General and Comparative Endocrinology 173 (2011) 419–427

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

MMP-2 and MMP-9 localization and activity in the female prostate during estrous cycle

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ARTICLE INFO

Article history:
Received 25 January 2011
Revised 22 June 2011
Accepted 28 June 2011
Available online 13 July 2011

Keywords:
Female prostate
Estrous cycle
MMPs
Steroid hormones
Ultrastructure

ABSTRACT

The gerbil female prostate undergoes morphological and physiological changes resulting from hormonal fluctuations that occur during the reproductive cycle. These repetitive cycles of glandular growth and regression are followed by an extensive reconstruction and remodeling of prostate stroma throughout the reproductive life of the female gerbil. The objective of this study was to evaluate the effect that the hormonal fluctuations of the reproductive cycle have on the stromal remodeling and the expression and activity of matrix metalloproteinases MMP-2 and -9 in the adult female gerbil prostate. For this, serological, ultrastructural, immunohistochemical and biochemical methods were employed. The results showed that the major stromal alteration coincide with the peak of estradiol, which occurs in estrus, and with the peak of progesterone, occurring during diestrus II. MMP-2 and -9 presented a similar pattern of expression and activity during estrous cycle. The estrus was the phase of greater expression and activity of MMP-2 and -9. On the other hand, in DI and DII, the tissue expression and activity of MMP-2 and -9 was very weak. These results are important since they suggest the involvement of estradiol and progesterone in regulating the expression and activity of MMP-2 and -9 in adult gerbil female prostate.

1. Introduction

The reproductive organs undergo dramatic changes in its structure and function throughout adult life. These changes, which are controlled by hormones and cytokines, involve extensive connective tissue remodeling [12].

Most of the processes of connective tissue remodeling are accomplished by extracellular components degradation by matrix metalloproteinases (MMPs). MMPs are zinc-dependent endopeptidases that degrade various extracellular matrix components such as collagen, elastin, laminin and proteoglycans [20,29].

The MMP family members are widely expressed in many reproductive processes, including menstruation, ovulation, and embryo implantation, and also during involutive processes of the uterus, breast and prostate [13,32,35]. In many organs, MMPs activity influences the cellular behavior by altering basic functions such as proliferation, differentiation, migration and apoptosis [13,21].

In normal prostate of male rodents, MMP-2 and -9 are produced by secretory epithelial cells and stromal cells. They act in the maintenance of glandular homeostasis and are also present in prostatic fluid [8,14,15,31,32]. Expression and activity of several MMPs have been described in early and late stages of progression of prostate cancer, and the increased activity of MMPs may be associated with poor prognosis of the disease [17,27].

Structural studies demonstrate that hormonal fluctuations that occur during the estrous cycle in gerbils (*Meriones unguiculatus*) drastically alter the morphophysiology of the female prostate [10]. These changes include glandular growth and increased secretory activity of the prostate during proestrus (P) and estrus (E), and glandular regression and decrease in secretory activity during diestrus I (DI) and II (DII).

These repetitive cycles of glandular development and regression cause extensive reconstruction and remodeling of prostate stroma throughout the reproductive life of the female gerbil. Thus, the objective of this study was to evaluate the effect that hormonal

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0016-6480
doi:10.1016/j.ygcen.2011.06.017

fluctuations of the reproductive cycle have on the stromal components of the adult gerbil female prostate, with special attention to expression and activity of MMP-2 and -9.

2. Materials and methods

2.1. Experimental design

Forty adult female gerbils (*M. unguiculatus*), with 90 days of age (early adulthood) and regular 4-days estrous cycle were used in this study. Five adult males were used as controls in the biochemical experiments. The animals were maintained in the vivarium of the Biology Department of the Institute of Biosciences, Letters and Sciences campus of São Jose do Rio Preto – SP, under adequate light and temperature conditions, with food and water *ad libitum*, according to internal rules of the Committee on Ethics and Animal Welfare.

The cycling procedure was performed by analysis of vaginal smear, always at 10:00 AM on the day of sacrifice. Ten adult females in each estrous cycle phase (P, E, DI and DII) [19] were anesthetized with ketamine/xylazine and then subjected to inhalation of CO_2. The prostates (removed along with the urethra and vagina) were fixed or frozen according to the methodology for each analysis. The prostates of male gerbils were dissected in ventral (VP), dorsolateral (DLP) and dorsal lobes (DP), and frozen in liquid nitrogen.

2.2. Hormonal profile

Five blood samples from each experimental group were collected in test tubes with 4 ml of separation gel, centrifuged at 3000 rpm, and serum levels of estradiol, progesterone and testosterone were measured. The hormonal profiles were performed by using chemiluminescence method in automated Vitros-ECi (Johnson & Johnson, Orthoclinical Diagnostics Division, Rochester, NY) The detection level was 0.1–3814 pg/ml for estradiol, 0.1–150 ng/ml for testosterone, and 0.1–100 ng/ml for progesterone. The statistical tests were performed with Statistica 6.0 Software (StatSoft, Inc., Tulsa, OK). The quantitative results are expressed as mean ± SE. The Kruskal–Wallis test were applied, with $p \leqslant 0.05$ considered statistically significant.

