Pregnancy-induced changes in substance P and neurokinin 1 receptor (NK1-R) expression in the rat uterus

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Adrenergic nerve fibres of the mammalian uterus degenerate during pregnancy. The behaviour of peptidergic fibres, such as substance P-positive fibres and of its preferred neurokinin 1 receptor (NK1-R), is poorly studied in the pregnant rat uterus. The present study analysed the changes in substance P immunoreactivity and in the expression of NK1-R protein in the uterus of non-pregnant, pregnant (days 7, 14 and 21) and postpartum rats (days 1, 8 and 22) by immunohistology, dot blot analysis and western blot analysis. In non-pregnant rats, substance P-positive fibres were localized to the myometrium; these fibres progressively disappeared during gestation and were almost absent at term (day 21). At day 22 post partum, substance P-positive fibres had recovered to numbers comparable with those in the non-pregnant uterus. Dot blot analysis revealed a significant decrease in the immunoreactivity of substance

P in the uterus at mid-pregnancy (day 14) and especially at term. Expression of the NK1-R protein showed a progressive increase throughout pregnancy reaching a peak on day 1 post partum; downregulation of NK1-R protein occurred on day 8 post partum. The low and high expressions of NK1-R protein were coincident with a large number of eosinophils and almost no eosinophils in the uterus at oestrus and at term, respectively. It was concluded that substance P immunoreactivity is inversely correlated with NK1-R protein expression in the pregnant and postpartum uterus. The marked upregulation of NK1-R protein at term and after birth indicates that the NK1-R may be involved in the complex regulation of labour and postpartum physiology. However, it is likely that the NK1-protein is not involved in the recruitment of eosinophils into the uterus at oestrus.

Introduction

In general, tissues and organs undergoing hypertrophy and hyperplasia show growth of blood vessels and nerve fibres; however, the pregnant uterus is an exception. At term the pregnant uterus weighs surprisingly more than it does before pregnancy, even though the nerve fibres are no longer present in the myometrium (Alm and Lundberg, 1988; Haase *et al.*, 1997; Zoubina and Smith, 2000). After parturition, the mammalian uterus is reinnervated. The physiological significance of uterine denervation may be one factor among others that support myometrial quiescence during growth of the fetus to prevent premature onset of labour. Furthermore, denervation of myometrial blood vessels impedes vasoconstriction and allows a regular placental blood flow. Although the denervation of adrenergic and cholinergic fibres in the non-pregnant and pregnant rat uterus has been reported by different groups (Alm and Lundberg, 1988; Haase *et al.*, 1997; Zoubina and Smith, 2000), there are conflicting reports on the behaviour of the peptidergic system. However, substance

P immunoreactivity appears to remain unchanged in the rat uterus during pregnancy (Taurig *et al.*, 1984), whereas it has been found to increase by threefold, as measured by radioimmunoassay (Amira *et al.*, 1995).

Retrograde tracing studies indicate that the perikarya of sensory intrauterine fibres are located in the upper lumbar ganglia and their axons in the superior ovarian nerve (Peters *et al.*, 1987; Majewski *et al.*, 1995; Papka *et al.*, 1996). These fibres behave in a dual manner; they act as afferent fibres for transmission of nociception and as neuroeffectors by the local release of neuropeptides, such as substance P, a member of the tachykinin family (Hökfelt *et al.*, 2001; Severini *et al.*, 2002). Substance P acts via its preferred neurokinin 1 receptor (NK1-R). As the receptor is located on vascular cells, it induces a so-called neurogenic inflammation reaction, characterized by an increase in vascular permeability, the recruitment of leucocytes and vasodilatation (Schäffer *et al.*, 1998; Lindsay De Vane, 2001). A significant increase in the content of NK1-R mRNA occurs during gestation in the rat uterus (Candenas *et al.*, 2001). The functional activity of NK1-R localized on smooth muscle cells within the uterus has been verified using functional organ bath studies of contractility in the presence of selective NK1-R agonists and antagonists (Hamlin *et al.*, 2000; Patak *et al.*, 2000; Crane *et al.*, 2002). However,

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evidence for the more significant occurrence of the NK1-R protein is lacking for the cyclic and pregnant uterus.

