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Gestational Protein Restriction: Study of the probable effects on Cardiac Muscle Structure and Function in Adult Rats

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Running title: gestational protein restriction affects cardiac muscle
Key words: dietary protein, cardiomyocyte, ultrastructure, iNOS, connexin 43
Abstract

Intrauterine growth restriction (IUGR) has been linked to heart disease in adulthood. This study aimed to examine the effect of gestational protein restriction during fetal and early postnatal life on the cardiac muscle structure and function in adult offspring. Pregnant female rats were randomly divided into two dietary groups: normal-protein diet (NP) and low-protein diet (LP). Fifteen male offspring from each group were included in the study. Offspring body weights were recorded at birth and monthly from weaning until 24 weeks of age while systolic blood pressure was measured weekly. At the end of the experiment, hearts were weighed and processed for light and electron microscopy and immunohistochemical study. Immunohistochemical staining for localization of inducible nitric oxide synthase (iNOS) and connexin 43 proteins was performed. The gestational protein restriction induced deleterious effects on adult offspring including decreased birth weight, heart weight, and heart rate, and increased systolic blood pressure. Histologically, the number of cardiomyocytes decreased and cardiac fibrosis increased. Signs of degeneration at both structural and ultrastructural levels of cardiomyocytes were also seen. The iNOS was up regulated in LP offspring which was a promoter for apoptosis, while connexin 43 was down regulated which would affect heart conductivity and contractility. Our results demonstrate that adult offspring body weight and cardiac muscle structure and function can be programmed by maternal gestational nutrition. These adverse outcomes suggest the criticality of dietary behavior during pregnancy on long-term offspring cardiac health.
Introduction:

The importance of maternal nutrition to growth of the fetus has long been recognized with inadequate maternal nutrition and induction of intrauterine growth restriction (IUGR). IUGR is linked to potential adverse impacts on lifelong health of the offspring (Zohdi et al., 2014). Importantly, an association between IUGR and a higher risk of cardiovascular disease later in life has been detected by both epidemiological and experimental studies (Lim et al., 2006; Menendez-Castro et al., 2014).

Cardiovascular disorders in most cases undergo long subclinical phases that can last decades before the first clinical symptoms appear. It is considered in the modern world as the leading cause of mortality in developed countries. There is growing evidence that in a proportion of cases, the predisposition to cardiovascular disease lies in prenatal life (Berenson, 2002; Nichols et al., 2012).

Moreover, a clear association between low birth weight and increased cardiovascular mortality in adulthood, including increased risk of hypertension, diabetes, dyslipidemia and coagulation disorders in children and adults was demonstrated by many studies (Crispi et al., 2008; Comas et al., 2010; Demicheva and Crispi, 2014).

There are critical periods in the differentiation and maturation of the tissues and cells involved in organogenesis throughout gestation and early postnatal life. This concept was illustrated using the examples of the kidney, heart, and pancreas, since their functional units are formed prenatally in the human fetus (Gluckman and Hanson, 2004; McMillen and Robinson, 2005). However, this is not a universal finding and requires clarification. This research seeks to address whether gestational protein restriction during fetal and early postnatal life might lead to influencing growth and maturation of offspring cardiac muscle and if such changes might affect long-term offspring cardiac health. Importantly, to date, little to no data about histological affection of cardiac muscle that drives these changes is well characterized. So, this study aimed to explore the effect of reduction of maternal protein diet on the cardiac muscle structure and function in their adult offspring.
Materials and methods

Animals and diet treatment:

This study was conducted according to the Institute Review Board Instruction of Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). All experimental procedures were approved and carried out in accordance with the guidelines of the Faculty of Medicine, Zagazig University, Egypt.

Twelve-week-old female and male albino rats were obtained from animal house, Zagazig University. Virgin female rats weighing between 175 and 225 g were mated with male rats; conception was confirmed by observation of a vaginal copulation plug or the presence of sperms in the vaginal flush. Two groups of pregnant rats were housed individually and randomly divided into two dietary groups, fed either an NPD (containing 20% casein) or a LPD (containing 8.7% casein) during pregnancy and for 2 wk after birth. The diets were commercially available, semipurified diets (Table 1). Initially, the rats were familiarized to the diets for 2 weeks before mating (Lim et al., 2006).

