

Structural and Morphological Changes in Rat Ventricular Myocardium Induced by Chronic Systemic Hypoxia

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ABSTRACT

Aim: to explore the effects of chronic systemic hypoxia on myocardial structure and morphology. In addition, the goal of present study is to develop a hypoxia-induced heart failure model in rats.

Methods: Sprague-Dawley male rats, weighing 220-250 g at the time of recruitment were randomly allocated into 7 groups ($n = 4$ per group), the control normoxia group was exposed to room air, while the hypoxia groups were caged in a plexiglas hypoxic chamber (8% O_2 and 92% N_2) for 28 days. Structural and morphological changes of ventricular myocardium were determined at day 28, while blood gas parameters were measured at day 1, 3, 7, 14, 21, and 28.

Results: histopathologic and morphologic evaluation showed massive hypertrophy accompanied by damage of the intercalated disk (ID) structure, angiogenesis, necrosis, fibrosis, and apoptosis as a hallmark of ventricular remodeling. At the end of treatment, there were increases of LV (2.79 vs 3.71) and RV (1.72 vs 2.54) wall thicknesses, and also in hypertrophy index (from 3.19 to 5.74). Blood gas analysis revealed metabolic acidosis compensated by respiratory alkalosis. There was an observed decrease of blood gas parameters in hypoxia group compared to control group: PO_2 (24.7 vs 96.4 mm Hg), PCO_2 (18.2 vs 40.4 mm Hg), O_2 saturation (25.5 vs 94.1 %), and HCO_3 (10.1 vs 23.4 mmol/L). On the other hand an increase in hemoglobin level (221.5 vs 120.3 g/L), haematocrit level (68.6 vs 45.2 %), and red blood cell count (10.4 vs 6.9 μ L/1000) could be observed.

Conclusion: our data clearly show that chronic systemic hypoxia causes massive ventricular hypertrophy accompanied by severe structural and morphological impairment of ventricular myocardium, which eventually results in cardiac failure.

Key words: chronic hypoxia, ventricular hypertrophy, fibrosis, apoptosis.

INTRODUCTION

The living organism ability to detect and response to hypoxia is crucial. In the absence of sufficient oxygen, cardiac energy demands are not met. Chronic exposure to hypoxia regulates the expression of numerous genes encoding enzymes, growth factors, or transporters, which induce molecular and histological modifications to reduce the cellular need and dependence on O_2 and to increase O_2 supply to the tissues.^{1,2}

Previous studies on chronic hypoxia have been limited to the effects on myocardial metabolism and function, and less is known about the effects on myocardial structure. Hypoxia participates critically in the pathogenesis of major causes of mortality, such as cancer, stroke, myocardial ischemia or infarction and chronic lung disease. Hypoxia also plays a pivotal role in many clinical cases, including: anemia, inflammation, diabetes, obstructive sleep apnea (OSA), sudden infant death syndrome (SIDS), wound healing, and altitude acclimation-related illnesses.¹⁻³

In the long term, chronic hypoxia causes pulmonary hypertension due to pulmonary vasoconstriction, arterial remodeling and polycythemia, which ultimately results in right ventricular hypertrophy, cardiac overload and then progress to cardiac failure.⁴

The role of the various mechanisms responsible in the development and progression of heart failure is difficult to detect in humans because of uncertainty of identification the timing when heart failure begins. Additional difficulty is associated with the influence of confounding such as concomitant pharmacological therapies. The understanding of left ventricular remodeling and dysfunction is fundamental to describe the natural history of the disease, as well as the

efficacy and timing of interventions needed to positively interfere with all the processes leading to heart failure and, ultimately, cardiac death.⁵ Because of these difficulties, animal models of heart failure are fundamental to describe the complex nature of this disease process and a number of experimental model systems have been generated in various species.⁶ In previous studies, animal models of hypoxia induced-heart failure in rats were performed by hypobaric-hypoxia⁷ or using drugs such as monocrotaline.⁸ We decided to study the effects of chronic systemic hypoxia on myocardial structure and morphology, and to develop hypoxia-induced heart failure model in rats.