2.3. Morphological analysis

The female prostatic complexes (three per experimental group) were fixed by immersion in 4% paraformaldehyde in phosphate buffer for 24 h. The tissues were then dehydrated in an ascending series of ethanol, cleared in xylene and embedded in paraffin (Histosec – MERK). Sections of 1–5 μm were produced on a rotary microtome (Leica RM2155, Nussloch, Germany). Histological sections were stained with the picrosirius–hematoxylin [1] to mark up the collagen fibers, and with hematoxylin–eosin for general analysis. The prostate tissue was examined under an Olympus BX60 light microscope (Olympus, Hamburg, Germany) and images were digitized by the Software Image-Pro Plus version 4.5 for Microsoft Windows™.

2.4. Immunohistochemical analysis

Histological sections of the three female prostatic complex for each experimental group were subjected to immunohistochemistry for detection of matrix metalloproteinases 2 (MMP-2) and -9 (MMP-9), as described in the protocols applied to the male prostate [6,14,15]. Primary antibodies for MMP-2 (SC6838/Santa Cruz) and MMP-9 (SC6840/Santa Cruz) were applied in a dilution of 1:20, overnight. The slides were incubated with secondary antibody labeled with peroxidase for 2 h and with avidin–biotin peroxidase for 45 min (Santa Cruz Biotechnology, Inc. – Santa Cruz, CA/USA). The reaction was revealed with diaminobenzidine (DAB) and the sections were counterstained with Harris hematoxylin.

2.5. Transmission Electron Microscopy (TEM)

Fragments of 0.5 mm from two female prostate glands at different stages of the estrous cycle were fixed in 3% glutaraldehyde diluted in Milloning buffer pH 7.3 containing 0.25% tannic acid for 24 h. The material was rinsed in buffer and post-fixed in 1% osmium tetroxide for 2 h. Subsequently, the fragments were dehydrated in acetone and embedded in Araldite resin [4]. Copper grids containing the ultrathin sections (50 nm) were contrasted with 2% uranyl acetate solution (20 min), washed in bi-distilled water, dipped in a solution of lead citrate at 2% (6 min) and washed again. The observations were performed in a Zeiss EM-910 transmission electron microscope.

2.6. Biochemical analysis

The biochemical evaluation of the activity of MMP-2 and -9 in the prostates of gerbil males and females during the estrous cycle were performed using frozen samples of five animals for each group. In males, the prostate was separated into its three lobes: ventral (VP), dorsal (DP) and dorsolateral (DLP). In females, the prostate was completely isolated from the urethra and adipose tissue associated. The study was conducted using the zymography technique. The prostates were homogenized in extraction buffer (30 mg tissue/100 μl solution) containing 50 mM Tris–HCl pH 7.4, NaCl 0.2 M, Triton X-100 0.1% and 0.1% protease inhibitor cocktail (P-8849-CO-Sigma, St. Louis, MO, USA). The homogenate was incubated for 2 h at 4 °C to increase the efficiency of extraction. After incubation, the homogenate was centrifuged at 4000 rpm and the supernatant was maintained at −80 °C. The protein concentration in each sample was determined using the Bradford method [2]. Aliquots of the extract were subjected to electrophoresis under non-reducing conditions (100 V at 4 °C) in 8% polyacrylamide gel containing 0.1% gelatin (denatured collagen). After electrophoresis, the gels were washed in Triton X-100 2.5% and in 50 mM Tris HCl pH 8.4. Subsequently, the gels were incubated for 12 h at 37 °C in the same buffer containing 5 mM $CaCl_2$ and 1 μM of $ZnCl_2$. After incubation, the gels were stained with Brilliant Blue Coomassie 0.25%. The bands obtained in zymography were scanned and analyzed by densitometry. The gelatinolytic activity of MMPs was analyzed obtaining the integrated optical density (IOD) of bands using a computer software (Image Master VDS, 3.0) coupled to the Image Master VDS apparatus. The values were plotted in histogram showing the ratio of the IOD groups of estrous cycle and IOD of male prostates.

3. Results

3.1. Serum hormone levels

Table 1 shows the hormone levels of estradiol, progesterone and testosterone in gerbil adult females during the estrous cycle. Serum levels of estradiol remained constant throughout the estrous cycle (33 ± 7.4–38.5 ± 9 pg/ml), with a peak concentration only in phase E (49.6 ± 15 pg/ml). Progesterone showed a peak concentration during DII (19.1 ± 10.7 ng/ml) and remained constant in the other phases of the cycle (10.1 ± 4.5–12.8 ± 4.1 ng/ml). Testosterone levels remained constant throughout the estrous cycle (0.3 ± 0.1–0.5 ± 0.1 ng/ml).

Table 1
Serum levels of estradiol, progesterone and testosterone in adult gerbil females during the phases of the estrous cycle (n=5 samples/group; mean ± SE).

	Proestrus	Estrus	Diestrus I	Diestrus II
Estradiol (pg/ml)*	$38.5^{a} \pm 9.0$	$49.6^{b} \pm 15$	$33.0^{a} \pm 7.4$	$34.5^{a} \pm 9.9$
Progesterone (ng/ml)*	$10.1^{a} \pm 4.5$	$10.6^{a} \pm 4.6$	$12.8^{a} \pm 4.1$	$19.1^{b} \pm 10.7$
Testosterone (ng/ml)	0.3 ± 0.1	0.5 ± 0.1	0.41 ± 0.1	0.3 ± 0.1

Superscript letters (a and b) represent statistically significant differences between the experimental groups.
* Statistically significant differences ($p \leqslant 0.05$).

