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Histological and immunohistochemical characterization of the Mongolian gerbil's mammary gland during gestation, lactation and involution

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ABSTRACT

The morphological description of normal tissues is fundamental for making comparisons and in order to identify injuries and lesions. The aim of this work was to describe the morphological characteristics of the female Mongolian gerbil's (*Meriones unguiculatus*) normal mammary gland, the average expression of hormone receptors, and the average proliferation rates in the epithelial cells during the periods of lactation, pregnancy and involution. Dams were euthanized on the 14th and 21st gestational days, 7 and 14 days after parturition, and 3 and 5 days after weaning. The dams' mammary tissues were processed and were submitted to haematoxylin and eosin staining, Periodic Acid Schiff (PAS) staining, and Gomori's Reticulin staining. Additionally, immunohistochemistry was performed for the characterization of myoepithelial cells with α-actin, the proliferation rates with proliferating cell nuclear antigen (PCNA), the estrogen hormonal receptors (ESR1 and ESR2), and progesterone receptor (PR) quantifications. It was observed that the abundant adipose tissues were replaced by glandular epithelia and there was an increase in the epithelial cell's height (from 5.97 to 32.4 μm in 14th and 21st gestational days and from 20.64 to 25.4 μm in 7th and 14th lactational days, respectively) and the acini diameters (from 24.88 to 69.92 μm in 14th and 21st gestational days and from 139.69 to 118.59 μm in 7th and 14th lactational days, respectively) with the progression of gestation and lactation. The PAS staining intensity varied throughout the glands and between the stages that were evaluated. The extracellular matrix showed different phenotypes too, with more of a presence of the Type I collagen during the early gestation and involution and with more reticular fibers (Type III collagen) during the late gestation period and lactation. The myoepithelial layers showed alterations in their distribution with thick patterns as verified by the α-actin labeling. The PCNA showed higher rates of the marked cells in 14th and 21st gestational days (40.25 and 60.28%) and in 7th and 14th lactational days (64.08 and 65.08%). The hormone receptor quantifications showed a high variation in the rates: the average PR staining decreased from 14th to 21st gestational days (from 42.3 to 8.54%), from 7th to 14th lactational days (from 59.83 to 23.18%) and from 3rd to 5th days after weaning (from 39.98 to 12.72%). There were higher averages of ESR1 staining in gestational days 14 and 21(from 58.06 to 30.02%). ESR2 staining decreased during gestation (25.7 and 12.94% in 14th and 21st gestational days)and involution (from 50.97 to 30.18% in 3rd and 5th days after weaning). The Mongolian gerbils showed similar morphological characteristics when they were compared to mice and rats. However, the higher proliferation rates with a smaller involution period compared to other murine characterized this species as being adequate for mammary pathologies studies.

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1. Introduction

The mammary gland, which distinguishes mammals from all other animals, produces and secretes milk in order to nourish offspring. It is classified as apocrine exocrine tubuloalveolar (Inman et al., 2015; Oftedal, 2002). Although there are structural differences between species, several researches use the mouse and rat

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mammary gland as a model to infer about developmental mechanisms (Macias and Hinck, 2012; Howlin et al., 2006). In general, the primary mammary gland structure originates from the ectoderm (epithelium) and the mesoderm (stroma). The interaction between these tissues promotes the formation of the mammary placodes which develop towards the anterior portion of the body (Balinsky, 1950). Generally after birth, the gland remains quiescent until the beginning of the cyclic reproductive life, in response to hormonal cues. Thus, the mammary gland provides a unique model for biologists to study development and organ specificity (Inman et al., 2015).

In rats, the parenchymal compartment of the mammary gland is composed of different epithelial structures with distinct morphological and functional activities, comprising the luminal epithelium of ducts, ductules, terminal end buds, alveolar buds, alveoli and the underlying myoepithelial layer (Líška et al., 2016; Masso-Welch et al., 2000). As in most glandular tissues, the adult mammary gland comprises multiple cell types, including epithelial, adipose, fibroblasts, immune, lymphatic and vascular cells that work together to sculpt and maintain a functional organ. These different cell types have been demonstrated to be of importance at specific stages of mammary gland development (Inman et al., 2015). The increase of estradiol secretion during puberty promotes the storage of dense connective tissues and adipocytes among the lobules (Sinowatz, 2012). Between the luminal epithelium and basement membrane the contractible myoepithelium is present, promoting the milk excretion under oxytocin stimulation during pregnancy (Masso-Welch et al., 2000).

The gland undergoes deep modifications with its structure during the reproductive cycles due to the hypothalamic-hypophysis-gonadal axis activity. In nonpregnant female, the development of the mammary gland is rigorously controlled by the ovary. In general, estrogens are responsible for mammary ducts growth and progesterone is necessary for lobuloalveolar development in the mouse (Russo and Russo, 1996). Nevertheless, the functional and structural development occurs in the dam exclusively during gestation and lactation, followed by involution (Sinowatz, 2012). During these different stages, the cells of the mammary gland proliferate, differentiate and go through apoptosis in response to stimuli, giving rise to significant remodeling of the glandular tissue architecture (Inman et al., 2015).

In the dam, early in gestation, the combined influences of ovarian estrogen, progesterone, and inhibin, with the production of rat chorionic gonadotropin contribute to stimulate the gland to undergo active cell proliferation (Ying, 1988). In the gestational and the lactational periods, the morphological alterations in the dam include an alveolar expansion of the secretory epithelium regions towards the fat pad, culminating in a considerable remodeling of adipose tissue (Collins and Schnitt, 2012). The end of gestation is the lobuloalveolar phase of the mammary gland's development (Richert et al., 2000). In C57BL/6 mice during lactation, the pre-secretory cells go through an intense activity, releasing their components, together with apical region pieces of the cytoplasm to the lumen. In this strain, after the end of lactation, the absence of a mechanical suckling stimulation promotes the mammary involution: apoptosis, epithelial cell detachment, and an alteration of the acini's shape are characteristics of the first stage of mammary involution (Inman et al., 2015; Monks et al., 2008). In rats, together with the secretory alveolar collapse, there is removal of active cells and secretions by macrophages (Helminen et al., 1968). In human mammary involution the extracellular matrix remodeling that is associated with a new wave of apoptosis then occurs, promoting the substitution of the epithelium by the adipose tissues (Macias and Hinck, 2012). This process of expansion and regression can occur across multiple gestations during the reproductive phase,

demonstrating that the mammary epithelial cells have considerable regenerative abilities (Arendt and Kuperwasser, 2015).

