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TOTAL TESTOSTERONE/PROGESTERONE RATE AND APPEARANCE OF SPONTANEOUS AGGRESSIVE BEHAVIOR IN MALE SWISS WEBSTER MICE

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RESUMO

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Violence is a serious public health problem and has a high impact on the quality of life of individuals across societies. Evolutionary, genetic, environmental, and cultural factors are closely interconnected as morbid causes. Animal models can be an important tool in the study of aggression. Regrouped Swiss Webster mice were selected based on mobility profile (Hypo, Normal; Hyper), which was defined by the tail suspension test. Our results demonstrate that Hyper mice (anxiety-like behavior) were more aggressive and violent than the other two categories, indicated by a pattern of aggressive behavior with a score 4+ in relation to score 1+ of Normal category. Hyper group, showed a decrease in total testosterone levels (-32.5% in relation to Norm group) and an increase in progesterone levels (+57.6% compared with the Norm group). We also observed a decrease in the weight of the testicles, the seminiferous tubules, Leydig and diploid spermatogenic cells number in hyper aggressive animals. Together, the results indicate an association between testosterone/progesterone and the appearance of highly aggressive behavior in male Swiss Webster mice. CEUA/FIOCRUZ, #LW-5/12.

Keywords: Mice. Aggressive Behavior. Sexual Hormones. Testosterone. Progesterone. Violence. Males.

1 INTRODUÇÃO

Aggressive behavior is defined by the World Health Organization (WHO) as the use of force (or power), threatened or actual, against oneself, another person, or a group. It may result in injury, death, psychological harm and developmental disability (physical or emotional)[1]. In recent years, violence has been linked to the death of approximately 1.6 million people worldwide[1]. Furthermore, violence accounted for 14% of all deaths in men and 7% in women (aged 15 to 44 years)[2]. Overal, total mortality due to violent causes is due to the following causes: 43.3% related to homicides, 27.2% road accidents, and 19.7% to various causes related to violent episodes[3].

Ethologically, various studies have related aggression to ontogenetic and phylogenetic adaptability of a species[4,5]. Despite systematic studies of animal behavior since the 1960's[6], there is still no consensus regarding the causes of aggression in laboratory animals. A complex network of factors related to genetics, biochemistry, physiology, and neuroanatomy appear to determine the presence of aggressive episodes among individuals of a particular group[7,8,9].

Brodkin et al (2002) suggested two possible chromosomal specific regions related to aggressive behavior (*Aggr1* and *Aggr2*)[10]. Other inter-

connected pathways have also been implicated in aggressive behavior, such as those related to androgen receptors, the serotonin system (5-HT)[11,12], increased expression of neurotransmitters and proteins such as Nerve Growth Factor (NGF)[13,14], vasopressin and nitric oxide (NO)[15], finally hypoglycemia[16]. With respect to biochemical systems, the vomeronasal organ, olfactory bulb, and hypothalamus appear to be major influences on the secretion and perception of pheromones and the excretion of gonadal hormones[17,18].

The steroids cortisol and testosterone have become well-established targets in the search of hormonal modulators of social aggression[19]. Moreover, it is hypothesized that the interaction between low serotonin and high testosterone levels in the central nervous system has a significant effect on the neural mechanisms involved in the expression of aggressive behavior. It seems that testosterone modulates serotonergic receptor activity in a way that directly affects aggression, fear and anxiety[11]. The functions of rapid increases in testosterone seem paradoxical because they can occur in response to different social contexts, such as male-male aggressive encounters and male-female sexual encounters. This suggests that context may impact the functional consequences of changes in testosterone, whether transient or long term[20].

Although testosterone is known to enhance aggression in various social contexts, Schneider et al (2003) have found that male progesterone receptor knockout (PRKO) mice exhibit no infanticidal behavior and little aggression toward young. Male PRKO mice also display significantly enhanced parental behaviors. In wild-type mice, blockade of PR induces a behavioral phenotype similar to that of the PRKO males, whereas progesterone exacerbates aggressive tendencies toward infants[21]. Aggressive behaviors directed toward adult males, by contrast, are unaffected by progesterone, PR antagonism, or PR gene deletion. Previously thought to be of diminished importance in male animals, PRs play a critical and specific role in modulating infant-directed behaviors in male mice[21,22].

