Research Article

Follicular Development and Secretion of Ovarian Hormones during the Juvenile and Adult Reproductive Lives of the Myelin Mutant taiep Rat: An Animal Model of Demyelinating Diseases

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Received 27 April 2018; Revised 26 June 2018; Accepted 5 August 2018; Published 16 September 2018

Academic Editor: Davide Francomano

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Infertility and reproductive problems have been reported in women with multiple sclerosis (MS), a demyelinating disease. MS is one of the most important demyelinating diseases. It is currently known that 70% of the cases of MS occur between 20 and 40 years of age, and that it has a higher prevalence in women than in men with a 3:1 ratio [1, 2]. Reproductive problems have been reported in women with MS, ranging from sexual dissatisfaction and the absence of orgasms [3, 4], to menstrual irregularity and hormonal and ovarian follicular development alterations [5, 6]. However, the relationship between the presence of ovarian change and demyelinating disease has not been clearly established.

It is well known that the functions of the ovary are regulated by gonadotropins secreted by the pituitary gland as a result of the stimulus from the hypothalamus by the gonadotropin-releasing hormone (GnRH). In the last five decades, there has been an accumulation of evidence demonstrating that, in addition to the regulation exerted by gonadotropins on the ovary, there are other types of signals coming from the autonomous innervation that participate in the regulation of gonadal functions [7]. The ovary receives and sends nervous information via the superior ovarian

1. Introduction

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nerve, the ovarian plexus, and the vagus nerve [8, 9]. These signals include classic neurotransmitters such as noradrenaline and acetylcholine, as well as several neuropeptides, such as NPY, VIP, and SP [10–12].

In this work, we proposed the use of the taiep rat as an animal model of demyelinating disease. This rat mutant is the result of a spontaneous mutation obtained in the F5 generation of the Sprague–Dawley strain obtained at the Benemérita Universidad Autónoma de Puebla (Mexico) in 1989 [13]. The taiep rat is characterized by various motor symptoms such as tremor, ataxia, immobility, epilepsy, and paralysis of the hind limbs. The name taiep is the acronym of these symptoms [13, 14].

We now know that this subline has an initial hypomyelination followed by a progressive demyelination of the CNS due to a mutation in chromosome 9. In fact, now we know that it is tubulin [15, 16]. It has prominent alterations in the electrical properties of neurons and in the electrical properties of the synaptic transmission in the spinal cord in the postnatal period [17]. Due to its symptoms, the taiep rat represents an important experimental model; Foote [18] proposed in 2005 that it as an animal model of demyelinating diseases. It has been shown to present a generalized epilepsy absence crises type, making it an ideal animal model for the study of several demyelinating pathologies [14, 18].

For these reasons, the aim of this study was to characterize the follicular development, secretion of steroid hormones, and presence of nerve fibers in the ovary on taiep rats in two stages of the life of the animal: juvenile and adult. In this way, we intend to contribute to knowledge about the ovarian level affectations that may be happening in an animal model of demyelinating disease.

2. Materials and Methods

2.1. Animals. We maintained Sprague–Dawley and taiep rat groups in our animal room facilities under controlled 12:12 hr cycle (lights on 07:00), relative humidity (30–45%), and temperature (22 ± 2 °C) conditions. All experiments were performed in strict accordance with the Mexican Law of Animal Treatment and Protection Guidelines and the specifications of the Mexican Official Standard (NOM-062-ZOO-1999) for production, care, and use of laboratory animals that are in accordance with the National Institute of Health (NIH) guide. Figure 1 shows the experimental design.

2.2. Assessment of the Vaginal Opening and Estrous Cycle. The juvenile animals were examined daily at 08:00 to recognize the vaginal opening. After the presence of vaginal canalization, the adult animals’ estrous cycle was monitored daily for two weeks by vaginal lavage.

2.3. Autopsy. The juvenile group was sacrificed on the 30th day. The adult group was sacrificed at the age of 90 days (vaginal estrus preceded by proestrus). Animals were subjected to an autopsy between 08:00 and 10:00 hrs with overdose of pentobarbital. After, the trunk blood was obtained by decapitation in a soundproof and isolated room to avoid stress. Serum was collected and kept at room temperature for 30 minutes, and then centrifuged at 3500 rpm for 15 min.
Serum was separated from cell buttons and stored at −20 °C in Eppendorf tubes until progesterone, 17β-estradiol, and testosterone quantifications were performed.

