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RESEARCH ARTICLE

EXPRESSION OF APELIN IS RELATED TO OXIDATIVE DAMAGE IN HEART TISSUE OF RATS DURING CHRONIC SYSTEMIC HYPOXIA

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ABSTRACT

Background: Chronic systemic hypoxia is severe environmental stress for the heart and might lead to the development of heart failure. Apelin is an endogenous peptide that has been shown to have various beneficial effects on cardiac function. Apelin appears to have a role to play in the ventricular dysfunction and maintaining the performance of the heart.

Objectives: In the present study we want to investigate the adaptive response of heart tissue to chronic systemic hypoxia and the correlation with apelin expression and oxidative stress in rat.

Methods: An experimental study was performed using 28 Sprague-Dawley male rats, 8 weeks of age. Rats were divided into 7 groups 4 each, namely control group; normoxia (O2 atmosphere) and the treatment group of hypoxia (8% O2) for 6 hours; 1;3;5;7 and 14 days respectively. Body weight and heart weight were measured at each treatment. Ventricular thickness was measured by caliper, Apelin mRNA was measured using real-time qRT-PCR with Livak formula and malondialdehyde (MDA) level was used to assess oxidative stress due to cardiac tissue hypoxia.

Results: Macroscopic exams showed hypertrophy at day 7th. The relative expression of Apelin mRNA in hypoxic heart is decreased at the beginning and then increased, starting from day-7 to day-14. The MDA levels were significantly increased from day-7 and were strongly correlated with relative expression Apelin.

Conclusion: It is concluded that the increase of Apelin expression is related to oxidative stress in heart tissue of rats during chronic systemic hypoxia.

Keywords : Apelin, Malondialdehyde, Chronic systemic hypoxia, Cardiac hypertrophy

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INTRODUCTION

Hypoxia is a pathological condition that characterized by an insufficiency of oxygen supply to fulfill cellular demand. The level of hypoxic injury in the cardiovascular system depends on the hypoxic intensity, the hypoxic stimuli duration and the cardiac tolerance to oxygen shortage.[1] The cardiac important responses to oxidative stress associated with hypoxia include pathological hypertrophy and ventricular remodeling. Cardiac hypertrophy is a maladaptive precursor to heart failure (HF).[2] Cardiomyocyte hypertrophy is induced by a variety of factors such as mechanical load,[3] neurohumoral factors,[4] and pathological mechanisms. Cardiac hypertrophy represents a typical feature of various cardiomyopathies including ischemic heart disease and arterial hypertension.[5] The hypertrophic responses include changes in the quality and quantity of various factors, such as cell size, gene expression, protein synthesis, and sarcomere organization.[6]

Heart failure is a global problem with an estimated prevalence of 38 million people worldwide, and a number that increases with aging population. Heart failure is a deadly and costly disease with a progressive syndrome, characterized by high mortality, frequent hospitalization, and a decrease in quality of life resulting from the inability of the heart to maintain sufficient cardiac output to meet tissue needs.[7] Current treatments mainly slow the development of this syndrome, and there is a need to develop new preventive and reparative therapies. For this case, we feel the need to search for new biomarkers to complement existing biomarkers using animal models. The study of HF requiring animal models that can live where chronic changes in the structure and function of myocardial can evolve and progression of HF and left ventricular dysfunction (LV) can be quantified.

In this study, the conditions of oxidative stress in rats were carried out by normobaric chronic systemic hypoxia, with the aim of developing an experimental model of HF.[8] One of the responses to cardiac stress is an increase in the expression of various peptides that help the heart compensate for stress. Adipose tissue can regulate cardiomyocyte hypertrophy by issuing various bioactive factors, through reactive oxygen species (ROS). The excessive ROS generation in cardiomyocytes has been shown to mediate the hypertrophic response to stretch or other hypertrophic stimuli, such as angiotensin II, endothelin-1, tumor necrosis factor-α (TNF-α).[9,10]

During hypoxia, ROS formation increases and causes oxidative stress conditions that play an important role in the development of left ventricular hypertrophy and HF.[9-11] If ROS formation exceeds antioxidant capacity, ROS produced in ischemic myocardium can directly cause cell death by damaging cell membrane lipids, protein, carbohydrates, and DNA, which leads to qualitative and quantitative changes in the myocardium that causes HF.[12,13] Reaction of ROS with lipid membrane, rich in polyunsaturated fatty acids will cause lipid peroxidation. Lipid peroxidation, which initiated with the presence of hydroxy radicals which results in the production of malondialdehyde (MDA), directly produces oxidative stress. Thus MDA can be measured by thiobarbituric acid test (TBARS test).[14]