Thus, the main objective of our study was to evaluate the expression of the sex steroid hormones, testosterone and progesterone, in highly aggressive mice in the laboratory environment. We believe it is essential to monitor the behavioral characteristics of each individual since weaning. In this context, we assessed the anxiety-like profile of juvenile mice by the tail suspension test (TST) and then grouped the animals by their performance and performed tests of aggression in adulthood. Thus, we have developed and applied the model of spontaneous aggressiveness (MSA). Finally, we also determined the serum levels of total testosterone and progesterone and correlated them with testicular histological evaluations and the aggressive behavior profile.

2 METHODS

Animals

One hundred (n=100 per assay) male albino Swiss mice (3 weeks) were maintained in our animal facilities at the Division of Animal Experimentation of the Cell Biology Laboratory, Instituto Oswaldo Cruz (SEA/LBC - IOC). They were adapted to the environment for 1 week in ventilated racks maintained under stable conditions of temperature, humidity and light/dark cycle (12:12; lights on at 6 am). Both food and water were available *ad libitum*, and routine cleaning was performed twice a week. The procedures performed during this project were approved by the Ethical Committee for the Use of Animals (CEUA/FIOCRUZ, #LW-5/12).

Model of spontaneous aggressiveness (MSA): The mice were separated in 10 groups (A1 to A10) (see Figure 1). Each group had 10 mice (identified as c1 to c10), which were housed together. Three behavioral assessments were then conducted (described below). The animals underwent each of these assessments once a week between the 4th and 8th weeks of life. At the 10th week, the animals were reassigned to new housing groups, based on the mobility profile defined by TST (low, medium

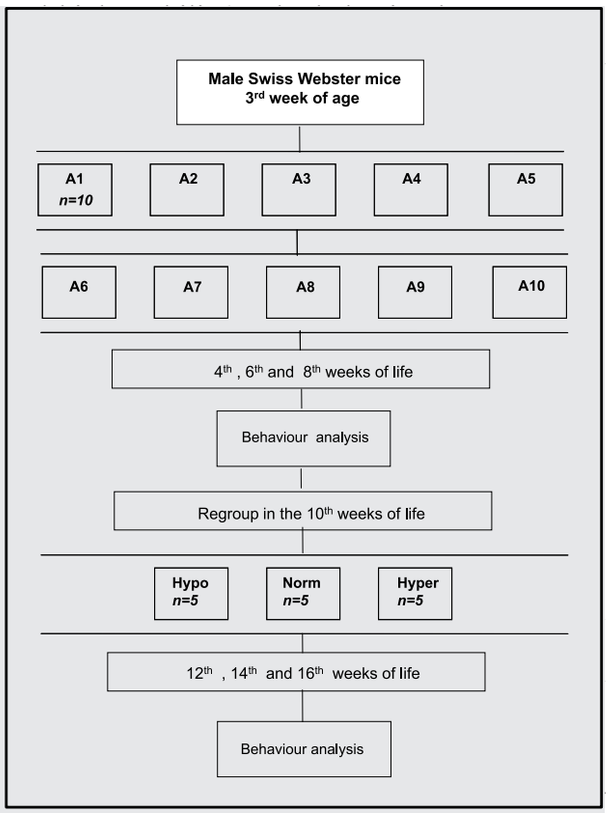

Figure 1: *Structuring and development of MSA:* Male Swiss Webster mice entered the study at 3 weeks of life. We identified and composed groups of 10 animals in order from A1 to A10. On the 4th, 6th and 8th week of life, we performed the ethogram and tail suspension test (TST) in all animals. The animals were then regrouped by TST mobility profile: Hypo (low), Norm (medium) and Hyper (high mobility) categories. In adulthood (10th week of life) we redistributed the mice into groups of only 5 mice per cage, for low population density. We then conducted behavioral tests (ethogram and TST) during the 12th, 14th and 16th week of life. During this same period, we evaluated the animals' metabolism through the use of metabolic cages. In the 16th week of life the animals were euthanized and blood (serum - steroids sexual hormone dosage) and the right and left testicles were collected.

test in each of our groups at the 4th, 6th and 8th week of life, and again after regrouping at 12th, 14th and 16th week of life. Each test lasted 5 minutes, and the time spent immobile was estimated. This immobility was characterized by the absence of torsion movements, rotation, or attempts to lift the body. We defined 3 categories related to the animal's physical reaction to the stress challenge based on immobility time: a) Hypoactivity (Hypo) as low mobility or depression-like behavior, with 104 to 150 seconds, b) Normal (Normal) as medium mobility, between 51 and 103 seconds, and c) Hyperactivity (Hyper) as high mobility or anxiety-like behavior, with between 0 and 50 seconds of immobility[9].