METHODS

Animals and Protocol

Sprague-Dawley male rats, weighing 220-250 g at the time of recruitment in the protocol were randomly divided into 7 groups ($n = 4$ per group): the control normoxia group was exposed to room air, while the hypoxia groups were caged in a hypoxic chamber, for 1, 3, 7, 14, 21, and 28 days, respectively. Control rats were raised in room air for 28 days before sacrificed. The oxygen tension inside the chambers was continuously monitored by an oxygen meter. All animals had free access to water and standard rat chow. Water and food consumption were assessed every 2 days. We used two types of chambers, the bigger was flushed with gas-mixture (8% O₂ and 92% N₂). When a cage opening was required for regular cleaning, replenish food and water, or sacrifice an animal, the small compensation chamber was first flushed with hypoxic gas before the animal is transferred into it. This study conformed to the Guide for the Care and Use of Laboratory Animals.

Blood Gas and Hematologic Analysis

Each rat was weighed and then anesthetized with ether. Immediately after induction of general anesthesia, the chest was opened through median sternomy. A blood sample was withdrawn into heparinized tube from aorta. After mixing, the sample was divided into two aliquots. Blood gas analysis was performed using Corning 165 (Corning Scientific Instrument), and hematologic analysis was performed using Sysmex KX21, Diamond Diagnostics.

Morphologic Measurement

The hearts were rapidly excised, excess blood was absorbed on tissue paper and then weighed. The ventricle was sliced perpendicular to the long axis, under atrioventricular junction and the thickness of

ventricular wall were measured by caliper digital.⁹

Histopathologic Examination

The hearts were fixed immediately after excision in 10% buffered formaldehyde and then dehydrated, impregnation and embedded in paraffin. Sections 4 μ m thick were cut on a microtome and stained with hematoxylin-eosin and periodic acid-Schiff. This last stain emphasizes the basal membrane, thus facilitating the microscopic measurement of the cardiomyocytes.⁹ Immunohistochemistry (IHC) procedure for detecting apoptotic cardiomyocytes were performed by TUNEL method according to the manufacturer's instruction (In Situ Cell Death Detection kit - Roche, USA).

Statistical Analysis

Statistical analysis was performed by using MedCalc v.10.2, MedCal Software, Belgium. Data are expressed as mean \pm SE. The significance level was $P = 0.05$ (two-tailed). To detect significant differences among the two groups, we performed *student t test*.

RESULTS

Blood Gas Analysis and Hematologic Examination

Table 1 shows changes in various parameters as a result of chronic hypoxia induction, stated as mean \pm SE. As seen in the table, gradual decrease in PO₂, PCO₂, and arterial O₂ saturation was found proportional to the duration of hypoxia treatment. The decrease could be observed since day one (P2) and continued until day 28 (P7). The significant decrease in PO₂ and O₂ saturations was significant day one, then gradually decreased until the end of the treatment period.

On the other hand, arterial blood pH was relatively unchanged up to day 14. Significant change was only observed on day 21, and continually decreased until the end of exposure. The HCO₃ level has gradually decreased since the first day, however significant decrease was observed on the seventh day of treatment. The decrease in HCO₃ persisted, reaching a very low level 10.1 mmol/l at the end of the treatment period.

Hypoxia treatment resulted in increases of hemoglobin, hematocrit, and red blood cell count. The increase of all three parameters persisted, causing an extremely high level at the end of the treatment. Hypoxia treatment also resulted in decreased food and water intake, and body weight. Decrease in weight was significant, and the mean body weight at the end of treatment was 168.0 g, while in the control group the BW increased to 254.0 g, which means there were

almost 20% decrease in BW.

Cardiac Morphology

Table 2 shows that the left ventricle (LV) and right ventricle (RV) wall thickness increases or becomes hypertrophic in proportion with the duration of treatment. Significant increase was found from the seventh day of treatment in both LV and RV, but the ventricular thickness increase ratio (hypertrophy index) of the RV wall is higher compared to LV. Rats at the 21-day hypoxia treatment (P6), hypertrophy index: 48.26% vs 32.26%, while in the 28-day hypoxia-treated group (P7), hypertrophy index: 48.8% vs 42.97%.

Heart weight (HW) gradually increased early in the course of treatment, so that at the 28-day hypoxia group (P7), HW had increased by 19% compared to the control group (normoxia). Due to BW decrease and HW increase, the HW/BW ratio increased, and at the end of treatment the ratio had increased by 80% compared to control group (5,74 vs 3,19).