3.2. Morphological analysis

In phase P, the stroma showed a large amount of collagen fibers arranged between the smooth muscle and the base of the alveolar epithelium (Fig. 1a and b). In the phases of E (Fig. 1c and d) and DI (Fig. 1e and f) the alveolar lumen became wider and collagen fibers were distributed as delicate fibrils interspersed with the smooth muscle cells. In the DII phase, prostatic alveoli were reduced and stroma became very dense, presenting a large amount of collagens fibers either in the epithelium basis or in deeper portions of stroma (Fig. 1g and h).

3.3. Ultrastructural analysis

In P, smooth muscle cells are characteristically fusiform and are closely associated to bundles of collagen fibers and other fibrillar and nonfibrillar components of the extracellular matrix (Fig. 2a). In E, the smooth muscle cells assume a spinous-like aspect, typical of contractile cells (Fig. 2b). At this stage, bundles of collagen fibrils and elastic system fibers can be observed in the spaces formed among the smooth muscle cells extensions (Fig. 2c). In DI, the smooth muscle cells appear less developed and interspersed by irregular bundles of collagen fibrils and elastic fibers (Fig. 2d), although in DII, the perialveolar stroma is densely filled by bundles of collagen fibrils interspersed by smooth muscle cells (Fig. 2e). In this phase, the cytoplasmic projections of smooth muscle cells show numerous caveolae, and its projections appeared associated with elastic fibers (Fig. 2f).

3.4. Immunohistochemical analysis of MMP-2 and MMP-9

Cytoplasmatic immunoexpression of MMP-2 was intense either in the epithelial (Fig. 3a–d and f–i) or smooth muscle cells (Fig. 3b and g) of female prostates in the phases P, E, DI and DII. MMP-2 and -9 immunostaining were also observed in some nuclei of epithelial cells and fibroblasts (Fig. 3h, i, l, and m). Deep regions of stromal compartment also presented MMP-2 immunoexpression in all phases of estrous cycle (Fig. 3a–d and f–i). Negative control has shown an absence of MMP-2 immunoexpression in the female prostates (Fig. 3e). Conversely, the immunoexpression of MMP-9 was more intense in P phase (Fig. 3j and k), and especially in E phase (Fig. 3l and m). The P phase was characterized by an intense immunoreaction of the fibroblasts and smooth muscle cells (Fig. 3j and k). In E, epithelial cells and fibroblasts of the deep portions of stroma showed very strong immunoreaction for MMP-9. Smooth muscle cells did not express this metalloproteinase in E (Fig. 3l and m). DI (Fig. 3n and o) and DII (Fig. 3p and q) phases have shown a weak immunoexpression of MMP-9. Moreover, for these phases (DI and DII) this reaction was restricted to the apical