Metabolic cage: Individual mice (per category) were placed in metabolic cages (Tecniplast, Milan, Italy) from 8:00 to 16:00 h, once a week during the 12th, 14th and 16th weeks. Food and water intake as well as feces and urinary excretion were determined in all categories. Body weight was monitored and estimated gain (or loss) of weight (grams) calculated.

Hormones: On the 16th week of life, blood was collected in glass tubes after euthanasia in a CO_2 saturated environment and stored by mobility category (pool). Afterwards the samples were centrifuged at 3018.4 *g* for 15 minutes at room temperature and the serum pipetted into a separate tube, which was then stored at -20ºC until hormonal determination. Levels of the two sex steroid hormones, testosterone (ng/dl) and progesterone (ng/ml), were measured through the chemiluminescent enzyme immmunoassay (Advia Centaur, Bayer Corporation, New York, USA). The ratio (index RTP) between total testosterone and progesterone was calculated by the formula: testosterone/(progesteroneX100). Duplicate serum aliquots were used.

Histopathological analysis

Qualitative evaluation: The right and left testicles were collected from each subject, weighed

(ratio: right/left testis divided by body weight – weight index: WT) and cleaved in longitudinal direction for observation of the parenchyma. Tissue fragments were fixed in Millonig-Rosman solution (10% formaldehyde in phosphate-buffered saline), and embedded in paraffin. Paraffin-embedded samples were further processed and stained with hematoxylin and eosin (HE) in 4-µm-thick slices. Slices were examined by light microscopy for possible alterations in the structural integrity of seminiferous tubules (ST) and Leydig cells (400x), sertoli and diploid spermatogenic (DS - specifically spermatogonia and primarily spermatocytes) cells (1000x). Edema, congestion, hemorrhagy, inflammatory infiltration, interstitial space integrity, and fibrosis in testicular tissue were also analyzed. Tissue representative changes were registered by photography using a computer-assisted plotting program (Neurolucida, version 10; MBF Bioscience, Colcehster, VT, USA) linked to a Zeiss Axioplan microscope using a 100x objective lens.

Quantitative evaluation: Cell quantification was performed by scanning the whole-tissue slice in a total of about 300 individual microscopic fields for each cell type (one field: 0.196 mm^2). ST and leydig cells were calculated by counting the total number of tubules/cells divided by the number total of fields. In a similar way, sertoli and DS cells was estimated by counting the number of cells per tubule and divided by the total number of fields, approximately.

Statistical analysis: The Mann-Whitney non-parametric test was used to compare the 2 groups (SPSS software, version 8.0) and p values are indicated in the Figure legends.

3 RESULTADOS

The intensity of aggression was clearly related to the profile of TST (Fig. 2). Animals ranked as Hypo (low mobility) showed a PBA score of 2+ (Fig. 2A). The animals rated as Normal (medium mobility) presented scores 0/1+, with the smallest evidence of aggressiveness behavior (Fig. 2B). On the other hand, the group Hyper (high mobility) showed PBA scores of 4+ with higher aggressivity (Fig. 2C). It was also noted that injury due to fights occurred in peculiar physical regions, in which, those in front legs, chest and muzzle region were the result of individual confrontations and of defense capabilities (Fig. 2D). It is important to stress that during the grouping between the 4 and 8^{th} weeks, PBA score was zero for all the groups.

All TST categories showed weight gain during our study, although to varying degrees. Normal mice showed a weight gain of 2.9±0.8 g/individual, which was significantly higher than the values

