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# Short- and long-term effects on reproductive parameters of female Wistar rats after exposure to rosuvastatin starting in pre-puberty

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ABSTRACT

Statins are a class of drugs that act lowering lipid levels by inhibiting cholesterol biosynthesis. Additionally, statins can act by "pleiotropic effects", related to the inhibition of synthesis of the other mevalonate pathway products. Rosuvastatin is a third-generation statin and has shown better results in reducing cholesterol concentrations when compared to other statins. Recent studies suggest that rosuvastatin may act as an endocrine disruptor that potentially damages the hormonal axis and, consequently reproductive development and function of male rats. However, the effects of rosuvastatin exposure on rat female reproductive parameters remain unknown. In this study female rats were exposed to rosuvastatin at the doses of 0 (control), 3, or 10 mg/Kg.bw$^{-1}$/day from pre-puberty to adulthood. No alterations in the female reproductive parameters were observed at a dose of 3 mg/Kg.bw$^{-1}$. However, females exposed to 10 mg/Kg.bw$^{-1}$ exhibited shorter estrous cycles, altered copulatory behavior, decreased serum prolactin level, and alterations in the liver, pituitary and placental weights, parameters to some extent influenced by the reproductive hormonal axis signaling pathway. On the other hand, pubertal onset, reproductive hormone levels, fertility, and histological parameters of the ovary, uterus, and placenta were unaltered by exposure to both doses of this statin. Thus, rosuvastatin exposure, at the higher dose, altered the reproductive function of female rats, probably due to the pleiotropic effects of this statin. Additional studies on the effects of this statin on female reproductive function and development are encouraged to better characterize its mode of action.

## 1. Introduction

Puberty is the period when the animals first become capable of reproducing sexually. During this time, genital organs mature and secondary sex characteristics develop in both male and female animals (Vidal, 2017). Puberty is considered a critical period of development and requires the action of different endocrine factors which are crucial to promoting adequate development of body systems (Mantovani and Fucic, 2014).

During pre-puberty, the hypothalamic-pituitary-gonadal axis is activated and secretes gonadotrophin-releasing hormone (GnRH) by the hypothalamus, which stimulates the adenohypophysis to release follicle-stimulating hormones (FSH) and luteinizing hormones (LH). The gonads gradually become more sensitive to the stimuli of these two gonadotrophins, and present considerable growth rate augmentation, releasing steroid hormones into the bloodstream. These events culminate in the onset of puberty (Rosenfield et al., 2014).

Different substances may act in these physiological events and disrupt normal reproductive development during this period. These substances are known as "endocrine disrupters", defined as an exogenous substance or mixture that alters the synthesis, secretion, transport, receptor binding, signal pathways, inactivation, metabolism, or elimination of endogenous hormones in the body and lead to adverse health effects in the organism (Solecki et al., 2017; Chou, 2019; Toppari, 2019).

In the last years, there is increased exposure to endocrine disruptors chemicals in children. These compounds are present in persistent organic pollutants, consumer goods, personal care products, food, drinking water, and pharmaceuticals (Botton et al., 2017; Silver and Meeker, 2015). Exposure to endocrine disruptors at childhood leads

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to alterations on developmental endpoints and compromise health during adulthood, these include damage to neurological function, alterations on puberty timing, and increased chances of metabolic and reproductive dysfunctions (Silver and Meeker, 2015).

Statins are a class of drugs whose main mechanism of action is the inhibition of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, a rate-limiting enzyme in cholesterol biosynthesis, consequently reducing lipid levels in the organism (Patel and Kothari, 2017). Furthermore, some of the beneficial effects associated with the administration of statins have been attributed to their "pleiotropic effects" that are independent of inhibition of cholesterol biosynthesis, and include endothelial protection, antioxidant and anti-inflammatory properties, reduction in thrombogenic response, pro-angiogenic (Girardi, 2014; Rohilla et al., 2016) and immunomodulatory effects (Ferri and Corsini, 2014).

The "pleiotropic effects" of statins are related to the inhibition of synthesis of other products derived from the mevalonate pathway, called isoprenoids, and are associated with different cellular and molecular events, which include post-translational modifications of proteins related to cell signaling, proliferation, migration, and differentiation (Alarcon and Marikawa, 2016; Lee et al., 2007; Eisa-Beygi et al., 2014).

One of the most recent additions to the statin class is rosuvastatin (Olsson et al., 2002), which has shown better results in reducing LDL cholesterol concentrations when compared to other statins (Cortese et al., 2016).

Recently, the use of statins has increased as a function of poor eating habits and a sedentary lifestyle, mainly during childhood and early adulthood, to reduce cholesterol levels (Kit et al., 2015; Ross, 2016).

Previous studies investigating *peri*-pubertal rosuvastatin exposure in a rodent experimental model revealed that this drug leads to reproductive impairments in male rats. Rosuvastatin can delay pubertal development, reduce sperm quality, impair spermatogenesis, and alter hormonal signaling (Leite et al., 2014, 2018, 2019). Also, rosuvastatin exposure in male rats might affect reproductive function in an intergenerational manner, by damaging ovarian and uterine histophysiology of female offspring (Leite et al., 2018).

These data indicate that rosuvastatin may act as an endocrine disruptor during the *peri*-puberal window of exposure, and can impair reproductive development and function. However, there are no data on direct exposure to rosuvastatin or other statins in the female *peri*-puberal period or its effects on female reproductive parameters. Therefore, this study aimed to investigate the effects of rosuvastatin exposure, using doses used in human clinics, starting in pre-puberty, on pubertal development and reproductive function of female Wistar rats.

## 2. Material and methods

### 2.1. Animals

Adult male and female Wistar rats (80 days old) were obtained from the Central Biotherium, São Paulo State University (UNESP), *campus* at Botucatu/SP, Brazil, and maintained under controlled conditions (12 h of light/12 h of darkness; average temperature of 23 °C) in the Small Mammal Biotherium at the UNESP Department of Structural and Functional Biology (Morphology), Institute of Biosciences in Botucatu, with food and water *ad libitum*. The animals were maintained according to the Ethical Principles for Animal Experimentation, adopted by the Brazilian College of Animal Experimentation. The project was filed under number 1089 with the Ethics Committee on Animal Experimentation of the UNESP Institute of Biosciences, in Botucatu.

To obtain pregnant females and pups, these animals were allowed to mate during the dark period of the cycle, with two females being placed in a cage containing one male. The gestational day (GD) 0 was determined by the presence of spermatozoa in vaginal smears of estrus females, who were then kept in individual cages. After birth, the number of pups per litter was standardized to 8, balancing 3 male and 5 female pups at postnatal day (PND) 1, based on the anogenital distance. Litters whose number of pups could not be balanced were not considered for this study. Male pups were maintained until adulthood without any previous treatment and were used to perform the copulatory behavior and reproductive performance assessment of treated females. The male rats were previously trained with non-treated female rats, to acquire copulatory experience. Additionally, the males were carefully selected in order to avoid mating with their respective sisters and consanguinity.

### 2.2. Experimental design

At weaning, on PND 21, female pups were randomly distributed among three experimental groups: control group, which received treatment with saline; and rosuvastatin groups, which received the statin at doses of 3 or 10 mg/Kg.bw$^{-1}$ (body weight) diluted in saline. The treatment was performed daily and by oral gavage starting on PND 22, and finished on the first estrus after PND 42 or 75. During the treatment period, rat body weights were measured weekly. A brief visual description of the experimental procedures performed in this study is presented in Fig. 1.

The rosuvastatin doses chosen for this study are based on the lowest and highest doses applied in human therapy for children (Leite et al., 2018a, 2019b, 2018c), adapted for rodents considering their body surface area, as proposed for Reagan-Shaw et al. (Reagan-Shaw et al., 2008).

