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Circadian Rhythm of Acylated Ghrelin, Leptin, Growth Hormone, IGF-1, IGFBP-1, and IGFBP-3 in Chronic Heart Failure Patients and Healthy Subjects

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ABSTRACT

Background: Following detection of receptors for ghrelin and growth hormone (GH) in the cardiovascular system, different clinical trials have used ghrelin or GH for the treatment of cardiac patients. While some of these trials reported improvements in the patients' situation, others reported deterioration.

Objectives: To clarify the contradictory outcomes, we designed this study to evaluate the circadian rhythms of acylated ghrelin, GH, and the related factors [Insulin-like Growth Factor-1 (IGF-1), Insulin-like Growth Factor Binding Proteins 1 and 3 (IGFBP-1 and IGFBP-3)], and leptin in patients with reduced ejection fraction (rEF).

Patients and Methods: Ten patients with rEF and an equal number of healthy control subjects matched for age and gender participated in this study according to inclusion criteria. All participants were hospitalized in the cardiac care unit (CCU), under identical conditions during collection of blood (every 2 hours). Primary processing of samples was carried out immediately and the plasma was stored at -20°C until evaluation of the aforementioned parameters using ELISA methods.

Results: Evaluation of the collected data showed that among aged participants only circulating leptin is gender-dependent, while the patients had significantly ($P < 0.001$) lower ghrelin, GH, IGF-1, and IGFBP-1, but a higher level of IGFBP-3 compared to the control group. In addition, except for GH that showed a mild circadian rhythm, the parameters we examined did not have a significant circadian rhythm. Correlation analysis of the data showed a positive correlation between ghrelin and GH or IGF-1, and significant negative or positive correlations between leptin and IGFBP-1, or IGFBP-3, respectively, in both groups.

Conclusions: Here, for the first time, we show that circulating ghrelin, GH, and IGF-1 levels are reduced in the patients with rEF, and the condition of patients is deteriorated not only due to reduced IGF-1 but also due to reduction of IGFBP-1 or increase of IGFBP-3, which may be influenced by circulating leptin. Finally, disturbance of the balance between ghrelin/GH/IGF-1 and leptin may be the cause of rEF, and thus evaluation of these parameters could provide diagnostic as well as prognostic tools for the treatment of these patients.

Implication for health policy/practice/research/medical education:
Disturbance of anabolic hormones such as ghrelin and leptin could be the cause of developing cardiovascular diseases. In addition, imbalance between ghrelin/GH/IGF-1 axis and leptin may be the reason for reduction of ejection fraction among cardiovascular patients.

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1. Background

Despite recent advances in therapy, chronic heart failure (CHF) remains a common and serious problem, particularly among the increasing proportion of the older population (1, 2). Left ventricular hypertrophy is a strong

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and independent predictor of cardiovascular morbidity and mortality, in hypertensive patients as well as in the general population, which is associated with a higher risk of myocardial infarction, stroke, sudden death, and death from other causes (3, 4).

It is known that growth hormone (GH) regulates growth in pediatric age as well as inducing anabolic actions directly, or insulin-like growth factor-1 (IGF-1)-mediated effects in adult age. Receptors for GH and IGF-1 have been observed in the cardiac myocyte membrane (5) and investigators in this field believe that GH modifies left ventricle structure and function (5-8), because patients with acromegaly have an increased propensity of developing cardiovascular complications, such as ventricular hypertrophy with interstitial fibrosis. Conversely, patients with GH deficiency can exhibit ventricular dysfunction, increased vascular thickness, and an increased number of atheromatous plaques (8, 9). Therefore, since 1995, many scientists have been investigating the beneficial effects of GH in the treatment of left ventricular hypertrophy (LVH); however, these studies have yielded contradictory results. While some studies suggest that addition of GH in the LVH treatment protocol improves patient outcomes others have found that GH is not advantageous or may even contribute negatively (6, 7, 10-16).

