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Mouse models for the study of postnatal cardiac hypertrophy

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ABSTRACT

The main objective of this study was to create a postnatal model for cardiac hypertrophy (CH), in order to explain the mechanisms that are present in childhood cardiac hypertrophy. Five days after implantation, intraperitoneal (IP) isoproterenol (ISO) was injected for 7 days to pregnant female mice. The fetuses were obtained at 15, 17 and 19 dpc from both groups, also newborns (NB), neonates (7–15 days) and young adults (6 weeks of age). Histopathological exams were done on the hearts. Immunohistochemistry and western blot demonstrated GATA4 and PCNA protein expression, qPCR real time the mRNA of adrenergic receptors (α-AR and β-AR), alpha and beta myosins (α-MHC, β-MHC) and GATA4. After the administration of ISO, there was no change in the number of offsprings. We observed significant structural changes in the size of the offspring hearts. Morphometric analysis revealed an increase in the size of the left ventricular wall and interventricular septum (IVS). Histopathological analysis demonstrated loss of cellular compaction and presence of left ventricular small fibrous foci after birth. Adrenergic receptors might be responsible for changing a physiological into a pathological hypertrophy. However GATA4 seemed to be the determining factor in the pathology. A new animal model was established for the study of pathologic CH in early postnatal stages.

1. Introduction

Pathologic cardiac hypertrophy (CH) is a process usually studied in adults, where the differentiated post mitotic myocardium is capable of responding to different adverse stimuli, genetic as well as environmental, increasing its size. Since this process can develop cardiac insufficiency and culminate in sudden death [1], it's diagnosis is considered a marker of poor prognosis. However this disease is not exclusive of adulthood. Approximately 95% of cases of CH are detected between the ages of 15 and 25 years [2]. It is estimated that the frequency of CH in the fetal stage is around 7% and probably higher. About 11% of the fetuses die in uterus due to this disease [3]. Physiologic CH is a key process for heart development during the fetal period, it increases while proliferation decreases and then, it decreases after birth as maturity is achieved [4]. However, what mechanisms turn a physiological into a pathological cardiac hypertrophy during fetal or postnatal life still remains a question, probably due to the lack of experimental in vivo models.

Among the most utilized adult in vivo experimental models to induce hypertrophy is the use of ISO. It is a simple, easy to administer and reproducible method with low mortality [5–7] compared with other techniques of short duration, with high stress or those that require surgical procedures such as coronary artery ligature or transverse aortic constriction that imply a high risk of morbidity and mortality [6,8].

ISO is a synthetic catecholamine with the addition of two methyl groups. It is structurally similar to adrenaline and is joined to β-adrenergic receptors (β-AR), producing a superior effect of up to ten times that of adrenaline itself [9,10]. Different studies in adult murine models have demonstrated the flexibility of ISO. A single subcutaneous (SC) dose of ISO between 10 and 85 mg/kg in adult rats causes myocardial necrosis and fibrosis [11–15]. Low doses of ISO (0.3 to 6 mg/kg) during the 1st, 2nd and 3rd weeks induce necrosis in areas of the myocardium [16–18]. In turn, moderate doses (35 mg/kg) administered SC for 3 days induced dilated cardiomyopathy. Chronic treatment (100 mg/kg) >2 weeks in adult rats produces diastolic dysfunction and causes decrease of fatty acids and glucose in the myocardium. Changes similar to those were observed in rat hearts after myocardial

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infarction of moderate severity [19,20]. Cardiotoxicity after ISO administration with osmotic mini-pumps is able to cause CH after 7 days; however, 14 days after treatment and once the mini-pumps have been removed, the pathology reverses itself [21]. On the other hand, concentrations >5 mg and up to 60 mg/kg/day for 7 days after IP administration, favors a pathological hypertrophic response, which affects the left ventricle (LV) in the adult rat [22,23]. Hypertrophy produced by ISO is very similar to CH in humans, determined by microarray analysis of mRNA expression. Genes found in the ISO model have a higher correlation than CH generated by active exercise in 8-week-old adult mice [23]. Finally, there is only one study that evidences the use of ISO during pregnancy. However, results are contradictory as the authors observed that CH reverses itself a few weeks after birth [24]. These studies all demonstrate that the mechanisms and time of ISO administration are important to obtain the degree of myocardial damage desired.

ISO is a β-adrenergic stimulant and is able to cross the placental barrier like epinephrine [25,26]. We hypothesized that prolonged IP administration of ISO in pregnant female mice is a stimulus capable of altering the myocyte physiology during fetal and neonatal stages, generating a pathologic CH with LV involvement, similar to experimental pathological models of hypertrophy generated in adulthood. For this reason our goal was to analyze the effect of IP administration of ISO in pregnant female mice for 7 days after the 5th day of implantation. We believe that the model proposed in this study not only raises new questions about the mechanisms of the evolution of this pathology, but can also be used to generate markers for early detection, as well as for evaluating the use of cell and/or regenerative therapy as treatment.