2.4. Steroid Hormone Quantification. Using the ELISA technique with commercial kits (DRG brand), we quantified the serum concentration of testosterone, estradiol, and progesterone. Kits for all hormones were used in accordance with manufacturer’s instructions. The intra- and interassay variabilities for the estradiol kit used were less than 9% and 14%, respectively, with a detection limit of 2000 pg/mL. The intra- and interassay variabilities for the progesterone kit were less than 7% and 9%, respectively, with a detection limit of 40 ng/mL. The intra- and interassay variabilities for the testosterone kit were less than 7% and 9%, respectively, with a detection limit of 16 ng/mL. The optical density of the wells was measured at 450 nm with a universal microplate reader BioTek ELx800. Hormone concentrations were calculated using KCjunior software (BioTek Instruments).

2.5. Ovarian Morphology. Ovaries were fixed in Bouin’s solution, dehydrated, and included in paraffin. Microtome sections 10 μm thick were obtained and stained with hematoxylin–eosin (H3136-E4009 Sigma-Aldrich). The follicles present in a section every 100 μm were classified according to their state as healthy or atretic. A follicle was considered atretic when it exhibited at least one of the following characteristics: the presence of oocyte with nuclear pycnosis, desquamation of the granulosa layers, and presence of fenestration in the granulosa layer. In addition, follicles were classified in relation to the thickness of the granulosa layer and the follicular diameter as follows: primary (P), secondary A (SA), secondary B (SB), preantral A (PA), preantral B (PB), antral A (AA), and antral B (AB), (Table 1). The criteria for reporting the presence of cysts are as follows: follicles that presented a wide antral cavity, a decrease of granulosa cell layer, thecal hyperplasia, and the absence of oocyte. The study of the follicular population was performed in the right and left ovaries of three animals per group taken randomly. Photographs of ovaries were taken every 100 μm using a Moticam3 camera. Images were analyzed using the program ImageJ 1.50i (NIH).

2.6. SPG Method. The presence of catecholamines were visualized using the glyoxylic acid technique [19, 20]. Ovaries were obtained fresh and were cut in a cryostat (Leica CM1850) every 20 μm. The cuts were treated with SPG solution (sucrose, potassium phosphate monobasic, and glyoxylic acid) and green malachite; then, they were dried with cold air and placed in an oven at 90 °C for 3 min. Subsequently, the cuts were assembled for observation under an epifluorescence or confocal microscope with an excitation of 450–490 nm. Thirty sections were analyzed per experimental and control groups. Images were analyzed in the program ImageJ 1.50i.

2.7. Statistical Analyses. Comparisons of more than two groups were analyzed with an ANOVA (multifactorial

| Initials | Follicle | Granulosa layer thickness (μm) | Follicular diameter (μm) | Ideal morphology |
|----------|----------|-------------------------------|-------------------------|-----------------|
| P        | Primary  | <20                           | <100                    |                 |
| SA       | Secondary A | 20–30                  | 100–150                  |                 |
| SB       | Secondary B | 30–40                   | 150–200                  |                 |
| PA       | Preantral A | 40–60                   | 200–300                  |                 |
| PB       | Preantral B | 60–80                   | 300–400                  |                 |
| AA       | Antral A    | 60–90                    | 400–500                  |                 |
| AB       | Antral B    | 60–100                   | >500                     |                 |
analysis of variance) followed by a Tukey’s test. Student’s t-test was used when we compare two groups. The Mann–Whitney U test was used for the follicular population analysis. Percentages were analyzed with a z-test. Differences in probabilities equal or less than 0.05 were considered statistically significant.

3. Results

3.1. Juvenile Stage. The body weight of taiep rats at the age of 30 days was 19% lower when compared to the body weight of Sprague–Dawley (SD) rats (77.2 ± 3 g of body weight vs. 99.2 ± 5.9 g of body weight, p < 0.05); this difference was preserved until the adult stage (Figure 2). A delay of 4 days in the vaginal opening of the taiep rat was observed (38.9 ± 0.3 days in taiep rat vs. 34 ± 0.2 days in SD rat; Student’s t-test, p ≤ 0.001).

