 1969-2002: the importance of reporting suspected reactions. Archives of internal medicine. 2005; 165(12): 1363.*

30. *Goodman L. Goodman & Gilman's the pharmacological basis of therapeutics () McGraw-Hill. NY, USA. 2011.*

31. *Bauman JW, Antal JM, Adams LM, Johnson BM, Murray SC, Peng B, et al. Effect of hepatic or renal impairment on the pharmacokinetics of casopitant, a NK-1 receptor antagonist. Investigational new drugs. 2012; 30(2): 662-71.*

32. *Singh B, Oh TJ, Sohng JK. Exploration of two epimerase homologs in Streptomyces peucetius ATCC 27952. Applied microbiology and biotechnology. 2013: 1-10.*

33. *McEvoy GK. AHFS Drug Information. Bethesda, MD: American Society of Health-System Pharmacists. Inc; 2007.*

34. *Staff P. Physicians' Desk Reference 2008 Supplement A: PDR Network; 2008.*

35. *Rashid S, Ali N, Nafees S, Ahmad ST, Arjumand W, Hasan SK, et al. Alleviation of doxorubicin-induced nephrotoxicity and hepatotoxicity by chrysin in Wistar rats. Toxicology mechanisms and methods. 2013; 23(5): 337-45.*

36. *Injac R, Boskovic M, Perse M, Koprivec-Furlan E, Cerar A, Djordjevic A, et al. Acute doxorubicin nephrotoxicity in rats with malignant neoplasm can be successfully treated with fullerenol C60 (OH) 24 via suppression of oxidative stress. Pharmacological Reports. 2008; 60(5): 742.*

37. *Klasco R. DRUGDEX® System (electronic version). Thomson Micromedex. Greenwood Village, Colorado, USA Accessed via http: //www thomsonhc com on. 2009; 1: 06-9.*

38. *Kalender Y, Yel M, Kalender S. Doxorubicin hepatotoxicity and hepatic free radical metabolism in rats: the effects of vitamin E and catechin. Toxicology. 2005; 209(1): 39-45.*

39. *Batist G, Ramakrishnan G, Rao CS, Chandrasekharan A, Gutheil J, Guthrie T, et al. Reduced cardiotoxicity and preserved antitumor efficacy of liposome-encapsulated doxorubicin and cyclophosphamide compared with conventional doxorubicin and cyclophosphamide in a randomized, multicenter trial of metastatic breast cancer. Journal of Clinical Oncology. 2001; 19(5): 1444-54.*

40. *Fisher B, Brown AM, Dimitrov NV, Poisson R, Redmond C, Margolese RG, et al. Two months of doxorubicin-cyclophosphamide with and without interval reinduction therapy compared with 6 months of cyclophosphamide, methotrexate, and fluorouracil in positive-node breast cancer patients with tamoxifen-nonresponsive tumors: results from the National Surgical Adjuvant Breast and Bowel Project B-15. Journal of Clinical Oncology. 1990; 8(9): 1483-96.*

41. *Wills S, Reid R, II I, Margolis J, editors. Hepatotoxicity in breast cancer patients post-chemotherapy with docetaxel, doxorubucin, and cyclophosphamide. J Clin Oncol (Meeting Abstracts); 2010.*

42. *Subramaniam SR, Cader RA, Mohd R, Yen KW, Ghafor HA. Low-dose cyclophosphamide-induced acute hepatotoxicity. The American journal of case reports. 2013; 14: 345.*

43. *Catimel G, Chauvin F, Guastalla J, Rebattu P, Biron P, Clavelt M. FAC (fluorouracil, doxorubicin, cyclophosphamide) as second line chemotherapy in patients with metastatic breast cancer progressing under FEC (fluorouracil, epirubicin, cyclophosphamide) chemotherapy. Annals of oncology. 1994; 5(1): 95-7.*

44. *Meyer RM, Gospodarowicz MK, Connors JM, Pearcey RG, Wells WA, Winter JN, et al. ABVD alone versus radiation-based therapy in limited-stage Hodgkin's lymphoma. New England Journal of Medicine. 2012; 366(5): 399-408.*

45. *Jaeschke H, McGill MR. Serum Glutamate Dehydrogenase—Biomarker for Liver Cell Death or Mitochondrial Dysfunction? Toxicological Sciences. 2013.*

46. *Pratt WB. The anticancer drugs: Oxford University Press; 1994.*

47. *Albini A, Pennesi G, Donatelli F, Cammarota R, De Flora S, Noonan DM. Cardiotoxicity of anticancer drugs: the need for cardio-oncology and cardio-oncological prevention. Journal of the National Cancer Institute. 2010; 102(1): 14-25.*

48. *Raschi E, Vasina V, Ursino MG, Boriani G, Martoni A, De Ponti F. Anticancer drugs and cardiotoxicity: insights and perspectives in the era of targeted therapy. Pharmacology & therapeutics. 2010; 125(2): 196-218.*

49. *Pratt DS, Kaplan MM. Evaluation of abnormal liver-enzyme results in asymptomatic patients. New England Journal of Medicine. 2000; 342(17): 1266-71.*

50. *Levels a. Evaluation of abnormal liver-enzyme results in asymptomatic patients. 2000.*

51. *Sallie R, Michael Tredger J, Williams R. Drugs and the liver Part 1: Testing liver function. Biopharmaceutics & drug disposition. 1991; 12(4): 251-9.*

52. *Teleman M, Chee C, Earnest A, Wang Y. Hepatotoxicity of tuberculosis chemotherapy under general programme conditions in Singapore. The International Journal of Tuberculosis and Lung Disease. 2002; 6(8): 699-705.*

53. *Phillips KA, Veenstra DL, Oren E, Lee JK, Sadee W. Potential role of pharmacogenomics in reducing adverse drug reactions. JAMA: the journal of the American Medical Association. 2001; 286(18): 2270-9.*

54. *Mancinelli L, Cronin M, Sadée W. Pharmacogenomics: the promise of personalized medicine. Aaps Pharmsci. 2000; 2(1): 29-41.*

55. *Wojnowski L, Kulle B, Schirmer M, Schlüter G, Schmidt A, Rosenberger A, et al. NAD (P) H oxidase and multidrug resistance protein genetic polymorphisms are associated with doxorubicin-induced cardiotoxicity. Circulation. 2005; 112(24): 3754-62.*

56. *Wolf MB, Baynes JW. The anti-cancer drug, doxorubicin, causes oxidant stress-induced endothelial dysfunction. Biochimica et Biophysica Acta (BBA)-General Subjects. 2006; 1760(2): 267-71.*

57. *Fuchs-Tarlovsky V. Role of antioxidants in cancer therapy. Nutrition. 2012.*

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Molecular detection of *Staphylococcus aureus* in sinusitis samples

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Abstract

Background: *Staphylococcus aureus (S. aureus)* is the most virulent bacterium of that causes a wide spectrum of diseases including sinusitis. Sinusitis is defined as an inflammation of the paranasal sinuses and it can occur with bacterial infection. Since traditional diagnosis of sinusitis such as bacterial culture is time-consuming, there is an urgent need to implement a rapid and sensitive method to detect *S. aureus* sinusitis.

Objectives: In the present study, application of PCR in detection of *S. aureus* in sinusitis samples was evaluated.

Materials and Methods: Polymerase chain reaction (PCR) was performed with species-specific primers set to identify the *S. aureus nuc* gene. Also, by providing serial dilution and doing PCR on DNA extracted from different micro-organisms, sensitivity and specificity of the primers were evaluated respectively.

Results: The product of 279 bp length correctly amplified by optimized PCR and observed on 1.5% agarose gel electrophoreses. Evaluating selected primers in various PCR tests with DNA extracted from other micro-organisms demonstrated 100% specificity of PCR technique. Moreover, sensitivity of the test was 10 CFU of bacteria. According to our PCR analysis, 18 out of 55 studied specimens (32.7%) from patients with sinusitis were positive for *S. aureus*.

Conclusion: Current study showed that the used PCR method is a high specific, sensitive and reliable tool for rapid detection of *S.aureus*-derived sinusitis.

Key words: *Staphylococcus aureus*, sinusitis, PCR, detection, nuc gene.

1. Background

S. aureus is an omnipresent bacterium and the most virulent of staphylococcal species in the micrococcaceae family, and its versatility has been shown by remaininga main cause of morbidity and mortality in spite of theavailability of tremendous potent anti-staphylococcal antibiotics(1). The major habitat of *S. aureus* is warm-blooded animals, including nasal membrane and skin of humans(2) and causes a wide range of diseasesuch as nosocomial and community-based infections either by toxin-mediated or non–toxin-mediated mechanisms(3). It has been found that *S. aureus* consistently exist in cultures from patients with sinusitis (4). Sinusitis is inflammation, bacterial or viral infections, allergy, and autoimmune problems in paranasal sinuses. Sinusitis has two major types, acute sinusitis and chronic sinusitis.According to the sinus cavity including maxillary, frontal, ethmoidal and sphenoidal, sinusitis causes pain in different parts of head and face.Sinusitis is counted as a common condition, with between 24–31 million cases occurring in the United States annually(5,6). According to the high frequency of patients who suffer from sinusitis, using rapid and efficient technique plays crucial role in managing the disease.

Culture-based testing is the main andconventional method fordiagnosis of bacterial infections. The diagnostic results which rely on these cultivations are usually time-consuming. In addition, sometimes due to unsuitable culturing conditions and methods, scientists face failure in cultivating of bacteria under laboratory condition. Molecular methods based on nucleic acid amplification can be used to overcome these problems and reach more rapid and

sensitive detection(7). These effectiveness and sensitivity has made PCR as an attractive tool in diagnostic laboratories and leaded fast diagnosis of infectious diseases. PCR-based methodsenable the direct detection of low concentrations of bacteria or bacterial products in clinical materials (8,9).

One of the most important pathogenic factors of *S. aureus* is its thermo-stable nuclease. There are two open reading frames (ORFs) in *S. aureus* genomes which have been predicted to encode nucleases, one of which named *nuc* and produces an extracellular thermo-nuclease (TNase) with a molecular weight of 17KDa(1,9).It is an endonuclease, degrading both DNA and RNA of the host by hydrolyzing phosphodiester bonds (10). The enzymatic activity of TNase resists at 100'C for at least 1 h (11).The identification of *S. aureus* isolates is carried out in different laboratories by enzymatic test for TNase production(12). However, streptococci and probably other bacteriacan produce nucleases whose enzymatic activities are similar to *S. aureus* TNase (13). On the other hand, earlier studies have demonstrated that *S. aureus* TNase has species-specific sequences(14)and therefore amplifying this unique sequence of thermo-nuclease gene can provides rapidand sensitive method fordiagnosis of *S. aureus*. In this research, we used PCR method by the S.aureus nuc gene amplification, as a rapid, specific and sensitive method for diagnosis of *S. aureus* in sinusitis samples.

2. Objectives

In the present study, the PCR method as a rapid, sensitive and specific method to detection of *S.aureus* in sinusitis samples was evaluated.

3. Materials and Methods

3.1. Samples and DNA extraction

55 specimens werecollectedfrom the secretion of maxillary, frontal and sphenoidal sinuses in Rasool Akram hospital (Tehran-Iran) by endoscopic method. Genomic DNA was extracted by DNG-plus™ DNA extraction kit (Cinagen, Iran). For DNA extraction, 100 µl sample was mixed with 400 µl of DNG solution and was vortexed (20 sec). 350 µl isopropanol was added and mixed by inverting the tube (10 times) gently, and then centrifuged at room temperature (12000 rpm/5 min). Supernatant was gently removed, 1 ml 75% ethanol was added to it and mixed by inverting the tube (10 times) and centrifuged (12,000 rpm/5 min). The supernatant was removed again. The ethanol was totally poured off, and the pellet was dried (65 °C for 5 min). The DNA pellet was dissolved in 100 µl of double distilled water (DDW), or in Tris 10 mM (pH = 8) for long time storage in -20°C.

In addition, the genomic DNA from *S. aureus ATCC 25923*, as a standard strain and control positive, was extracted using DNG-plus™ DNA extraction kit (Cinagen, Iran). The lyophilized *S. aureus* ATCC 25923 were resolved in 5 ml nutrient broth and incubated in 37°C 24hrs. The cultures of *S. aureus ATCC 25923* which were at the late-logarithmic phase were chosen for DNA extraction.

3.2. Primers and PCR

To evaluate the presence or absence of the selected gene, *nuc*, all PCR tests were carried out in Thermal Cycler using the species-specific primers set.The amplification conditions and primers for *nuc* gene are shown in Table 1. The PCR mixture included 14 µl DDW, 2.5 µlbuffer (10X), 0.75 µl MgCl2, 0.5 µl dNTP, 1 µl of each primer (forward and revers), 0.3 µl Taq DNA polymerase enzyme and 5 µl template DNA. Amplified DNA segments were analyzed by 1.5% agarose gel electrophoresis containing cyber green dye and were observed by UV light and trans illuminator machine.

3.3. Sensitivity and specificity test for detection

For testing the sensitivity of PCR reaction, the extracted DNA concentration of *S. aureus* was obtained using spectrophotometer in OD=260.

Table 1. The Primers and Condition of PCR Analysis

Gene	Primers Sequence(5---> 3)	Conditions			
		Denaturation	Annealing	Extension	Cycle(No)
nuc	F:5′ GCGATTGATGGTGATACGGTT3′ R:5′AGCCAAGCCTTGACGAACTAAAGC3′	94°c- 30s	58°c- 30s	72°c- 40s	35

Genomic DNA copy numbers were determined based on Genome Copy Number (GCN) formula and the genome size of *S. aureus*. Serial dilution was then provided from the template having known genome number by Kokh method and PCR analysis was performed on each dilution. In addition, the specificity of PCR reaction was assessed by analyzing the obtained DNA from *Haemophilus influenza, Escherichia coli, Mycoplasma pneumonia, Herpes Simplex Virus, Pseudomonas aeruginosa, ,Brucella abortus, Legionella pneumophila* and *S. aureus* using PCR.

4. Results

4.1. Result of PCR test on sinusitis specimens

After DNA extraction of 55 specimens collected from pus and biopsy samples from patients with sinusitis, PCR test was performed and PCR products were analyzed by 1.5% agaros gel electrophoresis. A total of 18 out of 55 specimens (32.7%) could produce a 279 bp band related to *S. aureus nuc* gene (As shown in Figure 1).

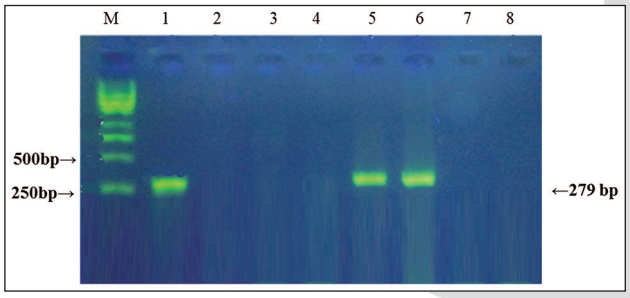

Figure 1. Gel electrophoresis of PCR products generated by direct testing of sinus specimens. M, Marker: 1 Kb DNA ladder;
lane 1: positive control (279 bp); lane 2 to 4 and 7, specimens which were not infected with S. aureus; lane 5 and 6, specimens infected with S. aureus; lane 8: negative control.

4.2. Result of sensitivity of PCR test

Assessing serial dilution of DNA extracted from*S. aureus* has shown that amplification of *nuc* gene could be performedeven by only 10 copy numbers of DNA using PCR. However, no band was observed after electrophoresis of PCR products obtained from dilutions with less than 10 copies of *S. aureus* DNA; and these results prove high sensitivity of PCR test (As shown in Figure 2).

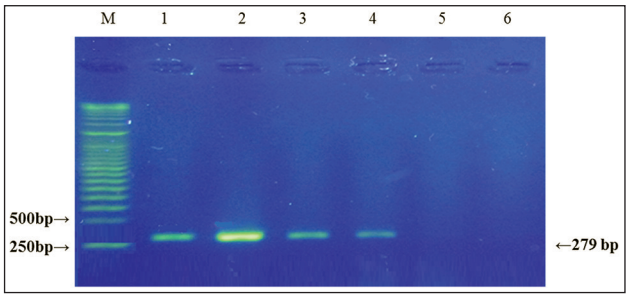

Figure 2. Gel electrophoresis of PCR products amplified from serial dilutions of S. aureus indicating sensitivity of PCR test. M, Marker: 1Kb DNA ladder (Fermentas , Germany);
lane 1-6, serial dilution of S. aureus DNA: lane1, positive control; lane 2: 10^{-1} dilution (10^{3} CFU); lane 3: 10^{-2} dilution (10^{2} CFU); lane 4: 10^{-3} dilution (10CFU); lane 5: 10^{-4} dilution (1 CFU); lane 6: negative control .

4.3. Result of specificity of PCR test

Specificity of the primers directed to the *nuc* gene was evaluated on different organisms including *Escherichia coli, Mycoplasma pneumonia,* Herpes Simplex Virus*, Haemophilus influenza, Pseudomonas aeruginosa*, *Legionella pneumophila* and *Brucella abortus*. The results of these assessments (As shown in Figure 3) have shown that *S. aureus* specific sequence was recognized and amplified by the utilized primer set, but none of the other micro-organisms were identified and amplified by the primers. Therefore, since only *S. aureus nuc* gene was amplified in this evaluation, primer specificity was shown to be 100%.

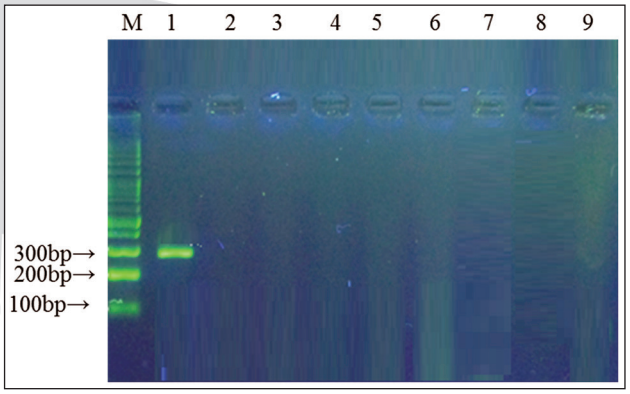

Figure 3. Specificity of PCR test. M, Marker: 100bp DNA ladder (Fermentas, Germany);
lane 1: positive control; lane 2: Haemophilus influenza DNA; lane 3: Herpes Simplex Virus DNA; lane 4: Mycoplasma pneumonia DNA; lane 5: Pseudomonas aeruginosa DNA; lane 6: Legionella pneumophila DNA; lane 7: Escherichia coli DNA; lane 8: Brucellaabortus DNA; lane 9: negative control.

5. Discussion

Classical methods such as bacterial culture and metabolic tests are widely used for bacterial identification in laboratories and hospitals (15). However, these processesare not ideal since they usually take days and in spite of great advances, some bacterialspecies grow with difficulty in culture media (16). Hence, several ultra-sensitive detection techniques have been developed to address these deficiencies(15). Among these high-sensitive techniques, there are efficient methods based on nucleic acid amplification, such as polymerase chain reaction (PCR) (17), strand displacement amplification (SDA) (18) and ligase chain reaction (LCR)(19).

Different studies have shown that PCR is a sensitive, rapid and reliable test (20,17) and therefore it has been used extensively in detection of different pathogenic and non-pathogenic micro-organisms such as viruses, bacteria, fungi, etc (21-23). The primary goal in this study was to implement PCR method as a dependable technique for detecting *S.aureus* in patients with sinusitis. To meet this goal, we used suitable primer set which identified species- specific sequences of *S. aureus nuc* gene encoding TNase. The *nuc* gene was amplified by our primers and amplification condition in sinus samples as well as *S. aureus ATCC 25923*, as a standard strain and control positive.

The sensitivity of *nuc* PCR test was 10 colony-forming unit (CFU) and could detect low levels (0.1pg) of extracted DNA. This sensitivity matches what described for PCR with other bacteria, being between 1 and 20 CFU (24,25) and is ten-fold more than that described for PCR with *S. aureus*, being between 1 and 100 pg DNA, in other studies (26,27).

The specificity of utilized primer was assessed by performing PCR on extracted DNA from different micro-organisms and it was shown that the designed primer set has 100% specificity for *S. aureus nuc* gene. Current findings are supported by *Brakstad et al*. who proved the potentiality of PCR test for the reliable detection of *S. aureus* infections(*9*)V. Atanassova et al. have also reported that specificity of PCR method in detection of *S.aureus* is more accurate in comparison to traditional methods (28,29). In addition, accumulating evidence has proved that PCR-based tests are more specific and sensitive than conventional method in detecting wide range of bacteria and micro-organism (30).

Frequency of detection *S.aureus* in sinusitis patients based on conventional method in world is different and wide range studies have reported that this amount is between 3% - 20% (31-33).According to our study, a total of 32.7%of collected samples could produce a 279 bp band related to *S. aureus nuc* gene.

In conclusion, the results of this study clearly demonstratesthat PCR technique based on species-specific sequences in *S. aureus nuc* gene, is a valuable method with high sensitivity, specificity and reliability for detecting *S. aureus* in patients with sinusitis.

References

1. *Tang J, Zhou R, Shi Z, Kang M, Wang H, Chen H. Two thermo stable nucleases coexisted in Staphylococcus aureus: evidence from mutagenesis and in vitro expression. FEMS Microbiol Lett 2008; 284(2): 176-183.*
2. *Chang W, Small DA, Toghrol F, Bentley WE. Global transcriptome analysis of Staphylococcus aureus response to hydrogen peroxide. J Bacteriol 2006; 188(4): 1648-1659.*
3. *Ashafa AO. Medicinal potential of Morella serata (Lam.) Killick (Myricaceae) root extracts: biological and pharmacological activities. BMC Complement Altern Med 2013; 13(1): 163.*
4. *Lin A, Busaba NY. Staphylococcus aureus and endoscopic sinus surgery. Curr Opin Otolaryngol Head Neck Surg 2006; 14(1): 19-22.*
5. *Anon JB. Upper respiratory infections.Am J Med 2010; 123 (4 Suppl): S16-25.*
6. *Mehle ME, Schreiber CP. Sinus headache, migraine, and the otolaryngologist. Otolaryngol Head Neck Surg 2005; 133(4): 489-496.*
7. *Jarvinen AK, Laakso S, Piiparinen P, Aittakorpi A, Lindfors M, et al. Rapid identification of bacterial pathogens using a PCR- and microarray-based assay. BMC Microbiol 2009; 9: 161.*
8. *Soejima T, Iida K, Qin T, Taniai H, Seki M, Yoshida S. Method to Detect Only Live Bacteria during PCR Amplification. J Clin Microbiol 2008; 46(7): 2305-2313.*
9. *Brakstad OG, Aasbakk K, Maeland JA.Detection of Staphylococcus aureus by polymerase chain reaction amplification of the nuc gene. J Clin Microbiol 1992; 30(7): 1654-1660.*
10. *Weber DJ, Mullen JP, Mildvan AS.Conformation of an enzyme-bound substrate of staphylococcal nucle-*

ase as determined by NMR. Biochemistry 1991; 30(30): 7425-7437.

11. *Chesneau O, Solh N. Primary structure and biological features of a thermostable nuclease isolated from Staphylococcus hyicus. Gene 1994; 145(1): 41-47.*
12. *Lachica RV, Genigeorgis C, Hoeprich PD. Metachromatic agar-diffusion methods for detecting staphylococcal nuclease activity. Appl Microbiol 1971; 21(4): 585-587.*
13. *Brakstad OG, Maeland JA. Detection of Staphylococcus aureus with biotinylated monoclonal antibodies directed against staphylococcal TNase complexed to avidin-peroxidase in a rapid sandwich enzyme-linked immunofiltration assay (sELIFA).J Med Microbiol 1993; 39(2): 128-134.*
14. *Gudding R. Differentiation of staphylococci on the basis of nuclease properties. J Clin Microbiol 1983; 18(5): 1098-1101.*
15. *Chang YC, Yang CY, Sun RL, Cheng YF, Kao WC, Yang PC. Rapid single cell detection of Staphylococcus aureus by aptamer-conjugated gold nanoparticles. Sci Rep 2013; 3: 1863.*
16. *Bolske G. Survey of Mycoplasma infections in cell cultures and a comparison of detection methods.Zentralbl Bakteriol Mikrobiol Hyg A 1988; 269(3): 331-340.*
17. *Cheng JC, Huang CL, Lin CC, Chen CC, Chang YC, Chang SS, Tseng CP. Rapid detection and identification of clinically important bacteria by high-resolution melting analysis after broad-range ribosomal RNA real-time PCR. Clin Chem 2006; 52(11): 1997-2004.*
18. *Edman CF, Mehta P, Press R, Sparg O, Walker GT, Nerenberg M. Pathogen analysis and genetic predisposition testing using microelectronic arrays and isothermal amplification J Investig Med 2000; 48(2): 93-101.*
19. *Moore DF, Curry JI. Detection and identification of Mycobacterium tuberculosis directly from sputum sediments by ligase chain reaction. J Clin Microbiol 1998; 36(4): 1028-1031.*
20. *Shahhosseiny MH, Hosseiny Z, Khoramkhorshid HR, Azari S, Shokrgozar MA. Rapid and sensitive detection of Mollicutes in cell culture by polymerase chain reaction. J Basic Microbiol 2010; 50(2): 171-178.*
21. *Yamamoto Y. PCR in diagnosis of infection: detection of bacteria in cerebrospinal fluids. Clin Diagn Lab Immunol 2002; 9(3): 508-514.*
22. *Clem AL, Sims J, Telang S, J. Eaton JW, Chesney J. Virus detection and identification using random multiplex (RT)-PCR with 3'-locked random primers. Virol J 2007; 4: 65.*
23. *Khot PD, Fredricks ND. PCR-based diagnosis of human fungal infections. Expert Rev Anti Infect Ther 2009; 7(10): 1201-1221.*
24. *Viitanen AM, Arstila TP, Lahesmaa R, Granfors K, Skurnik M, Toivanen P. Application of the polymerase chain reaction and immunofluorescence techniques to the detection of bacteria in Yersinia-triggered reactive arthritis. Arthritis Rheum 1991; 34(1): 89-96.*
25. *Olive DM. Detection of enterotoxigenic Escherichia coli after polymerase chain reaction amplification with a thermostable DNA polymerase. J Clin Microbiol 1989; 27(2): 261-265.*
26. *Wilson IG, Cooper GE, Gilmour E. Detection of enterotoxigenic Staphylococcus aureus in dried skimmed milk: use of the polymerase chain reaction for amplification and detection of staphylococcal enterotoxin genes entB and entC1 and the thermonuclease gene nuc. Appl Environ Microbiol 1991; 57(6): 1793-1798.*
27. *Johnson WM, Tyler SD, Ewan EP, Ashton FE, Pollard DR, Rozee KR. Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in Staphylococcus aureus by the polymerase chain reaction. J Clin Microbiol 1991; 29(3): 426-430.*
28. *Atanassova V, Meindl A, Ring C. Prevalence of Staphylococcus aureus and staphylococcal enterotoxins in raw pork and uncooked smoked ham-a comparison of classical culturing detection and RFLP-PCR.Int J Food Microbiol 2001; 68(1-2): 105-113.*
29. *Alarcon B, Vicedo B, Aznar R. PCR-based procedures for detection and quantification of Staphylococcus aureus and their application in food. J Appl Microbiol 2006; 100(2): 352-364.*
30. *Aly B. Hamad MS, Mohey M, Amen S. Polymerase Chain Reaction (PCR) Versus Bacterial Culture in Detection of Organisms in Otitis Media with Effusion (OME) in Children. Indian J Otolaryngol Head Neck Surg 2012; 64(1): 51-55.*
31. *Chan J, Hadley J. The microbiology of chronic rhinosinusitis: results of a community surveillance study. Ear Nose Throat J 2001; 80(3): 143-145.*
32. *Yildirim A, Oh C, Erdem H, Kunt T.Bacteriology in patients with chronic sinusitis who have been medically and surgically treated. Ear Nose Throat J 2004; 83(12): 836-838.*
33. *Brook I. Microbiology of Sinusitis. Proceedings of the American Thoracic Society 2011; 8(1): 90-100.*

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Evaluation of renal toxicity in chronic hepatitis B patients treated with tenofovir

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Abstract

Objective: To evaluate renal toxicity in chronic hepatitis B patients treated with tenofovir

Methods: A total of 54 patients (mean(SD) age: 47.2(13.8) years, 53.7% were males) diagnosed with chronic hepatitis B infection (CHB) and received tenofovir treatment for at least one year were included in this study based on retrospective evaluation of medical records of patients admitted to Department of Gastroenterology at Antalya Training and Research between 2008 and 2013. Data on patient demographics, co-morbid diseases and treatments, past history of renal diseases were collected along with fasting serum levels of creatinine, phosphate and calcium in each patient.

Results: When compared to baseline , no significant change in serum creatinine levels were determined during tenofovir treatment (0.7(0.12) for 1 year, 0.8(0.14) for 2 years and 0.9(0.17) for ≥ 3 years of treatment), while a clinically insignificant decrease occurred in serum phosphate levels (mg/dl) in parallel to duration of tenofovir treatment (from 3.5(0.5) to 3.3(0.4) for 1 year, 3.1(0.4) for 2 years and 2.8(0.4) for ≥ 3 years of treatment; $p<0.001$ for each).Serum creatinine levels were increased by 0.1 mg/dL in 17.0% of patients, hypophosphatemia (<2.5 mg/dl) was detected in 20.3%, while severe hypophosphatemia (<1 mg/dl) in none of patients.

Conclusion: Given the lack of a significant increase in serum creatinine levels regardless of the treatment duration, whereas a clinically insignificant decrease in phosphate levels in parallel to the treatment duration, our findings indicate tenofovir to be a safe therapeutic option among patients with CHB.

Key words: Renal complications; chronic hepatitis B; tenofovir treatment; safety; creatinine; phosphate

1. Introduction

Hepatitis B virus (HBV) has been associated with cirrhosis and hepatic failure and considered as the major cause of hepatocellular carcinoma (Lavanchy, 2004). Introduction of the HBV-polymerase inhibitors such as tenofovir disoproxil fumarate in the treatment of chronic hepatitis B (CHB) enabled increase in the proportion of treated patient by means of suppression of viral replication, restriction in the progression of liver disease and prevention of the onset of complications (Mauss et al., 2011; Si-Ahmed et al., 2011).

While the evidence on the convenience of tenofovir in patients with CHB as a once daily oral drug regimen has been reflected by the revision of the current treatment guidelines (Liaw et al., 2008, EASL, 2009), possible adverse events including renal function have gained more attention with increasing number of patients treated for years (Mauss et al., 2011).

Being actively accumulated in the proximal renal tubule via action of renal-specific organic anion transporters 1 (Cihlar et al., 2001), tenofovir has been associated with renal dysfunction with small but statistically significant changes in creatinine clearance (Gallant et al., 2006). Additionally, impairment of renal proximal tubular function under tenofovir therapy was reported leading to Fanconi or Fanconi-like syndrome with or without acute renal failure and characterized by decreased tubular handling of phosphate and the consequent hypophosphatemia (Badiou et al., 2006).

Although data in HIV infected patients revealed conflicting findings in terms of treatment tolerance and incidence of renal complications under tenofovir therapy, higher-than-normal levels of creatinine and less than-normal phosphorus levels in the blood

and anecdotal reports of kidney failure were reported in HIV patients under tenofovir treatment (Mauss et al., 2011, Wiercińska-Drapało et al., 2009).

Albeit much more limited, data from studies in HBV-monoinfected patients seem also inconsistent with data on a clear signal of renal impairment in the pivotal phase 3 studies in HBV-monoinfected patients (Marcellin et al., 2008, Heathcote et al., 2011), whereas no evidence of increased risk for renal- or bone-related complications as indicated in another study conducted in CHB patients with pre-existing mild renal impairment (Fung et al., 2013).

Given the limited data on the real incidence and prevalence of renal dysfunction, hypophosphatemia in relation to tubular reabsorption disorder in particular, among tenofovir-treated patients with CHB, the present study was designed to evaluate renal toxicity in CHB patients treated with tenofovir based on serum creatinine and phosphate levels measured within 1 to 5 years of follow up period.

2. Materials and Methods

2.1. Study population

A total of 54 patients (mean(SD) age: 47.2(13.8) years, 53.7% were males) diagnosed with CHB infection and received tenofovir treatment for at least one year were included in this study Data collection was based on retrospective evaluation of medical records of patients admitted to Department of Gastroenterology at Antalya Training and Research Hospital and attended to regular follow up visits from 2008 to 2013 including 1-year (n=14), 2-year (n=25) and ≥3 years (n=14) of treatment duration. Patients with a known kidney disease, under dialysis or receiving drug treatments effective on serum levels for calcium and phosphate were excluded from the study.

While the present study was exempt from the requirement of ethical approval in relation to its retrospective design, the permission was obtained from our institutional ethics committee for the use of patient data for publication purposes.

2.2. Study parameters

Data on patient demographics, co-morbid diseases and treatments, past history of renal diseases were collected along with fasting serum levels (mg/dl) of creatinine, phosphate, calcium and albumin in each patient. An increase of 0.5mg/dl in serum creatinine levels was considered significant. Based on serum levels for phosphate, patients were classified as normal (4.5-2.5 mg/dl) or with mild (2.5-2.0 mg/dl), moderate (2.0-1.0 mg/dl) or severe (<1.0 mg/dl) hypophosphatemia.

2.3. Statistical analysis

Statistical analysis was made using computer software (SPSS version 12.0, SPSS Inc. Chicago, IL, USA). Chi-square ($\chi 2$) test for the comparison of categorical data and Mann-Whitney U test was used for comparison of quantitative variables with non-normal distribution. Data were expressed as "mean (standard deviation; SD)", minimum-maximum and percent (%) where appropriate. $p<0.05$ was considered statistically significant

3. Results

3.1. Patient demographics, clinical and treatment related features

Males composed 53.7% of the study population and the mean(SD) age was 47.2(13.8) years with 9.3% (n=5) of patients aged ≥65 years. The percent of patients to receive tenofovir treatment only for one year was 27.8%, for 2 years was 46.3% and for ≥3 years was 25.9% (Table 1).

Co-morbid disease was evident in 51.9% of patients with hypertension alone in 20.4%. Concomitant treatment was identified in 44.4% with ACE inhibitor/Angiotensin receptor blocker treatment in 14.8% (Table 1).

3.2. Change in serum creatinine levels with respect to baseline

Baseline pre-treatment serum creatinine level was 0.8(0.15) mg/dl in the overall population with no significant change in serum creatinine levels during tenofovir treatment compared to baseline (0.7(0.12) for 1 year, 0.8(0.14) for 2 years and 0.9(0.17) for ≥ 3 years of treatment). Serum creatinine levels were determined to increase by 0.1 mg/dl in 17.0% of overall study population while none of patients had an increase in creatinine levels by 0.5mg/dl under tenofovir treatment (Table 2).

Table 1. Patient demographics, clinical and treatment related features

Age (years)	Mean(SD)		47.2(13.8)
	Median(min-max)		47.5(21-74)
	21-40years	n(%)	20(37.5)
	41-50 years		21(38.5)
	51-65years		8(14.8)
	≥65 years		5(9.3)
Gender			n(%)
Male			29(53.7)
Female			25(46.3)
Duration of tenofovir treatment			n(%)
1 year			15(27.8)
2 years			25(46.3)
≥3 years			14(25.9)
Duration of follow up (years)		Mean(SD)	2.0(0.7)
		Median(min-max)	1.98(1-5)
Co-morbid diseases			n(%)
Absent			26(48.1)
Present			28(51.9)
Hypertension			11(20.4)
Diabetes mellitus			6(11.1)
Hypertension + diabetes mellitus			3(5.6)
Concomitant treatment			n(%)
Absent			30(55.6)
Present			24(44.4)
ACE inhibitor/Angiotensin receptor blocker			8(14.8)
Lamivudin			7(12.9)
Calcium channel blocker-beta blocker			5(9.2)

Table 2. Change in serum creatinine levels (mg/dl) with respect to duration of tenofovir treatment

	Mean(SD)	**p value[1]** *vs. baseline*	**Increase by**				
			0.1 mg/dl	**0.2 mg/dl**	**0.3 mg/dl**	**0.4 mg/dl**	**0.5 mg/dl**
Baseline (n=54)	0.8(0.15)	-	-	-	-	-	-
1 year (n=15)	0.7(0.12)	p=0.121	4(7.4)	1(1.9)	-	1(1.9)	-
2 years (n=25)	0.8(0.14)	p=0.442	12(48.0)	-	1(4.0)	-	-
≥ 3 years (n=14)	0.9(0.17)	p=0.055	3(21.4)	-	-	-	-
Total			**19(17.0)**	**1(1.9)**	**1(1.9)**	**1(1.9)**	**-**

Table 3. Change in serum phosphate levels with respect to duration of tenofovir treatment

	Mean (SD)	**p value** *vs. baseline*	**Hypophosphatemia**			
			Overall (<2.5 mg/dl)	**Mild (2.5-2.0 mg/dl)**	**Moderate (2.0-1.0 mg/dl)**	**Severe (<1.0 mg /dl)**
Baseline (n=54)	3.5(0.5)	-				
1-year (n=15)	3.3(0.4)	p<0.001	2(3.7)	2(3.7)	-	-
2 years (n=25)	3.1(0.4)	p<0.001	5(12.8)	5(12.8)	-	-
≥3 years (n=14)	2.8(0.4)	p<0.001	4(28.8)	3(21.4)	1(7.1)	-
Total			11(20.3)	10(18.5)	1(1.9)	-

3.3. Change in serum phosphate levels during the entire study period

Baseline pre-treatment serum phosphate level was 3.5(0.5) mg/dl in the overall population. There was a significant gradual decrease in serum phosphate levels in parallel to duration of tenofovir treatment ((3.3(0.4) for 1 year, 3.1(0.4) for 2 years and 2.8(0.4) for ≥ 3 years of treatment; p<0.001 for each).

Hypophosphatemia (<2.5 mg/dl) was evident in 20.3% of patients within the entire 5-year study period, while none of patients was diagnosed with severe hypophosphatemia (<1 mg/dl) during the study (Table 3).

3.4. Duration of tenofovir treatment and laboratory findings with respect to change in serum phosphate levels

Pre-treatment levels for serum creatinine, albumin and calcium were similar in patients with increased or decreased serum levels for phosphate. Considering tenofovir treatment for 1, 2, and ≥3 years, decrease in serum phosphate levels was noted in significantly higher percentage of patients in each group compared to patients with increased serum phosphate levels (p<0.05 for each) (Table 4).

4. Discussion

Our findings related to renal toxicity in CHB patients treated with tenofovir revealed no significant change in serum creatinine levels as compared with pre-treatment levels regardless of the treatment duration. While increase in serum creatinine levels by 0.1 mg/dl was shown in 17.0% during the entire follow up period, none of patients was shown to have an increase in creatinine levels by 0.5mg/dl under tenofovir treatment. Likewise while a gradual decrease in serum phosphate levels was observed from baseline to 1, 2, and 3 years of tenofovir treatment, hypophosphatemia (<2.5 mg/dl) was noted only in 20.3% and severe hypophosphatemia (<1.0 mg /dl) in none of patients during the entire follow up period.

In relation to active accumulation of tenofovir in the proximal renal tubule via the action of renal-specific organic anion transporters 1 (Cihlar et al., 2001), renal dysfunction with small (6–10 ml/min) but statistically significant changes in creatinine clearance was reported under tenofovir therapy (Gallant et al., 2006).

Although a marked renal impairment was not reported amongst the general effects of antiviral therapy in controlled prospective studies with a variety of HBV-polymerase inhibitors including tenofovir (Mauss et al., 2011, Marcellin et al., 2008, Heathcote et al., 2011, a decline in renal function under tenofovir therapy was also noted (Wiercińska-Drapało et al., 2009).

Our findings related to increase in creatinine levels only by 0.1 mg/dl in 17.0%, up to 0.4mg/dl in only three patients and beyond 0.5 mg/dl in none of patients correspond well with the published data on good tolerance and low incidence of renal complications of tenofovir therapy (Mauss et al., 2011; Wiercińska-Drapało et al., 2009).

Indeed, use of HBV-polymerase therapy in patients with renal impairment or in combination with potentially nephrotoxic drugs has been considered likely to result in renal impairment in some

Table 4. Duration of tenofovir treatment and laboratory findings with respect to change in serum phosphate levels

	Serum phosphate level		p value
	Increased	Decreased	
Pretreatment levels	Mean(SD)		0.389
Creatinine (mg/dl)	0.8(0.1)	0.8(0.0)	0.319
Albumin (mg/dl)	4.1(0.1)	4.2(0.1)	0.836
Calcium (mg/dl)	9.2(0.2)	9.3(0.1)	
Tenofovir treatment	n(%)		
1 year (n=15)	5(33.3)	10(66.7)	**0.021**
2 years (n=25)	1(4.0)	24(96.0)	**0.031**
≥3 years (n=14)	1(7.1)	13(92.9)	**0.034**

patients in particular with pre-existing co-morbidities (Ha et al., 2009).

Exclusion of patients with concomitant renal disease and drug treatment that likely to affect serum levels of calcium and phosphate in the present study seems notable given that tenofovir related changes in serum have been usually considered to be clinically significant in the presence of normal renal function at baseline, whereas may become remarkable in case of concomitant renal disease (Hawkins, 2010). Nevertheless, given the rate of other concomitant diseases (51.9%) and treatments (44.4%) in our study population, lack of remarkable changes in serum creatinine and phosphate levels in the overall population and even in our patients who were under concomitant antihypertensive treatment with ACE/ARB medications (n=8) is worth noting. It should also be noted that no significant difference was determined in our study population between patients with increased or decreased serum levels for phosphate in terms of pre-treatment levels for serum creatinine, albumin and calcium.