Histopathologic Examination

Figures 1 to 4 are the microphotographic images of histology sections, taken using digital camera and light microscope.

Table 1. Blood values, intakes, and weights of animals

Parameters	Hypoxia						
	Normoxia	1 day	3 days	7 days	14 days	21 days	28 days
Arterial PO ₂ mmHg	96.4±4.8	56.2±6*	51.0±5.2*	44.6±4*	35.6±3.1*	30.1±4.5*	24.7±2.6*
Arterial PCO ₂ mmHg	40.4±2.7	36.2±2.2*	33.3±2.2*	29.0±3.3*	23.7±2.4*	20.1±1.9*	18.2±3.4*
Arterial O ₂ saturation, %	94.1±3.1	65.2±6.2*	60.2±5.5*	57.3±5.4*	52.9±8.6*	36.3±5.7*	25.2±5.4*
Arterial pH	7.39±0.01	7.40±0.02	7.40±0.01	7.40±0.02	7.37±0.01	7.36±0.02*	7.36±0.02*
Arterial HCO ₃ , mmol/L	24.3±2.5	22.2±2.3	20.4±1.7*	17.9±1.2*	14.1±1.1*	11.3±1.9*	10.1±2.1*
Hemoglobin, g/L	120.3±1.6	140.0±3.1*	150.2±3.7*	171.6±5.5*	199.3±3.9*	215.6±4.7*	221.5±5.2*
Hematocrit, %	45.2±2.5	47.8±3.6	52.5±5.1*	58.3±2.7*	63.2±2.6*	66.4±3.5*	68.5±4.3*
Red blood cell, μ L/1000	6.9±0.1	7.1±0.2	8.0±0.2*	8.5±0.5*	9.7±0.5*	10.2±0.2*	10.4±0.8
Ingested food, g/hr	25.6±0.4	20.4±0.8	18.2±0.2	17.6±0.3	14.8±0.5	10.2±0.2	7.4±0.2
Ingested water, mL/hr	35.3±0.5	32.0±0.1	27.5±0.3	23.4±0.3	20.2±0.5	17.3±0.5	15.6±0.3
Final body weights, g	254.0±3.9	206.3±9.7*	201.3±5.2*	195.0±5.4*	182.0±17.6*	173.0±1.2*	168.0±7.5*

Mean values \pm SE, * (Significant difference versus normoxia $P < 0.05$)

Table 2. Cardiac Morphologic Evaluations

Parameters	Hypoxia						
	Normoxia	1 day	3 days	7 days	14 days	21 days	28 days
LV wall thickness, mm	2.79±0.08	2.86±0.09	3.07±0.07	3.28±0.10*	3.51±0.11*	3.7±0.08*	3.71±0.06*
LVH (%)		2.51	10.04	17.56	25.81	32.26	32.97
RV wall thickness, mm	1.72±0.06	1.80±0.06	1.98±0.10	2.23±0.08*	2.33±0.06*	2.52±0.05*	2.54±0.08*
RVH		4.05	15.12	29.65	35.47	48.26	48.84
Heart weights, mg	811.5±42.6	844.3±65.4	863±26.2	917.0±68.1	937.8±75.1	958.0±22.5*	965.0±43.9*
Body weights, g	254.0±3.87	206.3±9.66*	201.3±5.15*	195.0±5.40*	182.0±17.55*	173.0±1.29*	168.0±7.51*
HW/BW, mg/g	3.19±0.011	4.09±0.007	4.29±0.005	4.70±0.123	5.15±0.043	5.54±0.017	5.74±0.006

Mean values \pm SE, * (Significant difference versus normoxia $P < 0.05$)

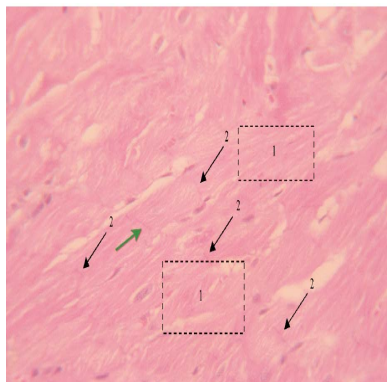


Figure 1. Normoxia: 400x magnification – HE staining. Myocardium with normal structure, Horizontal lines-1 and Intercalated disks (ID) are clearly seen-2. Normal nucleus, normal vascularization