The analysis of the ovaries showed a decrease of 44% in the total number of follicles present in the ovaries obtained from taiep in comparison to SD rats (334.33 ± 45.99 vs. 595 ± 17.04; Mann–Whitney U test with p ≤ 0.01) (Figure 3(a)). When analyzing the follicles present in the ovaries by each category, we observed that in the ovaries of the taiep rat there is a significant decrease in the percentage of P follicles and an increase in the percentage of PA follicles, in comparison with the follicles present in the ovaries of SD rats (Figure 3(b)).

Also, a decrease in the percentage of healthy follicles and an increase of 15% in atretic follicles were observed in the ovaries of taiep rats in comparison with SD rats (Figure 3(c)). When analyzing the follicles by category and stage (healthy or atretic), we observed that, with the exception of PB follicles, there was a decrease in the percentage of healthy follicles in all types of follicles present in the ovaries of the taiep rats when compared to SD rats (Table 2).

No significant differences were obtained in the serum levels of estradiol and progesterone, but there was an increase of 21% in the serum levels of testosterone observed in juvenile taiep with respect to SD rats (Figure 4(a)).

Histochemical analyses revealed the presence of catecholamines in the ovaries of taiep and SD rats; these fibers were located preferentially in the periphery of the ovarian follicles, cortex, and corpora lutea. Determining fluorescence intensity analysis, we obtained a significant decrease of 80% in the fluorescence intensity of catecholaminergic fibers in the ovaries of taiep rats when compared to SD rats (Figure 5(a)).

3.2. Adult Stage. Vaginal smears from 70 days of age show that taiep rats have irregular estrous cycles when compared to SD rats (Figure 6). Histological analyses revealed the presence of follicular cysts in the ovaries of taiep rats at 90 days of age (Figure 7(a)). There was no difference between both groups of rats in the total number of follicles present in the ovaries (taiep: 191.6 ± 22.0 vs. SD: 216 ± 25.3; Student’s t-test, p ≤ 0.51). When analyzing the follicles present in the ovaries by category, we observed that in the ovaries of the adult taiep rats there were significant changes in the percentages of P, SB, PA, and PB follicles, in comparison with the follicles evaluated in the ovaries of SD rats (Figure 7(b)).

We observed a decrease in the percentage of healthy follicles and concomitantly an increase in the percentage of atretic follicles in the ovaries of taiep rats when compared to SD rats (Figure 7(c)). When analyzing the follicles by category and stage (healthy or atretic), we observed that, with the exception of P follicles, there was a decrease in the percentage of healthy follicles with respect to all types of follicle population present in the ovaries of taiep rats with respect to SD rats (Table 2).

In adulthood, the serum levels of estradiol decreased by half in taiep rats when compared to SD rats. The serum levels of progesterone were increased 20 times, and no significant differences were observed in the serum levels of testosterone (Figure 4(b)).

Histochemical analyses revealed the presence of noradrenergic fibers in the ovaries from taiep and SD rats, similar to what was observed in the ovaries from the juvenile stage. The fluorescence intensity analysis showed a decrease of 50% in the fluorescence intensity of catecholamines in the ovaries of the taiep rat that was maintained in adult life (Figure 5(b)).

4. Discussion

The present work yields relevant information related to the ovarian physiology on taiep rats, an important myelin mutant rat. The first finding is a decrease in body weight observed throughout the life span of the taiep rat, which is probably a consequence of the progressive demyelination of the central nervous system, but not in the peripheral system [21, 22]. A decrease in weight has been reported in animal
models of demyelinating diseases, and in humans affected by Huntington’s disease [23, 24]. Since the 1980s, it has been known that the beginning of the reproductive stage is affected by body weight, specifically, by the proportion of fat with respect to the whole body mass [25, 26]. The low weight of \textit{taiep} rats may be one of the factors that result in the delay of the age of vaginal opening and the irregularity of their estrous cycles.