After birth, the offspring were housed with their mothers until weaning at 28 d, at which time they were housed two to three rats per cage and kept until 24 wk of age. No significant difference in litter size was observed between the groups; average number was 10.6± 0.4 in NP group and 10.4±0.5 in LP group. Male offspring only were included in the study with a total of 30 rats; half coming from mothers with NP diet and the others of LP feeding mothers. Diet intake was monitored daily. The breeder rats were housed individually and maintained at a constant temperature of 21°C. Food and water were administered ad libitum. Birth weight from offspring were recorded then to avoid the mothers becoming stressed, the offspring were not weighed until 2 weeks of age. Then body weight was measured monthly from weaning until 24 wk of age.

Blood pressure (BP) and heart rate (HR) measurements

Systolic blood pressure (SBP) was measured weekly in conscious, prewarmed, restrained rats by tail-cuff plethysmography (Kubota et al., 2006). At least seven determinations were made in every session and the mean of the lowest three values within 5 mmHg was taken as the
systolic blood pressure level. Heart rate (HR) was calculated from the physiological tracings obtained during BP measurements (Pauline et al., 2011).

At time of sacrifice and prior to perfusion, rats were anesthetized using a ketamine (up to 80 mg/kg body weight ketamine) administered via intraperitoneal injection. Subsequently, the animals were perfusion-fixed with a mixture of both aldehydes (10% neutral buffered formalin saline and 2.5% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.2). Each animal was perfused using a perfusion pressure of 80 mmHg, which increased gradually to 130 mmHg to maintain good perfusion (Thapare et al., 1980; Nasser Hajibagheri 1999).

After the end of perfusion, the hearts were excised and trimmed of excess adipose tissue. Hearts were weighed, and processed for histological and immunohistochemical study.

**Histological analysis**

For light microscope examination, specimens were immediately fixed in 10% neutral buffered formalin saline for 24 h. They were processed to prepare 5 µm thick paraffin sections for hematoxylin and eosin (H & E) stains, Masson’s trichrome (MT) and immunohistochemical staining for detection of iNos (inducible nitric oxide synthase) and Connexin 43 markers.

Immunohistochemistry was performed on normal deparaffinized heart tissue. To expose target proteins, heat induced antigen retrieval was performed using 10mM sodium citrate (pH 6.0) buffer, microwaved for 8-15 minutes. Following antigen retrieval tissues were blocked in 3% BSA-PBS for 30 minutes at room temperature. Tissues were then probed at a dilution of 1:200 with a rabbit polyclonal antibody recognizing inducible nitric oxide synthase(iNOS) (Product #PA1-036, Thermo scientific) and (Rabbit Polyclonal Anti-Connexin 43; Abcam), diluted at 1:100 overnight at 4°C in a humidified chamber. Then the slides were washed with phosphate buffer then incubated with the secondary anti-mouse antibodies universal kits obtained from Zymed Corporation. Staining was completed by incubation with substrate chromogen DAB (3,3’Diaminobenzidine) which resulted in brown-colored precipitate at the antigen sites and Mayer’s hematoxylin was used as a counter stain. Negative control sections were prepared without using the primary antibody (Bancroft and Gamble, 2002).
Specimens for electron microscope examination were immediately fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer at pH 7.4 for 2 hours at 4°C. Post fixed in 1% osmium tetroxide in the same buffer for one hour at 4°C. The specimens were processed, stained (Glauret and Lewis 1998) and examined with JEOL-JEM 1010 electron microscope at the electron microscope unit of Faculty of Medicine, Zagazig University.

