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Endocrine, Metabolic & Immune Disorders-Drug Targets, **XXXX,** ***XX*****, 1-7**

RESEARCH ARTICLE

Calcium-Regulating Hormonal System and HMGB1 in Cardiomyopathies

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ARTICLE HISTORY

Received: May 11, 2022
Revised: May 31, 2022
Accepted: June 03, 2022

DOI:
10.2174/1871530322666220817110538

Abstract: ***Background*****:** Calcium ions play a key role in the heart's functional activity. The steady-state levels of calcium are contingent on the calcium regulating hormonal system, impairment of which might result in the development of cardiac pathology. An important role in these processes is also attributed to the specific inflammatory mediator, HMGB1, one of the damage-associated molecular patterns (DAMPs) released by immune cells or cell damage.

Objective**:** This study investigated the cardioprotective potential of the calcium-regulating hormonal system in cardiomyopathies with an emphasis on the possible role of HMGB1.

Methods**:** Ca^{2+} and inorganic phosphate levels were determined in the serum using an electrolyte analyzer and spectrophotometric analyzer correspondingly. The 1-34 fragment of parathyroid hormone (PTH), calcitonin, vitamin D, and HMGB1 were detected using ELISA kits.

Results**:** The levels of PTH, calcitonin, phosphate, and HMGB1 were found elevated in females suffering from cardiomyopathy. The same tendency was observed in men; however, statistically significant changes were registered only for PTH and phosphate.

Conclusion**:** It can be suggested that among other reasons, the decrease of the left ventricular function in cardiomyopathy patients can be linked to the high HMGB1, whereas the activation of the calcium-regulating system as manifested by the elevated PTH aims at restoration of calcium homeostasis and thus have positive, *i.e.* cardioprotective consequences.

Keywords: Cardiomyopathy, calcium-regulating hormonal system, calcium, phosphate, parathyroid hormone, calcitonin, vitamin D, HMGB1.

1. INTRODUCTION

The most common causes of cardiomyopathy are heart ischemia, decreased perfusion, hypertrophy, myocardial infarction, gene mutations, and other heart pathologies [1-3]. Cardiomyopathies often culminate in heart failure and therefore, it is important to sustain the contractile function of the weakened myocardium, which is largely regulated by the calcium currents. The latter is important for the formation of the action potential in the cardiac pacemaker myocytes, the plateau phase of the action potential, and for the cardiac excitation-contraction [4-6].

The circulating levels of calcium are regulated by the calcium regulating hormonal system, including parathyroid hormone (PTH), parathyroid hormone-related protein (PTHrP), vitamin D, and calcitonin. Among these factors, PTH and PTHrP were shown to have a direct impact on Cardiomyocytes [7-9]. These effects are mediated by the PTH/PTHrP receptor, PTH1R expressed in the heart [10, 11].

In addition to the known factors affecting calcium homeostasis, growing evidence indicates an important role of the inflammatory component of various cardiac pathologies including cardiomyopathy, which involves the innate and/or adaptive cardiac immune responses [12, 13]. Innate immunity response is often mediated by the damage-associated molecular patterns (DAMPs) secreted actively or passively upon the cell [14] or by the pathogen-associated molecular patterns (PAMPs) upon the bacterial infection [15]. Whereas the effects of DAMPs and specifically of one of its key members, HMGB1, upon the acute phase of heart tissue injury are considered to be mostly cytoprotective [16], their prolonged action might be more negative. Thus, elevated serum levels of HMGB1 in sepsis were associated with the negative inotropic effect [17]. Treatment of feline cardiac myocytes with HMGB1 leads to the decrease of sarcomere shortening and a decrease in the height of the peak Ca^{2+} transient, which might be explained by the decreased calcium levels [13]. It can be therefore suggested that the extended action of HMGB1 can interfere with the calcium homeostasis in cardiac muscle and

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1389-2010/XX $65.00+.00

aggravate the pathogenesis of cardiac pathologies, including cardiomyopathy.

While high levels of DAMPs are considered detrimental to heart function, PTH, as well as PTHrP, are viewed as mostly cardioprotective as they facilitate an increase in heart rate, positive inotropic and chronotropic effects, and coronary arteries dilation [18-23]. However, this is supported mostly by the *in vitro* and animal studies, whereas the essential information regarding the circulating levels of the components of the calcium regulating system and also DAMPs, (HMGB1 in particular) in humans suffering from cardiac pathology (cardiomyopathy) is still missing.

