The effect of buserelin acetate on the uterus of adult rats: morphological aspects

C.R. Trindade[1], *M.D.*; A.F. Camargos[2], *M.D., Ph.D.*; F.E.L. Pereira[3], *Ph.D.*

[1]Department of Gynecology and Obstetrics, School of Medicine, School of Health Sciences, Espírito Santo
[2]Department of Gynecology and Obstetrics, School of Medicine, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais
[3]Department of Pathology, School of Medicine, Federal University of Espírito Santo, Espírito Santo (Brazil)

Summary

Purpose of investigation: To evaluate the effect of buserelin acetate on the morphology of the endometrium of adult, non-castrated, female Wistar rats. *Methods:* Female Wistar rats at estrus or diestrus (assessed by vaginal cytology) received daily subcutaneous injections of 20 mg buserelin acetate for four, eight or 12 days. Rats were sacrificed 24 hours or five days following final dosage. A control group received diluent for 12 days. *Results:* Progressive tissue hypotrophy occurred during treatment and was followed by estrogenic hyperactivity five days after the end of treatment. Vaginal cytology and endometrial histology revealed intense, vacuolized lining and glandular epithelia, brush borders and endometrial stroma densely infiltrated with eosinophils. *Conclusions:* Buserelin acetate appears to cause a progressive blockade of gonadotrophin secretion when administered to female rats for four, eight or 12 days, and an important rebound effect, with accentuated estrogen release already apparent in the first estrous cycle following treatment.

Key words: Buserelin; Gonadotrophin; Female rats; Estrous cycle; Cytology; Endometrium; Eosinophilia.

Introduction

Over the last 30 years, further research on GnRH analogs has confirmed the molecular stability of these substances, as well as their greater affinity for pituitary receptors, their longer half-life, enzymatic resistance and increase in ovulatory potential. Different effects can be achieved by varying their dosage, route of administration, duration of use and the posology of the drug. When administered intermittently, they induce the pulsatile release of the follicle stimulating hormone (FSH) and luteinizing hormone (LH). When used continuously, there is a strong initial stimulus followed by subsequent gonadotrophin desensitization by receptor endocytosis, resulting in reversible hypogonadotrophic hypogonadism [1, 2].

This capacity of gonadotrophins to display either a stimulatory or an inhibitory effect has permitted the ample clinical use of GnRH analogs (GnRH-a), particularly in *in vitro* fertilization [3], prostate cancer [4], breast cancer [5], uterine myomas, endometriosis [6] and precocious puberty [7].

With respect to side-effects, GnRH-a may cause hot flashes, flushing, weight gain, nausea, vaginal atrophy, discomfort, myalgia, cephalea, vomiting, depression, mood swings, acne, premenstrual syndrome, decreased libido, abdominal pain, mastalgia, asthenia, irritability, dizziness, dry skin, constipation, dyspepsia, gynecomastia and coughing [6].

In view of the important gonadotrophin stimulating and inhibiting capacity of GnRH analogs, the objective of this study was to evaluate the onset of these effects by assessing the morphological changes to the uterus and vagina induced by a GnRH-a. Female Wistar rats that reach sexual maturity at a mean of 92 days, have a gestation of 22 days and an estrous cycle of 3.7-5 days' duration, similar to that of humans with respect to variations in LH, FSH and prolactin [8], were used in the study. The estrous cycle of the rat is characterized by being regular, periodic and coordinated, and the histology of its reproductive tract is well-defined. The phases of diestrus, proestrus, estrus and metestrus are characteristic, with vaginal cytology, the basic structure of the uterine wall, vascular architecture and leukocyte and eosinophil infiltrates undergoing predictable periodic variations. The endometrium is the area in which the greatest physiological variations in reproductive activity occur (Table 1) [8-10].