To investigate the effects of rosuvastatin exposure on female reproductive function, the treatment period is based on the use of statins, which has become earlier in the child and adolescent population because of the risk of lipid abnormalities (Ross, 2016; Newman et al., 2019).

Throughout the experimental procedures, the rats were daily monitored for general toxicity and stress signs derived from the treatment, such as mortality and morbidity, excessive weight gain/loss, weakness, lethargy, presence of bristly hair, and abnormal behavior.

### 2.3. External physical signs of puberty onset

Starting on PND 30, female rats were evaluated daily for the complete vaginal opening. Then, rats were weighed and checked daily for the occurrence of the first estrus by assessing vaginal fluid, characterized by the presence of cornified epithelial cells (Marcondes et al., 2002). The vaginal fluid was collected from rats with a micropipette, which inserts 10 μL of saline into the vagina. The fluid was deposited on a slide and analyzed under light microscopy. Both procedures, vaginal opening, and detection of the first estrus were performed to determine the age of puberty onset in female rats (Laws et al., 2000).

### 2.4. Estrous cyclicity

On PND 60, the animals were evaluated daily for 15 consecutive days for estrous cyclicity based on the cellular composition of vaginal fluid. This procedure was employed to determine the lengths of each cycle and of each phase of the cycle (proestrus, estrus, metaestrus and diestrus). The proestrus phase consists of the predominance of nucleated epithelial cells; estrus phase is characterized by the presence of cornified epithelial cells; metaestrus present nucleated and cornified epithelial cells, and leukocytes; diestrus consists of the predominance of leukocytes (Marcondes et al., 2002).

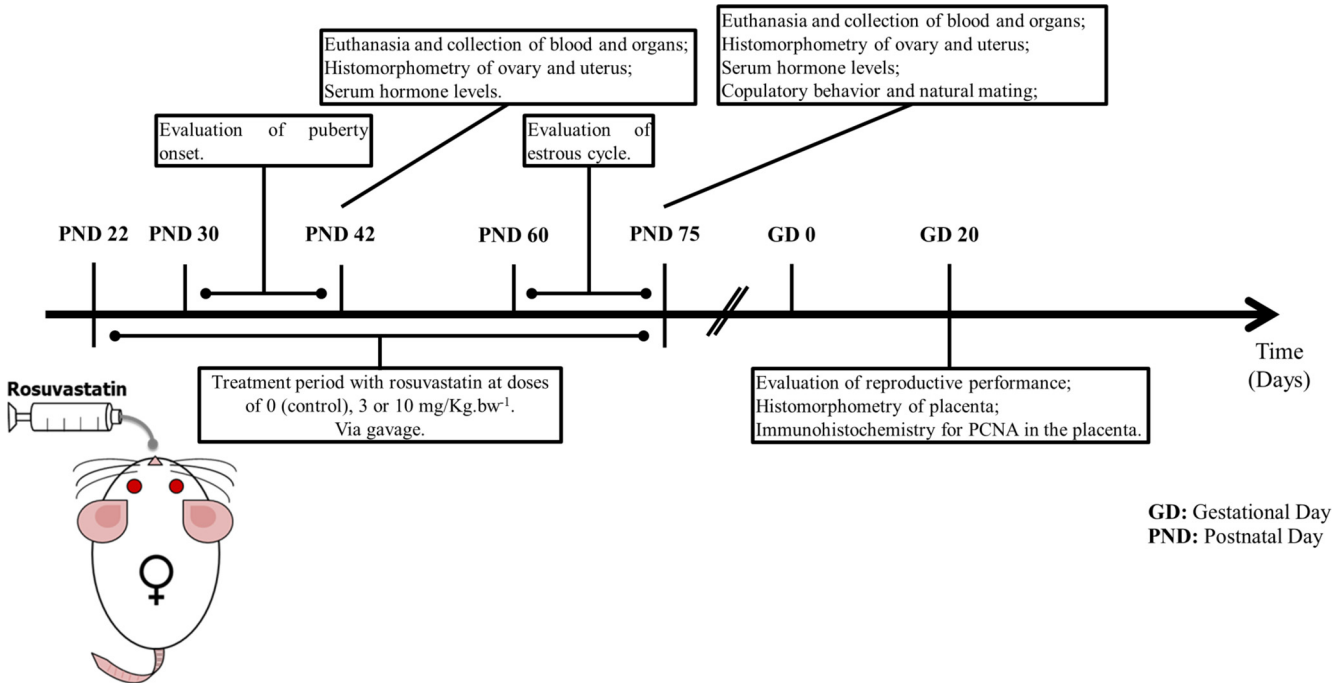


**Fig. 1.** Experimental design of the study.

### 2.5. Euthanasia of rats and organ collection

Rats from each experimental group were euthanized during the estrus phase at two different ages: at PND 42, right after the onset of puberty, as established by the U. S. Environmental Protection Agency (Agency, 2011), and at PND 75, during adulthood. Initially, animals were weighed and then euthanized by narcosis in $CO_2$. Blood samples for hormonal dosages were obtained by collecting blood from the inferior vena cava. Toxicological target (pituitary, thyroid, liver, adrenal, and kidney) and reproductive (ovaries and uterus) organs were collected and weighed. The left uterine horn and ovary were used for histological evaluation.

### 2.6. Serum reproductive hormone levels

Blood samples were centrifuged at 2400 rpm for 20 min at 4 °C and serum was obtained for hormonal dosages. The serum samples were immediately frozen and stored at −20 °C until the day of analysis. Serum levels of FSH, LH, prolactin were measured using a double-antibody radioimmunoassay with specific kits provided by the National Hormone and Peptide Program (Harbor-University of California at Los Angeles). Serum levels of progesterone, and testosterone were measured using a double-antibody radioimmunoassay with specific kits supplied by MP Biomedicals (ImmuChem™ Double Antibody, Progesterone: 07814701; Testosterone: 07814891. Orangeburg, NY, USA). All samples were measured in the same assay to avoid inter-assay errors.

### 2.7. Copulatory behavior and reproductive performance

During the first estrus after PND 75, female rats from experimental groups were submitted to the sexual behavior test. After detection of the estrous phase, female rats were put into cages of sexually experienced male rats, then allowed 10 mounts on the females while registering the presence of lordosis. Results were expressed as the lordosis quotient (lordosis number/10 mounts × 100) (Beach, 1976). All procedures were performed during the dark phase of the light/dark cycle, and females were used only once.

After the sexual behavior test, the females were maintained with the males for an additional 8 h. After finishing this period, rats were separated into individual cages and vaginal smears were collected for the detection of spermatozoa, to establish the initial gestational day (GD) 0. On GD 20, females were weighed and euthanized by $CO_2$. Gravid uteri and ovaries were collected, the number of corpora lutea were determined, implantation sites, resorptions, live fetuses, and weights of fetus and placentas were recorded. From these results, the following parameters were calculated: Gestational rate: number of pregnant females/number of inseminated females × 100; Fertility potential (efficiency of implantation): implantation sites/corpora lutea × 100; Rate of pre-implantation loss: (number of corpora lutea – number of implantations/number of corpora lutea) × 100; Rate of post-implantation loss: (number of implantations – number of live fetuses)/number of implantations × 100; and Sex ratio: number of female fetuses/number of male fetuses (Borges et al., 2017; Guerra et al., 2017).