In order to fill, at least partially, this gap of knowledge, we have monitored the levels of the selected blood electrolytes (Ca^{2+} and inorganic phosphate), and calcium-regulating factors, such as PTH, vitamin D, calcitonin in cardiomyopathy patients and healthy volunteers. In addition, we have determined the circulating levels of HMGB1 in the same groups of individuals.

2. MATERIALS AND METHODS

2.1. Ethics and Approval

All study subjects were informed in written form before enrolling in the study and each participant gave written consent. The study was approved by the Ethics Committee of the Yerevan State Medical University and followed the standards of the Declaration of Helsinki.

2.2. Study Subjects

Two groups of patients included 25 men and 21 women who suffered from cardiomyopathy as a result of ischemic heart disease or myocardial infarction. All patients underwent outpatient treatment in the Armenian Scientific Research Institute of Cardiology for 7-8 years. 14 male and 11 female patients had dilated cardiomyopathy accompanied by hypertension and aortic stenosis. Two male patients were suffering from hypertrophic cardiomyopathy, whereas 9 male and 10 female patients were diagnosed with idiopathic cardiomyopathy. Blood sampling for the current study was done when all patients were undergoing outpatient treatment and were prescribed antihypertensive drugs, such as ACE and calcium channel blockers and beta-blockers. Exclusion criteria included hyperparathyroidism, diabetes mellitus, and acute and chronic allergies.

Control groups consisted of 10 healthy men (age 54-65) and 10 healthy women (age 52-68) with no previous history of cardiac pathology and with the normal ECG and heart ultrasound data. Blood sample collection from the disease and control groups was carried out in March-May and September-October 2021. The levels of the hormones and electrolytes in the isolated sera were analyzed in November 2021.

Complete demographic data on all individuals involved in the study are presented in Table **1**.

2.3. Blood Sampling

4.5 ml of venous blood was collected into the Vacutainer tubes, which were left to coagulate on the bench at room temperature for 45 min and centrifuged at 3000 g for 10 min at 4 °C. Supernatants (sera) were stored at -80 °C until further use.

2.4. Biochemical Analyses

Ca^{2+} and inorganic phosphate levels were determined in the serum using electrolyte analyzer E-Lyte Plus (HTI Medical Inc., USA - Maryland) and spectrophotometric analyzer (Biosystems, Barcelona, Spain) correspondingly. The 1-34 fragment of PTH, calcitonin, and vitamin D were detected using ELISA kits from DRG International (Germany). HMGB1 serum levels were determined by the ELISA kit from IBL International (Germany). All analytical procedures followed the corresponding manufacturer's instructions. The performance characteristics (sensitivity, intra-, and inter-assay variability) for all the ELISA assays are presented in the Supplementary table.

Table 1. Demography of patients and healthy volunteers.

Sex	Healthy Volunteers	Age (Healthy Volunteers)	Patients	Age (Patients)	Groups of Patients		Myocardiopathy Classification					
					Angina	Myocardial Infarction (MI)	Dilated Cardiomyopathy		Hypertrophic Cardiomyopathy		Idiopathic Cardiomyopathy	
							Angina	MI	Angina	MI	Angina	MI
Male	10	54-65	25	51-80	8	17	4 (aortic stenosis, 3, hypertension, 1)	10 (aortic stenosis, 7, hypertension, 3)	-	2	4	5
Female	10	52-68	21	51-81	5	16	2 (aortic stenosis, 1, hypertension, 1)	9 (aortic stenosis, 7, hypertension, 2)	-	-	3	7

2.5. Statistics

Data were analyzed using two-way ANOVA and uncorrected Fisher's LSD test as implemented by the GraphPad Prism (v. 9.1.1) software. In case of missing values, data were analyzed by fitting a mixed model, rather than by repeated measures ANOVA. Data are presented as means ± SD. $P < 0.05$ was considered significant.

3. RESULTS AND DISCUSSION

One of the forms of cardiomyopathy, ischemic cardiomyopathy, develops due to a heart attack, hypertension, pathology of coronary arteries, and other cardiac disorders [24] often resulting in sudden death or congestive heart failure [3]. Our choice of the patient group was thus limited to the pathologies, such as myocardial infarction (33 patients) and coronary artery disease (13 patients (Table **1**). Dilated cardiomyopathy was observed in 14 male and 11 female patients. In this group, four male (three with aortic stenosis and one with hypertension) and two female patients (one with aortic stenosis and one with hypertension) developed cardiomyopathy in the background of myocardial ischemia. In 10 male patients (7 with the aortic stenosis and three with hypertension) and 9 female patients (7 with the aortic stenosis and two with hypertension) cardiomyopathy developed after the myocardial infarction. Hypertrophic cardiomyopathy was diagnosed in two males, whereas 7 males and 10 females were suffering from idiopathic cardiomyopathy.