The rat uterus at oestrus has an abundance of eosinophils (Tchernitchin *et al.*, 1976). These eosinophils disappear in the pregnant uterus and slowly reappear after term (Duchesne and Badia, 1992). Among other factors, recruitment of eosinophils depends on the presence of adhesion molecules, such as intercellular adhesion molecule and vascular cell adhesion molecule (VCAM) (Hogan and Foster, 1996). *In vitro*, VCAM is upregulated in endothelial cell cultures by a substance P-dependent stimulation of NK1-R causing strong adhesion of eosinophils (Dunzendorfer *et al.*, 1998; Quinlan *et al.*, 1999a,b). Therefore, it is of interest to compare the density of eosinophils, the amount of substance P immunoreactivity and expression of NK1-R protein in the non-pregnant, pregnant and postpartum rat uterus in a time course study.

The aims of the present study were to examine changes in substance P-positive intrauterine nerve fibres, to relate them to changes in substance P immunoreactivity by using dot blot analysis and to analyse changes in the expression of NK1-R protein by an immunoblot assay in the rat uterus throughout pregnancy and post partum compared with the non-pregnant uterus. Observations on the varying numbers of eosinophils are also included.

Materials and Methods

Animals

The experiments were approved by the local Institution for Ethical Guidelines of animals. Forty-nine female Wistar Fort rats were obtained from the breeding colony at the University of Leipzig. The rats had access to food and water *ad libitum*. Virgin sexually mature rats at oestrus, rats at days 7, 14 and 21 (term) of gestation and rats at days 1, 8 and 22 post partum were analysed ($n=7$ per group). Oestrus was determined by vaginal smears and mating was confirmed by the presence of spermatozoa in the vaginal smear. The rats were killed by an overdose of CO_2 . The uteri were removed and washed in sterile PBS (pH 7.4); the uterine horns were dissected and removed from connective tissue, pups and placenta, and the uterine horns from the left-hand side were immediately stored at -80°C until use.

Immunohistology for substance P and protein gene product 9.5 (PGP9.5), a general neuronal marker

The uterine horns from the right-hand side of each group ($n=7$ per group) were fixed in 4% (v/v) PBS-buffered formaldehyde at room temperature (21°C) overnight and embedded in paraffin wax. Implantation sites and interimplantation sites were studied separately. The uterine horns were cross-sectioned and then cut into

serial sections $7\text{ }\mu\text{m}$ in thickness and mounted on to object slides that had been coated with a paper glue. Deparaffinized tissue sections were treated with 3% (v/v) H_2O_2 in PBS for 3 min to quench endogenous peroxidase activity, washed in PBS and Tris-buffered saline (TBS, pH 7.4) (each buffer wash was for 2×5 min) and saturated with 1.5% normal goat serum (DAKO, Arhus) for 45 min. The sections were incubated immediately with the polyclonal anti-substance P antibody (Biotrend Chemicals, Cologne, 1:2000 working dilution) in PBS containing 0.25% BSA (Sigma, St Louis, MO) in a humidified chamber at 4°C overnight. Anti-substance P antibodies had been tested for specificity to substance P and other tachykinins using purified substance P, NKA and NKB peptides at various concentrations (range 10^{-6} to 10^{-9} mol l^{-1}). Dot blot analysis revealed antibodies that were specific for substance P. Negative controls were run with non-immune rabbit serum or PBS only.

After washing with buffer (0.05 mol TBS l^{-1} containing 0.12% (v/v) Tween 20) and with TBS only, each for 2×5 min, the sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted at 1:200 in 0.05 mol TBS l^{-1} containing 1% normal goat serum for 30 min at room temperature. After rinsing, sections were incubated with avidin-biotin-horseradish peroxidase-labelled complex (VECTASTAIN, ABC reagent, Vector Laboratories) for 30 min. The complex was detected with substrate solution containing 29 mg 3-amino-9-ethylcarbazole (AEC, Sigma) dissolved in 100 ml of 0.1 mol sodium acetate buffer l^{-1} , pH 5.2 containing 7.1 ml *N,N*-dimethylformamide and 0.03% (v/v) H_2O_2 . The sections were treated with AEC for 15–20 min at 37°C . After rinsing with distilled water and counterstaining with haematoxylin, the sections were embedded in water-soluble glycerine gelatine.