### 2.8. Histological procedures

The left uterine horn and ovary, during the estrous phase, and placenta of fetuses on GD 20 were collected and immersed in Bouin's fixative solution, histologically processed and enclosed in Paraplast®. Then, three sections of each organ were cut at a thickness of 5 μm, with an interval of 50 μm, and placed onto silanized slides and stained with hematoxylin and eosin (H & E). The ovarian analysis was performed by observing the histological aspect of this organ and by counting the number of corpora lutea and follicles in the different stages of follicular development, as described by Talsness et al. (Talsness et al., 2005) and Guerra et al. (Guerra et al., 2014). In the uterine sections, the heights of perimetrium, myometrium, endometrium, and luminal epithelium were measured, as described by Silva et al. (e Silva et al., 2016). General histological and histopathological aspects of ovaries and uteri were assessed qualitatively, based on the guidelines described by Dixon et al. (Dixon et al., 2014). Placental tissues were

evaluated in their general histological aspect and the height of the basal zone was measured at five different points of each section. Histological analyses were conducted under light microscopy, with the software packages Leica QWin 3 and ImageJ 1.48.

### 2.9. Immunohistochemistry for Proliferating Cell Nuclear Antigen (PCNA)

Immunohistochemistry assay for Proliferating Cell Nuclear Antigen (PCNA) was performed based on Borges et al. (Borges et al., 2016) and Barros, et al. (de Barros et al., 2018), with modifications. Initially, the placentas were sectioned at a thickness of 5 μm and placed on silanized slides. Then, the sections were dewaxed with xylol, hydrated with decreasing concentrations of alcohol, and washed with phosphate-buffered saline (PBS - pH 7.4). Antigenic recovery was performed with citrate buffer (pH 6.0) for 10 min in a microwave. After this step, the sections were incubated for 15 min with hydrogen peroxide (3.5%) and PBS, for blocking the endogenous peroxidase. In the next step, the sections were incubated for 30 min with Bovine Serum Albumin (BSA 3%) diluted in PBS, and then washed with PBS to be incubated overnight with the primary anti-PCNA antibody (PCNA PC10: sc-56, Monoclonal, Santa Cruz Biotechnology, CA, USA – 1:100). After the incubation period, the sections were washed again with PBS and incubated for 1 h with the secondary antibody (Goat Anti-Mouse peroxidase-labeled IgG, Catalog No. 474–1806, KPL Antibody – 1:200). Then, after further washing with PBS, the cuts were submitted, for 4 min, to diaminobenzidine (DAB) associated with hydrogen peroxide. After the reaction, the sections were washed with water and counterstained with hematoxylin. At the end of the procedure, the sections were dehydrated with increasing concentrations of alcohol and then immersed in xylol. The sections were covered with coverslips and analyzed under light microscope, coupled to a digital camera and a computer containing the software Leica Q-win (Version 3). Immunostaining for PCNA on the placental tissues was evaluated specifically in the basal zone, qualitatively, according to immunostaining intensity, and classified as “absent”, “weak”, “moderate” or “strong”.

### 2.10. Statistical analysis

Data are presented as mean ± standard error of mean (S.E.M.), median and interquartile range or percentage. Shapiro-Wilk’s normality test was applied in the intragroup values, then results were compared among groups by one-way analysis of variance (ANOVA) followed by Tukey’s test, for parametric variables, and by Kruskal-Wallis followed by Dunn’s test, for nonparametric variables. Categorical variables derived from the qualitative analysis were evaluated by the frequency/proportion of occurrence/observation of the alterations in the experimental group. Then, the proportions were analyzed by the Fisher exact test. Differences were considered statistically significant when $p \leq 0.05$. Statistical analyses were performed using the software GraphPad Prism (Version 6.0).

## 3. Results

### 3.1. General physical signs of toxicity throughout in vivo procedures

No evident signs of general toxicity and stress (mortality and morbidity, excessive weight gain/loss, weakness, lethargy, presence of bristly hair, and abnormal behavior) derived from the treatment were observed throughout the experimental procedures.

### 3.2. Short-term effects of exposure to rosuvastatin starting at pre-puberty

The results related to the short-term effects of exposure of female rats to rosuvastatin are summarized in Table 1. In this study, rosuvastatin exposure at doses of 3 or 10 mg/Kg.bw$^{-1}$ was unable to alter the age of puberty onset, herein analyzed by the vaginal opening followed by the detection of the first estrus (Fig. 2). Additionally, on PND 42, the experimental group exposed to 3 mg/Kg.bw$^{-1}$ of rosuvastatin did not exhibit alterations in body weight (Fig. 3) or the weights of reproductive (uterus and ovaries) and target organs (pituitary, thyroid, liver, adrenals, and kidneys), compared to the control group (Table 2). Similar results were seen for the group exposed to rosuvastatin at 10 mg/Kg.bw$^{-1}$ (Fig. 3; Table 2). However, there was an increase in liver weight and a decrease in pituitary weight (Table 2) in the animals from this group, compared to the control group.

**Table 1**
Summary results of the study.

| Short-term exposure (PND 22–42) | 3 mg/Kg. bw$^{-1}$ | 10 mg/Kg.bw$^{-1}$ |
|---|---|---|
| Body weight | n.o.e. | n.o.e. |
| Age of puberty onset | n.o.e. | n.o.e. |
| Weight of reproductive organs | n.o.e. | n.o.e. |
| Weight of target organs | n.o.e. | ↑Liver and ↓Pituitary |
| [1]Levels of reproductive steroid hormones | n.o.e. | n.o.e. |
| [2]Levels of reproductive pituitary hormones | n.o.e. | n.o.e. |
| Histological endpoints of ovaries | n.o.e. | n.o.e. |
| Histological endpoints of uteri | n.o.e. | n.o.e. |
| **Long-term exposure (PND 22–75)** | **3 mg/Kg. bw$^{-1}$** | **10 mg/Kg.bw$^{-1}$** |
| Body weight | n.o.e. | n.o.e. |
| Regularity of estrous cycle | n.o.e. | ↓Lenght of estrous cycles |
| Weight of reproductive organs | n.o.e. | n.o.e. |
| Weight of target organs | n.o.e. | n.o.e. |
| [1]Levels of reproductive steroid hormones | n.o.e. | n.o.e. |
| [2]Levels of reproductive pituitary hormones | n.o.e. | ↓Prolactin |
| Histological endpoints of ovaries | n.o.e. | n.o.e. |
| Histological endpoints of uteri | n.o.e. | n.o.e. |
| Copulatory behavior | n.o.e. | ↓Receptivity to male mounts |
| Reproductive performance | n.o.e. | n.o.e. |
| Pregnancy outcomes | n.o.e. | ↓Placental weight |

[1]Reproductive steroid hormones measured in this study were testosterone and progesterone. [2]Reproductive pituitary hormones measured in this study were FSH, LH, and prolactin. PND: Postnatal day. n.o.e.: No observed effects when compared with the control group.

On PND 42, serum levels of FSH, LH, prolactin, progesterone, and testosterone were similar among experimental groups (Fig. 4). Histological analysis of ovarian sections did not show evidence of alterations in the morphological aspect of both experimental groups exposed to rosuvastatin (Fig. 5.A-C). It was observed that both medulla and cortical regions of ovaries were histologically similar among experimental groups, and there was no increase in proliferative and non-proliferative lesions in this organ and its related structures at both doses tested. Also, the quantification of ovarian follicles, corpora lutea, and atretic follicles indicated a similar percentage of structures among the experimental groups (Fig. 5.G). Uterine tissues assessed histologically on PND 42 indicate a lack of morphological alterations promoted by rosuvastatin exposure on both doses tested (Fig. 5.D-F). Furthermore, morphometrical analyses of uterine layers showed no effects at this age by the exposure to the statin (Fig. 5.H).