In female mice offspring, on day 14 of gestation, the epithelium of the mammary gland responds to estrogen, thanks to the presence of estrogen receptors (ESR) (Sampayo et al., 2013). In the adult mammary gland, however, only a small population of epithelial cells expresses ESRs and progesterone receptors (PRs). It has been stated in several species and, although not all of their functions are clear, it is known that ESR1 is the most important receptor during the mammary ductal morphogenesis. PRs are only necessary during alveologenesis and throughout the gestation period (Macias and Hinck, 2012). Despite ESR1 is related to cellular proliferation, it is known that it exerts a paracrine effect, since the ESR1 positive cells have shown that they are not the same as those that are positive for bromodeoxyuridine in Balb/c mice (Zeps et al., 1998). A paracrine mechanism is also shown by the PR-positive cells in mammary gland of ROSA26 and RAG1 mice (Brisken et al., 1998). Moreover, estrogen receptor gene ESR2 knockout mice have shown no deficiencies in the mammary structure or with lactogenesis (Mehta et al., 2014; Krege et al., 1998). This suggests a greater presence of ESR1 and PR during the development stages (regulating ductal outgrowth and morphogenesis) while ESR2 is associated with the non-proliferative phases of the gland (Macias and Hinck, 2012). The ESR1-knockout mouse is infertile and it presents a rudimentary development of the mammary ductal system, because it lacks the terminal end buds. It seems that the growth of ducts depends on the presence of ESR1 in the stroma, and that the availability of epithelial cells expressing ESR2 is insufficient to evoke a mammary proliferation induced by estrogen (Hamilton et al., 2014; Musumeci et al., 2015). ESRs and PRs are also present in mesenchymal cells surrounding the mammary gland (Bigsby et al., 2004).

In both rats and mice, the mammary glands are aligned ventrolaterally along the mammary or milk lines from de cervical to the inguinal regions. Female mice have five pairs of mammary glands: one cervical, two thoracic, and two abdominal-inguinal pairs. The female rat has six pairs, the thoracic, abdominal, and inguinal glands that vary in their degree of development in the nulliparous rats, with the inguinal being the most differentiated, and the cervical glands being the least differentiated. The anatomic location and distribution of these paired organs is similar between these two species (Russo and Russo, 1996).

Female Mongolian gerbil (Meriones unguiculatus), a polyestrous species, have an estrous cycle of 4–6 days (Nishino and Totsukawa, 1996) and reaches puberty at 9–12 weeks. Gestation lasts 24–26 days and weaning happens 3 weeks after birth (Marston and Chang, 1965). They show four pairs of mammary gland: two thoracic and two inguinal (Fig. 1). Each of them show only one galactophore duct (Yu and Anderson, 1975).

A morpho-physiological description of the mammary gland has been made when related to several species (Bellatine et al., 2010; Chandra et al., 2010; Saji et al., 2000; Zeps et al., 1998). However, a descriptive study about the morphological variations of a Mongolian gerbil's mammary gland is not available in the literature. These experimental models have been increasingly used due to their relatively high development rates of spontaneous tumors (Salyards et al., 2013; Campos et al., 2008), what mimics the natural tumor environment and enables the development of research in this particular area. Thus, the aim of this study was to describe the structural characteristics of the female Mongolian gerbil's mammary gland, the average patterns of the hormone receptor expression, and the average proliferation rates in the epithelial cells during the periods of lactation, pregnancy and involution as a contribution to understanding the various aspects that involve the development and functionality of these glands.

Fig. 1. Schematic illustration of the inguinal mammary glands anatomic position of the female Mongolian gerbil.

2. Material and methods

2.1. Animals and experimental design

The animals were kept under standard temperature and humidity conditions (25 °C, relative humidity 40–70%, light/dark cycle 12/12 h) with access to chow (Rats and Mice, Presence®), and water *ad libitum.* The procedures were performed in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and was approved by the Ethics Committee on Animal Use (CEUA- IBILCE/UNESP 099/2014).

The study used 7 young adult (4 months old) female Mongolian gerbils (*Meriones unguiculatus*). One of them was a 4 month old female in estrous phase, whose mammary gland was used as resting control for comparison to other animals. The other six animals were housed with fertile males for the mating. All animals were euthanized at various stages of their reproductive life. The mating day was determined by the presence of sperm using a vaginal smear, indicating day 0 of pregnancy. One animal was euthanized at each of the following periods: the 14th and 21st days of pregnancy, 7 and 14 days after the birth of the puppies (lactation), and 3 and 5 days after the weaning (involution).

2.2. Histological analyses

Euthanasia was performed by a deepening anesthesia (xylazine 3 mg/kg and ketamin 10 mg/kg) followed by decapitation. After death, the abdominal area was shaved, enabling a visualization of the left caudal mammary glands, which were excised with the skin. The glandular tissue samples were fixed for 24 h in 4% paraformaldehyde that was diluted in a phosphate buffer solution. They were then processed for histology and cut with a microtome (4 μm thick longitudinal sections). Two sections were analyzed per gland. They were stained with Haematoxylin and Eosin (HE) for a general morphological analysis. A Periodic Acid Schiff (PAS) staining was performed, in order to identify the carbohydrates in the secretion. Gomori's Reticulin staining was performed too for the identification and the characterization of the reticular fibers in the stroma. The sections were analyzed under a light microscope (Olympus BX60, Japan) and the images were digitilized and analyzed by the Image Pro Plus software (Version 6.1 for Windows – Media Cybernetics, Silver Spring, MD, USA).

2.3. Morphometric analyzes

One section per animal was scanned using a B61VS camera (Olympus Corporation, Tokyo, Japan) coupled to an Olympus VS120® Virtual Microscopy Slide Scanning System (VS120-S5) from the same manufacturer and the total area of the section was measured. The ratio between the area occupied by the secretory epithelium and the area that was occupied by the fat cells and the stroma were calculated in μm^2 by the Image Pro-Plus software (Image ProPlus Media Cybernetics, MD, USA).

Representative fields scattered in the preparation (chosen randomly) of the HE stained sections were also analyzed in order to obtain the measurements of the acini diameters and the glandular secretory epithelium heights. It was analyzed two slides per animal. For the acini diameters, the mean and standard deviations of the values that were obtained for one mammary gland per animal were calculated from 60 measurements per gland/animal. To calculate the overall average of the epithelium height, 12 acini were evaluated and three cells of each were measured.