Materials & Methods

Female adult Wistar rats (Rattus norvegicus albinus), aged between 82 and 102 days, and weighing 250-330 grams were used in this study. They were maintained in confinement, isolated from males following gender identification, and fed with unrestricted chow and drinking water. The rats were introduced into the study during diestrus and estrus (Figure 1), identified using wet mount vaginal cytology. These phases were chosen because they coincide with, respectively, the lowest and highest synthesis and release of endogenous gonadotrophins. The study protocol was approved by the local ethics committee for the control of research studies involving laboratory animals in accordance with the IRB of the School of Medicine, Federal University of Minas Gerais.

For cytology, three drops of 30% toluidine blue were deposited in the vagina of the rats using an automatic pipette tip attached to a rubber dropper, and material was immediately col-

Revised manuscript accepted for publication July 30, 2007

Fig. 1

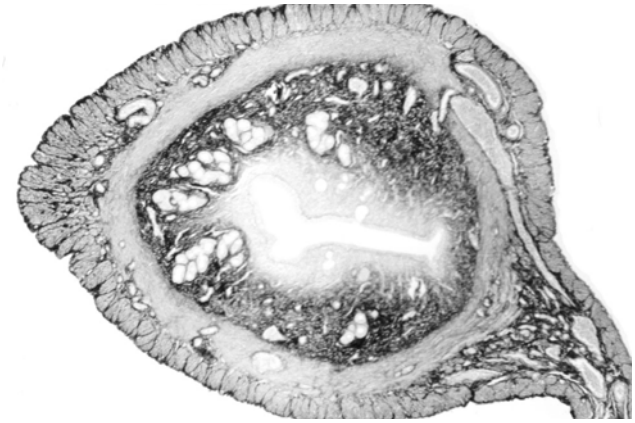

Fig. 2

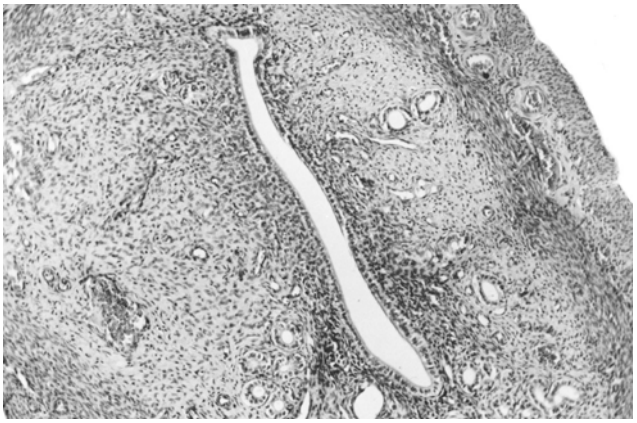

Figure 1. — Rat uterus in estrus: stained with picrosirius which reveals collagen and permits greater contrast and clarity between structures. Uterus with little dilatation, thick, folded lining epithelium, stroma with strong cell infiltrate close to the epithelium and collagen dominating at the periphery. Clear proliferation and vascular dilatation and muscle layer is well-defined (magnification x 100).

Figure 2. — Rat uterus after 12 days of use of buserelin acetate in the experiment initiated at diestrus. No dilatation, low lining epithelium with no folding, disperse glands with no dilatation. Vessels with little proliferation and no dilatation. Hematoxylin-eosin staining (magnification x 250).

Fig. 3

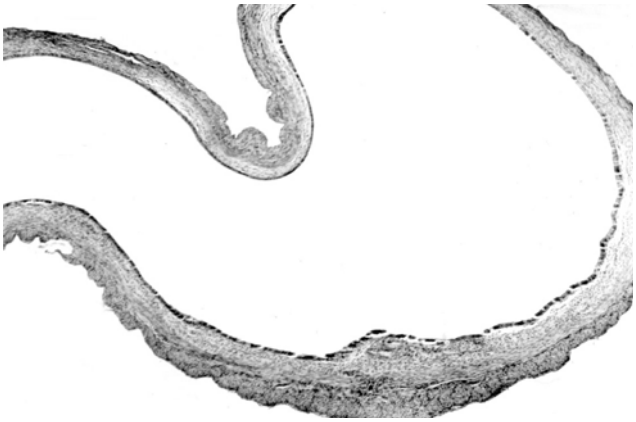

Figure 3. — Rat uterus at the end of the first estrous cycle following termination of buserelin acetate in the experiment initiated at diestrus. Uterus strongly dilatated with high lining epithelium and stroma compressed due to distention of the uterus (hematoxylin-eosin staining; magnification 100 x).

lected for examination using the thick drop method between a slide and a coverslip under an optical microscope. This procedure was repeated every three days until completion of the required number of rats in each study group, and then again on the day the animals were sacrificed. The definition of the estrous phase is based on the proportions between nucleated cells, keratinized cells and leukocytes obtained from the vaginal cavity (Table 1).