### 3.3. Long-term effects of exposure to rosuvastatin starting at pre-puberty

The results related to the long-term effects of exposure of female rats to rosuvastatin are summarized in Table 1. Reproductive cyclicity (frequency of each phase and number of estrous cycles) of animals from the experimental groups was not altered by exposure to rosuvas-

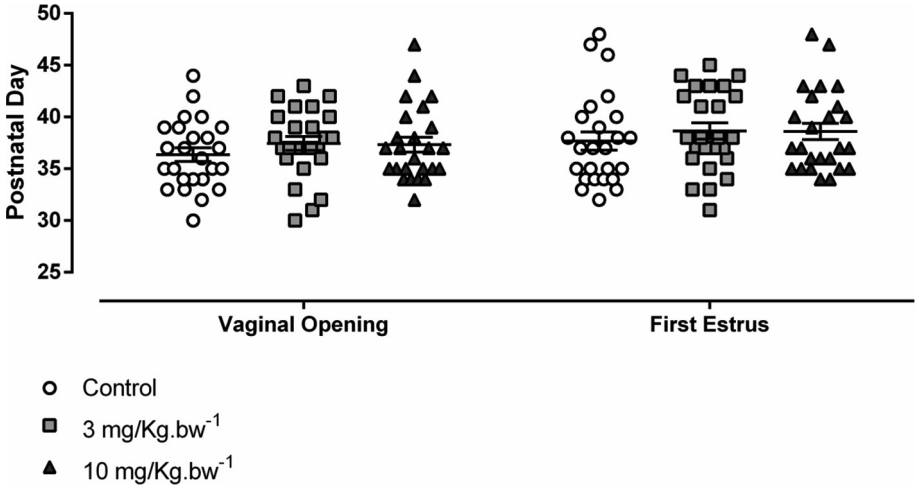


**Fig. 2.** Evaluation of external signs of puberty onset: Ages of vaginal opening and first estrus (n = 25/group). Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. $p > 0.05$.

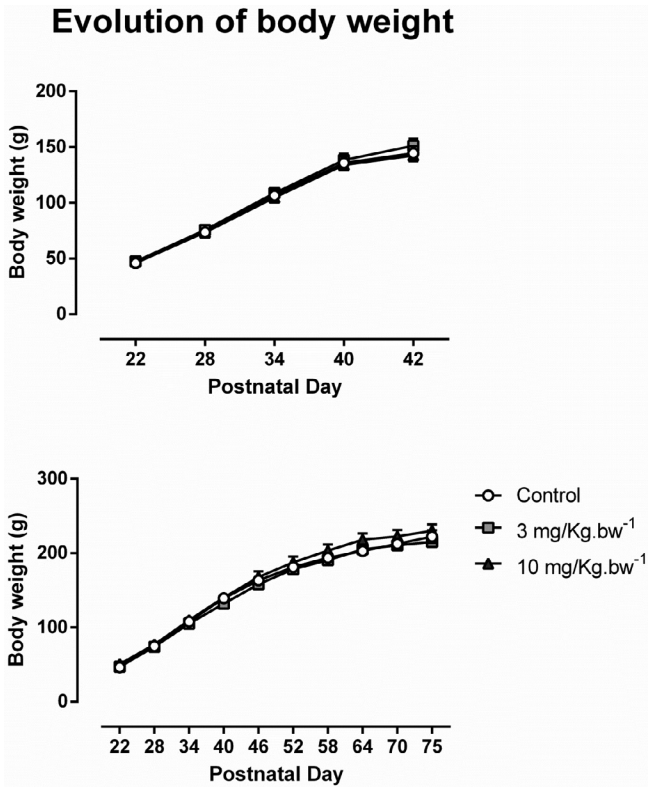


**Fig. 3.** Evolution of body weight of female rats euthanized on postnatal days 42 or 75, after treatment with rosuvastatin or vehicle (n = 6–7/group). Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. $p > 0.05$.

tatin, except those treated with rosuvastatin at doses of 10 mg/Kg.bw$^{-1}$, which presented shorter estrous cycles than the control group (Fig. 6). Despite that, on PND 75, weights of the whole body (Fig. 3) and organs (ovaries, uterus, pituitary, thyroid, liver, adrenals, and kidney) (Table 2) were similar in rosuvastatin at doses of 3 and 10 mg/Kg.bw$^{-1}$, compared to the control group. At this age, serum levels of FSH, LH, progesterone, and testosterone were similar among experimental groups (Fig. 4). However, serum prolactin levels were reduced in animals exposed to 10 mg/Kg.bw$^{-1}$ of rosuvastatin, but not at 3 mg/Kg.bw$^{-1}$.

**Table 2**
Body and organ weights of female rats during the estrous phase on postnatal days 42 and 75.

| Parameters | Experimental Groups | | |
|---|---|---|---|
| *Postnatal Day 42 (n = 6–7/group)* | Control | 3 mg/Kg. bw$^{-1}$ | 10 mg/Kg. bw$^{-1}$ |
| Body weight (g) | 144.6 ± 6.2 | 151.2 ± 6.3 | 142.4 ± 5.1 |
| *Relative organ weights* | | | |
| Ovaries (mg/100 g) | 38.06 ± 3.8 | 45.07 ± 7.0 | 45.33 ± 4.8 |
| Uterus with fluid (mg/100 g) | 187.8 ± 35.9 | 187.0 ± 19.1 | 170.8 ± 8.4 |
| Pituitary (mg/100 g) | 4.21 ± 0.2 | 4.73 ± 0.7 | 2.67 ± 0.4 * |
| Thyroid (mg/100 g) | 9.30 ± 1.4 | 8.48 ± 0.6 | 7.04 ± 0.7 |
| Liver (g/100 g) | 5.07 ± 0.07 | 5.38 ± 0.11 | 5.73 ± 0.14 ** |
| Adrenals (mg/100 g) | 36.35 ± 4.3 | 33.12 ± 4.2 | 34.32 ± 1.3 |
| Kidneys (g/100 g) | 1.01 ± 0.03 | 1.06 ± 0.01 | 1.06 ± 0.02 |
| *Postnatal Day 75 (n = 7/group)* | Control | 3 mg/Kg. bw$^{-1}$ | 10 mg/Kg. bw$^{-1}$ |
| Body weight (g) | 222.4 ± 8.7 | 215.3 ± 5.1 | 230.1 ± 8.6 |
| *Relative organ weights* | | | |
| Ovaries (mg/100 g) | 42.02 ± 4.1 | 38.76 ± 4.8 | 42.55 ± 1.9 |
| Uterus with fluid (mg/100 g) | 218.3 ± 38.8 | 218.7 ± 30.0 | 243.5 ± 40.7 |
| Pituitary (mg/100 g) | 5.99 ± 0.5 | 5.59 ± 0.4 | 6.02 ± 0.9 |
| Thyroid (mg/100 g) | 8.07 ± 1.2 | 7.43 ± 0.5 | 7.99 ± 0.7 |
| Liver (g/100 g) | 4.20 ± 0.07 | 4.11 ± 0.07 | 4.32 ± 0.08 |
| Adrenals (mg/100 g) | 45.33 ± 2.3 | 42.99 ± 0.6 | 46.82 ± 2.2 |
| Kidneys (g/100 g) | 0.88 ± 0.02 | 0.89 ± 0.03 | 0.92 ± 0.02 |

Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. *p ≤ 0.05, **p < 0.01 compared to control group.

Histopathological evaluation (Fig. 7.A-C) and quantification of ovarian follicles and structures (Fig. 7.G), on PND 75, showed no alterations associated with 3 and 10 mg/Kg.bw$^{-1}$ of rosuvastatin exposures. Uteri histological sections at this age did not present a sign of abnormal morphological alterations (Fig. 7.D-F) nor in height of the uterine layers in both doses tested in this study (Fig. 7.H).