Additionally, IGF-1 is synthesized by almost all tissues and is an important mediator of cell growth, differentiation, and transformation. It exerts all of its known physiologic effects by binding to the IGF-1 receptors and its effects are modulated by multiple IGF-1 binding proteins (IGFBPs) (17-20). The IGFBPs family has at least six members, which serve as transporter proteins and as storage pools for IGF-1 (21). The expressions of IGFBPs are tissue and developmental stage specific, and the concentrations of IGFBPs differ in different body compartments (19). All six IGFBPs have been shown to inhibit IGF-1 action, but IGFBP-1, -3, and -5 have also been shown to stimulate IGF-1 action, whereas some of the effects of IGFBPs might be IGF-1-independent (20).

Ghrelin was discovered in 1999 and it is now well documented that it stimulates GH release from the pituitary through its specific receptor, which is referred to as the growth hormone secretagogue receptor (22-25). Furthermore, the receptor for ghrelin was also detected in the blood vessels and ventricles of the heart (26, 27). Ghrelin also shows anti-cachectic effects through GH-dependent and -independent mechanisms (22, 23, 28-31).

2. Objectives

To our knowledge there is only one published report that has examined the circadian rhythm of GH among CHF patients (8 patients) (32), without considering other factors such as IGF-1, IGFBPs or ghrelin and leptin, which also in fluencing elements in cardiac function. There is also a report that shows leptin strongly predicts levels of GHBPs in CHF, regardless of a hyperleptinaemic state or severely altered body composition as in cardiac cachexia (33). Since there is a circadian rhythm for GH and related factors (IGFs and its carrier proteins) during different periods of life and for acylated ghrelin that depend on the state of feeding, and the lack of studies that have examined the relationship between GH and related proteins or hormones (IGF-1, IGFBP-1, IGFBP-3, ghrelin, and leptin), we sought to address this relationship in this study, and also to obtain data on the circadian rhythm of these factors among the aged patients with low ejection fraction (rEF).

3. Patients and Methods

The research approval and ethic committees of Kerman University of Medical Sciences approved the protocols for this study, which is in accordance with the internationally accepted principles found in the European Community Guidelines (EEC Directive of 1986; 86/609/EEC) or the US Guidelines (NIH publication #85-23, revised in 1985). Our cardiologist selected 10 CHF patients (five male and five female) as the CHF group and 10 healthy subjects as the control group. The inclusion criteria were non-diabetics, with normal lipid profile, and body mass index (BMI) close to 25 kg/m^2. A normal ejection fraction (EF) ($\geq$ 50) was considered for the control group, while the corresponding value for the patients was below 30, with a stable clinical condition for the previous 2 months. Exclusion criteria were the presence of chronic renal dysfunction (serum creatinine level $\geq$ 2.0 mg/dL), liver dysfunction, evidence of malignant diseases, active infection, hematologic abnormalities, or systolic blood pressure < 90 mm Hg.

Our cardiologist gave detailed explanations about the nature of the study to each participant and collected an informed written consent form from all participants. The day after the collection of the written statement, each precipitant was admitted to the cardiac care unit (CCU) under supervision of the cardiologist in the morning (8–9 AM). They were given identical meals throughout hospitalization (breakfast at 6:30, lunch at 12:30 and evening meal at 19:00) and the first sample collection started at 2 PM. For the sample collection, a butterfly needle was used for the first blood collection (3 mL) and repeated blood samples were taken, with 2-hour intervals between sample collections. The vein was kept open by connecting the needle to a normal saline solution (4 drops per minute). Collected samples were sent immediately to the hospitals' laboratory for primary processing, which included separation of sera by centrifuging at 800 × *g* for 5 minutes.

The samples were stored at -20° C, until further analysis. All parameters, including GH (Monobind, USA), IGF-1 (IDS,Uk), IGFBP-1 (Medix Biochemica, Finland), IGFBP-3 (Biosource, Belgium), acylated ghrelin (Biovender, Germany), and leptin (DBC, Canada) were evaluated in 1 day using ELISA methods according to the instructions provided by the manufactures of the ELISA kits. Coefficient of variation (CV %) for the medium range analytes, sen-

Table 1. Comparison of Ejection Fraction and Anthropometric Parameters in Patients and Healthy Subjects