The GnRH analog used was Suprefact®, supplied by Hoechst do Brasil Química e Farmacêutica S.A., São Paulo, SP, Brazil, for subcutaneous use, composed of 1 mg of buserelin acetate and 10 mg of benzyl alcohol diluted into 1 ml of vehicle. Each animal received 20 µg of buserelin acetate subcutaneously at a volume of 0.1 ml following dilution of the drug. The control group received 0.1 ml of the vehicle commercialized in Suprefact®, also supplied by Hoechst and labeled "placebo". The estrous cycle of four days and the dose of buserelin acetate used were based on observations in the literature from studies carried out by Long & Evans and Lobel *et al.* [9, 10]. Using a 1 ml disposable syringe and a 4x13 needle, the rear paw of the rat was transfixed for the injection in the subcutaneous region opposite the point of puncture, avoiding reflux and loss of the product. Table 2 describes the flowchart for vaginal cytology, administration of buserelin acetate and sacrifice of the rats in experiment A, beginning during diestrus, and experiment B, beginning during estrus.

The rats were sacrificed following inhalation of anesthesia with sulphuric ether. Samples were collected for vaginal cytology, after which the abdominal cavity of the rat was opened to remove the uterus, ovaries, bladder and the upper third of the vagina. Under macroscopy using a pachymeter, the length of the smallest uterine horn and the length of its mid-third were measured. Next, the uterus was placed on filter paper on a Petri dish and fixed in 10% formaldehyde. Three fragments were obtained from the mid-third of the smallest uterine horn, and these were embedded in paraffin and stained with hematoxylin-eosin and picrosirius, the latter for the detection of collagen in the tissue.

Using an optical microscope, the muscle layers of the uterus and the endometrium were examined, paying particular attention to the glandular and lining epithelia and to the stromal cellularity. Morphometry was used to measure the height of the lining and glandular epithelia, and the thickness of the endometrial stroma and the muscle layer, as well as the internal area of the uterus. All measurements were taken at the 12, 3, 6 and 9 o'clock positions using an ocular micrometer calibrated with a 1 mm rule divided into 100 subdivisions of 10 µg each. The measurement used was the arithmetical mean of the measurements taken at the four established points of reference. For the intensity of dilatation and vascular congestion of the uterus, the stromal cellularity, presence of intraepithelial leukocytes, eosinophils in the stroma and the proportion of nucleated cells, keratinized anucleated cells and leukocytes in the vaginal cytology, a score was established as follows: slight (+), moderate (++) and strong (+++).

Statistical analysis was performed using non-parametric tests for the comparison of different groups with each other, using the statistical software program GB-Stat Professional Statistics & Graphics, version 4.0 (Dynamic Microsystems, Inc., Silver Spring, USA). Differences were considered significant when $p < 0.05$.

Table 1. — *Uterine and vaginal characteristics of the estrous cycle of adult Wistar rats.*

Phasesof the cycle Duration	Vagina	Uterus	Endometrium
Diestrus 44-57 hours	1-7 layers of polyhedral cells. Few mitoses. Moderate number of epithelial cells and leukocytes.	Narrow cavity. Stroma with few leukocytes. Vessels with no dilatation.	Compact and cuboid. With mitotic figures. Low metabolism. No vacuolization. Nuclei situated at the base of the cell.
Proestrus 9-20 hours	8-12 cell layers. Many nucleated cells. Few keratinized cells. Absence of leukocytes.	Edema of the wall. Dilatation of the cavity, glands and vessels. Leukocyte infiltration.	Columnar epithelium. High metabolism. Few mitoses. Some vacuoles.
Estrus 12-30 hours	8-12 cell layers. Intense keratinization. Few nucleated cells. Absence of leukocytes.	Intense edema. Dilatation of the uterine cavity, glands and vessels. Great leukocyte and eosinophilic infiltration.	High columnar epithelium. Highly vacuolized. Intense eosinophilic infiltration. Brush border. Pseudo-stratification.
Metaestrus 3-12 hours	4-8 cell layers. Many nucleated keratinized cells and leukocytes.	No dilatation, edema or leukocyte infiltration.	Low and cuboid. Nuclei in the cell base. No vacuolization.