2.4. Immunohistochemistry

The sections that were obtained from each animal were submitted to immunohistochemical reactions with the primary antibodies for ESR1 (ERα, rabbit polyclonal, IgG, MC-20, sc-542, dilution 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA), ESR2 (ERβ, rabbit polyclonal, IgG, H-150, sc-8974, dilution 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and PR (rabbit polyclonal, IgG, C-19; sc-538, dilution 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) receptors. Also, Proliferating Cell Nuclear Antigen (PCNA) which is involved in cell proliferation and DNA repair antibody (mouse monoclonal, PC10, dilution 1: 100, sc-56, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. The antigen retrieval was performed by an immersion of the sections in 10 mM citrate buffer, pH 6.0, at 93 °C for 20 (for PCNA protocol) or 45 min (for hormone receptors protocols). A blockage of the nonspecific proteins was then carried out with a skimmed milk powder solution of 5% for 30 (for ESR1 and ESR2 protocols) to 60 min (for PCNA protocol), or with sniper background (Biocare Medical, Concord, CA, USA) for 15–20 min (for PR protocol). The sections were incubated overnight at 4 °C (for hormone receptors protocols) or for one hour at 36 °C (for PCNA protocol) with the primary antibodies and then incubated with the secondary antibodies for 1 h (Goat anti-rabbit IgG, ABC Staining Systems, Santa Cruz Biotechnology, CA, USA for hormone receptors protocols and goat anti-mouse IgG, sc-2039, dilution 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA for PCNA protocol). After that, an incubation with avidin/biotin (ABC Staining Systems, Santa Cruz Biotechnology, CA, USA) for hormone receptors protocols, or polymer (DAKO EnVision™, Dual Link System-HRP, Dako, CA, USA) for PCNA protocol, for 45 min at 22 °C, was performed. The sections were stained with a solution of 3–30 '-diaminobenzidine tetrahydrochloride (DAB, Sigma), and counterstained with Harris Haematoxylin.

2.5. Immunofluorescence

Sections were also submitted to immunofluorescence for the detection of the myoepithelial cells by α-actin (mouse monoclonal, IgG2a, 1A4, sc-32251, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Incubation with the primary antibody was

performed overnight at 4 °C, followed by an incubation with specific secondary fluorochrome-conjugated antibody (anti-mouse goat, IgG-FITC, sc-2010, dilution 1: 200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for two hours at 22 °C. DAPI (4′,6-diamino-2-phenylindol, UltraCruz™ Mounting Medium: sc-24941 for fluorescence with DAPI, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for a visualization of the cell nuclei. The sections were examined with a fluorescence microscope (Zeiss Imager M2, Zeiss, Gottingen, Germany).

2.6. Cellular proliferation rates and the quantification of ESR1, ESR2 and the PR positive cells

Six representative microscopic fields (40× magnification) were analyzed per animal in order to quantify the relative frequency of the positive cells for ESR1, ESR2, PR and PCNA in the secretory epithelium of the mammary gland. The percentages were calculated by a division of the number of labeled nuclei by the total number of cells counted in each field.

3. Results

3.1. The adipose tissue was partially replaced by the glandular epithelial cells. The extracellular matrix fibers underwent variations in their organization and the secretion compositions varied through the lobes during their functional differentiation

The mammary glands at different stages of the reproduction showed considerable variations in their morphology (Figs. 2 and 3).

Compared to the resting gland, it was observed that on the 14th gestational day, the mammary gland had a higher frequency of mammary epithelium (Fig. 3A–C). At this stage, there were small quantities of an eosinophilic secretion in few of the acini, with the presence of lipid droplets in their composition. The acini comprise a simple cuboidal epithelium. The final portion of the ducts was characterized by a simple squamous epithelium. The PAS staining was less intense in some acini when compared to the secretion that was present in the ducts. From 14 days of gestation on, the highly PAS positive secretion was associated with lipid droplets, characterizing the beginning of the milk production. The adipose tissue, which constituted the most part of the glands, was classified as unilocular. It was partially replaced by the secretory epithelium and loose and cellularized connective tissues, reducing the adipocytes contribution to the gland's composition. Gomori's Reticulin stain revealed thick bands of collagen fibers (Type I) amid the stroma, delimiting the areas of the glandular lobules. The acini and the ducts were externally delimited by a thin layer of black reticular fibers (collagen Type III).

In the last gestational week (21 days), there was a larger area occupied by the secretory epithelium, and consequently, there were less unilocular adipose tissues (Fig. 3D–F). The morphologic profile of epithelial cells altered, so that the acini were smaller at this stage of the development. The cells showed an extended cytoplasm, comprising a higher epithelium. The presence of blood capillaries in the acini became more evident, with an easy identification of red blood cells in the acinar baseline limits. The distribution of collagen Type I fibers at this stage was greatly reduced when compared to the previous phase and a thin layer of reticular fibers limited the alveoli. Nevertheless, the dense connective tissues retained their irregular characteristics as was seen at 14 days of gestation.

Considerable morphological changes occurred in the mammary gland during the perinatal period (Fig. 3G–M). A thin periglandular stromal layer filled the glands and a pronounced development of the secretory epithelium and the ductal structures was observed at 7 days postpartum – lactation. The adipose tissues were limited to small portions in the peripheral regions of the glands, with a considerable reduction in their size. The acini became considerably larger. The epithelial cell apical delimitations were not clear and this particular region of the cell consisted mostly of vesicles and lipid droplets. A small amount of loose and cellularized connective tissues delimiting the alveoli was richly vascularized. Cells with condensed chromatin corpuscles were identified in the lumen, corresponding to the detached cells that were released from the secretory epithelium. At this stage, the secretion stained by the PAS was less intense indicating a secretion with a lower carbohydrate concentration.

After the end of the lactational phase (Fig. 3N–T), the amount of detached epithelial cells in the lumen increased, becoming more frequent during the regression of the glands. Despite the absence of mechanical suction stimuli, three days of post-weaning acini remained morphologically similar to that which was shown during lactation, with stored secretion. Five days after the weaning, significant changes were observed. The acini showed a reduction in their size and frequency, the adipose tissues returned to occupy a considerable part of the glands and lumen showed remains of PAS positive secretion. In a similar manner to that which was observed during the resting phases, the Type I collagen fibers occupied much of the stroma. In contrast, the reticular fibers considerably reduced.