The sexual behavior test showed that females exposed to rosuvastatin at the dose of 10 mg/Kg.bw$^{-1}$ were less receptive to male mounting than control animals. In this test, 4 of the 9 females from the 10 mg/Kg.bw$^{-1}$ group used during the estrous phase of these animals (44.4%) were not receptive to male mounting. The other 5 females (55.6%) were receptive during the test [lordosis quotient 90.0% (75.0–100.0)], as were all the females from the control [n = 8; lordosis quotient 100.0% (90.0 – 100.0)] and 3 mg/Kg.bw$^{-1}$ [n = 9; lordosis quotient 90.0% (75.0 – 100.0)] groups (Kruskal-Wallis test, followed by Dunn's test. $p = 0.2612$).

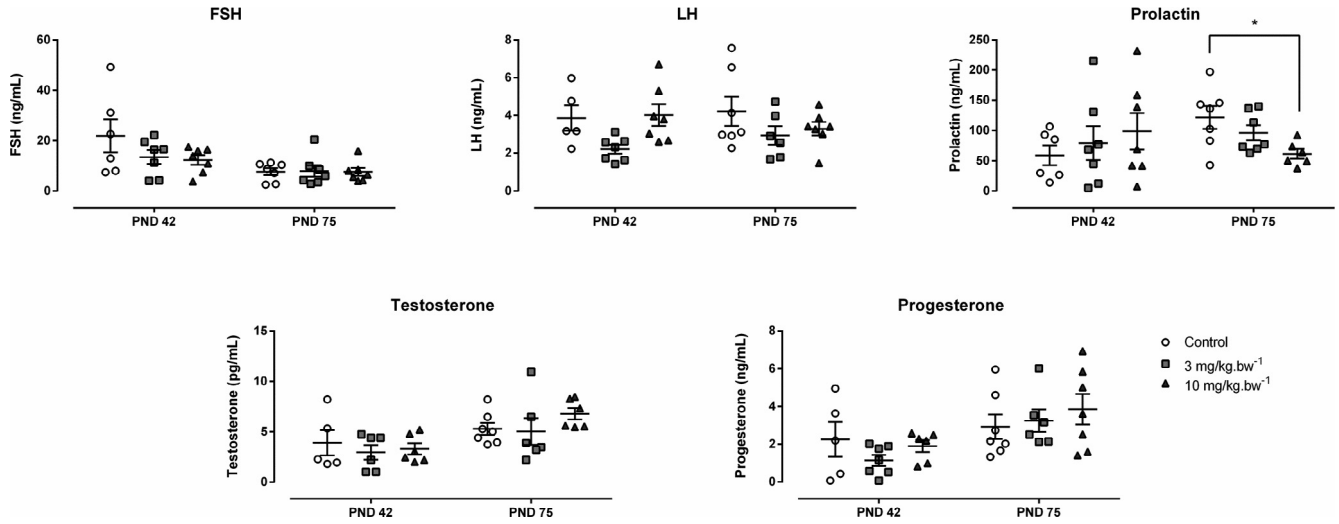


**Fig. 4.** Serum levels of FSH, LH, prolactin, testosterone, and progesterone of female rats, during the estrus phase on PND 42 (n = 6–7/group) and 75 (n = 7/group). Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. *$p < 0.05$ compared to the control group. PND (Postnatal Day).

Reproductive performance assessed on GD 20 by gestational rate, fertility potential, numbers of implantation sites, corpora lutea, fetus, and resorptions showed similar results among groups (Table 3). Also, there are no alterations associated with rosuvastatin at doses of 3 and 10 mg/Kg.bw$^{-1}$ in the percentage of pre- and post-implantation losses, as well as in fetal and gravid uterus weights, and sex ratio (Table 3). However, placental weight was decreased among the offspring of animals exposed to the highest dose of rosuvastatin, when compared to the control group (Table 3). Additionally, histopathological evaluation of placental tissues and morphometry of the basal zone did not show any evidence of alterations induced by rosuvastatin exposure at both doses (Fig. 8). Similarly, immunohistochemistry for PCNA in this transitory organ revealed a similar immunostaining pattern among experimental groups.

## 4. Discussion

Cholesterol is a molecule of great relevance in reproductive physiology, because of its importance as a precursor of sex steroid hormone synthesis. This study represents an initial general reproductive toxicity study in female rats exposed to rosuvastatin, a third-generation statin, a class of drugs that inhibit the biosynthesis of cholesterol. Besides the beneficial effects of statin therapy, some adverse effects should be considered before starting the treatment, such as myopathy, nephrotoxicity, neurologic manifestations, proinflammatory and immunogenic actions, and hepatotoxicity (Grover et al., 2014).

In the current study, rosuvastatin exposure showed no evident sign of toxicity in target organs for toxicological study, based on their weights. However, during puberty, on PND 42, liver weight was increased by rosuvastatin treatment at the highest dose. Statins present some hepatotoxicity potential, by increasing apoptosis in cultured human hepatocytes and oxidative stress in the liver (Beltowski et al., 2009), but in the current study, the increased liver weight might have been associated with its adaptation to the rosuvastatin exposure, because alterations in this parameter were not found after long-term exposure, on PND 75. Ahmadi et al. (Ahmadi et al., 2018) show that rosuvastatin exposure in rats is related to a compensatory mechanism in cholesterol biosynthesis and metabolism in the liver and extrahepatic tissues, which bolsters the idea of adaptive response by the liver to the statin.

On PND 42, female rats exposed to rosuvastatin at the dose of 10 mg/Kg.bw$^{-1}$ also presented reduced pituitary weight, an effect not found during adulthood. Statin exposure might affect pituitary function since it has the potential to inhibit the release of follicle-stimulating hormone in female rats (Guldvang et al., 2015), and most of the pituitary hormones cultured *in vitro* from baboon pituitary cells (Vázquez-Borrego et al., 2020). However, in the present study, serum gonadotropin hormone levels were not affected by the treatment. It is important to note that pituitary weight was not decreased in adult rats exposed to rosuvastatin, but their serum prolactin levels were reduced by the treatment. Therefore, it is reasonable to hypothesize that rosuvastatin might interact with lactotroph cells in the anterior pituitary and affect its proliferation and function processes. Additionally, given that in rodents, estrogen signaling is the major stimulatory factor for pituitary development (Cooper et al., 1989) and prolactin gene expression (Featherstone et al., 2012), rosuvastatin may exert its effects upon estrogen releasing and signaling, and consequently impair this organ.

During puberty, the hypothalamic-pituitary-ovarian axis becomes active and is crucial to the occurrence of normal morphological, physiological, behavioral, and psychological changes in the female (Parent et al., 2003). This period is also considered a biological sensor for abnormalities derived from genetic and environmental interactions during pre and postnatal development (Castellano et al., 2018). External markers of pubertal development are relevant for investigating the time of puberty onset. The vaginal opening and first occurrence of estrus in rats are indicators of puberty onset, derived from an increased level of estrogen in the blood (U.S. E.P.A., 1996). These events are also associated with the first ovulation in female rats (Castellano et al., 2018).

Rosuvastatin exposure starting in pre-puberty delays puberty onset in male rats as shown by Leite et al. (Leite et al., 2014). However, in the present study, this statin neither altered the timing of puberty in female rats nor affected the serum levels of reproductive hormones (testosterone and progesterone), ovarian follicular dynamics, or uterine morphology during puberty. These data indicate that rosuvastatin might lead to gender-specific alterations in pubertal development.