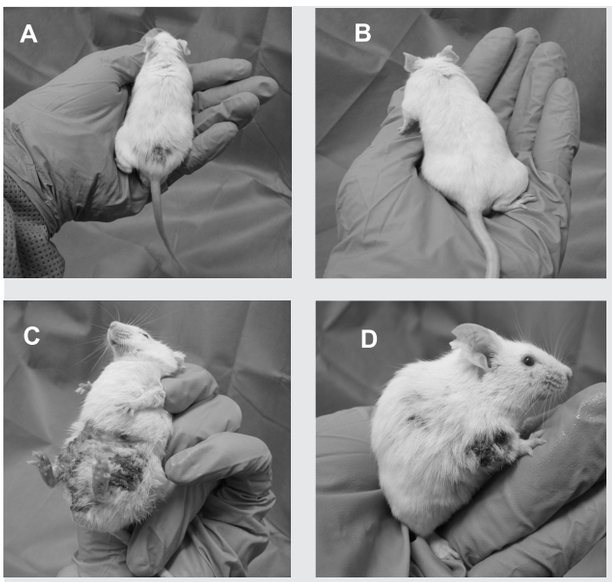

Figure 2: *Category by TST mobility profile and PBA score:* During the 10th week of life, we regrouped the Swiss Webster mice based on mobility profiles: Low-*Hypo* (dark gray); Medium-*Norm* (gray); High-*Hyper* (light gray) defined by tail suspension test (TST). Over the ethogram, Hypo mice showed 2+ (A), Norm 1+ (B) and Hyper 4+ relative to PBA score (C). Figure E described injuries on the animal defense posture and attempt to fight aggression.

from Hypo (0.8±0.4 g) and Hyper (1.6±0.3 g) (Normal *vs* Hyper: p=0.001) (Fig. 3A). Also chow intake was greater in Normal mice (0.83±0.1 g) than in the Hypo (0.53±0.1 g) and Hyper (0.56±0.1 g) categories (Normal *vs* Hyper: p=0.003). The Normal (0.64±0.1 g) group also produced significantly more feces than the Hypo (0.42±0.1 g) group (Normal *vs* Hyper: p=0.003) (Fig. 3B). No significant differences for water consumption were observed between categories. However, the Hypo group showed lower urine volume (0.27±0.1 ml) as compared with other groups (Normal: 0.69±0.1 ml and Hyper: 0.77±0.1 ml) (Normal *vs* Hyper: p=0.003) (Fig. 3C).

Serum total testosterone and progesterone levels are depicted in Fig. 4. Testosterone levels were lower when compared with normal categories in Hypo (870±40 ng/dl) and Hyper (1080±50 ng/dl) groups (Normal *vs* Hyper: p=0.001) (Fig. 4A). Progesterone levels were higher in Hyper (2.6±0.5 ng/ml) when compared with Normal (1.1±0.4 ng/ml) and Hypo (1.3±0.5 ng/ml) categories (Normal *vs* Hyper: p=0.001) (Fig. 4B). Of note, testosterone/progesterone ratio (RTP) indicated that the most aggressive animals (Hyper) presented a lower index (4.0±0.1) compared with the Hypo (7.0±0.1) and Normal (16.0±1.0) mice (Normal *vs* Hyper: p=0.001) (Fig. 4C).

Morphometric and histopathological analysis of the testicles also showed marked differences between the categories (Fig. 5), although no significant differences in weight were observed between the left and right testis was observed (data not shown). Animals in the Hyper group (index: 0.10±0.01) had testicles significantly smaller than those of the Normal (index: 0.20±0.01) and Hypo (index: 0.13±0.01) categories (Normal *vs* Hyper: p=0.001) (Fig. 5A). These results led us to consider the number of ST in the right and left testis of all categories. The ST value was similar on both left and right testis. Directly proportional to the weight of the testicles, the ST number for the Hyper category (right: 18.9±2 and left: 17.8±2 tubules) was lower in comparison with the 2 other groups: Normal (right: 47.9±2.0 and left: 44.9±2.0 tubules) and Hypo (right: 26.7±2.0 and