The left ventricular ejection fraction was decreased in all patients, with 42-48% in patients with coronary artery disease and 34-39% in patients with myocardial infarction in anamnesis. Both groups were diagnosed with class II and III heart failure (New York Heart Association's functional classification), respectively. The diagnostics were based on coronography, ECG, and heart ultrasound data.

The focus of the current study was the characterization of the circulating levels of the principal hallmarks of calcium regulating homeostatic systems, such as PTH, calcitonin, vitamin D and electrolytes Ca^{2+}, and inorganic phosphate. The levels of the main calcium regulating hormone, PTH were found to be significantly elevated in both men and women suffering from myocardiopathy (fold change (FC) = +4.7 and +3.07, correspondingly) (Fig. **1**, left panel). A similar trend was registered with calcitonin, however, only among females: cardiomyopathy patients had higher levels of this hormone in the blood (FC = +5.08) (Fig. **1**, right panel). The different levels of calcitonin in males and females of the control group reflect the known sex differences in the reference range of this hormone (females, <5 pg/ml; males, <12 pg/ml [25]).

At the same time, vitamin D was found unaffected in both sex groups of patients (Fig. **1**, middle panel). Interestingly, these levels are within or below the lower limit of its standard range in the blood (25-80 ng/ml), which could be interpreted as a negative prognosis for the further development of the disease [26, 27].

As the circulating ionized calcium and inorganic phosphate depend primarily on the activity of the calcium regulating system, we have decided to determine these electrolytes in the blood of cardiomyopathy patients. Whereas Ca^{2+} levels were not different from the control group, the inorganic phosphate was found elevated in both men and women with a cardiomyopathy diagnosis (Fig. **2**, left panel). However, although statistically significant, these differences were rather low in both sex groups (FC = +13.6, male and +17.9, female) (Fig. **2**, right panel).

One of the main findings of this study is significantly elevated levels of PTH and calcitonin (in females) in cardiomyopathy patients (Fig. **1**). The significance of the increased PTH levels for the development of various cardiac disorders is currently a highly debated issue. Thus, some studies associate both primary and secondary hyperparathyroidism with a negative development of cardiac disorders, as manifested by hypertrophy of the myocardium, calcification of valves, arrhythmia, and extrasystoles [28, 29]. It is considered that the high PTH level in elderly people is often associated with hypertension and increased lethality [30]. However, there are data that hypoparathyroidism can also contribute to the development of cardiac pathologies [31].

The higher levels of PTH in cardiomyopathy patients reported in this study cannot be considered as signaling true hyperparathyroidism because overall they remain within a standard range of circulating PTH values (9-94 pg/ml). It can be therefore suggested that this is an adaptive effect aiming at sustaining the weakened function of the myocardium. Consistent with this PTH and PTHrP were found to exert a vasodilating effect to improve microcirculation in coronary arteries, facilitating thus the myocardium's oxygenation and contractile function [32]. We have presented the hypothesis on the cardioprotective effect of the moderately elevated levels of PTH/PTHrP system in the recent review [33].

Increased levels of PTH and calcitonin might also be interpreted as a negative feedback response to the elevated inorganic phosphate (Fig. **2**), as both hormones are known to reduce phosphate [34]. At the same time, as PTH has a hypercalcemic and calcitonin – hypocalcemic effect, the cumulative effect of both hormones would lead to stable levels of Ca^{2+} as observed in this study (Fig. **2**).

Another novel finding of this study is the increase of HMGB1 in both sex groups with cardiomyopathy, which, however, only in females reached the level of statistical significance (FC = +2.37) (Fig. **3**).

The association of the elevated DAMPs (HMGB1 in particular) in circulation, with the impairment of the heart physiology, is well documented. It was reported that a high concentration of HMGB1 in blood negatively affects the heart contractile function [13, 17, 35]. In feline cardiac myocytes, HMGB1 decreases the sarcomere shortening and causes a negative inotropic effect by decreasing calcium availability in cardiac myocytes [13]. Similarly, it was shown that HMGB1 increases the frequency of Ca^{2+} sparks, reduces the sarcoplasmic reticulum Ca^{2+}, and decreases the myocyte contractility in the rat ventricular myocytes [36].