**Quantitative morphometric measures:**

Serial sections stained with H&E, MT and immunohistochemical reaction were morphometrically analyzed to evaluate cardiomyocyte numbers. Transverse sections of cardiac were scanned together with a microscale by a “Leica Quin” image analyser computer system (Leica Imaging System Ltd., Cambridge, England). The measuring frame of a standard area is equal to 7286.78 µm². Cardiomyocytes were counted at magnification X400 when they came into clear focus with a visible nucleus and no parts intersected the counting frame (Fiordaliso et al., 2004). Measurement of area percentage of blue-stained collagen fibers in MT-stained sections was done. Also, area percentage of immune reaction of iNOS and connexin 43 was measured at magnification X400 but the measuring frame was of area 118476. 6 µm². Ten various fields were chosen from each slide.

All statistical data were presented as mean ± standard deviation. The statistical analysis was carried out using SPSS statistical program version 17 and evaluated using paired sample t-test to detect the significance of difference between the scores of the different parameters in NP & LP groups. P value <0.05 was taken as significant.

**Results**

**Offspring Birth Weights**

Despite similar litter size of both NP and LP groups, the mean birth weight was significantly reduced in LP rats in comparison with NP rats. Both NP and LP offspring showed significant increases in body weight with age. Furthermore, the lower body weight of the LP group compared with NP was maintained until the end of the study (6 months) where mean ± SD of BW of NP group was 219.9 ± 2.7 g while that of LP group was 201.8 ± 4.17 g. Weight
differences between LP and NP were unlikely to be due to differences in food consumption since neither food nor water intake differed significantly between the 2 groups.

**Offspring heart weight, blood pressure, heart rate, cardiomyocyte numbers:**

Table (3) shows that mean ± SD of heart weight at the end of the study decreased significantly in rats with maternal protein restriction in LP offspring when compared with that of NP offspring (p value <0.05). Also, cardiomyocyte number/measured area decreased significantly in LP offspring when compared with NP offspring. Heart rate was significantly decreased in LP offspring when compared with that of NP offspring (p value <0.05) (table. 3).

Regarding measurement of systolic pressure, a significant increase in systolic blood pressure was detected in LP offspring when compared with NP offspring at different times of measurement until the end of study (fig. a).

**Histological results**

Light microscope examination of the cardiac muscle of male albino rats in control group (NP) revealed that it consists of long parallel cardiomyocytes with acidophilic cytoplasm and oval central nuclei (Fig. 1a). There are narrow interstitial spaces in-between these muscle fibers that contain scanty connective tissue and blood capillaries (Fig. 1a, 2a). The cardiac muscle fibers have transverse striations, branched and connected to each other by intercalated discs (Fig. 3a).

On the other hand, cardiac muscle fibers of the experimental (LP) group appeared swollen, disorganized and some of them had apoptotic nuclei. The interstitial spaces appeared to have extravasated RBCs and increased connective tissue cells (Figs. 1b and 1c). The interstitial spaces were wide and had perivascular fibrosis. Meanwhile, there was an increase in the fibrous tissue in-between the cardiomyocytes (Fig. 2 b) that was approved quantitatively in table (3) where significant increase in area% of collagen fibers in LP group when compared to NP group was observed (P<0.05). Furthermore, there were wide interstitium and extravasated red blood cells in-between cardiac muscle fibers. Also, some cardiomyocytes appeared to have striations, others appeared with loss of myofibrils alignment and lost striations. Degenerated and hypercontractile wavy myofibers were observed. Myofibers with contraction bands where sarcolemma is tightly folded were seen (Figs. 3 b, c and d).
Immunohistochemical examination of the cardiac muscle of rats in both groups revealed that the area % of immunoreaction of inducible nitric oxide synthase (iNOS) protein is more increased in cardiomyocytes of LP group than in cardiomyocytes NP group (Figs. 2c & 2d). This increase in iNOS is statistically significant as the P<0.05 (Table 3). On the other hand, the area % of immunoreaction of the connexin 43 protein is more decreased in cardiomyocytes of LP group than in cardiomyocytes NP group (Figs. 2e and 2f). This decrease in connexin43 is statistically significant as P<0.05 (Table 3).