Sustained HBV suppression and a favorable safety profile has been reported through 6 years of tenofovir therapy, while data in CHB patients with mild renal impairment has been emphasized to be scarce as they are excluded from most trials (Fung et al., 2013). However, in a past study concerning safety and tolerability of TDF in CBH patients with pre-existing mild renal impairment, patients with mild renal impairment and normal renal functions were shown to be similar with respect to safety and efficacy of tenofovir treatment with no evidence of increased risk for renal- or bone-related complications (Fung et al., 2013).

Age, gender, height, weight, concomitant disease and treatments and methods to assess renal function in individuals with normal or abnormal kidney function have been implicated in the documented variability related to tenofovir related renal toxicity (Mauss et al., 2011; Wiercińska-Drapało et al., 2009). Therefore, all of these factors have been recommended to be considered before and during tenofovir treatment to ensure the patients best possible treatment (Wiercińska-Drapało et al., 2009).

Tenofovir has been involved in tubulopathy leading to Fanconi or Fanconi-like syndrome with or without acute renal failure (Badiou et al., 2006) with hypophosphatemia reported in 32% of patients treated by antiretroviral therapy in a recent study (Day et al., 2005). Data on the proximal tubular reabsorption of phosphate in HIV-positive adults receiving a tenofovir containing regimen revealed hypophosphatemia (26.0%) and decreased proximal tubular reabsorption (47.0%) to be frequently observed (Badiou et al., 2006). However, a similar prevalence of these disorders (31% and 41%) was also reported in treatment experienced patients before introduction of tenofovir with no worsened effect 6 months after starting tenofovir (Badiou et al., 2006). Likewise, in naive CHB patients treated with tenofovir, hyperphosphaturia was reported to affect approximately one third of untreated patients and worsens in approximately one quarter of naïve patients treated with tenofovir, yet without causing significant hypophosphatemia or clinical consequences (Viganò et al., 2013). Accordingly, hypophosphatemia (<2.5 mg/dl) was evident in 20.3% of patients and more commonly in patients with longer duration of tenofovir treatment in the present study, while none of patients was diagnosed with severe hypophosphatemia during the tenofovir treatment.

Consistent with the recently reported independent association between hypophosphatemia and duration of highly active antiretroviral therapy (Jones et al., 2004), the likelihood of tenofovir related hypophosphatemia was positively associated with the duration of treatment in our study population. In this regard, our findings emphasize the need for longer term monitoring of proximal tubular function in larger scale studies (Viganò et al., 2013).

Tenofovir-induced toxicity was recently described in case reports to include tubulopathy, Fanconi syndrome and/or acute renal failure (Badiou et al., 2006). However, demonstration of mild and asymptomatic hypophosphatemia in 20.3% of patients under tenofovir therapy in the present study is in agreement with past studies indicated moderate and asymptomatic hypophosphatemia in HIV-positive patients under tenofovir therapy (Day et al., 2005, Izzedine et al., 2004, 2005) and renal safety both in treatment experienced (Izzedine et al., 2004) or treatment-naïve patients (Izzedine et al., 2005).

Indeed severe renal tubular dysfunction has been considered as an uncommon and reversible adverse effect of tenofovir-containing regimen (Peyriere et al., 2004; Gaspar et al., 2004; Malik et al., 2005). Moreover, even in cases with severe and symptomatic hypophosphatemia associated with a decreased creatinine clearance, an improvement of renal function was noted after discontinuation of tenofovir (Izzedine et al., 2004; Peyriere et al., 2004; Malik et al., 2005).

In an analysis of data from more than 5000 patients in the Tenofovir Expanded Access Program, grade 3 or 4 abnormalities in serum creatinine or phosphorus were observed only in 0.3% and 0.6% of patients, respectively (Gallais et al., 2004). Hence, given that serum creatinine levels increased by 0.1 mg/dl in 17.0% and by 0.5 mg/dl in none of our patients along with mild and asymptomatic hypophosphatemia in 20.3%, our findings correlate well with past clinical cohort studies indicating little evidence of nephrotoxicity associated with tenofovir (Wiercińska-Drapało et al., 2009;Gallais et al., 2004).

Alike to case in HIV positive adults receiving a tenofovir containing regimen (8), data on the tenofovir related renal toxicity and proximal tubular reabsorption of phosphate in HBV patients are limited and mostly based on short term retrospective studies with different criteria for evaluation of renal function and toxicity (EASL, 2009; Peyriere et al., 2004; Duarte-Rojo and Heathcote, 2010; Khungar and Han, 2010; Herlitz et al., 2010).

Accordingly, a renal tubular surveillance including phosphatemia, proteinuria and glycosuria as suggested in HIV-positive patients receiving tenofovir (Badiou et al., 2006) seems also to be of interest in HBV patients under tenofovir therapy to determine and avoid the risk of serious renal failure (De la Prada et al., 2006).

Based on our findings, we recommend evaluation of creatinine clearance in every patient prior to tenofovir treatment. If tenofovir is to be used in the presence of impaired renal function (creatinine clearance <50 ml/min), then it seems important to follow the prescribing guidelines with monitoring for renal function (Hawkins, 2010) based on measurement of creatinine clearance and serum phosphate levels monthly within the first year of therapy and then once every three months with consideration of every-other-day therapy in case of severe hypophosphatemia (EASL, 2009; Peyriere et al., 2004; Duarte-Rojo and Heathcote, 2010; Khungar and Han, 2010; Herlitz et al., 2010).

Nevertheless, in case of severe renal dysfunction (creatinine clearance <30 ml/min) and concomitant use of nephrotoxic drugs, tenofovir seems not to be a reasonable choice given the increased risk of adverse reactions.

5. Conclusions

In conclusion, given the lack of a significant increase in serum creatinine levels under tenofovir therapy regardless of the treatment duration, whereas a clinically insignificant decrease in phosphate levels in parallel to the treatment duration, our findings indicate tenofovir to be a safe therapeutic option among patients with CHB. However, based on the demonstration of 0.1 mg/dl increase in serum creatinine levels in 17.0% and mild-asymptomatic hypophosphatemia in 20.3% of our patients along with the likelihood of an association between tenofovir related hypophosphatemia and the duration of treatment, our findings emphasize the importance of adherence to prescribing guidelines with monitoring for renal function and to conduct longer term monitoring of proximal tubular function among larger numbers of tenofovir recipients.

References

1. *Badiou S, De Boever CM, Terrier N, Baillat V, Cristol JP, Reynes J. Is tenofovir involved in hypophosphatemia and decrease of tubular phosphate reabsorption in HIV-positive adults? J. Infect. 2006; 52: 335-338.*

2. *Cihlar T, Ho ES, Lin DC, Mulato AS. Human renal organic anion transporter 1 (hOAT1) and its role in the nephrotoxicity of antiviral nucleotide analogs. Nucleosides Nucleotides Nucleic Acids. 2001; 20: 641-648.*

3. *Day SL, Date HA, Bannister A, Hankins M, Fisher M. Serum hypophosphatemia in tenofovir disoproxil fumarate recipients is multifactorial in origin, questioning the utility of its monitoring in clinical practice. J. Acquir. Immune Defic. Syndr. 2005; 38: 301-304.*

4. *De la Prada FJ, Prados AM, Tugores A, Uriol M, Saus C, Morey A. Acute renal failure and proximal*

renal tubular dysfunction in a patient with acquired immunodeficiency syndrome treated with tenofovir. Nefrología 2006; 26: 626-630.

5. *Duarte-Rojo A, Heathcote EJ, 2010. Efficacy and safety of tenofovir disoproxil fumarate in patients with chronic hepatitis B. Therap. Adv. Gastroenterol. 2010; 3: 107-119.*

6. *EASL Clinical Practice Guidelines: Management of chronic hepatitis B., 2009. European Association for the Study of the Liver. J. Hepatol. 2009; 50: 227-242.*

7. *Fung S, Kwan P, Horban A, Pelemis M, Husa P, et al. Tenofovir DF (TDF) is safe and well tolerated in chronic hepatitis B (CHB) patients with pre-existing mild renal impairment. J. Hepatol. 2013; 58(Suppl 1): S301-S302.*

8. *Gallais H, Lazzarin A, Adam A, et al. The Viread Expanded Access Program (EAP) in Europe/Australia: summary of the safety and efficacy of tenofovir disoproxil fumarate (TDF) in antiretroviral treatment (ART) experience patients. Proceedings of the XV International AIDS Conference; July 11-16; Bangkok, Thailand. Abstract TuPeB4552. 2004.*

9. *Gallant JE, DeJesus E, Arribas JR, Pozniak AL, Gazzard B, et al. Study 934 Group., 2006. Tenofovir DF, emtricitabine, and efavirenz vs. zidovudine, lamivudine, and efavirenz for HIV. N. Engl. J. Med. 2006; 354: 251-260.*

10. *Gaspar G, Monereo A, Garcia-Reyne A, de Guzman M. Fanconi syndrome and acute renal failure in a patient treated with tenofovir: A call for caution. AIDS 2004; 18: 351-352.*

11. *Goicoechea M, Liu S, Best B, Sun S, Jain S, et al. California Collaborative Treatment Group 578 Team., 2008. Greater tenofovir-associated renal function decline with protease inhibitor-based versus nonnucleoside reverse-transcriptase inhibitor-based therapy. J. Infect. Dis. 2008; 197: 102-108.*

12. *Ha NB, Ha NB, Garcia RT, Trinh HN, Vu AA, et al. Renal dysfunction in chronic hepatitis B patients treated with adefovir dipivoxil. Hepatology 2009; 50: 727-734.*

13. *Hawkins T. Understanding and managing the adverse effects of antiretroviral therapy. Antiviral Res. 2010; 85: 201-209. Review.*

14. *Heathcote EJ, Marcellin P, Buti M, Gane E, De Man RA, et al. Three year efficacy and safety of tenofovir disoproxil fumarate treatment for chronic hepatitis B. Gastroenterology 2011; 140: 132-143.*

15. *Herlitz LC, Mohan S, Stokes MB, Radhakrishnan J, D'Agati VD, Markowitz GS. Tenofovir nephrotoxicity: acute tubular necrosis with distinctive clinical, pathological, and mitochondrial abnormalities. Kidney Int. 2010; 78: 1171-1177.*

16. *Izzedine H, Hulot JS, Vittecoq D, Gallant JE, Staszewski S, et al. Long-term renal safety of tenofovir disoproxil fumarate in antiretroviral-naive HIV-1-infected patients. Data from a double-blind randomized active controlled multicentre study. Nephrol Dial Transplant 2005; 20: 743-746.*

17. *Izzedine H, Isnard-Bagnis C, Hulot JS, Vittecoq D, Cheng A, et al. Renal safety of tenofovir in HIV treatment experienced patients. AIDS 2004; 18: 1074–6.*

18. *Jones R, Stebbing J, Nelson M, Moyle G, Bower M, Mandalia S, Gazzard B. Renal dysfunction with tenofovir disoproxil fumarate-containing highly active antiretroviral therapy regimens is not observed more frequently: A cohort and case–control study. J Acquir Immune Defic Syndr; 2004; 37: 1489-1495.*

19. *Khungar V, Han SH. A systematic review of side effects of nucleoside and nucleotide drugs used for treatment of chronic hepatitis B. Curr. Hepat. Rep. 2010; 9: 75-90.*

20. *Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J. Viral Hepat. 2004; 11: 97-107.*

21. *Liaw YF, Leung N, Kao JH, Piratvisuth T, Gane E, et al. Chronic Hepatitis B Guideline Working Party of the Asian-Pacific Association for the Study of the Liver., 2008. Asian–Pacific consensus statement on the management of chronic hepatitis B: a 2008 update. Hepatol Int. 2008; 2: 263-283.*

22. *Malik A, Abraham P, Malik N. Acute renal failure and Fanconi syndrome in an AIDS patient on tenofovir treatment—case report and review of literature. J. Infect. 2005; 51: E61-65.*

23. *Marcellin P, Heathcote EJ, Buti M, Gane E, de Man RA, et al. Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. N. Engl. J. Med. 2008; 359: 2442-2455.*

24. *Mauss S, Berger F, Filmann N, Hueppe D, Henke J, et al. Effect of HBV polymerase inhibitors on renal function in patients with chronic hepatitis B. J. Hepatol. 2011; 55: 1235-1240.*

25. *Peyriere H, Reynes J, Rouanet I, Daniel N, de Boever CM, et al. Renal tubular dysfunction associated with tenofovir therapy: Report of 7 cases. J. Acquir. Immune. Defic. Syndr. 2004; 35: 269-273.*

26. *Si-Ahmed SN, Pradat P, Zoutendijk R, Buti M, Mallet V, et al. Efficacy and tolerance of a combination of tenofovir disoproxil fumarate plus emtricitabine in patients with chronic hepatitis B: a European multicenter study. Antiviral Res. 2011; 92: 90-95.*

27. *Viganò M, Lampertico P, Mangia G, Soffredini R, Facchetti F, et al. Incidence and clinical consequences of reduced tubular phosphate re-absorption (TMPO4/GFR) in naïve chronic hepatitis B patients treated with tenofovir: a two-year field study J. Hepatol. 2013; 58 (Suppl 1): S316-S317.*

28. *Wiercińska-Drapało A, Mikuła T, Winiarczyk M, Sikora A. Nephrotoxicity of tenofovir true or myth? HIV AIDS Rev. 2009; 8: 17–20.*

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Intestinal anastomosis and diclofenac in rats counteracted by pentadecapeptide BPC 157

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Abstract

Although diclofenac application is considered to be postoperative rescue analgesia, the results on intestinal anastomosis healing are inconclusive. It is important to verify the very early post-surgical diclofenac administration along with the combined and mutually potentiated outcome in rats. Vice versa, a prompt reversal should be with stable anti-ulcer gastric pentadecapeptide BPC 157, proposed to be an antidote of NSAIDs toxicity that particularly counteracts diclofenac-toxicity and improves intestinal anastomosis healing. Macroscopical assessment, histological examination, and biomechanical testing were used to evaluate stomach, small intestine, large intestine and liver lesions, sphincter failure, anastomosis healing at 1, 3, 5, 7, 9 and 14 days after ileoileal anastomosis and diclofenac administration (6.25 mg/kg intraperitoneally immediately after anastomosis creation). As an antidote after anastomosis and after diclofenac, we applied the stable gastric pentadecapeptide BPC 157 (10 µg/kg, 10 ng/kg): (i) intraperitoneally, once every day, with the first application 30 min after surgery, and the final application at 24 h before sacrifice or (ii) in drinking water (0.16 µg/mL, 0.16 ng/mL) until the end of the experiment.

Indicative were progressive anastomosis dehiscence and loss of anastomotic strength, the leading diclofenac ulcerogenic effect that expands invading the stomach, small and large intestine, lower esophageal and pyloric sphincter failure; progressing intestinal wound healing inability (i.e., adhesion presentation mostly with neighboring small intestine loops also included, stomach, liver ―packed ‖ , advanced intestinal passage obstruction, poor blood filling of the anastomosis arcade vessels, presenting vessels empty); and increasing hepatotoxicity. Specifically, when any of regimens of BPC 157 was given, the much smaller number of mucosal lesions in all of regions, preserved sphincter function, advanced anastomosis healing (only small adhesions presentation, no passage obstruction, increased anastomotic strength exhibited no anastomotic dehiscence, preserved blood filling of the anastomosis arcade vessels, all intestine wall layers more vigorously adapt) and liver lesions recovery since very beginning (i.e., a higher number of binucleated hepatocytes, less fatty liver, no necrosis).

Therefore, it seems that BPC 157 combines the activated mucosal protection, wound (anastomosis) healing and hepatoprotection, which all together have an effect against combined intestinal anastomosis with diclofenac-toxicity.

Key words: Intestinal anastomosis, diclofenac, BPC 157

1. Introduction

Regardless their possible limitation due to their side effects, non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used analgesics for minor surgery and are useful adjunctive analgesics in patients undergoing major surgery, including abdominal surgery as well [1-3].

Thereby, with stable gastric pentadecapeptide BPC 157, we further challenge diclofenac, a NSAID associated with adverse drug reactions in humans, increased number of clinically significant anastomotic leakages in patients, and in particular, the still inconclusive results of diclofenac application on anastomosis healing [4-9].

Here, we attempt to resolve this apparent inconsistency in intestinal anastomosis/diclofenac search (i.e., no adverse effect [6] vs. expected greater fatality risk than diclofenac application or intestinal anastomosis *per se* (apparent worsening, i.e., anastomotic dehiscence followed by peritoni-

tis and death)[7]). Thus, advanced diclofenac toxicity (marked stomach-, intestinal-and liver-lesions [8] and sphincteric failure [10]) and poor healing of intestinal anastomosis [9] were together combined in rat. Diclofenac application was early after surgery and anastomosis creation.

To solve this complex combined diclofenac/intestinal anastomosis issue, BPC 157, given parenterally or perorally, is particularly interesting since it improves intestinal anastomosis healing [9,11], and especially, since therapy with BPC 157 (for review see, [12-16]) is recently proposed to be an antidote of NSAIDs toxicity [16]. Pentadecapeptide BPC 157 is an original anti-ulcer peptide (GEPPPGKPADDAGLV, M.W. 1419, PL-10, PLD-116, PL 14736) since stable in human gastric juice more than 24 hours, in trials for inflammatory bowel disease [12-16], wound treatment (i.e., stimulating the expression of the early growth response 1 (egr-1) gene and egr-1 repressor nerve growth factor 1-A binding protein-2 (nab2)) [17], no toxicity or side effects reported, LD1 could be not achieved, effective alone without carrier [for review see, i.e., [12-16]], particularly interacting with NO-system [18-24]. Also, its particular interaction with NSAIDs [8,25-28] implies it`s special role in cytoprotection as a novel mediator of Robert's cytoprotection [13], and thereby, exhibiting special counteracting potential for different NSAIDs and different NSAIDs lesions (i.e., gastrointestinal tract-, liver- and brain- lesions [8,25-27], and finally, counteraction of aspirin-prolonged bleeding and thrombocytopenia[28]).

2. Materials and Methods

We used male Albino Wistar rats kept under normal conditions, weighing from 250 to 280 g, at least 10 rats per experimental group and period, randomly assigned for all experiments, approved by the Local Ethics Committee.

2.1. Surgery

Under deep anesthesia, as described before [9], the peritoneal cavity was entered through a 4-cm midline incision, the ileum divided above 10 cm from the ileocecal valve, and a single-layer ileoileal anastomosis performed with 7-0 polypropylene (Prolene; Ethicon, Hamburg, Germany) continuous sutures; the abdominal incision was closed with 3-0 silk sutures. The evaluation (at 1, 3, 5, 7, 9 and 14 days after ileoileal anastomosis) included a macroscopical assessment, histological examination, biochemical, and biomechanical testing as described before.

2.2. Drugs

Medication, without carrier or peptidase inhibitor, included pentadecapeptide BPC 157 (a partial sequence of the human gastric juice protein BPC, freely soluble in water at pH 7.0 and in saline). It was prepared as a peptide with 99% (HPLC) purity (1-des-Gly peptide was the main impurity; manufactured by Diagen, Ljubljana, Slovenia, GEPPPGKPADDAGLV, M.W. 1419).

Diclofenac (Voltaren®, Pliva) was prepared as described before [8]. Diclofenac (6.25 mg/kg) was given intraperitoneally once immediately after anastomosis creation. As an antidote after anastomosis and after diclofenac, we applied the stable gastric pentadecapeptide BPC 157 (10 μg/kg, 10 ng/kg) as follows: (i) intraperitoneally, once every day, with the first application 30 minutes after surgery, and the final application at 24 h before sacrifice or (ii) in drinking water (0.16 μg/mL, 0.16 ng/mL) until the end of the experiment while corresponding controls received at the same time (i) an equivolume of saline (5ml/kg i.p.) or (ii) drinking water only (12ml/rat/day).

2.3. Anastomosis assessment

In general, the macroscopy and microscopy assessment was carried out as described before [9]. Briefly, adhesion presentation [9] was scored 0–7: 0, no adhesion; 1, thin adhesions covering less than one half of anastomosis; 2, more prominent adhesions with more than half of anastomosis; 3, exaggerated adhesions with whole anastomosis; 4, the mesenterial part of small bowel also included; 5 neighboring small intestine loop also included; 6, many neighboring small intestine loops included; 7, neighboring loops, stomach, liver —packed. Intestinal passage obstruction [6] was scored 0–3, according to the loop diameters ratio close to anastomosis. In other words, if loop diameters at 2 cm

orally/loop diameters at 2 cm aborally = 1 passage is normal (score 0), between 1 and 1.33 is the sign of mild obstruction (score 1), between 1.33 and 1.66 is moderate obstruction (score 2), and more than 1.66 is severe obstruction (score 3). Blood filling the anastomosis arcade vessels (between 10 cm orally and 10 cm aborally) [6] was scored 0–2: 0, vessels completely filled with blood; 1, vessels only partly filled; 2, vessels empty.

For microscopy assessment, the tissue specimens were immediately fixed in buffered formalin (pH 7.4), for 24 h, dehydrated, and embedded in paraffin wax. The samples were stained with hematoxylin–eosin (for the evaluation of epithelization, necrosis, edema, granulation tissue, and inflammatory cells), Gommori (for reticulin), Giemsa (for collagen), or immunohistochemically for desmin (Dako, Glostrup, Denmark) (newly formed muscle) and were examined in a blinded fashion. For the morphometrical analysis special software programs SFORM and ISSA (VAMSTEC, Zagreb, Croatia) were used. Five high powerfields were randomly selected for the analysis. We assessed the number of rats with progressing epithelization (surface epithelium regeneration present), or with newly formed muscle unlike the muscularis (both mucosae and propriae) abrupt ending. We determined the scores for edema [i.e., none (0); gap not exceeding one third or one thickness of muscle layer area (1); gap exceeding one thickness, but not two thicknesses of muscle layer area (2); gap two thicknesses of the muscle layer area (3). Necrosis and granulation tissue formation were scored as follows: none (0); presentation less than 20% of the anastomosis area (1); presentation between 20% and 60% of the anastomosis area (2); presentation more than 60% of the anastomosis area (3). Morphometrical analyses were used to determine the total number of inflammatory cells, and reticulin and collagen presentation (expressed as a percentage of total area).

To assess adaptation of small intestine, as described previously [9,11] (portion 3 cm proximal to 3 cm distal to the anastomosis), villus height (from villus base to villus tip), crypt depth (from crypt base to villus base), and muscle thickness (inner, circular, and outer, longitudinal muscular layer) were measured in blinded fashion using an objective mounted micrometer at 200x magnification and an optical microscope at 10x100 magnification. Measured data for mucosal height (villus height and depth) and muscle thickness (inner, circular, and outer, longitudinal, muscular layer) are averages of eight measurements for each animal. Accordingly with previous findings [11] villus height 307.4±13μm, crypt depth 145.2±14.7μm, and muscle thickness (inner, circular 22.6±5.8μm, and outer, longitudinal muscular 12.5±4.6μmlayer) were considered as normal.

2.4. Biomechanics

Anastomosis dehiscence was biomechanically assessed in separate groups of animals, under deep anesthesia. We studied the volume (ml) infused through a syringe perfusion pump system (Argus 600, Argus Medical A6,Heimberg, Switzerland) (1 ml/10 s) to leak induction using catheter (BD Careflow 5-F 200 mm, Becton Dickinson, Franklin Lakes, NJ, USA).

2.5. Gastrointestinal lesions assay

Injury severity was assessed immediately after sacrifice. The sum of the longest lesion diameters was assessed as described previously [8], and tissues were processed for routine microscopy analysis as described previously.

2.6. Lower esophageal sphincter pressure assessment and pyloric sphincter pressure assessment

As described before [29-32], in all rats manometrical evaluation (cmH_2O) was performed with a water manometer connected to the drainage port of the Foley catheter as described (the values of 68-76 cmH2O for lower esophageal sphincter and 68-74 cm H_2O for pyloric sphincter were considered to be normal as determined before). The proximal side of the esophageal or distal side of the duodenal incision was ligated to prevent regurgitation.

2.7. Bilirubin and enzyme activity

To determine the serum values of aspartate transaminase (AST), alanine transaminase (ALT) (IU/L) and total bilirubin (μmol/L), blood samples were obtained immediately after sacrifice

and were centrifuged for 15 min at 3000 rpm. All tests were measured using an OlympusAU2700 analyzer with original test reagents (Olympus Diagnostica, Lismeehan, Ireland) [8,25,33,34]).

2.8. Weight assessment

Animals were weighed before protocol initiation and before sacrifice. After sacrifice, livers were removed and weighed. The relative liver weight was assessed.

2.9. Liver lesions

Liver tissue was immediately placed in 10% neutral buffered formalin for 24 h and subsequently embedded in paraffin. Hematoxylin–eosin stained sections were analyzed on three high-power fields. The number of nuclei as well as their diameter was measured using the ISSA program (Vamstec, Zagreb, Croatia), and the number of binucleated cells was also counted. Microvesicular steatosis was scored from 1–3: 1–less than 20% of hepatocytes showing microvesicular steatosis, 2 – 20–60% of hepatocytes showing microvesicular steatosis and 3 – over 60% of hepatocytes showing microvesicular steatosis. Parenchymal necrosis, eosinophilic cytoplasm, pyknotic nuclei and conspicuous nucleoli were scored semiquantitatively as follows: 0 -showing no changes, 1 – minimum, 2 – moderate and 3 – maximum changes [8,25,33,34].

2.10. Statistical analysis

Nonparametric Kruskall–Wallis and Mann–Whithney U-tests were used for the statistical analysis. The number of rats presenting with epithelization or newly formed muscle was compared using Fisher's two-tailed exact probability test. Values of $P<0.05$ were considered to be statistically significant.

3. Results

Commonly, diclofenac-treated rats with intestinal anastomosis presented with downhill course that was apparently attenuated and even reversed with BPC 157 regimens (Table 1, Table 2, Table 3, Figure 1, Figure 2, Figure 3, Figure 4, Figure 5, Figure 6).

3.1. Anastomosis assessment

In general, macroscopically and microscopically, diclofenac-treated rats with intestinal anastomosis (Table 1, Table 2, Figure 1, Figure 5) clearly showed increased adhesion presentation mostly with neighboring small intestine loops also included, stomach, liver – packed ‖ , resulting with advanced intestinal passage obstruction. Blood filling of the anastomosis arcade vessels was poor, presenting vessels empty (Table 2, Figure 5). Epithelization, necrosis, edema, granulation tissue, and inflammatory cells, reticulin, collagen presentation (Table 1) accords with poor gross presentation despite adaptation presented as villus height, twofold increase in crypt depth and fourfold increase in muscle thickness within the first week (Figure 1).This threatening course was completely reversed by BPC 157 regimens, intraperitoneal and peroral, µg-and ng-regimens. Macroscopically, the adhesion formation remained attenuated, blood vessels filled with blood, and mild intestinal passage obstruction was only temporarily observed. Microscopically, edema was markedly attenuated and the number of granulocytes decreased, and subsequently, necrosis was attenuated, while granulation tissue, reticulin, and collagen formation substantially increased.

Microscopy anastomosis assessment (min/med/max) (days) in rats with ileoileal anastomosis and diclofenac (6.25mg/kg, i.p.) application after surgery that received pentadecapeptide BPC 157 (10 µg/kg, 10 ng/kg) (i) intraperitoneally, once every day, with the first application 30 min after surgery, and the final application at 24 h before sacrifice or (ii) in drinking water (0.16 µg/mL, 0.16 ng/mL) until the end of the experiment while corresponding controls received at the same time (i) an equivolume of saline (5ml/kg ip) or (ii) drinking water only (12ml/rat/day). * $P<0.05$, at least vs. corresponding control.

Gross anastomosis assessment (min/med/max) (days) in rats with ileoileal anastomosis and diclofenac (6.25mg/kg, i.p.) application after surgery that received pentadecapeptide BPC 157 (10 µg/kg, 10 ng/kg) (i) intraperitoneally, once every day, with the first application 30 min after surgery, and the final application at 24 h before sacrifice or (ii) in drinking water (0.16 µg/mL, 0.16 ng/mL) until the end of the experiment while corresponding

Table 1. Microscopy anastomosis assessment (min/med/max)(days) in rats with ileoileal anastomosis and after surgery diclofenac (6.25mg/kg i.p.) application

Assessed parameters	Treatment /kg b.w. i.p.	Microscopy anastomosis assessment (min/med/max) (days) in rats with ileoileal anastomosis and after surgery diclofenac 6.25mg/kg i.p. application					
		1st day	3rd day	5th day	7th day	9th day	14th day
Edema (scored 0-3)	0.9%NaCl 5ml	3/3/3	2/3/3	1/2/3	0/1/2	0/0/1	0/0/0
	BPC 157 10 μg	1/1/2*	0/1/2*	0/0/0*	0/0/0*	0/0/0	0/0/0
	BPC 157 10 ng	1/2/2*	0/1/2*	0/0/1*	0/0/0*	0/0/0	0/0/0
	Drinking water	3/3/3	2/3/3	1/2/3	0/1/2	0/0/1	0/0/0
	BPC 157 10 μg	1/1/2*	0/1/2*	0/0/0*	0/0/0*	0/0/0	0/0/0
	BPC 157 10 ng	1/2/2*	0/1/2*	0/0/1*	0/0/0*	0/0/0	0/0/0
Granulation tissue (scored 0-3)	0.9%NaCl 5ml	0/0/0	0/0/0	0/0/1	0/0/1	1/1/2	1/2/3
	BPC 157 10 μg	0/0/0	0/0/0	1/1/2*	1/2/3*	1/3/3*	3/3/3*
	BPC 157 10 ng	0/0/0	0/0/0	0/1/2*	0/1/2*	1/2/3*	2/3/3*
	Drinking water	0/0/0	0/0/0	0/0/1	0/0/1	1/1/2	1/2/3
	BPC 157 10 μg	0/0/0	0/0/0	1/1/2*	1/2/3*	1/3/3*	3/3/3*
	BPC 157 10 ng	0/0/0	0/0/0	0/1/2*	0/1/2*	1/2/3*	2/3/3*
Necrosis (scored 0-3)	0.9%NaCl 5ml	3/3/3/	3/3/3	1/2/3	0/2/2	0/1/2	0/0/0
	BPC 157 10 μg	2/2/2*	2/2/2*	0/1/2*	0/0/2*	0/0/1	0/0/0
	BPC 157 10 ng	2/2/2*	2/2/2*	0/2/2*	0/1/2	0/0/1	0/0/0
	Drinking water	3/3/3/	3/3/3	1/2/3	0/2/2	0/1/2	0/0/0
	BPC 157 10 μg	2/2/2*	2/2/2*	0/1/2*	0/0/2*	0/0/1	0/0/0
	BPC 157 10 ng	2/2/2*	2/2/2*	0/2/2*	0/1/2	0/0.5/1	0/0/0
Number inflammatory cells	0.9%NaCl 5ml	140/149/155	60/66/75	78/88/94	78/90/98	60/70/78	58/65/70
	BPC 157 10 μg	138/150/154	40/44/45*	39/42/45*	33/36/42*	31/36/40*	27/31/34*
	BPC 157 10 ng	135/152/158	40/45/48*	35/41/48*	36/40/43*	34/40/42*	31/36/39*
	Drinking water	138/148/153	62/68/73	73/80/84	70/77/80	64/66/71	54/55/58
	BPC 157 10 μg	132/151/154	38/44/49*	35/39/44*	33/36/42*	30/38/43*	25/33/36*
	BPC 157 10 ng	138/152/158	39/45/51*	38/43/47*	36/42/45*	34/41/45*	29/38/40*

Table 2. Gross anastomosis assessment (min/med/max)(days) in rats with ileoileal anastomosis and after surgery diclofenac (6.25mg/kg i.p.) application.

Assessed parameters	Treatment /kg b.w. i.p.	Gross ansstomosis assessment (min/med/max)(days) in rats with ileoileal anastomosis and after surgery diclofenac 6.25mg/kg i.p. application					
		1st day	3rd day	5th day	7th day	9th day	14th day
Intestinal obstruction (0-3)	0.9%NaCl 5ml	1/1/1	2/2/3	2/2/3	2/2/3	2/2/3	2/2/3
	BPC 157 10 μg	0/0/0*	0/0/0*	0/0/1*	0/0/1*	0/0/1*	0/0/1*
	BPC 157 10 ng	0/0/0*	0/0/1*	0/0/1*	0/0/1*	0/0/1*	0/0/1*
	Drinking water	1/1/1	2/3/3	2/2/3	2/2/3	2/2/3	2/2/3
	BPC 157 10 μg	0/0/0*	0/0/0*	0/0/1*	0/0/1*	0/0/1*	0/0/1*
	BPC 157 10 ng	0/0/0*	0/0/1*	0/0/1*	0/0/1*	0/0/1*	0/0/1*
Adhesions presentation (scored 0-7)	0.9%NaCl 5ml	1/1/1	3/3/4	4/4/5	4/5/6	4/5/6	4/5/6
	BPC 157 10 μg	0/0/0*	1/1/1*	1/1/1*	1/1/1*	1/1/2*	1/1/2*
	BPC 157 10 ng	0/0/0*	1/1/1*	1/1/1*	1/1/1*	1/1/2*	1/1/2*
	Drinking water	1/1/1	3/3/4	4/4/5	4/5/6	4/5/6	4/5/6
	BPC 157 10 μg	0/0/0*	0/1/1*	1/1/1*	1/1/1*	1/1/2*	1/1/2*
	BPC 157 10 ng	0/0/0*	1/1/1*	1/1/1*	1/1/1*	1/1/2*	1/1/2*
Fullfiling anastomosis arcade vesseles scored (0-2)	0.9%NaCl 5ml	2/2/2	2/2/2	2/2/2	2/2/2	2/2/2	2/2/2
	BPC 157 10 μg	0/0/0*	0/0/0*	0/0/0*	0/0/0*	0/0/0*	0/0/0*
	BPC 157 10 ng	0/0/0*	0/0/1*	0/0/1*	0/0/1*	0/0/1*	0/0/1*
	Drinking water	2/2/2	2/2/2	2/2/2	2/2/2	2/2/2	2/2/2
	BPC 157 10 μg	0/0/0*	0/0/0*	0/0/0*	0/0/0*	0/0/0*	0/0/0*
	BPC 157 10 ng	0/0/0*	0/0/1*	0/0/1*	0/0/1*	0/0/1*	0/0/1*

controls received at the same time (i) an equivolume of saline (5ml/kg ip) or (ii) drinking water only (12ml/rat/day). * P<0.05, at least vs. corresponding control.

Finally, increased epithelization progressed in all rates treated with higher doses contrasts with the absence of epithelial regeneration in controls (P < 0.05, at least). In addition, BPC 157 rats presented strands of newly formed muscle, in contrast to muscularis (both mucosae and propriae) ending abruptly in the controls (versus control P < 0.05, at least). Likewise, with the respect to the intestine adaptation after anastomosis and diclofenac administration injury, presenting increased villus height, crypt depth, and muscle thickness values, with BPC 157 therapy, both perorally and parenterally, adaptation may be advanced, and villus height, crypt depth, and muscle thickness (particularly inner (cir-

cular), and then outer (longitudinal) muscular layer) also additionally increased (Figure 1).

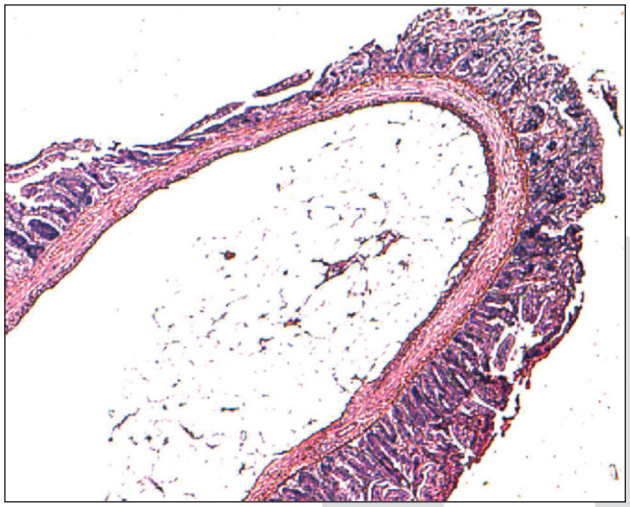

Figure 1. Ileum crypt depth, villus height, and muscle

Crypt depth, villus height, and muscle thickness (inner (circular), outer (longitudinal) muscular layer) in rats with ileoileal anastomosis and diclofenac 6.25 mg/kg intraperitoneal application after surgery that received pentadecapeptide BPC 157 (10 µg/kg, 10 ng/kg) (i) intraperitoneally, once every day, with the first application 30 min after surgery, and the final application at 24 h before sacrifice or (ii) in drinking water (0.16 µg/mL, 0.16 ng/mL) until the end of the experiment while corresponding controls received at the same time (i) an equivolume of saline (5ml/kg ip) or (ii) drinking water only (12ml/rat/day). *P<0.05, at least vs. corresponding control.

3.2. Biomechanics

Biomechanically, anastomosis dehiscence was evidenced in diclofenac-rats with intestinal anastomosis throughout the end of the observation period (Figure 2). In contrast, all BPC 157 regimens, intraperitoneal and peroral, µg-and ng-regimens increased anastomotic strength and exhibited no anastomotic dehiscence.

Volume (ml) to anastomosis leak induction, pressure within lower esophageal sphincter (LES) and pressure within pyloric sphincter pressure (PS) (cmH_2O) in rats with ileoileal anastomosis and diclofenac 6.25mg/kg intraperitoneal application after surgery that received pentadecapeptide BPC 157 (10 µg/kg, 10 ng/kg) (i) intraperitoneally, once every day, with the first application 30 min after surgery, and the final application at 24 h before sacrifice or (ii) in drinking water (0.16 µg/mL, 0.16 ng/mL) until the end of the experiment while corresponding controls received at the same time (i) an equivolume of saline (5ml/kg ip) or (ii) drinking water only (12ml/rat/day). * P<0.05, at least vs. corresponding control.

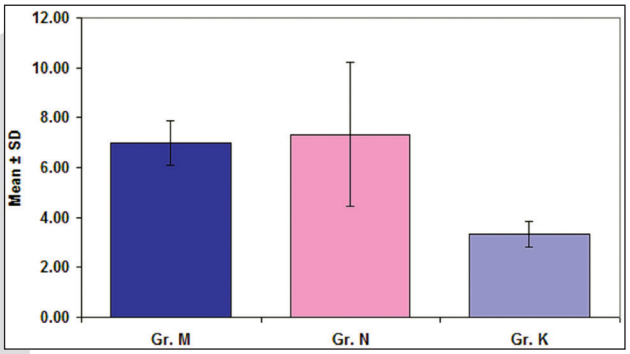

Figure 2. Anastomotic biomechanics

3.3. Gastrointestinal assay

Gastric lesions, small and large intestinal lesions were consistently presented in all diclofenac-rats with intestinal anastomosis (Fig. 3). In contrast, all BPC 157 regimens, intraperitoneal and peroral, µg- and ng-regimens presented only minute lesions.

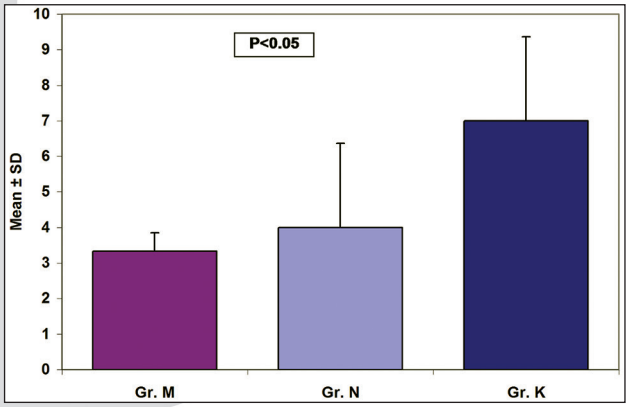

Figure 3. Anastomotic biomechanics

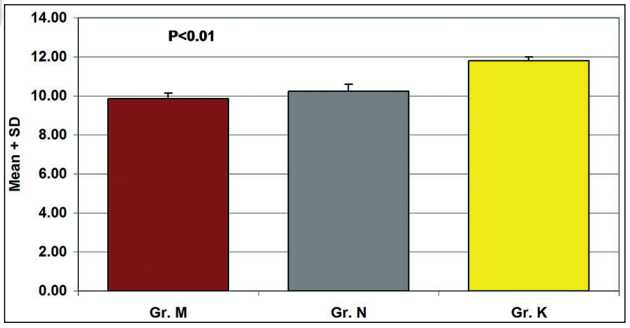

Figure 3A. Gastric

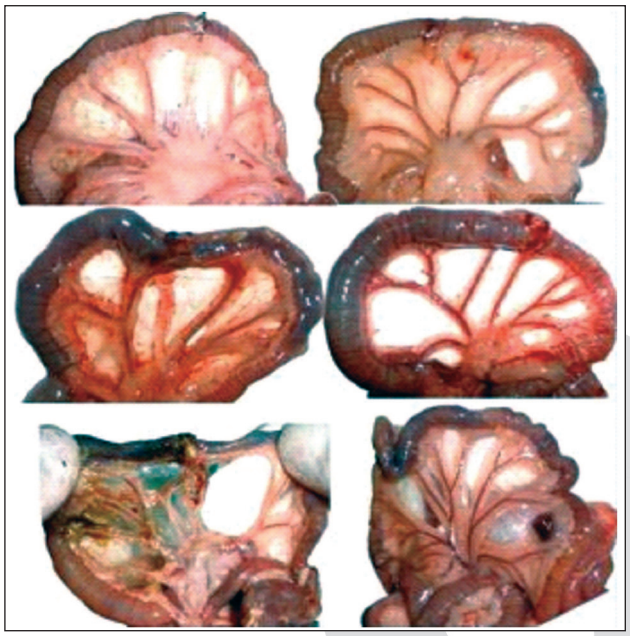

Figure 4. Intestinal lesions

Gastric lesions, small and large intestinal lesions were consistently presented in rats with ileoileal anastomosis and diclofenac 6.25 mg/kg intraperitoneal application after surgery that received pentadecapeptide BPC 157 (10 µg/kg, 10 ng/kg) (i) intraperitoneally, once every day, with the first application 30 min after surgery, and the final application at 24 h before sacrifice or (ii) in drinking water (0.16 µg/mL, 0.16 ng/mL) until the end of the experiment while corresponding controls received at the same time (i) an equivolume of saline (5 ml/kg ip) or (ii) drinking water only (12ml/rat/day). * P<0.05, at least vs. corresponding control.