It should be also noted that the study is not devoid of certain shortcomings. Thus, despite the clearly similar tendencies of changes in calcitonin and HMGB1 levels in both sex groups, statistically significant were only data in the female group, which might reflect the insufficient sizes of both patient and control groups. Another potential confounding factor

was the selection of healthy volunteers, which was limited by the age factor that had to correspond to the average age of patients, and the absence of various pathologies, the cardiovascular in particular. However, it cannot be ruled out that even such healthy subjects might have slightly altered levels of various circulating hormones, and electrolytes due to a variety of additional factors. For instance, the HMGB1 levels may vary depending on sex, body mass, smoking, *etc.* [37-41]. It can be therefore speculated that this was the reason for the statistically insignificant results for HMGB1 in the male group (Fig. **3**).

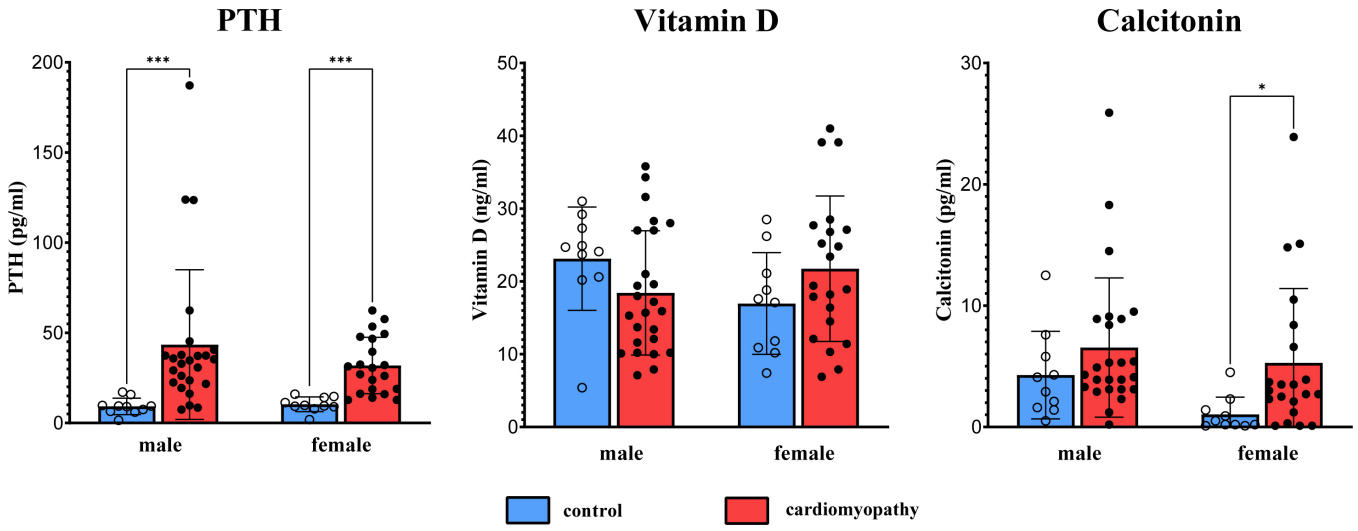

Fig. (1). Calcium regulating hormones and vitamin D in cardiomyopathy patients. The circulating levels of PTH, calcitonin and vitamin D were determined as described in Materials and Methods. *, $p<0.05$; ***. $P<0.001$. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

Fig. (2). Circulating electrolytes in cardiomyopathy patients. Ca^{+2} and inorganic phosphate were determined as described in Materials and Methods. ***. $P<0.001$. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

