

Heart-type fatty acid-binding protein and its relation with morphological changes in rat myocardial damage model induced by isoproterenol

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ABSTRACT

We have investigated heart type fatty acid binding protein (H-FABP) rat serum values at different time point following subcutaneous (s.c) isoproterenol (ISO) administration and their correlation with severity of myocardial lesion. Thirty adult, male, Wistar rats were used for this study. Six rats per group were treated with a single dose of either ISO (ISO groups, dose 100 mg/kg, s.c.) at different time point (30', 60', 120', 240') or with saline (control group). Serum H-FABP was determined by enzyme-linked immunosorbent assay (ELISA) and histological analysis was performed by hematoxylin-eosin (HE) method of staining. The first serum H-FABP increase was obtained 30' following ISO administration, but maximal value was reached after 240'. Myocardial histological changes were time-dependent and correlated with serum H-FABP values ($p < 0.001$). The results of the study suggest that H-FABP is sensitive marker for acute rat myocardial injury and its possible inclusion in myocardial injury screening studies in rats.

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KEY WORDS: H-FABP, isoproterenol, histology, myocardial lesion score, rat

INTRODUCTION

Cardiac biomarkers are protein macromolecules that are found in myocardial cells, and their serum increasing is indicative in cell damage or loss of cell membranes integrity [1, 2]. As additive to traditional markers of myocardial damage, among proposed new biomarkers of myocardial injury is heart fatty acid binding protein (H-FABP) [3]. H-FABP is 14.5 kDa protein, consisted of 132 amino acid residues. It comprises 4-8% of the total cytoplasmic protein of cardiac myocytes and its function is hydrophobic long-chain fatty acids transport from the cell membrane to their intracellular sites of metabolism in the mitochondria. In rat, as in human, H-FABP is distributed in heart but also in skeletal muscles, kidneys, brain but with lower expression [4, 5]. Heart content of H-FABP is approximately 10-fold greater than in most skeletal muscles [6].

Isoproterenol (ISO), a nonselective beta-agonist, is widely used in toxicological studies for induction of cardiac muscle injury [7]. Excessive amounts of catecholamines produce cardiac dysfunction by inducing intracellular Ca^{2+} overload in cardiomyocytes. As additive mechanism of catecholamines cardiotoxicity is their oxidation and generation oxyradicals. These oxidation products of catecholamines produce coronary spasm, arrhythmias, and cardiac dysfunction by inducing Ca^{2+} -handling abnormalities in both sarcolemmal and sarcoplasmic reticulum, defects in energy production by mitochondria, and myocardial cell damage. Previous described change in heart exposed to toxic levels of catecholamines is coagulative myocytolysis [8]. These ISO-induced changes in the heart are associated with cardiomyocyte proteins releasing and their increasing in serum. We have hypothesized that rat model of myocardial injury induced by subcutaneous ISO application can be suitable model for investigation of H-FABP kinetic biomarker of myocardial injury and that H-FABP correlates with myocardial lesion severity. The aims of the study were: investigation of rat H-FABP serum values at different time point following subcutaneous ISO administration, the examination of histological myocardial changes and their correlation with serum H-FABP.

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MATERIALS AND METHODS

Animals

Wistar albino male rats (n=30), average body weight 277 ± 4.03 g were used for the study. They were raised in air-conditioned, humidity-controlled cages. Rats had free access to water and commercial food during experimental period. Ethical Committee of Medical Faculty, University of Sarajevo, approved the study protocol. We distributed rats in two groups: isoproterenol group (ISO; n=24) and control group (CG; n=6). ISO rats were divided, according to time points, into 4 groups: ISO I (30 minutes), ISO II (60 minutes), ISO III (120 minutes) and IV group (240 minutes), each group consisted of 6 rats. ISO dissolved in saline, dose 100 mg/kg of rat body weight was used for administration to ISO groups [9] and 0.95% NaCl for control group. We have administered subcutaneously volume of solution that corresponds to the species and animals' body weight [10]. Isoproterenol hydrochloride was manufactured by Sigma Chemical Company, USA. In order to determine baseline level of H-FABP (control) we have drawn tail vein blood from the conscious animals before the ISO or saline injection. After injection of ISO or saline solution, blood samples were taken from abdominal aorta after scheduled time for each group. Blood and heart samples were taken from ether-anaesthetized rats and after which rats were euthanized by decapitation. The blood was centrifuged for 10 minutes at 4000 r.p.m. The sera were frozen and stored at -20° until determination.