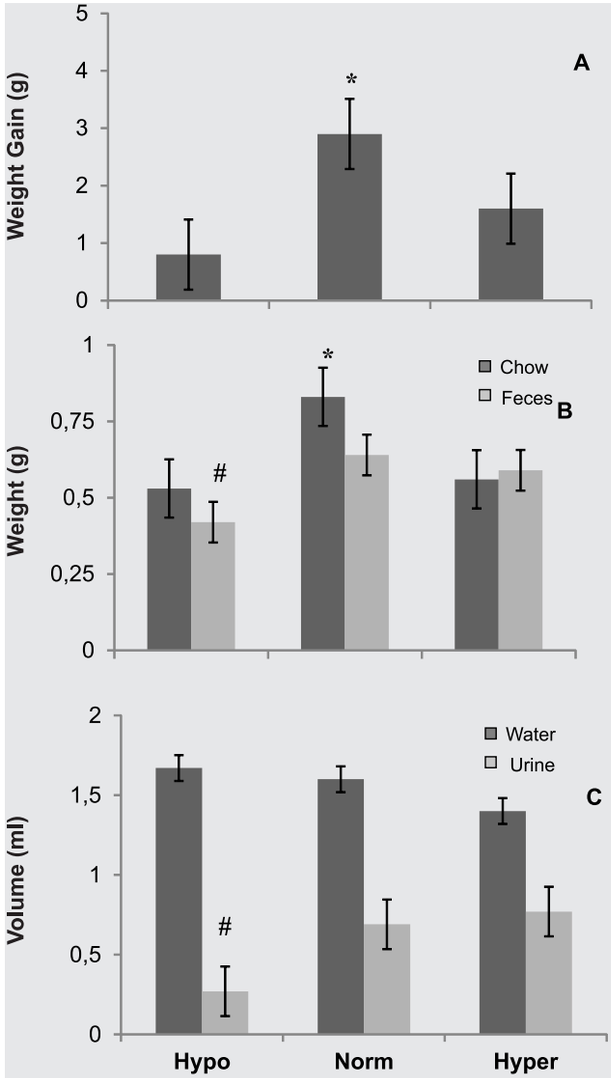

Figure 3: *Metabolic parameters:* The average weight of the animals showed no significant differences among categories. However, in relation to weight gain (grams) per mobility profile observed a increase for the Normal and decrease value for Hypo mice category (A). Food intake (grams - dark gray bar) was also higher for the Normal (B). Hypo mice showed fewer feces excreted (grams - light gray bar) compared to Norm category. There was no difference in the volume of water intake (milliliters – dark gray), but the Hypo category had reduced volume of urine (milliliter – light gray) (C). Values correspond to means ± standard deviation of three independent experiments. *p<0.05 between Normal and other categories; #p< 0.05 between Hypo and Normal category.

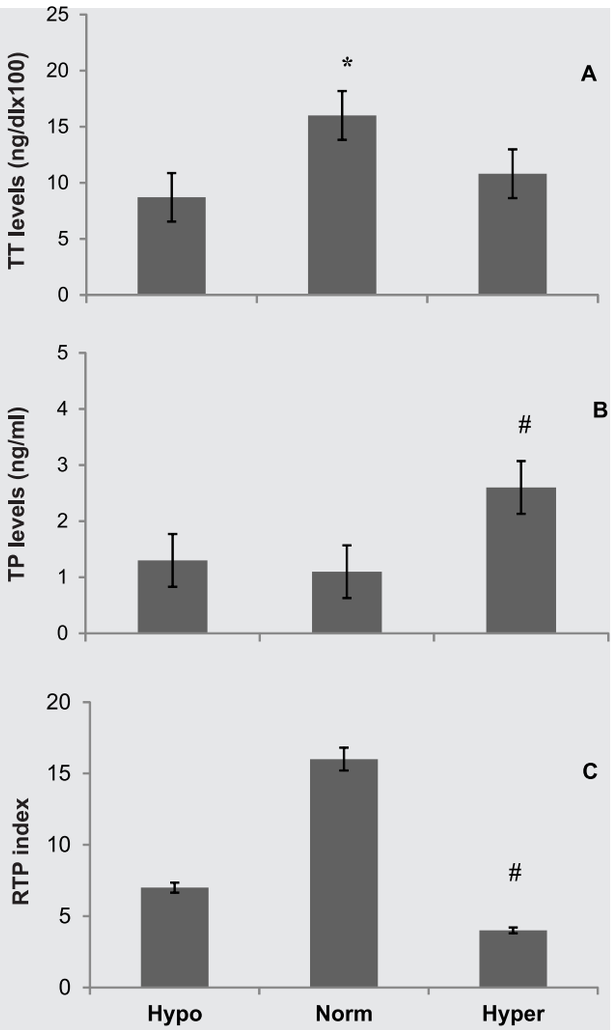

Figure 4: *Sex hormone levels:* Serum total testosterone (TT – ng/dl) and progesterone (TP – ng/ml) were measured relative overall categories at the 16th week of life of animals. TT showed a decrease in levels in Hyper and Hypo when compared to the Normal categories (A). TP showed increased levels specifically in the Hyper compared to other categories (B). The ratio TT/TP (RTP index) was significantly reduced in Hyper mice (C). Values correspond to means ± standard deviation of three independent experiments. *p<0.05 between Normal and other categories; #p< 0.05 between Hyper and other categories.

