EFFECT OF LEPTIN ON FOLLICULOGENESIS DURING DEVELOPMENT IN FEMALE WISTAR RAT

GHOURI[1, 2)*] Imane, Hanane BOUCHEFA[2)], Amina BOUBEKRI[3)], Amel GHOZLANI[2)], Mohamed BACHIR PACHA[1)], Rachid KAIDI[1)], Fatima HADJ-BEKKOUCHE[2)]

[1)]Laboratory of Biotechnologies related to Animal Reproduction, Institute of Veterinary Sciences, University of Blida-1, Route de Soumâa, BP 270, 09 000, Blida, Algeria;
[2)]Laboratory of Endocrinology, Faculty of Biological Sciences, University of Sciences and Technology Houari Boumediene (USTHB), BP 32, El Alia, Bab Ezzouar, 16 111, Algiers, Algeria; [3] Laboratory of Research on Arid Lands (LRZA), Faculty of Biological Sciences, University of Sciences and Technology Houari Boumediene (USTHB), BP 32, El Alia, Bab Ezzouar, 16 111, Algiers, Algeria
** Corresponding author: imaneghouri@yahoo.fr*

Abstract. This study aims to investigate the effect of leptin on ovaries in female Wistar rat during development. Rats were divided into two groups: 30 and 60-day-old females. Experimental treated females were administrated 8 µg/100 g body weight intraperitoneal leptin injections daily for 5 days, while controls received the same volume of saline solution. Leptin treatment resulted in a significant decrease in body weight (150.50 ± 4.02 vs 157.78 ± 3.51 g, $p < 0.05$*) and a very significant decrease in food intake (14.75 ± 0.31 vs 16.33 ± 1.22 g, $p < 0.01$**) in the 60-day group. Vaginal smears did not reveal an estrus stage after treatment. Histological examination revealed a suspension of ovulation and an acceleration of the luteinization of granulosa cells in adult treated group in relation to the elevation of LH (0.301 ± 0.032 vs 0.189 ± 0.020 IU/L, $p < 0.05$*) and progesterone (41.60 ± 4.58 vs 33.39 ± 5.13 ng/mL, $p > 0.05$). For FSH, the difference between controls and treated females was not significant in both ages ($p > 0.05$). Leptin appears to be involved in the regulation of body weight, folliculogenesis, the ovulatory process and luteogenesis by control of gonadotropic hormones and progesterone at the age of 60 days but not at 30 days, when leptin receptors are probably not expressed yet.

Keywords: Leptin, Body weight, Folliculogenesis, Gonadotropins, Progesterone, Rat

INTRODUCTION

Leptin is a non-glycosylated protein of 16 kDa essentially secreted by white adipose tissue (Zhang et al., 1994). At the central level, it regulates energy metabolism and body weight, as well as appetite by controlling the sensation of satiety (Ahima and Flier, 2000). Leptin intervenes in many physiological processes. At the peripheral level, leptin is involved in the immune response, inflammation, stimulation of angiogenesis, control of blood pressure, hematopoiesis, osteogenesis and also in the onset of puberty and reproduction (Chehab et al., 1996; Ahima et al., 1997; Umemoto et al., 1997; Fantuzzi and Faggioni, 2000; Mantzoros, 2000; Park et al., 2001; Sagawa et al., 2002). These effects are related to the ubiquitous distribution of mRNAs of these Ob-R receptors that have been localized in the central nervous system and in peripheral tissues, with a predominant expression in the hypothalamus (Mercer et al., 1996; Fei et al., 1997; Zamorano et al., 1997).

Leptin was the first adipokine relating nutritional status to reproductive function. It plays a crucial role in the physiology of the reproductive system as "a messenger" informing the hypothalamic-pituitary axis on the available energy reserves that allow for fertilization, pregnancy and lactation. It regulates the secretion of GnRH (Gonadotropin Releasing Hormone) in the hypothalamus, modulates the secretion of LH (Luteinizing Hormone) and FSH (Folliculo-Stimulating Hormone) in the anterior pituitary (Jin et al., 1999) and acts directly on the ovaries controlling ovulation by an endocrine and paracrine effect (Cioffi et al., 1997; Karlsson et al., 1997). The aim of this work was to study the effects of leptin on folliculogenesis in female Wistar rats aged of 30 days (immatures) and 60 days (adults).