Assessment of estrous cyclicity is a means of evaluating the integrity of the hypothalamic-pituitary-ovarian axis and consequently female reproductive function (Goldman et al., 2007). In the latter study, the estrous cycle was altered by rosuvastatin exposure, given that animals treated with the highest dose of the statin presented shorter cycles than the control group. On the other hand, during adulthood, serum levels of FSH, LH, progesterone, and testosterone were not affected by the rosuvastatin treatment, as well as ovarian and uterine histological endpoints and reproductive organ weights. Guerra

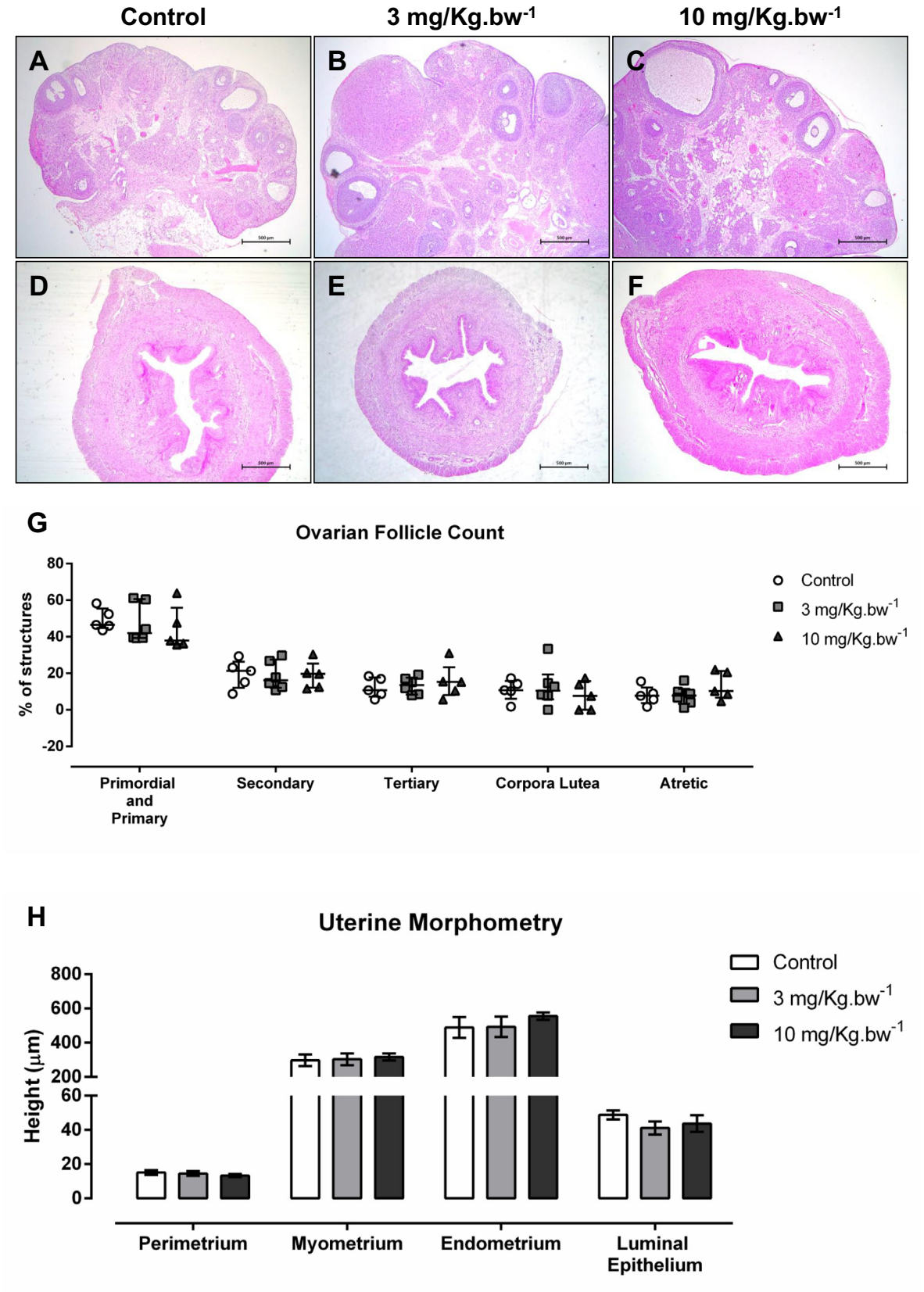


**Fig. 5.** Histological evaluation of ovary and uterus of female rats, during the estrus phase on postnatal day 42. (A-C) Representative histological aspect of ovaries. (D-F) Representative histological aspect of uteri. (G) Follicular count in ovarian sections (n = 5–6/group). Values expressed as median and interquartile range. Kruskal-Wallis test, followed by Dunn's test. $p > 0.05$. (H) Histomorphometric measurements of uterine layers (n = 6–7/group). Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. $p > 0.05$.

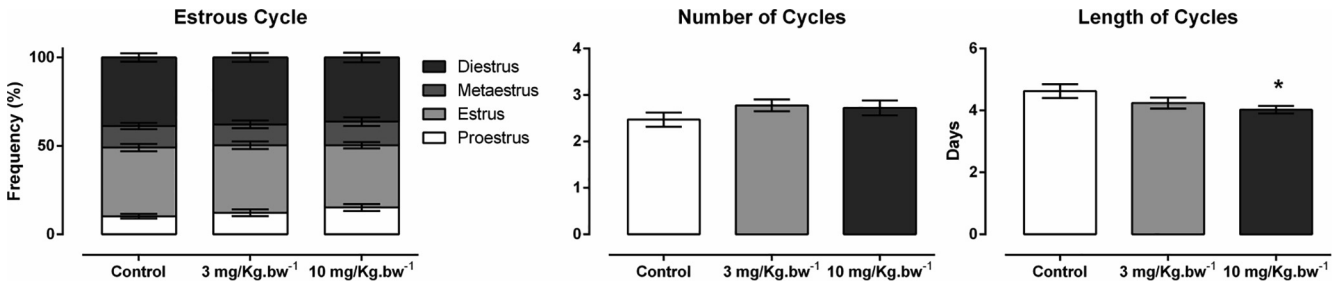


**Fig. 6.** Evaluation of estrous cycle for 15 consecutive days (n = 17–18/group). Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. *$p \leq 0.05$ compared to the control group.

et al. (Guerra et al., 2014, 2013) demonstrated that reproductive function is not necessarily affected by the alterations in serum levels of steroid hormones, but is influenced by their signaling pathways mediated by cell receptors, whose expression pattern can be altered in different cells and tissues. Thus, impaired reproductive cyclicity of female rats exposed to rosuvastatin might originate from an abnormal hormonal axis signaling.

Alterations in hormonal signaling caused by rosuvastatin exposure can also impair sexual behavior, as suggested by the fact that females presented disrupted reproductive cycles. Furthermore, another factor that could be influencing reproductive behavior in female rats treated with the highest dose of rosuvastatin is the uterine physiology, which is highly dependent on steroid hormones (Taylor and Gomel, 2008). Studies of ovariectomized rats show that impaired hormone binding capacity in the uterine tissue is related to inhibitory effects in female receptivity (AHDIEH, 1984), which may explain the absence of a lordosis quotient in some of the female rats as observed in this study. Thus, further studies regarding uterine physiology after statin exposure might clarify the possible mechanisms of rosuvastatin upon female reproductive function.

A recent study from our research group showed that reproductive parameters such as sexual behavior and epididymal morphology were affected after long-term exposure to rosuvastatin with no effect on serum testosterone levels (Silva et al., 2020), which corroborates the idea that rosuvastatin might disrupt hormonal signaling through interactions with steroid receptors.

In the current study, treatment with rosuvastatin in female rats was initiated in the pre-pubertal period and concluded in adulthood, before confirmation of pregnancy, because statin therapy is not indicated during gestation (Zarek et al., 2013). By the end of the gestational period, in this study, no evident signs of maternal toxicity or fertility impairment from rosuvastatin exposure were found, nor any evidence of fetal growth restriction or impairment of offspring intrauterine development. However, the mean placental weight of animals exposed to the highest dose of this statin, in this experimental design, was lower than that of the control group. Studies of rosuvastatin exposure in male rats show that post-implantation losses are increased by this statin due to decreased sperm quality (Leite et al., 2017); however, one study by Dostal et al. (Dostal et al., 1996) of atorvastatin, another statin, did not find evidence of fertility impairment in either male or female rats. Considering the latter finding, it is reasonable to infer that rosuvastatin might lead to fertility impairments in a gender-specific manner.