left: 29.5±2.0) (Normal *vs* Hyper: p=0.003) (Fig. 5B). Qualitative pathological analysis showed no evident alteration in the structural integrity of ST and absence of edema, congestion, hemorrhage, inflammatory infiltration and fibrosis in testicular parenchyma. However, an expansion in ST length was observed (Fig. 5C). This finding is best seen when comparing to the groups Normal (Fig. 5Ca) and Hyper (Fig. 5Cb). Regarding the reproductive cells (Fig. 6), we observed no significant difference in the values for Sertoli cells (data not shown). On the other hand, the average number of Leydig cells (Fig. 6A) in Hyper mice (right: 17±2 and left: 17±2 cells/field) was lower when compared with the Normal (right: 21±2 and left: 22±2 cells/field) group (Normal *vs* Hyper: p=0.003). Likewise a lower number of DS cells (Fig. 6B) was observed in the Hyper (right: 84±5 and left: 74±6 cells/field) in relation to Normal category (right: 107±7 and left: 83±5 cells/field) (Normal *vs* Hyper: p=0.002).

4 DISCUSSÃO

Our goal was to address the trigger of aggressive events using experimental models for the study of aggression in mice[8,9]. Our results demonstrate that in the Normal and Hypo categories, aggressive behavior was relative smaller. Furthermore, it was clearly noted that male Swiss Webster mice with anxiety-like profiles were highly aggressive when regrouped in adulthood.

Hypotheses about territorialism, disputes over females, and access to food as the cause of such behavior do not satisfactorily explain the intensity of aggression[24,25,26,27]. These factors associated to the anxiety state impair the ability to establish a linear hierarchy[9]. Thereby, maintenance of leadership (or dominance) is imposed by increasing the number and severity of aggressions. Furthermore, the severity of lesions also appears to be related to the individual's defensive ability. Animals with defensive attitudes have bites located on the front legs, chest and muzzle. Metabolic parameters

