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Gradual decrease in spermatogenesis caused by chronic stress

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ABSTRACT

Chronic stress induces decreased sperm motility, viability and concentration in stressed males. Also, stress modifies oxidative status and causes apoptosis in testes, as well as a decrease in the epithelial area of seminiferous tubules. However, there are no studies that analyze the alterations caused by stress in testicular cells. Thus, in this study, alterations in the morphology of testicular germ cells caused by different days of chronic stress were assessed. Adult male rats were exposed to stress by immersion in cold water (ICW) daily for 3, 8, 20 or 50 consecutive days. Plasma testosterone and corticosterone were also assessed. Results showed that chronic stress causes loss of germ cells, and alteration of spermatogenesis. Seminiferous tubules from stressed males showed several degenerative signs, such as vacuoles in the basal epithelium, with picnotic indicia; moderate to severe exfoliation of degenerative germinal cells in the tubule lumen was also observed. These alterations were observed in all days of stress in a gradual way, from day 3–50. Testosterone levels were decreased at all those times, and corticosterone concentrations were increased on the same days. These results show that chronic stress causes severe damage to germ cells, which can account for infertility problems in males. These alterations are related to a decrease in testosterone as well as an increase in corticosterone caused by stress.

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1. Introduction

Male fertility depends on the production of a large number of spermatozoa by spermatogenesis (de Kretser et al., 1998). This process occurs in testicular seminiferous epithelium, where germ cells are produced in a clonal way by mitotic and meiotic divisions followed by their differentiation to structural and functional spermatozoid. Spermatogenesis occurs continuously, closely associated to Sertoli cells (Mesquita-Horn and Fritsch, 2012) and this process depends on pituitary gonadotropin, follicle stimulant hormone (FSH) and luteinizing hormone (LH) (Hess and de Franca, 2008), as well as adequate testosterone concentrations (O'Donnell et al., 2006).

Both acute (Fukuda et al., 1996) and chronic stress (Sheiner et al., 2003; Wingfield and Sapolsky, 2003) are capable to cause disruption of male reproduction. Aside to the increase in adrenal corticosteroids (Nicolaidis et al., 2015), chronic stress induces suppression of testosterone secretion, libido and spermatogenesis in males (Johnson et al., 1992; Retana-Márquez et al., 2014). Although

stress is not the only factor in the etiology of infertility, there is growing evidence that chronic stress is an additional risk factor for infertility (Sheiner et al., 2003; Collodel et al., 2008). In men, stress causes sperm morphological defects (Auger et al., 2001), as well as a decrease in sperm motility (Fukuda et al., 1996) and count (Lenzi et al., 2003; Collodel et al., 2008), thus lowering fecundity in men (Hjollund et al., 2004).

Animal models have been used to evaluate the effects of chronic stress on male fertility, with special emphasis in the effects on testes and sperm quality. It has been reported that the number of mature spermatids and Sertoli cells decrease in the seminiferous epithelium in rats exposed chronically to immobilization stress (6 h for 60 days) (Almeida et al., 1998). Mature spermatids decrease in male rats exposed to forced swimming stress (3 min for 15 days); however, fertility was not disrupted (Mingoti et al., 2003), probably because the duration of the stress was shorter than the spermatogenic cycle (48–53 days in the rat; Clermont et al., 1959). Chronic forced swimming (15 min) after immobilization (1 h) stress for 60 days in male rats causes loss of germinal cells, mainly type A spermatogonia, paquitene primary spermatocytes, round and elongated spermatids in stage VII of the seminiferous epithelium cycle (Nirupama et al., 2012). All these effects are associated with a decrease in plasma testosterone (Sapolsky, 1985).

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Also, unpredictable chronic stress for 21 or 35 consecutive days causes a decrease in the diameter of seminiferous tubules, germ cell numbers (Hou et al., 2014) and Leydig cell loss by apoptosis (Chen et al., 2012), thus decreasing testosterone. Moreover, seminiferous epithelial area decreases in testes of male rats exposed to immersion in cold water (15 min for 20 or 50 days) (Retana-Márquez et al., 2014). Furthermore, acute and chronic stress can induce apoptosis in testicular germ and Leydig cells by activating both intrinsic and extrinsic apoptotic pathways (Juárez-Rojas et al., 2015). It has also been reported that chronic stress alters oxidative status or induces oxidative stress by increasing reactive oxygen species (ROS) production and lipid peroxidation (LPO), as well as decreasing the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx-4) in testes (Nirupama et al., 2012; Al-Damegh, 2014; García-Díaz et al., 2015). Both, oxidative stress and apoptosis, are associated with high plasma levels of corticosterone and low levels of testosterone (Al-Damegh, 2014; García-Díaz et al., 2015; Juárez-Rojas et al., 2015), reducing sperm viability, motility and concentration in epididymal sperm (Juárez-Rojas et al., 2015; García-Díaz et al., 2015). Additionally, it has been demonstrated that corticosterone is capable of directly inducing apoptosis in testes during stress (Chen et al., 2012). Chronic stress causes testicular alterations such as decreased numbers and diameter of seminiferous tubules, as well as a decreased number and size of Leydig and spermatogenic cells (Hou et al., 2014), and a decreased seminiferous epithelial area at stages I–VIII of the spermatogenic cycle (Retana-Márquez et al., 2014). Moreover, the number of offspring from females copulating with males stressed for 50 days was significantly lower than control (Retana-Márquez et al., 2014).