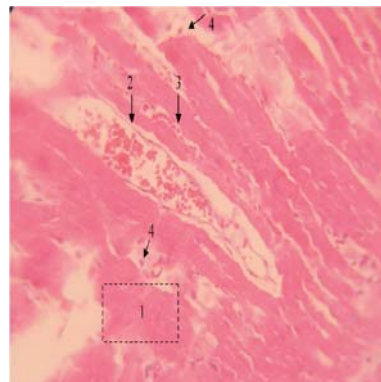


Figure 2. Hypoxia 21 days 400x magnification – HE staining. Horizontal lines and ID structure fade-1, angiogenesis with congestion-2, bleeding spot-3, and signs of inflammatory cell infiltration-4

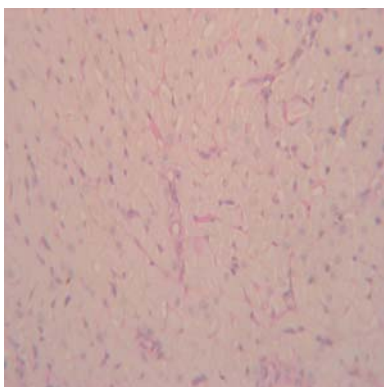


Figure 3. Normoxia: 400x magnification – PAS staining. Normal cell size, clear nucleus, no signs of nuclear degeneration or necrosis.

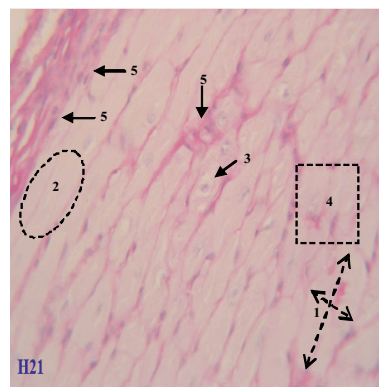


Figure 4. Hypoxia 21 days - 400x magnification -PAS staining. Massive hypertrophic-1, some nucleus disappeared and degenerated-2, perinuclear space-3, necrosis-4 and fibrosis in a number of locations-5.

Impression: Histopathologic features are in line with cardiomyositic hypertrophy with signs of angiogenesis, congestion, necrosis, and fibrosis (ventricular remodeling), due to chronic systemic hypoxia.

DISCUSSION

In this experimental study using rats which are conditioned to hypoxia for 28 days, there was significant decrease in all gas parameters such as PO_2 , PCO_2 , O_2 saturation, and HCO_3 after one day of hypoxia. This result proves that the treatment provided caused systemic hypoxia and the experimental animal was in a severe metabolic stress state. Similar study by Witt *et al*¹⁰ reported that the significant decrease from the same parameters could be found from the first hour of hypoxia exposure (O_2 6%). Comparable result was also reported by Corno *et al*⁹ who provided hypoxia treatment (O_2 8%) for 14 days and Carraway *et al*¹¹ who provided hypoxia-hypobaric treatment (with

atmospheric pressure similar to 17.000 feet height), with 1, 3, 7, 14, and 21-day treatment scheme. The rapid decrease of the parameter could be explained because O_2 is the main element in the energy metabolism of all aerobic organisms.¹

Starting from day 3, the PO_2 was significantly decreased until the end of treatment (day 28), when the rats were in severe hypoxia ($PO_2 = 27.4$ mmHg). In human, PO_2 level <40 mmHg is classified as severe hypoxia. Significant decrease in PCO_2 having begun since the first day and continuously decreased, was due to compensatory effort to dyspnea because of O_2 depletion, by triggering breathing (hyperventilation). On day 3 the experimental animals was already in metabolic acidosis compensated with respiratory alkalosis, although in relatively low level. This view was supported by significant HCO_3 decrease from the third day of treatment. Although the pH was still in normal range at that time, pH is known as a parameter that in

long term will only undergo slight changes. That means, the pH could decrease drastically, for example 6.4, but only for a very short period. All mammals in general will not be able to live in $\text{pH} < 7.1$.¹ In this experiment, significant decrease in pH was only found on the 21st day of treatment and remained at 7.36 at the end of treatment. At that time the experimental animals were in severe metabolic acidosis with severe alkalosis compensation (hyperventilation), each of these conditions could be observed from the severely low HCO_3^- and PCO_2 values. The disturbance in acid-base balance (decreased pH) became more significant along with the duration of hypoxia.