MATERIELS AND METHODS

Ethical approval. Experimental procedures were approved by the Institutional Animal Care Committee of the National Administration of the Algerian Higher Education and Scientific Research (Ethical approval number: 98-11, Law of August 22, 1998) and were conducted according to recommendations edited in the "Guide for the Care and Use of Laboratory Animals" (NIH Publications No. 8023, revised 1978). All efforts were made to minimize the suffering of rats.

Animals. The experiments were carried out on female albinos Wistar rats (*Rattus norvegicus*). The animals were divided into two groups: 30 and 60-day-old females. Each group included two subgroups: controls (C) and leptin-treated females (T). The control and its corresponding experimental treated female had almost the same initial body weight (b.w.): 55.58 ± 4.17 g *vs* 55.42 ± 4.64 g (for the 30-day group) and 150.89 ± 2.83 g *vs* 150.50 ± 4.22 g (for the 60-day group). Rats were kept at ambient temperature and hygrometry. The laboratory having a fully glazed facade, allowed the rats' exposure to external lighting.

Experimentation. 30 g of "Rats and Mice" food and 200 mL of drinking water with 0.5 mL of vitamins were distributed daily. Rats' body weights and food intake were measured daily during the experimental period. Leptin (Leptin rat, Lyophilised powder, Sigma-Aldrich®) was diluted in NaCl (9‰). 8 µg of leptin / 100 g b.w. were injected intraperitoneally daily at 9:00 am for 5 days. The same volume of NaCl solution was injected into the control females. Rats were euthanized 24 hours after the last injection by decapitation. Blood was collected in a dry tube supplemented with EDTA (2%) and centrifuged. Plasma was distributed in eppendorf tubes and immediately frozen at - 20 °C for LH, FSH and progesterone assays. The ovaries were removed and fixed in 10% formaldehyde for histological study.

Vaginal smear. In order to determine the stage of the estrous cycle at the moment of sacrifice, a vaginal smear was performed on adult females. The observations were made either directly under a photonic microscope or after May-Grünwald Giemsa (MGG) staining.

Hormonal assays. The immunoradiometric assay of LH and FSH was performed using IRMA Kits, while the radioimmunoassay of progesterone was performed using RIA kits (Immunotech®).

Histology. After dehydration, clarification and inclusion of the ovaries in paraffin, serial sections of 2 µm thick were made using a microtome along a sagittal

plan. Two topographic histological stains were used: Hematoxylin Eosin (H&E) stain and modified Masson Trichrome stain. Photographs were taken using a Hirocam® camera. Histological images were directly recorded on a computer via TSView software before being analyzed.

Statistical analysis. The results were expressed in mean ± SEM. Statistical analysis was performed using the Student's test (t-test). The level of significance is: $p < 0.05$ (p = P-value).

RESULTS AND DISCUSSION

Body weight. For the 30-day-old females, the body weight and the food intake curves were similar between control and leptin-treated females ($p > 0.05$). For the 60-day old rats, a significant decrease in body weight ($p < 0.05$*) as well as a very significant decrease in the quantity of food ingested ($p < 0.01$**) were observed in the 5th day in treated females compared to controls (Table 1 and Figure 1).