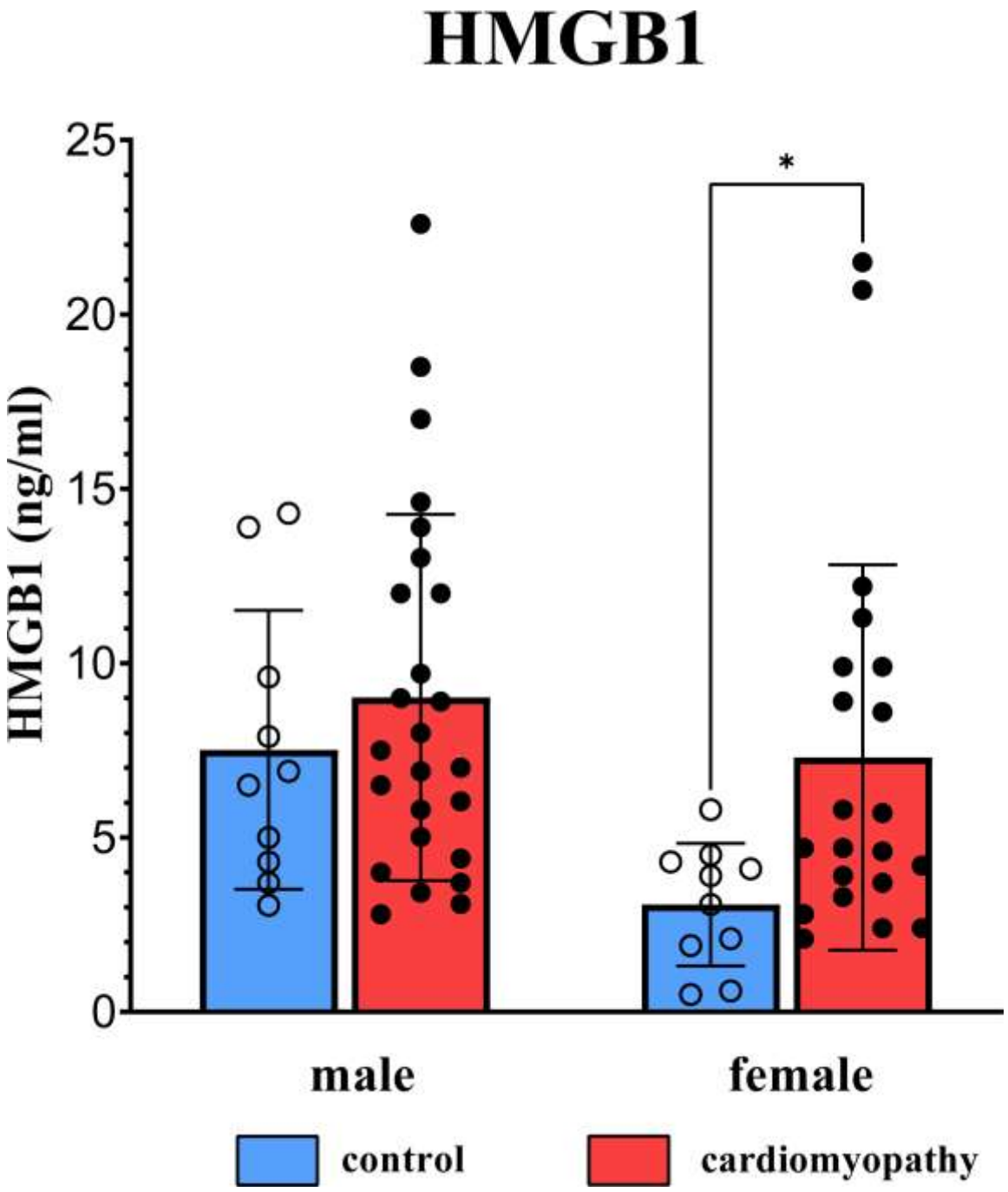

Fig. (3). Circulating HMGB1 in cardiomyopathy patients. HMGB1 was determined as described in Materials and Methods. *, $p<0.05$. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

CONCLUSION

Based on the data presented here and other findings (reviewed in [33]) it can be suggested that HMGB1 might interfere with calcium homeostasis in the myocardium, aggravating thus, the cardiac pathology. Therefore, it is conceivable that among other reasons, the decrease of the left ventricular function in cardiomyopathy patients can be linked to the high HMGB1, whereas the activation of the calcium-regulating system as manifested by the elevated PTH aims at restoration of calcium homeostasis and thus have positive, *i.e*. cardioprotective consequences.

LIST OF ABBREVIATIONS

DAMPs	=	Damage-Associated Molecular Patterns
PTH	=	Parathyroid Hormone
PTHrP	=	Parathyroid Hormone-Related Protein

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Institutional Ethics Committee, Yerevan State Medical University after M. Heratsi,

Armenia (protocol code 5-1/20 and date of approval 13.02.2020).

HUMAN AND ANIMAL RIGHTS

No animals were used in this study. The study was conducted in accordance with the Declaration of Helsinki.

CONSENT FOR PUBLICATION

All study subjects were informed in written form before enrolling in the study and each participant gave written consent.

STANDARD OF REPORTING

STROBE guidelines were followed.

AVAILABILITY OF DATA AND MATERIALS

None.

FUNDING

This research was funded by the Science Committee of MESCS RA, in the frames of research project No. 21T-3A112.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

Declared none.

SUPPLEMENTARY MATERIALS

Supplementary material is available on the publisher's website along with the published article.

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