Another set of sections was similarly stained by using the rabbit polyclonal antibody against the PGP9.5 (Biotrend Chemicals) at a dilution of 1:4000. The PGP9.5 is considered as a pan-neuronal marker (Lundberg *et al.*, 1988). ABC reagent complexes were detected by 0.02% (w/v) 3,3'-diaminobenzidine (DAB, Aldrich, Basel) in TBS and containing 0.03% H_2O_2 before use. Sections were embedded in Histokitt (Roth, Karlsruhe). Staining for substance P and PGP9.5 was repeated twice for the uterus of each group and was performed on independent days.

Protein extraction

The uterine horn from the left-hand side was minced with a pestle and mortar in liquid nitrogen. Powder was resuspended in 1 ml ice-cold homogenization buffer (20.0 mmol Hepes l^{-1} , 1.0 mmol EDTA l^{-1} , 0.2 mol sucrose l^{-1} , 20.0 μg soybean trypsin inhibitor ml^{-1} , 20.0 μg leupeptin ml^{-1} , 5.0 μg pepstatin A ml^{-1} , 5.0 mmol dithiothreitol l^{-1} , 5.0 μg E-64 ml^{-1} , 5.0 μg bestatin ml^{-1} , 5.0 μg aprotinin ml^{-1} , 5.0 μg antipain ml^{-1}

and 0.1 mmol equine chorionic gonadotrophin l⁻¹) containing 1.66% (v/v) SDS as described by Cellek *et al.* (1999). After homogenization by ultrasonification the samples were centrifuged twice at 16 060 g for 15 min at 4°C. The supernatants were pooled and the total protein concentration determined using the protein assay based on bicinchoninic acid and BSA as a standard (BCA-method, Pierce, Rockford, IL).

Immuno-dot blot analysis

Proteins extracted from the left-hand side of the uterine horns of different groups were blotted at various dilutions on to a nitrocellulose membrane (BA85, Schleicher and Schuell, Dassel) (Reibiger *et al.*, 2001). Before blotting, the optimal protein concentration of the primary and secondary antibodies was determined. A specificity control was performed involving pre-adsorption of substance P antibodies with the peptide substance P (100 µmol l⁻¹) for 16 h at 4°C as well as the use of non-immune rabbit serum. A protein extract from rat cerebellum was used as a positive substance P immunoreactivity control. The membranes were saturated with 5% (v/v) milk powder in wash buffer (0.3% (v/v) Tween 20, 0.05% (v/v) Triton-X100 in PBS), followed by immediate incubation with the anti-substance P antibody (1:1000) in buffer with 0.5% BSA overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase-labelled anti-rabbit IgG (Vector Laboratories) at a dilution of 1:5000 for 90 min. Immunolabelling was detected with the enhanced chemoluminescence kit (ECL, Amersham Biosciences UK Limited, Little Chalfont) followed by exposure of nitrocellulose to Hyperfilm ECL. The densitometrical analysis of autoradiograms was done with the Alphamager™ 2000 program (Alpha Innotech Corp., San Leandro, CA). The data were normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Immunoblot analysis of NK1-R

Constituent proteins from uterine preparations were resolved on 12.5% SDS-PAGE and western blot analysis was carried out as described by Gounni *et al.* (2001). Specific binding of the anti-NK1-R antibodies at a dilution of 1:15 000 (Biotrend Chemicals) to the blots was performed overnight. The membranes were washed (6 × 10 min) and then incubated for 1 h with goat anti-rabbit antibody conjugated to horseradish peroxidase (Vector Laboratories, 1:5000). Immunolabelling was visualized with an ECL kit according to the manufacturer's instructions (Amersham). Exposed films were analysed as described above. Loading of equal total protein and the band densitometrical analysis were normalized using the expression of GAPDH.

Number of eosinophils

For each uterine horn, deparaffinized cross-sections were stained with Sirius red (Bayer AB, Leverkusen) solution as described by Bogomoletz (1980) and Reibiger and Spanel-Borowski (2000). Eosinophils with a distinctly red stained cytoplasm and a bilobed nucleus were counted under × 40 magnification using an ocular grid of 0.05 mm². Ten areas of the endometrium were examined starting close to the myometrium and evaluating the basal zone at regular intervals. The number of eosinophils was calculated per mm².