3.2. The acini diameters and the epithelium heights increased with the progression of pregnancy and reduced considerably during involution

The mean and the standard deviations of the secretory acini diameters and the epithelial heights are shown in Fig. 4.

At 21 days of gestation, the acini increased their diameter by about three times when compared to the 14 day of this period. The average values of the secretory epithelium heights showed an increase of 6 times over gestation. The measures doubled after the onset of lactation and they remained relatively high until the lactational end. However, a slight decrease in the epithelium height averages, from the late pregnancy (32.4 μm) to the first week of lactation (20.64 μm), was observed. After the end of lactation, the parameters became similar to those that were observed at the beginning of lactation.

The values of the ratios between the areas occupied by the secretory glandular epithelium and the stroma are shown in Table 1 and are given in μm². It was observed that there was a gradual replacement of the fat pad by the secreting epithelial tissues during pregnancy and lactation. After weaning, involution occurred in a fast period, reaching a ratio epithelium/adipocytes and stroma similar to that observed at 14 days of gestation five days after weaning.

3.3. Immunohistochemistry

3.3.1. The Myoepithelial Cell Layer was more Remarkable during Pregnancy.

The images of the α-actin labeled cells that were obtained by the fluorescence microscope are shown in Fig. 5. The immunofluorescence for α-actin revealed that the myoepithelial cells were arranged concentrically around the acini and ducts.

The images of the hormonal receptors and PCNA positive cells are shown in Fig. 6.

3.3.2. The PCNA expression increased in the epithelial cells at the end of pregnancy and during lactation

An increase in the epithelial cell proliferation rates was observed at 21 days of gestation (60.28%) and during lactation (64.08 and 65.08%) when compared to the 14 days of gestation (40.25%).
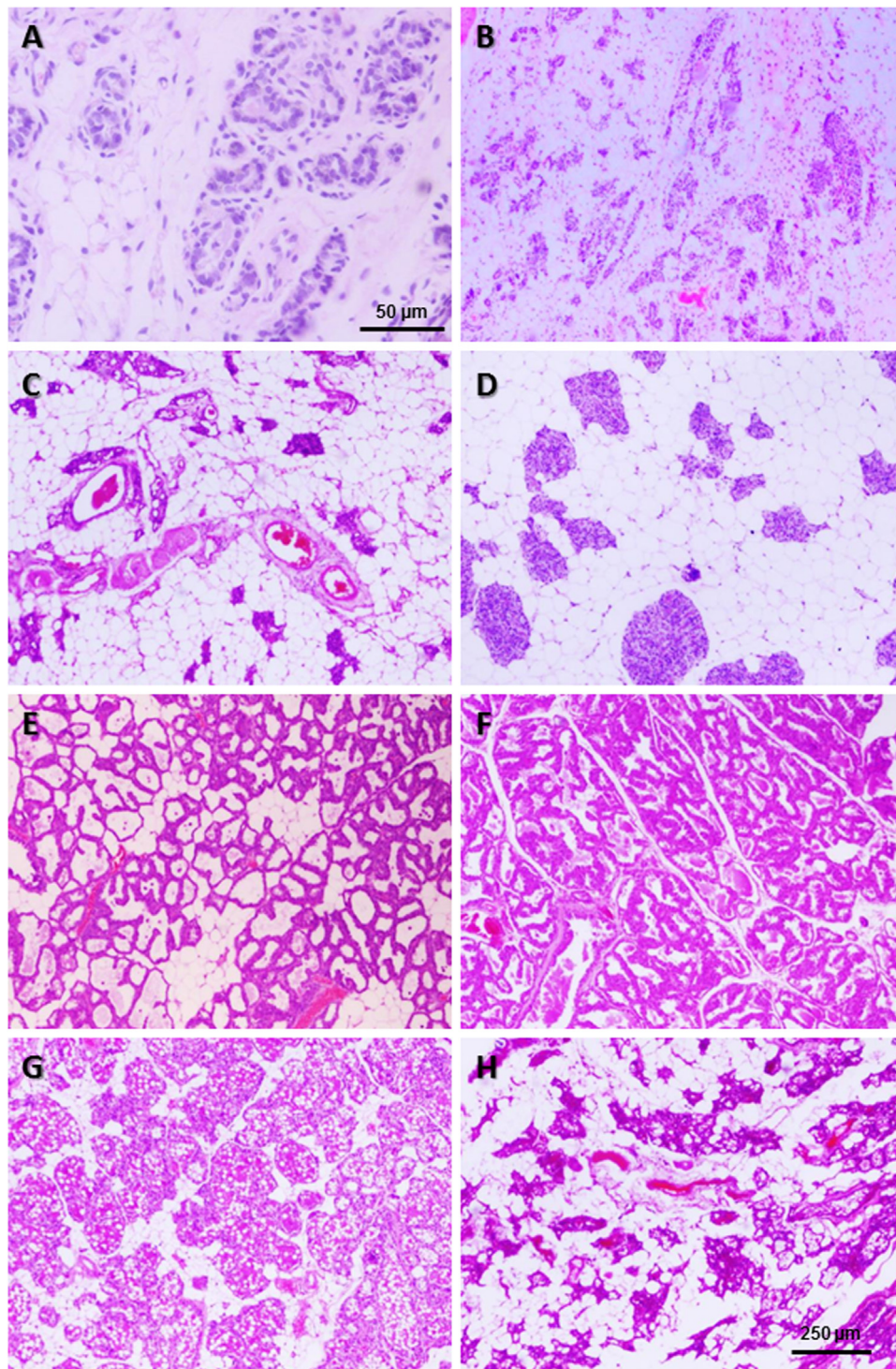
Fig. 2. Morphological aspects of female gerbil mammary gland in low magnifications. Resting gland from female at estrous (A and B), 14 days of gestation (C), 21 days of gestation (D), 7 days of lactation (E), 14 days of lactation (F), 3 days after weaning (G) and 5 days after weaning (H) stained with haematoxylin and eosin are shown. The main difference noticed between the stages is the frequency of epithelial structures amid the adipose tissue. Magnifications: A-G: 4x; H:20x.

Table 1
Values in μm^2 of the secreting epithelium and the stroma area that were obtained in the histological sections from the female gerbil's mammary gland at different stages of the reproduction and the relationship between the parameters (n = 1).