Table 1

Evolution of food intake and body weight at 30 and 60 days of age

Variables	30 / C (n=12)	30 / E (n= 12)	60 / C (n=9)	60 / E (n=8)
Food intake at D1 (g)	8.63 ± 0.47	8.63 ± 0.63	16.11 ± 0.74	15.88 ± 0.70
Food intake at D5 (g)	10.75 ± 0.70	10.25 ± 0.48	16.33 ± 1.22	14.75 ± 0.31
Body weight at D1 (g)	55.58 ± 4.17	55.42 ± 4.64	150.89 ± 2.83	150.50 ± 4.22
Body weight at D5 (g)	69.83 ± 4.53	70.42 ± 4.75	157.78 ± 3.51	150.50 ± 4.02

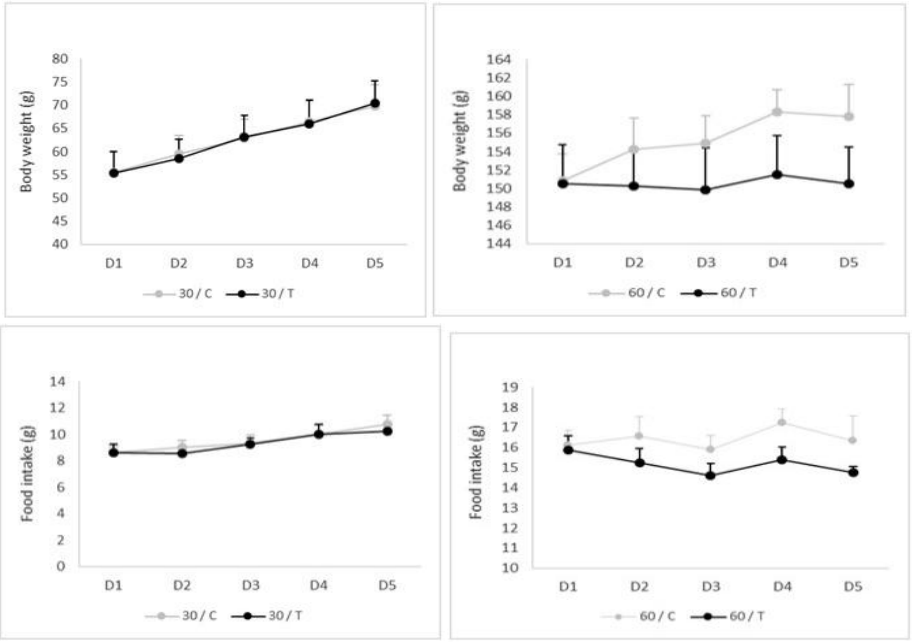
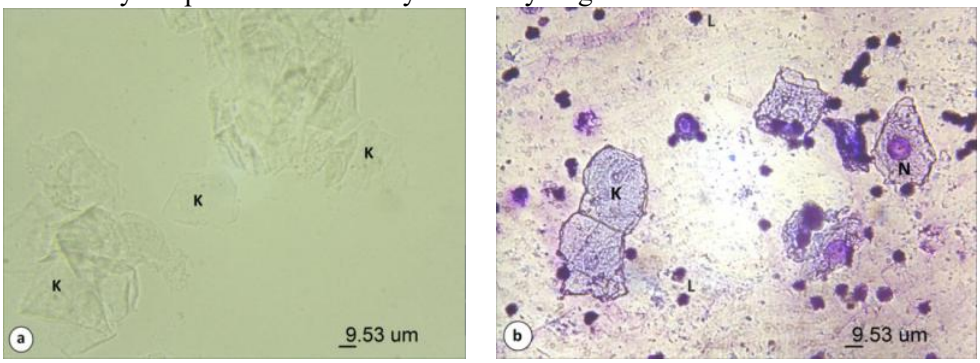


Fig. 1. Evolution of body weight and food intake in control (C) and leptin-treated (T) females at the age of 30 days (left) and 60 days (right)