Methods

We used rat H-FABP quantitative test based on a solid phase enzyme-linked immunosorbent assay (ELISA) developed by Life Diagnostics Inc. West. Chester PA, USA. Samples and standards were incubated in microtiter wells coated with antibodies (capture antibody) with horseradish peroxidase-conjugated labeled antibody. During incubation time, the immune complexes consisted of antibody-antigen-enzyme conjugated antibody were formed. The peroxidase labeled antibodies reacts with the substrate tetramethylbenzidine (TMB). We measured absorbance at 450 nm spectrophotometrically by using immunoanalyzer STAT FAX 2100, USA. Values of H-FABP were given in ng/ml. Rat hearts were dissected for histological examination, shortly after blood samples were taken. Left ventricular tissue was placed in 10% buffered formalin solution, embedded in paraffin, sectioned at 4-5 μ m and stained with hematoxylin-eosin (HE) and observed microscopically. We performed a histological analysis by using microscope Nikon type 400E with installed digital camera for characterization of myocardial lesion. The semi-quantitative analysis included analysis of myocardial lesion severity in subendocardial area of myocardium.

The severity of cardiac lesions was graded on a scale of 0 to 4 and based on the number of injured myofibres. For this purpose the following criteria, described by York [11], were used: 0 (no abnormalities detected); minimal (grade 1; occasional individual myofibre injury), mild (grade 2; multiple individual myofibre injury), moderate (grade 3; larger focal to locally extensive areas of myofibre injury), or moderately severe (grade 4; the majority of fibres in the myocardium affected).

Statistical analysis

We presented obtained data as mean \pm standard error of mean (SEM). Nonparametric Kruskal-Wallis was used for testing between-group differences. A post hoc comparison was performed by the Mann-Whitney *U* test. Comparison of the two means of H-FABP before and after ISO or saline application was carried out by using paired *t*-test. Pearson's correlation test was used for examination of relationship between serum H-FABP and myocardial lesion score. We considered *p* value less than 0.05 ($p < 0.05$) statistically significant.

RESULTS

Mean values of serum H-FABP were presented in Table 1. We measured baseline H-FABP value in all rats before saline or ISO administration (control). Mean baseline serum value of rat H-FABP in the groups was from 0.73-1.35 ng/ml. Serum H-FABP rising was noted after both saline and ISO application. There wasn't statistical significant difference between serum H-FABP values prior and after saline application ($p > 0.05$). ISO administration to rats was followed by H-FABP releasing from cardiomyocytes into circulation. We recorded its statistically significant increase

TABLE 1. Mean serum H-FABP value in rats of experimental groups

Groups	Number of animals in each group	H-FABP (ng/ml) (baseline level-control)	H-FABP (ng/ml) (following saline or ISO)	Time post dosing (minutes)
Control group	6	0.73 \pm 0.31	*1.55 \pm 1.04 ^{NS}	240'
ISO I group	6	1.35 \pm 0.21	*20.45 \pm 2.78 [†]	30'
ISO II group	6	1.25 \pm 0.13	*17.51 \pm 1.41 [†]	60'
ISO III group	6	0.73 \pm 0.066	37.56 \pm 2.63 [†]	120'
ISO IV group	6	1.11 \pm 0.24	51.88 \pm 6.81 [†]	240'