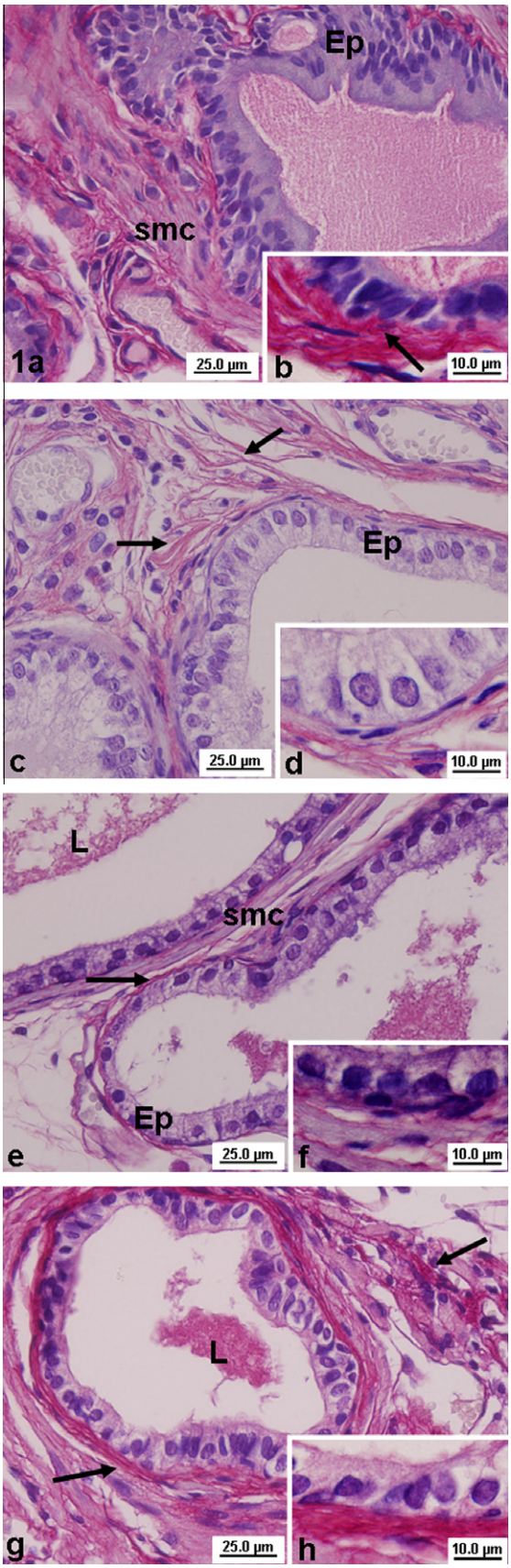

Fig. 1. Histological sections of the gerbil female prostate stained with picrosirius–hematoxylin. (a and b) Phase P: Dense stroma with compact bundles of collagen fibers (arrows) at the base of the epithelium (Ep). Smooth muscle cells (smc). (c and d) Phase E: Thin collagen fibers around of the developed prostatic alveoli. (e and f) Phase DI: Alveolar expansion leads to rearrangement of collagen fibers (arrows) in thin fibrils arranged around the prostatic alveoli. Lumen (L). (g and h) Phase DII: Reduced prostat ic alveoli are presented surrounded by compact layers of collagen fibers (arrows).

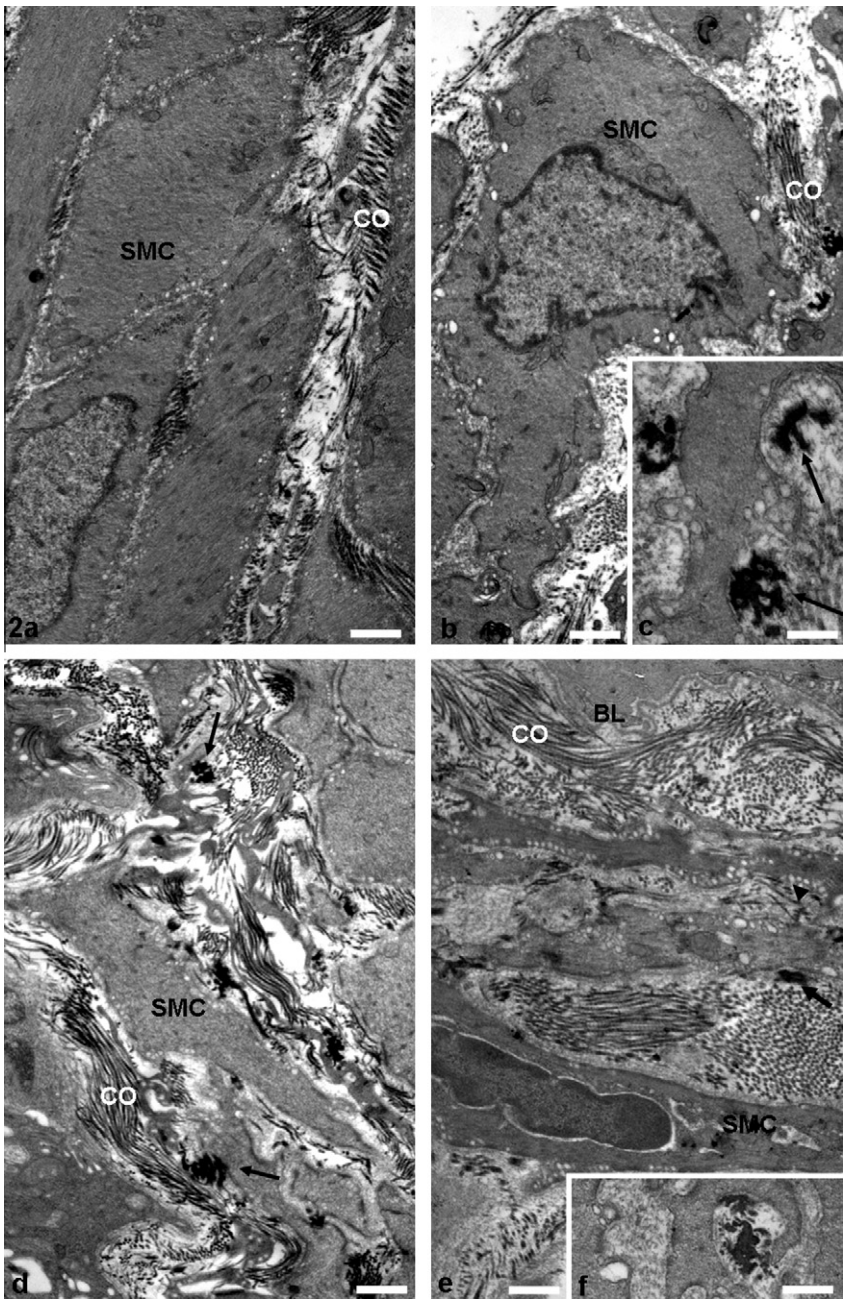

Fig. 2. Ultrastructure of the stroma of the gerbil female prostate in all phases of the estrous cycle. (a) Phase P: Regular smooth muscle cells (SMC) separated with collagen fibers (CO). Bar = 2 μm. (b and c) Phase E: Smooth muscle cell (SMC) with contractile aspect associated to collagen (CO) and elastic fibers (arrows). Bars: b = 2 μm, c = 700 nm. (d) Phase DI: Smooth muscle cells (SMC) presenting numerous extensions in association with the extracellular matrix elements. Elastic fibers (arrows). Bar = 2 μm. (e and f) Phase DII: Dense layers of collagen fibers (CO) interspersed with smooth muscle cells (SMC). The extensions of the smooth muscle cells exhibit numerous caveolae (arrowhead), and are associated with elastic fibers (arrows). Basal lamina (BL). Bars: e = 2 μm and f = 700 nm.

cytoplasm of secretory epithelial cells (Fig. 3n and q) and fibroblasts (Fig. 3o and q).