Our results showed that daily intraperitoneally administration of 8 μg of leptin / 100 g b.w. to female Wistar rats did not seem to have any effect on body weight and food intake at 30-day age. Yuan et al. (2000) observed a significant decrease in body weight after daily administration of leptin at 0.3 μg/g and 1 μg/g peripherally in neonatal rats during the breastfeeding. However, we noted a significant decrease in body weight and food intake in adult females confirming the role of leptin, which acts directly on the hypothalamus, limiting food intake and increasing energy expenditure and thermogenesis (Friedman and Halaas, 1998). In humans, patients with a mutation of leptin or its receptor exhibit severe obesity and hyperphagia (Montague et al., 1997) corrected by daily injections of recombinant leptin. In adult Sprague Dawly rats, a non-significant decrease in body weight has also been reported after intracerebroventricular infusion of 3 μg of leptin per day for 4 days (Peters et al., 2007). Nevertheless, leptin bolus injections in obese Wistar rats or chronic elevation of leptinemia in the mouse hypothalamus after weight reduction cause insulin resistance; these rats become insensitive to leptin and regain weight comparing to controls (Buison et al., 2004; Zhang and Scarpace, 2006).

Leptin has different effects on the neurons of the arched, ventromedial, lateral and ventral premammillary hypothalamic nuclei, which are involved in the regulation of body weight and reproductive function. It works by inhibiting the orexigenic factors represented mainly by neuropeptide Y (NPY) and by stimulating those with anorectic actions (Sawchenko, 1998; Elmquist et al., 1999; Schwartz et al., 2000). Leptin acts on kisspeptin neurons that play a direct role in fertility related to metabolism (Luo et al., 2016).

Vaginal smear. The examination of vaginal smear revealed the presence of all stages of the estrous cycle in the control females: the proestrus is characterized by the presence of round epithelial cells and sometimes a few rare polynuclear cells. Estrus is characterized by the presence of anucleated keratinized epithelial cells (Figure 2a). The postestrus (Figure 2b) is marked by the presence of polynuclear cells with or without keratinized epithelial cells, and is divided into two successive phases: the metestrus in which we note the presence of keratinized cells and leucocytes, and the diestrus dominated by the presence of leucocytes in very large numbers.



K: Keratinized cells, L: Leucocytes, N: Nucleated cells

Fig. 2. Vaginal smear in estrus stage in a control female (a: Direct observation), Vaginal smear in postestrous stage in a treated female, a few rare nucleated cells are observed (b: MGG staining)

Vaginal smears performed on adult females treated with leptin mainly allowed the highlighting of diestrus and metestrus stages, while the estrus stage was not observed.

Hormonology. The gonadotropic hormone assay revealed a non-significant increase in LH after leptin treatment at the age of 30 days ($p > 0.05$) but significant at 60 days ($p < 0.05$*) compared to controls. For FSH, no difference between controls and treated rats was noted in the immature females ($p > 0.05$); a non-significant decrease was however observed in the adults after leptin treatment. Progesterone levels revealed a non-significant increase after treatment in 30-day-old females ($p > 0.05$) as well as at 60 days ($p > 0.05$) compared to controls (Table 2 and Figure 3).

Table 2

Plasma levels of LH, FSH and progesterone in control females and leptin-treated females at the age of 30 and 60 days

LH (IU/L)	30 / C (n=7)	30 / T (n=7)	60 / C (n=6)	60 / T (n=7)
	0.247 ± 0.030	0.310 ± 0.026	0.189 ± 0.020	0.301 ± 0.032
FSH (IU/L)	30 / C (n=6)	30 / T (n=6)	60 / C (n=5)	60 / T (n=5)
	3.949 ± 0.365	3.994 ± 0.636	4.307 ± 0.817	2.639 ± 0.297
Progesterone (ng/mL)	30 / T (n=7)	30 / E (n=8)	60 / T (n=9)	60 / E (n=7)
	18.62 ± 2.84	22.11 ± 3.50	33.39 ± 5.13	41.60 ± 4.58