3.4. Lower esophageal sphincter pressure assessment and pyloric sphincter pressure assessment

A decrease was consistently noted in lower esophageal sphincter pressure and pyloric sphincter pressure assessment in diclofenac-rats with intestinal anastomosis (Figure 2). This was markedly counteracted in rats that received BPC 157 regimens, intraperitoneal and peroral, µg-and ng-regimens.

3.5. Liver lesions

Accordingly with presentation of ALT and AST serum values, we observed parenchymal necrosis at the end of two week period, more conspicuous nucleoli, more eosinophilic hepatocytes with pyknotic nuclei, extensive microvesicular steatosis and sinusoidal dilation. Liver weight considerably increased (Table 3, Figure 4, Figure 6).

Microscopy liver assessment (min/med/max) (days) in rats with ileoileal anastomosis and diclofenac (6.25mg/kg, i.p.) application after surgery that received pentadecapeptide BPC 157 (10 µg/kg, 10 ng/kg) (i) intraperitoneally, once every day, with the first application 30 min after surgery, and the final application at 24 h before sacrifice or (ii) in drinking water (0.16 µg/mL, 0.16 ng/mL) until the end of the experiment while correspon88ding controls received at the same time (i) an equivolume of saline (5ml/kg ip) or (ii) drinking water only (12ml/rat/day). * P<0.05, at least vs. corresponding control.

Serum values of aspartate transaminase (AST), alanine transaminase (ALT) (IU/L) and total bilirubin (µmol/L), body weight (g) (animals were weighed before protocol initiation and before sacrifice), relative liver weight, the number of binucleated cells in rats with ileoileal anastomosis and diclofenac 6.25mg/kg intraperitoneal application after surgery that received pentadecapeptide BPC 157 (10 µg/kg, 10 ng/kg) (i) intraperitoneally, once every day, with the first application 30 min after surgery, and the final application at 24 h before sacrifice or (ii) in drinking water (0.16 µg/mL, 0.16 ng/mL) until the end of the experiment while corresponding controls received at the same time (i) an equivolume of saline (5ml/kg ip) or (ii) drinking water only (12ml/rat/day). * P<0.05, at least vs. corresponding control.

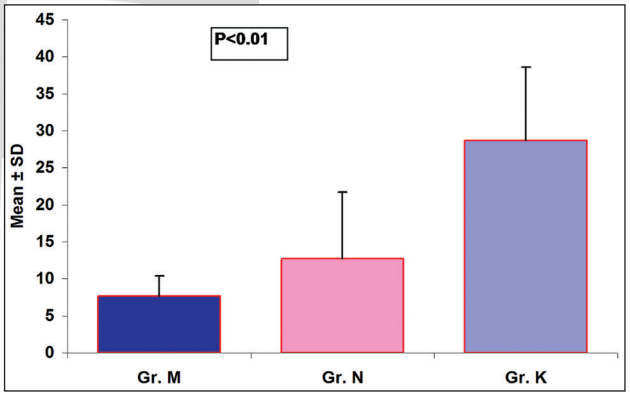

Figure 5. Liver function

Left; In control group (day 1 (upper), day 3 (middle), day 9(lower)) blood filling of the anastomosis arcade vessels was progressively poor,

Table 3. Microscopy liver assessment (min/med/max)(days) in rats with ileoileal anastomosis and diclofenac (6.25mg/kg, i.p.) application after surgery.

Assessed parameters	Treatment /kg b.w. i.p.	Microscopy liver assessment (min/med/max)(days) in rats with ileoileal anastomosis and after surgery diclofenac 6.25mg/kg i.p. application					
		1st day	3rd day	5th day	7th day	9th day	14th day
Steatosis, scored (0-3)	0.9%NaCl 5ml	1/1/1	2/2/2	2/2/2	3/3/3	3/3/3	3/3/3
	BPC 157 10 µg	0/0/0*	1/1/1*	1/1/1*	1/1/1*	1/1/1*	1/1/1*
	BPC 157 10 ng	0/0/0*	1/1/1*	1/1/1*	1/1/1*	1/1/1*	1/1/1*
	Drinking water	1/1/1	2/2/2	2/2/2	3/3/3	3/3/3	3/3/3
	BPC 157 10 µg	0/0/0*	1/1/1*	1/1/1*	1/1/1*	1/1/1*	1/1/1*
	BPC 157 10 ng	0/0/0*	1/1/1*	1/1/1*	1/1/1*	1/1/1*	1/1/1*
Necrosis, scored (0-3)	0.9%NaCl 5ml	0/0/0	0/0/0	0/0/0	0/0/0	1/1/1	1/1/1
	BPC 157 10 µg	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0*	0/0/0*
	BPC 157 10 ng	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0*	0/0/0*
	Drinking water	0/0/0	0/0/0	0/0/0	0/0/0	1/1/1	1/1/1
	BPC 157 10 µg	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0*	0/0/0*
	BPC 157 10 ng	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0*	0/0/0*
Sinusoidal dilation, scored (0-3)	0.9%NaCl 5ml	3/3/3	3/3/3	3/3/3	3/3/3	3/3/3	3/3/3
	BPC 157 10 µg	1/2/2	1/2/2*	2/2/2*	2/2/2*	2/2/2*	2/2/2*
	BPC 157 10 ng	2/2/2*	2/2/2*	2/2/2*	2/2/2*	2/2/2*	2/2/2*
	Drinking water	3/3/3	3/3/3	3/3/3	3/3/3	3/3/3	3/3/3
	BPC 157 10 µg	1/2/2	1/2/2*	2/2/2*	2/2/2*	2/2/2*	2/2/2*
	BPC 157 10 ng	2/2/2*	2/2/2*	2/2/2*	2/2/2*	2/2/2*	2/2/2*
Hepatocytes with Eosinophilic cytoplasm and pyknotic nuclei, scored (0-3)	0.9%NaCl 5ml	1/1/3	2/2/3	2/2/3	2/2/3	2/2/3	2/2/3
	BPC 157 10 µg	0/0/1*	0/0/1*	0/0/1*	0/0/1*	0/0/1*	0/0/1*
	BPC 157 10 ng	0/0/1*	0/0/1*	0/0/1*	0/0/1*	0/0/1*	0/0/1*
	Drinking water	1/1/3	2/2/3	2/2/3	2/2/3	2/2/3	2/2/3
	BPC 157 10 µg	0/0/1*	0/0/1*	0/0/1*	0/0/1*	0/0/1*	0/0/1*

presenting vessels empty, passage obstruction, and anastomosis dehiscience (lower). Right; This threatening course was completely reversed by BPC 157 regimens, intraperitoneal and peroral, µg-and ng-regimens. Macroscopically, the adhesion formation remained attenuated, blood vessels filled with blood, and mild intestinal passage obstruction was only temporarily observed.

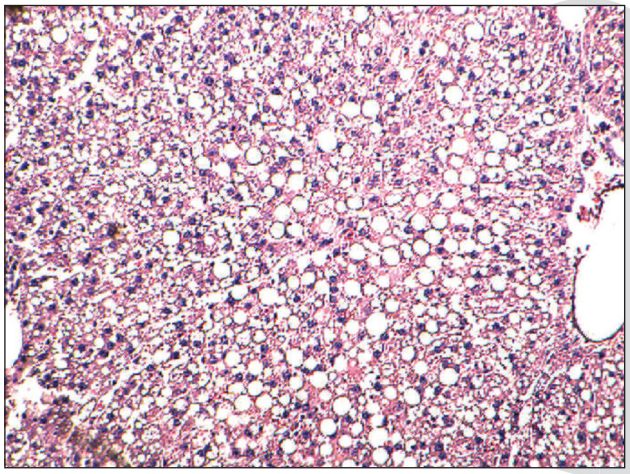

Figure 6. Liver lesions

Characteristic liver lesions course, microscopy presentation. HEx20. Day 3 (upper), 7 (middle), 14 (lower) Left. Control rats presented with progressing course - moderate microvesicular steatosis, >30% cells affected,severe expansion of sinusoids, without necrosis (left, upper, day 3), severe microvesicular, >60% cells affected, severe expansion of sinusoids,without necrosis(left, middle, day 7) reaching severe microvesicular and macrovesicular steatosis, >60% cells affected, severe expansion of sinusoids, focal necrosis (left, lower, day 14). Right. This threatening course was markedly attenuated by BPC 157 regimens, intraperitoneal and peroral, µg-and ng-regimens. Mild microvesicular steatosis, <30% cells affected, moderate expansion of sinusoids,without necrosis (day 3 (right, upper), day 7 (right, middle), day 14 (right, lower)).

Diclofenac-treated rats with intestinal anastomosis that received pentadecapeptide BPC 157 exhibited decreased ALT and AST serum values, no liver weight increase and no parenchymal necrosis. Morphometric analyses indicated a smaller average hepatocyte area, with a greater number of nuclei per high power visual field (HVF) and no sinusoidal dilation. Interestingly, hepatocytes in the BPC 157-treated animals demonstrated a higher number of binucleated hepatocytes (Table 3, Figure 4, Figure 6).

There was neither fibrosis nor mononuclear inflammatory infiltrate in the portal areas of both groups, and there were no obvious differences in the bile duct numbers of both groups.

4. Discussion

Early post-surgical diclofenac administration induced the combined intestinal anastomosis with diclofenac-toxicity, and most likely, mutual potentiation in rats. Hopefully, this may be important in practice with intestinal anastomosis and diclofenac. Vice versa, a prompt reversal due to stable gastric pentadecapeptide BPC 157 application established the consequent background for reciprocal, combined and mutually potentiating therapeutic effect. Accordingly, in previous separate studies [8, 9] BPC 157 exhibited mucosal protection, wound healing and hepatoprotective effect, and sphincters function rescue [10], all demonstrated separately [8-10].

Now all these effects successfully combined appear as a practical solution for up to now unsolved complexity of combined intestinal anastomosis failure with diclofenac-toxicity progression, whatever may be the prime cause of poor healing course deterioration (i.e., diclofenac toxicity specifically depended on intestinal bacteria [37]; progressive anastomosis dehiscence and loss of anastomotic strength and degradation of collagen [38] imply more toxins and more liver lesions [37]) or many in combination: the leading diclofenac ulcerogenic effect that expands invading the stomach, small and large intestine, lower esophageal and pyloric sphincter failure; progressing intestinal wound healing inability (i.e., adhesion presentation mostly with neighboring small intestine loops also included, stomach, liver —packed ‖ , advanced intestinal passage obstruction, poor blood filling of the anastomosis arcade vessels, presenting vessels empty) or still increasing hepatotoxicity.

Specifically, when any of regimens of BPC 157 was given, the much smaller number of mucosal lesions in all gastrointestinal regions, preserved sphincter function, advanced anastomosis healing (only small adhesions presentation, no passage obstruction, increased anastomotic strength exhibited no anastomotic dehiscence, preserved blood filling

of the anastomosis arcade vessels, all intestine wall layers more vigorously adapt) and liver lesions recovery since very beginning (i.e., a higher number of binucleated hepatocytes, less fatty liver, no necrosis). Therefore, it seems that against combined intestinal anastomosis with diclofenac-toxicity, the activated mucosal protection, wound (anastomosis) healing and hepatoprotection are the effects of their own combined all together by BPC 157.

In particular, in intestinal anastomosis with diclofenac-toxicity, it may be that the BPC 157 activated mucosal protection [12-15]. Very likely, it implemented Robert's concept of cytoprotection [17] (i.e., at least for 24 hours stable in human gastric juice [12-15], playing the role of novel mediator of Robert's cytoprotection [13,21, 39, 40]) versus a NSAID (diclofenac) application, even with increased, more aggressive lesions since occupying lower parts of gastrointestinal tract as well. BPC 157 cures the gastric/intestinal lesions induced by a variety of NSAIDs, i.e., aspirin [28], indomethacin [39], diclofenac [8] and ibuprofen [26]. Accordingly, more sphincteric failure [29-31] in more damaged-diclofenac rats with intestinal anastomosis while less damaged gastric mucosa and preserved pressure in sphincters should be along with regularly maintained and rescued sphincter function [29-31] after BPC 157 administration. Likewise for liver lesions counteraction, as a secondary effect (i.e., less gastrointestinal lesions, less toxins, less liver damage [37]) or as a primary effect, BPC 157 recent counteraction of NSAIDs-(paracetamol-, diclofenac- and ibuprofen-) hepatotoxicity [8, 25, 26] neutralizes here increased hepatotoxicity after intestinal anastomosis and diclofenac. Thus, it obviously does imply distinctive hepatic lesions and quite different mechanisms (i.e., unlike that of gastrointestinal lesions (COX-1/COX-2 inhibition), the hepatotoxic NSAIDs background is more diverse (i.e., intrinsic, immunoallergic, idiosyncratic and in particular metabolic idiosyncratic like that of diclofenac (for review see, i.e., [62])).

Also, we found convincing evidence that BPC 157 activates in intestinal anastomosis with diclofenac-toxicity an additional innate ―wound healing effect‖ [11, 13, 14, 36] (previously, after massive resection the remaining intestine quickly functions better and reaches more adaptive capacity [11], weight gain, bringing rats with short bowel to the weight of normal, non-operated rats [11] and likewise here, as before [11], all intestine wall layers more adapt), all of the major healing events that occur in a set order following the loss of tissue integrity realized, step by step [9]. Namely, an advanced intestinal anastomosis healing that occurs with only small adhesions formation and preserved blood filling of the anastomosis arcade vessels supports special BPC 157's ―wound healing effect‖ [11, 13, 14, 36] that includes different tissues [35, 41-55], in particular, gastrointestinal tract [9, 11, 18, 20] and blood vessels [56] and potent anti-inflammatory effects [54]. And most importantly, BPC 157 stimulates the expression of the egr-1 repressor nerve growth factor 1-A binding protein-2 (nab2) [17], a feedback mechanism that serves to regulate egr-1-mediated gene transcription responsible for collagen and blood vessel formation, but also the development of liver lesions, chronic ethanol-induced steatosis [58], hepatic injury during acute inflammation and/or hepatitis [59], gastrointestinal (cysteamine) ulcers, while NSAIDs delay ulcer healing and hinder angiogenesis by inhibiting egr-1 [37, 60, 61].

5. Conclusion

A final argument that promotes BPC 157 as a practical solution of particular complex problem of intestinal anastomosis after diclofenac administration, should be that all these beneficial and counteracting effects of BPC 157 were consistently obtained using an equipotent dosage (μg, ng/kg) in parenteral or peroral regimens .

References

1. *Wattchow DA, Fontgalland DD, Bampton PA, Leach PL, McLaughlin K, Costa M, Clinical trial: the impact of cyclooxygenase inhibitors on gastrointestinal recovery after major surgery - a randomized double blind controlled trial of celecoxib or diclofenac vs. placebo, Aliment. Pharmacol. Ther. 2009; 30: 987-998.*

2. *Aram FO. Preoperative use of analgesia in appendicitis, Saudi Med. J. 2008; 29: 859-62.*

3. *Ekstein P, Szold A, Sagie B, Werbin N, Klausner JM, Weinbroum AA. Laparoscopic surgery may be associated with severe pain and high analgesia requirements in the HYPERLINK "http://www.ncbi.nlm.nih.gov/pubmed/16371735" immediate postoperative period, Ann. Surg. 2006; 243: 41-46.*

4. *Klein M. Postoperative non-steroidal anti-inflammatory drugs and colorectal anastomotic leakage. NSAIDs and anastomotic leakage, Dan. Med. J. 2012; 59: B4420.*

5. *Rushfeldt CF, Sveinbjørnsson B, Søreide K, Vonen B. Risk of anastomotic leakage with use of NSAIDs after gastrointestinal surgery, Int. J. Colorectal. Dis. 2011; 26: 1501-1509.*

6. *Klein M, Krarup PM, Kongsbak MB, Agren MS, Gögenur I, et al. Effect of postoperative diclofenac on anastomotic healing, skin wounds and subcutaneous collagen accumulation: a randomized, blinded, placebo-controlled, experimental study, Eur. Surg. Res. 2012; 48: 73-78.*

7. *de Sousa JB, Soares EG, Aprilli F. Effects of diclofenac sodium on intestinal anastomotic healing. Experimental study on the small intestine of rabbits, Dis. Colon Rectum, 1991; 34: 613-617.*

8. *Ilic S, Drmic D, Franjic S, Kolenc D, Coric M, et al. Pentadecapeptide BPC 157 and its effects on a NSAID toxicity model: diclofenac-induced gastrointestinal, liver, and encephalopathy lesions, Life Sci. 2011; 88: 535-542.*

9. *Vuksic T, Zoricic I, Brcic L, Sever M, Klicek R. et al. Stable gastric pentadecapeptide BPC 157 in trials for inflammatory bowel disease (PL-10, PLD-116, PL14736, Pliva, Croatia) heals ileoileal anastomosis in the rat, Surg. Today 2007; 37: 768-777.*

10. *Franjic S, Drmic D, Bauk L, Stupnisek M, Becejac T, et al. Pentadecapeptide BPC 157 counteracts failure of lower esophageal and pyloric sphincter induced by NSAIDs in rats, Gastroenterology, 2011; 140: 232.*

11. *Sever M, Klicek R, Radic B, Brcic L, Zoricic I, et al. Gastric pentadecapeptide BPC 157 and short bowel syndrome in rats, Dig. Dis. Sci. 2009; 54: 2070-2083.*

12. *Sikirić P, Petek M, Rucman R, Seiwerth S, Grabarević Z, et al. A new gastric juice peptide, BPC. An overview of the stomach-stress-organoprotection hypothesis and beneficial effects of BPC, J. Physiol. Paris, 1993; 87: 313-327.*

13. *Sikiric P, Seiwerth S, Brcic L, Sever M, Klicek R. et al. Revised Robert's cytoprotection and adaptive cytoprotection and stable gastric pentadecapeptide BPC 157. Possible significance and implications for novel mediator, Curr. Pharm. Des. 2010; 16: 1224-1234.*

14. *Sikiric P, Seiwerth S, Rucman R, Turkovic B, Rokotov DS, et al. Stable gastric pentadecapeptide BPC 157: Novel therapy in gastrointestinal tract, Curr. Pharm. Des. 2011; 17: 1612-1632.*

15. *Sikiric P, Seiwerth S, Rucman R, Turkovic B, Rokotov DS, et al. Focus on ulcerative colitis: Stable gastric pentadecapeptide BPC 157, Curr. Med. Chem. 2012; 19: 126-132.*

16. *Sikiric P, Seiwerth S, Rucman R, Turkovic B, Rokotov DS, et al. Toxicity by NSAIDs. Counteraction by stable gastric pentadecapeptide BPC 157, Curr. Pharm. Des. 2013; 19: 76-83.*

17. *Tkalcevic VI, Cuzic S, Brajsa K, Mildner B, Bokulic A, et al. Enhancement by PL 14736 of granulation and collagen organization in healing wounds and the potential role of egr-1 expression, Eur. J. Pharmacol. 2007; 570: 212-221.*

18. *Cesarec V, Becejac T, Misic M, Djakovic Y, Olujic D, et al. Pentadecapeptide BPC157 and the esophagocutaneous fistula healing therapy, Eur. J. Pharmacol. 2013; 701: 203-212.*

19. *Balenovic D, Bencic ML, Udovicic M, Simonji K, Hanzevacki JS, et al. Inhibition of methyldigoxin-induced arrhythmias by pentadecapeptide BPC 157: a relation with NO-system, Regul. Pept. 2009; 156: 83-89.*

20. *Klicek R, Sever M, Radic B, Drmic D, Kocman I, et al. Pentadecapeptide BPC 157, in clinical trials as a therapy for inflammatory bowel disease (PL14736), is effective in the healing of colocutaneous fistulas in rats: role of the nitric oxide-system, J. Pharmacol. Sci. 2008; 108: 7-17.*

21. *Boban-Blagaic A, Blagaic V, Romic Z, Jelovac N, Dodig G, et al. The influence of gastric pentadecapeptide BPC 157 on acute and chronic ethanol administration in mice. The effect of N(G)-nitro-L-arginine methylester and L-arginine, Med. Sci. Monit. 2006; 12: 36-45.*

22. *Bencic ML, Sikiric P, Hanzevacki JS, Seiwerth S, Rogic D, et al. Doxorubicine-congestive heart failure-increased big endothelin-1 plasma concentration: reversal by amlodipine, losartan, and gastric pentadecapeptide BPC157 in rat and mouse, J. Pharmacol. Sci. 2004; 95: 19-26.*

23. *Sikiric P, Seiwerth S, Grabarevic Z, Rucman R, Petek M, et al. The influence of a novel pentadecapeptide, BPC 157, on N(G)-nitro-L-arginine methylester and L-arginine effects on stomach mucosa integrity and blood pressure, Eur. J. Pharmacol. 1997; 332: 23-33.*

24. *Grabarevic Z, Tisljar M, Artukovic B, Bratulic M, Dzaja P, et al. The influence of BPC 157 on nitric oxide agonist and antagonist induced lesions in broiler chicks, J. Physiol. Paris, 1997; 91: 139-149.*

25. *Ilic S, Drmic D, Zarkovic K, Kolenc D, Coric M, et al. High hepatotoxic dose of paracetamol produces generalized convulsions and brain damage in rats. A counteraction with the stable gastric pentadecapeptide BPC 157 (PL 14736), J. Physiol. Pharmacol. 2010; 61: 241-250.*

26. *Ilic S, Drmic D, Zarkovic K, Kolenc D, Brcic L, et al. Ibuprofen hepatic encephalopathy, hepatomegaly, gastric lesion and gastric pentadecapeptide BPC 157 in rats, Eur. J. Pharmacol. 2011; 667: 322-329.*

27. *Sikiric P, Seiwerth S, Grabarevic Z, Rucman R, Petek M, et al. Pentadecapeptide BPC 157 positively affects both non-steroidal anti- inflammatory agent-induced gastrointestinal lesions and adjuvant arthritis in rats, J Physiol. Paris, 1997; 91: 113-122.*

28. *Stupnisek M, Franjic S, Drmic D, Hrelec M, Kolenc D, et al. Pentadecapeptide BPC 157 reduces bleeding time and thrombocytopenia after amputation in rats treated with heparin, warfarin or aspirin, Thromb. Res. 2012; 129: 652-659.*

29. *Petrovic I, Dobric I, Drvis P, Shejbal D, Brcic L, et al. An experimental model of prolonged esophagitis with sphincter failure in the rat and the therapeutic potential of gastric pentadecapeptide BPC 157, J. Pharmacol. Sci. 2006; 102: 269-277.*

30. *Dobric I, Drvis P, Petrovic I, Shejbal D, Brcic L, et al. Prolonged esophagitis after primary dysfunction of the pyloric sphincter in the rat and therapeutic potential of the gastric pentadecapeptide BPC 157, J. Pharmacol. Sci. 2007; 104: 7-18.*

31. *Petrovic I, Dobric I, Drmic D, Sever M, Klicek R, et al. BPC 157 therapy to detriment sphincters failure-esophagitis-pancreatitis in rat and acute pancreatitis patients HYPERLINK "http://www.ncbi.nlm.nih.gov/pubmed/22204800" low sphincters pressure, J. Physiol. Pharmacol. 2011; 62: 527-534.*

32. *Barisic I, Balenovic D, Klicek R, Radic B, Nikitovic B, et al. Mortal hyperkalemia disturbances in rats are NO-system related. The life saving effect of pentadecapeptide BPC 157, Reg. Pept. 2013; 181: 50-66.*

33. *Prkacin I, Separovic J, Aralica G, Perovic D, Gjurasin M, et al. Portal hypertension and liver lesions in chronically alcohol drinking rats prevented and reversed by stable gastric pentadecapeptide BPC 157 (PL-10, PLD-116), and propranolol, but not ranitidine, J. Physiol. Paris, 2001; 95: 315-324.*

34. *Sikiric P, Seiwerth S, Grabarevic Z, Rucman R, Petek M, et al. Hepatoprotective effect of BPC 157, a 15-amino acid peptide, on liver lesions induced by either restraint stress or bile duct and hepatic artery ligation or CCl4 administration. A comparative study with dopamine agonists and somatostatin, Life Sci. 53 (1993) 291-296.*

35. *Mikus D, Sikiric P, Seiwerth S, Petricevic A, Aralica G, et al. Pentadecapeptide BPC 157 cream improves burn-wound healing and attenuates burn-gastric lesions in mice, Burns, 2001; 27: 817-827.*

36. *Sikiric P, Seiwerth S, Brcic L, Blagaic AB, Zoricic I. et al. HYPERLINK "http://www.ncbi.nlm.nih.gov/pubmed/17186181" Stable gastric pentadecapeptide BPC 157 in trials for inflammatory bowel disease (PL-10, PLD- 116, PL 14736, Pliva, Croatia). Full and distended stomach and vascular response, Inflammopharmacology, 2006; 14: 214-221.*

37. *Deng X, Liguori MJ, Sparkenbaugh EM, Waring JF, Blomme EA, et al. Gene expression profiles in livers from diclofenac-treated rats reveal intestinal bacteria-dependent and -independent pathways associated with liver injury, J. Pharmacol. Exp. Ther. 2008; 327: 634-644.*

38. *Hendriks T, Mastboom WJB. Healing of experimental intestinal anastomoses. Parameters for repair, Dis. Colon Rectum, 1990; 33: 891–901.*

39. *Sikirić P, Seiwerth S, Desković S, Grabarević Z, Marović A, et al. New model of cytoprotection/adaptive cytoprotection in rats: endogenous small irritants, antiulcer agents and indomethacin, Eur. J. Pharmacol. 1999; 364: 23-31.*

40. *Sikirić P, Seiwerth S, Grabarević Z, Rucman R, Petek M, et al. Beneficial effect of a novel pentadecapeptide BPC 157 on gastric lesions induced by restraint stress, ethanol, indomethacin, and capsaicin neurotoxicity, Dig. Dis. Sci. 1996; 41: 1604-1614.*

41. *Seiwerth S, Sikiric P, Grabarevic Z, Zoricic I, Hanzevacki M, et al. BPC 157's effect on healing, J. Physiol. Paris 1997; 91: 173-178.*

42. *Sikiric P, Seiwerth S, Mise S, Staresinic M, Bedekovic V, et al. Corticosteroid-impairment of healing and gastric pentadecapeptide BPC-157 creams in burned mice, Burns 2003; 329: 323-334.*

43. *Staresinic M, Petrovic I, Novinscak T, Jukic I, Pevec D, et al. Effective therapy of transected quadriceps muscle in rat: Gastric pentadecapeptide BPC 157, J. Orthop. Res. 2006; 24: 1109-1117.*

44. *Novinscak T, Brcic L, Staresinic M, Jukic I, Radic B, et al. Gastric pentadecapeptide BPC 157 as an effective therapy for muscle crush injury in the rat, Surg. Today, 2008; 38: 716-725.*

45. *Pevec D, Novinscak T, Brcic L, Sipos K, Jukic I, et al. Impact of pentadecapeptide BPC 157 on muscle healing impaired by systemic corticosteroid application, Med. Sci. Monit. 2010; 16: 81-88.*

46. *Staresinic M, Sebecic B, Patrlj L, Jadrijevic S, Suknaic S, et al. Gastric pentadecapeptide BPC 157 accelerates healing of transected rat Achilles tendon and in vitro stimulates tendocytes growth, J. Orthop. Res. 2003; 21: 976-983.*

47. *Chang CH, Tsai WC, Lin MS, Hsu YH, Pang JH, The promoting effect of pentadecapeptide BPC 157 on tendon healing involves tendon outgrowth, cell survival, and cell migration, J. Appl. Physiol. 2011; 110: 774-780.*

48. *Krivic A, Majerovic M, Jelic I, Seiwerth S, Sikiric P. Modulation of early functional recovery of Achilles tendon to HYPERLINK "http://www.ncbi.nlm.nih.gov/pubmed/18594781"bone unit after transection by BPC 157 and methylprednisolone, Inflamm. Res. 2008; 57: 205-210.*

49. *Krivic A, Anic T, Seiwerth S, Huljev D, Sikiric P. Achilles detachment in rat and stable gastric pentadecapeptide BPC 157: Promoted tendon-to-bone healing and opposed corticosteroid aggravation, J. Orthop. Res. 2006; 24: 982-989.*

50. *Cerovecki T, Bojanic I, Brcic L, Radic B, Vukoja I, et al. Pentadecapeptide BPC 157 (PL 14736) improves ligament healing in the rat, J. Orthop. Res. 2010; 28: 1155-1161.*

51. *Gjurasin M, Miklic P, Zupancic B, Perovic D, Zarkovic K, et al. Peptide therapy with pentadecapeptide BPC 157 in traumatic nerve injury, Regul. Pept. 2010; 160: 33-41.*

52. *Tudor M, Jandric I, Marovic A, Gjurasin M, Perovic D, et al. Traumatic brain injury in mice and pentadecapeptide BPC 157 effect, Regul. Pept. 2010; 160: 26-32.*

53. *Sebecic B, Nikolic V, Sikiric P, Seiwerth S, Sosa T, et al. Osteogenic effect of a gastric pentadecapeptide, BPC-157, on the healing of segmental bone defect in rabbits: a comparison with bone marrow and autologous cortical bone implantation, 1999; Bone 24: 195-202.*

54. *Keremi B, Lohinai Z, Komora P, Duhaj S, Borsi K, et al. Antiinflammatory effect of BPC 157 on experimental periodontitis in rats, J. Physiol. Pharmacol. 2009; 60: 115-122.*

55. *Lazić R, Gabrić N, Dekaris I, Bosnar D, Boban-Blagaić A, Sikirić P. Gastric pentadecapeptide BPC 157 promotes corneal epithelial defects healing in rats, Coll. Antropol. 2005; 29: 321-325.*

56. *Hrelec M, Klicek R, Brcic L, Brcic I, Cvjetko I, et al. Abdominal aorta anastomosis in rats and stable gastric pentadecapeptide BPC 157, prophylaxis and therapy, J. Physiol. Pharmacol. 2009; 60: 161-165.*

57. *Silverman ES, Khachigian LM, Santiago FS, Williams AJ, Lindner V, Collins T. Vascular smooth muscle cells express the transcriptional corepressor NAB2 in response to injury, Am. J. Pathol. 1999; 155: 1311–1317.*

58. *Donohue Jr TM. Alcohol-induced steatosis in liver cells, World. J. Gastroenterol. 2007; 13: 4974–4978.*

59. *Pritchard MT, Roychowdhury S, McMullen MR, Guo L, Arteel GE, Nagy LE. Early growth response-1 contributes to galactosamine/lipopolysaccharide induced acute liver injury in mice, Am. J. Physiol. Gastrointest. Liver Physiol. 2007; 293: 1124–1133.*

60. *Deng X, Szabo S, Khomenko T, Jadus MR, Yoshida M, Chen L. Detection of duodenal ulcer-associated genes in rats, Dig. Dis. Sci. 2008; 53: 375–384.*

61. *Szabó IL, Pai R, Soreghan B, Jones MK, Baatar D, Kawanaka H. NSAIDs inhibit the activation of egr-1 gene in microvascular endothelial cells. A key to inhibition of angiogenesis? J. Physiol. Paris, 2001; 95: 379–383.*

62. *Manov I, Motanis H, Frumin I, Iancu TC. Hepatotoxicity of anti-inflammatory and analgesic drugs: ultrastructural aspects, Acta Pharmacol. Sin. 2006; 27: 259-272.*

63. *Collier HO, The story of aspirin, in: M.J. Parnham, J. Bruinvels (Eds.), Discovery in Pharmacology. Haemodynamics, Hormones & Inflammation. Elsevier Science Publishers, Amsterdam, New York, Oxford, 1984; 555-593.*

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The tobacco smoking habits with the observed side effects in the student population

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Abstract

The sample have been made of student group with age of 18 to 26 years, that filled questionare for participation in the study, voluntarily. This study consisted of two groups with an equal number of participants: 60 smokers and 60 non smokers.

The study was conducted at the JU HP Institute for health care of students, at the University of Sarajevo. The 120 students were divided into two groupes: 60 smokers and 60 non smokers. Trend of increasing rates of morbidity and mortality from smoking diseases is demonstrated in the Federation of Bosnia and Herzegovina, according to regular statistical surveys. The appearance of human desire to improve the lifes quality is result of the development of civilization, increased knowledge and various experiences in the fight against disease. Tobacco smoking is very serious and difficult social problem in many countries of the modern world. Analysis and attempts to eliminate the causes of the greatest number of diseases of modern society pointed to tobacco as one of the causes. Smoking habits have negative multivariate character, because of smokers threaten their own health and life, but and the lives of those who are close to them. It is quite clear today that tobacco plants, the nature of tobacco leaf, the nature of the land, harvest, process of preparation, aging and fermentation have a great impact on tobacco ingredients. Poison that tobacco smoker entered into his body is his choice, but his community will put up with him, because they will have to be borne by, among others, the cost of treating tobacco smokers. Erythema, pain and frequent interruptions in the continuity of the oral mucosa represent a clinical manifestation of heat effect of nicotine on the oral mucosa. Papillary changes, with the unpleasant odour and a reduced sense of taste and smell, dominate. The World Health Organization has placed special emphasis on the promotion of healthier lifestyles and care for a healthier environment. Lifestyle is associated with the values, priorities and practical possibilities and obstacles specific cultural, social and economic situation.

Key words: smoking, bitterness in the mouth, bad breath

Introduction

Smoking and tobacco use are frequent occurrences in our society. Smoking habits are acquired very early causing many diseases and it affects the quality and length of life. Smoking refers to four million lives worldwide, every year, according to the World Health Organization, because it is rightly called "killer" number one in the world.(1)(2) Smoking as a socio-pathological phenomena can have medical, psychological, political, ideological and other dimensions which are expressed in young people. (3)(5)

Unfortunately, there are more and more children and young people among the users of this poison. The way of life of each individual depends on the interpersonal relationship, social attitudes which are connected and depend on the social environment.(3) Use of tobacco meets all these criteria, and because of that, compelling urge to smoke occurs, which causes a chronic need for tobacco. So we can say that nicotine is addictive similar to heroin! For this reason, only a small number of smokers succeed in the first attempt to stop smoking, and two-thirds of that number, do not want to.(4)(7)

Aim of research

We intend to determine the structure of smokers and non-smokers according to the number of ciga-

rettes smoked per day and identify side effects as a result of smoking with a sense of bitterness in the mouth and bad breath that observed by smokers.

Materials and methods

This is clinical, applicative and controlled study with experimental- prospective character and randomly selected sample. We used questionnaire cardboard to determine side effects: feeling of bitterness in the mouth and feeling bad breath in patients. We have introduced into the data specifically designed questionnaire card, which the patients filled out voluntarily. We determine the age and sex of smoking addicts, amount of cigarettes smoked per day by the survey. We sort smokers into three groups according to the amount of cigarettes smoked per day, based on previous research. These are the groups:

1. Moderate smokers up to 10 cigarettes per day.
2. Medium moderate of 11-20 cigarettes per day.
3. Heavy smokers more than 21 cigarettes per day

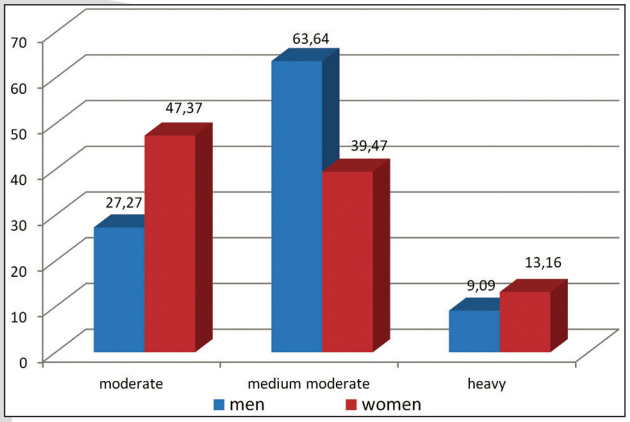
Results

We sort smokers into three groups according to the amount of cigarettes smoked per day: moderate smokers up to 10 cigarettes per day, medium moderate of 11-20 cigarettes per day, heavy smokers more than 21 cigarettes per day (table 1).

There were 40.0% moderate smokers (1-10 cigarettes per day), medium moderate 83.3% (11-20 cigarettes per day) and heavy smokers, 11.7% (21 or more cigarettes per day) in the sample

Differences in smoking status were statistically significant. Medium moderate smokers - 43.3% are the most represented, followed by moderate smokers - 40.0%, and heavy smokers - 11.7%. Are least represented (table 2).

We found difference in the male smokers between medium moderate smokers and heavy smokers, on the level p <0.05 (t= 2.27), between smokers of both genders on the level p<0,05 (t=2,43), and signifficant difference on the level p<0,0001 (t=3,58) between medium moderate male smokers and all female smokers in total, by testing difference in smoking habits on the model of Student's t-test.



Graph 1. Smokers status gender

Table 1. Smoking status according to gender

Smokers	moderate		medium moderate		heavy		Total	
	N	%	N	%	N	%	N	%
Men	6	27,27	14	63,64	2	9,09	22	100
Women	18	47,37	15	39,47	5	13,16	38	100
Total	24	40,00	29	43,33	7	11,67	60	100

$X^2=16.604$ $p<0.005$

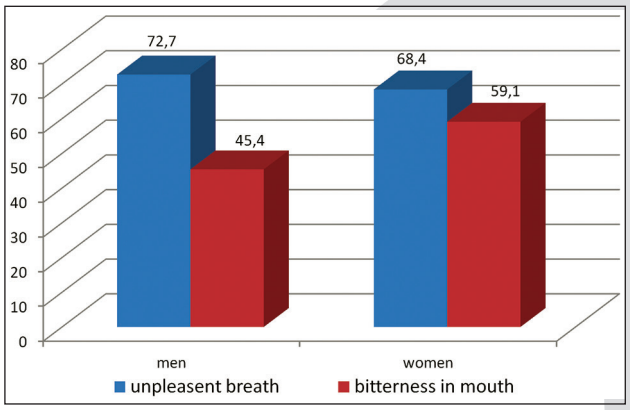
Table 2. The difference in smoking habits between the genders was tested on the model of Student's t-test

Smokers	moderate smokers*	medium moderate smokers **	Heavy smokers ***
Men	1,63	2,27	0,67
Women	0,45	1,34	1,79
Total	2,09	3,58	2,43

** moderate smokers 1-10 cigarettes per day,*
*** medium moderate smokers 11-20 cigarettes per day*
**** heavy smoker 21 or more cigarettes per day*

The highest percentage of moderate smokers was among female students - 47,4%, the highest percentage of moderate medium was among male students - 63,6%, while heavy smokers were among female students 13,2%, and among male students 9,1%.

The smokers themselves, perceive side effects of smoking on oral cavity. Cognition about the presence of bad breath and bitterness in the mouth is particularly present. There are differences in relation to gender, actually bad breath more bothers to men, but in addition to bad breath, and bitterness in the mouth bothers to women.



Graph 2. Side effects of smoking observed by smokers

Discussion and conclusion

The students group, with the age range between 18-26 years, presented a sample, and they voluntarily participated in the study, filling out the questionnaire. There were 120 students , involved in the study, with equal number of smokers and non-smokers.The smokers had significantly more frequent changes in the oral mucosa than non-smokers. The changes in the oral mucosa were found in 50% of smokers, and in 10 % of non-smokers.

The results of our study show that in the Federation of Bosnia and Herzegovina primary health care system is not yet able to satisfy all the population needs that would have a preventive character and lead to a better quality of life. Analyzing data on the use of health care we see that a larger number of visits do not lead to improvement of health which is in contrast with the results of most authors.[11][12]

The study included the 120 students at the University of Sarajevo, aged 18 to 26 years. They are classified into two groups: 60 students of smokers and 60 nonsmoker's students. This research represents an initial study that examines the influence of the harmful effects of nicotine. The smokers were divided into moderate (1-10 cigarettes per day), medium moderate (11-20 cigarettes per day) and heavy smokers (21 or more cigarettes per day), according to the number of cigarettes that they smoked per day. Differences in smoking status were statistically significant. The presence of medium moderate smokers is the largest 43.3%, it was lower the incidence of moderate smokers (40,0), while the prevalence of heavy smokers was lowest 11.7%

We found difference in the male smokers between medium moderate smokers and heavy smokers, on the level $p < 0.05$ ($t = 2.27$), between smokers of both genders on the level $p<0,05$ ($t=2,43$), and high significant difference on the level $p<0,0001$ ($t=3,58$) between medium moderate male smokers and all female smokers in total, by testing difference in smoking habits on the model of Student's t-test.