By analyzing the vaginal tissue closely associated to the prostate (Fig. 4) it could be noticed that MMP-2 expression occurs especially in the epithelium and in the vaginal muscle layer. This immunoexpression pattern was observed in phases P, DI and DII. However, in E the expression of MMP-2 was more pronounced and observed in all layers of the vaginal wall (Fig. 4b). The immunoexpression of MMP-9 was observed in the lining epithelial cells and diffusely in the fibroblasts of the lamina propria and in the vagina muscle layer of gerbil female prostates in the phases P, DI and DII (Fig. 4e, g, and h). In E phase, MMP-9 immunoexpression was found being more intense and in all tissues that make up the vaginal wall (Fig. 4f).

3.5. Zymography

The zymography gel of the female and male gerbil prostates showed the typical clear bands of MMP-2 (pro, intermediate and active enzyme) and MMP-9 (pro and active enzyme) (Fig. 5a). All forms of MMP-2 and -9 showed higher gelatinase activity in the prostate of females than in the three prostatic lobes of the male (Fig. 5b–f).

In the female prostate, all forms of MMP-2 and -9 presented strong activity in P and, especially, in E (Fig. 5b–f). Phase DI was the one with the lower gelatinase activity both for MMP-2 and -9 (Fig. 5b–f). In DII, pro and active MMP-9 and pro-MMP-2 showed a recovery in its activity, whereas intermediate and active MMP-2 maintained a lower activity according to IOD analysis.