Apelin is a new adipocytokine, initially isolated from bovine stomach tissue extracts by Tatemoto in 1998.[15] The apelin precursor is translated as a

77–amino acid preproapelin and the apelin propeptide has many potential proteolytic cleavage sites forming apelin-36, apelin-19, apelin-17, apelin-16 apelin-13, apelin-12 and also the pyroglutamate (Pyr1) apelin-13 subtypes.[15-17] Apelin is a newly endogenous peptide that is a ligand for the apelin receptor, which was described as an orphan G-protein-coupled receptor (GPCR, APJ).[15-18] All apelin isoforms may function through the unique APLNR, their tissue specificity, binding affinity to APLNR and efficacy in APLNR recycling may lead to differential functions of isoforms. It has been shown that apelin signaling pathways are widely represented in the cardiovascular system and it is an important regulator of cardiac function.[19] Apelin and its receptor APJ are expressed in several tissues (stomach, heart, lung, skeletal muscle, etc.) and in the brain, including the hypothalamus.[20] The apelin is expressed in the endothelium via paracrine and endocrine signaling to activate APJ on myocardial cells, endothelial cells, and some smooth muscle cells.[16-21] Apelin has a functional role in cardiovascular development and may also participate in cardiovascular pathological processes.[22-24] In preclinical models, apelin causes nitric oxide-dependent vasodilatation and increases cardiac contractility in rats with normal and failing hearts.[15,25] Foldes *et al*. showed that in human HF Apelin expression levels were higher than those of normal tissue, and suggested that apelin might be involved in the pathophysiology of human HF.[26] Acute Apelin infusion increases cardiac output and lowers blood pressure and peripheral vascular resistance in patients with HF.[27] Foussal et al. reported that administration of exogenous apelin can maintain heart function through reduced oxidative stress and increase catalase activity. This suggests that apelin is a powerful regulator of cardiomyocyte antioxidant defense against oxidative stress in hypertrophic myocardial remodeling.[13]

Tatemoto et al. have described a novel adipokine, produced and secreted by human and mouse adipocytes, apelin,15 and reported that reactive oxygen species (ROS) and oxidative stress may increase the apelin levels.[28,29] The aim of this study is to analyze the adaptive response of heart tissue during chronic systemic hypoxia by investigating the relative expression of Apelin gene in rat and its relation with oxidative stress caused by hypoxia.

MATERIAL AND METHODS

Animal

All procedures were approved by the Ethical Research Committee FMUI No 354/UN.2F1/ETIK/2015 This study was an in vivo experimental study, using 28 male healthy Sprague-Dawley rats weighing 200–250 g. Rats were allowed to access food and water ad libitum. The animals were adapted for at least 7 days before experiments. After a period of adaptation, the rats were divided into 7 groups randomly. Group 1 (Normoxia) was control group within atmospheric air. Six other groups (Hypoxia: H1-H6), were the treatment groups, exposed to hypoxia (8% O2, 92% N2) for 6 hours; 1; 3; 5; 7 and 14 days respectively. All groups were sacrificed at respected time by mean of decapitation after ketamine anesthesia. The thoracic cavity was opened and the heart was quickly excised from the aortic root. Heart tissues were weighed and cut transversely in the subangular region, below the atrial-ventricular junction in two parts.[30] The lower part of the heart is measured by the thickness of the ventricular wall in the thickest part using

caliper (precision 0.01 mm) and then heart tissue placed into microtube frozen immediately and stored at -80ºC for analysis of Apelin expressions and MDA content.

Quantitative real-time RT-PCR

Total RNA was isolated from 25 mg of heart tissue using the Total RNA Mini Kit, Tissue (Geneaid) according to the manufacturer's instructions. Tissue samples were homogenized in a microcentrifuge tube using micropestle. After washing with absolute ethanol, the final RNA was elected in 50 µl of RNase-free water. The purity and concentration of RNA were verified by optical density (OD) absorption ratio OD260 nm/OD280 nm between 1.8 and 2.0 using Varioskan (Thermo Fisher).