Electron microscope examination of the cardiac muscle in both NP and LP groups revealed that the cardiomyocytes of the NP group appeared to have sarcoplasm that contains longitudinally arranged myofibrils with mitochondria arranged in rows in-between them. Each myofibril consists of successive dark (A) and light (I) bands. Each I band is bisected by dark Z-line and sarcomere is located between two successive Z lines. The A band is bisected by a pale zone (H-zone) that is bisected by a dark line (M-line) (Fig. 4a). In contrast, in LP group the ultrastructural changes of cardiomyocytes were in the form of signs of cellular degeneration. These were swelling of mitochondria (Fig. 4b), presence of concentric lamellar or myelin bodies, the myofibrils were disorganized (which explains the disappearance of striations) and sarcomeres were hypercontractile (Fig. 4c). Also, sarcomeres appeared telescoped due to hypercontractility which in turn led to undulation of the sarcolemma. The intercellular spaces appeared to have numerous extravasated RBCs while mitochondria were aggregated in-groups in-between the myofibrils (Fig. 4d).

Discussion

Both prenatal life and early postnatal life are “critical periods” that are characterized by a high degree of plasticity (Gluckman et al., 2011) and a high cell proliferation rate in the developing tissues (Godfrey and Barker, 2000; Symonds et al., 2009). So, any in utero insult leads to functional and structural changes will remain in postnatal life and may persist into adult life (Berenson, 2002; Gluckman, and Hanson, 2004; Palinski and Napoli, 2008).

In this study, we used an experimental model of maternal low-protein (LP) diet to study cardiac structure and function, wherein protein is restricted during fetal and early postnatal life by reducing the mothers’ protein diet during 1st two weeks of life. Previous studies confirmed the
appropriateness of this experimental model of maternal protein restriction (Cheema et al. 2005; Lim et al., 2006; Aroutiounova et al. 2009; Remacle et al. 2011; Tappia et al. 2013).

The offspring male albino rats of both NP and LP groups were used in this study. This choice has been supported by the work of Woods et al. (2005) who found that the severity of the hypertension in rodent models appears to depend on sex, with males having higher risk. Also, previous studies established significant changes in the hearts of female offspring exposed to maternal protein restriction (Corstius et al., 2005 and Lim et al., 2006). So, further work on male offspring was needed.

Different structural and functional changes were detected in offspring of LP diet of the current study. There was a significant decrease in body weight of LP offspring at birth and until the end of the study when compared with NP offspring. Similar findings were made by Langley-Evans (2014).

In the current study, histological changes in the myocardiocytes of LP adult offspring on both structural and ultrastructural levels have occurred. The myocardiocytes appeared swollen and disorganized. Ultrastructural changes were aggregation and swelling of mitochondria, and formation of lamellar bodies. These changes indicate that the cells are in apoptosis which is considered as one important finding of this work. Concurrently, with these structural changes in cardiac muscle of LP offspring there was a significant increase in inducible nitric oxide synthase (iNOS). These results could be explained by Pinsky et al. (1999) who indicated that exposure of purified adult rat ventricular myocytes to an nitrous oxide (NO) donor caused iNOS induction and induced the apoptosis of cardiac myocytes. Moreover, Haywood et al. (1996) revealed that elevated iNOS levels are frequently associated with myocarditis, ischemic heart disease, and valvular heart disease. Similarly, Zhang et al. (2007) confirmed that(iNOS) protein is expressed in cardiac myocytes of patients and experimental animals with congestive heart failure (CHF). Lagranha et al. (2012) recorded myocardial insult, including mitochondrial swelling and loss of cristae in LP offspring.