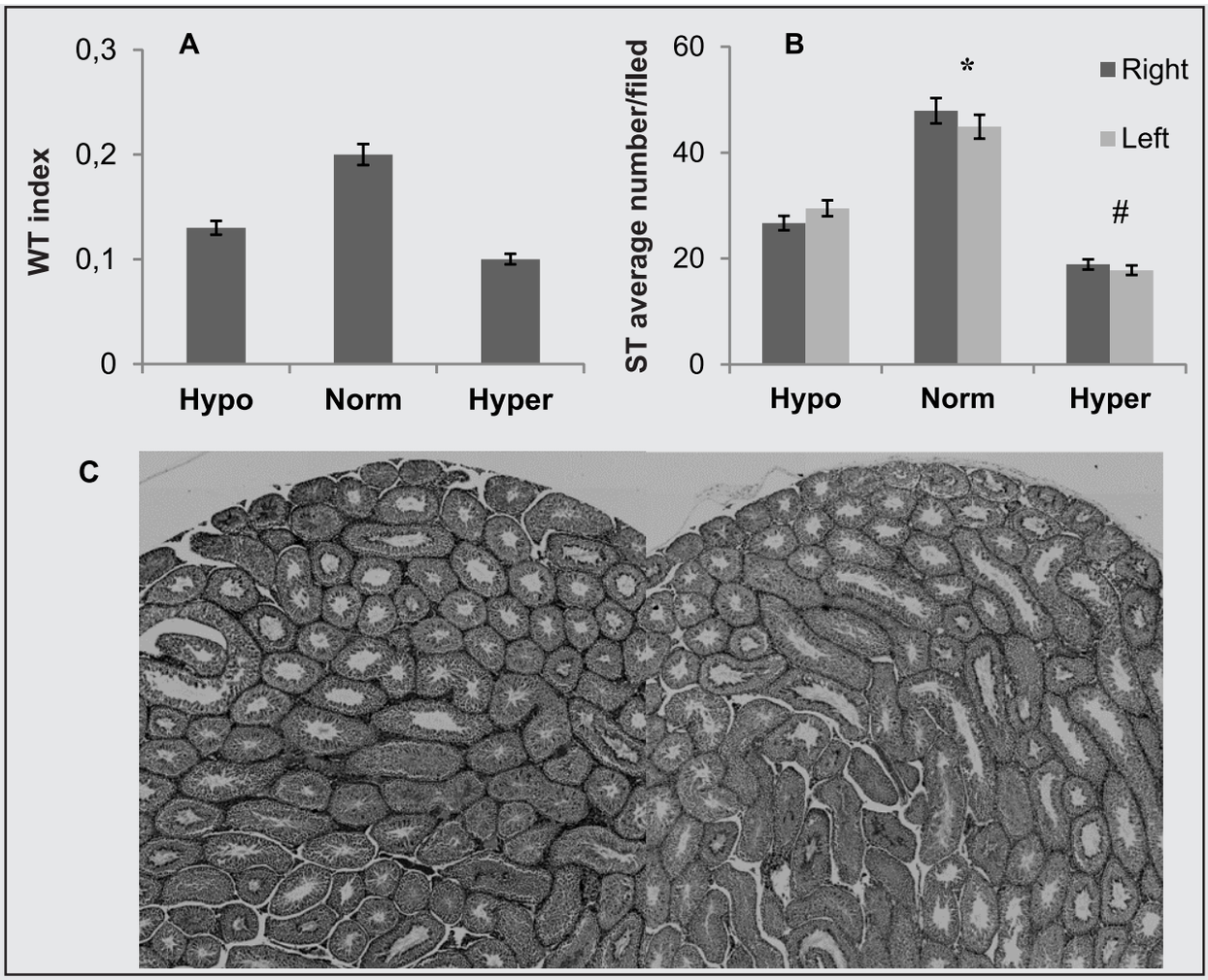

Figure 5: *Morphometric evaluation of the testicles:* No statistical difference in the total weight and the seminiferous tubules number between the right (dark gray) and left (light gray) testicles. Hyper mice had smaller testicles than the other categories (A). The number of seminiferous tubules was greater in Norm and lower in Hyper category (B). Total magnification is 100x – black bar. *p<0.05 between Normal and other categories; #p < 0.05 between Hyper and other categories.

demonstrated that the aggressiveness decreased food intake, weight gain, and volume of feces. These data support the connection of the stress condition with the presence of a PBA. Interestingly, the Hypo category showed some peculiar results: a) alteration in TST mobility profile of some animals; b) lower weight gain associated with decreased chow consumption and weight of feces (relative to Normal) and c) urine volume reduction. The Hypo category was has also been considered to represent a depression-like phenotype for mice. In this phenomenon, it is possible that changes in neurotransmitters and hormones in this depressive-like state lead to decreased metabolism in the animals[6,28,29].

Physiologically, aggressiveness in several species has been associated with elevated levels of testosterone[20,30,31]. This male sex steroid hormone is conserved through most vertebrates indicating its importance in evolution and development of

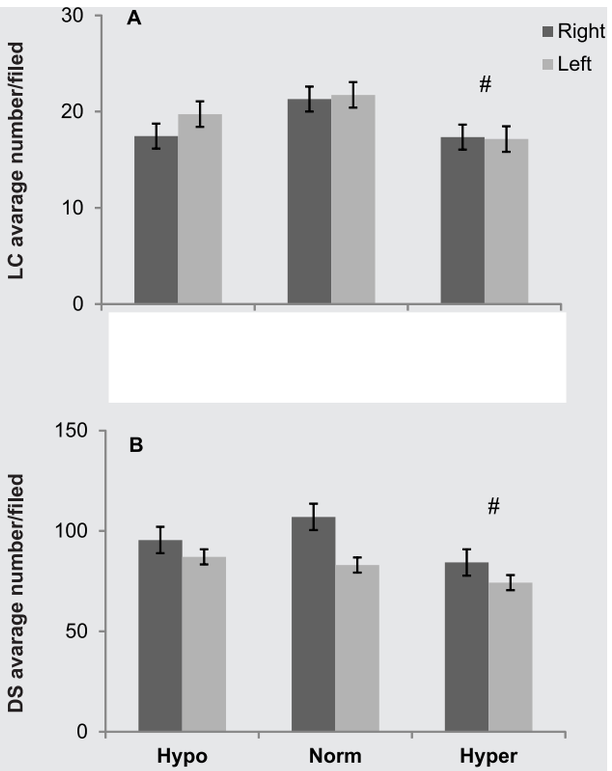

Figure 6: *Quantitative histopathology evaluate of reproductive cells*: Optical microscopic evaluation of testicular tissue processed and stained with hematoxylin and eosin (HE). Comparing the number of cells in the right (dark gray) and left (light gray) testicle. We calculated the average number per field of Leydig (A) and diploid spermatogenic cells (B) between specific categories. #p<0.05 between Hyper and Normal mobility profile.