Hematologic parameters, Hb, Ht, and RBC significantly increased early in the treatment, increase in Hb could be observed from the first day, while Ht and RBC started to increase from day 3. All of these happened as a compensatory effort to PO_2 decrease in cells and tissues, in order to increase O_2 transport through the increase of all parameters. Decreased O_2 saturation was found from the first day, proportional to the decrease of PO_2 . The low O_2 saturation at the end of treatment showed that the experimental animal was in severe hypoxia at that time, since O_2 saturation is an indicator of hypoxia.

As stated previously, the molecular mechanism which lay behind the adaptation to chronic hypoxia is mediated by a transcriptional factor called hypoxia-inducible factor-1 (HIF-1) which activates a myriad of target genes. Here, the Ht and RBC increase were found as a result of erythropoietin (EPO) gene upregulation by HIF-1.^{3,13,14} Food and water intake of the hypoxic rats continually decreased since the first days of treatment and proportional with the duration of treatment, while in the rats in control group, 5 gram/week increase in body weight was observed. This shows that hypoxia condition causes severe stress. An interesting phenomenon was the considerable decrease in BW that persisted, causing hypoxic cachexia at the end of treatment. The decrease in BW could not only be explained by decreased food and water intake or stress. In chronic hypoxic condition, there is a shift in metabolism pattern to anaerobic, decreasing ATP as a product. However whether the phenomenon serves as an adaptation mechanism with specific purpose still needs further investigation. Other investigator reported this event as a hypoxia-induced sickness behaviour (SB),¹¹ a disease with nonspecific symptoms as a response to trauma or infection, which causes complex interaction between the nervous system, endocrine system, and the immune system.

The cardiac morphometric evaluation shows increased RV and LV wall thickness and cardiac weight. The heart also experienced a relatively severe cardiac hypertrophy, because at the end of treatment the HW/BW ratio increased to almost 80%. Similar changes in cardiac morphometry were also found by Corno *et al.*⁹ Although both ventricular walls were hypertrophied, the RV hypertrophy was more dominant compared to the LV. This is thought to be due to pulmonary hypertension. Hypoxia triggered adaptation mechanism through stimulation of sympathetic nerve system (SNS), which objective is to cause vasoconstriction in the peripheral tissue including lungs, and on the other hand causing vasodilatation to vital organs such as the heart and the brain. Stimulation of SNS causes an increase of renin-angiotensin system (RAS), especially angiotensin II which causes vasoconstriction, thickening and remodeling of pulmonary vasculature, which at the end causes pulmonary hypertension.¹² Pulmonary hypertension will subsequently increase the RV burden in pumping blood to the lungs, which in turn will cause hypertrophy. If this condition lasted for a long time, it would cause an increase in LV burden in the effort to pump the blood to systemic circulation, which at the end causes LV hypertrophy as well.¹³ Besides, the increase in Ht and RBC (polycythemia) that initially occurred in order to increase oxygen transport, will further increase the RV burden due to increased blood viscosity.¹⁴

There are several other important mechanisms that could explain ventricular hypertrophy as a result of hypoxia, among them are oxidative stress due to increased ROS formation. Increased ROS beyond the capacity of antioxidants could cause a number of changes in cell components, such as protein, lipid, and nucleotides, causing cell damage and even cell death, both through apoptosis mechanism or as a result of necrosis or autophagia.¹⁵ In addition, chronic hypoxia is known to increase the formation and activity of growth factors, and activate the hypertrophic signal. Furthermore, hypoxia causes fibrosis of the vascular endothelium, both as a direct effect or through stimulation of Ang II secretion from SNS and the one produced by the heart.¹⁶