Table 1. Primer sequences used by real-time quantitative RT-PCR

Gene	Primer sequences
Apelin	Left: 5'-GTGAAGCCCAGAACTTCGAG-3'
	Right: 5'-CAGCGATAACAGGTGCAAGA-3'
18srRNA	Left: 5'-CGCGGTTCTATTTTGTTGGT-3'
	Right:5'-AGTCGGCATCGTTTATGGTC-3'

The homogeneous cDNA was generated by the reverse transcription of RNA samples using the AccuPower® CycleScript RT PreMix (dN12) (Bioneer) Thermocycler. Real-time PCR was performed using a detector (Exicycler) and then subjected to PCR with SYBR Green I dye in real-time monitoring as the detected PCR products. The PCR solution contained specific left primer and right primer (1 µL each), 5 µl cDNA template, and adjust by DEPC-distilled water to a final volume of 20 µL. The PCR primers designed with Primer3 based on GeneBank were depicted in Table 1. The reaction conditions for amplifying DNA were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15s, 61 °C for 30 s, and 72 °C for 30 s. The mRNA expression was normalized to the expression level of 18s rRNA and was calculated using the following equation: Fold change = $2^{-\Delta\Delta Ct}$

Assessment of oxidative stress by TBARS estimation

To assess the oxidative damage, MDA level (a marker of lipid peroxidation) was measured by reaction with thiobarbituric acid (TBA) methods as described Wills. MDA and TBA react to produce a pink pigment with maximum absorption at 532 nm. 400 µL of homogenate was added 200 µL of trichloroacetic acid (TCA) 20%, then centrifuged. The supernatant was added 400 µL of a solution of TBA. Furthermore, it was incubated in a water bath for 10 minutes at a temperature of 80-100ºC, then cooled to room temperature. The absorbance was measured against a reference blank at 532 nm by spectrophotometer Genesys 10 UV scanning thermoscientific. 1,1,3,3-tetramethoxypropane (Sigma Chemicals, USA) were used as a standard. The levels of MDA in heart tissue were expressed as nmol/mL.

Statistical Analysis

All data were presented as means ± SEM (n = 4 per group) and analyzed using GraphPad software (Version 6, for windows). A one-way analysis of variance (ANOVA) was used to detect statistical differences between groups. A post hoc test (LSD) was performed to determine differences between groups. Significant differences were considered at $p < 0.05$.

RESULTS

Table 1 shows the results of blood gas analysis and hematology. The changes in various parameters as induced by hypoxia, which was expressed as mean ± SEM (standard error of the mean). Table 1 shows the pO_2, pCO_2, arterial O_2 saturation, and HCO_3^- decreased gradually in line with the duration of treatment of hypoxia, while blood pH decreased at the end of exposure. On the other hand, hypoxia led to an increase in hemoglobin, hematocrit and red blood cells (RBC). The increase in all three parameters will continue until the end of the treatment and the concentration or amount to be very high.

Figure 1 shows macroscopic features of the normal and chronic systemic hypoxia cross-section of rats ventricular after hypoxia of 6 hours 3, 5,7 and 14 days respectively. The ventricular wall thickness was significantly increased at right ventricular on 7 days hypoxia and left ventricular on 5 days hypoxia, which indicated an occurrence of ventricular hypertrophy induced hypoxia.

Table 2 shows morphometric evaluation of the normal and chronic systemic hypoxia of rats heart ventricular in hypoxia 6 hours, 1 day, 3 days, 5 days, 7 days and 14 days. A significant increase was observed in both the right ventricular after 7-days of hypoxia and left ventricular after 5-days of hypoxia. The ratio of ventricular thickness (hypertrophy index) of the right ventricular wall was greater than the left ventricular, as seen after 5 days of hypoxia.