*H-FABP-heart type fatty acid binding protein; ISO I,II,III,IV group-ISO treated rats according to different time points (0,30,60,120, 240'); Control group-saline treated rats; Control-rats before saline or ISO administration; Values are expressed as mean \pm standard error of mean ($\bar{X} \pm SEM$); * $p < 0.01$ control group vs. ISO group; [†] $p < 0.01$ ISO I vs. ISO III and ISO IV; ISO II vs. ISO III and ISO IV; ^{NS} $p < 0.01$ ISO group vs. control; NS-not significantly different $p > 0.05$ control group vs. control;*

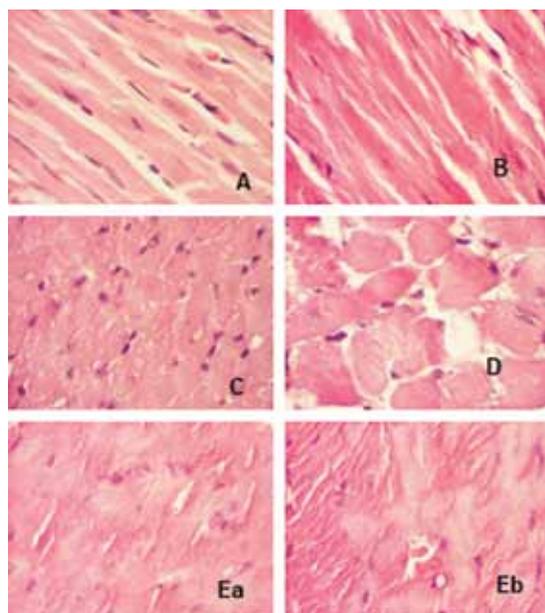


FIGURE 1. Histological characteristics of myocardial tissue in experimental rats. (HE stained sections of left ventricular myocardium; magnifications for all images-400X) A) Control rats showing the normal appearance of cardiac myofibres; B) Early changes in the myocardium at 0.5 hours post ISO application showing myofibrillar eosinophilia, granular degeneration and partial loss of normal myofibrillar striation pattern; C) Swollen cardiomyocytes with cytoplasm vacuolization and piknotic nuclei at 60' following ISO application; D) Myonecrosis at 2 hours after the administration of ISO with the nuclei vacuolization and disappearance; Ea, Eb) Myonecrosis at 4 hours after the administration of ISO with completely disappearance of cell nuclei. Necrotic muscle fibers show weak affinity for staining.

in all ISO groups in comparison with control group and control baseline value before application ($p < 0.01$). Serum H-FABP had tendency of rising in all ISO groups. In ISO II group we noted the lowest values which were statistically significant in comparison with value of control and control group. There was statistically significant difference between ISO I and II versus ISO III and IV groups ($p < 0.01$). Control rats, which received saline, showed normal composition of myocardial tissue. The earliest changes in the myocardium were evident at 30 minutes following ISO administration. More intensive changes of subendocardial and perivascular myocardium were noted. Change of myocardial tissue in groups ISO II and I were in form of myofibrillar degeneration with myofibrillar eosinophilia, oedema, vacuolization and granular degeneration. In addition, hyperchromatic nuclei were presented. We have registered changes from individual to multiple myofibre injury in these groups. Coagulative myocytolysis or contraction band necrosis was noted in

groups ISO III and IV. Beside intensive changes in cytoplasm, there were vacuolization of nuclei and completely disappearance. Myocardial lesions of ISO III group were in form of larger focal to locally extensive areas of myofibre injury. By myocardial examination of ISO IV rats, we have noted that the majority of fibres in the myocardium were affected (Figure 1). Figure 2 presents the semi-quantitative score for myocardial injury. Thirty minutes after ISO administration, severity of myocardial lesions was 1.33 ± 0.21 on score scale. These histological changes were followed by increasing serum H-FABP value from 1.35 ± 0.21 before application to 20.45 ± 2.78 ng/ml. Rats sacrificed 60' after ISO application developed myocardial lesion grade 2.16 ± 0.47 . Mean serum H-FABP in this group was 17.51 ± 1.41 ng/ml that was a lower than ISO I group value. Baseline value of H-FABP for ISO II was 1.25 ± 0.13 ng/ml. We have noted disconnection between lesion severity and serum H-FABP value. A further development