Statistical analysis

Values are expressed as the mean ± SEM and ± SD, respectively. Statistical analysis was performed using StatView Version 5.01. Significant differences among all group means were assessed by the non-parametric Kruskal–Wallis test followed by the Mann–Witney U test. Differences with a *P* value of < 0.01 were considered significant.

Results

Pregnancy induced changes in the density of substance P-positive nerve fibres and PGP9.5-positive fibres, as deduced from serial sections of the pregnant rat uterus in comparison with the non-pregnant and postpartum uterus. At oestrus, the density of the fibres was at its maximum (Fig. 1a,b). The nerve fibres entered the uterus from the mesometrial side together with blood vessels. Fibres were detected in close association with blood vessels in the stratum vasculare at the anti-mesometrial side. The fibres were distributed from the stratum vasculare between the inner and the outer myometrial layers and were often running parallel to the longitudinal axis of smooth muscle cells. There appeared to be fewer substance P-positive fibres than PGP9.5-positive fibres. A decrease in substance P-positive fibres was detected on day 14 of gestation (data not shown). This diminished density of nerve fibres between the myometrial fibres paralleled the loss in PGP9.5-positive fibres. On day 21 of pregnancy, neither substance P-positive nor PGP9.5-positive fibres were apparent at the anti-mesometrial side either at the implantation area or the interimplantation area as verified in whole cross-sections and serial sections (Fig. 1c,d). Some PGP9.5-positive fibres remained at the mesometrial entrance side, although clearly reduced in number. A remarkable reappearance of substance P-positive fibres in the uterus was observed after birth; the number and distribution pattern of substance P-positive nerve fibres were almost similar to those in the uterus by day 22 post partum (Fig. 1e). PGP9.5-positive fibres showed a similar pattern of distribution (Fig. 1f). The negative controls performed using serum from pre-immune rabbits did not

Fig. 1. Localization of substance P- and protein gene product 9.5 (PGP9.5)-positive nerve fibres in the non-pregnant, pregnant and postpartum rat uterus by indirect immunohistology. The tissue sections ($n=7$ per group) were processed twice and probed with a polyclonal rabbit antiserum to (a,c,e) substance P or to (b,d,f) PGP9.5. (a) In the non-pregnant rat uterus, substance P-positive staining is present in nerve fibres of the myometrium both in the circular and longitudinal smooth muscle layers (arrows). A positive response appears close to blood vessels in the stratum vasculare. (b) In the non-pregnant uterus, positive fibres are distinctly located with the panneuronal marker PGP9.5. (c,d) Fibres are prominent in the stratum vasculare. On day 21 of gestation, note absence of substance P-positive nerve fibres in (c) and of PGP9.5 positive fibres in (d). (e,f) In the postpartum uterus on day 22, both the substance P-positive fibres (arrows in e) and PGP9.5-positive fibres (arrow in f) reappear. E: endometrium, M: myometrium, V: blood vessel. Scale bars represent (a,c,e) 50 μm and (b,d,f) 100 μm .

demonstrate any recognition of similar structures (data not shown). These results give clear evidence that the presence of substance P-positive nerve fibres is distinctly modulated in the myometrium during gestation and after parturition.

Semi-quantitative analysis to determine substance P immunoreactivity in the rat uterus during gestation and post partum was carried out by dot-blot analysis (Fig. 2). Densitometric examination revealed that the immunoreactivity of substance P in the uterus from virgin and

Fig. 2. Densitometric analysis of changes in substance P immunoreactivity in the rat uterus during pregnancy (days 7, 14 and 21, ■) and after parturition (days 1, 8 and 22, □). Equal amounts of uterine proteins (5 µg) from non-pregnant, pregnant and postpartum rats were subjected to dot-blot analysis. Signals have been normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels and are expressed as arbitrary units (AU). The histograms represent mean ± SEM from five to seven different rats in each group. * $P < 0.01$ versus the other days. The data are representative of three separate experiments.