Table 1. Blood gases and hematology Values

Parameter	Normoxia	Hypoxia					
		6 hours	1 day	3 days	5 days	7 days	14 days
pO_2 (mmHg)	95.9 ± 2.0	85.1 ± 0.9	64.2 ± 3.5	59.0 ± 2.6*	53.7 ± 0.9*	44.4 ± 2.1*	35.1 ± 1.9*
pCO_2 (mmHg)	40.4 ± 1.3	38.3 ± 0.5	36.2 ± 1.3	33.3 ± 1.0*	31.3 ± 0.8*	29.2 ± 1.6*	23.7 ± 1.1*
Ph	7.40 ± 0.01	7.40 ± 0.01	7.39 ± 0.00	7.39 ± 0.0	7.38 ± 0.00	7.38 ± 0.01*	7.38 ± 0.01*
HCO_3^- (mmol/L)	24.3 ± 1.3	23.2 ± 0.7	22.1 ± 1.1	20.4 ± 0.9	19.1 ± 0.2	17.9 ± 0.5*	14.9 ± 0.6*
O_2 Saturation (%)	94.1 ± 1.5	80.8 ± 1.0	67.5 ± 3.1	60.7 ± 2.7*	58.0 ± 0.7*	55.3 ± 2.1*	52.1 ± 4.2*
Hemoglobin (g/L)	12.01 ± 0.06	12.03±0.08	13.1 ± 0.1	15.01± 0.2*	16.09± 0.1*	17.2 ± 0.2*	19.9 ± 0.2*
Hematocrit (%)	45.3 ± 1.2	46.5 ± 0.4	47.7 ± 1.7	52.5 ± 1.8*	55.4 ± 0.4*	58.3 ± 1.1*	63.2 ± 1.2*
RBC (ml/1000)	6.8 ± 0.1	6.9 ± 0.0	7.1 ± 0.1	8.0 ± 0.0*	8.3 ± 0.1*	8.5 ± 0.2*	9.7 ± 0.2*

Mean values ± SE, * (Significant difference versus normoxia $P<0.05$,
pO_2, pCO_2, O_2 saturation and HCO_3^- fall dramatically, this means that the treatment provided is causing systemic hypoxia. In Advance hypoxia created severe metabolic acidosis (decrease in pH and HCO_3^-) with the compensation for severe respiratory alkalosis (pCO_2 decreased).

Table 2. Evaluation of cardiac morphometric

Parameter	Normoxia	Hypoxia					
		6 hours	1 day	3 days	5 days	7 days	14 days
Right ventricular thickness (mm)	1.36±0.02	1.38±0.06	1.4±0.05	1.43±0.03	1.61±0.02	1.74±0.03*	1.9±0.09*
Right ventricular hypertophy (%)		0.92%	2.75%	4.59%	18.35%	27.52%	39.45%
Left ventricular thickness (mm)	2.23±0.03	2.28±0.03	2.30±0.02	2.34±0.02	2.4±0.02*	2.61±0.06*	2.79±0.06*
Left ventricular hypertophy (%)		2.25%	3.37%	5.06%	7.87%	17.42%	25.28%

Mean Value ± SEM, * significant difference versus normoxia ($p < 0.05$)
Index Hypertrophy (%) = ((Ventricular Thickness Hypoxia - Ventricular Thickness normoxia) / Ventricular Thickness normoxia) x 100%

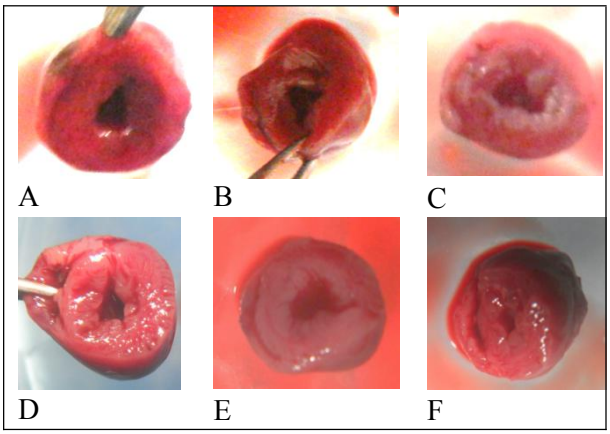

Figure 1. The macroscopic features of the normal and chronic systemic hypoxia cross-section of rats heart ventricular. Transverse sections of the hearts. A. Normoxia, B. 6 hour hypoxia, C. 3 day hypoxia, D. 5 days hypoxia, E. 7 days hypoxia, and F. 14 days hypoxia. The left ventricular and right ventricular wall thickness increases in proportion with the duration of hypoxia treatment.

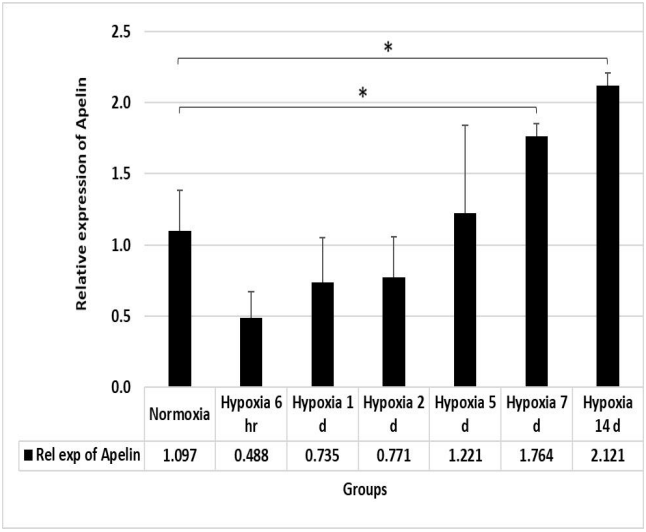

Figure 2. The expression of mRNA Apelin in the heart tissue. There were significant differences between normoxia and the hypoxic groups 7th and 14th days.