Another possible cause of the alteration of the estrous cycle in \textit{taiep} rats is the high concentration of testosterone obtained by these rats at an early age. In Wistar rats, it has been reported that postnatal dihydrotestosterone administration delays vaginal opening and alters the estrous cycle [27]. In 21-day-old Sprague–Dawley rats given systemic testosterone, there is a delay in the age of vaginal opening and also an irregularity in the estrous cycle [28].

Figure 3: Follicle population in Sprague–Dawley and \textit{taiep} rats in the juvenile stage. (a) Representative histological slice of the ovary. (b) Percentages of follicles in the different categories. (c) Percentages of total atretic and healthy follicles in the ovary. SD: Sprague–Dawley; AF: antral follicle; PF: preantral follicle; P: primary; SA: secondary A; SB: secondary B; PA: preantral A; PB: preantral B; AA: antral A; AB: antral B. *\(p \leq 0.01\) z-test and \(^\#\)\(p \leq 0.001\) z-test.
Table 2: Follicle population in the ovaries from the juvenile and adult taiep and Sprague–Dawley rats.

|          | Total number of follicles | P (%) | SA (%) | SB (%) | PA (%) | PB (%) | AA (%) |
|----------|---------------------------|-------|--------|--------|--------|--------|--------|
| Juvenile |                           |       |        |        |        |        |        |
| SD       | 595.00 ± 17.04            | 93    | 7      | 88     | 12     | 74     | 26     | 70     | 30     | 85     | 15     |
| taiep    | 334.33 ± 45.99 *          | 87Δ   | 13Δ    | 74Δ    | 26Δ    | 63Δ    | 37Δ    | 62Δ    | 38Δ    | 71     | 29Δ    |
| Adult    |                           |       |        |        |        |        |        |
| SD       | 216.00 ± 25.33            | 98    | 2      | 95     | 5      | 85     | 15     | 66     | 34     | 74     | 26     |
| taiep    | 191.67 ± 22.06            | 96    | 4      | 86θ    | 14θ    | 43Δ    | 57Δ    | 42Δ    | 58Δ    | 33Δ    | 67Δ    |

SD: Sprague–Dawley; P: primary; SA: secondary A; SB: secondary B; PA: preantral A; PB: preantral B; AA: antral A; AB: antral B; A: atretic follicles; H: healthy follicles. *p ≤ 0.01 Mann–Whitney U; for $ p ≤ 0.004 z$-test, for # p ≤ 0.005 z-test, and for Δ p ≤ 0.001 z-test.

Figure 4: Serum levels of ovarian hormones in the juvenile and adult Sprague–Dawley and taiep rats. (a) Serum levels of estradiol, progesterone, and testosterone in the juvenile Sprague–Dawley and taiep rats. The error bars represent the standard error of measurements for 6 Sprague–Dawley rats (SD) or taiep rats. (b) Serum levels of estradiol, progesterone, and testosterone in adult Sprague–Dawley and taiep rats. The error bars represent the standard error of measurements for 6 Sprague–Dawley rats (SD) or taiep rats. SD: Sprague–Dawley. *p ≤ 0.033, #p ≤ 0.009, and &p ≤ 0.017; Student’s t-test.
Given that a decrease in the follicular population and reserve has been reported in women with multiple sclerosis [6], in this study we characterized follicular development in the juvenile and adult stages of this myelin mutant.

During the juvenile stage, the taiep rat showed a significant decrease in the follicular population, with a decrease in the primary follicles and an increase in the preantral follicles and follicular atresia. This may be a consequence of the increase in the plasma concentration of testosterone [29]. It has been reported that administration of testosterone in 23-day-old Sprague–Dawley rats results in an increase in follicular atresia [30]. When characterizing the adult stage, we observed an increase of primary follicles and a decrease of growing follicles, along with an increase in follicular atresia and the presence of follicular cysts. All changes can be due to the high concentrations of testosterone present in the juvenile stage or due to the demyelinating process. In 21-day-old Wistar rats which do not present demyelination of the central nervous system, the implantation of dihydrotestosterone for 11–13 weeks produces an increase in follicular atresia and causes the development of follicular cysts [31]. In Wistar rats, it has been reported that postnatal administration of dihydrotestosterone affects follicular development in the adult stage, causing the increase of preantral follicles, decrease of antral follicles, and appearance of follicular cysts [27].