Table 2. — *Flowchart of vaginal cytology, administration of buserelin acetate, and sacrifice of the rats in the experiments, beginning either during diestrus (A) or during estrus (B).*

Experiments	A and B				
Medication	Buserelin Acetate			Diluent	
Groups	I	II	III	IV	Untreated
Days on medication	4	8	12	12	12
Day on which vaginal cytology was performed	1st & 5th	1st & 9th	1st & 13th	1st & 17th	1st & 13th
Day of sacrifice of rats	5th	9th	13th	17th	13th

Results

When initiated at diestrus or estrus, buserelin acetate induced a blockade of gonadotrophic secretion, establishing characteristics similar to diestrus, with a tendency towards progressive hypotrophy when the drug was administered for four to 12 consecutive days and the animals sacrificed 24 hours following the end of treatment. Vaginal cytology revealed moderate epithelial cells and leukocytosis, few mitoses and scarce keratinized anucleated cells; uterus with a narrow internal area, stroma with few leukocytes, vessels with no dilatation, endometrium with compact cuboid epithelium, mitosis figures, nuclei positioned at the base and absence of vacuolization (Figure 2).

On the contrary, in the rats sacrificed at the end of the first estrous cycle following 12 days of treatment, irrespective of whether the experiment was initiated in the diestrus or in the estrus, the results of cytology and uterine morphology were characteristic of estrogenic hyperactivity. Vaginal cytology showed intense cell keratinization (anucleated cells), few nucleated cells and absence of leukocytes. There was strong uterine dilatation, intense edema in the vessels and glands, strong leukocyte and eosinophilic infiltrate, high columnar endometrium, with highly vacuolized brush borders, pseudo-stratified, and strong infiltration of eosinophils (Figure 3).

Discussion

In the groups treated with buserelin acetate for a period of approximately one to three estrous cycles (4-12 days) and sacrificed 24 hours following administration of the final dose, cytology revealed a great number of leukocytes, nucleated cells and scarce or no keratinized anucleated cells. There was a progressive reduction in estrogenic activity. In the groups sacrificed after one estrous cycle following the end of treatment, cytology was characterized by an abundance of keratinized, anucleated cells that corresponded to practically all the cells in the smear. This aspect of cytology is compatible with strong estrogenic activity. It emphasizes not only the immediate return of gonadotrophic secretory activity following the use of buserelin acetate but also an intense gonadotrophic response, typical of a rebound effect.

In the first four days of treatment, the results of the two experiments initiated during diestrus and estrus were similar, with a slight estrogenic stimulus and a small increase in the height of the glandular epithelia and epithelial lining and in the thickness of the muscle layer. In the animals treated for more than four days, the uterus tended towards diestrus, i.e., with no dilatation in the cavity, no vascular congestion, and a cuboid endometrial and glandular epithelium, with scarce cell exudate in the stroma. This confirms that buserelin acetate effectively induces a progressive blockade of gonadotrophin production, giving the uteri the morphological characteristics of organs that have not undergone hormonal stimulation. This progressive blockade was very marked in the animals treated for 12 days, where values of epithelial height were lower, indexes of eosinophilic exudation were lower in the endometrium and vaginal cytology was typical of diestrus.