Although several studies exist regarding the effects of chronic stress on the testes, as far as we know, there are no studies in which testicular histopathologic analysis of seminiferous epithelial germ cells has been made. Therefore, the aim of this study was to quantify the damage in seminiferous germ cells caused by chronic stress exposure. Body weight, gonadosomatic index (FSI), adrenal somatic index (ASI), as well as testosterone and corticosterone plasma levels were also assessed.

2. Materials and methods

2.1. Animals

Three month old adult male Wistar rats, 300–350 g body weight were housed in cages (50 × 30 × 20 cm) each with five animals, under standard vivarium conditions. A 12:12 reversed light cycle (lights off: 0900 h) and controlled temperature (24 ± 1 °C) were maintained in the colony room. Food and water were available *ad libitum* throughout the experiments. The rodent diet used was “2018 Teklad global” from Harlan Laboratories. The composition was: Crude Protein (18.6%), Fat (6.2%), Carbohydrate (44.2%), Crude Fiber (3.5%), Neutral Detergent Fiber (14.7%), Ash (5.3%), Vitamins A, D, E, K, B, minerals (Ca, P, Na, K, Cl, Zn, Mg, Mn, Cu, I, Se), Aminoacids and fatty acids (www.harlan.com).

2.2. Ethical declaration

Pain or discomfort to animals was minimized by suitable measures. Handling of the rats throughout the experiments, as well as the method of euthanasia was in accordance to Mexican Official Rules (NOM-062-ZOO-1999), and Domestic and Laboratory Animal Regulation Guidelines for Ethical Conduct in Research, Teaching and Diffusion, Division of Biological and Health Sciences, 2010. The approval for the use of animals in the experiments presented in this study was obtained from the Ethics Committee of the Divi-

sion of Biological and Health Sciences, Autonomous Metropolitan University.

Intact male rats (n = 40) were randomly assigned to one of the following groups (n = 8, each): (1) control; (2) stress for 3 days; (3) stress for 8 days; (4) stress for 20 days; (5) stress for 50 days.

2.3. Stress method

The cages of stressed rats were transferred to another room for stress. The order of the cages being taken to the stressor area was random and balanced from day to day. The stressors were applied at the onset of the dark phase of the cycle to avoid circadian variations of corticosterone.

Stress by immersion in cold water (ICW): Rats were placed individually in a covered tank of cold water (depth = 15.5 cm; temperature = 15 °C), where they either swam or remained in an upright position, standing on their hind limbs, and keeping their head above water level. This situation lasted for 15 min. After this time, rats were picked up from water and towel dried. This procedure involves the combination of two well-known physical stressors: cold (15 °C) and immersion in water. Thus, the additive components of the two stressful situations, and the duration of the exposure to this stressor are enough to cause an intense response of adrenal axis (Retana-Márquez et al., 1996).

Male rats from control groups remained undisturbed in their home cages throughout the experiment and were maintained away from stressed rats.

At the end of each experimental time, rats were anesthetized by an intraperitoneal dose of sodium pentobarbital (25 mg/kg body weight, Sedalphorte Reg SAGARPA Q-7503-003) at the end of the last exposure to the stressor, according to the group. This was 30–60 min after the onset of the dark phase.

2.4. Processing of biological material

Blood was collected by cardiac puncture in heparinized tubes and plasma was separated by centrifugation. Afterward, corticosterone and testosterone were evaluated by HPLC.

2.5. Testes

Testes of all males were dissected and weighed washed with 0.9% saline solution. Gonadosomatic index (GSI) was calculated with the following formula:

$$GSI = [\text{Gonadal Weight} / \text{Total Body Weight}] \times 100 \quad (\text{Zeyl et al., 2014}).$$

Afterward, testes were fixed, processed and embedded in EPON for histological analysis. The rutin of tissue specimens processing has been reported earlier (Vigueras-Villaseñor et al., 2009). Testes were fixed with Karnovsky's solution without Ca²⁺, pH 7.4 (Karnovsky, 1965). Subsequently, they were washed 3 times with sodium cacodylate 0.1 M, dehydrated in a graded series of ethanol, postfixed with 1% OsO₄ (Merck KgaA, Darmstadt, Germany) in Zetterqvist's buffer (Brandes et al., 1956) for one hour and finally embedded in EPON 812 (Ted Pella, Inc., CA, USA). Semithin 1-mm-thick sections were cut with an Ultracut UCT microtome (Leica, Vienna, Austria) and then stained with 0.5% toluidine blue.

2.6. Histopathological analysis

Fifteen transverse sections of seminiferous tubules were randomly chosen from each testis. A total of 240 seminiferous tubules were analyzed per group. Each section was analyzed in order to identify specific alterations according to the method reported by Vigueras-Villaseñor et al. (2009). The histological analysis was performed using an Axiostar light microscope (Zeiss).

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