	Control [a]		Patients [a]	
	Male	Female	Male (n = 5)	Female (n = 5)
Age, y	70.6 ± 8.96	66.0 ± 6.82	68.2 ± 10.38	60.6 ± 5.73
Weight, kg	69.0 ± 6.56	64.2 ± 6.02	63.8 ± 6.06	63.2 ± 2.68
Height, m	1.71 ± 0.09	1.59 ± 0.03	1.75 ± 0.04	1.59 ± 0.02
BMI, kg/m^2	23.61 ± 2.60	25.51 ± 2.84	20.77 ± 1.59	24.95 ± 1.17
Ejection Fraction	60.4 ± 1.67	62.8 ± 2.17	21.0 ± 7.42 b	26.2 ± 1.64 [b]

[a] Data shown are the Mean ± SD.
[b] Significant difference ($P < 0.001$) between patients and their corresponding control groups

sitivity, and dynamic range of different commercial kits used are presented in *Table 3*.

3.1. Statistical Analysis

Data were analyzed using SPSS software (Version 19, IBM). After checking for normal distribution using the Shapiro-Wilk test, we used two-way repeated-measures ANOVA for detection of the circadian rhythms of the evaluated parameters. Independent sample t-tests were used for comparisons between the two groups. Determination of correlations was carried out using Pearson's two-tailed bivariate model. *P*-values less than 0.05 were considered as statistically significant differences.

4. Results

Average age, weight, height, BMI, and EF of patients and corresponding control subjects, who were divided according to their gender, are presented in *Table 1* and the different chemistries evaluated, irrespective of their gender, are presented in *Table 2*. Neither age nor BMI (compared according to control group of the corresponding gender) were significantly different between patients and control subjects. As expected, the EF of patients were significantly lower ($P < 0.001$) than those of the control subjects. Comparison of evaluated data for the male and female participants of both groups showed only values obtained for leptin was gender-dependent (which depended on their BMI) and the sex difference did not influence the circulating values obtained for their fasting glucose (FBS), urea, creatinine, lipid profiles, liver function tests, lactate dehydrogenase (LDH), creatine kinase (CPK), hemoglobin, white blood cell count (WBC), GH, IGF-1, IGFBP-1, IGFBP-3, or acylated ghrelin. For this reason, values obtained for both male and female subjects were combined into a single group when comparing data ob-

Table 2. Comparison of Biochemical and Hematological Parameters in Patients and Healthy Subjects

Chemistry [a, b]	Patients	Control	*P* value
FBS [a], mg/dL	87.70 (9.53)	89.10 (5.92)	0.70
Urea, mg/dL	33.80 (6.53)	32.60 (6.67)	0.68
Creatinine, mg/dL	1.38 (0.31)	1.26 (0.23)	0.34
Total Bilirubin, mg/dL	1.01(0.21)	0.96 (0.22)	0.61
Direct Bilirubin, mg/dL	0.23 (0.12)	0.27 (0.11)	0.45
AST [c], IU/L	22.90 (6.86)	23.60 (7.07)	0.81
ALT [c], IU/L	23.30 (4.35)	23.40 (4.38)	0.96
Alk.P [c], IU/L	118.4 (16.9)	112.0 (19.4)	0.44
Cholesterol, mg/dL	184.1 (13.7)	180.2 (9.9)	0.48
Triglycerides, mg/dL	144.6 (14.4)	151.1 (16.8)	0.37
HDL-c [c], mg/dL	32.60 (4.12)	31.40 (4.01)	0.52
LDL-c [c], mg/dL	133.90 (14.7)	135.20 (6.3)	0.80
LDH [c], IU/L	97.20 (14.1)	88.70 (15.1)	0.21
CPK [c], IU/L	72.40 (13.3)	67.50 (14.0)	0.43
PT [c], second	13.50 (1.18)	13.00 (0.82)	0.29
WBC [c] ×103/μL	6.86 (1.10)	7.46 (0.56)	0.14
Hemoglobin, g/dL	14.01 (0.83)	14.57 (0.77)	0.15

[a] Number of subjects in both groups was equal (n =10 for both groups).
[b] Values presented are calculated mean ± standard deviations, which are indicated in parentheses.
[c] Abbriviation: FBS, Fasting blood glucose; AST, Aspartic transaminase; ALT, Alanine transaminase;
Alk.p,Alkaline phosphatase; HDL-c, High-density lipoprotein cholesterol; LDL-c, Low-density lipoprotein cholesterol; LDH, Lactate dehydrogenase; CPK, Creatine kinase; PT, prothrombin time; WBC, White blood cell count