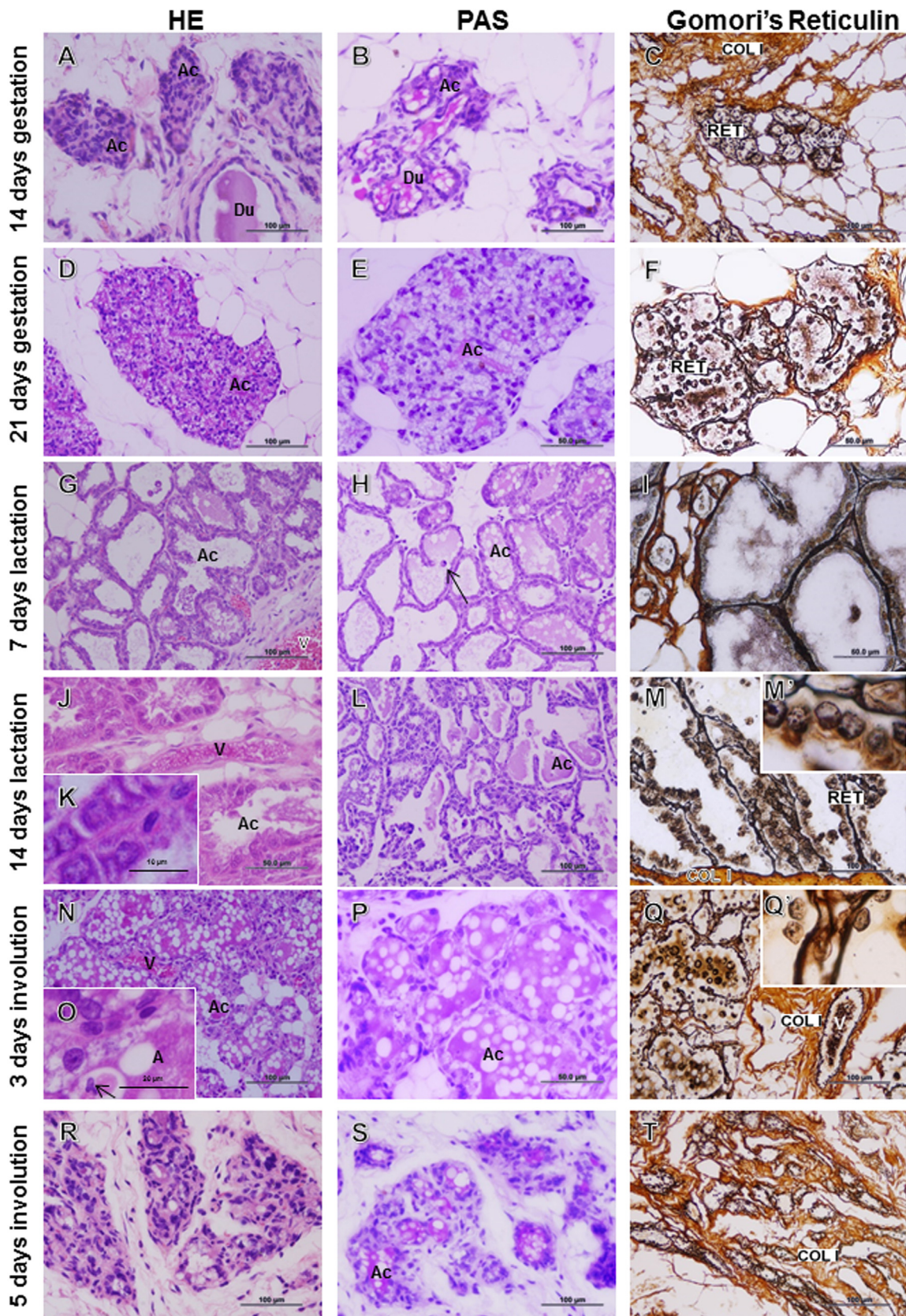| Stage	Total Area	Secreting Epithelium Area	Stroma Area	Ratio Epithelium/adipocytes and stroma
14 days gestation	30.18	6.40 (21.2%)	23.77 (78.8%)	0.26
21 days gestation	33.72	7.62 (22.6%)	26.09 (77.4%)	0.29
7 days lactation	22.25	15.70 (70.6%)	6.54 (29.4%)	2.4
14 days lactation	23.05	16.68 (72.4%)	6.36 (27.6%)	2.62
3 days involution	94.75	37.59 (39.7%)	57.16 (60.3%)	0.65
5 days involution	44.95	9.64 (21.5%)	35.3 (78.5%)	0.27

Fig. 3. Morphological aspects of the female gerbil's mammary gland stained with Haematoxylin & Eosin (HE), the Periodic Acid-Schiff (PAS) staining, and Gomori's Reticulin staining, during gestation (A–F), lactation (G–M), and involution (N–T). The acini (Ac), the ducts (Du), the blood vessels (V), and the stroma components, such as the collagen fibers Type I (stained in brown, COL I), the reticular collagen fibers Type III (stained in black, RET) and the detached epithelial cells in the acini (arrow), are shown.
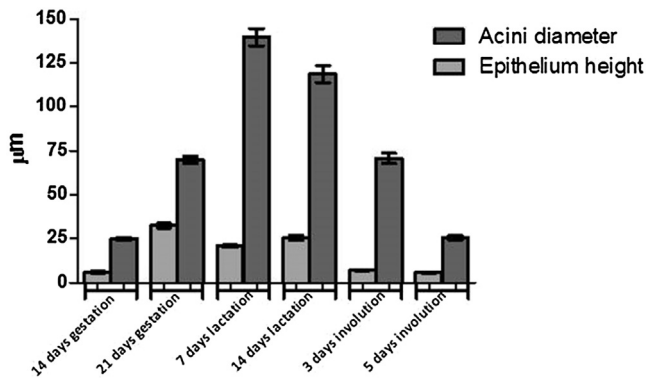
Fig. 4. The mean and standard deviations of the biometric values of the secretory portion of the female gerbil's mammary gland at different stages of the reproduction: pregnancy, lactation and involution (n = 1).

The PCNA labeling rates reduced in involution period (57.34 and 52.38%) compared to late gestation and lactation, but without reaching the levels that were presented during the early gestation (Fig. 6A).

3.3.3. The hormone receptor expressions varied at different stages of the mammary gland development

The hormone receptors positive epithelial cells were identified by immunohistochemistry as a brown nucleus in appearance and quantified (Fig. 6B–D).