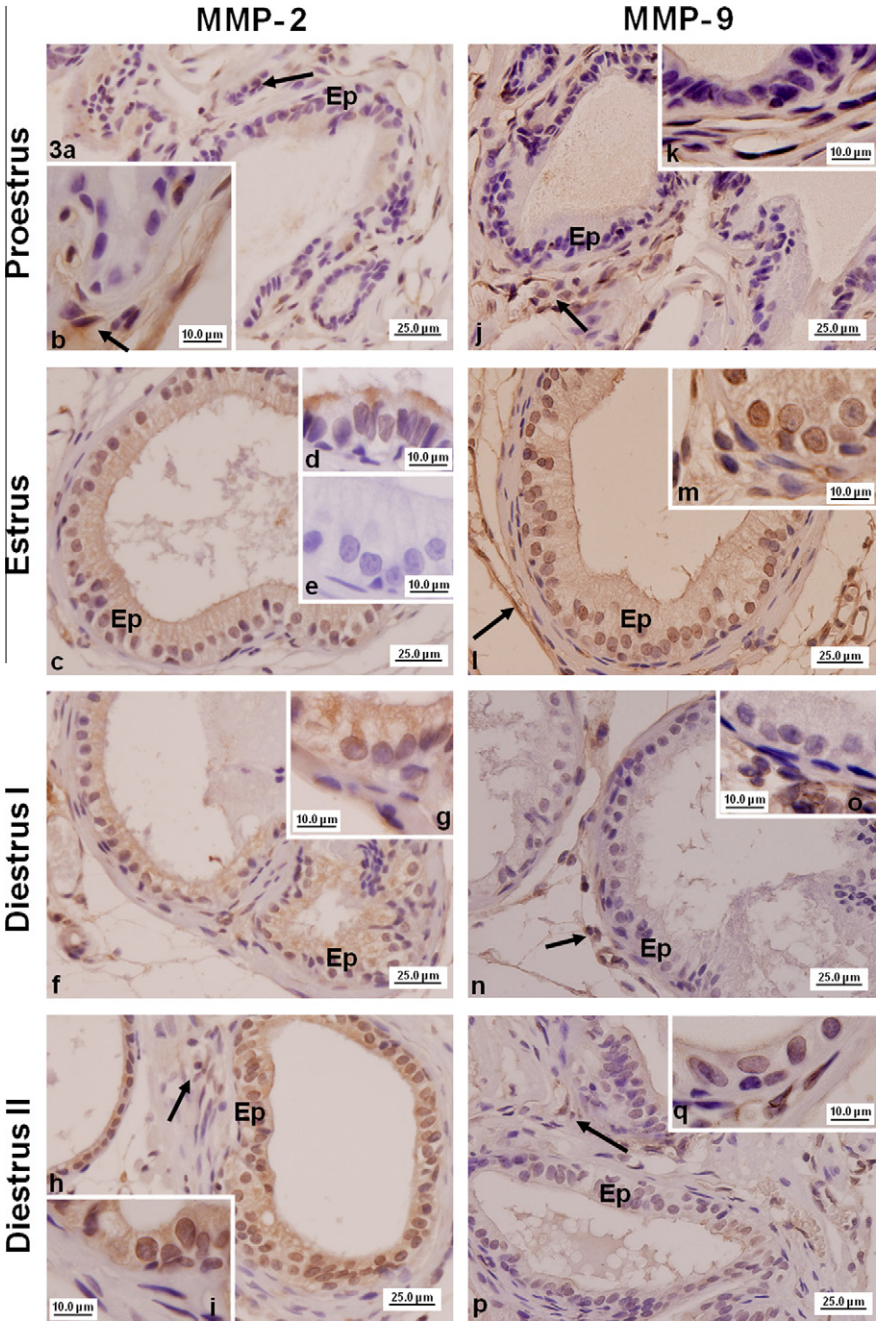

Fig. 3. Immunoexpression of MMP-2 and MMP-9 in the gerbil female prostate during the estrous cycle. Immunoreaction of MMP-2 is observed in the cytoplasm of secretory epithelial cells (Ep) and fibroblasts (arrows) in deep regions of the prostatic stroma of females in phases P, E, DI and DII. The immunoexpression of MMP-9 is more intense in the epithelial cells (Ep) and in fibroblasts (arrows) of the prostates in the phases P and, especially, E. In DI and DII this immunoexpression was more evident in the fibroblasts placed next to the smooth muscle cells (arrows).

4. Discussion

Several studies have reported the tissue structure and composition of the female prostate in rodents [5,9,10,11,24,25] and in humans [26,34]. However, this is the first study detailing the stroma profile and the expression and activity of MMPs in the female prostate.

The morphophysiological pattern of the prostate glands analyzed here is consistent with data previously published [10], where the gerbil female prostate shows a well developed and active secretory phenotype in the phases P and E, and a glandular involution in DI and DII. However, data from serum levels of estradiol and progesterone described by Fochi et al. [10] are conflicting to those obtained by this research. The formation of additional experimental groups, as well as the supplementary assays for hormonal analysis can explain this divergence of result. Importantly, the hormonal results presented here were confirmed by three repetitions and are consistent with hormonal pattern showed by females of other rodent species [7,30].

Our serological data showed that the major hormonal alterations of the reproductive cycle in the female gerbil occur during E, with a peak of estradiol, and in DII, with a peak of progesterone. These hormonal variations coincide with major stromal alterations observed in this study, indicating the importance of estradiol and