The highest percentage of moderate smokers was among female students - 47,4%, the highest percentage of moderate medium was among male students - 63,6%, while heavy smokers were among female students 13,2%, and among male students 9,1%. We examined the effect of cigarette smoke on the oral health of the young population of students of the University of Sarajevo. Results of Muhammad Asif and colleagues (2002) study are interesting. The study included 1,100 residents. The data show that 40.02% of respondents smoked different types of tobacco.[7] In their study, 36.66% of smokers among the 442 participants, smoked only for the company, while 57.01% of them smoked because of concerns and anxieties. There were 292 (66.06%) smokers who had a cough at night, 96 (21,72%) smokers had a cough during the day, but 54 (12,22%) had cough during the day and night. There were 125 (28,28%) smokers with unchanged sputum, while 43 (9,73%) smokers had occasionally or frequently blood-colored sputum. They found that 290 smokers (63.61%) smoked in public places.[9] The side effects of smoking on oral cavity and the presence of bad breath and bitterness in the mouth, especially in women are pointed out in our work. The smokers, themselves, noticed side effects of smoking on the oral cavity. Cognition about the

presence of bad breath and bitterness in the mouth is particularly present. There are differences in relation to gender, actually bad breath more bothers to men, but in addition to bad breath, and bitterness in the mouth bothers to women. There is bitterness in the mouth, in addition to bad breath, in the both genders. Hadžović Emir, in his master"s study: Predisposition and socio-demographic characteristics of smoking habits among the students of the University of Sarajevo "(1990)., found that 2.000 students of both sex had prevalence of smoking 46,8%,the percentage for male smokers was 46,8%, and 46,0%.[7] for female smokers. We investigated, in our study, student"s population with the same age, both sexes.

References

1. *Anonymous. Smoking. Sarajevo: Institute of Public Health of F BiH, 2009: 1-9.*
2. *Zalihić A, Mulaosmanović-Zalihić M. Tobacco smoking disease that kills. Sarajevo: Ministry of Canton Sarajevo, 2008; 4-28.*
3. *Omanić A, Omanić J, Pećanac N. Guide to Quitting Smoking. Sarajevo: Faculty of Medicine in Sarajevo; Institute of Social medicine, 2004.*
4. *Kecmanović D. Combating harmful and development of health lifestyles. Sarajevo: Svjetlost, 1989.*
5. *Tahirović S, Omanić A, Omanić J. Oral health, monitoring knowledge, attitudes, thinking and practice behaviors on samples of the population in Canton of Sarajevo. Sarajevo: Institute of Public Health of FBiH, 2004.*
6. *Omanić A, Mašić I, Kurtagić E. Programme for the prevention of smoking medicine students, dentistry, pharmacy and more medical schools, UMC-A in Sarajevo. III Symposium on Health Education, Proceedings - Pula in 1987.*
7. *Hadžović E. Predisposition and socio-demographic characteristics of smoking habits among students of Sarajevo University: Master's thesis. Sarajevo: Faculty of Medicine, University of Sarajevo, in 1990.*
8. *Pilav A. Risk factors and addictive habit of smoking, drinking alcohol and psychoactive substances among students of high schools in the Federation. "Health, human rights and the reform process." Book of abstracts; VI Social Medicine, Public health of the Federation of Bosnia and Herzegovina Sarajevo, 8-10. October 2006*
9. *Javorik N. Smoking as a risk factor for reduced mental and physical health of students of the Zagreb University: Doctoral dissertation. Zagreb:medical faculty of the University of Zagreb, 1988 Dimitrijević I. The Century Drugs: A guide to family and school. Beograd: s.n., 2007: str. 141.sr.scribd.com › Books - Nonfiction › Health & Lifestyle*
10. *Jaleel MA, Nooreen R, Parveen A, Abdul Hameed FN. Comparison of Population Survey of Multan about Cigarette Smoking with survey of Abbottabad. J Ayub Med Coll Abottabad 2002; 14(1): 16-9.*
11. *Public Health Institute of the Federation. A report on the health status of the population in the Federation of Bosnia and Herzegovina; 2008.*
12. *Džubur A, Kurspahić Mujčić A, Dilić M. Journal of the Institute for Research and Development Clinical Center University of Sarajevo – Impact of tobacco smoking status and presence of acute disease on the respondents quality of life; 2013.*

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Human lipopolysaccharide-responsive gene *HLrp*: characterization of the coding sequence and stimulation of expression by LPS and TNF-α

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Abstract

Inflammation plays a key role in many pathophysiological conditions. Exposure of human cells to bacterial lipopolysaccharide (LPS) potently initiates an inflammatory response, but the underlying mechanisms are unclear. Coding sequences of the human LPS-responsive gene *HLrp* were obtained from LPS-treated HEK293 cells. HLrp protein contains leucine-zipper domains and is thus a potential regulator of transcription. *HLrp* expression was robustly upregulated in cell culture following LPS or TNF-α treatment, and the protein was localized predominantly in cytoplasm, notably in association with the nuclear membrane. Expression levels were low in Hela cells and HepG2 cells, but were high in human dental papilla cells and in HEK293 cells. High levels of HLrp protein were detected in diverse primary human tissues including the liver, lung, the epithelia of the esophagus, stomach, colon, rectum, and small intestine, and in smooth muscles of the esophagus and rectum. Only low levels of expression were detected in breast and kidney. Because HLrp is probable regulator of transcription, and is induced by LPS and TNF-α, HLrp could modulate the cascade of inflammatory response gene expression.

Key words: Functional characterization; Lipopolysaccharide; Molecular cloning; Tissue expression

Introduction

Inflammation plays a key role in several pathophysiological conditions [1] and is thought to be induced by innate immune cells in response to infection and/or tissue damage [2]. The induction of an inflammatory response results in extensive recruitment of macrophages that participate in defense against foreign agents and tissue repair [3]. Lipopolysaccharide (LPS), an outer membrane component of the Gram-negative bacteria (GNB), has been widely used to induce inflammation and macrophage activation [4] and provides a well-established model for studies on the innate immune response [5]. Although LPS is a robust molecular signal for host recognition of Gram-negative pathogens [6], intense inflammatory responses to LPS can lead to endotoxic shock and death [7], and the risk of such adverse effects is dependent both on the level of LPS and on host susceptibility factors [7].

Inflammation is initiated and maintained by a network of chemokines, pro-inflammatory cytokines and anti-inflammatory mediators, including mitogen-activated protein kinase (MAPK) and nuclear factor-kB (NF-kB), that play distinct or shared biological activities [8]. Upon activation, macrophages produce cytokines [9] such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6, [10]. Excess production of these cytokines and pro-inflammatory mediators has been implicated in the pathoetiology of several inflammatory diseases including atherosclerosis, rheumatoid arthritis, asthma, pulmonary fibrosis, and septic shock [11]. Although the mechanisms underlying LPS-mediated induction of inflammation and cytokine expression remain obscure, it has been reported that several host gene loci modulate the LPS response, and also that inflammation is associated with changes in the expression levels of several genes that are likely to reflect host adaptation to bacterial infection and/or LPS [12, 13]. Given the importance of inflammatory disease, we sought to investigate the molecular mechanisms mediating the induction of inflammation by LPS. We report the characterization of the

cDNA sequence of the human LPS-responsive gene, *HLrp*, and the properties of the encoded protein.

Materials and methods

Materials

DEPC, HEPES, LPS (*E. coli*) and TNF-α were purchased from Sigma (Texas, USA). Plasmids pUC19, Triazol and DNA purification reagents were purchased from Invitrogen (California, USA). The reverse transcriptase kit was purchased from Promega (Madison, US) and the plasmid extraction kit (endotoxin-free) from Sangon (Shang'hai, China). Restriction endonucleases, *Taq* DNA Polymerase and T4 ligase were from Santa Cruz (Santa Cruz, USA). The enhanced chemiluminescence detection system was from Pierce (Rockford, USA). Human dental papilla cells HDPC, the HEK293, Hela and HepG2 cell lines, the BL21 (DE3) cell strain and plasmid pcTAT were provided by the Biochemistry and Molecular Biology Laboratory, Fourth Military Medical University (Xi'an, China). The protein chip of normal human tissues was purchased from ChaoYing company (Xi'an, China).

Primer sequences for amplifying HLrp gene

The *HLrp* gene was amplified using the upstream primer P1: (TCGGGTACCATGGCGA CGCGGGTAGAGG) and the downstream primer P2: (TCGTCTAGACACATCAAGGAACCA TCGTC). For cloning purposes the upstream and downstream primers contained *Kpn*I and *Xba*I restriction sites (underlined).

Cell culture

Human HEK293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) at 37°C under humidified 5% CO_2. Cells were exposed to LPS (100 mg/L) for 24 h in logarithmic growth phase.

RT-PCR amplification of HLrp cDNA

Total RNA was extracted from LPS-treated HEK293 cells using the TRIzol reagent. cDNA synthesis employed reverse transcriptase and oligo-dT. PCR with primers P1 and P2 (above) was for 30 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 40 s, and extension at 72°C for 40 s; a further extension step was performed at 72°C for 30 min. PCR products were separated by electrophoresis on 1% agarose, excised from the gel and recovered using a DNA purification kit. PCR products were digested with *Kpn*I and *Xba*I and inserted between the *Kpn*I and *Xba*I sites of vector pUC19 prior to transformation into *E. coli* BL21 cells. Transformants were confirmed by DNA sequencing. The insert of pUC19-*HLrp* was excised with *Kpn*I and *Sph*I and inserted between the *Kpn*I and *Sph*I sites of vector pcTAT prior to transformation into *E. coli* BL21 cells. The nucleotide sequence and the deduced amino acid sequence of *HLrp* were analyzed using software from the UCSC Genome Bioinformatics Site (http://genome.ucsc.edu).

Expression of HLrp and preparation of immune serum

Plasmid pcTAT-*HLrp* was transformed into *E. coli* BL21 cells and gene expression was induced with IPTG. 6His-TAT fusion proteins were purified by nickel-nitrilotriacetic acid (Ni-NTA) column chromatography and SDS-PAGE. Rabbits were immunized according to standard procedures with recombinant *HLrp* polypetide, boosted three times, and antisera to *HLrp* were collected.

Evaluation of LPS- and TNF-α-induced changes in HLrp expression by Western blotting

HEK293 cells were exposed to LPS (1mg/L) for 24 h or to TNF-α (1×10^6 U/L) for 2 h. Cells were lysed and protein extracts were prepared by standard techniques. Aliquots (50 mg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Rabbit polyclonal antibodies were used to detect *HLrp* polypeptides; blots were developed using horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and an enhanced chemiluminescence detection system.

Distribution of HLrp expression in normal human tissues

To determine the expression pattern of *HLrp* protein, a chip containing an array of immobilized extracts of normal human tissues was employed. Tissues represented included liver, kidney, lung, breast, epithelia from esophagus, stomach, colon, small intestine and rectum, and smooth muscle from esophagus and rectum. The presence of

HLrp was detected by immunohistochemistry. Tissues were fixed and blocked prior to the addition of rabbit anti-*HLrp* polyclonal antisera.

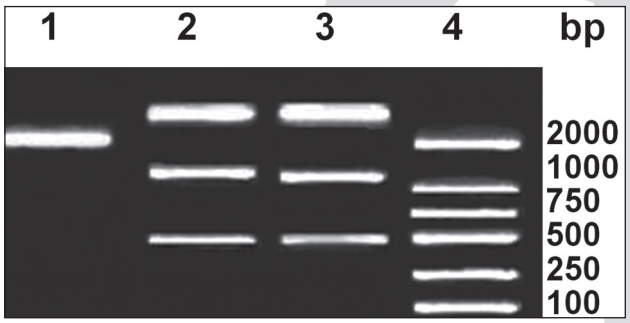
Detection of Hlrp expression in cell lines

Human dental papilla cells (HDPC), Hela cells, HepG2 cells and HEK293 cells in logarithmic growth phase were fixed and blocked prior to the addition of rabbit anti-HLrp polyclonal antisera. Bound antibody was detected using biotinylated goat anti-rabbit IgG, stained with streptavidin-biotin peroxidase complex (SABC), developed with FITC, and imaged using laser-scanning confocal microscopy.

Results

Isolation and analysis of HLrp cDNA

Primers for the amplification of *HLrp* cDNA were designed based on the GenBank sequence (accession NM_018360) and were used to PCR amplify total oligo-dT-primed cDNA from LPS-treated HKE293 cells. The full-length (2045 bp) cDNA for *HLrp* was cloned and sequenced. This was found to correspond to the Genbank sequence and contained the complete HLrp open reading frame (ORF) and the 5' and 3' untranslated regions (Figure 1A). The UCSC Genome Bioinformatics Database (http://genome.ucsc.edu) permitted



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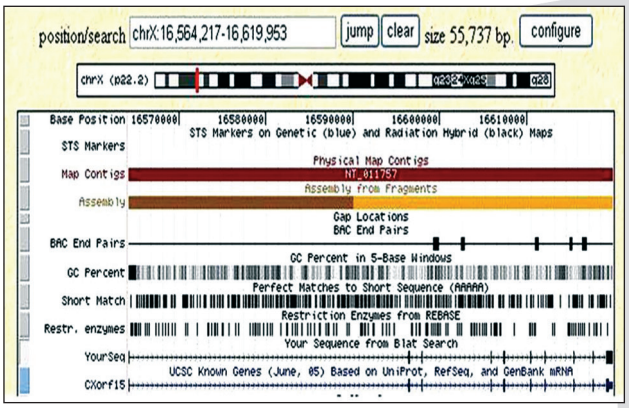

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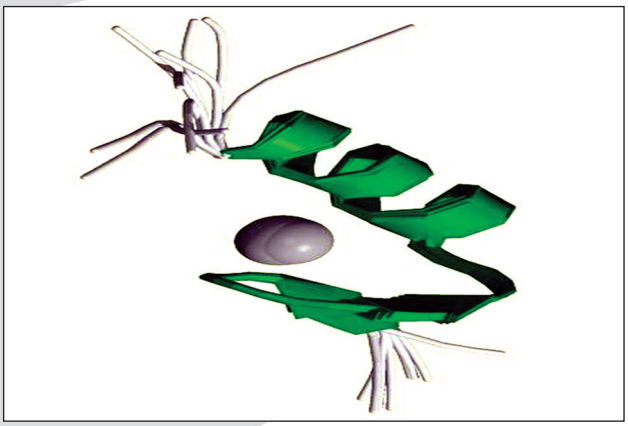

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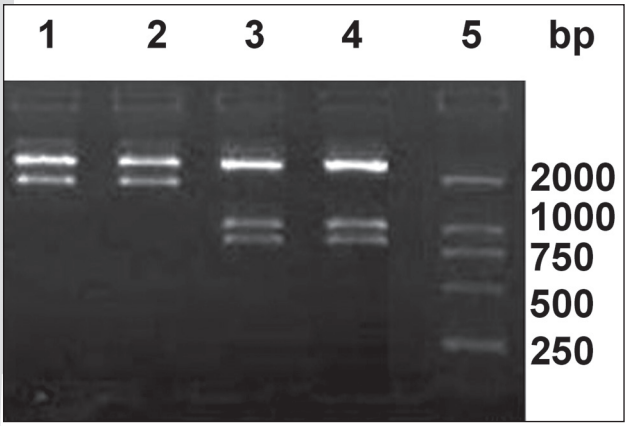

D

Figure 1. (A) Full-length HLrp cDNA sequence was amplified by RT-PCR from a cDNA prepared from HEK293 cells and confirmed by restriction enzyme digestion and gel electrophoresis. Lane 1, HLrp gene amplified from HEK293 cells; Lanes 2,3, pUC19-HLrp digested with PstI; Lane 4, TaKaRa DL2000 DNA marker. (B) Chromosomal location of the HLrp gene. The UCSC Genome Bioinformatics Database (http://genome.ucsc.edu) identified the location of the HLrp gene at p22.2 on the X chromosome X. (C) Analysis of functional domains with conserved leucine-zipper motifs in HLrp polypeptide (D) Confirmation of recombinant plasmids containing HLrp cDNAs by restriction enzyme digestion and gel electrophoresis. Lanes 1,2, pcTAT-HLrp digested with KpnI and SphI; Lanes 3,4, pcTAT-HLrp digested with KpnI and EcoRI; Lane 5: TaKaRa DL2000 DNA marker.

localization of the *HLrp* gene to p22.2 on the X chromosome (Figure 1B). The full length *HLrp* cDNA ORF encodes a protein of 528 amino acids (Figure 1B) containing leucine-zipper regions (L-x(6)-L-x(6)-L-x(6)-L) (Figure 1C).

Expression of HLrp protein in E.coli

To characterize the HLrp polypeptide, the full-length cDNA was introduced into the pcTAT expression vector to generate a hybrid coding sequence in which *HLrp* sequences were fused in frame to 6His-TAT. The integrity of recombinant plasmid pcTAT-*HLrp* was confirmed by restriction enzyme digestion with *Kpn*I and *Sph*I (or *Kpn*I and *Eco*RI) (Figure 1D). The 6His-TAT-HLrp fusion protein was then expressed in *E. coli* where the expressed products were present in inclusion bodies (Figure 2A). The polypeptides were purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography and used to immunize rabbits. Titres of the antisera obtained exceeded 1:1000 (Figure 2B).

Effects of LPS and TNF-α on HLrp expression

HEK293 cell lines were treated with LPS or TNF-α and levels of HLrp were quantitated by Western blotting using antisera directed against the recombinant protein. Expression levels of HLrp were very low in untreated control cells, but HLrp was strongly upregulated both after LPS treatment (Figure 2C) and after TNF-α treatment (Figure 2D)

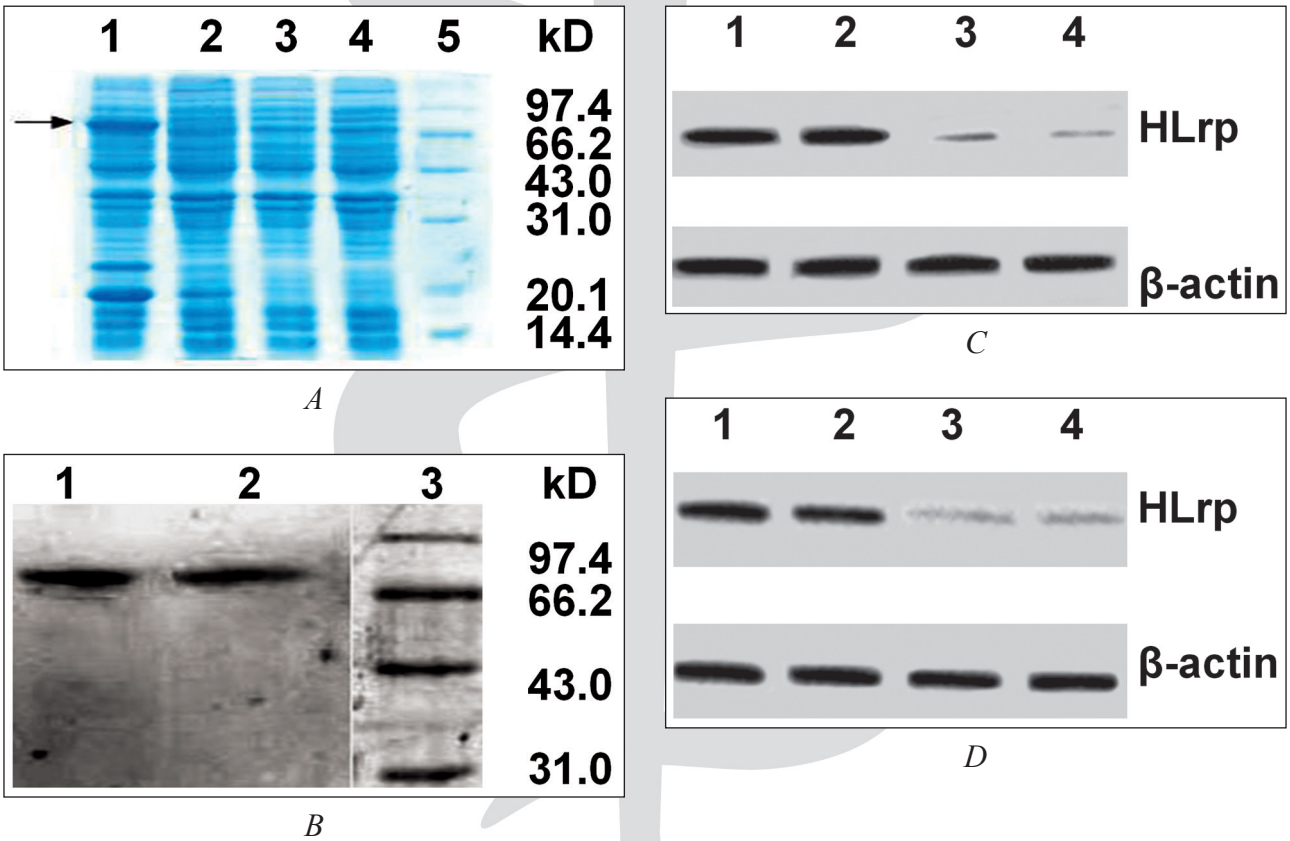

Figure 2. (A) Expression of 6His-TAT-HLrp in E. coli. The 6His-TAT coding sequence was fused in-frame with the HLrp cDNA ORF; the fusion protein was expressed in E. coli and purified by a Ni-NTA column. Lane 1, pcTAT-HLrp/BL21 (DE3) with IPTG induction; lane 2, pcTAT-HLrp/BL21 (DE3) without IPTG induction; lane 3, pcTAT/BL21 (DE3) with IPTG induction; lane 4, pcTAT/BL21 (DE3) without induction; lane 5, protein markers. (B) Western blot analysis of HLrp expression. Lanes 1,2, pcTAT-HLrp/BL21(DE3) with IPTG induction; lane 3, protein markers; (C) Western blotting of HLrp proteins in HEK293 cells exposed to LPS. Lanes 1,2, HEK293 cells exposed to LPS; Lanes 3,4, untreated HEK293 cells. (D) Western blot analysis of HLrp proteins in HEK293 cells exposed to TNF-α. Lanes 1,2, HEK293 cells exposed to TNF-α; lanes 3,4, untreated HEK293 cells.

Distribution of HLrp expression in human tissues

The tissue profile of HLrp expression was determined using a tissue microarray technique and immunohistochemistry. High levels of HLrp protein were detected in diverse primary human tissues including liver, lung, the epithelia of the esophagus, stomach, colon, rectum, lung, and small intestine, and in smooth muscles of the esophagus and rectum (Figure 3). Only low levels of expression were detected in breast and kidney.

Expression and subcellular localization of HLrp in different cell lines

To determine the subcellar distribution HLrp protein, different cell lines were probed with anti-HLrp polyclonal antisera (Materials and Methods). HLrp was found to predominantly located in the cytoplasm but appeared to be most densely distributed around the nuclear membrane in HDPC (Figure 4A), Hela (Figure 4B), HepG2 (Figure 4C) and HEK293 (Figure 4D) cells. Expression levels of HLrp were low in Hela and HepG2 cells, but were high in human dental papilla cells and HEK293 cells.

Discussion

Tissue invasion by microorganisms is known to activate a potent inflammatory response [14]. Detection of bacterial molecular markers, including the pathogen-associated molecular pattern (PAMP), is mediated by innate pattern recognition receptors (PRRs) [14], and PRR activation leads to upregulation of the transcription of genes encoding inflammatory mediators that coordinate the elimination of pathogens and infected cells [14].

Host defense against invading Gram-negative bacteria depends on innate immune recognition of LPS [15]. LPS induces an increase in body tempe-

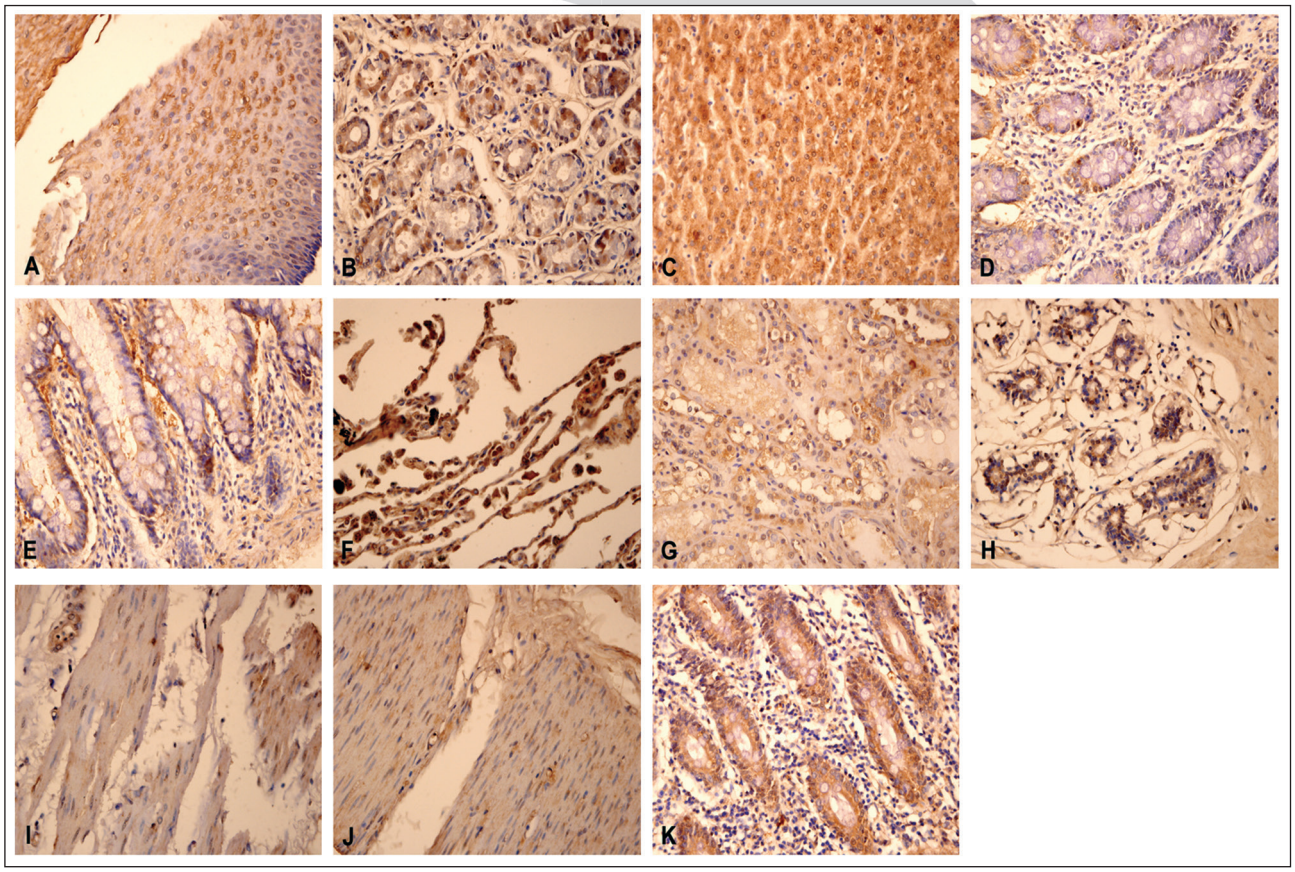

Figure 3. Expression profile of HLrp protein in normal human tissues. (A) Esophagus epithelium, (B) stomach epithelium, (C) liver, (D) colon epithelium, (E) small intestine epithelium, (F) lung, (G) kidney, (H) breast, (I) esophagus smooth muscle, (J) rectum smooth muscle, (K) rectum epithelium. Original magnification, ×200. Expression levels were high in all tissues with the exception of kidney and breast where only low levels of expression were detected.

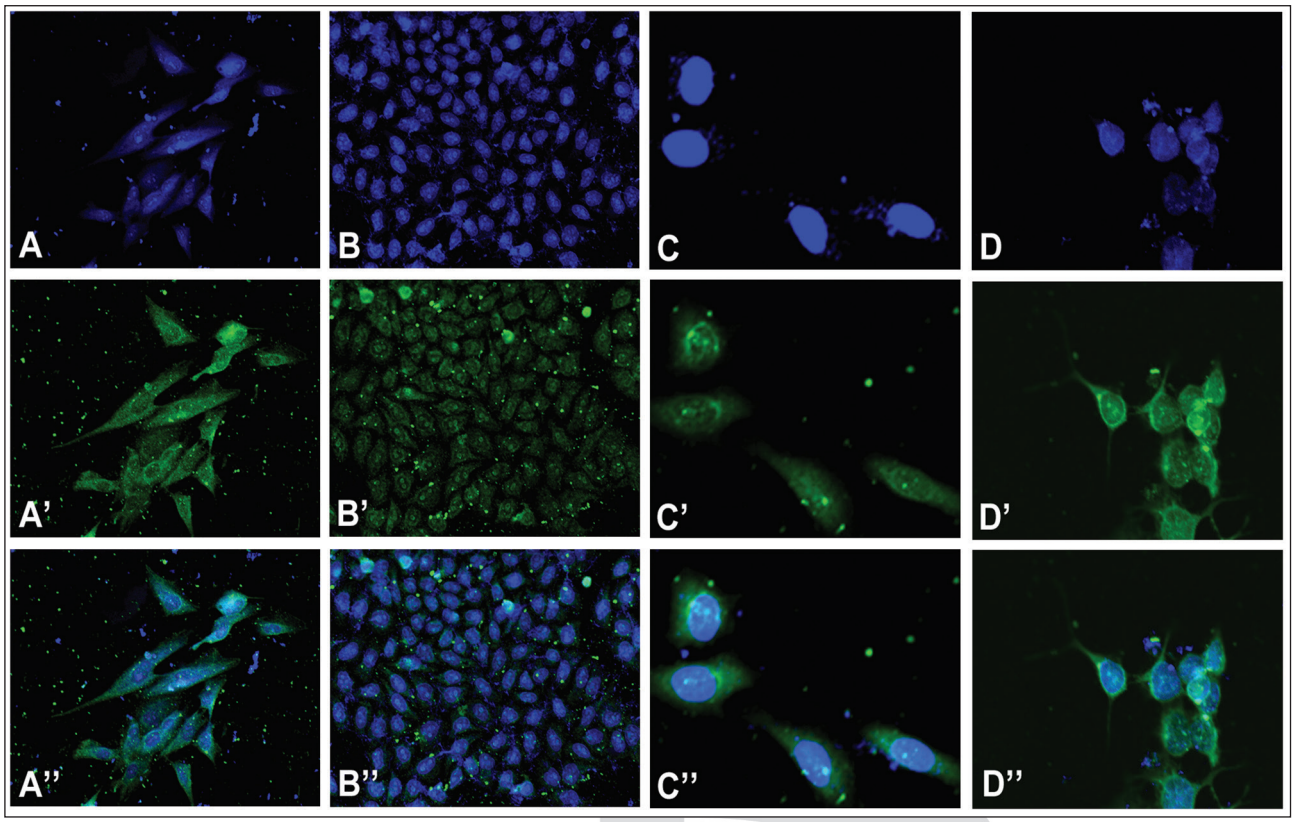

Figure 4. Intracellular location HLrp protein in four cell lines by laser scanning confocal fluorescence microscope. (A) human dental papilla cells cells, (B) HeLa cells, (C) HepG2 cells, (D) HEK293 cells. Original magnification, ×400. A–D: blue fluorescence, nucleus; A'–D': green fluorescence, HLrp protein; A''–D'': superimposition of green and blue fluorescence.

rature, malaise, and increased secretion of cortisol and cytokines [5]. LPS stimulation of mammalian cells takes place via multiple pathways [16], and macrophage activation leads to the secretion of proinflammatory cytokines including TNF-α, IL-6, and IL-1β [17]. Although these cytokines play an important role in mediating host defense, persistent overexpression of TNF-α, IL-6, and IL-1β in chronic inflammatory disease leads to a spectrum of adverse effects including changes in cell adhesion molecules and angiogenesis [18].

The mechanisms underlying the induction of inflammation by LPS are not well understood, and the extent of the inflammatory response is modulated by both environmental factors and the genetic susceptibility of each individual [19]. We report the cloning and characterization of human sequences for *HLrp*, a gene whose expression is induced by LPS and TNF-α. The *HLrp* gene is located at p22.2 on the X chromosome and the full length *HLrp* cDNA encompasses an ORF encoding a protein of 528 amino acids.

Sequence analysis revealed that the HLrp protein contains a leucine-zipper region, a a common 3D structural motif that has been implicated in the regulation of diverse aspects of physiology including cell survival, learning and memory, cancer progression, lipid metabolism, and development [20, 21]. Leucine-zipper factors constitute one of the most important classes of enhancer-type transcription factors [22].

HLrp protein is strongly upregulated by LPS treatment and, in view of its conserved leucine-zipper domains, is a potential transcriptional regulator. It is therefore possible that *HLrp* is involved in transcriptional regulation following activation of the LPS signaling pathway. However, it is not known whether HLrp upregulation is involved in the induction of the inflammatory response or is a downstream consequence of inflammation. However, we also report that HLrp expression is rapidly stimulated by TNF-α, suggesting that HLrp is likely to play a role in TNF-α-induced inflammation. TNF-α is known to stimulate the production

of many proinflammatory cytokines [23,24], and high plasma levels of TNF-α are associated with an increased risk of developing inflammatory disease and of morbidity and mortality [25,26].

Immunohistochemistry revealed that HLrp is predominantly localized in the nuclear membrane and cytoplasm of human dental papilla cells and of Hela, HepG2 and HEK293 cell lines. Expression levels of HLrp were low in Hela and HepG2, but high in dental papilla cells and HEK293. High levels of HLrp protein were detected in diverse primary human tissues including liver, lung, the epithelia of the esophagus, stomach, colon, rectum, lung, and small intestine, and in smooth muscles of the esophagus and rectum. Only low levels of expression were detected in breast and kidney. In inflammatory bowel disease it has been proposed that the production of TNF-α, IL-6, and IL-1β is a consequence of infiltration of inflammatory cells [27], and the widespread distribution of HLrp suggests that it plays a role in LPS- and/or TNF-α-induced inflammation.

Previous studies revealed that the sequence of HLrp polypeptide is highly homologous to γ-taxilin/FIAT, molecules described by other groups [28, 29]. Taxilin is a novel syntaxin-binding protein that contains a long coiled-coil region in its C-terminal half. Database searching revealed the presence of two other molecules with a long coiled-coil region homologous to that of taxilin in mammals, arguing that there are three distint members of the mammalian taxilin family [28]. FIAT, a nuclear leucine-zipper protein described previously, was reported to interact with ATF4 to inhibit ATF4 binding to DNA, thereby inhibiting ATF4-mediated transcription of the osteocalcin gene *in vitro*. Transgenic mice overexpressing FIAT in osteoblasts had reduced osteocalcin expression and decreased bone mineral density [20, 29]. Although members of the taxilin family have been implicated in transcriptional and translational control [30, 31], few studies have addressed the potential involvement of taxilins in inflammation.

In summary, we report cDNA and polypeptide sequences for the human LPS-inducible gene *HLrp*. HLrp polypeptide is predominantly localized in the cytoplasm and nuclear membrane, and contains conserved leucine-zipper domains, a structural motif commonly found in transcriptional regulators. This suggests that HLrp expression induced by LPS could potentially contribute to the inflammatory response. Further research will be required to determine if pharmacologic intervention in HLrp pathways might be of therapeutic value in septic shock induced by endotoxin exposure. To our knowledge, this is the first evidence of *HLrp* involvement in LPS and/or TNF-α inflammatory pathways and provides a foundation for further in-depth studies on the function of *HLrp*.

Acknowledgements

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Abbreviations used are: FBS, fetal bovine serum; GNB, Gram-negative bacterium; HLrp, human lipopolysaccharide responsive gene; IL-1β, interleukin-1β; IL-6, interleukin-1; LBP, LPS-binding protein; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-kB, nuclear factor-kB; PAMP, pathogen-associated molecular pattern; PRRs, pattern recognition receptors; SABC–FITC, streptavidin-biotin peroxidase complex–fluorescein isothiocyanate; TNF-α, tumor necrosis factor-α.

References

1. *Nathan C. Points of control in inflammation. Nature. 2002; 420: 846–852.*
2. *Foster SL, Medzhitov R. Gene-specific control of the TLR-induced inflammatory response. Clin Immunol. (Orlando, FLA). 2009; 130: 7–15.*
3. *Molloy RG, Mannick JA, Rodrick ML. Cytokines, sepsis and immunomodulation. Br J Surg. 1993; 80: 289–297.*
4. *Nagy G, Pál T. Lipopolysaccharide: a tool and target in enterobacterial vaccine development. Biol. Chem. 2008; 389: 513–520.*
5. *Bahador M, Cross AS. From therapy to experimental model: a hundred years of endotoxin administration to human subjects. J Endotoxin Res. 2007; 13: 251-279.*
6. *Beutler B, Hoebe K, Du X, Ulevitch RJ. How we detect microbes and respond to them: the Toll-like receptors and their transducers. J Leukoc Biol. 2003; 74: 479–485.*

7. *Freudenberga MA, Tchaptchet S, Kecka S. Lipopolysaccharide sensing an important factor in the innate immune response to Gram-negative bacterial infections: benefits and hazards of LPS hypersensitivity. Immunobiology. 2008; 213: 193–203.*

8. *Schultz H, Weiss JP. The bactericidal/permeability-increasing protein (BPI) in infection and inflammatory disease. Clin Chim Acta. 2007; 384: 12–23.*

9. *Opal SM. The host response to endotoxin, antilipopolysaccharide strategies, and the management of severe sepsis. Int J Med Microbiol. 2007; 297: 365–377.*

10. *Rovina P, et al. Transcriptional repression of ceramide kinase in LPS-challenged macrophages. Biochem Biophys Res Commun. (2010), doi:10.1016/j.bbrc.2010.09.041*

11. *Beutler B, Moresco EM. The forward genetic dissection of afferent innate immunity. Curr Top Microbiol Immunol. 2008; 321: 3–26.*

12. *Zhu F, Yan W, Zhao ZL. Improved PCR-based subtractive hybridization strategy for cloning differentially expressed genes. Biotechniques. 2000; 29: 310–313.*

13. *Du KJ, Chai YB, Hou LC, et al. Over-expression and siRNA of a novel environmental lipopolysaccharide-responding gene on the cell cycle of the human hepatoma-derived cell line HepG2. Toxicology 2008; 243: 303–310.*

14. *Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell 2010: 140; 805-20.*

15. *Gioannini TL, Weiss JP. Regulation of interactions of Gram-negative bacterial endotoxins with mammalian cells. Immunol Res. 2007; 39: 249–260.*

16. *Miyake K. Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. Semin Immunol. 2007; 19: 3–10.*

17. *Lin WW, Karin M. A cytokine-mediated link between innate immunity inflammation, and cancer. J Clin Invest. 2007; 117: 1175–1183.*

18. *Surh YJ, Chun KS, Cha HH, et al. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. Mutat Res. 2001; 480–481: 243–268.*

19. *LeVan TD, Bloom JW, Bailey TJ. A common single nucleotide polymorphism in the CD14 promoter decreases the affinity of Sp protein binding and enhances transcriptional activity. J Immunol. 2001; 167: 5838–5844.*

20. *St-Arnaud R, Elchaarani B. Identification of additional dimerization partners of FIAT, the factor inhibiting ATF4-mediated transcription. Ann NY Acad Sci. 2007; 1116: 208–215.*

21. *Ameri K, Harris AL. Activating transcription factor 4. Int J Biochem Cell Biol. 2008; 40: 14–21.*

22. *Deppmann CD, Alvania RS, Taparowsky EJ. Cross-species annotation of basic leucine zipper factor interactions: insight into the evolution of closed interaction networks. Mol Biol Evol. 2006; 23: 1480–1492.*

23. *Thiemermann C, Wu CC, Szabo C, et al. Role of tumor of tumor necrosis factor in the induction of nitric oxide synthase in a rat model of endotoxin shock. Br J Pharmacol. 1993; 110: 177-182.*

24. *Tracey KJ, Cerami A. Tumor necrosis factor, other cytokines and disease. Annu Rev Cell Biol. 1993; 9: 317-343.*

25. *Samuel IM, Robert KE, Martin WB. LPS, TLR4 and infectious disease diversity. Nat Rev Microbiol. 2005; 3: 36–46.*

26. *Noguchi M, Hiwatashi N, Liu Z, Toyota T. Secretion imbalance between tumour necrosis factor and its inhibitor in inflammatory bowel disease. Gut. 1998; 43: 203–209.*

27. *Trent MS, Stead CM, Tran AX, Hankins JV. Diversity of endotoxin and its impact on pathogenesis. J Endotoxin Res. 2006; 12: 205–223.*

28. *Satoru N, Sachie S, Sawako TN. Identification and characterization of taxilin isoforms. Biochem Biophys Res Commun. 2004; 319: 936–943.*

29. *Vionnie WCY, Gourgen A, Lieve V. FIAT represses ATF4-mediated transcription to regulate bone mass in transgenic mice. J Cell Biol. 2005; 169: 591-601*

30. *Yoshida K, Nogami S, Satoh S, et al. Interaction of the taxilin family with the nascent polypeptide-associated complex that is involved in the transcriptional and translational process. Genes Cells. 2005; 10: 465-476.*

31. *Oba-Shinjo SM, Bengtson MH, Winnisehofer SM, et al. Identification of novel differentially expressed genes in human astroeytomas by cDNA representational difference analysis. Brain Res Mol Brain Res. 2005; 140: 25-33.*

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High incidence of preventable deaths during childhood in Porto Velho, Rondonia - Brazil

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Abstract

Background: We aimed to estimate and analyze epidemiological profile of deaths during childhood.

Method: We used the databases of deaths provided by Information System of mortality between 2006 and 2010. We selected only those records where the occurrence of deaths corresponded to Porto Velho city and those who were aged between 12 and 48 months old at the moment of death. We examined only deaths from preventable causes and poorly defined.