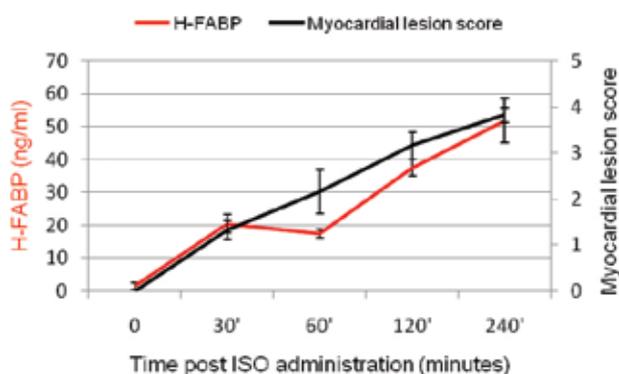


FIGURE 2. Myocardial lesion score and serum H-FABP level in rats of experimental groups. Serum H-FABP value (ng/ml) and myocardial lesion score are presented as mean \pm standard error of mean ($\bar{x} \pm \text{SEM}$); H-FABP-heart type fatty acid binding protein.

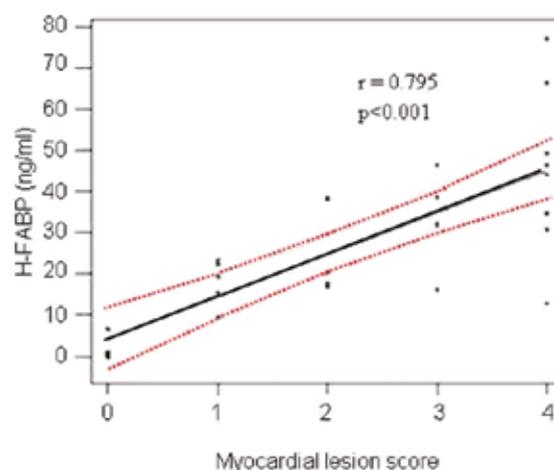


FIGURE 3. The correlation between rat myocardial lesion score and serum H-FABP in experimental groups. Myocardial lesions were graded as: 0 (no abnormalities detected); minimal (grade 1-occasional individual myofibre injury), mild (grade 2-multiple individual myofibre injury), moderate (grade 3-larger focal to locally extensive areas of myofibre injury), or moderately severe (grade 4-the majority of fibres in the myocardium affected). Myocardial lesion score is presented as mean value in groups during experimental period (4 hours). Line presents regression; dotted line presents 95% confidence interval of regression; r- Pearson's correlation coefficient; p value-level of statistical significance.

of myocardial lesions two hours after ISO application we characterized as 3.16 ± 0.3 and followed by increasing of serum H-FABP (37.56 ± 2.63 ng/ml). Baseline serum H-FABP value of ISO III rats was 0.73 ± 0.066 ng/ml. Four hours after ISO administration, we described myocardial lesion severity as mean group value 3.83 ± 0.16 . This severity score was followed by serum H-FABP increasing from 1.11 ± 0.24 before and 51.88 ± 6.81 ng/ml after ISO administration. The correlation between the onset of histopathological lesions and serum levels of H-FABP are illustrated in Figure 3. It was noted significant positive correlation between myocardial lesion score and rat serum H-FABP value ($p < 0.001$; $r = 0.795$).