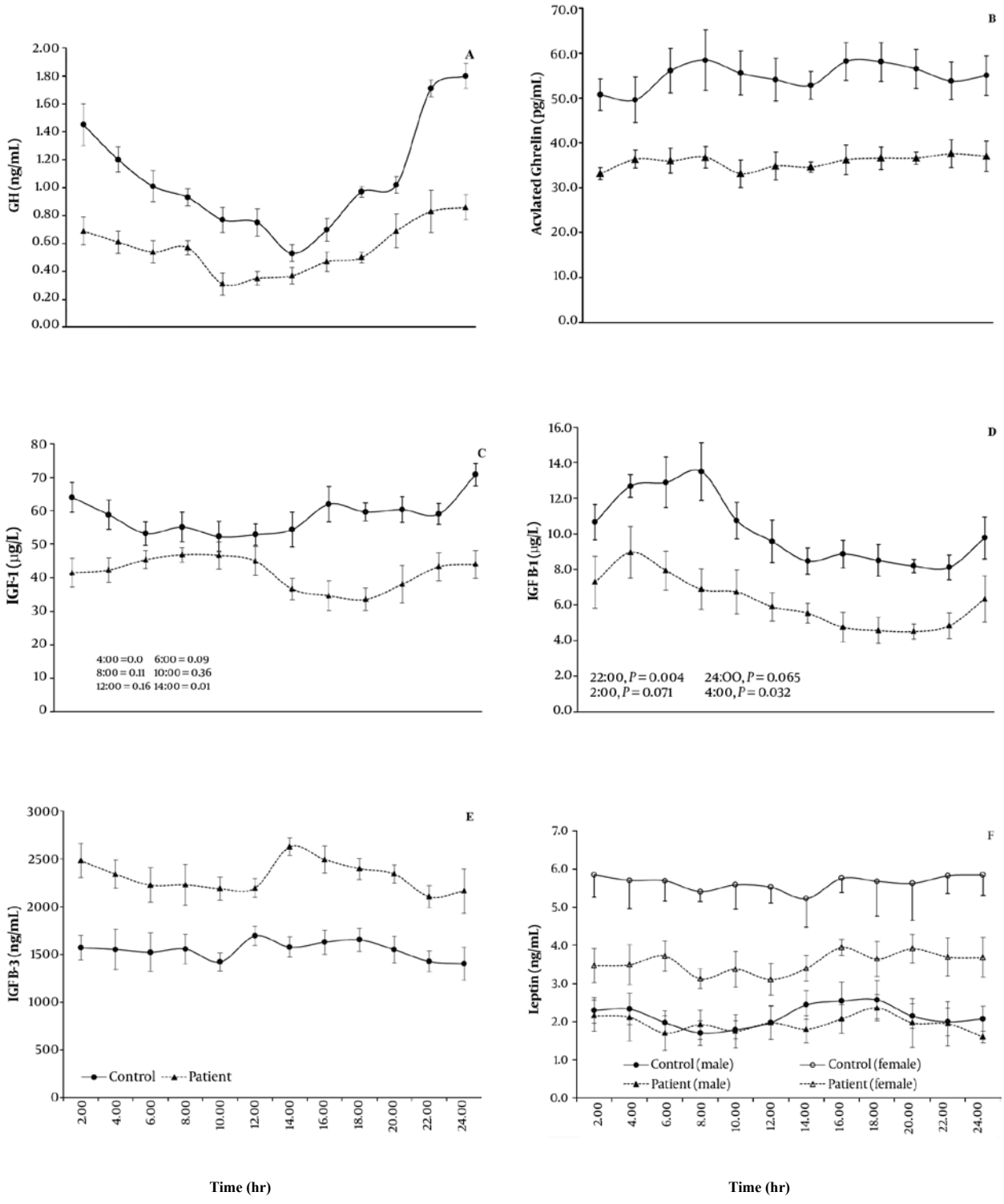

Figure 1. Analysis of ghrelin, GH, and GH-Related Factors in cardiac Patients and Healthy Control Subjects.
Data for patients with reduced ejection fraction (n = 10, 5 male and 5 female), and corresponding age- and gender-matched control subjects (n = 10). Data shown are the mean ± standard error of mean; A, Circadian rhythm of GH (ng/mL) among two groups of participants. Except for two time points, at 14:00 and 16:00, that were not significant ($P > 0.05$), patients had lower GH than the control group at all the remaining time points; B, Circulating acylated ghrelin (pg/mL) of patients at all time points (P from 0.025 to 0.001) was lower than that of control subjects; C, Data for IGF-1 (ng/mL). From 6:00 to 12:00, there was no significant difference between patients and control subjects, while at remaining time points, differences were significant (P <0.01–0,001); D, IGFBP-1 (ng/mL) levels of the two groups. Except for two time points, at midnight and 2:00 AM, that were not difference between the two groups, patients had lower IGFBP-1 ($P < 0.05$–0.001) at the remaining time points; E, At all time points, patients had significantly higher circulating IGFBP-3 (ng/mL) than the control group (P <0.05–0.001); F: Leptin (ng/mL) levels of male subjects were similar, while female participants had significantly higher levels of leptin. In addition, female patients had significantly lower circulating leptin levels ($P < 0.05$) than corresponding control females.

Table 3. Quality Control Information of Commercial Kits Used in the Study

	Ghrelin, pg/mL	Leptin, ng/mL	GH, ng/mL	IGF-1, μg/L	IGFBP-1, μg/L	IGFBP-3, ng/mL
CV (medium level), %	6.7	4.3	5	2.3	4.4	6.9
Sensitivity	0.3	0.34	0.008	1.9	0.4	10.5
Dynamic range	0.3–250	0.34–100	0–150	1.9–1070	0.4–180	10.5–6902

tained for the mentioned parameters.

The data showed that at all 11 time points (except time 14:00), the circulating GH levels of patients were significantly lower ($P < 0.02$–0.001) than those of the corresponding control subjects (*Figure 1A*). Their acylated ghrelin were also significantly lower ($P < 0.01$–0.001) (*Figure 1B*), throughout the 24-hour sampling period. Additionally, both