Results: The total number of deaths was estimated to be 103, 48.6% male. High frequencies of deaths occurred (39.8%) in children before reaching two years old. Vast majority of deaths (66.9%) was due to preventable causes, 18.4% by poorly defined causes and nearly one in three by external causes. Approximately one in four were due to traffic accidents, 41.9% by drowning and submersion. Also, there were significant frequencies of deaths associated with respiratory diseases (17.5%) and infectious and parasitic diseases (16.6%).

Conclusion: These findings reinforce the importance of studies of infant mortality, drawing attention to the debate on policy design to reduce childhood deaths, especially in acting on preventable causes.

Key words: Mortality; Child Health; Public Policy.

Background

One way to evaluate the policies and actions of health care is through studies regarding the distribution of deaths in a population, the identification of the most vulnerable issue to health problems and definition of the main problems. These aspects guide the allocation of resources and assist the planning, management and health evaluation. [1]

Wagstaff, [2] studying the magnitude and causes of socioeconomic inequalities in mortality of children under five years old from nine countries found that inequalities were higher than the corresponding mortality of infants, and were quite pronounced in Brazil and relatively high in Nicaragua and the Philippines. Inequalities in mortality in children under 5 years old were lower in Cote d'lvoire, Ghana, Pakistan and Vietnam. These results reflect intense inequalities in mortality between socioeconomic groups, with deaths concentrated among the most disadvantaged.

The biological determinants of death in childhood are subject to environmental, social and economic conditions and the availability of health services. [3] Rutstein et al. [4] indicate that the death is linked to the economic structure, social health of the country, and it is also associated with medical care. These authors proposed a method for evaluating the quality of medical care and created the concept of preventable death.

The preventable deaths in infancy occur for lack of adoption of individual and social actions, when compared to other observed populations, whose indexes are shown in low or reduction process. [5] Avoidable death is an excellent indicator of the quality of access to health services and indicates the perverse face of mortality manifestation, especially in children. [6]

The prevention of deaths is crucial to understanding the changes that occur in the structure of mor-

tality, as to distinguish the causes that are preventable by certain actions of health sector interventions for those preventable through public policies, as well as to evaluate access to health services and provide support for interventions priority problems observed. [7] Similarly, another study claims that the estimate and profile analysis of infant mortality may subsidize aims for good health planning. [8]

In view of the above considerations, this study aimed to estimate and analyze the epidemiological profile of deaths during childhood in Porto Velho, RO, Brazil.

Method

This is a descriptive study, we used the databases of deaths provided by the Information System of mortality between 2006 and 2010. The study was approved by the Ethical Committee in Research (Protocol CAAE No. 042/2010). We selected only those records where the deaths corresponded to the Porto Velho city and those who were aged 12 to 48 months old at the moment of death. We examined only deaths from preventable causes and poorly defined.

In this study we considered as the causes of avoidable deaths, only the following classifications: (I) – reduced through appropriate actions to diagnosis and treatment, and (II) reduced through appropriate actions to promote health, linked to appropriate actions to attention of health. To the poorly defined causes of death we considered: (I) signs, symptoms and abnormal findings of clinical and laboratory not elsewhere classified. In others cases, the classification considered the list of preventable deaths of the Brazilian Health System proposed by Malta et al. [9]

The descriptive analysis of deaths was presented by frequency tables.

Results

The total number of deaths by cause was estimated to be 103, 51.4% female. There were high rates of deaths (39.8%) in children before reaching two years old. Vast majority of deaths (66.9%) was due to preventable causes, poorly defined causes comprised 18.4%. We observed that around one in four was due to traffic accidents, 41.9% by drowning and submersion. There were large discrepancies between the extremes of the years studied, i.e. the coefficient from 5.7 deaths in 2006 to 8.1 (per 10,000 inhabitants) in 2010, an increase of 70%. Table 1 shows the results of distribution of deaths of children.

The epidemiological profile of deaths remained during the first three consecutive years. On the other hand, it appeared generally decreased during 2010. Table 2 shows the results of distribution of deaths of children aged 1 to 4 years old, according to specific causes.

Table 1. Distribution of deaths of children aged 1 to 4 years old, according to the variables investigated

Socio-demographic variables	Year										Total	
	2006		2007		2008		2009		2010			
	n*	%	n*	%	n*	%	n*	%	n*	%	n*	%
Age (year)												
1	10	50.0	5	45.5	11	39.3	11	52.4	4	17.4	41	39.8
2	3	15.0	4	36.4	6	21.4	5	23.8	4	17.4	22	21.4
3	1	5.0	2	18.1	8	28.6	4	19.0	9	39.1	24	23.3
4	6	30.0	-	-	3	10.7	1	4.8	6	26.1	16	15.5
Gender												
Male	12	60.0	7	63.6	16	57.1	10	47.6	5	21.7	50	48.5
Female	8	40.0	4	36.4	12	42.9	11	52.4	18	78.3	53	51.5
Race												
White	8	42.1	4	44.4	11	47.8	4	22.2	10	45.5	37	40.7
Black	11	57.9	5	55.6	12	52.2	14	77.8	12	54.5	54	59.3

Source: SIM – Dept. Epidemiology/SEMUSA, 2012. We used the rate per 10,000 inhabitants.

Table 2. Distribution of deaths of children aged 1 to 4 years old, according to specific causes

Specific Cause	Year									
	2006		2007		2008		2009		2010	
	n*	%	n*	%	n*	%	n*	%	n*	%
Respiratory diseases	5	25.0	2	18.2	-	-	5	23.8	6	26.0
Infectious and parasitic diseases	3	15.0	3	27.3	2	7.1	6	28.6	3	13.1
Poorly defined causes	4	20.0	-	-	7	25.0	2	9.5	6	26.0
External Causes	6	30.0	4	36.3	15	53.6	3	14.3	3	13.1
Neoplasias	-	-	2	18.2	3	10.7	3	14.3	1	4.5
Others	2	10.0	-	-	1	3.6	2	9.5	4	17.3
Total	**10**	**100.0**	**11**	**100.0**	**28**	**100.0**	**21**	**100.0**	**23**	**100.0**

Source: SIM – Dept. of Epidemiology/SEMUSA, 2012. Underlying cause of death according to the International Statistical Classification of Diseases - Tenth Revision (ICD-10). We used the rate per 10,000 inhabitants.

Table 3. Distribution of deaths of children aged 1 to 4 years old, according to external causes

Specific Causes	Year										Total	
	2006		2007		2008		2009		2010			
	n*	%	n*	%	n*	%	n*	%	n*	%	n*	%
Transport accidents	2	28.6	1	25.0	4	26.7	-		1	33.3	8	25.8
Accidental drowning and submersion	3	42.9	1	25.0	6	40.0	2	66.7	1	33.3	13	41.9
Exposure to smoke, fire and flames	1	14.3	1	25.0		0.0	-		-	-	2	6.5
Others	1	14.3	1	25.0	5	33.3	1	33.3	1	33.3	8	25.8
Total	**7**	**100.0**	**4**	**100.0**	**15**	**100.0**	**3**	**100.0**	**3**	**100.0**	**31**	**100.0**

Source: SIM – Dept. of Epidemiology/SEMUSA, 2012. Underlying cause of death according to the International Statistical Classification of Diseases - Tenth Revision (ICD-10). We used the rate per 10,000 inhabitants.

Table 4. Distribution of proportional deaths in 1-4 years old children

Deaths	Year										Total	
	2006		2007		2008		2009		2010			
	n*	%	n*	%	n*	%	n*	%	n*	%	n*	%
Preventable	14	70.0	9	81.8	17	60.7	15	71.4	14	60.8	69	67.0
Poorly defined	4	20.0	-	-	7	25.0	2	9.6	6	26.0	19	18.4
Not preventable	2	10.0	2	18.2	4	14.3	4	19.0	3	13.2	15	14.6
Total	**7**	**100.0**	**4**	**100.0**	**15**	**100.0**	**3**	**100**	**3**	**100.0**	**31**	**100.0**

Source: SIM – Dept. of Epidemiology/SEMUSA, 2012. Brazilian List of Preventable Deaths. We used the rate per 10,000 inhabitants.

The data in Table 3 suggest that the vast majority of deaths from external causes occurred in children between one and two years old, with decreasing trend in the period of the study.

Table 4 presents the results of proportional distribution of preventable, poorly defended and not preventable deaths in children between 1 and 4 years old.

Discussion

Considering that there are few studies that explore death data provided by the Information System of mortality in Brazil, mainly in Rondônia, this investigation was undertaken to evaluated epidemiological profile of deaths during childhood in Rondônia. In the city of Porto Velho, in the period studied, more black children died from one to

four years old compared to white. This finding is in agreement with the socially expected in many countries of the world, particularly in Brazil [10] and the U.S. [11] In these countries there are a significant number of black people whose families have low income and poor health care services and medication, which may be part of explaining mortality in black children.

Moreover, the present study found that more female children died when compared to male, which is against with what is biologically expected, i.e. there are more deaths in male gender compared to females, in all age groups. In this perspective, research asserts that the male gender is a risk factor for mortality in all stages of human life. [12-14]

Besides this differential mortality between the genders of children in the study, the low number of deaths in 2007 compared to other years is typical of underreporting cases of information, suggesting the need for a commitment to provide complete and trustful data by professionals qualified to perform such function. Also, according to Duarte (1992), [15] mortality statistics are affected by the low coverage of the information system and / or the under-reporting of information or poor quality of death certificates, specifically regarding the causes of death. The death declarations when not properly filled preclude analyzes of mortality that serve to delineate the health profile of the population in a particular region.

In this study, we found that the one of the main causes of deaths were caused by external causes (30%) and by infectious and / or parasitic (16.6%) in children of Porto Velho. These findings corroborate the data in others Brazilian states [16-17] and in Porto Velho city. [9]

Almost half of the deaths due to external causes in this study were by drowning and / or submersion. Accidents affect children because they are more exposed to factors risk such as, for example, swimming pools and rivers baths. The high incidence of deaths from this accident in particular the deaths of children in swimming pools and rivers baths, can be understood as a public health issue, and can be avoided when protective and monitoring measures are taken with the child by responsible and / or caregiver. [18]

In this direction, another research performed in one year old regarding drowning indicates that it is a result of lack of skill in the aquatic environment added with parents and guardians carelessness. [19] High percentage of deaths (67.0%) are estimated as preventable, in this study, if health promotion actions were adopted and / or if they had adequate actions of diagnosis and treatment, and according to a previous study, these high percentages of deaths suggest problems of access to health services, coverage and / or quality of care. [20]

It was also high (18.4%) the incidence of deaths due to poorly defined causes in this study. This incidence of deaths is considered as an indicator of quality of information about death causes and their occurrence, pointing to the need for investments in diagnostic and therapeutic resources, as well as the training of professionals for the correct completion of death certificates. [21] Moreover, in some developed countries the incidence of deaths due to poorly defined causes is less than 1%. Brazil is considered by the World Health Organization as having mid-level quality of mortality information system. [22]

There was a very important increase (70%) in mortality rate between 2006 and 2010 despite the improvement in living conditions in Porto Velho, from 2004, with improvements in urbanization, sanitation, nutrition and education, as well as improvements in the performance of health services with the expansion of the Family Health Strategy, the increased number of hospital beds and the quality of care.

Therefore, one may consider that Porto Velho city showed no improvements on economic health required for satisfaction of its population in the researched period. However, there was a reduction in infant mortality in Brazil, although there are regional disparities, which persist in the high rates of mortality in children under five years old in some regions of the country, such as Porto Velho city, Rondônia. [23, 24]

Although our study presents limitations because it was based on secondary data, our findings seem to reflect the present situation in other cities with characteristics of health services similar to Porto Velho, Rondônia, reiterating the need to evaluate the effectiveness of care provided, especially if it is considered that deaths could be avoided by health care measures. The absence of a network of verification services structured deaths makes the basic causes still poorly defined persistent in the city.

Conclusion

This study offers further evidence that reinforce the importance of studies of mortality infant, drawing attention to the debate on policy design to reduce deaths in childhood, working in particular on preventable causes.

Author's contribution

All authors participated in the revision of the manuscript. All authors determined the design, interpreted the text and drafted the manuscript. All authors read and gave final approval for the version submitted for publication.

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References

1. *Macmahon B, Trichopoulos D. Epidemiology principles and methods. NY: Little Brown and Company; 1996.*
2. *Wagstaff A. Socioeconomic inequalities in child mortality: comparisons across nine developing countries. Bull World Health Organ 2011; 78: 19-29.*
3. *Afolabi BM, Clement CO, Ekundayo A, Dolapo D. A hospital-based estimate of major causes of death among under-five children from a health facility in Lagos, Southwest Nigeria: possible indicators of health inequality. Int J Equity Health 2012; 11: 39.*
4. *Rutstein DD, et al. Measuring the quality of medical care. New England J Medic 1976; 294: 582-98.*
5. *Xavier PB. Avoidable mortality in Hunter-SC, from 1980 to 2003. Profile and trends. Joacaba: SC Press; 2005.*
6. *Samico I, Hartz ZMA, Felisberto E, Frias PG. The situation room of the family health unit: the Information System of Primary Care (SIAB) as a tool for strategic planning site. Saúde Debate 2002; 26: 236-244.*
7. *Vermelho LL, Monteiro MFG. Demographic and epidemiological transition. Sao Paulo: Atheneu; 2005.*
8. *Chiavegatto Filho ADP: Mortality from hypertensive disease in women 20 to 49 years in São Paulo, SP, Brazil. Rev Brasil Epidemiolog 2004; 7: 252-258.*
9. *Malta DC. List of causes of avoidable mortality interventions Health System of Brazil. Epidemiol Serv Saúde 2007; 16: 233-244.*
10. *Barros FC, Victora CG, Horta BL. Ethnicity and infant health in Southern Brazil. A birth cohort study. Int J Epidemiol 2001; 30: 1001-8.*
11. *Pearce N. Genetics, race, ethnicity and health. BMJ 2004; 328: 1070-2.*
12. *Ferreira CEC. Infant mortality and social inequality in São Paulo. thesis. Universidade de São Paulo, Faculdade de Saúde Pública da USP; 1990.*
13. *LaurentI R: Health Statistics. São Paulo: EPU/ EDUSP; 1985.*
14. *Pereira MG. Epidemiology: Theory and Practice. Rio de Janeiro: Guanabara Koogan; 2002.*
15. *Duarte MCR. Quality of life and health indicators: Aspects of infant mortality in the state of Rio de Janeiro and its regions. Cad Saúde Púb 1992; 8: 414-427.*
16. *Jodas AD. Avoidable mortality in children under five years: Sentinel of the quality of primary care in Maringá-PR. PhD thesis. Universidade Estadual de Maringá, Health Sciences; 2010.*
17. *Murakami GF, Guimarães MJB, Sarinho SW. Sociodemographic inequalities and causes of death in children under five years in the state of Pernambuco. Rev. Brasileira Materno infantile 2011; 11: 139-152.*
18. *Sekii H, Ohtsu T, Shirasawa T, Ochiai H, Shimizu T, Kokaze A. Childhood Mortality Due to Unintentional Injuries in Japan, 2000–2009. Int. J. Environ. Res. Public Health 2013; 10: 528-540.*
19. *Paes CEN, Gaspar VLV. Safety, prevention of accidents, injuries, home accidents, home safety, injury control. Ped 2005; 81: 146-154.*
20. *Vidal AS. Avoidable infant deaths in a state referral hospital in Northeast Brazil. Rev Bras Saude Mater Infant 2003; 3: 281-89.*
21. *Pires-Alves FA, Paiva CH, Santana JP. The internationalization of health: contextual elements and institutional frameworks of Brazilian cooperation. Rev Panam Salud Publica 2012; 32: 444-50.*
22. *Mathers CD, et al. Counting the dead and what they died from: an assessment of the global status of cause of death data. Bull World health Organization 2005; 83: 171-177.*
23. *Simões CCS, Monteiro CA. Secular trends and regional differentials in infant mortality in Brazil. São Paulo: Hucitec; 1995.*
24. *State of Brazil's Children. 2006. http://www.unicef.org/brazil/pt/Pags_152_153_IDI_Estados.pdf. Accessed 12 May 2013.*

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Antibacterial activity of Neem (*Azadirachta indica*) leaf extracts against wound causing bacteria

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Abstract

The antibacterial potential of crude leaves extracts of *Azadirachta indica* against some bacteria associated with surgical wound infections was investigated. Leaves were extracted using water and ethanol. Aqueous extract did not show any inhibitory activity. Ethanolic extract inhibited the growth of all the pathogens to varying degree except *Klebsiella* spp. The gram-positive bacteria were more susceptible to the effect of ethanolic extract. By agar well diffusion method maximum in vitro inhibition was obtained at 100% concentration against *Staphylococcus aureus* with zone size of 22mm±0, followed by MRSA. The minimum inhibitory concentration (MIC) values for the tested bacteria ranged between 1-128mg/ml. The highest MIC value was obtained against 6 strains of *Acinetobacter* species. While lowest MIC was observed for *Staphylococcus aureus* and MRSA, all strains inhibiting at 2mg/ml. The study recommended trials to investigate the invivo activity of plant for management of wound infections.

Key words: *Azadirachta indica,* leaf extracts, wound infections, antimicrobial assay.

Introduction

Many times human body has to deal with open wounds. Trauma is the most common cause of wound which can be nosocomial (hospital acquired) or intentional [1]. When an individual develops an open wound, the nature attempts to cover the wound by re-epithelialization. This is a slow process and invasion of the wound site by opportunistic bacteria is common [2]. If infection is not treated properly complications like chronic open sores, sepsis and even death can occur [3]. The surgical site infections (SSIs) are the second most common nosocomial infection [4]. The 2002 survey report by Nosocomial Infection National Surveillance System (NINSS) which covers the period between October 1997 till September 2001 surgical site infection rate was 10% in UK. In Pakistan high rate of wound infection can be demonstrated by a study carried out at the department of general surgery, Shaikh Zayed Hospital, Lahore. Wound infection rate was 3.3% corresponding to the infection rate of 2.6% of international figures (Nur et al., 2000).

Some of the microorganisms frequently isolated from infected wounds are *Staphylococci, Streptococci, Escherichia coli, Pseudomonas, Klebsiella, Proteus, Acinetobacter* and *Candida.* These pathogens are resistant to most of the commonly used antibiotics (Nester et al., 2004). The indiscriminate use of antimicrobials is the main cause of failure in the management of wound infection [5]. Plants are a good source of phytochemicals and produce a diverse range of agents which have structure similar to many synthetic drugs [6]. Plant extracts have a great potential to act against multi-resistant bacteria for management and treatment of wounds [7]. Medicinal use of plants is the oldest form of healthcare [8]. Although modern medicinal science has been developed to a great extent, many rural people of Pakistan still depend on plant products and herbal remedies for treating their ailments. Treatment of diseases through medicinal plants is also a characteristic feature of Islamic teachings.

Neem tree (*Azadirachta indica*) specie of family Meliaceae is evergreen tree found in most tropical countries (Pakistan, Bangladesh, India). Every part of the *A.indica* can be used to treat different conditions [8]. It was the Pakistani scientist "**Salimuzzaman Siddiqui**" who pioneered the isolation of unique chemical compounds from neem**.** He extracted 3 bitter compounds from neem oil which he named as nimbin, nimbinin and nimbidin (Dr.Kazim, 2011). Since then more than 135 compounds have been isolated from different parts of *A.indica* [9][8][10]. The compounds have

been divided into major classes: isoprenoids and non-isoprenoids [11]. The isoprenoids include diterpenoids and triterpenoids. The non-isoprenoids include proteins (amino acids) and carbohydrates (polysaccharides), sulphurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds etc [12]. Biological activities of compounds of *A.indica* proved by different researchers are; anti-inflammatory, anti-arthritic, anti-pyretic, hypoglycemic, anti-gastric ulcer, spermicidal, anti-fungal, anti-bacterial, diuretic, anti-malarial, anti-tumor, immunomodulatory etc [13]. Since rural people have developed techniques for treating their cuts and wounds by application of a paste made from fresh or dried leaves of neem, it will be interesting to investigate usefulness of plant in treating these infections. The present study is aimed at evaluating the antimicrobial potential of crude extracts of neem against pathogenic bacteria associated with wound infections.

Materials and methods

Plant material

Neem leaves were collected from grounds of PCSIR (Pakistan Council of Scientific and Industrial Research). The collected leaves were identified and authenticated by Department of Botany, Agriculture University, Faisalabad. The leaves were washed in fresh water and dried under shade at room temperature. The leaves were crushed to small pieces using pestle and mortar and grinded in an electric grinder to obtain 1kg of dried powder.

Preparation of extract

500g of powder was soaked in 1000 ml of distilled water. The preparation was left overnight at room temperature; the suspension was sieved through a muslin cloth and then centrifuged at 4000rpm for 20mins. The supernatant containing the plant extract was then transferred to a pre-weighed beaker, concentrated and stored in sterile bottle at 4^0C until further use. The same experimental procedure was followed when 95% ethanol was used as extract. The sterility of all the extracts was checked by spreading a loopful quantity on blood agar plate and incubated overnight. No growth was obtained on blood culture medium after 24 hours.

Test Microorganisms

The test bacteria were *Staphylococcus aureus*, MRSA, *Staphylococcus epidermidis*, *E.coli*, *Enterobacter* spp, *Proteus* spp, *Provedencia* spp., *Klebsiella* spp., *Pseudomonas* spp. and *Acinetobacter* spp. isolated from clinical specimens obtained from patients diagnosed with surgical wound infection at the Services Hospital (tertiary care hospital), Lahore, Pakistan. Collection, transportation of specimens and isolation of bacteria were performed following standard microbiological procedures [14].These bacteria were gram stained and identified using methods described by Mackie & McCartney (2006) and then confirmed by API 20E and API 20NE kits wherever indicated and feasible (Bio Merieux, Inc).

Screening for antibacterial activity

The effect of plant extracts on the bacteria isolated was assayed by Agar well diffusion method according to CLSI (2006). The bacterial cultures used in this study were regularly sub cultured and maintained at 4^0C. The freshly cultured bacterial colonies were transferred into nutrient broth for 6hrs at 37^0C. The turbidity of the actively growing broth culture was adjusted with sterile broth to obtain turbidity optically comparable to that of the 0.5 Mc Farland standards. This resulted in suspension containing approximately 1x 10^8 CFU/ml. The required quantity of the medium was prepared according to the manufacturer's instruction. The medium was sterilized and poured into a sterile petri dish and allowed to solidify at room temperature. Using a sterile cotton swab lawn cultures of the test organisms were made on the Muller Hinton agar plates. A sterile cork borer of 6mm diameter was used to cut four wells in the agar. For each type of plant extract (aqueous and ethanol) four concentrations were made; 100%, 50%, 25% and 12.5%. Against each gram-positive and gram-negative bacteria first the activity of aqueous and then ethanolic extract was determined. The plates were incubated at 37^0C for 24 hours. Each sample was assayed in triplicate and the mean values were observed. The anti-bacterial activity was interpreted from the size of the diameter in mm of the clear zones surrounding the wells [15][16].

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the extract was then determined using agar dilution method. One ATCC reference strain of *Staphylococcus aureus* (ATCC 25923) and one ATCC reference strain of *E.coli (*ATCC 25922) were also tested for MIC. This assay is more sensitive and provides quantitative results which are closer to clinical scenario. The agar (MH agar) was mixed with measured quantity of extract at 50 ^{0}C. Plates at different concentrations of the extracts were made, i.e. 128, 64, 32, 16, 8, 4, 2 and 1 mg/ml [17]. They were allowed to solidify at room temperature and kept in refrigerator till use. The MIC was recorded as the lowest concentration of extract at which visible bacterial growth was completely inhibited. This experiment was performed in triplicate to ensure the reproducibility of the results [18].

Results and discussion

In this study, wound swabs were collected from 90 patients showing signs of local infection. Among the samples collected, 17 (19%) samples showed mixed growth while majority (81%) yielded growth of single organism. The gram-positive bacteria isolated were *Staphlycoccus aureus* (MSSA and MRSA), *Staphylococcus epidermidis* and gram-negative bacteria were *E-coli*, *Enterobacter* spp., *Proteus* spp., *Acinetobacter* spp., *Pseudomonas* spp., *Providencia* spp. and *Klebsiella* spp. (Figure 1). The results of the antibacterial screening of the aqueous and ethanolic extracts of neem (Figure 2 and Table 1).

Ethanolic extract showed good activity against all the tested bacteria except *Klebsiella*. *Staphylococcus aureus* was the most susceptible bacteria with zone of 22mm. The inhibition zone of tested organisms was observed to increase with increasing extract concentration. Positive control, 6 % phenol (w/v) exhibited inhibition of 6mm -15mm zone range. Ethanolic extract produced zone diameters, between 8.97-22mm.The aqueous extract of neem leaves showed no inhibitory effect on both gram-positive and gram-negative bacteria.

The MIC values of the ethanolic extract are represented in Table 2. The MIC of the test organisms ranged between 1 - 128 mg/ml. All the strains of *Staphylococcus aureus* (MSSA) were inhibited at 2 mg/ml. *Acinetobacter* spp showed high MIC value and inhibited at 128mg/ml whereas *Klebsiella* spp. were not inhibited even at 128mg/ml. The MIC values for the control bacteria were lower than the test organisms.

Plants have the great potential for the management and treatment of wounds. Most of the plant extracts had shown antibacterial activity which is comparable to antibiotics commonly used [19]. In many countries plants are considered very effective for the treatment of wounds and burns [20]. These natural agents possess not only healing and regenerative properties [21] but are also safe and affordable [22]. The herbal extracts have effectively arrested bleeding from fresh wounds, inhibited microbial growth and accelerated wound healing [23]. *Azadirachta indica* phytochemicals have been established in previous studies and these include tannins, saponins, alkaloids, carbohydrate, phenols, flavonoid, anthraquinones, cardiac glycosides, sterols and resins [8]. Several studies have linked these bioactive compounds to its antimicrobial activity. These compounds protect the plants against infection by micro-organism, predation by insects while some give plants their odors, flavors and pigments [17]. These active compounds can be used in the synthesis of many useful drugs [24].

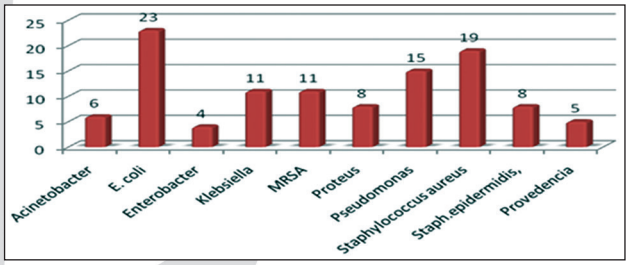

Figure 1. Bacteria isolated from wound samples

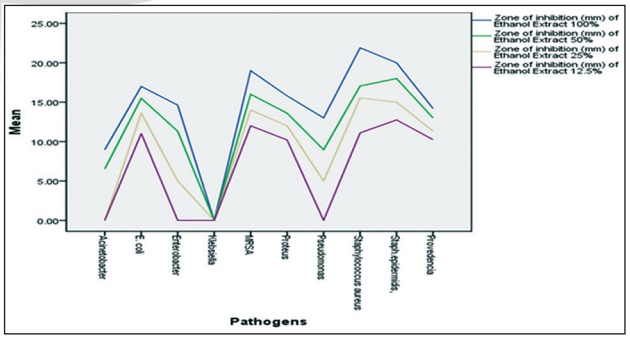

Figure 2. Comparison of zones of inhibition of bacteria isolated at different extract concentrations

In this study 110 pathogenic bacteria were isolated from infected wounds and antibacterial activity of the crude extracts of neem leaves was determined against these bacteria.

As reported in this study *E.coli* were the predominant bacteria causing wound infection followed by *Staph. aureus*. Both *E.coli* and *Staph. aureus* are one of the common pathogens isolated from surgical wounds. *E.coli* is not only an inhabitant of human gastrointestinal tract but also associated with many extra intestinal infections. Another study carried out in a tertiary care hospital in Peshawar from January 2009 till December 2009. In which 100 patients who developed SSIs after operation were sampled and the common organism involved in the SSIs was *E.coli* [25].

The ethanolic extract of neem leaves inhibited the growth of both the test (except *Klebsiella* spp.) and the control bacteria. The aqueous extract did not show any antibacterial activity. The organic solvent give superior results than aqueous because the components of the plants are organic compounds which easily dissolve in an organic solvent and secondly the temperature used in the organic extraction is much higher than in water extraction. Several other studies have also shown similar results; a study [26] involving some dermatophites, no activity was recorded for aqueous extract of neem.

Table 1. Antibacterial activity of leaves extract of Azadirachta indica by agar well diffusion assay

Bacteria	Zone of Inhibition of Ethanolic and aqueous extracts of neem in mm				
	12.5% Mean ± S.D	25% Mean ± S.D	50% Mean ± S.D	100% Mean ± S.D	Aqueous extract
Acinetobacter spp	0	0	6.53 ± 0.01	8.97 ± 0.02	N/A
E-coli	10.6±0.46	13.6±0.43	15.5±0.4	17.86 ± 0.36	N/A
Enterobacter spp	0	5±0	11.33 ± 0.88	14.66 ± 0.45	N/A
Klebsiella spp	0	0	0	0	N/A
MRSA	12±0	14.76±0.31	15.83±0.2	18.96±0.03	N/A
Proteus spp	10.16±0.45	12±0	13.6±0.43	15.86±0.18	N/A
Pseudomonas spp	0	5±0	8.96±0.19	13±0.36	N/A
Staphylococcus aureus	11±0	15.16±0.22	17.1±0.26	22±0	N/A
Staphylococcus epidermidis	13±0.5	15±1.0	18±0.5	19.16±0.28	N/A
Provedencia spp	10.16±0.45	11.33±0.88	13.03±0.36	14.2±0.52	N/A

N/A= no activity observed

Table 2. The MIC values of ethanolic extract of Azadirachta indica

Bacteria	N	No. of isolates susceptible at MIC (mg/ml)							
		1	2	4	8	16	32	64	128
Staphylococcus aureus	19	*10	19	** -	-	-	-	-	-
Staphylococcus epidermidis	8	5	7	8	-	-	-	-	-
MRSA	11	5	11	-	-	-	-	-	-
Escherichia coli	23	*** 0	0	* 13	20	23	-	-	-
Proteus spp	8	0	4	8	-	-	-	-	-
Enterobacter spp	4	0	0	1	3	4	-	-	-
Provedencia spp	5	0	0	3	5	-	-	-	-
Klebsiella spp	11	0	0	0	0	0	0	0	0
Pseudomonas spp	15	0	0	0	9	13	15	-	-
Acinetobacter spp	6	0	0	0	0	0	3	3	6

**Staphylococcus aureus (ATCC 25923) was also inhibited at 1mg/ml.*
E-coli (ATCC 25922) was inhibited at 4mg/ml. ** No growth observed. * Not inhibited*

In another study no antibacterial activity was shown by aqueous extracts of both the bark and the leaves of neem. *Staphylococcus aureus* (MSSA) and MRSA were the most sensitive bacteria with inhibition zone of 22 mm and 18.96 mm at 100% concentration respectively. All of the strains of both were inhibited at MIC of 2mg/ml. *Staph. aureus* and MRSA infections are one of the main factors contributing to impaired wound healing. In the past decade there has been increase in the wound infections caused by MRSA and are the leading cause of SSIs in hospitals [27]. If not treated properly many complications such as osteomyelitis, bacteremia and sepsis may occur. Treating the cases of MRSA successfully can save financial and human costs. *Staph. aureus* and MRSA are also intranasal and skin commensals and patients who carry them are at greater risk of developing wound infections. Further work can be done to evaluate its role as a possible topical body decolonizing agent. In vitro antibacterial activity of ethanol extract of neem against *Staphylococcus aurues* and MRSA was strongly suggested in another study [28]. *Staph. epidermidis* is often regarded as a culture contaminant but in recent years its importance as a pathogen has been recognized. All the clinical isolates obtained in this study of *Staph. epidermidis* were inhibited effectively. All the bacteria against which neem activity was tested were bacteria commonly associated with nosocomial infections and are known to possess resistance against several antibiotics. The gram-negative bacteria *E.coli, Pseudomonas* spp and *Acinetobacter* spp showed higher MIC values, meaning that higher concentration of extracts were required to inhibit their growth. This finding was also observed by another author [17]. Inhibitory activity of extract against bacteria of same species ranged widely from strain to strain. This may be because the strains of same species may differ in their resistance pattern. This observation is in agreement with another study [29] in which bactericidal activities of six commonly used disinfectants was determined against seven different species of clinically isolated bacteria. However, the results of this study contradict the findings of [30] where the extracts of four plants were found to be ineffective against *E.coli.* The method of extraction used in their study was different from ours and may be this is the reason why they didn't find any activity against *E.coli.*

The neem extract didn't show any activity against *Klebsiella* spp. *Klebsiella* spp. possess capsule (outer protective covering) that help in developing resistance against different plant extracts. Also these strains were of hospital isolates which are usually MDR, so they might have developed resistance to different plant extracts as well. The work published by an author [31] showed activity of acetone extract of neem against *Klebsiella* but the sizes of zone of inhibition were small. The neem extract in this research showed better activity against gram-positive bacteria as compared to gram-negative bacteria. Several other authors have also reported that plant extracts are more effective against gram-positive than gram-negative bacteria. They linked this selective toxicity to the differences in the cell wall of gram-positive and gram-negative bacteria [32][17]. In another work on the antibacterial activity of the phytoconstituents of neem , the authors have reported that the extract of neem leaves was very effective against *Staphlococcus aureus* and *E-coli* [23]. On the contrary, a study carried out on four different bacteria using three extracts of neem leaves, fairly high degree of activity against gram-negative bacteria was observed as compared to gram-positive [33]. The ATCC strains of *Staph. aureus and E.coli* were inhibited at lower MIC values then the test bacteria. The sensitivity of control bacteria to toxic effects of crude neem extracts was shown by other authors as well [17]. This study supports the use of neem by traditional health workers for treatment of wound infection.

Conclusions

The results of this study support the use of crude neem leaves extract to treat wound infection. Neem leaves could be a source of compounds useful in treating mono and poly microbial infections and resistant bacteria. However, further studies; about the absence of toxicity in plant extracts and its activity in vivo, are important to propose this plant as alternative approach for wound management.

References

1. *Singhal H, Kaur K, Zammit C. Wound infection. Emedicine. 2008.*

2. *Collier M. Recognition and management of wound infections. World wide wounds: 2004; 1-10.*

3. *Jahan T, Begum ZA, Sultana S. Effect of neem oil on some pathogenic bacteria. Bangladesh Journal of Pharmacology 2007; 2: 71-72.*

4. *Ahmed M, Alam N, Manzar S. Postoperative wound infection: a surgeon's dilemma. Pakistan J Surg 2007; 23: 41-47.*

5. *Anderson DJ, Sexton DJ, Kanafani ZA, Auten G, Kaye KS. Severe surgical site infection in community hospitals: epidemiology, key procedures, and the changing prevalence of methicillin-resistant Staphylococcus aureus. infection control and hospital epidemiology 2007; 28: 1047-1053.*

6. *Jagessar R, Mars A, Gomes G. Selective Antimicrobial properties of Phyllanthus acidus leaf extract against Candida albicans, Escherichia coli and Staphylococcus aureus using Stokes Disc diffusion, Well diffusion, Streak plate and a dilution method. Nature and Science 2008; 6: 24-38.*

7. *Sukanya S, Sudisha J, Hariprasad P, Niranjana S, Prakash H, et al. Antimicrobial activity of leaf extracts of Indian medicinal plants against clinical and phytopathogenic bacteria. African journal of biotechnology 2009; 8.*

8. *Biswas K, Chattopadhyay I, Banerjee RK, Bandyopadhyay U. Biological activities and medicinal properties of neem (Azadirachta indica). Current science 2002; 82: 1336-1345.*

9. *Das B, Mukherjee S, Sahu B, Murjani G. Neem (Azadirachta indica) extract as an antibacterial agent against fish pathogenic bacteria. Indian journal of experimental biology 1999; 37: 1097-1100.*

10. *Subapriya R, Nagini S. Medicinal properties of neem leaves: a review. Current Medicinal Chemistry-Anti-Cancer Agents 2005; 5: 149-156.*

11. *Chaturvedi MP, Bag A, Rawat V, Jyala N, Satyavali V, et al. Antibacterial Effects of Azadirachta indica Leaf and Bark Extracts in Clinical Isolates of Diabetic Patients. National Journal of Integrated Research in Medicine 2011; 2: 5-9.*

12. *Perumal Samy R, Gopalakrishnakone P. Therapeutic potential of plants as anti-microbials for drug discovery. Evidence-Based Complementary and Alternative Medicine 2010; 7: 283-294.*

13. *Schumacher M, Cerella C, Reuter S, Dicato M, Diederich M. Anti-inflammatory, pro-apoptotic, and anti-proliferative effects of a methanolic neem (Azadirachta indica) leaf extract are mediated via modulation of the nuclear factor-κB pathway. Genes & nutrition 2011; 6: 149-160.*

14. *Collee J. Mackie and McCartney practical medical microbiology, 1989.*

15. *Shankar GS, Navneet M, Sanjay K, Prabhat M. Screening Of Antibacterial Activity Of Nepeta Ciliaris Benth. Against Respiratory Tract Pathogens. Kathmandu University Journal of Science, Engineering and Technology 2012; 8: 100-103.*

16. *Valgas C, Souza SMd, Smânia EF, Smânia Jr A. Screening methods to determine antibacterial activity of natural products. Brazilian Journal of Microbiology 2007; 38: 369-380.*

17. *El-Mahmood A, Ogbonna O, Raji M. The antibacterial activity of Azadarichta indica (neem) seeds extracts against bacterial pathogens associated with eye and ear infections. J Med Plant Res 2010; 4: 1414-1421.*

18. *Ertürk Ö. Antibacterial and antifungal activity of ethanolic extracts from eleven spice plants. Biologia 2006; 61: 275-278.*

19. *Malik F, Hussain S, Mirza T, Hameed A, Ahmad S, et al. Screening for antimicrobial activity of thirty-three medicinal plants used in the traditional system of medicine in Pakistan. J Med Plant Re 2011; 5: 3052-3060.*

20. *Thakur R, Jain N, Pathak R, Sandhu SS. Practices in wound healing studies of plants. Evidence-Based Complementary and Alternative Medicine 2011.*

21. *Benli M, Güney K, Bingöl Ü, Geven F. Antimicrobial activity of some endemic plant species from Turkey. African journal of biotechnology 2007; 6.*

22. *Raina R, Parwez S, Verma P, Pankaj N. Medicinal plants and their role in wound healing. Online Veterinary J 2008; 3: 21.*

23. *Aslam F, Rehman K, Asghar M, Sarwar M. Antibacterial activity of various phytoconstituents of neem. Pak J Agri Sci 2009; 46: 3.*

24. *Ogbonnia S, Enwuru N, Onyemenem E, Oyedele G, Enwuru C. Phytochemical evaluation and antibacterial profile of Treculia africana Decne bark extract on gastrointestinal bacterial pathogens. African journal of biotechnology 2008; 7.*

25. *Jan W, Khan S, Jehanzeb M. Surgical site infection and pattern of antibiotic use in a tertiary care hospital in Peshawar. Journal of Ayub Medical College, Abbottabad: JAMC 2010; 22: 141.*

26. *Natarajan V, Venugopal P, Menon T. Effect of Azadirachta indica (neem) on the growth pattern of dermatophytes. Indian journal of medical microbiology 2003; 21: 98.*

27. *Anderson DJ, Kaye KS, Chen LF, Schmader KE, Choi Y, et al. Clinical and financial outcomes due to methicillin resistant Staphylococcus aureus surgical site infection: a multi-center matched outcomes study. PLoS One 2009; 4: e8305.*

28. *Sarmiento WC, Maramba CC, Gonzales MLM. An in-vitro study on the antibacterial effect of neem (azadirachta indica) leaf extract on methicillin-sensitive and methicillin-resistant staphylococcus aureus. PIDSP Journal, 2011; 12.*

29. *Namba Y, Suzuki A, Takeshima N, Kato N. Comparative study of bactericidal activities of six different disinfectants. Nagoya Journal of Medical Science, 1985; 47: 101-112.*

30. *Joshi B, Sah GP, Basnet BB, Bhatt MR, Sharma D, et al. Phytochemical extraction and antimicrobial properties of different medicinal plants: Ocimum sanctum (Tulsi), Eugenia caryophyllata (Clove), Achyranthes bidentata (Datiwan) and Azadirachta indica (Neem). J Microbiol Antimicrob, 2011; 3: 1-7.*

31. *Irshad S, Butt M, Younus H. In-Vitro Antibacterial Activity of Two Medicinal Plants Neem (Azadirachta indica) and Peppermint.*

32. *Parekh J, Chanda S. Antibacterial and phytochemical studies on twelve species of Indian medicinal plants. African Journal of Biomedical Research 10, 2007.*

33. *Koona S, Budida S. Antibacterial Potential of the Extracts of the Leaves of Azadirachta indica Linn. Notulae Scientia Biologicae, 2011; 3: 65-69.*

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The Effects of Injection of Sterile Water, Normal Saline & Xylocaine on the Changes of Low Back Pain in Primiparous Women

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Abstract

Background: During the first stage of labor many women suffer from low back pain. Lower back pain during labor may be associated with different etiology.

Objectives: To compare the effect of, sterile water, normal saline and xylocaine in the management of low back pain during labor.

Materials & Methods: This intervening research was conducted on primiparous women who referred to hospitals of Shiraz University of Medical Sciences. Pain intensity was assessed on a visual Analogue Scale, before, 45 & 90 minute after injection.