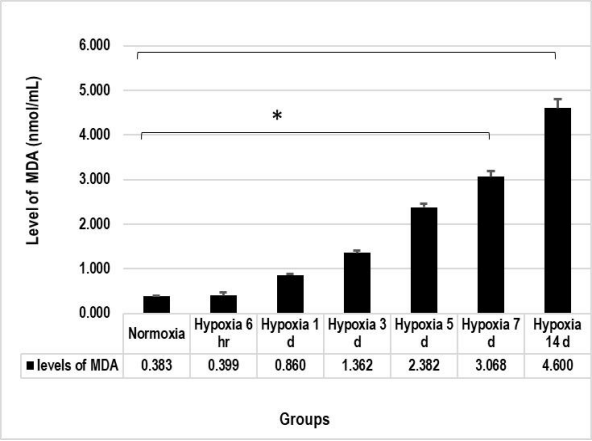

Figure 3. MDA levels in Heart Tissue. There were significant differences between normoxia and the hypoxic groups 7th and 14th days.

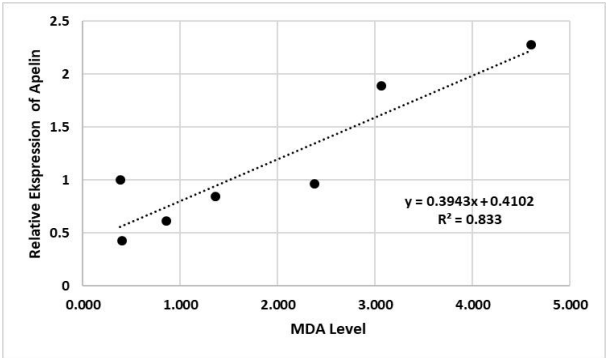

Figure 4. Correlation of MDA levels and Apelin relative expression in heart tissue of rat-induced by systemic hypoxia. The level of MDA and relative expression Apelin is strongly correlated and significant ($r = 0.667$; $p = 0.001$).

Figure 2 shows relative expression of apelin as a result of chronic hypoxia induction, in mean±SE. Product of real-time RT-PCR was detected as fluorescence absorbance of SYBR Green. Threshold of fluorescence curve was set-up to achieve the optimum efficiency of expression. The relative expression of Apelin mRNA in heart hypoxic was decreased at the beginning and dramatically increased with the duration of treatment, starting from 7-days until the end of the treatment period. Total RNA was extracted from the heart, and measured by UV light absorption wavelength of 260 nm.

MDA, a biomarker of cardiac oxidative injury, is a product of lipid peroxidation that can be produced by a variety of oxidative damages. As shown in Figure 3, the hypoxia significantly increased MDA levels at days 7 and 14 ($P < 0.001$) in comparison with normoxia group. The increase of MDA occurred from the beginning (h 6 hours) until the end of the treatment. Figure 4 showed that the increase of Apelin expression is related to oxidative damage in heart tissue of rats during chronic systemic hypoxia.

DISCUSSION

The experimental study conducted in rats which were conditioned to hypoxia for 14 days using 8% O_2 and 92% N_2. All parameters such as blood gases pO_2, pCO_2, O_2 saturation, and HCO_3^- fall dramatically, this means that the treatment provided is causing systemic hypoxia. In Advance hypoxia created severe metabolic acidosis (decrease in pH and HCO_3^-) as the compensation for the shortness of breath due to lack of O_2 breathing accelerated and pCO_2 decreased causing respiratory alkalosis. Witt *et al*.[31] reported that the striking decline from the same parameters has occurred since the first hours of exposure to hypoxia. Comparable results were also reported by other researchers.[1,32] The increased in hemoglobin, hematocrit and RBC occurred as compensation for the decrease of pO_2 level in the network, so that through the increase in all parameters, improved O_2 transport. However, this causes an increase in blood viscosity, making the heart work harder.