species[32,33,34]. High levels of testosterone promote the lack of serotonin. Furthermore, increased antidiuretic hormone (ADH) levels in the medial amygdala, lateral hypothalamus, and preoptical medial area are involved in aggressive behaviors[30]. Our results demonstrate that the aggressiveness exacerbated in Hyper mice was associated with a decrease in testosterone and increase in progesterone levels. We suggest that highly aggressive behavior is related to the ratio between testosterone and progesterone. The smaller the difference in the testosterone/progesterone ratio, the greater was the propensity to aggression between animals. These results are in agreement with the histopathological evaluation of testicular tissue. The Hyper mice showed a decrease in testis weight (body weight relative) and lower number of ST, DS and Leydig cells. The series testis tubule-cells are involved in the mechanisms of production and maturation of sperm[35]. We believe that the aggressiveness could be related to sexual selection, as these parameters suggest reduced fertility and the need of a greater number of females to fecundate[36,37].

In a recent study investigating the effects of early stress on the development of social behaviors in mice, aggressive behaviors were examined in peripubertal male mice exposed to maternal separation during the first 2 weeks of life[38]. This study demonstrated that mice who underwent maternal separation showed reduced testosterone levels, decreased arginine/vasopressin, and increased oxytocin immunoreactivity in the paraventricular nucleus of peripubertal males[38]. These results collectively suggest that early life stress disrupts the development of male aggressive behaviors and associated neuroendocrine systems[38,39]. Another recent study demonstrated that progesterone and its receptor have inhibitory effects on parental behavior and increase infant-directed aggression in male mice[21]. Previously thought to be of diminished importance in male animals, PRs play a critical and specific role in modulating infant directed behaviors in male mice[22].

In humans, early life stress (child and adolescent abuse, neglect and trauma) induces robust alterations in the emotional and social functioning resulting in an enhanced risk for the development of psychopathologies such as aggressive disorders[39]. We believe that the cause of aggression is related to a complex and integrated network of genetic, behavioral, neurological and biochemical factors. In Swiss Webster mice, increased aggressive behavior may be related to disruption due to maternal separation, anxiety-like profiles and neuroendocrine deregulation. In conclusion, our

results relate decreased total testosterone/progesterone ratio and appearance of highly aggressive behavior in male Swiss Webster mice.

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ESTUDO SOBRE A RELAÇÃO ENTRE TESTOTERONA/ PROGESTERONE E O SURGIMENTO DE COMPORTAMENTO AGRESSIVO DE CAMUNDONGOS MACHOS DA LINHAGEM SWISS WEBSTER

ABSTRACT

A violência é um problema de saúde pública e tem um grande impacto sobre a qualidade de vida dos indivíduos e na sociedade. Fatores evolutivos, genéticos, ambientais e culturais estão intimamente interligados como causas do comportamento agressivo. Os modelos animais podem ser uma importante ferramenta no estudo de agressividade. Camundongos Swiss Webster foram selecionados com base no perfil de atividade (Hypo, Normal, Hiper), definida pelo teste de suspensão pela cauda. Nossos resultados demonstram que os ratos Hiper (ansiedade -like) foram mais agressivos e violentos do que as outras duas categorias, indicado por um padrão de comportamento agressivo, com uma pontuação de 4+ em relação ao escore 1+ da categoria Normal. Além disso, Hiper, mostraram uma diminuição nos níveis de testosterona total (32,5% em relação ao grupo Normal) e um aumento nos níveis de progesterona (57,6% em comparação com o grupo de Normal). Observou-se também uma diminuição no peso dos testículos, os túbulos seminíferos. Também do número de células de Leydig, diplóide e o número células espermatogênicas em animais hiper-agressivo. Em conjunto, os resultados indicam uma associação entre a testosterona/progesterona e o aparecimento do comportamento altamente agressivo em camundongos Swiss Webster machos. CEUA/FIOCRUZ, #LW-5/12.

Palavras-chave: Camundongos. Comportamento agressivo. Hormônios sexuais. Testosterona. Progesterona. Violência. Machos.

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