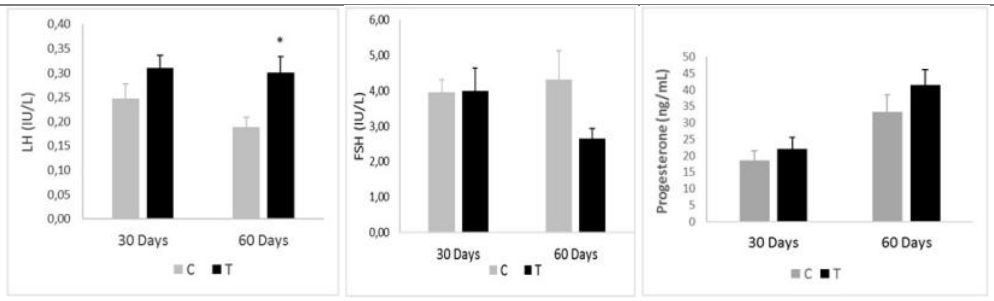


Fig. 3. Plasma levels of LH, FSH and progesterone in control and leptin-treated females

Gonadotropic hormone assays results revealed a significant increase in plasma LH levels in adult females. The direct role of leptin at the central level has been demonstrated (Ahima et al., 1996) and specific levels of leptin would be required to maintain normal ovarian function. In both males and females, leptin injection in ob/ob mice causes hypothalamic-pituitary and reproductive system maturation with increased FSH and LH plasma concentrations associated with reproductive organs development (Barash et al., 1996; Chehab et al., 1996). The dose used by Barash et al. (1996) was 50 µg leptin (0.5 mL intraperitoneal injections twice daily for 14 days).

Histology

30-day ovaries. We noticed the presence of different stages of follicular development in both control and leptin-treated ovaries. The pre-antral follicles are represented by primordial, primary and secondary follicles, while antral follicles

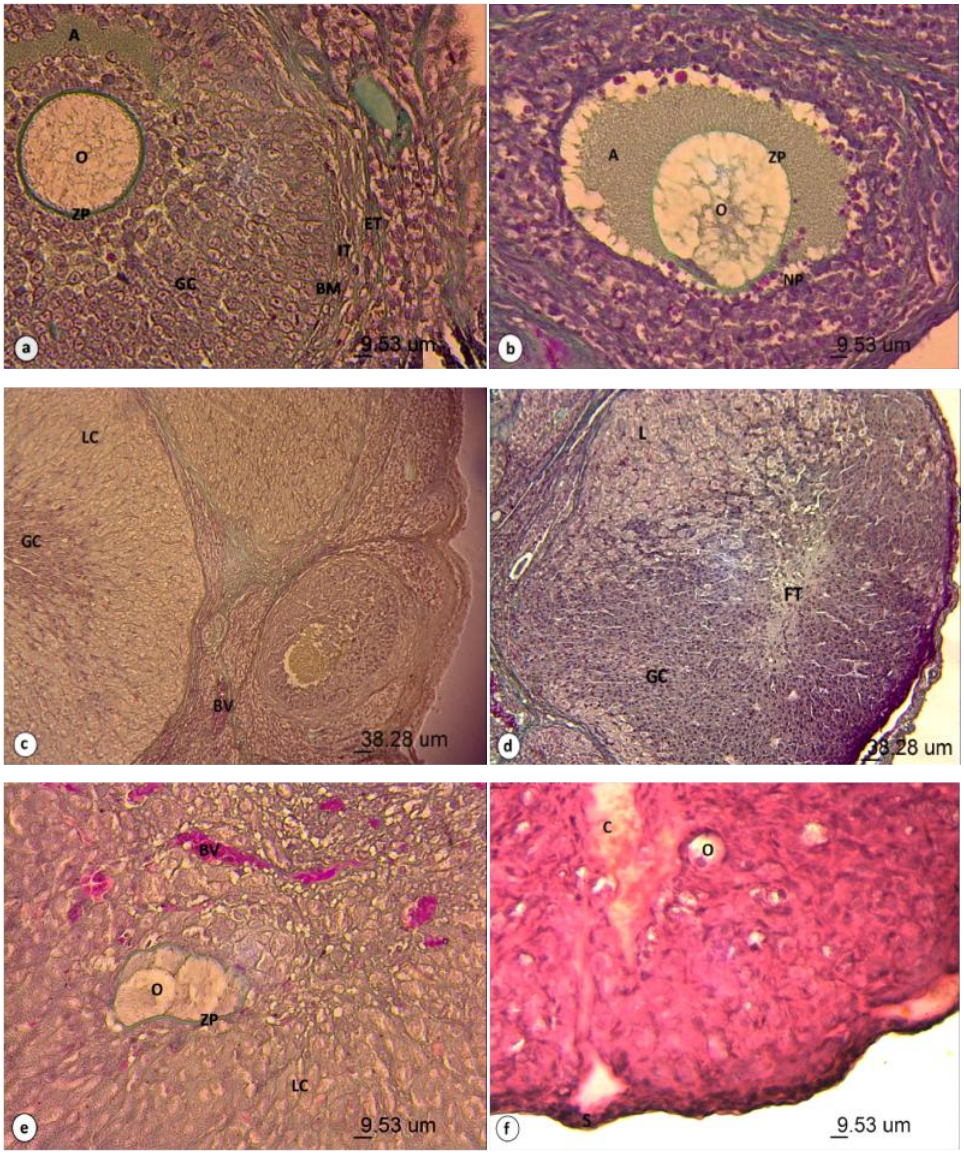
regroup tertiary (Figure 4a) and pre-ovulatory (De Graaf) follicles. The polyoocyte follicle usually contains two oocytes. Atretic follicles are characterized by the degeneration of granulosa cells (Ia and Ib stages) and / or the degeneration of oocyte (IIa and IIb stages: Figure 4b) (Osman, 1985). In our study, on 30-day ovaries, pre-ovulatory and polyoocyte follicles were all atretic.