the patient and control groups showed a circadian rhythm for GH, such that higher levels were detected during the nighttime, while lower levels were observed during the daytime. Furthermore, due to low circulating GH among the patients, the difference of the highest levels (at midnight) was significantly greater ($P < 0.05$) than values obtained for 10:00, 12:00, and 14:00 hours, while control subjects had a more prominent diurnal variation ($P < 0.001$). On the other hand, neither the patient nor control groups showed a significant circadian rhythm for acylated ghrelin, though a non-significant variation was detected.

Circulating level of total IGF-1 was also lower among the patients when compared to the values obtained for the control subjects, throughout the 24-hour sampling period. Additionally, while the total IGF-1 of the patients did not fluctuate significantly during 24 hours (uppermost level between 8.00 and 12.00 AM and lowest level between 16.00 and 18.00 PM), this pattern was the converse in the control subjects. The divergence in the patterns of circulating IGF-1 among the two groups did not cause significant differences between the two groups during the morning (6.00–12.00), while marked differences ($P < 0.001$) were detected during the evening (14.00–24.00) (*Figure 1C*). The data for IGFBP-1 showed that the patients had lower levels of circulating IGFBP-1, while both patients and control groups had the same pattern during day and night. In addition, both groups had an apparent and non-significant peak during the night (2.00–8.00), while low levels were detected between 18.00 and 22.00 hours. Statistically, except for the data obtained for two time points of 00.00 and 24.00 that were not significant, patients had significantly lower ($P < 0.05$–0.001) IGFBP-1 levels than the control subjects at other time points (*Figure 1D*).

During the 24 hours of sample collection, circulating levels of IGFBP-3 of both patient and control groups had approximately the same pattern, without significant changes during day and night. However, the values obtained for the patients were higher than those for the control group ($P < 0.01$) at all time points (*Figure 1E*).