The percentages of the PR positive cells showed large variations between the animals. During pregnancy, the values showed a sharp drop in PR positive cells from 14 days (42.3%) to 21 days (8.5%), then going up during lactation (59.8 and 23.18) and involution of the glands (39.9 and 12.72%).

The ESR1 positive cells showed increases and reductions, similar to the PR. There was a decrement from 14 days (58.0%) to 21 days during gestation (30.0%). Lactation showed a precipitous drop on day 7 (7.1%), followed by an increase on day 14 (31.9%), with declination during involution periods (26.6 and 9.5%). The ESR2 positive cell percentages showed lower values during pregnancy (25.7 and 12.9%), which increased during lactation (60.6 and 68, 2%) and involution (50.9 and 30.1%).

4. Discussion

Although the anatomical features and a little of the Mongolian gerbil's mammary gland histology have been previously described
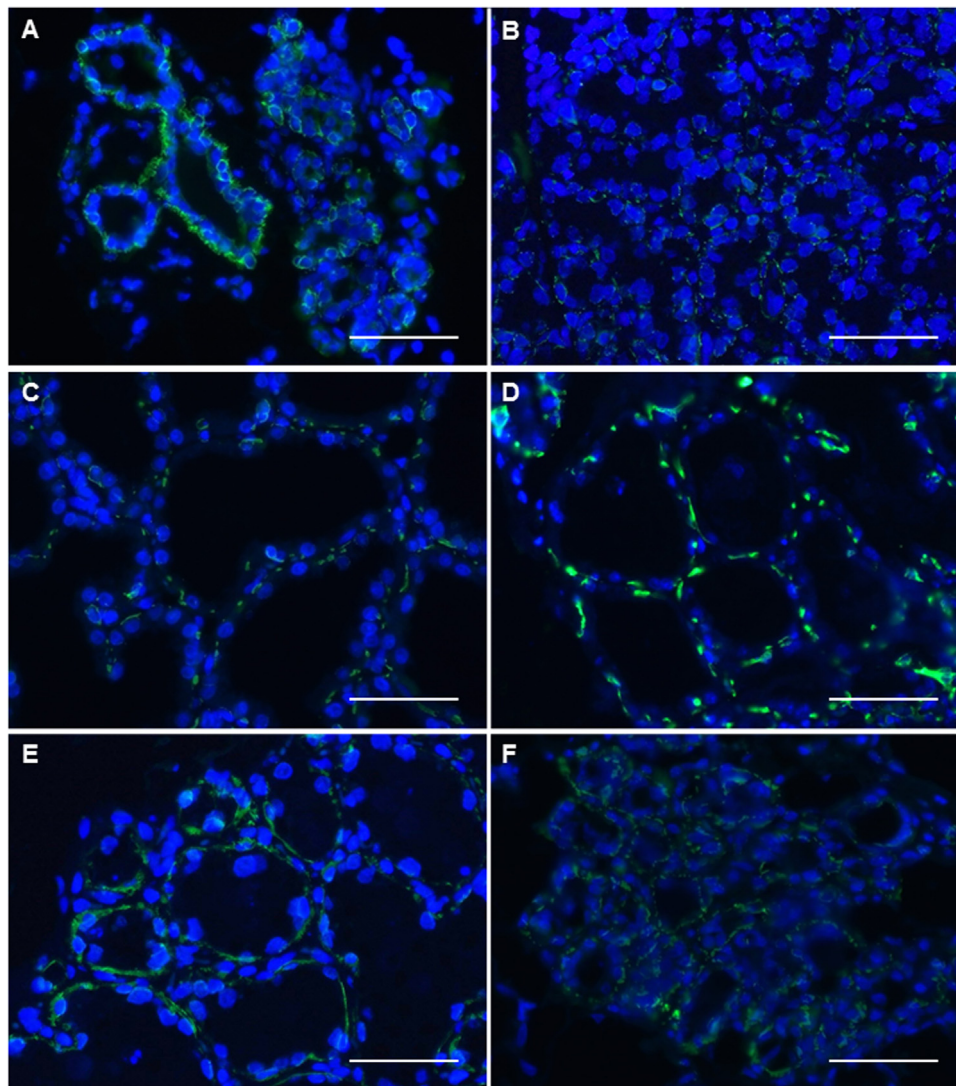