60-days ovaries. The ovary had irregular sections and was much larger than at the age of 30 days. All stages of follicular development were observed in both controls and treated females. The pre-ovulatory follicles and the corpus luteum protrude on the surface of the ovary. The process of luteinization occurs from the outside to the inside and results in the formation of a corpus luteum (Figure 4c). After ovulation, the granulosa cells are loaded with lipid droplets and are transformed into large luteal cells while the internal theca cells give small luteal cells (or paraluteal cells), a coagulum can be observed. In control females, during the estrus stage, the corpus luteum was small, with a central liquid and basophilic cytoplasmic cell, smaller in the metœstrus stage. In the diestrus stage, the corpus luteum was large and sometimes vacuolized with the onset of formation of a central fibrous tissue. In the proestrus stage, the degenerated corpus luteum had cytoplasmic vacuoles with proliferation of central fibrous tissue. In treated females, the corpus luteum often had an irregular shape and the luteinization of granulosa cells was disorganized (Figure 4d). A fully degenerated oocyte with a very irregular zona pellucida was observed inside a corpus luteum in a treated female (Figure 4e). On another ovary, oocyte maturation did not take place, the oocyte remained trapped within the corpus luteum, and the newly formed stigma was clearly visible (Figure 4f). All stages of atresia were found but mainly observed in tertiary and pre-ovulatory follicles in both control and treated females.

Our histological study revealed a suspension of ovulation as well as an acceleration of the luteinization of granulosa cells in relation to the elevation of LH levels in treated adult females. Indeed, while the follicle is in full development, the high level of leptin induces a sharp rise in the level of LH responsible of the rapid luteinization of granulosa cells that turn into luteal cells synthesizing lutein and developing progesterone, with formation of the corpus luteum even before ovulation has occurred. It results in a degeneration of the oocyte inside the newly formed luteal body. A study has shown that leptin replacement therapy in leptin deficient corpus luteum accelerates tissue development, increasing overall tissue mass and forming a structure that resembled a mature corpus luteum during the early stages of development, which suggest that leptin contributes to the development of the corpus luteum (Garcia, 2017).