The leptin levels of male patients did not differ from the values obtained for the control group (male). On the other hand, female subjects had higher levels of circulating leptin ($P < 0.01$) when compared with the respective values obtained for the male groups (both patients and control subjects). Furthermore, female patients showed a significant decrease of circulating leptin compared to the respective female control group ($P < 0.05$). Here also, we did not detect a significant change during day or night, and both groups had approximately the same pattern (*Figure 1F*).

The fluctuations detected for IGF-1, IGFBP-1, IGFBP-3, and leptin in both groups did not fit the pattern of a circadian rhythm, as determined by statistical analysis of the data using repeated measure of variances. Since we did not detect a considerable circadian rhythm for the evaluated parameters, we carried out a two-tailed bivariate correlation between different parameters. The two-tailed bivariate analysis of the data showed that ghrelin had a positive correlation with total IGF-1 ($r = 0.33$, $P < 0.01$) and a negative correlation with leptin ($r = -0.28$, $P < 0.01$) in the patient group. On the other hand, the circulating leptin in the patient group showed a negative correlation with GH ($r = -0.19$, $P < 0.05$), IGF-1 ($r = -0.38$, $P < 0.01$) and IGFBP-1 ($r = -0.38$, $P < 0.01$), while leptin had a positive correlation with IGFBP-3 ($r = 0.32$, $P < 0.01$). Data for the control group also showed that ghrelin had negative correlations with leptin ($r = -0.38$, $P < 0.01$), while leptin showed negative correlations with IGF-1 ($r = -0.33$, $P < 0.01$) and IGFBP-1 ($r = -0.31$, $P < 0.01$) and a positive correlation with IGFBP-3 ($r = 0.361$, $P < 0.01$). Combining the data of both groups and re-analyzing the Pearson's two-tailed bivariate correlation showed remarkable results. These set of data indicated that ghrelin correlates positively with GH ($r = 0.35$, $P < 0.01$), IGF-1 ($r = 0.42$, $P < 0.01$), and IGFBP-1 ($r = 0.36$, $P < 0.01$), and correlates negatively with IGFBP-3 ($r = -0.48$, $P < 0.01$). In addition, GH also showed a significant correlation with circulating IGF-1 ($r = 0.47$, $P < 0.01$), and IGFBP-1($r = 0.38$, $P < 0.01$), while it showed a negative correlation with IGFBP-3 ($r = -0.43$, $P < 0.01$).

5. Discussion

Except for GH, which showed a circadian rhythm in both groups, other GH-related parameters evaluated in this study did not exhibit a circadian rhythm, although the values obtained for acylated ghrelin, IGF-1, IGFBP-1 and IGFBP-3 in patients were different from those obtained in the control group. Furthermore, while the circulating leptin levels of male participants were the same, female patients had lower leptin levels that control females. The differences detected for the female participants could be

due to their higher BMI, as indicated in previous studies (33, 34).

Acylated ghrelin, which is the active form of ghrelin, plays a critical role in a variety of physiological processes, including the stimulation of GH secretion, regulation of energy homeostasis by stimulating food intake, and promoting adiposity via a GH-independent mechanism (34). Growth hormone also regulates IGF-1 levels, which is an anabolic hormone that spares protein stores at the expense of fat utilization during conditions of caloric restriction (22). In the present study, patients had lower circulating acylated ghrelin than corresponding control subjects. They also had lower GH levels with diminished or a decreased circadian rhythm that could result in lower levels of IGF-1, without a significant circadian rhythm.