Results: A total of 399 pregnant women were observed. Low back pain intensity in group (I), group (II) and group (III) prior to injection was different from in the same group 45 and 90 minutes after the injection ($P<0.001$). There was a statistically significant difference between low back intensity at the time of 3-4 centimeter dilatation and 45 and 90 minutes after injection in the 3 groups to compared the control group.

Conclusion: Similar to xylocaine, normal saline has the least pain at the site of injection, and is most effective in reducing pain with a high satisfactory rate (84.3%).

Key Words: Injection, Pain, Women

Introduction

Lower back pain during labor may associated with different degree of fetal malpositioning occipito-posterior position that apply pressure on pain sensitive structures inside the pelvic which cause greater analgesic requirement(5)

During the first stage of labor many women suffer from low back pain. The back pain associated with more pharmacological analgesia uses that had negative outcomes(1).

The technique for sterile water injections is very old as it mentioned in 1885(6). The sterile water causes osmotic and mechanical irritation resulting in a brief and significant sharp pain .pain relief that cause by sterile water injection follows immediately and last for up to 2 hour(5).

Several techniques tries in studies for relieving low back pain in that happen during the first step of labor such as stimulation or anesthesia of the lumbosacral area, acupuncture and transcutaneous electrical nerve stimulation but some contradicatory finding been presented. Midwife must be able to communicate with a women in labor about her pain about pain (2) Intradermal sterile water injections produce skin nociceptors stimulation to relieve referred pain (3). Varieties of blinded randomized controlled trials have previously done. Some of them compared the four injection of technique using sterile water against normal saline placebo controls, and others compare a single intradermal sterile water injection with the four injection technique in the degree and duration of analgesic effect.

Aim of this study is to find a non-pharmacological analgesic techniquethat is simple, safe and effective without serious side effect that compare a single intradermal sterile water injection with the four injection technique in the degree and duration of analgesic effect in Iranian population that never done before.sterile water injection provide all of these option suitable for all maternity care setting and models of care all around the world.

Materials and Method

This intervening research was conducted on the primiparous women who referred to the labor rooms of hospitals affiliated to Shiraz University of

Medical Sciences. There were 399 women whom were randomly divided into 4 groups. The first group included 99 subjects which received 0.4c.c of xylocaine injection, the second group with 102 subjects received 0.4c.c of normal saline and the third group with 100 subjects received 0.0.4c.c of sterile water through intra-cutaneous injection in the lumbo sacral area. The fourth group including 96 subjects was the control group. The severity of low back pain in all subjects was carefully studied in three stages. In the first stage the intensity of the pain was evaluated prior to the injection, during the 3-4 cm dilation of the cervix.

During the second and third stages i.e. 45 and 90 minutes after injection, the severity of the low back pain was measured by using the visual analog scale.

ANOVA and Turkey multiple comparison tests were applied to compare the average severity of the low back pain before and after injection at 45 and 90 minute intervals in all groups. Moreover, repeated measurement test was used to compare the intensity of the pain at the mentioned time intervals separately in each group. No form of analgesia was given before and during of injections. Twelve hours after injections, all mothers filled out a simple questionnaire, regarding their satisfaction of the analgesia they had received during labor .All patients were further followed for any probable side effects.

Results

Three hundred ninety nine women received the blocks and complete the questionnaire forms. Sterile water blocks were given to 100; Saline solution was given to 102 women and xylocaine to 99 women. Patients in all groups were matched regarding to the number of precipitous, maternal age, and the mean cervical dilatation, when the blocks were given.

In the sterile water group 54 (54.5%) of the women noted an analgesic effect of the blocks, compared with 86(84.3%) in the saline solution group and 80(80.8%) in the xylocaine group ($P<0.001$).

Initial pain scoring did not show any significant difference between the groups. ($P=0.27$) (table I), but 45 and 90 minutes after the blocks were given, there were highly significant differences (tab II, III)(Fig I, II, III) ($P<0.001$). Except for the initial burning sensation, lasting about 30 seconds, no local side effects were noted. The result of the questionnaire answered by the mothers after delivery are given in (tab I) of the 399 women 220 (73.3%) found this technique sufficient during the first stage.

Discussion

While sterile water injectionsare a good treatment for back pain during labor, there is a lack of knowledge among midwives about thismethod of pain relief during labor and an interest in knowing more.

Our study shows that the pain level in all 3 groups was lower 45 and 90 minutes after injections. Our finding reflect statistically significant difference in outcome of low back pain intensity prior to injection and 45 and 90 minutes after injections, and normal saline with the least pain at the site of injection and a high satisfactory rate (84.3%) compared to xylocaine (80.8%) and sterile water (54.5%), Therefore we could consider normal saline as the most effective route in reducing low back pain during delivery compared to other blocking methods.

Some studies examined acupuncture for the prenatal back pain and the results shows acupuncture group reported significant improvement of pain(4).

Two studies examined acupuncture for the relief of prenatal back pain and pelvic pain(7)(8).In a study comparing physiotherapy to acupuncture, participants attended ten sessions of either physiotherapy or acupuncture at a variety of acupoints(7). The acupuncture group reported significant improvement of pain and perception of disability compared to physiotherapy but disproportionate dropout and the dissimilarity of the type of back pain limit interpretation. In another trial compared to physiotherapy but disproportionate dropout and the dissimilarity of the type of back pain limit interpretation. In another trial of acupuncture at a variety of points specific to each subject, pain scores decreased in 60% of the intervention group and 14% of the control group. Pain with various activities was found to decrease in 43% and 9% of controls, both results were statistically significant(8).

The opportunity to relieve or reduce the amount of pain and suffering experienced by women during the birthing process has long been the goal

of midwifes, and the normal saline injections clearly provide a tool to safety and effectively achieve this end.

References

1. *Lee N, Webster J, Beckmann M, Gibbons K, Smith T, et al. Comparison of a single vs. a four intradermal sterile water injection for relief of lower back pain for women in labour: a randomised controlled trial. Midwifery. 2013 Jun; 29(6): 585-91.*

2. *Bergh IH, Ek K, Mårtensson LB. Midwifery students attribute different quantitative meanings to hurt, ache and pain: a cross-sectional survey.Women Birth. 2013 Jun; 26(2): 143-6.*

3. *Fogarty V. Intradermal sterile water injections for the relief of low back pain in labour-A systematic review of the literature. women and birth, 2008; 21: 157-163.*

4. *Wedenberg K, Moen B, Norling A. A prospective randomized study comparing acupuncture with physiotherapy for lowbackand pelvic pain in pregnancy. ActaObstetGynecolScand 2000; 79(5): 331 –5.*

5. *Lee N, Martensson LB, Kildea S. Cross sectional study of Australian midwivesknowledge and use of sterile water injectionsfor pain relief in labour: Women and birth 2012; 25: e75-e80.*

6. *Martensson L, McSwiggin M, Mercer JS. US Midwives' Knowledge and Use of Sterile WaterInjections for Labor Pain.*

7. *Wedenberg K, Moen B, Norling A. A prospective randomized study comparing acupuncturing with physiotherapy for low back and pelvic pain in pregnancy, ActaObstetGynecolScand 2000; 79(5): 331-5.*

8. *Kvorning N, Holmberg C, Grennert L, Aberg A, Akeson J. Acupuncture relieves pelvic and low back in late pregnancy. ActaObstetGynecolScand 2004; 83(3): 246-50.*

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Complete ureteral duplication with upper pole exclusion at left: Literature review

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Abstract

Duplication of collecting duct system is a frequent abnormality of urinary tract, often asymptomatic. It occurs due to a division of mesonephric diverticulum and its extension depends on the amplitude of this boundary. Duplication can be complete or partial (more common). Upper-pole exclusion is frequent. Treatment consists in a superior polar heminephrectomy with resection of correspondent ureter.

Key words: Congenital urogenital malformations; ureteral duplication; heminephrectomy.

Background

Congenital abnormalities in human beings affects about 3% of all newborns[1,2]. Studies affirm that overall incidence of congenital anomalies in Latin America is not significantly different from what is seen in other regions[3].

The study of congenital anomalies is relevant, because this deffects are one of most important causes of children mortality[4]. Children mortality is recognized as a sensitive indicator of the health situation in a determined population. It results mainly of socioeconomic conditions[5].

Another important information regarding to congenital anomalies, beside mortality, is a greater morbidity, defined as a higher risk for developing clinical complications and, consequently, increasing the amount of internments and the gravity of recurrencies[3].

Urinary tract is the third system most affected by congenital malformations, after central nervous system and cardiovascular system. These malformations can vary since anomalies without clinical significance to severe and potentially lethal dismorphic abnormalities, like renal agenesis[6].

Materials and methods

Our study is based in the search and analysis of medical literature about congenital abnormalities of the urinary tract, especially the complete ureteral duplication with exclusion of the upper pole of the kidney.

Virtual Health Library (VHL) was surveyed between september and november of 2011. We consulted two databases: Latinoamerican and Caribbean Health Sciences Library (LILACS) and Scientific Electronic Library Online (SciELO). Keywords used in search were selected from specific terminology found in the Health Sciences Keywords (DECS-BIREME). They are: "congenital urogenital anomalies", "ureteral duplication" and "heminephrectomy". We did no restrictions regarding to publications' languages or dates.

Results and discussion

Congenital anomalies of urinary tracts are relatively frequent and found in about 3-4% of overall population[7]. These abnormalities of kidney or lower urinary tract, as its complications, are important causes of death during childhood, notably due to renal failure[6]. Anomalies of genital and urinary tract can occur simultaneously, due to interrelations in their embrionary development[7].

Duplication of collector duct system is a frequent deffect of urinary tract and it is often asymptomatic. This kind of anomalies results from a wrong division of mesonephric diverticulum – the ureteric bud[8].

Ureteric bud is an epithelial tube that grows in extension and gives rise to ramifications which promote the development of ureter and kidney's collector ducts. While many genes that controls this process are known, events of the cells com-

prehended in renal morphogenesis are unknown. Several cell changes which can contribute to ureteric bud morphogenesis, like migration and morphologic changes, involve actin cytoskeleton. Actin-depolymerising factor (ADF) proteins are important for these changes in the organization of cytoskeleton in cultivated cells, but their role in vivo were not completely comprehended[9].

The extension of duplication depends on how complete is the division of mesonephric diverticulum and can be divided in partial or complete. Partial duplication is more frequent (present in 1/500 people)[7]. Ureteric bud partial duplication usually results in a divided kidney with a bifid ureter. Complete division results in double kidney with bifid ureter of duplicated ureters[8].

When the division is complete, it can ocurrs an obstruction of the upper segment due to an ectopic ureteric inserction or to the presence of an ectopic ureterocele and reflux towards lower segment. The ureter draining upper segment is inserted in a position more inferior and medial than its normal location and ureter draining lower components is inserted more superior and laterally than usual[7].

Ureteral duplication is present in about 75% of patients with ureteroceles and is more frequent in the ureter draining upper unity, due to its usually ectopic insertion[7]. Duplication is often associated to a malfunction on the middle of the upper pole and this malfunction is frequently caused by an obstruction which leads to an important hydronephrosis of this unity[10].

There are rare cases, like ureteric duplication with blind-ending bifid ureter, in which deplicated ureter is connected to urinary tract by a fibrous wire or has not any connection[11]. Complete bilateral ureteral duplication and ureteral triplication are very unfrequent in adults[12,13].

Anatomic changes of urinary system cause urinary stasis, leading to a delay in the crystal carrying and higher risk of urinary tract infection (UTI), predisposing to the development of renal calculi. Pyeloureteral duplication is the most common anomaly observed when there is calculus formation[14].

In addition to this predisposition to calculi formation, ureteral duplication is related to recurrent UTI with fever, vesicoureteral reflux (VUR), hydronephrosis, hematuria, abdominal pain and other urinary symptoms[11,15]. Late investigation represents an important cost, considering potential sequels of recurrent UTI and sepsis[15].

Avaliable imaging examinations for the investigation of anatomic changes of urinary tract are renal ultrasonography, excretory urography, computed tomography, mictional urethrocystography, renal scintigraphy and magnetic resonance[16].

Ultrasonography is the first method due to its lower cost and the absence of risks during the procedure[16]. In adition to this, ultrasonographic trial is important to detect other associated anomalies which could indicate some syndrome or chromosomal abnormality[2,7]. Excretory urography measures bilateral renal function and surveys urinary tract anatomy. It can diagnose nephrolithiasis, obstructions, duplication and position anomalies, but the use of iodine contrast presents risk of allergic and nephrotoxic reactions[16].

Computed tomography evaluates urinary tract anatomy even in patients with renal failure and can give a tridimensional reconstruction with additional information. It can also evaluate nephrolithiasis without endovenous contrast and it is not affected by the superposition of bones and bowels, like excretory urography[16,17]. Mictional urethrocystography assesses the anatomy of urinary bladder and urethra and if there is any vesicoureteral reflux. Renal scintigraphy gives information about kidney's function and anatomy. Magnetic resonance allows potential advantages in children and is a good method to appreciate multiple congenital anomalies[16].

Treatment for symptomatic duplications of urinary tract in children has high complexity and stills very controversial[15]. When there is ureteral duplication and upper pole is not functional, standard tratment is a segmentar heminephrectomy with the resection of the correspondent ureter, in the larger possible extension, without another incision, and ligature of residual segment of ureter[10,18].

In children, this surgery is often performed through a classic approach (lombotomy), with the isolation of renal vascular pedicle, ligature of upper segmental branch of the renal artery and excision of ischemic segment. However, arterial dissection extends surgical time and the arterial isolament is, in many times, not possible, due to an additional risk for vascular injury. Thus, standard surgical technique is performed with risk of causing malfunction on the lower pole in about 5% of cases[18].

Jednak and colleagues[19] described a simplified technique of upper-pole heminephrectomy, in which renal vascular pedicle dissection is avoided. So there is a reduction of surgical time and surgical risk, in addition to minimizing risk of injury to lower segment blood supply. There is not, however, other reports of this technique.

"Classic" open approach means a significantly more painful recovering and some scars[10]. In pediatric practice, the use of minimally invasive surgery stills ascending, thanks to its several advantages when compared to open surgery. Nephrectomy was one of the first laparoscopic procedures performed in children and gained notable acception due to minimal morbidity, lower hospitalar permanence and better recovering[20]. Since the first report, by Jordan and Winslow[21], laparoscopic approach became the best option for heminephrectomy.

Many other therapeutic proposals are recent, especially those using systematic endoscopic incision for ureteroceles as initial therapy, with incomplete long-term evaluation. This methodology, very attractive thanks to its simplicity, at the first sight, can cause, in many cases, severe vesicoureteral reflux with difficult tratment[15].

Main controverses involve the dilemma between endoscopic therapeutics and definitive cure capacity or high-risk methods (extense vesical reconstruction surgeries) against lower-morbidity methods (heminephrectomy) with a lower efficiency for definitive cure in a single time. We should highlight that diagnostic endoscopy has its classic indications and the referred controverse about therapeutic endoscopy cannot be applied to cases of vesical obstruction or perinatal infection refractory to optimal antibiotic therapy, in which initial endoscopic treatment, when avaliable, is not argued[15].

High levels of success can be achived with the injection of dextranomer/hyaluronic acid (DHA) copolymer in children with complete ureteral duplication. DHA injection is more probable to have success for older female children with vesicoureteral reflux (VUR) and complete ureteral duplication. If initial injection does not result in VUR resolution, a second injection can give success. For doctors and families who look for an alternative to surgical treatment, DHA represents an attractive option for selected children with complete ureteral duplication[22].

More recently, there was an advance in surgery with single-site laparoendoscopic and natural orifice transluminal endoscopic surgery (NOTES). Term hybrid-NOTES refers to a NOTES approach with a transumbilical port. Usually, vaginal mean is employed to introduce cameras and instruments, as well as organ removal[10].

References

1. *Leite JC, Schüler-Faccini L. Defeitos congênitos em uma região de mineração de carvão. Rev Saude Publica. 2001; 35: 136-41.*

2. *Barini R, Stella JH, Ribeiro ST, Botcher F, Isfer EV, Sanchez RC et al. Performance of Prenatal Ultrasound in the Diagnosis of Fetal Chromosomal Abnormalities in a Tertiary Center. Rev Bras Ginecol Obstet. 2002; 24(2): 121-127.*

3. *Horovitz DDG, Llerena-Junior JC, Mattos RA. Birth defects and health strategies in Brazil: an overview. Cad Saúde Pública. 2005; 21(4): 1055-1064.*

4. *Nurdan N, Mattar R, Camano L. Stillbirth in a microrregion of Minas Gerais State: causes and associated factors. Rev Bras Ginecol Obstet. 2003; 25(2): 103-107.*

5. *Alves AC, França E, Mendonça ML, Rezende EM, Ishitani LH, Cortes MCJW. Leading causes of post-neonatal infant deaths in Belo Horizonte, State of Minas Gerais, Brazil, 1996 to 2004. Rev Bras Saude Mater Infant. 2008; 8(1): 27-33.*

6. *Noronha L, Reichert A, Martins VDM, Sampaio GA, Cat I, Seramião MJ. Study of urinary tract congenital malformations: analysis of 6,245 pediatric autopsies. J Bras Patol Med Lab. 2003; 39(3): 237-243.*

7. *Kim MH, Sameshima YT, Francisco-Neto MJ, Funari MBG. Ultrasonographic findings in genitourinary tract malformations: a pictorial essay. Einstein. 2009; 7: 494-502.*

8. *Moore KL, Persaud TVN. Embriologia Clínica. 5th edition. Rio de Janeiro: Guanabara Koogan, 1994.*

9. *Kuure S, Cebrian C, Machingo Q, Lu BC, Chi X, Hyink D, et al. Actin depolymerizing factors cofilin1 and destrin are required for ureteric bud branching morphogenesis. PLoS Genet. 2010; 6(10): 1–11.*

10. *Sotelo R, Giedelman C, Carmona O, Andrade R, Ramirez D. Heminefrectomía NOTES-híbrido transvaginal por duplicidad del sistema colector renal en el paciente adulto. Actas Urol Esp. 2011; 35(6): 363-367.*

11. *Chattopadhyay A, Mitra SK, Dutta S, Chakraborty H. Gastric, pancreatic, and ureteric duplication. J Indian Assoc Pediatr Surg. 2010; 15(1): 25–27.*

12. *Anceschi U, Torcia C, Lauretti S, Amici A. Single-setting laparoscopic treatment of complete bilateral ureteral duplication in adult women. J Laparoendosc Adv Surg Tech A. 2011; 21(2): 125-129.*

13. *Mubarak MY, Zainun AR, Rohaya M. Ureter triplication with contra-lateral partial duplex system. Med J Malaysia. 2009; 64(3): 236-237.*

14. *Peres LAB, Langer SS, Schmidt RC, Nacke RAB, Francescon PVM, Almeida RC et al. Nephrolithiasis in pediatric patients: metabolic and anatomical investigation. J Bras Nefrol. 2011; 33(1): 34-38.*

15. *Jesus LE, Judice MM, Mello EG. Ureteral duplications and ureteroceles - surgical treatment. J Pediatr Rio J. 2003; 79(2): 173-180.*

16. *Peres LAB, Ferreira JRL, Beppu APK, Araujo-Junior ER, Vicenzi G, Yamamoto RYT. Alterações anatômicas em pacientes com nefrolitíase. J Bras Nefrol. 2010; 32(1): 35-38.*

17. *Freire-Filho EO, Leão ARS, Capobianco J, Szejnfeld J, D'Ippolito G. Definição do nível da junção ureterovesical pela tomografia computadorizada. Radiol Bras. 2006; 39(6): 419-423.*

18. *Barroso-Junior U, Vinhaes AJ, Barros MS, Calado AA, Macedo-Junior A, Srougi M. Simplified upper pole nephrectomy: initial experience. Int Braz J Urol. 2005; 31(2): 157-160.*

19. *Jednak R, Kryger JV, Barthold JS, Gonzalez R. A simplified technique of upper pole heminephrectomy for duplex kidney. J Urol. 2000; 164: 1326-1328.*

20. *Miranda ML, Oliveira-Filho AG,Carvalho PT, Ungersbock E, Olimpio H,Bustorff-Silva JM. Laparoscopic upper-pole nephroureterectomy in infants. International Braz J Urol. 2007; 33(1): 87-93.*

21. *Jordan GH, Winslow BH. Laparoendoscopic upper pole partial nephrectomy with ureterectomy. J Urol. 1993; 150: 940-943.*

22. *Bayne AP, Roth DR. Dextranomer/hyaluronic injection for the management of vesicoureteric reflux in complete ureteral duplication: should age and gender be factors in decision making? J Endourol. 2010; 24(6): 1013-1016.*

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Cervical cancer and pregnancy: late diagnosis

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Abstract

Cervical cancer is the second most prevalent cancer in women worldwide and is thought to be a sexually transmitted cancer. Cervical cancer is pregnant women has been seen in early stages. Here we discussed a 37-year-old woman with vaginal spotting started at 21st weeks and diagnosis of cervical cancer.

Key words: Cervical cancer, pregnancy, Gorgan

Introduction

Cervical cancer is the second most prevalent cancer in women worldwide, with about 500000 cases and over 270000 estimated deaths annually. Infection with human papilloma virus (HPV) is believed to be the main etiology of the cervical cancer (1, 2). Cervical cancer is one of the possible malignancies occur in pregnancy estimated to be one per 1,000 to 5,000 pregnancies (1-3). In the north of Iran, this malignancy is as common as stomach and esophagus cancers (4). Most of the pregnant women with cervical cancer are in early stages and higher stages are rare (1). Here we represent a case of cervical cancer during pregnancy with late diagnosis.

Case definition

A 37-year-old woman at 21st weeks and 3 days of gestation (based on ultrasound examination) presented to another gynecologic clinic with intermittent self-limiting vaginal spotting and anemia. Spotting was no related to the sexual activity. In her past medical history, she was reported to be opium addict and had multiple sexual partners. Her obstetrics history was 8 pregnancies and 3 abortions (induced abortions). At her first admission, she received 1 unit of packed cell and her ultrasound examination reported placenta previa. Second ultrasound evaluation in three days revealed low anterior marginal placenta previa. At second admission in the next month that was for anemia and continuous spotting, she received 3 units of packed cell again. Two ultrasonic examinations by two different radiologists were performed ,one reporting anterior placenta with 5cm distance from Cervix and the other reporting a 49*29 cm hematoma in inferior margin and no distance between placenta and endocervix (anterior marginal placenta). In the third admission after 2 months ultrasound examination reported anterior 2nd grade placenta, 3cm from the endocervix. Cesarean section was done the next month. Four days after Cesarean section she received the Depo injection.

Three months later she was visited because of continued vaginal spotting. Estrogen tabs were prescribed and suggested to return after the end of the treatment course.

Two months later she had vaginal spotting yet and was visited in our clinic for the first time. In this session a vaginal examination was done and a large disrupted cervical mass was seen. Because of bleeding tendency she was transferred to operating room for biopsy. It was impossible to perform a diagnostic curettage. Histopathology reported a stage III SCC of cervix (involvement of pelvic wall, distal third of vagina and hydronephrosis).

Radiotherapy was started immediately and continued for 7 weeks. She is now cancer free and a Pap smear test is done every 6 months.

Discussion

The prevalence of cervical cancer is higher in low socioeconomic status and is considered mostly a sexually transmitted disease. In the United States, vaccination has been helping in reducing its prevalence but it is yet the second-leading cause of death in women worldwide (3, 5, 6).

The main etiology of cervical cancer is HPV infection especially sexual acquisition of HPV (5). Our patient had multiple sex partners.

Current studies have shown that cervical cancer in pregnant women is diagnosed in earlier stages and 76% of these lesions are in stage 1B and 3-8% are in stage III (1). Our patient was not diagnosed with cancer until after the delivery and it was mostly because she was not examined until then. It is not clear if she was in the earlier stages before referring to us or not.

Radiation therapy, surgery and adjuvant chemotherapy are standard treatments for cervical cancer and radiation therapy is the first line treatment but many people do not respond to it (7). Chun et al described 3 cases with early cervical cancer in pregnancy, 2 of them were referred because of cervical mass that were found by physical examination and 1 patient because of vaginal spotting. Two of these patients developed recurrences and one died due to progressive disease (2).Our patient received radiotherapy alone but her response was excellent and she survived.

References

1. *Gonçalves CV, Duarte G, Costa JSD, Marcolin AC, Bianchi MS, Dias D. Diagnosis and treatment of cervical cancer during pregnancy. Sao Paulo Medical Journal. 2009; 127(6): 359-65.*

2. *Chun K-C, Kim D-Y, Kim J-H, Kim Y-M, Kim Y-T, Nam J-H. Neoadjuvant chemotherapy with paclitaxel plus platinum followed by radical surgery in early cervical cancer during pregnancy: three case reports. Japanese journal of clinical oncology. 2010; 40(7): 694-8.*

3. *Jin Xwen, Sikon A, Yen-Lieberman B. Cervical cancer screening: Less testing, smarter testing. Cleveland Clinic Journal of Medicine. 2011; 78(11): 737-47.*

4. *Zarchi MK, Akhavan A, Gholami H, Dehghani A, Naghshi M, Mohseni F. Evaluation of Cervical Cancer Risk-factors in Women referred to Yazd-Iran Hospitals from 2002 to 2009. Asian Pacific Journal of Cancer Prevention. 2010; 11: 537-8.*

5. *Ibeanu OA. Molecular pathogenesis of cervical cancer. Cancer biology & therapy. 2011; 11(3): 295-306.*

6. *Franceschi S, Plummer M, Clifford G, de Sanjose S, Bosch X, Herrero R, et al. Differences in the risk of cervical cancer and human papillomavirus infection by education level. British journal of cancer. 2009; 101(5): 865-70.*

7. *Maranga IO, Hampson L, Oliver AW, Gamal A, Gichangi P, Opiyo A, et al. Analysis of Factors Contributing to the Low Survival of Cervical Cancer Patients Undergoing Radiotherapy in Kenya. PloS one. 2013; 8(10): e78411.*

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Rapid evolution of hepatocellular carcinoma in HIV-HBV co-infection. Autopsy case report

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Abstract

Viral hepatitis B is common among human immunodeficiency virus (HIV) infected patients due to the similar routes of transmission and it is one of the main risk factor for developing hepatocellular carcinoma (HCC). Co-infections may accelerate the progression from chronic viral hepatitis to cirrhosis and/or to HCC.

We describe a case of HCC developing over underlying liver cirrhosis of hepatitis B virus (HBV) etiology in a 21-year-old young man infected with HIV. He was known with HIV-HBV coinfections from childhood and he was in treatment with antiviral drugs for both conditions. Despite good therapy compliance over the time and favorable clinical and biological periodic evaluation, he developed during a few months' cirrhosis and then fulminant HCC. Multiple lungs metastasize were considered at the beginning primary neoplastic tumor leading finally to death.

Our case showed very rapid clinical deterioration despite highly active antiretroviral therapy.

Key words: hepatocellular carcinoma, pulmonary metastasis, HIV, HVB, autopsy, immunohistochemistry

Introduction

Nowadays, the hope of life of HIV infected patients has increased, first of all due to use of combined antiretroviral therapy (cART). Coinfection with hepatitis B virus (HBV) is frequently seen in HIV-positive subjects due to the similar routes of transmission. Progression to end-stage liver disease seems to occur faster in these patients and cirrhosis and hepatocellular carcinoma (HCC) may be recognized at an increasing rate in patients coinfected with HIV-HBV [1, 2]. Overall, 80% of HCC are attributed to chronic hepatitis B and C infection [3].

HCC rarely occurs before the age of 40 years and reaches a peak at approximately 70 years of age [4].

A majority of HCC patients are considered as advanced or even at end-stage at their diagnostic time, with extensive tumor status, for example, macroscopic vascular invasion or extra-hepatic metastasis [5, 6].

HCC shows also intrahepatic multiple occurrence and intrahepatic metastasis in 75% of the time [7].

The usual route of metastatic spread from HCC is hematogenous, with the most common extrahepatic site being the lung, followed by lymph node, bone and brain [5]. The incidence of lung metastases is high, up to 43%, in HCC [8, 9].

In one retrospective study conducted by Zhang *et al.*, the survival time of patients with lung metastases from hepatocellular carcinoma was 264 days (8.8 months) [10].

Nowadays, there are various treatment modalities for both intrahepatic tumor and extrahepatic metastatic foci of HCC, including surgical resection, transcatheter arterial chemoembolization (TACE), radiotherapy, chemotherapeutics, and recent molecular targeted therapeutic drugs. Surgical resection with complete extirpation of tumor gives the best chance of a cure for patients with HCC. [3]

Material and metod

Ethic comity agreement was obtained in order to publish patient data and accompanying images in scientific point of view. Written informed consent of patient was obtained before last hospitalization regarding participation in medical teaching process and scientific interest.

Pathological autopsy was performed, according to legal provisions.

Tissue samples were collected from internal organs and fixed in 10% formalin.

Light microscopic study was performed after paraffin-embedded tissue samples were cut at a 4 μm thickness and stained with hematoxylin-eosin (HE).

Immunohistochemical analysis was based on a panel of antibodies:

- monoclonal mouse anti-Glypican 3 (clone IG12, Biocare Medical);
- monoclonal mouse anti-hepatocyte specific antigen (HSA, clone OCH1E5, Biocare Medical);
- policlonal rabbit anti-alpha 1 fetoprotein (AFP, Biocare Medical);
- policlonal rabbit anti-carcinoembryonic antigen (CEAp, Biocare Medical);
- monoclonal mouse anti-Ki 67 (clone MM1, Biocare Medical);
- monoclonal mouse anti-CD 34 (clone QBEnd/10, Biocare Medical);
- monoclonal rabbit anti-cytokeratine 7 (CK7, clone BC1, Biocare Medical);
- monoclonal mouse anti-neuron specific enolase (NSE, clone DT01+BC100, Biocare Medical);
- monoclonal mouse anti-chromogranine (clone LK2H10+PHE5, Biocare Medical).

Microscopic images were taken with a 518 CU 5. OM CMOS camera using an Optech light microscope.

DNA–HBV and RNA–HIV were detected by polymerase chaine reaction (PCR), using a real-time PCR method, Roche Amplicor.

Case description

A 21-year-old young man, diagnosed with HIV and HBV infections at the age of 4 and who was under periodical clinical surveillance and compliant to combine antiretroviral therapy (cART), effective for both conditions, was admitted for pains at right upper quadrant and asthenia. Under cART his CD4 cell count was above 800 cells/mmc and the level of HIV-RNA became undetectable. In all these years, levels of liver transaminases were normal, as well as alpha-fetoprotein level and abdominal ultrasound evaluation.

About one month before dying he performed a radiological examination of the thorax which evidenced multiple micro- and macronodular lesions (0.3-9 cm) of the lungs. Abdominal ultrasound revealed features of cirrhosis with hepatosplenomegaly and ascites.

Laboratory evaluation of blood cells revealed a decreased platelet count, a prolonged Quick time, bilirubin level and liver transaminases increased. HIV-RNA was undetectable but HBV-DNA level increased at 8410 copies/ml (one month earlier HBV-DNA level was 4648 copies/ml).

Computed tomography (CT) examination of thorax revealed bilateral lung micro- and macronodular lesions of possibly granulomatous bacillary etiology (figure 1); abdominal magnetic resonance imaging showed hepatosplenomegaly and an irregular outlined, voluminous tumoral mass located in segments VII, VIII, IV and VI of liver.

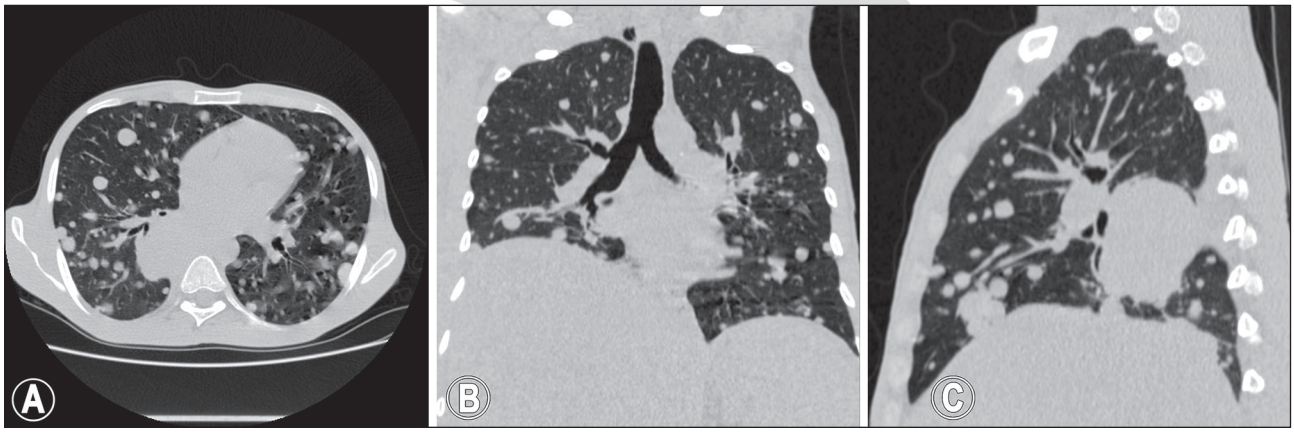

Figure 1. Toraco-pulmonary computed tomography (a. axial view, b. coronal view, c. transversal view) show relatively well defined voluminous tissue mass, with maximum axial dimensions of 59/46 mm situated infrahilar medial-basal on the right side, in contact with the mediastinal and costo-vertebral pleura; and also multiple nodular and micronodular formations, clearly defined, with axial dimensions between 4 and 28 mm, disseminated in both lung fields

His clinical outcome has worsened rapidly leading to death in 20 days. The cause of death was respiratory failure due to lung metastatic lesions from an advanced HCC.

Autopsy and histopathological findings

The autopsy was conducted in Pathology Department of Constanta Clinical Infectious Diseases Hospital. Internal examination revealed hepatomegaly and an ill-defined large tumor located in the liver's right lobe of 15 cm in greatest dimension with multiple satellite nodular lesions of 0.5-3 cm with intraparenchymal and subcapsular disposition, all of them replacing more than 90% of the right lobe and extending to the left lobe; the cut surface of tumoral lesions showed light yellow color with areas of necrosis and hemorrhages; tumoral mass invaded portal vein (Figure 2a). The remaining nontumoral liver has a mixed micro- and macro-nodular cirrhosis aspect. Peritoneal effusion of 2000 ml was found in abdominal cavity.

Multiple nodular masses of 0,5-8 cm were found in the lungs (Figure 2b).

Histological examination with hematoxylin-eosin staining (HE) revealed a malignant proliferation of epithelial type in liver, composed of variable size neoplastic cells with trabecular (Figure 3a), pseudoglandular and solid disposition, nuclear hyperchromatism, prominent nucleoli and atypical mitotic activity. In poorly differentiated areas, tumoral cells displayed sarcomatoid features with spindle shaped cells (Figure 3b), small cells patterns (Figure 3c), and clear cells (Figure 3d); we identified also peliotic-like changes (Figure 3e). It was identified also intratumoral cholestasis (Figure 3f) and intracellular hyaline Mallory bodies. Large areas of necrosis of tumoral mass and invasion of portal vein were also noticed.

Microscopic examination established diagnosis of HCC moderately to poorly differentiate with intrahepatic, vascular and pulmonary metastasis arising in a background of micro- and macro-nodular cirrhosis.

Immunohistochemical tests confirmed diagnosis by revealing diffuse positivity in tumoral cells for Glypican 3 (figure 4a) and alpha 1 fetoprotein (figure 4b). HSA (figure 4c) was focally positive in tumoral cells and CEAp (figure 4d) was weakly positive. Nuclear proliferation index Ki67 was positive in 5% of tumoral cells. CD34 was completely positive in sinusoids and CK7, Chromogranine and NSE were negatives in tumoral cells both in liver and lungs.

Discussion

Although HIV-infected persons have a higher incidence of certain cancers than noninfected persons, up until a few years ago, HCC was not one of them. [11] More studies suggested that HIV infection raises the risk for HCC. [12, 13] HCC as much as cervical cancer or anal cancer has becoming an increasingly significant clinical problem in the HIV population [14, 15].

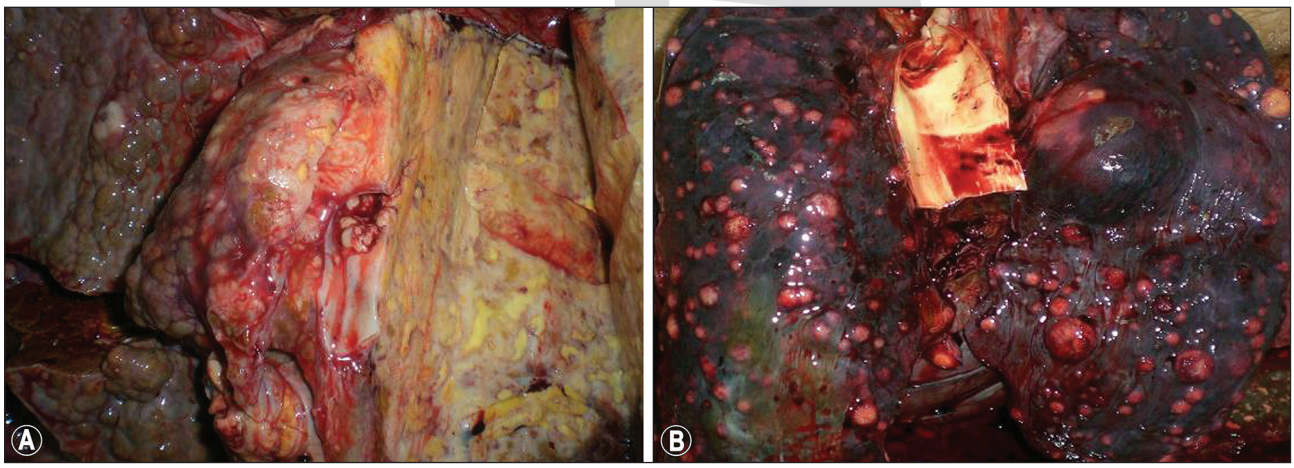

Figure 2. Autopsy findings (a) Gross appearance of liver showing a large tumoral mass developed on a cirrhotic liver; invasion of portal vein, multifocality and areas of tumoral necrosis can be noticed. (b) Multiple nodular lesions on both lungs

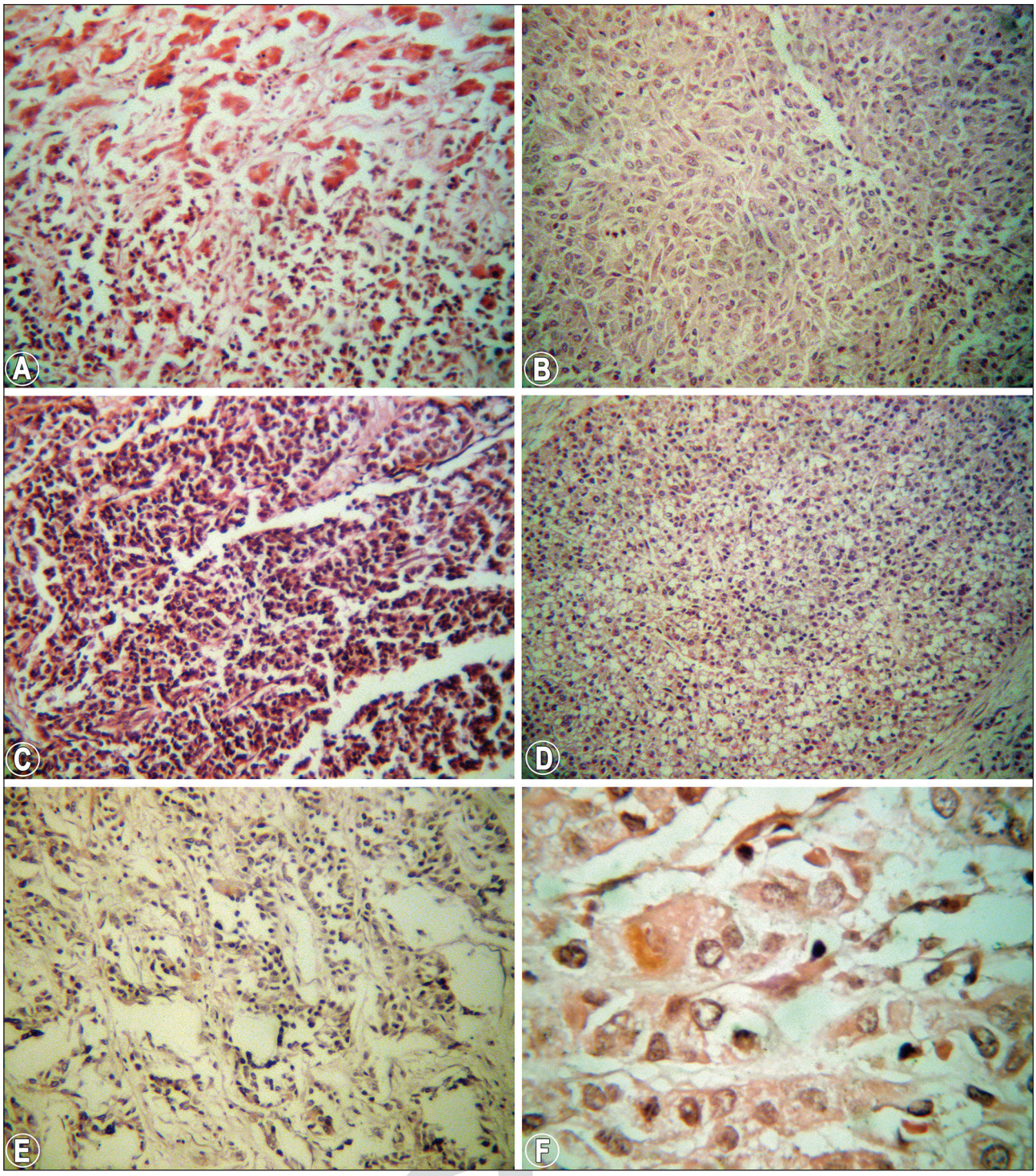

Figure 3. Histological aspects of hepatocellular carcinoma (a) Interface of liver parenchyma with malignant proliferation displaying small cells pattern. (b) Sarcomatoid pattern of hepatocellular carcinoma. (c) Small cells features of malignant proliferation. (d) Clear cells pattern of tumor (e) Area with peliotic-like changes of tumoral proliferation. (f) Intratumoral cholestasis.