2. Methods

2.1. Mouse model for postnatal pathologic cardiac hypertrophy

To produce postnatal cardiac hypertrophy (p-CH), female Mus musculus Balb/C strain mice, previously paired with males of the same strain, were utilized. Observation of the vaginal plug was regarded as day 0 of gestation. From the fifth day postcoitus (dpc) shortly after the implantation of the embryo, pregnant female mice were injected IP with ISO 50 μg/kg/day (Sigma Aldrich, St. Louis, MO) in phosphate-buffered saline solution (PBS) for 7 days. The fetuses were obtained at (age groups) 15, 17 and 19 dpc from both groups, also newborns (NB), neonates (7–15 days) and young adults 6 weeks of age.

Another group was formed with pathologic CH mice induced in adult stage (CH-ad). The hypertrophy was induced in 6 week old mice injected IP with 50 mg/kg/day of ISO for 7 days. They were sacrificed 8 days after the end of ISO administration [27,28].

A control group (CTR) for each group, was injected with PBS during the same time period as the experimental groups. All the animals were bred in the laboratory animal sources of the Hospital Infantil de México Federico Gómez, under the Mexican Official Norm NOM-062-ZOO-1999. They were kept in of 12 hour light/dark cycles.

2.2. Evaluation of the effects of ISO in pregnant female mice

To confirm the effects of ISO in the offspring of treated pregnant female mice, the maternal weight was recorded from the first day of gestation until the time of sacrifice. The number of reabsorptions by litter and the number of offsprings were recorded. After labor or cesarean delivery, maternal hearts were washed by perfusion with PBS and fixed in 3.5% paraformaldehyde. In both groups ISO and PBS, dissections of the heart chambers and transverse dissections were performed. Images were analyzed with a stereoscopic microscope (Olympus, Tokyo, Japan). Micrographs and measurements of the dissected hearts along the free walls of both ventricles were done using ImageJ 1.46 software (National Institutes of Health, Bethesda, MD).

2.3. Cardiac hemodynamics

Echocardiography was carried out at 6 weeks to supervise the cardiac hemodynamics with a VisualSonics (Toronto, Canada) Vevo 770 echocardiograph, with a linear transducer (30″) in B and M modes. M mode was used to measure the LV ejection fraction (LVEF) in five 6 week mice for of each group. Each mouse was anesthetized with inhaled isoflurane (2% for induction and 1.5% for maintenance) [29].

2.4. Morphometric analysis

After mice were sacrificed, five hearts were taken from each age group, from both study groups and washed by perfusion with PBS, stopped in diastole with KCl, and fixed in paraformaldehyde. The ratio of the heart weight with the body weight (HW/BW) was determined using the body weight in g and the heart weight in mg [28].

Morphometric analysis of the heart was done with micrographs taken on a clear field with a stereoscopic microscope using ImageJ 1.46 software. The following were determined: a) thickness of each ventricular wall and IVS; b) ventricular lumen; c) total surface of the heart, and d) left and right ventricular cavities.

2.5. Histological analysis

Evaluation of myocardial fibrosis and interstitial space index was done. Dissected hearts were dehydrated with alcohol (30–100%), made transparent with cedar oil and embedded in paraffin. Transverse dissections were done to obtain 5-μ-thick sections. Histological cuts were deparaffinized and rehydrated with alcohol until water. Nuclei were stained with hematoxylin and eosin. Three hearts from each age group were analyzed and micrographs were taken at 40× using an optical microscope. Three sections of each heart were evaluated in the LV and IVS. At each level, three fields were selected. The analysis was carried out using UTHSCSA Image Tool v.3.0. The area of muscle tissue was determined and the remainder was considered to be interstitial space [7,30].

2.6. Indirect immunofluorescence

To establish the expression levels of the GATA4 transcription factor and cell proliferation, immunofluorescence studies were done using an anti-GATA4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and proliferating cell nuclear antigen (PCNA, Dako, U.S.A.). Three hearts were used from each age group. Each trial was carried out in triplicate. Tissue slides were treated with citrate solution at a 15 lb/in^2 pressure for antigen release. Nonspecific activity was treated with protein blocking solution (Biogenex, Fremont, CA). The primary antibody was visualized with anti-mouse Zenon Alexa Fluor 488 (Life Technologies, Grand Island, NY). After washing, nuclei were contrasted with Draq7 (Biostatus, Leicestershire, UK). Finally, Vectashield was used as mounting media (Vector Laboratories, Burlingame, CA). Histopathological cuts were observed in a confocal microscope (Zen 2009, Carl Zeiss, Dublin, CA). Micrographs were captured at 40× and 15 fields were evaluated for each region (LV and IVS). ImageJ 1.46 program was used for quantification. Percentage of expression was calculated.

2.7. Western blot

Hearts were treated with lysis solution T-PER (Pierce, Rockford, IL). For total protein extraction, protein quantification was done according to the Bradford method (Bio-Rad, Hercules, CA). Each sample was diluted in protein loading buffer (30 μg) and polyacrylamide gel electrophoresis (10%) was carried out. Proteins were transferred to a PVDF membrane, previously treated with blocking solution (5% nonfat dry milk powder in TBS) for 1 h to prevent nonspecificity. Anti-GATA4 primary antibody was incubated in blocking buffer for 12 h at 4 °C.