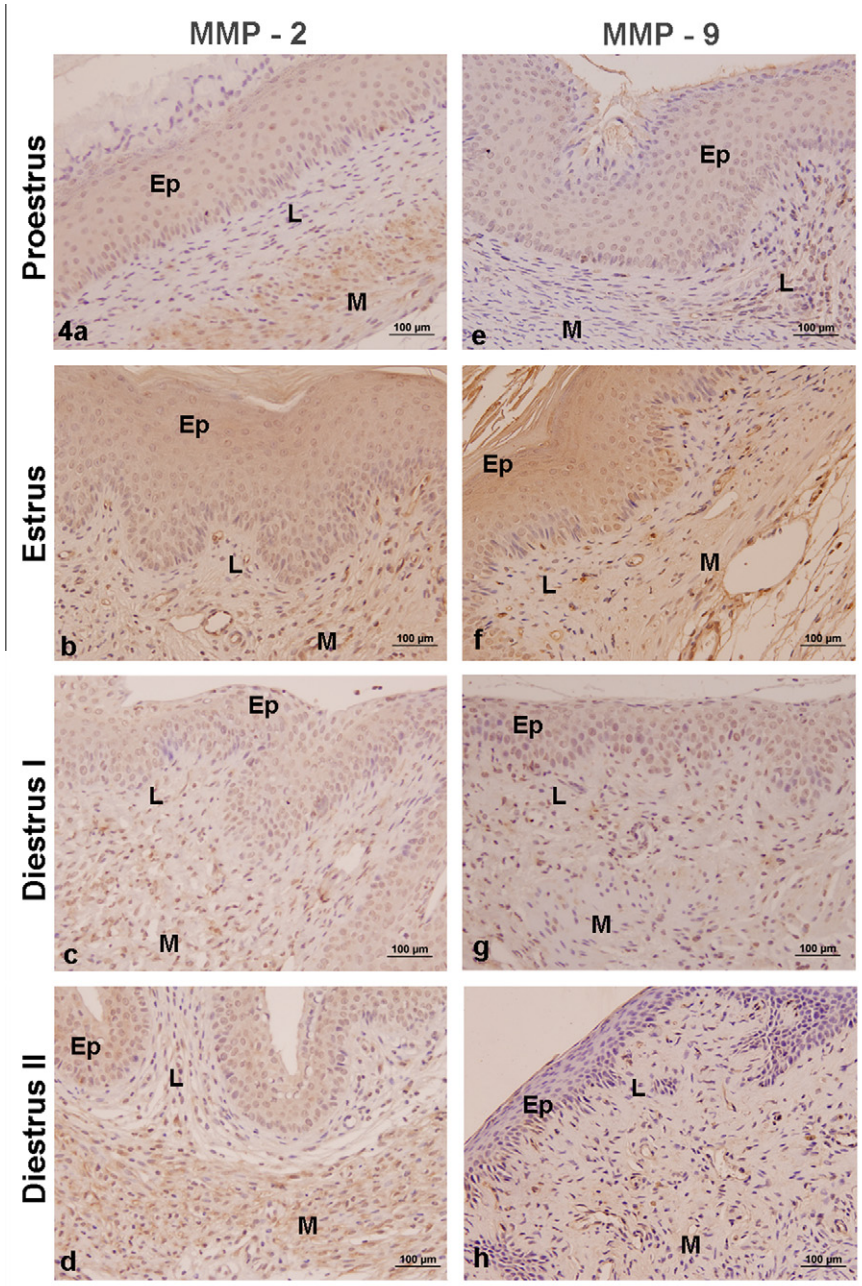

Fig. 4. Immunoexpression MMP-2 and MMP-9 in the female gerbil vagina, adjacent to the prostatic tissue, during the estrous cycle. The immunoexpression of MMP-2 is observed in the epithelial (Ep) and muscle (M) layer of the vagina of females in phases P, DI and DII. In phase E the expression of MMP-2 is very intense in all layers of the vaginal wall. Lamina propria (L). The immunoexpression of MMP-9 is observed in the luminal epithelium (Ep) and diffusely in the lamina propria (L) and smooth muscle layer (M) at stages P, DI and DII. In E, the immunoexpression of MMP-9 is very intense in the epithelium (Ep) and the muscular layer (M).

progesterone in the regulation of components that promote stromal remodeling during the estrous cycle in gerbils. However this affirmative is only correlative, further experimental studies will be needed to reinforce this hypothesis.

The ultrastructural analysis of the prostatic stroma during the estrous cycle revealed that smooth muscle cells alternate their ultrastructural aspect from a fusiform in phases P and spinous-like in the E phase to a contractile state in phase DI and, especially, DII. In DII, the epithelial involution is accompanied by a reduction in the glandular lumen and an intense stromal rearrangement. In this respect, the contractility of smooth muscle cells in this phase seem to participate in the remodeling of collagen fibers in the compact bundles that were observed around the prostatic alveoli. Analog situation of alternating ultrastructural features in the smooth muscle cells was previously studied by our research group, which suggested that in the rat male prostate after androgen deprivation, the smooth muscle cells undergo a contractile to synthetic phenotype without changing their differentiation state [28]. In current experimental model the synthetic ultrastructural aspect of smooth muscle cells is discrete, but the contractile activity is very intense and occurs in the DI and DII phases for stromal remodeling. It is important reinforce that synthesis and degradation of extracellular matrix components in the prostate stroma is the primary effects in the remodeling dynamic, and, in this model, may be associated to the stromal contraction by smooth muscle cells activity.

However, the main finding of our study was about MMPs immunoexpression in the gerbil female prostate during estrous cycle. The immunohistochemistry technique demonstrated that