In many retrospective studies were described the fact that a late diagnosis of HCC is less treatable and with lower survival. [16] There is moderately strong evidence that antiviral therapy that controls HBV infection substantially reduces but does not eliminate the risk of HCC in patients with viral hepatitis. [4]

As further discusses Gramenzi *et al.*, HIV infection *per se* worsens the prognosis of patients with virus-related HCC by doubling the risk of dying with a median survival of 16 months in HIV infected patients versus 30 months in seronegatives. [17]

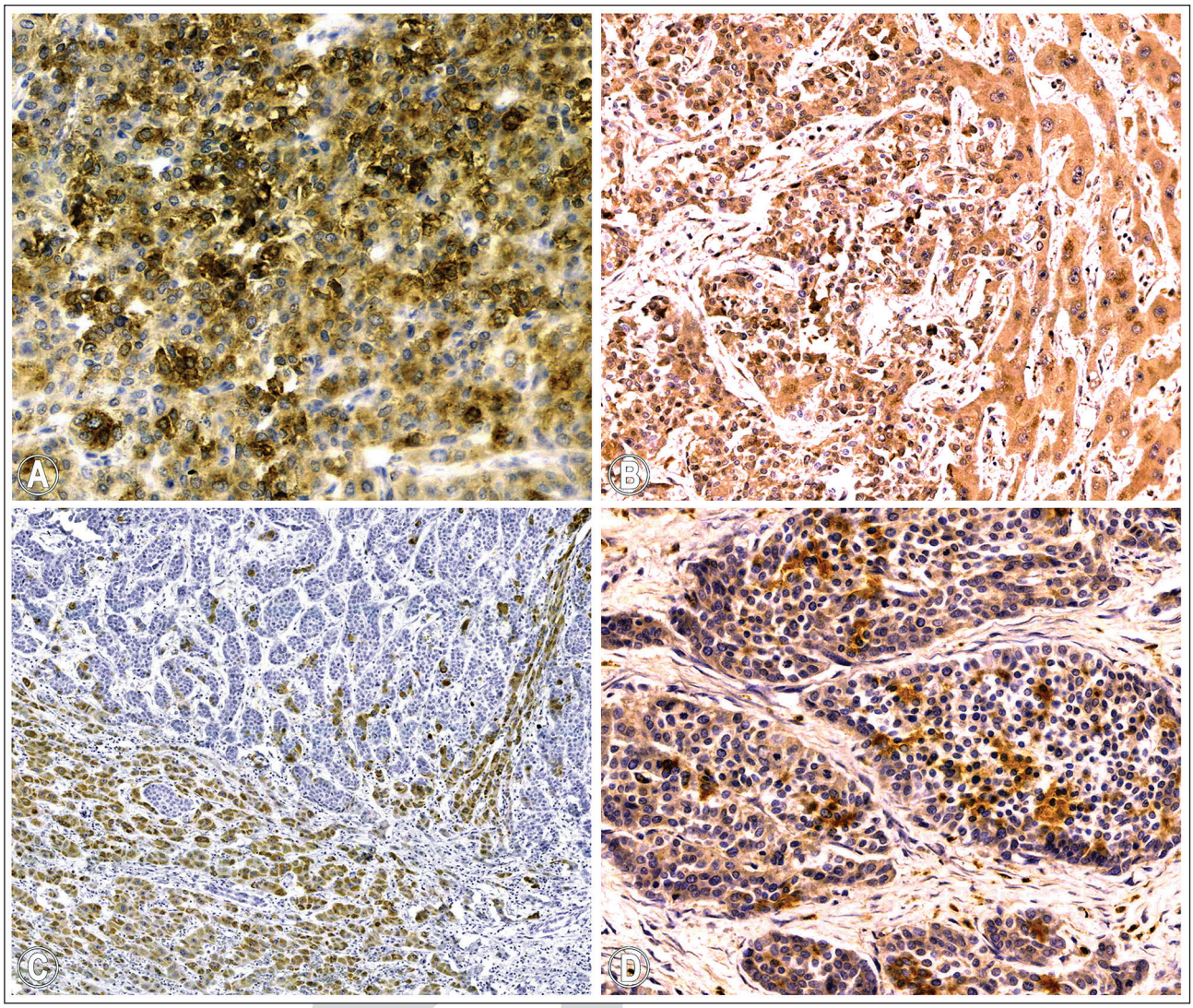

Figure 4. Immunohistochemical findings. (a) Diffuse positivity for Glypican3 in tumoral cells. (b) Diffuse positivity for AFP in tumoral cells and hepatocytes. (c) Focal positivity for HSA in poorly differentiated areas of proliferation. (d) pCEA is focal weakly positive in tumoral cells.

Bräu N *et al.* showed in one retrospective analysis conducted on 63 HIV infected HCC patients that in untreated patients, undetectable HIV RNA independently predicts better survival. [18] In our case, although the HIV RNA level was undetectable, clinical evolution was aggressive and survival was short (20 days).

From pathological point of view, HCC exhibits many histological features, and it varies architecturally and cytologically.

Generally, architectural patterns of HCC are trabecular, pseudoglandular (acinar) and compact. Cytological variants include sarcomatous change, clear cells, pleomorphic cells and pelioid type. It has been demonstrated that tumor cells are accompanied also with bile pigment, glycogen, fat, globular hyaline bodies, Mallory hyaline bodies or ground glass inclusions.

Different architectural patterns and cytological variations may occur in combination in the same tumor as we found in this case. Besides classical and frequently encountered trabecular, pseudoglandular and solid patterns we find also areas displaying rare cytological variants such as sarcomatoid, clear cells, small cells and areas with peliotic-like changes.

The lung is the most commonly affected organ of the extrahepatic metastases. Once the pulmonary metastases occur, the tumor is considered automatically to have entered the advanced stage. [10]

Unfortunately, HCC is frequently diagnosed at an advanced stage, and mortality continues to be

very high, with no significant changes in recent years.

New treatment strategies are available for advanced HCC but there are few data available in HIV associated HCC.

Patients co-infected with HIV and hepatitis viruses require heightened attention because they face a more rapid progression to HCC. [19]

Since our patient clinical course has worsened rapidly with extensive liver lesions and multiple pulmonary nodules, there was no effective physical time for any surgical treatment or palliative chemotherapy.

The differential diagnosis in lifetime between a possible pulmonary tuberculosis and lung cancer, even drug abuse was taken into consideration, represented one of the difficulty of the present case. Final results of the autopsy established diagnosis of hepatocellular carcinoma with intrahepatic, vascular and pulmonary metastasis, and fulminant evolution in a patient with HIV-HBV coinfection.

This case report describes an unfortunate clinical course of HCC in a young HIV-HVB co-infected patient, compliant to antiviral treatments. Although he had few symptoms and the immune status was relatively compensate, at the moment of diagnosis, the tumor was already at an advanced stage, with multiple pulmonary metastases leading to death in a short time.

In the last years we noticed that HCC occurs at a younger age in HIV-HBV coinfections. Despite cART, evolution to cirrhosis, HCC and death was unavoidable in this case. Therefore, a more attentively clinical and biological monitoring is necessary in HIV-HBV coinfections with periodic evaluation of alpha-fetoprotein and abdominal ultrasonography which may allow an earlier diagnosis and a potentially curative therapy. This case highlights also the importance of performing autopsy, even in cases with chronic diseases, to accurately determine the final diagnosis.

References

1. *MacDonald DC, Nelson M, Bower M, Powles T. Hepatocellular carcinoma, human immunodeficiency virus and viral hepatitis in the HAART era. World J Gastroenterol 2008; 14(11): 1657-63.*

2. *Salmon-Ceron D, Rosenthal E, Lewden C, et al. the ANRS EN19 Mortalité Study Group and Mortavic. Emerging role of hepatocellular carcinoma among liver-related causes of deaths in HIV-infected patients. The French National Mortalité 2005 study. Journal of Hepatology. 2009; 50(4): 736-45.*

3. *Rahbari NN, Mehrabi A, Mollberg NM, Müller SA, Koch M, et al. Hepatocellular carcinoma: current management and perspectives for the future. Ann Surg. 2011; 253(3): 453–69. doi: 10.1097/SLA.0b013e31820d944f.*

4. *El-Serag HB. Hepatocellular Carcinoma. N Engl J Med. 2011; 365(12): 111827. doi: 10.1056/NEJMra10016835.*

5. *Natsuizaka M, Omura T, Akaike T, et al. Clinical features of hepatocellular carcinoma with extrahepatic metastases. J Gastroenterol Hepatol. 2005; 20(11): 1781-87.*

6. *Uchida R, Masugi Y, Nishizawa T, Kimura T, Yamada T. Extensive endobronchial growth of metastatic hepatocellular carcinoma resulting in respiratory failure: A case report. Pathol Res Pract; 2010; 206(2): 116-20.*

7. *Terada T, Maruo H. Unusual extrahepatic metastatic sites from hepatocellular carcinoma. Int J Clin Exp Pathol. 2013; 6(5): 816–20. Published online 2013 A. PMCID: PMC3638091. Accesed: Oct 7, 2013*

8. *Kakumu S. Trends in liver cancer researched by the Liver Cancer Study Group of Japan. Hepatol Res. 2002; 24(Suppl 1): S21–7.*

9. *Ikai I, Itai Y, Okita K, et al. Report of the 15th follow-up survey of primary liver cancer. Hepatol Res. 2004; 28(1): 21–9.*

10. *Zhang SM, Zeng ZC, Tang ZY, et al. Prognostic analysis of pulmonary metastases from hepatocellular carcinoma. Hepatol Int. 2008; 2(2): 237–43. doi: 10.1007/s12072-008-9052-7.*

11. *Smukler AJ, Ratner L. Hepatitis viruses and hepatocellular carcinoma in HIV-infected patients. Curr Opin Oncol. 2002; 14(5): 538–42.*

12. *Garcia-Samaniego J, Rodriguez M, Berenguer J, et al. Hepatocellular carcinoma in HIV-infected patients with chronic hepatitis C. Am J Gastroenterol. 2001; 96(1): 179–83.*

13. *Puoti M, Bruno R, Soriano V, et al. For the HIV HCC Cooperative Italian–Spanish Group. Hepatocellular carcinoma in HIV-infected patients: epidemiological features, clinical presentation and outcome. AIDS. 2004; 18(7): 2285–93.*

14. *Bruno R, Sacchi P, Filice C, Puoti M, Filice G. Hepatocellular carcinoma in HIV-infected patients with chronic hepatitis: an emerging issue. J Acquir Immune Defic Syndr. 2002; 30(5): 535–36.*

15. *Halichidis S, Cambrea SC, Resul G, et al. Correlations between Babes-Papanicolau's findings and immunosuppression in Human Immunodeficiency virus infected women. Gineco.eu.2013; 33(3): 122-27.*

16. *Merchante N, Merino E, López-Aldeguer J, et al. Increasing Incidence of Hepatocellular Carcinoma in HIV-Infected Patients in Spain Clinical Infectious Diseases. 2013; 56(1): 143-50. doi: 10.1093/cid/cis777*

17. *Gramenzi A, Tedeschi S, Cantarini MC, et al. Outcome of hepatocellular carcinoma in human immunodeficiency virus-infected patients. Dig Liver Dis. 2013; 45(6): 516-22. doi: 10.1016/j.dld.2012.12.010.*

18. *Bräu N, Fox RK, Xiao P, et al; North American Liver Cancer in HIV Study Group. Presentation and outcome of hepatocellular carcinoma in HIV-infected patients: a U.S.-Canadian multicenter study. J Hepatol. 2007; 47(4): 527-37.*

19. *Bruno R, Puoti M, Sacchi P, Filice C, Carosi G, Filice G. Management of hepatocellular carcinoma in human immunodeficiency virus-infected patients. J Hepatol. 2006; 44(1 Suppl): S146-50.*

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Complete ureteral duplication with upper pole exclusion at left: Case report

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Abstract

Background: Duplication of collector duct system is a frequent abnormality of urinary tract, often asymptomatic. This kind of anomaly is caused by a division of the mesonephric diverticulum or ureteric bud.

Case Report: A male 9 year-old child was admitted complaining about pain in left flank in association with macroscopic hematuria and dysuria. He underwent to an ultrassonography, which showed a dilatation of pyelocaliceal system and a volumous liquid image around the left kidney. Urotomography disclosed ureteral duplication with upper-pole exclusion at left. A polar heminephrectomy was performed, with resection of correspondent ureter.

Discussion: Extension of duplicity depends on how complete is the division of ureteric bud. It can be classified as complete or partial (more common). Ureteral duplication is present in about 75% of patients with ureteroceles and is more frequent in the ureter draining the upper pole, generally ectopic.

Key words: Urogenital congenital malformations; ureteral duplication; heminephrectomy.

Background

Congenital malformation is an structural abnormality present at birth. It includes any functional or morphological anomalies of fetal development due to genetical, environmental or idiopathic causes, even when these defects are not apparent in the newborn child, becoming evident later[1].

According to Moore and Persaud[2], abnormalities in the urinary tract are the third most common type of congenital anomalies. About 0,5-1% of newborns have prenatal diagnosis of these urinary anomalies, but these children are often asymptomatic. Diagnosis is frequently given after patients present some complications. Congenital anomalies of the urinary tract are responsible for 20-35% of all situations that lead to chronic insufficiency of renal failure in childhood[3].

Duplication of ureter and renal pelvis is a relatively common congenital abnormality of genital and urinary tract[4]. This kind of anomalies results from a wrong division of mesonephric diverticulum or ureteric bud – the origin of ureter and renal pelvis. The amplitude of ureteral duplication depends on how complete is the embrionary division of ureteric bud. Bifid pelvis and ureter can be unilateral or bilateral. Separated openings in the bladder are common. A partial division of ureteric bud results in bifid ureter, while a complete division results in supernumerary kidney[5].

This is a case report from a nine year-old male patient complaining about pain in left flank, in association with macroscopic hematuria and dysuria. Imaging examinations diagnosed an complete ureteral duplication with exclusion of upper pole of the kidney, and its further surgical tratment.

Case report

K. B. L. A. S., a nine year-old male patient, was admitted at Hospital São Francisco de Assis, a public hospital located in Crato, in the northeast region of Brazil. He was complaining about an intense pain in left flank, associated with macroscopic hematuria and dysuria for the last five days. According to patient's mother, he had not presented any related symptoms before. At physical examination, patient was pallid (++/4+) and presented pain on deep palpation of left flank and hypochondrium, without signals of peritonitis or other changes. His mother denied comorbidities, previous surgeries or blood transfusions. He also used no continuous medications.

Laboratorial evaluation was requested (Tables 1, 2 and 3). An ultrasonography of abdomen showed an irregular-shaped kidney on the left side, with heterogeneous texture, enlarged pyelocaliceal system and a volumous liquid collection occupying perirenal space, parietocolic gutter and extended towards the pelvis. A computed tomography of urinary tract disclosed ureteropelvic duplication on the left side, in association with an important hydronephrosis of the upper unity and a hydroureter also significant. This ureter was enlarged until the bladder. Contrast excretion was seen through the lower unity, but upper unity demonstrated functional exclusion (Images 2 and 3).

Surgical procedure occurred in 05/03/2011 and was conducted through open medial approach, under general anesthesia. A upper-pole heminephrectomy at left and resection of the correspondent ureter were performed (Image 4). Patient had hospitalar discharge six days after surgery, without complaints. Surgical specimen (Image 5) was sent to anatomopathological study. The segment of ureter, measuring 14 centimeters in lenght and 2 centimeters in its larger width, presented hypertrofic muscular layer, chronic and unspecific inflamatory process and nodular mucose hemorrhagia. The segment of kidney, measuring 7,5 per 2,7 centimeters, had a contracted terminal standard and chronic and unspecific pyelonephritis, with presence of tubular leukocyte (segmented neutrophils) cylinders.

Nowadays, patient stills under ambulatorial follow-up, but presents neither symptoms os clinical signals.

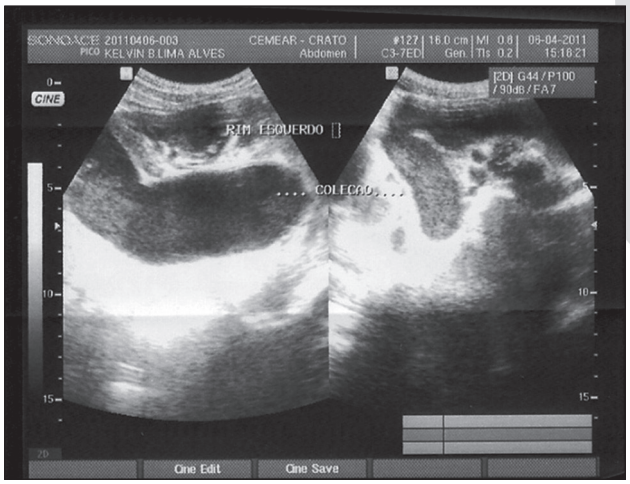

Image 1. Ultrasonographic image of left kidney and ureter

Table 1. Laboratorial examination (hematology)

	04/05/2011	**04/19/2011**
Hemoglobin	10.6 g/dL	14.1g/dl
Hematocrit	31.8%	33.4%
Leukocytes	14,300/mL	4,800/mL
Eosinophils	0%	4%
Stab neutrophils	0%	0%
Segmented neutrophils	90%	48%
Lymphocytes	7%	40%
Monocytes	3%	8%
Platelets	304,000/mm³	694,000/mm³
Coagulation time (Lee White)		3.15 min
Bleeding time (Duke)		2.3 min
Tourniquet test		Negative
Stephanini		48%
PT		13seg
PA		100%
INR		1.00
aPTT		36seg

Table 2. Laboratorial examination (biochemistry)

04/19/2011	
Urea	15
Creatinine	0.5
Fasting glucose	78

Table 3. Laboratorial examination (urinalysis)

04/05/2011	
Biochemistry	
Proteines	Negative
Glucose	Negative
Leukocytes	Positive (++)
Ketone bodies	Negative
Hemoglobin	Positive (++)
Bilirubin	Negative
Urobilinogen	Normal
Nitrite	Negative
Sedimentoscopy	
Leukocytes	Many
Red blood cells	6-8/HPF
Bacterial casts	Negative
Urate crystals	Negative
Uric acid crystals	Negative
Oxalate crystals	Negative

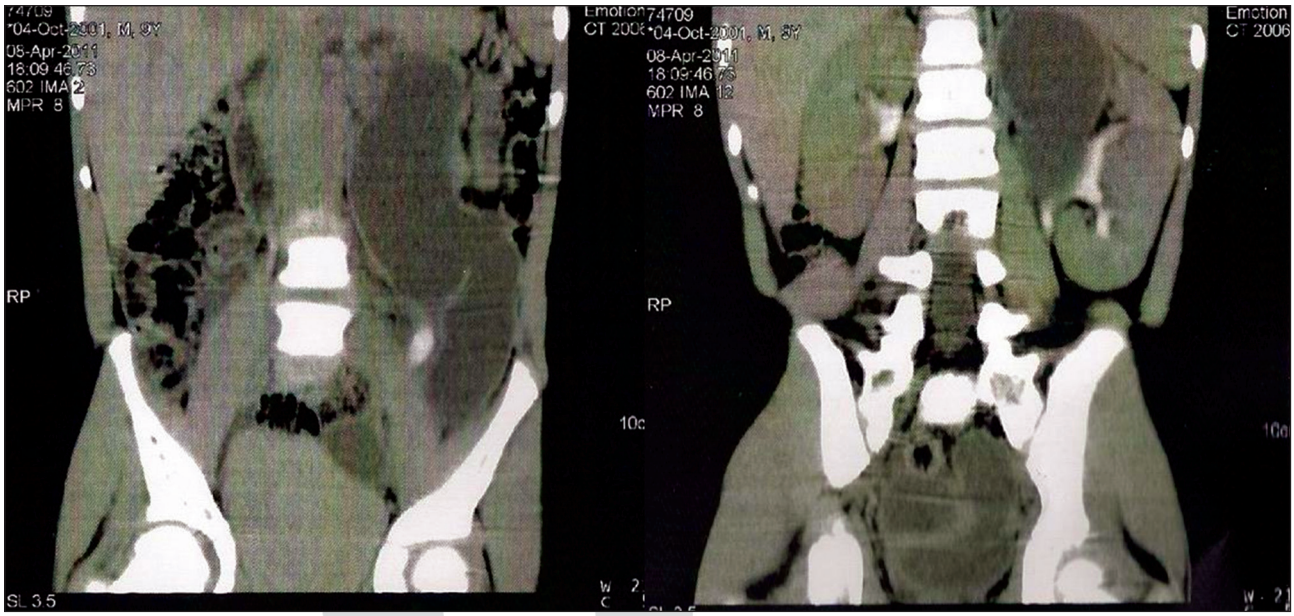

Image 2. Computed tomography showing hydroureter and and left kidney

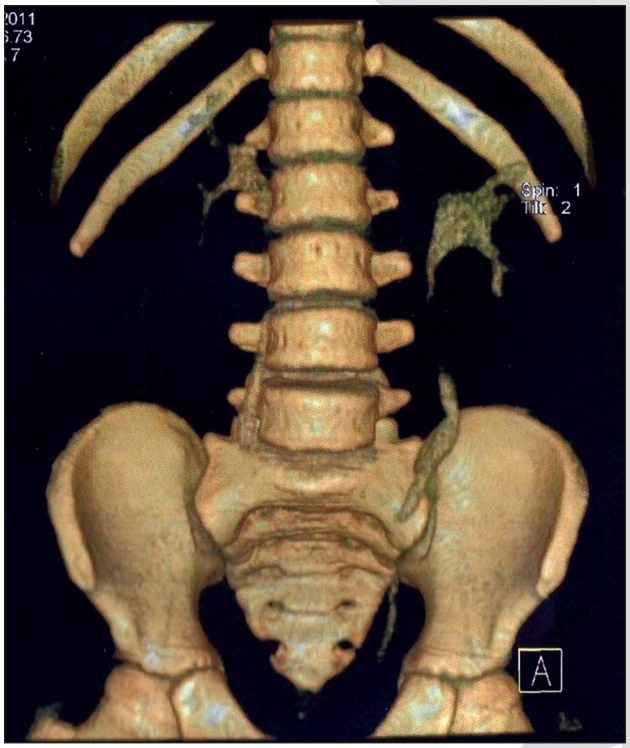

Image 3. Tridimensional reconstruction of computed tomography disclosing contrast leaking on left kidney's lower pole

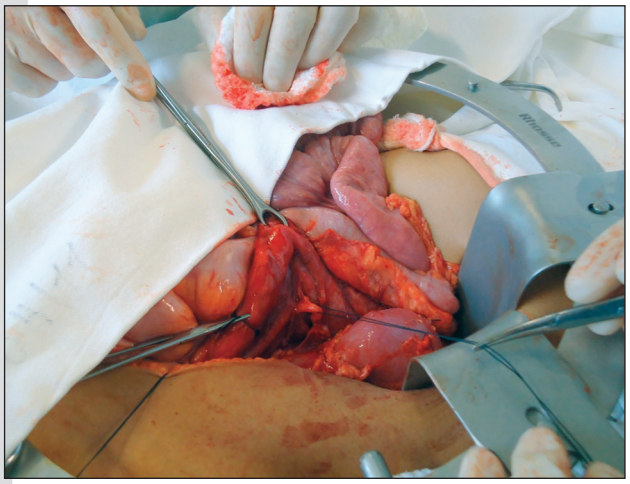

Image 4. Ureteral duplication: enlarged supernumerary ureter (hydroureter)

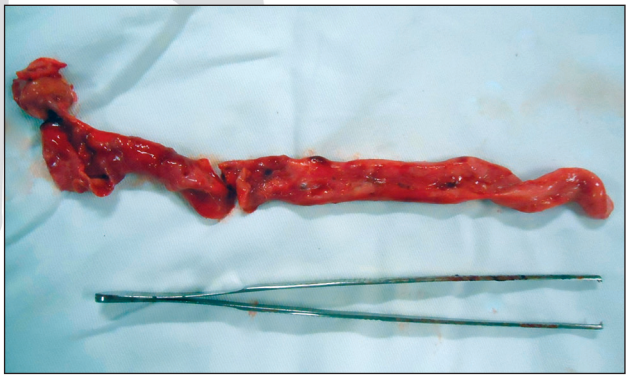

Image 5. Surgical specimen: upper pole of left kidney and correspondent ureter

Discussion

This study reports a case of ureteral total duplication with kidney's upper-pole functional exclusion on the left side in a nine year-old male patient, who only presented symptoms after the establishment of a complication from the existing anomaly. This information is compatible to literature. According to previous reports, children are often asymptomatic early after birth and the diagnosis is frequently achieved when patient presents any complication[3]. This patient's clinical presentation, like reported in the literature, consisted in pain (usually located in the flank), macroscopic hematuria and dysuria[6].

Aiming to support surgical procedure, patient underwent to some imaging examinations, standardized by recent studies about ureteral duplication. An abdominal ultrasonography showed a dilatation of pyelocaliceal system and discarded other kind of abdominal anomalies as suggested by some authors[7,8]. Urotomography disclosed a duplication of ureter and renal pelvis at left, with important hydronephrosis in the upper unity, making clear its functional exclusion, and hydroureter. The anatomy of urinary tract was assessed by computed tomography and tridimensional reconstruction, what made possible to improve surgical planning.

Surgery was programmed as a left upper-pole heminephrectomy, in association with the resection of correspondent ureter. This surgical technique is described in literature as the gold-standard treatment[4,9].

Surgical procedure was performed through the open medial approach, what permitted a better visualization and an improved management of the duplicated ureter and its ureteropelvic junction. Literature, however, reports lombotomy as the best open approach9. Videolaparoscopic heminephrectomy, when possible, is nowadays considered the best choice for treatment[10].

Conclusion

Ureteral duplication with upper-pole exclusion is a relatively frequent abnormality of urinary tract. It may be diagnosed through imaging assessment, even in utero. The definitive treatment is surgical: upper-pole heminephrectomy and the resection of correspondent ureteric segment.

References

1. *Carvalho QCM, Cardoso MVLML, Oliveira MMC, Lúcio IML. Congenital malformation: the significance of the parents' experience. Cienc Cuid Saude. 2006; 5(3): 389-397.*
2. *Moore KL, Persaud TVN. Embriologia Clínica. 5th edition. Rio de Janeiro: Guanabara Koogan, 1994.*
3. *Oliveira EA, Viana MCFB, Diniz JSS, Pereira AK, Pezzuti ILS, Santarosa RC. Postnatal approach of the urinary tract anomalies identified in prenatal diagnosis by ultra-sonography. Rev Med Minas Gerais. 2001; 11(2): 77-83.*
4. *Sotelo R, Giedelman C, Carmona O, Andrade R, Ramirez D. Heminefrectomía NOTES-híbrido transvaginal por duplicidad del sistema colector renal en el paciente adulto. Actas Urol Esp. 2011; 35(6): 363-367.*
5. *Moore KL, Dalley AF, Agur AMR. Anatomia orientada para a clínica. 6th edition. Rio de Janeiro: Guanabara Koogan, 2011.*
6. *Chattopadhyay A, Mitra SK, Dutta S, Chakraborty H. Gastric, pancreatic, and ureteric duplication. J Indian Assoc Pediatr Surg. 2010; 15(1): 25–27.*
7. *Kim MH, Sameshima YT, Francisco-Neto MJ, Funari MBG. Ultrasonographic findings in genitourinary tract malformations: a pictorial essay. Einstein. 2009; 7: 494-502.*
8. *Peres LAB, Langer SS, Schmidt RC, Nacke RAB, Francescon PVM, Almeida RC et al. Nephrolithiasis in pediatric patients: metabolic and anatomical investigation. J Bras Nefrol. 2011; 33(1): 34-38.*
9. *Barroso-Junior U, Vinhaes AJ, Barros MS, Calado AA, Macedo-Junior A, Srougi M. Simplified upper pole nephrectomy: initial experience. Int Braz J Urol. 2005; 31(2): 157-160.*
10. *Miranda ML, Oliveira-Filho AG,Carvalho PT, Ungersbock E, Olimpio H,Bustorff-Silva JM. Laparoscopic upper-pole nephroureterectomy in infants. International Braz J Urol. 2007; 33(1): 87-93.*

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Effect of Vaginal Isosorbide Mononitrate Pill and Low-Dose Syntocinon on type of delivery: A comparative Randomized Controlled Trial study in Iran

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Abstract

Increased rate of cesarean delivery is an important matter in developing countries. Lack of enough study regarding cervical ripening in Iran, this study was designed to assess the effect of vaginal Iisosorbide Mononitrate pills with low-dose Syntocinon in type of delivery. Randomized Controlled Trial study was conducted in 2009 in Shahid Sadoughi University of Yazd, Iran. Convenience sampling was used to select the participants, including primigravida women who needed labor induction. One hundred Participants were randomly allocated to group 1 receivers of 40 mg Iisosorbide Mononitrate pills and group 2 receivers of low-dose Syntocinon. Data were collecteded by questionnaire and were analyzed using t-student test. There was not significant statistical difference between two groups in mean of age (P=0.671), mean of Gestational Age (P=0.254), mean of Bishop Score (P=0.221), mean length of labor induction (P=0.876), mean of neonate's Apgar Score in the first (P=0.424) and fifth minutes (P=0.656). 72% in group 1 and 78% in group 2 delivered vaginally (P=0.645). The most reason for cesarean delivery was arresting in labor progress (P=0.468). Two groups were similar in participants, satisfaction regarding treatment procedure (P=0.99). Vaginal Iisosorbide Mononitrate pill is effective, safe and available as low-dose Syntocinon in type of delivery.

Key words: Iisosorbide Mononitrate, low-dose Syntocinon, induction of labor, delivery

Introduction

The nitric oxide donors including Isosorbide Mononitrate Donor (IMD) has been recently considered as one of the valuable agent in obstetrics and gynecology. They are used as a treatment method for disorders in reproductive age and postmenopausal women too.1,2

Oxide nitric is a free radical gas considered as releasing agent of flat vessel muscles, flat stomach muscles, and also myometrium.3, 4 In obstetrics, oxide nitric donors relax the myometrium5, 6 and have been used for preterm labor treatment 7,8, manual placenta removal and improvement of fetal blood circulation.2,9

Labor induction is a common intervention before delivery and is defined as stimulation of uterine contractions prior to initiation of spontaneously labor pain.10 Labor induction is used for nearly 9.7 to 32.7 percent of all pregnancies.11 Since labor induction without suitable cervix is considered as one of the most complex situations in obstetrics and failure to adequate ripening the cervix leads to increase the rate of Cesarean operation, it seems vital to apply clinical measures which are accompanied with less materno-fetal complications besides having desirable influence.2,5,12

Cervical ripening is achieved through administration of several drugs such as Syntocinon and Prostaglandin E2 before induction of labor. Although Syntocinon has been known as a usual method in cervical ripening but adverse side effects like uterine hypertonisity may be occurred after administration both of Syntocinon and Prostaglandin. Uterine hypertonisity produces painful contractions in

mother and also fetal distress in the fetus. This situation often increases emergency Cesarean delivery.2, 4, 13 The best agent for cervical ripening is a drug with producing adequate cervical changes and with the least feto-maternal adverse side effects. IMD seems might be such agents for cervical ripening with unserious feto-maternal unwanted side effects and low-risk method of labor induction for delivery.5,14,15,16 Several studies approved vaginal application of oxide nitric donors are effective for cervical ripening before induction of labor.2,16,17 In Nunes et al. (2006) study, the results showed that if glycerin trinitrate is vaginally administered together with Dinoprostone for cervical ripening, uterine tachysystole will be reduced as compared to vaginal use of Dinoprostone and placebo. This situation indicates antagonistic effect of oxide nitric donors on myometrium which is stimulated by prostaglandin, leading to loosing of myometrium and therefore reducing uterine tachysystole.18 Several research have been recently conducted on use of Isosorbide Mononitrate (IM) pill in cervical ripening during first three months of pregnancy before termination of pregnancy and cervical ripening prior to labor induction. In addition, a considerable number of women given IM pill for cervical ripening at term go into labor within the next 24 hours.19 Vaginally IM pill is used in 20 mg and also 40 mg doses for cervical ripening; these doses can be replicated once or twice in some studies based on Bishop Score (BS).2 Bullarbo et al. (2007) reported IM pill is an effective, safe, and well tolerated agent for cervical ripening.4 In another study was conducted by Abdellah et al. (2011), administering a combination of IM and Misoprostol was reported more effective than Misoprostol alone, fast cervical ripening was occurred and the length of labour induction was shorter.20 According to Nicoll et al. (2001) study vaginal administration of 20 or 40 mg IMN to pregnant women at term improved both maternal and fetal hemodynamics, but this effect was not clinically significant.2 In Haghighi et al. (2013) study Significant statistical difference was not observed in BS eight hours after administration of intravaginal Iso Sorbide Di Nitrate (ISDN,40 mg) and Misoprostol (25 μg), but in the ISDN receiver group, labor induction was reported significantly longer than Misoprostol receiver group with more frequently need induction of labor.17

While, rate of Cesarean delivery is an important matter in the world, especially in developing countries for its high cost and burden, reported rate of Cesarean delivery nearly threefold more than of its, world average rate (5-15%) in Iran (40%) revealed that many strategies should be applied to decrease the rate of Cesarean delivery.21, 22 Although Syntocinon infusion has been introduced as a routine method for cervical ripening but according to Krening et al. (2012) the use of Syntocinon, for induction of labor has long been a source of argument because of increased obstetrical legal claims following tachysystole occurring after the administration of Syntocinon.23 Also this treatment protocol needs a closed monitoring of mother and increases workload on labor and delivery units, searching for a perfect, safe and effective method for cervical ripening in order to manage cervical ripening process and a normal vaginal delivery is necessary.4

Although Mansour Ghanei et al. (2013) and Yazdizadeh et al. (2013) conducted Randomized Controlled Trial (RCT) study for comparing effect of administration of IM in compare with placebo on cervical ripening and success of labor induction; and Haghighi et al. (2013) carried out a research concerning intravaginal ISDN or Misoprostol for cervical ripening; however, vaginal administration of isosorbide mononitrate (IM) is considered a low-risk method of labor induction for pregnant women at full term.12, 16, 17 Considering lack of fully agreement upon the most appropriate method for cervical ripening and induction of labor by practitioners 16, and absence of enough studies regarding comparison of vaginal IM pills with administration of Syntocinon (as a routine and usual method for induction of labor in maternity units) 10 in Iran, this study was designed to assess the effect of vaginal IM pills in compare with low-dose Syntocinon in type of delivery.

Methods

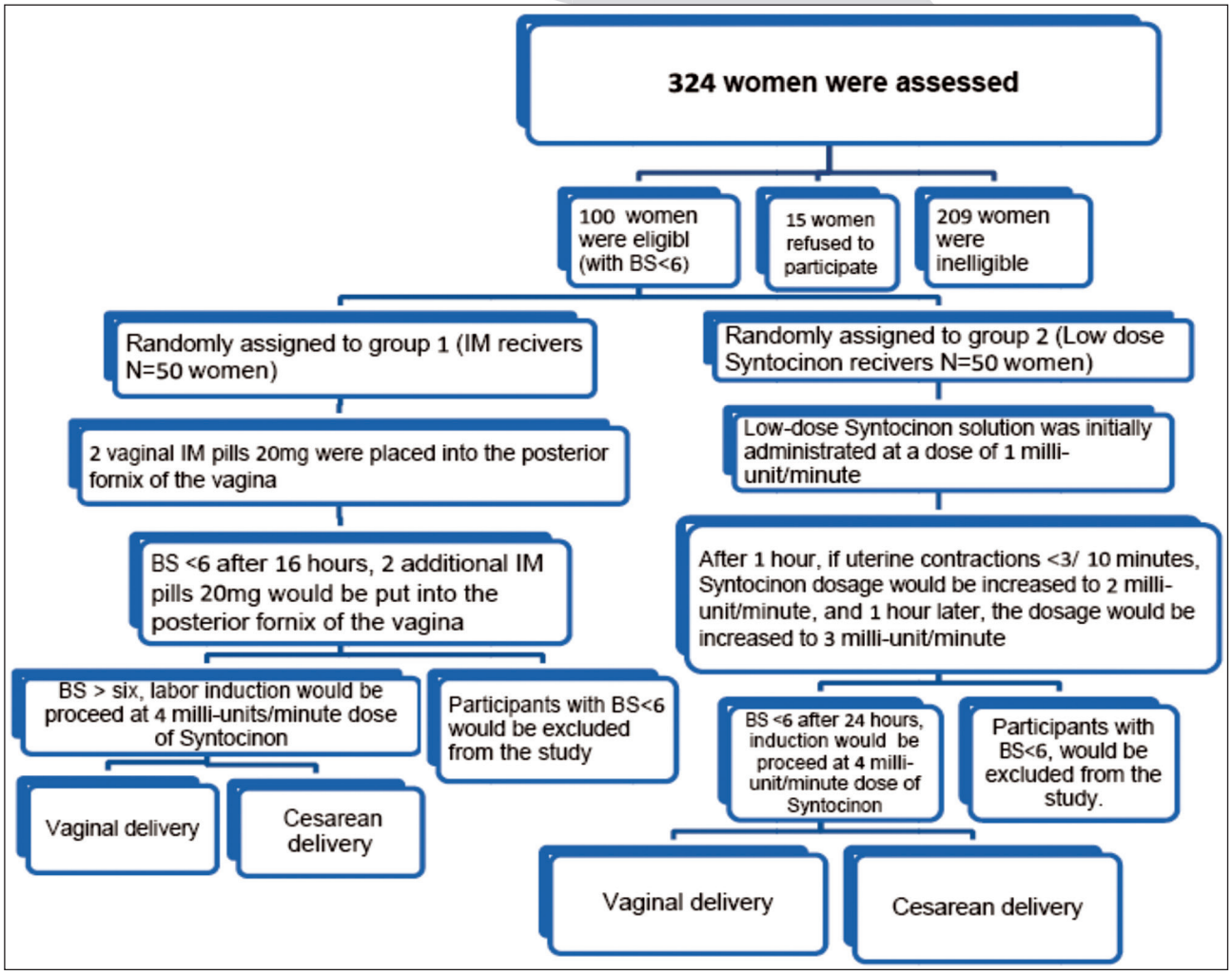
This study was a comparative RCT research. It was conducted from March 2009 to August 2010 in Shahid Sadoughi University of Medical Sciences of Yazd, Iran. Ethical approval was obtained from the ethics committee of Shahid Sadoughi University of Medical Sciences before initiating

the study protocol with code 113-54. Convenience sampling was used to selecting the participants. Sample size was determined 86 women according to statistical calculation in 95% confidence level, but because of probability of exclusion from the study or emergency condition during treatment, one hundred women were considered as participants. Participants included primigravida women who needed labor induction due to termination of pregnancy (Postterm pregnancy, Oligohydramnios, Preeclampsia) with unsuitable cervix condition who were admitted in Shahid Sadoughi referral hospital of Yazd during 2009-2010. Informed consent was obtained from eligible women. Inclusion criteria were singleton pregnancy, Cephalic presentation, intact embryo membranes, BS <6, reactive results of fetal wellbeing monitoring test, lack of uterine contractions, lack of Cephalo Pelvic Disproportion (CPD), Polyhydramnios, vaginal delivery contraindication (including previous uterine scar, history of cervix conization), absence of unexplained antepartum vaginal hemorrhage and absence of systematic problems for clinical treatment.

Allergic reaction to administrated drugs after participation in the trial and BS<6 were determined as exclusion criteria. At the beginning of the study, clinical examinations including BS evaluation, checking of vital signs (Blood Pressure, temperature, and mother's puls rate), systematic examinations, Fetal Heart Rate (FHR) monitoring, uterine contractions and Trans Abdominal Sonography (TAS) were all done by the investigator.

Assessment of the cervix including an measurement of consistency, length, dilatation, position, and station of the fetal presenting part as described by Bishop in 1964 and modified by Calder et al. in 1977 were performed.2, 14

Participants were randomly allocated in two groups using a computer-generated random num-



Algorithm 1. Sampling process and treatment protocol in participants

ber table. Sampling process and treatment protocol were showed in algorithm 1.

In the group 1, two vaginal IM pills 20 mg were placed into the posterior fornix of the vagina and BS was again measured after 16 hours. Then, two additional other IM pills would be put into the posterior fornix of the vagina if BS was less than six, and BS was once again assessed 24 hours after the first dosage administration. If BS exceeded six, labor induction would proceed at four milli-unit/minute dose of Syntocinon. Participants with BS<6 would be excluded from the study. During the treatment, participants, heart rate and BP were measured every four hours. In group 2 (receivers of low-dose Syntocinon), the solution was initially administrated at a dose of one milli-unit/minute. After one hour, if frequency of uterine contractions were less than three contractions per 10 minutes, Syntocinon dosage would be increased to two milli-unit/minute, and the dosage would be increased to three milli-unit/minute in the case of continuity of this condition. The last dosage would be kept until fully cervical ripening if FHR and uterine contractions frequency were ideal. If frequency of contractions in 10 minutes were more than five (tachysystole) or contraction lasted longer than 90 seconds (hypertonus), hyperstimulation would be diagnosed and Syntocinon administration would be stopped. Cervical examinations were done every four hours by the investigator and other maternity assistant coworkers. The investigator checked and compared the results of its BS measurement and other maternity assistant coworkers. Because maternity assistant coworkers were trained in the same method on BS measurement, the results of their examination were similar to the investigator.