The placenta corresponds to the maternal-fetal interface for the exchange of substances (Charest et al., 2018). In rats, the placenta becomes completely functional at mid-gestation and grows continuously up to the last few days before parturition, when the placental weight stays relatively stable (Cline et al., 2014). Alterations in the placental weight might compromise fetal development, which is associated with fetal reprogramming effects (Longtine and Nelson, 2011).

Histologically, the placenta is composed of four distinct parts, two of which constitute the fetal components while the other two correspond to the maternal components of the placenta, namely the decidua and metrial gland, both derived from the endometrium. The fetal placental components are the labyrinth zone, where maternal and fetal exchanges occur; and the basal zone (also known as Trophospongium), formed by three different types of trophoblastic cells: the spongiotrophoblast cells, the glycogen cells, and the trophoblast giant cells. The latter cells perform important roles for the maintenance of pregnancy, such as endocrine function and releasing of factors and molecules that promote local and systemic physiological maternal adaptations throughout pregnancy (Charest et al., 2018; Cline et al., 2014).

In the present study, the placental histology of rosuvastatin-exposed animals was not altered. Even cell proliferation status, assessed by immunostaining with PCNA marker, did not show any signs of placental tissue impairment, especially in the basal zone. Alarcon & Marikawa (Alarcon and Marikawa, 2016) show that statin exposure in mouse embryos is related to impaired trophoblast initial differentiation and consequently inhibition of blastocyst formation due to cellular mechanisms associated with inhibition of geranylgeranylation processes, a post-translational process of modification of proteins. This suggests that statins affect trophoblast cells, and have the potential to compromise placental development. Here, despite the absence of alterations in fetus weight, further investigations are necessary to conclude whether rosuvastatin has the potential to promote fetal reprogramming through placental damage related to apoptosis, proliferation, or other cellular mechanisms.

The estrogen signaling pathway is crucial for the development and physiology of female reproductive parameters (48). Lack of estradiol serum levels represents a limitation of this study. In a previous study with female offspring of rats whose fathers were exposed to rosuvastatin, the rats exhibited altered morphology of luminal epithelium of uterus, indicative of reduced estradiol concentrations, and the same limitation with estradiol measurements was found (20). The results obtained in this study also indicate that estradiol concentrations and/or signaling were affected by direct rosuvastatin exposure in female rats, by alterations promoted in the estrous cyclicity, copulatory behavior, pituitary weight, prolactin concentrations, endpoints highly dependent on estrogenic signaling.

It is not possible to ignore the fact that inhibition of HMG-CoA reductase promoted by statins also presents influences in the mevalonate pathway, not only by cholesterol biosynthesis but by the other derivates from this pathway called isoprenoids (Mo and Elson, 2006). Intermediates isoprenoids of the mevalonate pathway include farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which are involved in different cell events, such as cell signaling, differentiation, proliferation, and cytoskeleton dynamics (Mo and Elson, 2006; Buhaescu and Izzedine, 2007).

The alterations in the reproductive parameters observed in this study with the highest dose of rosuvastatin are indicative of a possible endocrine disruption mechanism of action related to the estrogen signaling pathways. However, many reproductive endpoints closely

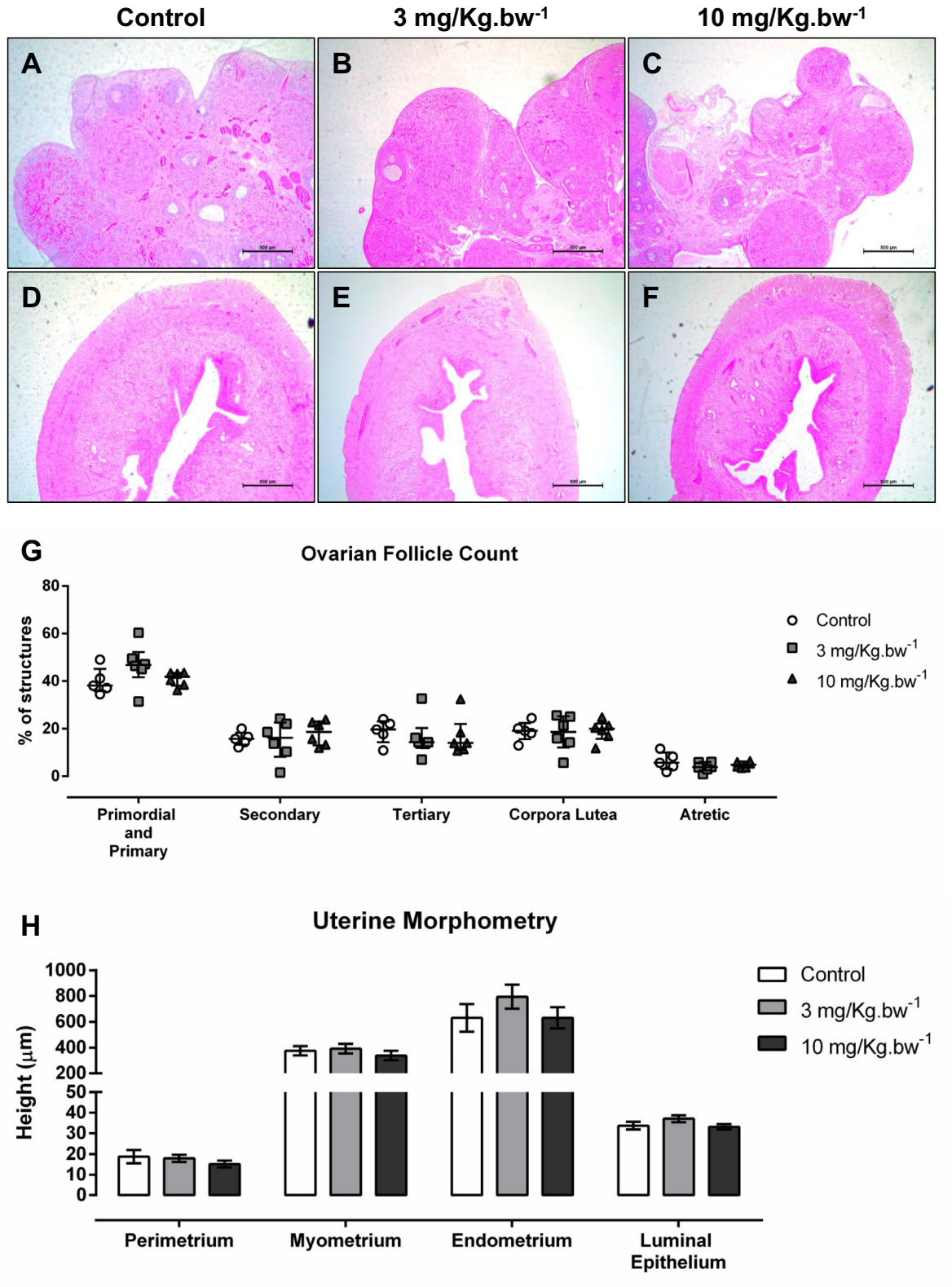


**Fig. 7.** Histological evaluation of ovaries and uteri of female rats, during the estrus phase on postnatal day 75. (A-C) Representative histological aspect of ovaries. (D-F) Representative histological aspect of uteri. (G) Follicular count in ovarian sections (n = 5–6/group). Values expressed as median and interquartile range. Kruskal-Wallis test, followed by Dunn's test. $p > 0.05$. (H) Histomorphometric measurements of uterine layers (n = 7/group). Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. $p > 0.05$.

related to this steroid hormone, such as puberty timing and reproductive organ morphology were not affected by the treatment in both doses tested.