Systemic chronic hypoxia causes an increase of ventricular wall thickness. The left and right ventricular wall thickness were increased in proportion with the duration of treatment. Similar changes in cardiac morphometry were also found by Ferdinal *et al*.[32] In physiologic levels, ROS act as signaling molecules in several cellular functions; on the other hand, in pathologic conditions overproduction of ROS have deleterious effects by damages to the several cellular components.[33] An important mechanism that can explain the occurrence of ventricular hypertrophy as a result of hypoxia is oxidative stress as a result of increased ROS production that leads to cell death, either through the mechanism of apoptosis or by necrosis or autophagy. Furthermore, hypoxia causes fibrosis of the vascular endothelium, either as a direct effect or by stimulating the secretion of Angiotensin II are derived from the sympathetic nervous system and resulting in heart.[8]

Apelin is a peptide ligand of the APJ receptor implicated in cardiovascular diseases. Apelin has many functions such as a positive inotropic, vasodilator and diuretic. The apelin and its receptor can be found in the adipose tissue, with higher concentrations in the lung and cardiovascular system.[34] This study reports that apelin myocardial expression

and secretion are activated by hypoxia. We observe an early upregulation of the rat apelin gene expression under hypoxia (8% O_2) in the heart cells. Rokainen *et al.*[28] said the hypoxia increases the apelin expression in cultured cardiomyocytes.

To determine hypoxia-induced regulation of apelin, we measured mRNA expression after treatment of the rats with hypoxia (8% O_2, 92% N_2).for different time periods. Total RNA was isolated from the heart of a rat exposed to normoxic and hypoxic conditions (8% O_2). After 7 days, the apelin mRNA was significantly increased in the heart of the rat exposed to hypoxia. The expression of Apelin mRNA in heart tissue is shown in Figure 2. Hypoxia and various other stimuli induce HIF-1α signaling cascade, and then transcriptionally activate multiple genes.[35]

It has been reported that apelin is present in human plasma and the myocardium.[16,28] In chronic HF due to ischemic heart disease or dilated cardiomyopathy there is an increase the apelin mRNA levels and apelin plasma levels are reported to increase in the early stage of left ventricular dysfunction.[25,36] Ronkainen et al. said the most presumable candidate for the regulation of apelin gene expression would be hypoxia-inducible factor-1 (HIF-1).[28] HIF-1 is activated in hypoxia due to the prevention of oxygen-dependent HIF prolyl-4-hydroxylase (HIF-P4H)-mediated proline hydroxylation, which in normoxia targets the HIF-1 subunit for ubiquitination and proteasomal degradation.

Hypoxia leads to increased formation of free radicals. MDA is produced in the process of lipid peroxidation by ROS, thus that the measurement of concentration, is used as a biochemical marker of oxidative stress. In hypoxia, ROS production in the respiratory chain increased particularly superoxide anion, as a result of leakage of electrons, resulting in a partial reduction of oxygen. In this study, the treatment of hypoxia comparable to the increased concentration of MDA. The high concentration of unsaturated fatty acids in cell membranes makes cell membranes the main target of free radicals that are formed lipid peroxide with solving the main results in the form of MDA. We demonstrated that the level of MDA in heart tissue had increased since day-1 of hypoxia. The MDA levels increased with the length of exposure to hypoxia and reach the maximum level at the day-14 with a significant increase on day-7 and day-14 of hypoxia The increased level of MDA in heart tissue also proved by Folden *et al*.[37] which found that oxidative stress caused to an increase in MDA level. Increased ROS formation from mitochondria will trigger the redox signaling cascade. ROS will activate signaling pathways and in turn activate transcription factor and expression of target gene.[2,38,39]

Correlation between MDA of rat heart with relative expression of mRNA Apelin of heart tissue got result of correlation coefficient equal to 0,667 which showed a strong positive correlation. This shows that Apelin is expressed under hypoxia/oxidative stress. This is consistent with the increase in MDA concentrations were positively correlated strongly to increase the Apelin relative expression.

CONCLUSION

We created an experimental rat heart damage model with chronic systemic hypoxia. This damage is supported by a significant increase in the relative

expression of Apelin mRNA in chronic systemic hypoxia starting from the 7th day. This shows structural damage due to pressure overload in the ventricular cavity and the ventricular wall stretching resulting in increased ventricular wall thickness. Induction of chronic systemic hypoxia causes an increase in formation of MDA in rat heart tissue because the formation of ROS increases during hypoxia. This study shows that Apelin mRNA levels can be candidates as biochemical markers of heart damage.

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