Participants, heart rate and BP were checked every four hours. BS was measured again 24 hours after treatment initiation. If BS exceeded six, labor induction would proceed at four milli-unit/minute dose of Syntocinon and in the case of BS<6, the participant would be excluded from the study. Three criteria were taken into account concerning duration of cervical ripening. Cervical ripening would be terminated as long as any of these three criteria were observed and the decision on participant would be taken depending on the situation. The criteria includeing BS≥7 in vaginal examinations, spontaneous rupture of embryo membranes and failure to ripe the cervix 24 hours after start of treatment. Five milli-unit/milliliter Syntocinon ampoule produced by Abidi Company and 20 mg IM pill produced by Schwartz Company were used in this study.

All data related to personal characteristics, history of pregnancy, results of vital signs measuring and monitoring, other performed clinical examinations, BS assessment and other treatment protocol were recorded at the beginning of the study until the end of the research by investigator and other maternity assistant coworkers by means of questionnaire. Data were analyzed using descriptive statistical tests and t-student tests. P value <0.05 was determined as significance level.

Results

The results showed that mean age of participants was 24 ± 3.9 years in group1 and 24.3 ± 4.3 years in group 2. There was not any significant statistical difference between two groups in mean of participants, age (P=0.671) (Table 1). Mean of Gestational Age based on the first day of Last Menstrual Period (LMP) were 40.2 ± 1.4 weeks in group1 and 39.9 ± 1.5 weeks in group 2 (P=0.254) (Table 1). Mean of Gestational Age based on Trans Abdominal Sonography were 40.1±1.3 in weeks in group1 and 39.7±1.4 weeks in group 2 respectively (P=0.211) (Table 1). Significant statistical difference was not observed between two groups in mean of Gestational Age (based on LMP and Trans Abdominal Sonography). Frequency of previous abortion were 12 (24%) in group1 and 10 (20%) in group 2 (P=0.577) (Table 1).

One of the objectives of the study was to compare effect of two treatment methods, namely IM and low-dose Syntocinon on cervical ripening for labor induction in two groups. Significant statistical difference were not observed between two groups in mean of BS at the beginning of the intervention, mean length of labor induction, mean of neonate, s Apgar Score in the first and the fifth minutes. The results showed that mean of BS at the beginning of the intervention were 3.5 ± 1.0 in group 1 and 3.3 ± 1.0 in group 2 (P=0.221) (Table 2). Mean length of cervical ripening until delivery were 36.13 ± 4.05 hours in group 1 and 36.28 ± 3.88 hours in group 2, which suggests insignificant statistical differences (P=0.876) (Table 2).

Mean of neonate, s Apgar Score in the first minute were 8.8 ± 0.3 in group1 and 8.7 ± 0.6 in group 2 (P=0.424) (Table 2). Mean of neonate, s Apgar Score of the fifth minute were 9.9 ± 0.2 in group 1and 9.1 ± 0.3 in group 2 (P=0.656) (Table 2).

Assessment the type of delivery, indicated that most women in two groups (72% in group 1 and 78% in group 2) delivered vaginally following cervical ripening protocol, which they were similar according to P = 0.645 (Table 3).

Table 1. Comparison of participants' personal characteristics and obstetrics parameters in two groups

	group 1 (IM pill receivers)	group 2 (Low-dose Syntocinon receivers)	P-value
Mean of participants age (year)	24 ± 3.9	24.3 ± 4.3	0.671 (NS)
Mean of Gestational Age based on LMP (week)	40.2 ± 1.4	39.9 ± 1.5	0.254 (NS)
Mean of Gestational Age based on Trans Abdominal Sonography (week)	40.1±1.3	39.7±1.4	0.211 (NS)
Frequency of previous abortion	12 (24%)	10 (20%)	0.577 (NS)

**NS: Not statistically significant*

Table 2. Comparison of mean length of labor induction and Apgar Score in two groups

Treatment methods results	group 1 (IM pill receivers)	group 2 (Low-dose Syntocinon receivers)	P-value
Mean of BS at the beginning of the intervention	3.5 ± 1.0	3.3 ± 1.0	0.221
Mean length of labor induction until delivery (hour)	36.13 ± 4.05	36.28 ± 3.88	0.876 (NS)
Mean of neonate, s Apgar Score in the first minute	8.8 ± 0.3	8.7 ± 0.6	0.424 (NS)
Mean of neonate, s Apgar Score in the fifth minute	9.9 ± 0.2	9.1 ± 0.3	0.656 (NS)

**NS: Not statistically significant*

Table 3. Frequency Comparison of type of delivery in two groups

Type of delivery	group 1 (IM pill receivers)	group 2 (Low-dose Syntocinon receivers)	P-value
Vaginal delivery	36 (72%)	39 (78%)	0.645 (NS)
Cesarean delivery	14 (28%)	11 (22%)	

**NS: Not statistically significant*

Table 4. Comparison of reasons for Cesarean delivery in two groups

Reasons for Cesarean delivery	group 1 (IM pill receivers)	group 2 (Low-dose Syntocinon receivers)	P-value
Arresting in labor progress	13 (92.9%)	9 (81.9%)	0.468 (NS)
Meconium expulsion	1 (7.1%)	2 (18.8%)	

**NS: Not statistically significant*

Table 5. Comparison of participants, satisfaction concerning treatment procedure in two groups

participants, satisfaction concerning treatment procedure	group 1 (IM pill receivers)	group 2 (Low-dose Syntocinon receivers)	P-value
	46 (92. %)	45 (90%)	0.99 (NS)

NS: Not statistically significant

The main reason of Cesarean delivery in two groups was arresting in delivery progress in two groups (92.9% in group1 and 81.9% in group 2). No significant statistical difference was observed between two groups (P= 0.468) (Table 4).

One of the aims of this study was to compare participants, satisfaction concerning treatment procedure. According to the results of the study participants in group 1 were more satisfied with treatment protocol (92%) than group 2 (90%), but this difference was not statistically significant (P= 0.99) (Table 5).

Discussion

According to International Federation of Gynecology and Obstetrics (Figo, 2009) unnecessary Cesarean delivery is not ethically and legally accepted and several strategies in order to decrease it's worldwide rate should be performed.24 New appropriate treatment methods for cervical ripening without unwanted serious side effects have been located in obstetrics research.15,20,21 The main objective of this study was to compare the effect of vaginal IM pill and low-dose Syntocinon for cervical ripening during labor on type of delivery.

The results of this study showed that mean length of cervical ripening until delivery was similar in two groups. In the similar studies the mean of BS was greater and the admission until delivery interval was shorter for the IM group in compare with placebo group, with statistically significant differences. 4, 12,16, 20, 25, 26,27 Also in Bollapragada et al. (2009) study, the effect of two aforementioned drugs (IM and placebo) on labor duration was reported no statistically significant. 28 In Haghighi et al. (2013) study labor induction in the ISDN recievers group, was required more repeatedly and the time from the beginning of intervention to the initiation of active phase of labor in compare with Misoprostol receiver group was significantly longer.17 The results of current study showed that vaginally administration of IM is as effective as Syntocinon on cervical ripening and length of intervention to delivery. This finding approved the ripening effect of IM on cervix cells.

Mean of Apgar Score in the first and the fifth minute were similar in two groups in this study. The results implied that neonatal outcomes after vaginally IM administration were comparable with Syntocinon group. The same results reported in similar studies.2, 4, 12, 15, 16, 18, 20, 25, 26, 28, 29, 30 According to this study an acceptable mean of Apgar Score approved vaginally administration of IM pill is as safe as Syntocinon without adverse side effects on neonate's Apgar Score.

The results of this study showed that percent of vaginal delivery were similar in two groups. The findings of the study regarding the rate of Cesarean delivery was in accordance with findings of other related studies.4, 12, 16, 25, 26, 27, 28 The most reason for Cesarean delivery in this study was reported arresting in labor process in two groups. In similar studies reasons for Cesarean delivery were not reported. One of findings of this study was to compare participants, satisfaction regarding treatment procedure. According to results of the study participants in IM receiver group were more satisfied with treatment protocol than Syntocinon receiver group without statistically significant difference. In Agarwal and Bollapragada et al. (2009), Bullarbo studies, participants, satisfaction concerning treatment procedure was reported similar in IM receiver group and placebo receiver group without any statistically significant differences. 4, 26, 28Although this difference was not statistically significant, it seems that more participants, satisfaction in IM receiver group in this study is related to their less painful contractions in compare with Syntocinon receiver group.

BS measurement by other maternity assistant coworkers was the limitation of conducting this research because of determination of BS needed a long time period attending in the hospital from mother admission in labor to after delivery, which it is impossible to be done by one person. Fortunately, maternity assistant coworkers trained in the same method and the results of the examination were similar to investigator. Also this limitation was observed in other related studies.

Conclusion

The results of this study approved Vaginal Iisosorbide Mononitrate pill is effective, safe and available as low-dose Syntocinon in type of delivery and can be used as simple and inexpensive treatment for delivery.

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Conflict of interest

The authors verify that this project has been supported financially by Shahid Sadoughi University of Medical Sciences, Yazd, Iran. There is no conflict of interest in this article.

References

1. *Nabhan AF, Noha H Rabie. Isosorbide mononitrate versus alendronate for postmenopausal osteoporosis. Int J Gynecol Obstet .2008; 103: 213-6.*
2. *Nicoll AE, Mackenzie F, Greer IA, Norman JE. Vaginal application of the nitric oxide donor isosorbide mononitrate for preinduction cervical ripening: a randomized controlled trial to determine effects on maternal and fetal hemodynamics. Am J obstet Gynecol. 2001; 184: 958-64.*
3. *Chwalisz K, Garfield RE. Nitric oxide as the final mediator of cervical ripening. Hum Reprod. 1998; 13: 245-52.*
4. *Bullarbo M, Orrskog ME, Andersch B, Granström L, Norström A, Ekerhovd E. Outpatient vaginal administration of the nitric oxide donor isosorbide mononitrate for cervical ripening and labor induction postterm: a randomized controlled study. Am J Obstet Gynecol. 2007; 196: 50.e1-5.*
5. *Norman J, Thomson A, Greer I. Cervical ripening after nitric oxide. Hum Reprod. 1998; 13: 251-2.*
6. *Izumi H, Yallampalli C, Garfield R. Gestational changes in L-arginine–induced relaxation of pregnant rat and human myometrial smooth muscle. Am J Obstet Gynecol. 1993; 169: 1327-37.*
7. *Rowlands S, Trudinger B, Visva-Lingam S. Treatment of preterm cervical dilatation with glyceryl trinitrate, a nitric oxide donor. Aust N Z J Obstet Gynaecol. 1996; 36: 377-81.*
8. *Lees C, Campbell S, Jauniaux E, et al. Arrest of preterm labour and prolongation of gestation with glyceryl trinitrate, a nitric oxide donor. Lancet. 1994; 343: 1325-6.*
9. *Mercier F, Dounas M, Bouaziz H, Lhuissier C, Benhamou D. Intravenous nitroglycerin to relieve intrapartum fetal distress related to uterine hyperactivity: a prospective observational study. Anaesth Analg. 1997; 84: 1117-20.*
10. *Cunningham F, Kenneth L, Bloom S, Hauth J, Gilstrap L, wenstrom K. Williams Obstetrics. 23th ed. New York: McGraw Hill; 2010.*
11. *Glantz JC. Labor induction rate variation in upstate New York: what is the difference? Birth. 2003; 30: 168-74.*
12. *Mansour Ghanaie M, Mirblouk F, Godarzi R, Shakiba M. Effect of outpatient Isosorbide Mononitrate on success of labor induction. J Babol Univ Med Sci. 2013; 15: 12-17.*
13. *Keirse M. Any prostaglandin/any route for cervical ripening. Oxford, (UK): Cochrane Collaboration; 1995.*
14. *Calder A, Embrey M, Tait T. Ripening of the cervix with extraamniotic prostaglandin E2 in viscous gel before induction of labour. BJOG. 1977; 84: 264-8.*
15. *Wo¨lfler MM, Facchinetti F, Venturini P, et al .Induction of labor at term using isosorbide mononitrate simultaneously with dinoprostone compared to dinoprostone treatment alone: A randomized, controlled trial. Am J Obstet Gynecol .2006; 195: 1617–22.*
16. *Yazdizadeh H, Abedi P, Najar S, Angali KA. The impact of isosorbide mononitrate on cervical ripening and labor induction in primiparous women with term pregnancy: A double-blind, randomized, controlled trial. Iran J Nurs Midwifery Res. 2013; 18: 246-50.*
17. *Haghighi L, Homam H, Raoofi Z, Najmi Z. Intravaginal isosorbide dinitrate or misoprostol for cervical ripening prior to induction of labour: a randomised controlled trial. J Obstet Gynaecol. 2013; 33: 272-6.*
18. *Nunes P F, Campos PR, Manvel M. Intravaginal glyceryl trinitrate and dinoprostone for cervical ripening and induction of labor. Am J Obstet Gynecol. 2006; 194: 1022-6.*
19. *Chanrachakul B, Herabutya Y, Punyavachira P. Potential efficacy of nitric oxide for cervical ripening in pregnancy at term. Int J Gynecol Obstet. 2000; 71: 217-9.*
20. *Abdellah MS, Hussien M, Aboalhassan A. Intravaginal administration of isosorbide mononitrate and misoprostol for cervical ripening and induction of labour: a randomized controlled trial. Arch Gynecol Obstet. 2011; 284: 25-30.*

21. *Najmi RS, Rehan N. Prevalence and determinants of caesarean section in a teaching hospital of Pakistan. J Obstet Gynaecol. 2000; 20: 479-483.*

22. *Ministry of Health and Medical Education. Rate of Cesarean delivery. 2013. [Online access]. http:// behdasht.gov.ir/index.aspx?siteid=1&pageid=27 467&newsview=12392&pro=nobak.[September 2013]*

23. *Krening, C F, Rehling-Anthony K, Garko C. Oxytocin administration. The transition to a safer model of care. J Perinat Neonat Nurs. 2012; 26: 15–24.*

24. *International Federation of Gynecology and Obstetrics. Ethical Issues in Obstetrics and Gynecology by the FIGO Committee for the Study of Ethical Aspects of Human Reproduction and Women's Health. (2009). [Online access]. http: //www.figo.org/files/ figo-corp/Ethical%20Issues%20-%20English.pdf. [September 2013]*

25. *Habib SM, Emam SS, Saber AS. Outpatient cervical ripening with nitric oxide donor Isosorbide Mononitrate prior to induction of labor. Int J Gynaecol Obstet. 2008; 101: 57-61.*

26. *Agarwal K, Batra A, Batra A, Dabral A, Aggarwal A. Evaluation of isosorbide mononitrate for cervical ripening prior to induction of labor for postdated pregnancy in an outpatient setting. Int J Gynecol Obstet. 2012; 118 : 205–9.*

27. *Rameez MF, Goonewardene IM. Nitric oxide donor isosorbide mononitrate for pre-induction cervical ripening at 41 weeks' gestation: A randomized controlled trial. J Obstet Gynecol Res. 2007; 33: 452-6.*

28. *Bollapragada SS, MacKenzie F, Norrie JD, et al. Randomised placebo-controlled trial of outpatient (at home) cervical ripening with isosorbide mononitrate (IMN) prior to induction of labor: Clinical trial with analyses of efficacy and acceptability. The IMOP study. BJOG. 2009; 116: 1185-95.*

29. *Ekerhovd E, Bullarbo M, Andersch B, Norström A. Vaginal administration of the nitric oxide donor isosorbide mononitrate for cervical ripening at term. Am J Obstet Gynecol. 2003; 189: 1692-7.*

30. *Osman I, Mockenzie F, Norrie J, Murray HM, Greer IA, Norman JE. A randomized comparison of PGE2 gel with the nitric oxide donor ISOSORBIDE MONONITRATE for cervical ripening before the induction of labor at term. Am J Obstet Gynecol. 2006; 194: 1012-21.*

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Advantages of eProblem-based learning in healthcare education: A literature review

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Abstract

Objectives: This review aimed to explore the advantages of eProblem-Based Learning in healthcare education in terms of group work and communication. The addressed research questions were: What are the evident advantages of eProblem-Based Learning? Does the available evidence provide information about eProblem-Based Learning in healthcare education? Does virtual learning environment support Problem-Based Learning interactions between students, and between students and teachers? Does virtual learning environment support Problem-Based Learning group work?

Data sources and Review methods: all major electronic sources of relevant information were systematically searched to identify peer-reviewed English language papers published between 2000 and 2013. To identify all relevant studies for the review, the search strategy comprised searches of the following databases: CINAHL, Proquest, Medline, Science Direct, DOAJ and Internet source (www.google.com) using the key words: Problem-Based Learning, eProblem-Based Learning, online Problem-Based Learning, LMS, helthcare education. Included were studies which trials PBL strategy practice online especially in healthcare education.

Results: This review has shown that PBL can be successfully implemented in an online format. It affords increased flexibility in time and location of learning. Adults learners with children or those who juggle work with studies, are likely to benefit the most from this added flexibility. Another potential innovation of ePBL could be interdisciplinary teams as small learning groups. A team made up of a nursing student, a medical student, a social work student, and a pharmacy student could operate differently from the PBL tutorial group as currently constituted.

Conclusions: Evidently ePBL research in healthcare and nursing education is still at its beginning. Additional research with larger sample size and high quality is needed to ascertain real effectiveness within healthcare and nursing educational context. Design recommendations include an emphasis on incorporating a rich multimedia background, realistic communication, and project management tools.

Key words: Problem-Based Learning, eProblem-Based Learning, online Problem-Based Learning, Virtual Learning Environment, Health Education, Communication, Group work.

Introduction

Problem-based learning (PBL) has its origins within the medical education of North America during the late 1960s (Barrows, 1996). Therefore PBL is most widely associated with the large body of literature coming out of medical education. PBL is based on assumptions that learning is an active, integrated, and constructive process influenced by social and contextual factors. While the content and structure of PBL courses may differ, the general goals and learning objectives tend to be similar. Howard Barrows (1996) lists the six original characteristics for the problem-based learning model employed in the medical school as follows: learning is student centered; learning occurs in small student groups; teachers are facilitators or guides; problems form the original focus and stimulus for learning; problems are a vehicle for the development of clinical problem solving skills; new information is acquired through self-directed learning. PBL is not merely a teaching and learning technique, but a total approach to education. There are four important components of PBL: PBL curriculum design, PBL tutorials, PBL compatible assessments and philosophical principles underpinning PBL (Barrett, 2005). Evidently students learn as many facts as those in traditional classes, but they enjoy their studies more and become lifelong learners (Aspy

et al., 1993; Biley and Smith, 1998; Cooke and Moyle, 2002; Dolmans et al., 2005). Groupwork is also an essential aspect of PBL for several reasons. It is interesting and motivating for students because they become actively involved in the work and are held accountable for their actions by group members (Cohen et al., 1994). Groupwork helps develop learning communities in which students feel comfortable developing new ideas and raising questions about the material (Allen et al., 1996), groupwork also enhances communication skills and students' ability to manage group dynamics. For these reasons, groupwork can enhance student achievement. Through the creative exchange of ideas, groups can solve problems and construct knowledge beyond the capacity of single member. So it is possible to talk about the concept of group learning (Cohen et al., 2002). It is generally agreed that effective PBL groups may be valued not only for the content of their work, but also for the way they work (Lycke, 2002). Well designed, open-ended problems that require the input and skills of all group members also are essential to positive groupwork experiences (Cohen, 1994). However, groups do not always work effectively without guidance. Usually the instructor facilitates and monitors group interactions. Many students have had little experience working in groups in an academic setting. The role of the facilitator is therefore central to the process, and without adequate understanding of the processes and philosophies involved, a PBL programme or any other innovations cannot function (Biley and Smith, 1998). Small-group work protects against dropout and encourages students to study regularly (Schmidt et al., 2011).

Disadvantages of PBL

Despite of evident success of PBL, areas of weakness have been identified (Biley and Smith, 1998): time-consuming when compared to the average, crucial literature and expert resources were sometimes missed and the end result somewhat superficial, many students feel unduly pressurized and anxious when embarking on a PBL curriculum.

Students are familiar with traditional methods and may feel threatened if the system is changed. Due to lack of fixed curriculum and textbooks these students, confronted with messy, real-world problems, often lack the skills towards self-directed learning (Wood, 1994; Hoffman and Ritchie, 1997). PBL turns the teacher, as one with authority and ownership of knowledge, into a facilitator who does not necessarily know the answer, a situation which is less comfortable (Wood, 1994).

Learning technology and PBL

PBL offers some advantages over traditional forms of instructional delivery, but it brings with it a variety of obstacles. Hallinger (2005) takes the position that the PBL and learning technology have the potential to enhance to each other's strengths. Computer technologies can provide learners with access to knowledge relevant to a given problematic situation in ways that are more flexible and powerful than traditional searching through books and journal articles (Hallinger, 2005). Hoffman and Ritchie (1997) discuss how multimedia might be used to address some problems with PBL. Multimedia as a tool for PBL activities holds the potential to help practitioners overcome numerous weaknesses and pitfalls of PBL. Camp (1996) has predicted potential coming PBL innovation and real possiblity computer based or "virtual" PBL groups. Online learning practices usually follow constructivist perspectives and make use of the Internet to facilitate personalized learning regardless of time and space boundaries. Moreover, as online learning environments are flexible, attractive and interactive, it is more convenient to implement constructivist and PBL practices through online learning tools. Online collaboration, case learning and PBL were the preferred instructional methods during the coming decade for online instructors in colleges and universities. When asked to select four pedagogical techniques that would be used most widely online during the next few years from a list of twelve instructional methods, over 65 percent selected group problem-solving and collaborative tasks, while 58 percent chose problem-based learning (Bonk et al., 2004). However, there is still confusion about the models, media and environments being used to support various kinds of PBL that use technology in some way (Savin-Baden, 2006). Depending on media and the method of delivery, partially or fully online, the following terms are used in the lit-

erature: web based PBL, online PBL, ePBL, virtual PBL, distributed PBL (dPBL), and blended PBL. Bowdish et al. (2003) in their research focused on exploring the effectiveness of prototypic virtual PBL (via world wide web), where a careful combination of digital video, images, text, question and radio button prompts, and text boxes for posting group responses was implemented. PBL online is the incorporation of PBL strategy with web-based technology. This combination provides a more flexible mode of education delivery, while retaining the key beneficial features of PBL. Online PBL also has the advantage of reducing the sense of isolation, which reduces motivation that pervades much of distance education. Students work cooperatively in virtual learning environmments such as Lotus Notes, WebCT, Blackboard, Moodle, etc. Distributed PBL (dPBL) is a version of PLB that can be offered to distance learners. Wheeler (2006) stated that in dPBL learning is mediated through computer technology, and a shared, 'virtual' distributed learning environment is used to enable students to collaborate. McConnell (2006) introduced two types of distributed PBL: collaborative dPBL and cooperative dPBL. Cooperative and collaborative learning essentially provide a structured way of sharing responsibilities for learning in groups. In collaborative dPBL students work in small group to define a problem relating to their personal or professional practice. A blended PBL approach could contribute to overcoming some existing problems experienced in conventional PBL by providing a complementary pedagogical tool. Blending e-learning with conventional PBL may help overcome student-perceived shortcomings of conventional PBL and improve the learning experience, making learning more interactive and interesting (Alamro and Schofield, 2012). Technology helps teacher implement PBL, but PBL also helps teachers integrate technology by providing reasons for its use (Ravitz and Blazevski, 2010). Savin-Baden (2006) emphasises that the aim of online PBL is to develop and supplement what has already been achieved rather than replace it. Then also notes some concerns whether ePBL will affect the existence of facetoface problem-based learning since ePBL will be seen as being more cost effective, will destroy some of the original aims of problem-based learning since some forms of online problem-based learning tend to focus on solving narrowly defined problems that fail to encourage students to be independent inquirers who own their learning.

PBL online vs. PBL f2F

Cheaney and Ingebritsen (2005) in their study note a major difference between online PBL and PBL in a traditional face-to-face learning environment. There are obvious differences in the way of interaction between group members, with regards to scheduling, interpersonal relationships, student motivation, timeliness, and technical problems with hardware, software, or infrastructure. Findings indicated that online PBL application increased students' achievement levels and retention scores (Bedard et al., 2012; Chagas et al., 2012). The advantages that are often unnoticed in discussion about PBL online are (Savin-Baden, 2007):

changes in university learning and teaching culture, new type of learning community, in some cases easier computed mediated communication than face-to-face with no social, pressure and greater freedom, virtual environment provide more opportunity for reflective analysis. Most of the studies when review effectiveness of online instruction compared with the traditional classroom are focused on measurable student outcomes (Ramage, 2002). Clark (1994) presents the idea that selection of the media has little to do with learner outcomes, rather the method that the media share in delivering content. How faithfully can PBL methodologies be applied online? An important element in PBL group processing and collaborative learning is building a sense of learning community. Given current technology, a low degree of intimacy and immediacy is inherent in online environments; thus, how do learning groups collaborate effectively to negotiate meaning? How can tutor effectively nurture and guide learning online? How can we support selfdirected learning online? What compromises, if any, are required to engage learners in PBL online? (Hung et al., 2008).

PBL Group work and communication

Chiriac (2008) notes that there are a few study conducted on the core of PBL, the tutorial group. These studies are focused on the role of problems,

the cognitive process and their effects on achievement, motivational influences and the influence of the tutor on students'learning. Students have different levels of communication apprehension and these can affect their performance in group discussions. Students' participation in PBL is affected by their comfort with communicating in groups. Although a student may be less involved in PBL discussions, this is not necessarily linked to an inability to acquire and process the information (Blue et al., 1998). Moving PBL online can provide scaffolding to further support collaborative knowledge construction. Most studies recommended the provision of pre-intervention training for both the learners and facilitators, who are novice to the online PBL platform; adequate preparation of facilitators prior to assuming their roles; and clear learning objectives and course expectations to be conveyed to the learners prior to the commencement of the course (Lien, 2010). The key to the efficacy of collaborative learning is social interaction (Kreijns et al., 2003). Northrup (2001) propose a framework of interaction that includes interaction with content, collaboration, conversation, intrapersonal interaction, and performance support. Collaborative group skills needed careful teaching and use of as many forms of communication as possible, both synchronous and asynchronous, helped develop social presence and collaborative communities online (Stacey, 2004). It has been found that in online settings a smaller number of group members makes online communication and collaboration more effective and active and transforms groups into teams more rapidly (Novak, 1998; Donnelly, 2010). Synchronous collaboration tolls are vital for the effective use of PBL online because tools such Chat, shared whiteboards, video conferencing or collaborative web surfing for groups (group surf the same website same time) are crucial to ensuring collaboration within the PBL (Savin-Baden, 2007). Baturay and Bay (2010) study measured the effects of problem-based learning on higher education students' web-based classroom community perceptions and achievement. The results indicated that students who worked on problem-based projects felt much more connected to other class members when compared to the control group. Education is a social process therefore, online learning environments must be able to support the social practice and process of learning. The construct and research on social presence help explain how the social practice and process of learning takes place online. Establishing social presence is an important aspect for effective online interaction and learning (Stacey, 2002). Social presence is defined as the ability of participants in a community to project themselves, socially and emotionally, as real people through a medium of communication (Garrison and Anderson, 2003). Richardson and Swan (2003) study indicates that students perceive the presence of others in their online learning experience as an essential part of it and that students' perceptions of satisfaction with an instructor are related to their perceptions of social presence. Furthermore their study indicates that the intensity of social presence students' perceived in their online courses, from both their instructor and their peers, was directly related to their perceived learning. Barrows (2002) and Orrill (2002) are certain that collaboration suffered in online PBL environments. Orrill (2002) highlighted some difficulties in the transition from face-to-face to online PBL, related to the need for appropriate tools to support collaborative problem solving in a distributed context. Barrows (2002) has raised concerns regarding the application of authentic PBL in a distributed form. These concerns include questioning about the need for synchronous communication, the need of new skills for the dPBL tutor as facilitator and the development of adequate mediating technology. Facilitating virtual teams in dPBL courses takes time, effort and requires significant staff training.

PBL in healthcare education

The expectations of PBL teaching strategy are that it will enable nurses to develop skills required for professional practice that including enquiry, reasoning, interpersonal and lifelong learning skills. The problems are used as a focus for learning basic science and clinical knowledge along with clinical reasoning skills. PBL approaches are claimed to be more effective in preparing student nurses for this role than the more traditional educational methods, so PBL has been adopted by a number of nursing and midwifery faculties (Barrow et al., 2002). Biley and Smith (1998) noted that mostly

benefits of the PBL method have been extrapolated from research into the medical experience and limited evidences of PBL implementation in nursing education. They stated also that would be a mistake to wholeheartedly embrace the concept of PBL only on the supporting evidence of the medical literature. The suitability of PBL as a teaching method in health care and nursing will be shown only on the basis of the relevant research. Yuan et al. (2008) conducted a systematic review to provide the available evidence on developing nursing students' critical thinking through PBL. But the available evidence did not provide supportive database to research. Baker et al. (2007) in their research study contributed some evidence regarding the influence of PBL methods on learning styles of master's students in Nursing Administration.

ePBL in healthcare education

The results of Chagas et al. (2012) research showed a great variability in the degree of participation of each member of the group as well as the development of a group dynamic. Despite the individual variability observed, most students kept actively involved in the work. Not all, but some groups revealed a very good capability for self-regulation. Different digital skills possessed by each individual student presenting a great imbalance in the level of participation of each group

Table 1. Mapping of reviewed articles – PBL online in health and nurse education

Author (year)	Design, method	Sample, participants	Duration	Intervention	Findings, results
Chagas et al. (2012)	Qualitative study	30 students of an online course on health education	3 years	online PBL	participation, group functioning
King et al. (2010)	Qualitative study	20 students in interprofessional Health Science course	10 class period	online PBL	interaction between the social context (group PBL) and the environmental context (online)
Candela et al (2009)	Qualitative study	6 graduated students in doctoral nursing program	semester-long	online PBL	positive perception, improvement in leadership abilities and confidence
Valaitis, Sword, Jones & Hodges (2005)	Qualitative study	22 undergraduate nursing and midwifery students, graduate students in a neonatal nurse practitioner program	6 weeks	online PBL	deeply process content, difficulties making group decisions online
Spinelo et al. (2004)	Qualitative study	28 undergraduate public health education students	semester course	web-based computer simulation as an implementation of PBL	web- based simulation as motivation
Choi (2003)	Qualitative study	109 nursing students	4 weeks	Online PBL	the role of interaction between tutor and student in web-based PBL
Price (2000)	Qualitative study	69 students of distance learning modul of the Bachelor of Science with honours degree in Nursing Studies	Semester-long	PBL within distance	The role of learning materials and tutors as facilitators

member. King et al. (2010) noticed a good interaction between the social context (group PBL) and the environmental context (online). Candela et al. (2009) described the use and positive students' perception of a semester-long problem-based learning activity in an online doctoral course focusing on nurse educator leadership. Valaitis et al. (2005) in their project investigated the health sciences students' perceptions of their experiences in online PBL, focusing on the learning and group process in the online environment. They exposed the importance of tutors and students online literacy skills development to smooth their transition to an online PBL environment. The study of Spinello et al. (20004) suggested that a PBL experience based on a community simulation may be effective in providing a motivating and interesting PBL tool for instructing undergraduate public health students. Price (2000) reported experiences with introducing PBL into one module of the Bachelor of Science with honours degree in Nursing Studies by distance learning degree (table 1).

Conclusions

This review has shown that PBL can be successfully implemented in an online format. It affords increased flexibility in time and location of learning. Adults learners with children or those who juggle work with studies, are likely to benefit the most from this added flexibility (Valaitis et al., 2005).

Another potential innovation of ePBL could be interdisciplinary teams as small learning groups. A team made up of a nursing student, a medical student, a social work student, and a pharmacy student could operate differently from the PBL tutorial group as currently constituted (Camp, 1996).

Evidently ePBL research in healthcare and nursing education is still at its beginning. Additional research with larger sample size and high quality is needed to ascertain real effectiveness within healthcare and nursing educational context. Design recommendations include an emphasis on incorporating a rich multimedia background, realistic communication, and project management tools.

References

1. *Alamro AS, Schofield S. Supporting traditional PBL with online discussion forums: A study from Qassim Medical School. Medical Teacher, 2012; 34(1): 20-24.*
2. *Allen DE, Duch BJ, Groh SE. The Power of Problem-based Learning in Teaching Introductory Science Courses. New Directions for Teaching and Learning, 1996; 68: 43-52.*
3. *Aspy DN. What doctors can teach teachers about problem-based learning. Educational Leadership, 1993; 50(7): 22-24.*
4. *Baker CM, Pesut DJ, McDaniel AM, Fisher ML. Evaluating the impact of problem-based learning on learning styles of master's students in nursing administration. Journal of Professional Nursing, 2007; 23(4): 214-219.*
5. *Barrett T. Understanding Problem-based learing. In Barrett, T., Mac Labhrainn, I., Fallon, H. (Eds). Handbook of Enquiry & Problem Based Learning, 2005; 13-25.*
6. *Barrow EJ, Lyte G, Buterworth T. An evaluation of problem-based learning in a nursing theory and practice module. Nurse Education in Practice, 2002; (2): 55-62.*
7. *Barrows HS. Problem-based learning in medicine and beyond: a brief overview. New Directions for Teaching and Learning, 1996; 68: 3-12.*
8. *Barrows H. Is it truly possible to have such a thing as dPBL? Distance Education, 2002; 23(1): 119122.*
9. *Baturay MH, Bay OF. The effects of problem-based learning on the classroom community perceptions and achievement of web-based education students. Computers & Education, 2010; 55(1): 43-52.*
10. *Biley FC, Smith KL. Exploring the potential of problem-based learning in nurse education. Nurse Education Today, 1998; 18(5): 353-361.*
11. *Blue AV, Stratton TD, Donnelly MB, Nash PP, Schwartz RW. Students' communication apprehension and its effects on PBL performance. Medical Teacher, 1998; 20(3): 217-221.*
12. *Bowdish BE, Chauvin SW, Kreisman N, Britt M. Travels towards Problem Based Learning in Medical Education (VPBL). Instructional Science, 2003; 31(4-5): 231-253.*
13. *Bonk CJ, Kim K-J, Zeng T. Future directions of Blended Learning in Higher Education, In Bonk, C. J. & Graham, C. R. (Ed.) Handbook of Blended Learning: Global Perspectives, Local Designs, 2004; 550-568.*

14. *Camp G. Problem-based learning: A paradigm shift or a passing fad? Medical Education Online, 1996; 1.*

15. *Candela L, Carver L, Diaz A, Edmunds J, Talusan R, Tarrant TA. An online doctoral education course using problem-based learning. The Journal of nursing education, 2009; 48(2): 116.*

16. *Chagas I, Faria C, Mourato D, Pereira G, Santos A. Problem-based Learning in an Online Course of Health Education. European Journal of Open, Distance and E-Learning, N1, 2012. http://www.eurodl.org/materials/contrib/2012/Chagas_et_al.pdf*

17. *Cheaney J, Ingebritsen TS. Problem-based Learning in an Online Course: A case study. The International Review of Research in Open and Distance Learning, 2005; 6: (3).*

18. *Chiriac EH. A scheme for understanding group processes in problem-based learning. Higher Education, 2008; 55(5): 505-518.*

19. *Choi H. A problem-based learning trial on the Internet involving undergraduate nursing students. The Journal of nursing education, 2003; 42(8): 359-363.*

20. *Clark RE. Media will never influence learning. Educational Technologa Research and Development, 1994; 42(2): 21-29.*

21. *Cohen EG. Restructuring the Classroom: Conditions for Productive Small Groups. Review Of Educational Research, 1994; (64)1: 1-35*

22. *Cohen EG, Lotar RA, Abram PL, Scarloss BA, Schultz SE. Can groups learn? Teachers College Record, 2002; 104(6): 1045-1068.*

23. *Cooke M, Moyle K. Students' evaluation of problem-based learning. Nurse Education Today, 2002; 22(4): 330-339.*

24. *Dolmans DH, De Grave W, Wolfhagen IH, Van Der Vleuten CP. Problem-based learning: future challenges for educational practice and research. Medical education, 20-05; 39(7): 732-741.*

25. *Donnelly R. Harmonizing technology with interaction in blended problem-based learning. Computers & Education, 2010; 54(2): 350-359.*

26. *Garrison DR, Anderson T. E-learning in the 21st century: A framework for research and practice. New York: Routledge Falmer, 2003.*

27. *Hallinger P. Integrating Learning Technologies and Problem-based Learning. In Proceedings of the Second International Conference on eLearning for Knowledge-Based Society. Bangkok, Thailand, 2005.*

28. *Hoffman BOB, Ritchie D. Using multimedia to overcome the problems with problem based learning. Instructional Science, 1997; 25(2): 97-115.*

29. *Hung W, Jonassen DH, Liu R. Problem-based learning. Handbook of research on educational communications and technology, 2008; 3: 485-506.*

30. *King S, Greidanus E, Carbonaro M, Drummond J, Boechler P, Kahlke R. Synchronous problem-based e-Learning (ePBL) in interprofessional health science education. Journal of Interactive Online Learning, 2010; 9(2): 133-150.*

31. *Kreijns K, Kirschner PA, Jochems W. Identifying the pitfalls for social interaction in computer-supported collaborative learning environments: a review of the research. Computers in human behavior, 2003; 19(3): 335-353.*

32. *Lien PC. Learning From Problem-Based Learning In A Web-Based Environment: A Systematic Review. Reflections on problem-based learning, 2010; 9: 12-19.*

33. *Lycke KH. Inside PBL groups: Observation, confirmations and challenges. Education For Health, 2002; 15(3): 326-334.*

34. *McConnell D. E-Learning Groups and communities, Open University Press, McGraw Hill, 2006.*

35. *Northrup P. A framework for designing interactivity in web-based instruction. Educational Technology, 2001; 41(2): 31–39.*

36. *Novak JD. Learning how to learn. Cambridge University Press. 1998.*

37. *Orrill CH. Supporting online PBL: Design considerations for supporting distributed problem solving, Distance Education, 2002; 23(1): 41-57.*

38. *Price B. Problem-based learning the distance learning way: a bridge too far? Nurse Education Today, 2000; 20: 98–105.*

39. *Ramage TR. The'No Significant Difference' Phenomenon: A Literature Review. Journal of Instructional Science and Technology, 2002; 5(1).*

40. *Ravitz J, Blazevski J. Assessing the impact of online technologies on PBL use in US high schools. Paper presented at Annual Meetings of the Association for Educational Communications and Technology. Anaheim, CA, 2010.*

41. *Richardson JC, Swan K. Examining social presence in online courses in relation to students' perceived learning and satisfaction. Journal of Asynchronous learning networks, 2003; 7(1): 68-88.*

42. *Savin-Baden M. The challenge of using problem-based learning online. Savin-Baden, M., & Wilkie, K. (Eds.). Problem-based learning online. Open University Press, 2006.*

43. *Savin-Baden M. Challenging models and perspectives of problem-based learning. Management of change: Implementation of problem-based and project-based learning in engineering, 2007; 9-30.*

44. *Schmidt HG, Rotgans JI, Yew EH, The process of problem-based learning: what works and why. Medical Education, 2011; 45: 792–806.*

45. *Spinello E, Fischbach R. Problem-based learning in public health instruction: A pilot study of an online simulation as a problem-based learning approach. Education for health-abingdon-carfax publishing limited, 2004; 17: 365-373.*

46. *Stacey E. Social presence online: Networking learners at a distance. Education and Information Technologies, 2002; 7(4): 287-294.*

47. *Stacey E. Problem-based learning online, in Research in Distance Education 6: selected refereed papers from the 2004 Research in Distance Education conference, Deakin University, Geelong, Vic., 2004; 59-70.*

48. *Valaitis RK, Sword WA, Jones B, Hodges A. Problem-based learning online: perceptions of health science students. Advances in Health Sciences Education, 2005; 10(3): 231-252.*

49. *Wheeler S. Learner support needs in online problem-based learning, The Quarterly Reviw of Distance Education, 2006; 7(2): 175-184*

50. *Wood EJ. The problems of problem-based learning. Biochemical Education, 1994; 22(2): 78-82.*

51. *Yuan H, Williams BA, Fan L. A systematic review of selected evidence on developing nursing students' critical thinking through problem-based learning. Nurse Education Today, 2008; 28(6): 657-663.*

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Abstract

In this paper the instructions for preparing camera ready paper for the Journal are given. The recommended, but not limited text processor is Microsoft Word. Insert an abstract of 50-100 words, giving a brief account of the most relevant aspects of the paper. It is recommended to use up to 5 key words.

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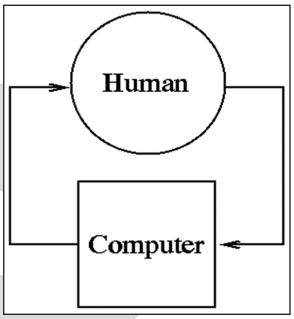

Figure 1. Text here

Conclusion

Be brief and give most important conclusion from your paper. Do not use equations and figures here.

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References

1. *Sakane T, Takeno M, Suzuki N, Inaba G. Behcet's disease. N Engl J Med 1999; 341: 1284–1291.*
2. *Stewart SM, Lam TH, Beston CL, et al. A Prospective Analysis of Stress and Academic Performance in the first two years of Medical School. Med Educ 1999; 33(4): 243- 50.*

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