Fig. 5. Fluorescence images for the identification of the myoepithelial cells in the female gerbil's mammary gland through the immunohistochemistry for the α–actin. A: 14 days gestation; B: 21 days gestation; C: 7 days lactation; D: 14 days lactation; E: 3 days involution; F: 5 days involution. Bars: 50 μm.
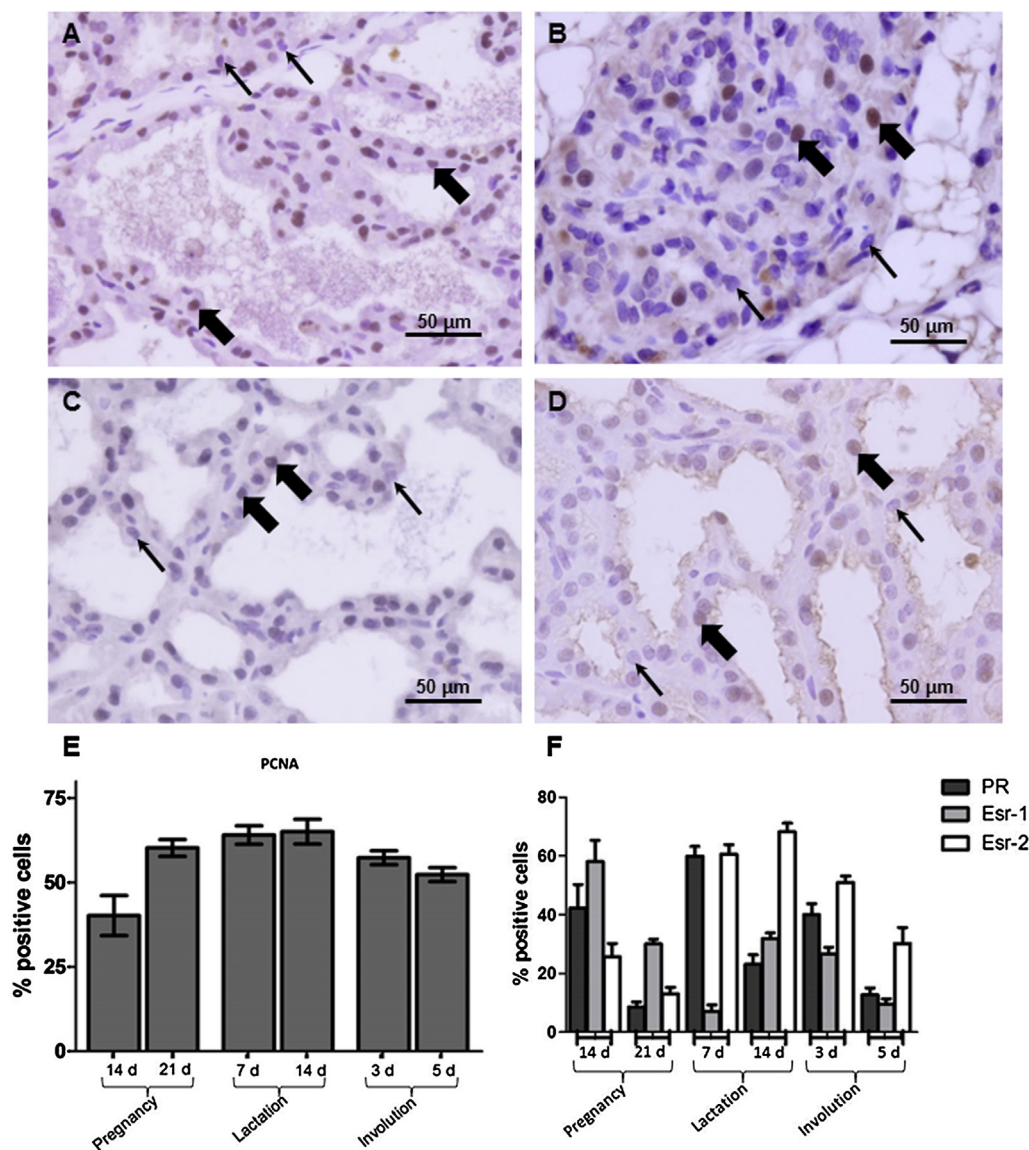
Fig. 6. Immunohistochemistry for the PCNA, the PR, the ESR1 and the ESR2 positive cell detections in the female gerbil's mammary gland. Gland marked with PCNA at gestational day 7 (A), PR at involution day 5 (B), ESR1 at lactation day 14 (C) and ESR2 at gestational day 21 (D). The figures show few positive cells (thick arrows), and negative cells (thin arrows). The percentages of PCNA (E) and the receptor positive cells (F) are also shown (n = 1). Bars: 50 μm.

(Yu and Anderson, 1975), the present study adds a considerable morphophysiological description of this species. It was described the structural characteristics of the gerbil's mammary gland, the hormone receptor expression pattern, and the proliferation rates in the epithelial cells during the periods of lactation, pregnancy and involution.

There was a clear change in the glands that provided development of their secreting potential during the periods of pregnancy and lactation. During the early stages of pregnancy, implantation of the embryo in the uterine wall induced the maintenance of the corpus luteum in the ovary and increased the levels of progesterone. As a consequence, the gland now develops itself, from its rudimentary structures, to exert its function. Firstly, the ductal tree develops from the activation and the proliferation of the TEBs, where there was a differentiation of the secretory epithelium, thus developing the acinar structure. To this aim, the unilocular fat cells showed a reduction in their size, since they provided energy and substrates for the synthesis of the growth factors and the hormones necessary for the differentiation and lactation, apart from yielding space for

the development of the ducts and the acini (Hovey and Aimo, 2010). As in several rat strains (Líška et al., 2016), the gerbil's mammary gland had a higher amount of fat in their non-secretory portions.

It was observed that the growth of the secretory portions during gestation was accompanied by extracellular matrix remodeling, as in mice strains (Silberstein, 2001). Also, the epithelial cells were always surrounded externally by a collagen layer, which was thicker in the early pregnancy period and less developed in late gestation and early lactation. The epithelium of the ducts that were observed in gerbils varied from a cuboid monolayer, similar to that observed in dogs (Chandra et al., 2010) and pigs (Ji et al., 2006), to a simple squamous, when it was closer to the secretory acini. Also, the acini epithelium in the active gland of gerbils was classified as a simple cuboidal epithelium.

Once the alveoli were developed and the epithelium differentiated as secretory cells, the epithelial height, and consequently, the cell volume increased with gestation. The results that were observed during the analyses of the epithelial height measurements and the acini diameters led one to conclude that a higher epithelial

height was observed during pregnancy, the same as in rats (Líška et al., 2016). A deposition of fat droplets and secretion vesicles in the cytoplasm of the secretory cells was responsible for the increase in its volume. During pregnancy, the ovarian progesterone production suppressed the milk secretion (Neville et al., 2002), which was activated in the perinatal period with the production of colostrum and milk. In late gestation, the accumulated content in the cytoplasm was transferred to the lumen and the myoepithelial cells underwent a multiplication process and reach their peak during the early stages of lactation, as has been described in Wistar rats (Jin et al., 2000). From now on, their morphology was changed to a thicker aspect that surrounded the largely developed acini during the late gestation and early lactation. During pregnancy, it was observed that the myoepithelial cell layers were continuous in the ducts and discontinuous in the acini, providing a greater contractility for the excretory portion of the gland, similar to that seen in Sprague-Dawley rats and canines (Sánchez-Céspedes et al., 2016; Masso-Welch et al., 2000).

The percentages of the PCNA positive cells increased during pregnancy. This marker has also been related to the DNA repair (Shivji et al., 1992), but the increase in its rates during pregnancy may be associated with cell proliferations. Although ESR1 and PR are related to proliferation, the quantitative analyses showed a reduction in these receptor rates during the periods of increased positivity for the PCNA. As described by Shigehira et al. (2000), the presence of estrogen receptors in epithelial cells is not a prerequisite for proliferation. Therefore, these results have corroborated the assumption of the paracrine proliferation effects as shown by the PR (Brisken et al., 1998) and the ESR1 (Zeps et al., 1998) positive cells in the mammary glands of mice. ESR2 knockout mice have shown that female fertility and the development of mammary glands during pregnancy and lactation are not altered when in comparison to wild animals (Krege et al., 1998). Also, it is known that the ESR2 expression naturally exceeds the ESR1 expression in mammary glands and has an antiproliferative action, as in other tissues (Mehta et al., 2014). According to Cheng et al. (2004), ESR1 is directly associated with the cellular proliferation because it initiates the DNA synthesis, and thereafter, it ceases to be expressed in the nucleus. ESR2, in turn, serves to facilitate the ESR1's return to the nucleus and resumes the responsiveness to the hormone. This may explain the high rates of ESR2 positive cells in times of a low expression of ESR1.