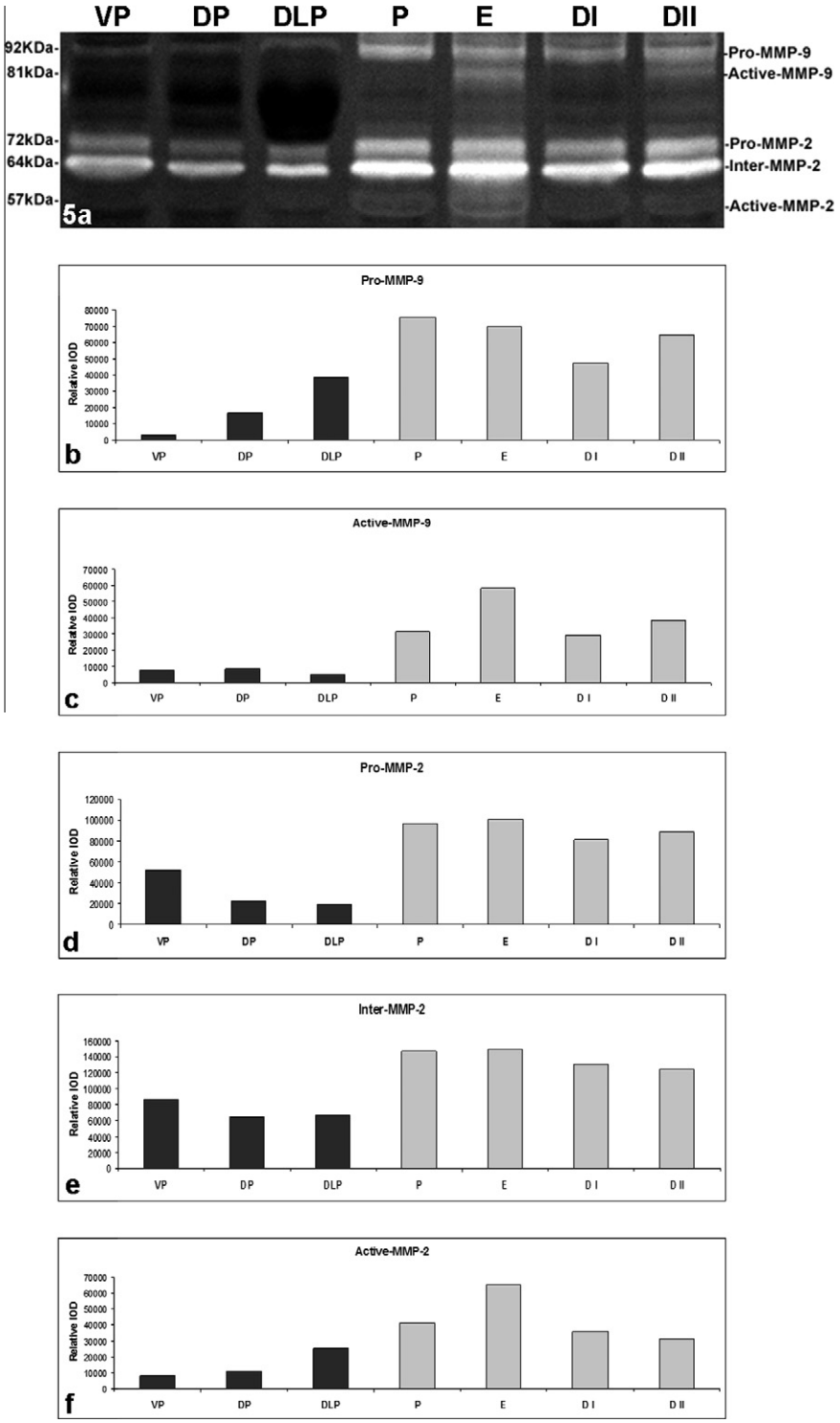

Fig. 5. Zymography gel showing the typical clear bands of MMP-2: proenzyme (72 kDa), intermediate (64 kDa) and active enzyme (57 kDa); and MMP-9: proenzyme (92 kDa) and active enzyme (81 kDa). In the gel and in the quantification graphs are represented the male prostate lobes: ventral (VP), dorsal (DP) and dorsolateral (DLP), and female prostate in all phases of the estrous cycle: proestrus (P), estrus (E), diestrus I (DI) and diestrus II (DII). Quantification of gelatinase activity of MMPs is expressed as IOD (integrated optical density). The male prostatic lobes are represented in black and the female prostate in the different estrous cycle phases is shown in gray.

MMP-2 had a high immunoexpression in all phases of estrous cycle. However, the zymography technique demonstrated that although MMP-2 was produced continuously, it was more active in E than other phases. These results suggest that MMP-2 can be produced constitutively without hormonal control, while its activity is regulated by steroid hormones.

The E was the phase of greater immunoexpression and activity of MMP-2 and -9. On the other hand, in DI and DII, the tissue expression of MMP-9 and activity of MMP-2 and -9 was very weak. These results suggest that MMPs activity in female prostate follows a similar pattern described for uterine endometrium, which estrogen has a stimulatory action and progesterone has an inhibitory

role [11,18,22]. This finding is very important, since the vast majority of studies about MMPs expression and activity indicate the testosterone as key hormone in the regulation of these peptidases in male prostate [8,15,16,31].

In our study, some nuclei from female prostate epithelial cells presented positive immunostaining for MMP-2 and -9. New locations and substrates form MMPs have been described, including nuclear compartment [3]. However, the function of MMPs in the nuclei from female prostate epithelial remains to be determined.

Furthermore, analysis of the vaginal tissue adjacent to the urethra and prostate showed that even though at the same stage of estrus cycle, the female prostate and the vagina have distinct MMPs patterns of expression. In the vagina, the tissue expression of MMP-2 and -9 increases during the E phases, and maintained a constant pattern of expression in other cycle phases.

Several previous studies have demonstrated that the rodent female prostate is homologous to the ventral lobe of male rodent prostate [11,23,33]. However, one important feature observed in this study was that adult gerbil female prostate, in all phases of the estrous cycle, presented activities of MMP-2 and -9 up to 12 times higher than any adult male prostatic lobes. These differences possibly reflect the repetitive cycles of development and involution that the gerbil female prostate undergoes every 4–5 days. These modifications require constant involvement of MMPs in the degradation and reconstruction of the extracellular matrix components, such as basement membranes and collagen system fibers.

Studies with senile gerbils (12–18 month-aged) demonstrated that the female prostate presents signs of aging and spontaneous neoplastic foci earlier than males of the same age [5]. In addition, studies involving the expression and activity of MMPs in rodents prostate cancer have demonstrated that the increased activity of these enzymes is related to the high indexes of invasiveness and indifferentiation of the tumor cells [17]. In this manner, the high and constant activity of MMP-2 and -9 in the female prostate of gerbil may be associated to the appearance of early spontaneous lesions in this gland.

5. Conclusions

Thus we can conclude that MMP-2 and MMP-9 exhibit a pattern of expression and activity in the gerbil female prostate during the estrous cycle similar as observed in uterine endometrium, actively participating in the tissue remodeling during glandular growth that occurs in P and E phases. Furthermore, the expression and activity of these metalloproteinases in gerbil female prostate is up to 12 times higher than adult male prostate.

Acknowledgments

This work was supported by Grants from the Brazilian Agencies FAPESP – São Paulo Research Foundation (Procs. Nos. 06/06952-0; 06/06985-5; 06/06876-1) and CNPq – Brazilian National Research and Development Council (Procs. Nos. 301111/05-7 and 300163/2008-8 research fellowships to S.R.T.). This paper is part of the thesis presented by S.S.R.M. to the Institute of Biology, UNICAMP, in partial fulfillment of the requirement for a Doctoral degree and part of the post doctoral fellow of FCAS. The authors wish to thank to Mr. Luiz Roberto Falleiros Júnior for technical assistance, as well as all other researchers at the Microscopy and Microanalysis Laboratory.

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