Hormonal stimuli are not the only important element for ovarian function, since the presence of adequate noradrenergic neurons and their axons has been demonstrated in the ovaries of Wistar rats as well as the presence of noradrenergic fibers in the ovaries of Sprague–Dawley rats [11]. The main source of ovarian noradrenaline comes from the superior...
Figure 6: Estrous cycle of Sprague–Dawley and taiep rats from 70 days of age until sacrifice. Each graph represents the estrous cycle of one Sprague–Dawley and one taiep rat. The estrous cycle comprises four stages: estrus, diestrus 1, diestrus 2, and proestrus. Red arrows indicate the moments when the estrous cycle of the taiep rat is irregular. SD: Sprague–Dawley; E: estrus; D1: diestrus 1; D2: diestrus 2; P: proestrus.
ovarian nerve [8], which is well known for participating in the processes of steroidogenesis and folliculogenesis, and also for stimulating the expression of FSH receptors in primordial follicles [12, 32].

In taiep rats, we observed a decrease in the fluorescence of catecholaminergic fibers at the juvenile stage and in adult animals. These changes in catecholaminergic fiber innervation may be due to the progressive demyelinating process that the taiep rat experiences. It has been reported that noradrenaline levels at the central level are altered in patients with multiple sclerosis and in animal models with experimental autoimmune encephalomyelitis, a model of MS which presents damage at the locus coeruleus, causing an increase of inflammation that can induce neuronal damage [33]. A decrease in noradrenaline in peripheral blood mononuclear cells of patients with MS has also been observed when the disease is inactive and when norepinephrine levels are increased, causing failure in apoptosis [34].

In taiep rats, the activation of astrocytes, along with the activation of the glial fibrilar acidic protein [22, 35] and

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**Figure 7:** Follicle population in Sprague–Dawley and taiep rats in the adult stage. (a) Representative histological slice of the ovary. (b) Percentages of follicles in the different categories. (c) Percentages of atretic and healthy follicles in the ovary. SD: Sprague–Dawley; CF: cystic follicle; CL: corpora lutea; P: primary; SA: secondary A; SB: secondary B; PA: preantral A; PB: preantral B; AA: antral A; AB: antral B. *p ≤ 0.01 z-test and †p ≤ 0.001 z-test.
concomitantly the activation of nitric oxide synthase, has been demonstrated to induce neuronal damage and endothelial dysfunction, which are probably due to a neuroinflammation process [35]. There is also an increased production of some chemokines [36] supporting an active inflammatory process that adds to progressive demyelination [22].

5. Conclusions

The results of the present work show alterations in ovarian functions in two stages of the life of the taiep rat, an animal model of demyelinating disease. These results allow us to conclude that demyelination at the level of the central nervous system in taiep rats affects follicular development and steroidogenesis in the early stages of the animal’s life, and this is maintained until adulthood. Our results clearly contribute to the knowledge about the ovarian level affectations that may be happening in an animal model of demyelinating disease. This allows us to postulate that the taiep rat is a good animal model for continuing the analysis of alterations in ovarian physiology in animals with central demyelination. These studies will help in proposing possible reproductive strategies to improve fertility and ovarian function in women who suffer such diseases.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no financial or any other disclosures.

Acknowledgments

We are very grateful to Omar Isidro (Bachelor in Veterinary Medicine) for taking care of the animals, and to Biol. Jose Luis Córdova de la Luz for his assistance in the use of the microscope. This study was partially supported by the following grant sponsors: VIEP-BUAP-TRHA-NAT16-G, VIEP-BUAP-TRHA-NAT17-G, and 100274222-VIEP2018. The authors also acknowledge the support of the academic group of neuroendocrinology BUAP-CA-288, as well as CONACYT Grant nos. 243247 to J. R. Eguibar and 243333 to C. Cortés. L. P. Muñoz-de-la-Torre acknowledges the fellowship from CONACYT (no. 594397) for the author’s masters in physiological science studies.

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