The synthesis of mevalonate pathway products inhibited by the rosuvastatin exposure could be involved with the alterations observed in the reproductive parameters of female rats exposed to 10 mg/Kg.

**Table 3**
Reproductive performance and fertility test assessed on gestational day 20.

| Parameters | Experimental Groups | | |
|---|---|---|---|
| | Control (n = 9) | 3 mg/Kg.bw$^{-1}$ (n = 8) | 10 mg/Kg.bw$^{-1}$ (n = 5) |
| Gestational rate (%) | 88.8 | 75.0 | 80.0 |
| [2]Fertility potential (%) | 96.88 (92.98–100.00) | 96.16 (87.98–100.00) | 96.16 (90.58–100.00) |
| [1]Number of Implantations | 12.7 ± 0.7 | 12.5 ± 0.8 | 10.7 ± 0.6 |
| [1]Number of corpora lutea | 13.5 ± 0.8 | 13.3 ± 0.4 | 11.2 ± 0.6 |
| [1]Number of fetuses | 12.2 ± 0.6 | 12.2 ± 0.6 | 10.7 ± 0.6 |
| [1]Number of resorptions | 0.5 ± 0.3 | 0.3 ± 0.3 | 0.0 ± 0.0 |
| [2]Pre-implantation loss (%) | 3.1 (0.0–7.0) | 3.8 (0.0–12.0) | 3.8 (0.0–9.4) |
| [2]Post-implantation loss (%) | 0.0 (0.0–6.8) | 0.0 (0.0–3.3) | 0.0 (0.0–0.0) |
| [1]Gravid uterus weight (g) | 71.8 ± 4.5 | 70.0 ± 3.9 | 63.3 ± 2.7 |
| [1]Fetal weight (g) | 4.05 ± 0.10 | 3.89 ± 0.14 | 4.05 ± 0.09 |
| [1]Placental weight (g) | 0.66 ± 0.02 | 0.62 ± 0.03 | 0.56 ± 0.02* |
| [1]Sex ratio (F:M) | 0.91 ± 0.1 | 1.12 ± 0.3 | 0.71 ± 0.2 |

Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. *$p \leq 0.05$ compared to control group. [2]Values expressed as median and interquartile intervals. Kruskal-Wallis test, followed by Dunn's test. $p > 0.05$.

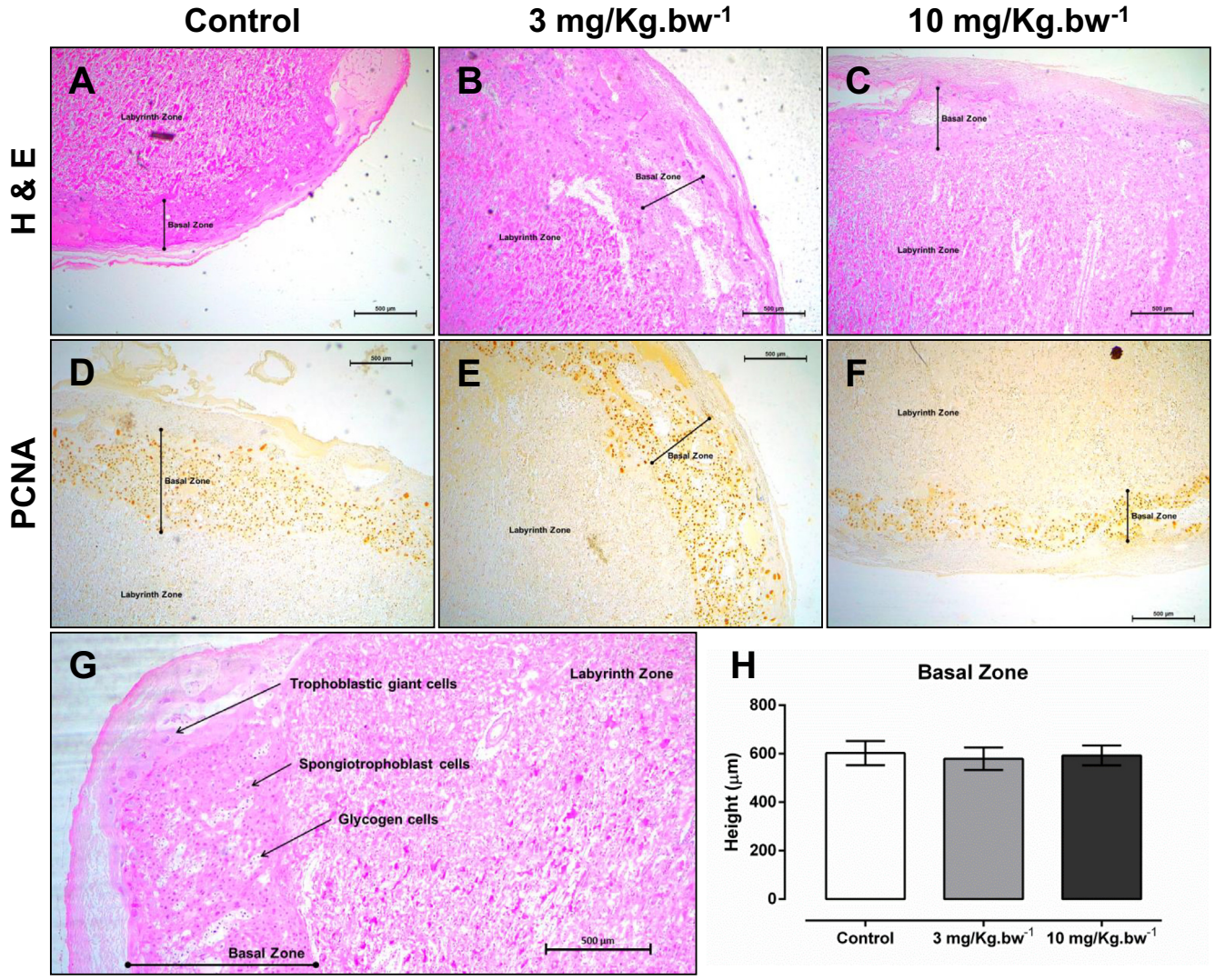


**Fig. 8.** Histological evaluation of placentas of pregnant female rats on gestational day 20. (A-C) Representative histological aspect of placentas. (D-F) Immunostaining for Proliferating Cell Nuclear Antigen (PCNA) in placental basal zone cells. (G) Histological organization of a basal zone cell. (H) Histomorphometric measurement of basal zone (n = 4–5/group). Values expressed as mean ± S.E.M. ANOVA followed by Tukey's test. $p > 0.05$.

$bw^{-1}$ of rosuvastatin, once the effects found in this study do not show a clear relationship or clues that they are derived from the disruption in the hypothalamic-pituitary-ovarian axis.

In conclusion, the present study shows that the exposure of prepubertal female rats to rosuvastatin, at a dose used in human clinics, affected reproductive endpoints at adulthood. The results suggest that non-endocrine mechanisms might be involved, by altering cell and tissue dynamics, probably due to the pleiotropic effects of this statin. Additional studies on the effects of this statin on female reproductive function and development are encouraged to better characterize its mode of action.

## CRediT authorship contribution statement

**Jorge W.F. Barros:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Karolina S. Tonon:** Methodology, Formal analysis, Investigation. **Cibele S. Borges:** Methodology, Formal analysis. **Patrícia V. Silva:** Methodology, Formal analysis. **Ana F.Q. Lozano:** Methodology. **Tainá L. Pacheco:** Methodology. **Janete A. Anselmo-Franci:** Methodology, Resources. **Wilma G. Kempinas:** Conceptualization, Validation, Formal analysis, Resources, Writing - review & editing, Supervision, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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