Mitochondrial changes and lamellar bodies’ formation that observed in myocardiocytes of LP offspring in this study could be explained by Hruban et al. (1963). They recorded that membrane phospholipids are particularly difficult to digest and accumulate because appropriate
lipases are not present. When large amounts of membrane are present, these structures become arranged in a laminated, concentric myelin figure. Further, Phaneuf and Leeuwenburgh, (2002) suggested that an increase in apoptosis may be a consequence of decreased mitochondrial membrane stability and permeability transition pore formation, which leads to cytochrome c release into the cytosol, resulting in activation of caspase-3 and -9 which are considered as the executioners of apoptosis. Several changes in mitochondrial structure and function resulting from early maternal protein restriction could persist into adulthood. Cardiac muscle cells are non-dividing. So, it is expected to maintain certain subcellular organelles for far longer periods than would be observed in regularly dividing cells and mitochondria may have a much longer half-life than their counterparts in dividing populations (Chan, 2006). Recently Nickel et al (2013) confirmed the concept that mitochondria are considered as gatekeepers of life and death. Any defects in the mitochondria of cardiomyocytes are strongly interrelated with cardiac structure and function deficits.

Ultrastructural changes associated with contractility such as contraction bands, telescoped sarcomeres and undulating sarcolemma were obvious in cardiomyocytes of LP offspring in the current work. Iruretagoyena et al. (2011) recorded decreased sarcomere length associated with less efficient contraction in IUGR fetuses that died in the perinatal period. In addition, these alterations persist in adulthood as well due to altered gene expression of the sarcomere regulatory proteins (Bijnens et al., 2012). The previously detected cardiac alterations are in line with that described in other cardiac disease models caused by hypertension or hypervolemia (Bijnens et al., 2012). As mentioned previously the iNOS is upregulated in cardiomyocytes of LP offspring, and Heger et al. (2003) stated that the experimental and clinical results led to the concept that an enhanced production of NO by iNOS is causally related to contractile dysfunction in heart failure. On the other hand, connexin 43 which is the major structural protein of ventricular gap junctions was significantly decreased in LP offspring. Down-regulation of Cx43 is a typical feature of myocardial remodeling (Dupont et al., 2001), and a significant decrease in Cx43 caused by cardiac renin angiotensin system (RAS) activation (Teo et al., 2004), which will affect the conductivity and consequently the contractility of the cardiomyocytes in LP group rats resulting in sudden arrhythmic death (Van Norstrand et al., 2012).
Histological analysis in the present study revealed a reduction of cardiomyocyte numbers in cardiac muscle of LP offspring, which explains the reduction in heart weight. Similar results were found in heart by Corstius et al. (2005) and in kidney by Awazuff and Hidaff (2015) where maternal protein restriction caused reduced nephron number. Almeida, and Mandarim-de-Lacerda, (2005) found that rats from prenatal protein–calorie restriction developed hypertension and cardiomyocytes apoptosis which might clarify the reduction of cardiomyocytes in LP offspring in this study. These findings support the concept that an adverse intrauterine environment may permanently reduce the numbers of cells/functional units in vital organs, which in turn will affect postnatal organ function (Woods et al., 2001). In support of this idea, it was found that spontaneously hypertensive rats that show spontaneous heart failure between 18 and 24 months of age are born with a lower number of ventricular cardiomyocytes than normotensive rats (van der Laarse et al., 1987; Boluyt et al., 1995).

The salient finding of this study is the significant increase of interstitial fibrosis in cardiac muscle of LP offspring. Menendez-Castro et al. (2014) found that the expression of microfibrillar matrix proteins was 3 to 5-fold increase in the myocardium of LP rats than in that of NP rats. Lim et al. (2012) and Zohdi et al. (2015) detected a similar increase in interstitial fibrosis in LPD offspring that was exacerbated with age. In support of this concept, maternal protein restriction in rats leads to fewer cardiomyocytes in the heart at birth (Corstius et al., 2005), and this is associated with an increased deposition of interstitial fibrosis within the myocardium by early adulthood (Lim et al., 2006). Accordingly, one of clinically relevant findings in this study was the decrease in heart rate of LP offspring. This could be explained by Fleg et al. (1990) and Fleg and Lakatta (2007) who found that increasing interstitial fibrosis by aging has an impact on the electrical properties of the conduction system and the occurrence of sinus bradycardia.