Indeed, ghrelin inhibits cardiomyocyte and endothelial cell apoptosis (35, 36) and improves left ventricular function (3, 27, 37). Acylated ghrelin also exerts vasodilatory effects in humans, improves cardiac function, and decreases systemic vascular resistance in patients with chronic heart failure (3, 27, 37, 38). Reduction in circulating acylated ghrelin with diminished circadian rhythm (39) not only reduces the functions mentioned, it also decreases the appetite (22, 23) of aged patients. Furthermore, a decrease in circulating ghrelin may be the cause of the decreased circulating GH and, consequently, reduced circulating IGF-1, as observed in the present study and suggested by previously reported data (2, 40, 41). The positive correlation between acylated ghrelin and GH or IGF-1 among our rEF patients may also support the validity of the clinical trials that used ghrelin (37, 42, 43) or GH (9, 13, 14, 44) for treatment of their patients, and observed improvement in the patients' condition, such as increasing exercising capacity, left ventricular function and reduces proinflamatory cytokins. On the other hand, while our data for ghrelin (45), GH (32, 44), and IGF-1 (46) are consistent with earlier reports, there are reports that showed circulating ghrelin (47, 48) and GH (27, 49) increase in patients with cardiac cachexia and observed deterioration of their patients' health. The discrepancy in the results have prompted experts in the field to stress the need for evaluating the basal endocrine status of the patients before treating them with GH, and for paying particular attention to those patients who present with a peripheral GH resistance (5). Additionally, patients with GH deficiency exhibit ventricular dysfunction, increased vascular thickness, and an increased number of atheromatous plaques (8). However, most of GH action is via production of IGFs that constitute a system of peptides that promote mitosis, growth, and organ development by both paracrine and endocrine pathways (2, 8, 50). One study has demonstrated a significant correlation between left ventricular ejection fraction and the mean IGF-1 concentrations of the patients ($r = 0.24$, $P = 0.02$) (51). In the present study, we observed reduced circulating total IGF-1 among our patients that was coupled with low levels of IGFBP-1 and high levels of IGFBP-3. Previous studies have also reported lower circulating levels of IGFBP-1 (21), while there are reports that showed no change (52) or a decrease (46) in IGFBP-3 among CHF patients, without mentioning the state of ejection fraction among their patients. Under normal circumstances, less than 1% of IGF-1 circulates in free form and is degraded within a few minutes (50, 53). Bioavailability of IGFs in circulation is modulated by at least six specific IGFBPs (53). Thus, circulating levels of these binding proteins act as the main regulators of IGFs bioavailability in circulation (54). Furthermore, while the majority of IGF-1 is bound to IGFBP-3 that is unable to cross the endothelium, IGFBP-1 is comparatively small and has been proposed to be the pivotal acute regulator of IGF-1 activity. IGFBP-1 is also capable of crossing the endothelium and acutely modulating access of IGF to tissues and cell surface receptors (54). Our data indicate that patients with low ejection fraction have lower bioavailability of total IGF-1, not only due to a reduction in acylated ghrelin and/or GH but also due to reduced levels of circulating IGFBP-1 and increased levels of circulating IGFPB-3.

Finally, leptin was the only parameter that showed a difference between male and females of both groups (patient and control groups). Furthermore, while circulating levels of leptin among male participants were the same, female patients had lower levels of leptin compared to the female control patients ($P < 0.05$). The discrepancy could be due to the higher BMI for the female participants (33, 34). Furthermore, previous studies also did not find significant differences among the control and cardiac patients with identical BMIs (55).

Circulating leptin regulates energy homeostasis and is elevated in obesity, and it influences ventricular and vascular remodeling (56). Here, we did not detect considerable differences between circulating leptin levels of our patients and the corresponding control group, since BMI was less than 25 kg/m^2, but detected a negative correlation between circulating leptin and IGFBP-1 and a positive correlation with IGFBP-3 needs more attention. Considering reduced levels of ghrelin, GH, and IGF-1 with unchanged leptin levels suggest that there should be a balance between leptin and ghrelin or GH /IGF-1, to maintain an appropriate level of IGFBPs in circulation. Since this is the first study that demonstrates a relationship between IGFBPs and leptin, further investigation is necessary to confirm this.

In conclusion, in this study, for the first time, we examined the circadian rhythm of acylated ghrelin, leptin, and related components (GH, IGF-1, IGFBP-1, and IGFBP-3) among the patients with low ejection fraction, and showed that except for GH, which showed a decreased circadian rhythm, the remaining factors do not exhibit a circadian rhythm in this age group. Furthermore, while circulating levels of acylated ghrelin, GH, and IGF-1 are reduced among these patients, a reduction of circulating IGFBP-1 and an increase in IGFBP-3 probably worsens the condition of patients by reducing the bioavailability

of IGF-1. Finally, the correlation detected between circulating leptin and IGFBPs or reduced ghrelin and its association with GH/IGF-1 may provide diagnostic as well as prognostic tools for the treatment of patients with low ejection fraction, as suggested recently (2).

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