early pregnancy (day 7) exhibited approximately equal intensities in the dot-blot assay with no significant differences. In contrast, significant differences in substance P immunoreactivity were observed on days 14 and 21 (full term) of gestation in comparison with the non-pregnant uterus ($P < 0.001$). The immunoreactivity of substance P in the uterus remained low after parturition and was still significantly decreased when compared with that in the non-pregnant uterus ($P < 0.001$), but a rapid recovery in substance P immunoreactivity occurred from day 8 post partum onwards and was significantly different from the expression during pregnancy and on day 1 post partum ($P < 0.001$). Immunoblot analysis with anti-NK1-R antibody was performed to examine NK1-R expression at the protein level in the non-pregnant, pregnant and postpartum uterus (Fig. 3a). The antibody against the NK1-R specifically recognized a protein migrating at approximately 50 kDa that corresponds to the known molecular mass of the rat NK1-R protein (Hershey and Krause, 1990). This protein did not react with pre-immune serum. Compared with the rat uterus at oestrus, a clear upregulation of NK1-R protein expression was demonstrated with increasing days of pregnancy and on day 1 post partum. Densitometric analysis of the signals after normalization to GAPDH levels (Fig. 3b) demonstrated a progressive increase in NK1-R protein expression on days 7, 14 and 21 of gestation ($P < 0.01$ for all compared with the oestrous uterus). The amounts of NK1-R protein in the pregnant uterus were about 1.7 to 2.0-fold higher than the amounts of NK1-R protein in the non-pregnant uterus. Unexpectedly, densitometric analysis revealed that NK1-R protein content was the

Fig. 3. (a) Immunoblot analysis of the expression of neurokinin 1 receptor (NK1-R) protein in the rat uterus during pregnancy and after parturition. Similar amounts of proteins (30 µg) from uterine extracts of individual samples on various days of pregnancy or after parturition were separated by 12.5% SDS-PAGE and immunoblotted using the anti-NK1-R antibody. Lanes are indicated from left to right: oestrus, days 7, 14 and 21 (term) of pregnancy, days 1, 8 and 22 post partum, non-immune serum. The relative position of the molecular mass markers in kDa is indicated on the left. The nitrocellulose membranes were also probed with anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an indicator of equal loading (data not shown). (b) Densitometric analysis of NK1-R protein expression during pregnancy (days 7, 14 and 21, ■) and after parturition (days 1, 8 and 22, □). Signals were normalized to GAPDH levels and expressed as arbitrary units (AU). Histograms represent the mean ± SEM from four to five different rats in each group. * $P < 0.01$ versus oestrus and day 22 post partum.

highest on day 1 post partum showing a 2.4-fold increase when compared with the non-pregnant uterus. The expression of NK1-R was at its lowest on day 8 post partum.

Eosinophils populated the cyclic uterus more densely in the basal zone of the endometrium than in the myometrium (Fig. 4). They were undetectable in uterus at mid-pregnancy and at term. On day 1 post partum, eosinophils started to migrate into the postpartum uterus, yet on day 22 the number remained below the number detected in the cyclic uterus (Fig. 5).

Discussion

Studies on substance P immunoreactivity in the rat uterus throughout pregnancy are contradictory. Some

Fig. 4. Eosinophils (arrows) are stained with the Sirius red solution and located in the rat uterus. (a) Oestrous stage, (b) at term, that is day 21 of pregnancy, (c) day 8 post partum and (d) day 22 post partum. M: myometrium. Scale bars represent 50 μm .

Fig. 5. Number of eosinophils per mm^2 of rat endometrium adjacent to the myometrium at oestrus, on days 14 and 21 of pregnancy, and on days 1, 8 and 22 post partum. Histograms represent the mean $\pm$ SD from six different rats in each group. * $P < 0.001$ compared with oestrus.