Leptin is able to directly modulate the development of the ovarian follicle and depending on its circulating levels; it can exert both stimulating and inhibitory effects on the ovulatory process (Bilbao et al., 2015). At a physiological serum concentration, leptin stimulates steroidogenesis in granulosa and theca cells (Argawal et al., 1999; Brannian et al., 1999). This effect appears to be reversed for a concentration higher than 10 ng/mL with a significant decrease in the number of ovulations by a direct effect on the ovary (Duggal et al., 2000). At a supraphysiological concentration, leptin could thus be responsible of anovulation by inhibiting the production of estradiol,

interfering with the development of dominant follicles and the maturation of oocytes (Pérez-Pérez et al., 2015).



a: A normal antral follicle at tertiary stage with integral zona pellucida in a control adult female, b: An atretic antral follicle in a 30-day old treated female (Stage IIb), the degenerated oocyte has an irregular zona pellucida in direct contact with the antrum which has at its periphery numerous pycnotic nuclei and macrophages, c: A normal corpus luteum in a control adult female, d: Disorganized corpus luteum in a treated adult female, presence of large pale-vacuolated luteinized cells, and acidophilic cells reminding granulosa, e: A degenerated oocyte trapped inside a luteal body in a treated adult female, note the deformation of the oocyte and the zona pellucida, and the disorganization of the ooplasm, f: A non-expelled oocyte trapped in a luteal body in a treated adult female, the furrow of the follicular rupture is still visible with the presence of a coagulum. A: Antrum, BM: Basal membrane, C: Coagulum, ET: External theca,

FT: Fibrous tissue, GC: Granulosa cells, IT: Internal theca, LC: Luteinized cells, NP: Nuclear particles, O: Oocyte, S: Stigma, ZP: Zona pellucida, BV: Blood Vessel

Fig. 4. Ovarian histological sections in female Wistar rats (a, b, c, d, e: Modified Masson Trichrome stain, f: H&E stain)

Leptin deficiency is responsible of the folliculogenesis alteration by the increase of follicular atrophy that can cause infertility. The ovarian expression of leptin and its gonadotropin-regulated receptor is maximal at ovulation, whence its possible involvement in oocyte maturation, angiogenesis, follicle rupture or subsequent formation of the corpus luteum (Ryan et al., 2003; Craig et al., 2005). Leptin receptors would induce tyrosine phosphorylation of STAT 3 (major intracellular protein of leptin signal transcription in oocytes at metaphase II stage) (Matsuoka et al., 1999). In rats, transcription of leptin occurs in the early stages of follicular development, whereas leptin protein appears only in mature follicles (Archanco et al., 2003) and intervenes in the subsequent luteal phase. It can modulate the folliculogenesis process by altering directly gonadotropin sensitivity of the ovary (Olatinwo et al., 2005). It can also induce the expression of the neuropeptic transcription of the CART (Cocaine and Amphetamine Regulated Transcript) in granulosa cells of ovarian follicles *in vitro* as *in vivo*, leading to a lower estradiol synthesis and the alteration of folliculogenesis (Ma et al., 2016). The effect of leptin on cellular apoptosis remains controversial. For some, leptin attenuates follicular atresia (Brown and Dunmore, 2007; Dineva et al., 2007; Lam et al., 2010). We previously noticed that leptin seemed stimulating follicular apoptosis at immature age by preventing the occurrence of too early ovulation, before the maturation of the hypothalamic-pituitary-ovarian axis (Ghouri et al., 2018). For Bilbao et al. (2015), the anti-apopototic effect of leptin depends on the type of treatment (acute or daily).

CONCLUSION

Leptin injected at a dose of 8 µg/100 g b.w. does not seem to influence the evolution of body weight and reproductive system in 30-day-old female Wistar rats, probably due to the lack of receptors on the studied systems. At 60 days, leptin induces a decrease in body weight and food intake and appears to alter folliculogenesis, the process of ovulation and luteogenesis. At supra-physiological serum concentration, it induces an acceleration of follicle maturation by a significant increase in circulating LH level, a suspension of ovulation and an early luteinization of granulosa cells with an increase in progesterone level.

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