Histopathologic examination showed ventricular hypertrophy and cardiomyocytes accompanied by structural changes that could be observed as fading of the horizontal line structure and intercalated disk (ID). ID is a vital structure in the heart, which serves as a connector between heart cells, forming a kind of

syncytium. Damaged or faded ID structure is an evidence of ventricular remodelling, which could be caused by angiogenesis and fibrosis due to continuous RAS (Ang II) stimulation, as a result of hypoxia treatment.¹⁷ This also shows that the hypertrophy was no longer an adaptative-compensatory mechanism, but more likely a maladaptation due to chronic overload burden to the heart. The molecular mechanism of the event is because the ID consists of 3 main structures: gap junction, adherence junction, and desmosome. In cardiac hypertrophy due to pressure overload such as in hypoxia, the number of gap junctions decreases, while the number of adherens junctions increases, causing communication disturbance between the heart cells and heart stiffness.¹⁸

Lemler *et al*⁷ performed a hypobaric-hypoxia study in one- to two-day old neonate calves exposed to 430 mm Hg barometer pressure (~ 4570 meters). The calves experienced RV hypertrophy, and subsequently cardiac failure after 15 days of treatment. In the histopathology, perinuclear space was found, along with ID and horizontal line structure damage and fibrosis, while the echocardiography examination found RV dilatation. They also studied neonate calves born in the lowlands, which were then raised in a 3000-meter highland. The calves experienced RV hypertrophy and cardiac failure, causing death after 9 months of age.

Besides severe hypertrophy, structural damage, and fibrosis, histochemistry evaluation also found apoptosis of cardiomyocytes (positive TUNEL test). As known before, cardiomyocyte differentiation ends soon after birth. The growth stimulus by RAS as an effort to minimize the overload burden of the heart stimulated a compensatory hypertrophy response, not hyperplasia, and causing apoptosis in the future. Therefore every apoptosis event will reduce the number of cardiomyocytes, causing further decrease of cardiac function. One of the causes of apoptosis in the experimental animals in this study was due to oxidative stress.¹⁹

A number of studies stated that fibrosis and apoptosis is a sign of transition from adaptive hypertrophy to maladaptive hypertrophy that progress towards cardiac failure, and some studies even stated that apoptosis was found in advanced cardiac failure.²⁰ Ritter & Neyses²¹ stated that 4 characteristic histopathologic changes in cardiac failure are: cardiomyocyte hypertrophy, fibrosis, apoptosis, and cardiomyocyte slippage. Because these four alterations were also found in this study, we might be able to conclude that the rats in P6 group (21-day hypoxia)

and P7 group (28-day hypoxia) in this study, histopathologically experienced cardiac failure. As supported data, on the same experimental animal, a drastic increase in plasma BNP levels and ventricular BNP mRNA concentration were observed, and these finding has been reported in our previous study,²² while BNP has been proved as biomarkers of heart failure by FDA and ACC/AHA.²³

As a comparison, Hessel *et al*⁸ reported that hypobaric hypoxia with 30 mg/kgBW monocrotaline (MCT) injection for 4 weeks causes compensated hypertrophy, while the 80 mg/kgBW dose administration causes cardiac failure. From the two major results of this study we could conclude that there is a dose-response relationship in the pathophysiology of hypoxia-induced cardiac failure. That means, once a pathologic hypertrophy happens, or more precisely, once the pathologic hypertrophy signaling pathway is activated, sooner or later it will lead to cardiac failure. This fact also proves the progressive nature of the pathogenesis of cardiac failure.²⁴

CONCLUSION

Induction of chronic systemic hypoxia causes ventricular hypertrophy, starting with pulmonary hypertension, RV hypertrophy, ventricle remodeling and then progress to cardiac failure. This is supported mainly by histopathological features of heart failure due to pressure cardiac-overload, which shows massive cardiomyocyte hypertrophy associated by structural damage, fading of ID structure, angiogenesis, fibrosis and apoptosis. Induction of chronic hypoxia causes various blood gas parameter changes, especially decrease in PO₂, PCO₂, HCO₃⁻ and O₂ saturation, which in turn causes metabolic disturbance in form of severe metabolic acidosis with severe respiratory alkalosis compensation. Changes in hematologic parameters were found in increased Hb, Ht, and RBC count, as a compensatory mechanism to increase oxygen supply to the tissues. Extreme decrease of body weight which causes hypoxic cachexia is observed.

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