studies report no change, whereas others report an increase in substance P immunoreactivity (Traurig *et al.*, 1984; Amira *et al.*, 1995). The normal and increased substance P immunoreactivity may be explained by different sensitivities of two different immunological

assays. The assumption is that the substance P-positive fibres remain stable intrauterine elements despite the presence of high concentrations of sex steroids or may even increase in density in the pregnant rat uterus. This assumption is not in agreement with the results of the present study, which clearly show the disappearance of myometrial and perivascular substance P-positive fibres during pregnancy. Immuno-dot blot analysis also depicts a decrease in substance P immunoreactivity during pregnancy. The immunoreactivity of substance P in the present study is related per mg protein, because there is an uneven nerve fibre distribution between regions of the rat uterus (Richeri *et al.*, 2002). For this reason, substance P immunoreactivity that has been obtained from a small uterine portion cannot be translated to total uterine horn. The decrease in peptidergic nerve fibres in the pregnant uterus observed in the present study may be explained by the presence of serum oestrogens at increased concentrations at mid- and late pregnancy. This assumption is supported by the finding that chronic oestrogen treatment causes a complete loss of sympathetic nerve fibres in the prepubertal rat uterus (Brauer *et al.*, 1995). The high oestrogen concentrations of pregnancy may affect dorsal root ganglion neurones which express both mRNA and protein of oestrogen receptor- α and - β (Papka *et al.*, 2001). Furthermore, the

growing fetus may cause a stretch-induced intrauterine nerve fibre degeneration and be responsible for the retraction of axon terminals (Lundberg *et al.*, 1989).

The present study demonstrates a significant variation of substance P immunoreactivity and changes in the occurrence of NK1 protein in the rat uterus during gestation. It is evident that the decrease in substance P immunoreactivity in the pregnant rat uterus is negatively correlated with NK1-R protein synthesis during pregnancy, at term or on day 1 post partum. The reverse is stated on day 8 post partum when substance P immunoreactivity reappears and the lowest NK1-R protein expression is seen. So far only the functional activity of tachykinin receptors has been investigated by testing myometrial contractions under the treatment of receptor agonist and antagonist either in rat uterus (Pinto *et al.*, 1999; Hamlin *et al.*, 2000; Candenas *et al.*, 2001; Crane *et al.*, 2002) or in human uterus (Patak *et al.*, 2000).

Pinto *et al.* (1999) reported that the mRNA content of NK1-R is upregulated by oestrogens in the uterus of ovariectomized rats. Little is known about the mechanism by which NK1-R protein synthesis is increased in the uterus at term or on day 1 post partum. This observation may be explained by basic sensitization, that is a moderate receptor internalization because the local amount of substance P and, thus, substance P binding is low. In comparison, sites with neurogenic inflammation and a high amount of substance P rapidly internalize the receptor–ligand complex to limit the inflammatory response (Bowden *et al.*, 1994). Furthermore, the high NK1-R protein synthesis observed here may be influenced by interferon- γ secreted from the pregnancy-associated natural killer cells (Askar and Croy, 2001). This cytokine is able to increase the synthesis of the NK1-R protein on peritoneal macrophages (Marriott and Bost, 2000). Thus, the pregnant rat uterus may become a useful animal model to get new insights into the regulation of NK1-R protein synthesis.

The demand for myometrial contraction at term depends on a high density of gap junctions which transform the myometrium into a functional syncytium. At the onset of labour, connexin 43, the major myometrial gap junction protein, is upregulated by receptors, for example oxytocin, prostaglandins and endothelin 1 (Carbillon *et al.*, 2001). The high amount of the NK1-R protein at term and on day 1 post partum observed in the present study indicates communication between NK1-R protein synthesis and gap junction formation in support of myometrial contractions at term. If this hypothesis is correct, other tachykinins rather than substance P might interact with NK1-R, because the tachykinin system is very redundant (Höckfelt *et al.*, 2001; Severini *et al.*, 2002).

One of the substance P-dependent functions corresponds to the NK1-R mediated upregulation of VCAM expression on cultured endothelial cells and to stimulat-

ory chemotactic effect on eosinophil migration *in vitro* (Dunzendorfer *et al.*, 1998; Quinlan *et al.*, 1999a,b). Here the NK1-R protein content is very low in the non-pregnant uterus, which is densely populated by eosinophils. Yet the receptor increases in the pregnant rat uterus when eosinophils have disappeared. This indicates that NK1-R is not involved in eosinophil recruitment into the rat uterus. Oestrogens remain as the major candidates; they greatly modulate endothelial cell adhesion molecules (Cid *et al.*, 2002) and are known to cause the recruitment of eosinophils into the uterus of ovariectomized rats (Tchernitchin *et al.*, 1976; Lee *et al.*, 1989).

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