During lactation, a drop in the serum progesterone that is associated with other endocrine changes leads to a milk secretion. For the milk production, the loss of cytoplasm in the epithelial cells is necessary, featuring a mixed apocrine secretion, containing lipids, lactose and proteins. This cell content loss causes a decrease in the average heights of the secretory epithelium. The difficulty in defining the limits of the apical secretory cells at this stage confirms this proposition. The different PAS staining shades that were observed throughout the mammary glands may be related to the changes in the molecular composition of secretion during gestation, lactation and involution. According to Grigor et al. (1986), carbohydrates concentration in mammary gland secretion may vary through the glands according to the suckling frequency, which may explain these differences in PAS staining. In C57BL/6J and SEG/Pas mice, the glycosylation of the proteins secreted by the mammary gland in the beginning of lactation is more intense than that observed in the end (Boumahrou et al., 2011). This is an indicative that the stage of lactation affects the composition of the secretion. In lactation, between adjacent secretory epithelial cells, permeable tight junctions are formed to prevent the leakage of milk components after parturition, concurrent with lactogenesis (Kobayashi et al., 2016). These junctions are formed during lactogenesis and are instrumental in establishing and maintaining milk synthesis and secretion. However its integrity is compromised during mammary involution (Stelwagen and Singh, 2014). The myoepithelial cells had a full development during lactation and their appearance at this stage was similar to that described for rats: increased cytoplasmic processes for a greater coverage of ducts during lactation (Jin et al., 2000), however, being discontinuous around the acini (Masso-Welch et al., 2000).

The increase in the PCNA rates throughout lactation indicated cell proliferation during this period, as has been described for mice (Traurig, 1967), with a peak reached in early lactation (Líška et al., 2016). In contrast, apoptosis was present in the gerbils and was identified by the presence of apoptotic bodies in the lumen, showing cell renewal.

During lactation, the ESR2 expression exceeded that of the ESR1 expression. This showed that at this stage of the mammary gland's development, although the proliferation rates were high, the return of the ESR1 receptors to the nucleus was probably occurring, as described by Cheng et al. (2004), also indicating a differentiation of the epithelial cells. As in cows (Schams et al., 2003) and sheep (Colitti and Parillo, 2013), the ESR1 receptors were also present in the cytoplasm. In sheep, the peak percentages of the PR positive cells also occurred in early lactation (Colitti and Parillo, 2013).

The mammary involution after weaning was associated with the epithelium compression, due to the milk accumulation in the lobes, together with ischemia and with an absence of mechanical stimuli suction, and even, the action of digestive enzymes present in the basal membrane (Masso-Welch et al., 2000). According to Macias and Hinck (2012), the rodent mammary involution is divided in two stages, which may be applicable for involution process observed in gerbils. The first stage is reversible and lactation can be resumed with the return of the suction stimuli. The second stage is irreversible, as the alveoli begin to collapse and the return of suction does not resume lactation. According to our results, the second phase of involution in the gerbil's mammary glands started between the third and fifth days of post-weaning, since the morphological changes were not showed on the third day, but were identified on the fifth day. The secondary involution process was more evident after 5 days of weaning and this has also been described in rats, with a greater increase in the proportion of fat when in relation to the secretory epithelium (Líška et al., 2016). According to Líška et al. (2016), each development stage in the rat's mammary gland has a specific composition of the extracellular matrix, which aids in the mammary differentiation, justifying the variation of the fiber types that were observed during the period that was assessed for the gerbils. The mammary regression procedure in the gerbils occurred faster than the development that was observed during pregnancy, since the epithelium predominance that was observed at regression day 5 was very close to that observed at gestational day 14. The values for the acini diameter, the epithelium height, and the epithelium area, supported the conclusion that at post weaning day 5, the mammary involution was already in advanced stages, with similar aspects to those that were shown in early pregnancy. Watson (2006) stated that in mice, involution of the mammary epithelium was concluded six days after the 10-day post-partum forced weaning. In rats, the adipose tissue percentages at involution day 5, is statistically similar to that observed at the beginning of lactation (Leite et al., 2007). Thus, the mammary involution in the gerbils was quicker than in mice and rats, since the adipose tissue area percentages at post-weaning day 5 were very close to those shown at the beginning of gestation. For most species, the loss of cell–cell communication may initiate involution and apoptosis of mammary epithelial cells and is a localized intramammary event, occurring only in non-suckled glands (Phyn et al., 2016). The neighboring mammary epithelial cells themselves appear to be the primary cell type responsible for apoptotic cell clearance during involution. Although there is increasing evidence that phagocytosis by mammary epithelial cells has a crucial role

in maintaining tissue homeostasis in the involuting murine mammary gland, little is known about how mammary epithelial cells become phagocytic during postpartum involution (Fornetti et al., 2016).

The hormone receptors showed a small increase in their expression during the mammary involution when compared to the lactational period. In bovine mammary glands, the expression of ESR1 and PR is similar to that observed in the present experiment, with a decrease during lactation and small increase during the mammary involution (Schams et al., 2003). These observations have also inferred about the possible role of estrogen signaling during the gland involution period.

The mammary glands underwent a considerable morphological change, which was based on a reduction of the unilocular adipose tissues during pregnancy and that was associated with the development of the mammary secretion epithelium. The cell proliferation rates remained high until the end of lactation and the expression of the hormone receptors varied considerably between pregnancy and involution. At the end of the intense production season, the glands rapidly restored their standards, substantially reducing the amount of mammary ducts and alveoli, and presenting again, a large unilocular fat deposition. Considering the regenerative abilities of the mammary gland during the reproductive phases and its high propensity to develop neoplastic lesions, new experimental models may help the understanding of molecular processes that happen in this organ. Thus, once the number of published work about Mongolian gerbils is scarce, this work helps the elucidation of its morphological characteristics. In conclusion, the Mongolian gerbil's mammary gland morphophysiology shows similarities to that of other rodents. However, a faster involution period and high proliferation rates have featured this animal as an excellent experimental model for mammary gland research. Our results have proved that Mongolian gerbils may be suitable for clarifying the molecular and morphological aspects of healthy and pathologic mammary gland.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.acthis.2017.02.003.

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