The animals sacrificed after one estrous cycle without medication had morphological data indicative of intense estrogenic uterine activity. The lining and glandular epithelium were high, vacuolized, with brush borders,

and the endometrial stroma was intensely infiltrated by eosinophils. The presence of eosinophils is a morphological finding strongly indicative of estrogenic hyperactivity in the rat [10]. Dilatation and the accumulation of liquid are also morphological findings typical of uteri under the influence of estrogen. These observations permit us to conclude that the inhibition of gonadotrophic secretion induced by buserelin acetate may, when the drug is withdrawn, go through a typical rebound phenomenon.

Conclusion

The experiments show that buserelin acetate injected subcutaneously in the female rat at a dose of 20 µg/day for more than one estrous cycle tends to result in vaginal cytology and uterine morphology compatible with a state of low gonadotrophic stimulation, resembling diestrus.

In contrast, four days after cessation of the treatment, there seems to be a release of gonadotrophins apparently greater than that of a normal cycle, a fact confirmed by the pronounced alterations in vaginal cytology and in uterine morphology that are compatible with estrogenic hyperactivity.

In the experiment initiated during diestrus, the weight gain curve followed the same pattern as during estrus, but percentages of weight gain were clearly greater in the first group. On the other hand, the rats in the untreated groups in both experiments underwent no variations with respect to percentage of weight gain, confirming that buserelin acetate may result in a greater weight gain in rats when administration is initiated during diestrus. We were unable to find any satisfactory explanation for this finding in the literature.

Acknowledgements

The GnRH analog and the vehicle used in Suprefact® were supplied by Hoechst do Brasil Química e Farmacêutica S.A., São Paulo, SP, Brazil. The animal laboratory was made available by the School of Medicine, School of Health Sciences, Vitória, Espírito Santo, Brazil.

References

[1] Yaman C., Ebner T., Sommergruber M., Hartl J., Pölz W., Tews G.: "Three-dimensional endometrial volume estimation as a predictor of pituitary down-regulation in an IVF-embryo transfer programme". *Hum. Reprod.,* 2000, *15,* 1698.

[2] Al-Inany H., Aboulghar M.: "GnRH antagonist in assisted reproduction: a Cochrane review". *Hum. Reprod.*, 2002, *17,* 874.

[3] Simon C., Oberye J., Bellver J., Vidal C., Bosch E., Horcajadas J.A. *et al.*: "Similar endometrial development in oocyte donors treated with either high- or standard-dose GnRH antagonist compared to treatment with a GnRH agonist or in natural cycles". *Hum. Reprod.*, 2005, *20,* 3318.

[4] Labrie F., Belanger A., Simard J., Cusan L., Labrie C., Gomez J., *et al.*: "Gonadotropin-releasing hormone agonists in the treatment of prostate cancer". *Endocr. Rev*., 2005, *26,* 361.

[5] Klijn J.G., Beex L.V., Mauriac L., van Zijl J.A., Veyret C., Wildiers J. *et al.*: "Combined treatment with buserelin and tamoxifen in premenopausal metastatic breast cancer: a randomized study". *J. Natl Cancer Inst*., 2000, *92,* 903.

[6] Prentice A.: "Regular review: endometriosis". *Br. Med. J.,* 2001, *323*, 93.

[7] Carel J.C., Lahlou N., Roger M., Chaussain J.L.: "Precocious puberty and statural growth". *Hum. Reprod. Update,* 2004, *10,* 135.

[8] Schwartz N.B.: "A model for the regulation of ovulation in the rat". *Recent Prog. Horm. Res.,* 1969, *25*, 1.

[9] Long J.A., Evans H.M.: "The oestrous cycle in the rat and its associated phenomena". *Mem. Univ. Calif.*, 1922, *6,* 1.

[10] Lobel B.L., Tic L., Shelesnyak M.C.: "Studies on the mechanism of nidation XVII. Histochemical analysis of decidualization in the rat. I. Framework: oestrous cycle and pseudopregnancy". *Acta Endocrinol. (Copenh.),* 1965, *50*, 452.

Address reprint requests to:
C.R. TRINDADE, M.D.
Rua Portinari, 27/705 Ed. River Center
Barro Vermelho
Vitória-ES, CEP: 29045-415 (Brazil)
e-mail: celiart@terra.com.br