DISCUSSION

Laboratory evaluation of cardiac injury by protein biomarkers measurement is of great importance in clinical and non-clinical studies because of lack specificity and sensitivity of traditional enzyme markers. The study was performed to investigate possibility of ISO induced rat myocardial injury detection by measurement serum H-FABP as maker of myocardial damage. The baseline H-FABP level in blood is likely to be due to the continuous release of this protein from skeletal muscle cells [12]. Serum H-FABP concentration measured in apparently healthy individuals is result of continuous releasing from damaged skeletal muscle cells and its concentration is subject to biological variations like age, sex, circadian rhythm [13, 14, 15]. We used commercial test kit for rat H-FABP determination because of dissimilarity of human and rat H-FABP [16] and we have observed the lower H-FABP baseline level in circulation of rats than in previous investigation, included human samples [17]. Subcutaneous saline administration to rats of control group has induced H-FABP increasing in serum but there was not statistical significant difference compared to baseline level ($p > 0.05$). Because of low H-FABP expression in intramuscular and subcutaneous adipocytes [18], we have considered that increased H-FABP serum level in control group partially originates from these adipocytes. Tachycardia and relative myocardial ischemia induced by stressful effect of handling can be cause of possible H-FABP releasing of cardiac cells' origin. The results of our study have showed that as early as 30 minutes after ISO administration to rat, serum H-FABP level was significantly higher than the baseline level that indicates its high diagnostic sensitivity for myocardial injury detection. Meng and associates in their study have used animal model of acute myocardial infarction induced by ligation of left anterior descending (LAD) coronary artery [19]. As small cytosolic protein, H-FABP is released quickly from ischemia-damaged cardiomyocytes. Its circulation half-life is about 27 minutes [20]. Short H-FABP half-life, small molecular mass and rapid elimination

through the kidneys have caused decreasing of H-FABP serum value at 60 minutes following ISO administration like in Meng's study [19]. They have considered that amount of released H-FABP from cardiomyocytes varies depend on post ischemia interval. Maximal values of H-FABP have reached at four hours after ISO application but minimal serum values have noted at two hours following ISO application. It points at biphasic kinetic of serum H-FABP observed in our study. H-FABP kinetic like in our study has been noted in the study of Meng and associates despite the different method of myocardial damage induction [19]. We have explained this serum values by progression of myocardial lesions and impaired renal function followed by slowly H-FABP elimination that has also been described in human myocardial infarction [21, 22, 23]. We have considered that small portion of serum H-FABP in ISO groups originates from kidneys because Maatman and coworkers described presence of liver (L-FABP) and heart type H-FABP in the kidney [24]. In our study, we haven't noted mild myocardial changes of control group rats induced by stress, tachycardia and myocardial hypoperfusion compared with previous published studies [25, 26]. Histological myocardial changes showed time dependency like serum H-FABP values. We have noted a more intensive changes in groups ISO III and IV in comparison with ISO I and II groups. We explained the distribution of more severe myocardial lesions by ISO influence on adrenergic receptors followed by consequences in form of blood pressure reduction and myocardial necrosis development. The adrenergic stimulation results by increasing of the rate and force of myocardial contraction followed by hypoxia of the least perfused area-subendocardial area [27]. The serum H-FABP levels showed an association with the development of cardiac lesions. Increased HFABP serum level preceded maximal lesion severity development. It points at high sensitivity of H-FABP in detecting of minimal myocardial damage caused by ISO application. Our results are in concordance with Clement and coworker study results [28]. They have measured serum troponins and H-FABP in dose dependent study and noted that release of cardiac troponins and H-FABP begin early in myocardial injury, prior necrosis development and correlate well with myocardial injury morphologically detected. In the Meng and associates study serum H-FABP correlated with myocardial infarction size as in our study [18]. Limitation of the study is lack of H-FABP specificity that indicates the need for combined measurement with cardiac specific marker like cardiac troponin I.

CONCLUSIONS

The conducted study demonstrates that H-FABP is sensitive marker of myocardial injury in rats treated with ISO

and that H-FABP serum values correlate with myocardial lesions development. Release of H-FABP into circulation begins prior the myocardial necrosis development. The early appearance of a marker released into the bloodstream, soon after an injury, may facilitate early myocardial injury detection in toxicological studies.

DECLARATION OF INTEREST

There is no conflict of interest.

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