In comparison to the normal protein diet, our results showed that restriction of maternal protein in rats caused a rise in blood pressure by adult life. Accordingly, the present study provides empirical assistance for the hypothesis that gestational protein restriction during fetal and early postnatal life can influence the growth and maturation of cardiac muscle which in turn predisposes to cardiovascular dysfunction and thereby leads to hypertension in adult offspring of LP rats. Diez, et al. (1998) concluded that hypertension is an established risk factor for
pathologic changes in the heart, including loss of cardiomyocytes and increased fibrosis. Many studies have shown that a maternal low-protein diet during the gestational period leads to lower birth weight, hypertension, vascular dysfunction, increased angiotensin-converting enzyme activity (McMillen and Robinson, 2005; Nuyt and Alexander, 2009, Watkins et al., 2010; Tarry-Adkins and Ozanne, 2011; de Brito Alves et al. 2014). IUGR caused hypertension and hypervolemia in fetuses as a result of hemodynamic redistribution and adaptation to hypoxia and insufficient nutrition (Kiserud et al., 2006). This was affected by the duration and timing of maternal exposure to low protein diet (Nishina et al., 2003) and not corrected even after consumption of a normal-protein diet throughout the remainder of development and adulthood (Bol et al., 2010). In utero stress conditions caused cardiovascular remodeling that persist in postnatal life (Tintu et al., 2009) including dilated cardiomyopathy-like heart remodeling, and hypertension (Iruretagoyena et al., 2011).

In conclusion, exposure to low maternal dietary protein during gestation and early postnatal life induces upregulation of iNOS, cellular apoptosis, a decrease in cardiomyocyte numbers, an increase in interstitial fibrosis, downregulation of Cx 43, and ultrastructural changes in the structure of cardiac muscle that are potential primary precursors of hypertension. These results point to the possible importance of proper dietary protein supply during gestation and lactation.
References:

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suppresses the newborn renin-angiotensin system and programs adult hypertension in
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Table 1: Composition of semipurified diets fed to dams during pregnancy and for two weeks after delivery

<table>
<thead>
<tr>
<th>Diet composition (% by weight)</th>
<th>LPD</th>
<th>NPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (acid)</td>
<td>8.7</td>
<td>20</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Starch (total)</td>
<td>64.41</td>
<td>53.11</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.14</td>
<td>0.14</td>
</tr>
<tr>
<td>Minerals (AIN_93_G)</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Vitamins(AIN_93_G)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Choline chloride 50% wt/wt</td>
<td>0.25</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Table (2): Offspring birth weight of both NP and LP groups at different times during the experiment. Data are represented as mean ± SD.

<table>
<thead>
<tr>
<th></th>
<th>NP offspring Mean ± SD(n=15 male)</th>
<th>LP offspring Mean ± SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>At birth</td>
<td>6.7±0.10</td>
<td>4.6±0.8</td>
<td>&lt; 0.05*</td>
</tr>
<tr>
<td>At weaning</td>
<td>50.02±0.99</td>
<td>41.5±1.53</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>At 8 weeks</td>
<td>177.8±2.97</td>
<td>170.2±1.9</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>At 24 weeks</td>
<td>219.9±2.7</td>
<td>201.8±4.17</td>
<td>&lt;0.05*</td>
</tr>
</tbody>
</table>

* Significant difference between groups (p<0.05)
Table (3): showing heart weight (HW), heart weight to body weight ratio (HW/BW), blood pressure and histomorphometric results of studied groups at time of sacrifice. Data are represented as mean ± SD.

<table>
<thead>
<tr>
<th></th>
<th>NP offspring (n=15 male)</th>
<th>LP offspring (n=15 male)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HW (g/100g BW)</td>
<td>0.310±0.040</td>
<td>0.258±0.001</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HW/BW (×10⁻³)</td>
<td>1.36±0.14</td>
<td>1.25±0.017</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td>123±3.9</td>
<td>164±5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Heart rate (beat/minute)</td>
<td>199±2</td>
<td>165±10</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Cardiomyocytes number</td>
<td>25.9±1.9×10⁸</td>
<td>20.6±1.5×10⁸</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Area% of collagen fibers</td>
<td>2.82±0.04</td>
<td>7.15±0.09</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Area % of iNOS immunoreaction</td>
<td>1.02±0.05</td>
<td>6.59±0.89</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Area % of Connexin 43 immunoreaction</td>
<td>3.68±0.12</td>
<td>2.31±0.18</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
Figure legends:

**Figure (a):** Systolic blood pressure measurement in LPD (■) and NP (▲) offspring from the time of birth until 24 weeks of age. Data are represented as mean ± SD. *significant from NP group (p<0.05)

**Figure (1):** H&E stained sections. Fig. 1a: A photomicrograph of the cardiac muscle of male albino rats in control NP group showing long parallel cardiomyocytes with acidophilic cytoplasm, and oval central nuclei (arrowheads). Narrow interstitial spaces with few connective tissue cells (arrows) and blood capillaries (C) are seen X 400.

Fig. 1b & 1c: Photomicrographs of the cardiac muscle of male albino rats in LP group showing swollen and disorganized cardiomyocytes (arrowheads), and some of them have small and dark nuclei (wavy arrows). The interstitial spaces appeared to be wide and have extravasated RBCs (Er) and connective tissue cells (arrows) X 400.

**Figure (2):** (2a, 2b): Photomicrographs of MT stained cardiac muscle of control NP group (2a) showing a small area of blue stained interstitial collagen fibers (arrows) and LP group (2b) showing blue stained interstitial collagen fibers (arrows) with apparent increase in 2b more than in 2a.
Fig. (2c, 2d): photomicrographs of iNOS immunohistochemical reaction in the cardiac muscle of control NP group (2c) and LP group (2d) showing brown stained cytoplasmic reaction (arrows) in cardiomyocytes with apparent increase in fig. (2d).

Fig. (2e, 2f): photomicrographs of connexin 43 immunohistochemical reaction in the cardiac muscle of male albino rats in control NP group (2e) and LP group (2d) showing brown stained cytoplasmic reaction (arrows) in cardiomyocytes with apparent decrease in fig. 2f. (X 400)

**Figure (3):** toluidine blue semi thin sections cardiac muscle of the studied groups. Fig. (3a): photomicrograph of control NP group showing the blood capillaries (C) arranged in close proximity to cardiac muscle fibers and transverse striations (arrowheads) in cardiomyocytes are also seen. Theses cardiomyocytes branch and interconnect with each other at intercalated discs (arrows). Figs. (3b, c, d): photomicrographs of LP group showing in (3b): wide interstitium (asterisk) and congestion by extravasated red blood cells (circle). Fig. (3c) showing: degenerated myofibers (D), misalignment and loss of striated appearance (arrowheads). Fig. (3d) showing wavy myofibers (W) with contraction bands, and tightly folded sarcolemma (wavy arrows) (X 1000).

**Figure (4):** An electron micrograph of the cardiac muscle fiber of the studied groups. Fig. (4a): control NP group showing sarcoplasm of cardiac muscle fibers packed with longitudinally arranged myofibrils (f) interrupted by rows of mitochondria (m). The myofibrils show multiple sarcomeres (S) that extend between successive Z lines. These sarcomeres have alternating A-I bands and are intersected by pale H zones which are also intersected by dark M lines. In-between myofibrils, mitochondria (m) appeared well preserved with organized cristae and intact membranes ;(EM, X 4000) (scale bar= 2µm). Figs. (4b, c, d): cardiac muscle fiber of LP group showing in 4b: cardiac myofibrils with abundant mitochondria (m) in affected areas and mitochondrial swelling (sm). Fig. (4c) showing: concentric lamellar body (arrows), disrupted organization of myofibrils (Circle), and hypercontractile sarcomeres (S). Fig. (4d) showing contraction bands with hypercontractile sarcomeres (S). Note the undulating sarcolemma (arrows), aggregation of mitochondria (m) and extravasation of red blood cells (RBC) in the interstitium ;(4 a, b, d: EM X 4000, scale bar= 2µm) ;(4c: EM X 8000, scale bar= 1µm).