*Original article*

# Effect of vaginal administration of prostaglandin E$_2$ and/or 17β-estradiol on luteal function and histological characteristics of the cervix in cyclic pigs

## E. Przygrodzka[1], A. Andronowska[1], T. Janowski[2], A.J. Zięcik[1]

[1] Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences,
Tuwima 10, 10-748 Olsztyn, Poland
[2] Department of Animal Reproduction with Clinic, Faculty of Veterinary Medicine,
University of Warmia and Mazury, Oczapowskiego 14, 10-719 Olsztyn, Poland

## Abstract

The overall objective of this study was to examine the effect of vaginal administration of prostaglandin E$_2$ (PGE$_2$) and/or17β-estradiol (E$_2$) on luteal function maintenance and histological properties of the porcine cervix. For this purpose, crossbred gilts were divided into three groups (n=5 per group) supplied on days 11-16 of the estrous cycle with suppositories containing: (1) placebo (Group I, Control); (2) 0.4 mg of E$_2$ (Group II); (3) 0.4 mg of E$_2$ and 2 mg of PGE$_2$ (Group III). Blood samples were collected on days 11-19 of the estrous cycle to determine the concentration of progesterone (P$_4$). Additionally, to examine local effects of the hormones applied, segments from the uterine and vaginal parts of the cervix and from the ovaries were collected *post-mortem*. Prolonged luteal function and extended synthesis of P$_4$ were observed in 2 of 5 gilts receiving PGE$_2$ and E$_2$ simultaneously (Group III). Then, these gilts were subdivided into Group IIIA (n=2; presence of corpora lutea on the ovaries) and Group IIIB (n=3; lack of corpora lutea). Increased levels of plasma P$_4$ were observed in Group IIIA on days 15-19 compared to Group IIIB and on days 16-19 compared to Group I and Group II (P<0.05; P<0.01; P<0.001, respectively). In the cervix of gilts in Groups II and III, enlarged blood vessels in the lamina propria of both parts of the cervix were observed. Furthermore, in Group II the epithelium of the uterine part of the cervix was thicker (P<0.001). Our study confirmed the proposed luteotrophic/antiluteolytic actions of E$_2$ and PGE$_2$ applied intravaginally. These results are significant considering that very low doses of E$_2$ were used when compared to previous attempts. Despite the inadequate response to treatments in some of the gilts, the local effects of these hormones on the histological properties of the porcine cervix suggest that further improvements in the vaginal administration route might help to elaborate new methods for enhancing the luteal function in the pig.

**Key words**: prostaglandin E$_2$, 17β-estradiol, corpus luteum, cervix

Correspondence to: A.J. Zięcik, e-mail: a.ziecik@pan.olsztyn.pl, tel.: +48 89 535 74 22

## Introduction

Prostaglandin $E_2$ ($PGE_2$) and 17β-estradiol ($E_2$) are the main conceptus signals in the pig, overcoming luteolysis and extending the luteal function, thereby providing continuous synthesis of progesterone during pregnancy (Ziecik et al. 2002, Waclawik and Ziecik 2007). Consequently, exogenous administration of $PGE_2$ (Akinlosotu et al. 1986) or $E_2$ (Ford et al. 1982) in the mid luteal phase prolongs the luteal function.

Over the past several decades, pseudopregnancy has been induced in pigs using intramuscular injections of 5 mg of estrogens daily on days 11-16 of the estrous cycle (Geisert et al. 1987). However, this treatment led to 3-4 fold higher concentration of plasma $E_2$ than that normally occurring during the estrous cycle (Ziecik et al. 1986). Moreover, a high dose of $E_2$ affected the expression of estrogen receptors in the endometrium (Rosser et al. 1993). Some studies also emphasized negative effects on embryo development after premature exposure of the uterus to $E_2$ (Long and Diekman 1986, Geisert et al. 2006).

Einer-Jensen (1993) demonstrated rapid absorption of progesterone ($P_4$) from the vaginal lumen and its transfer into the arterial blood supplying the uterus and ovary in the pig. It resulted in 2- to 4-fold higher $P_4$ levels in the uterine artery than in systemic blood. Further studies revealed the local transfer of steroids and prostaglandins (PGs) in the porcine reproductive tract (Stefanczyk-Krzymowska et al. 2006). Interestingly, vaginal administration of suppositories containing $E_2$ and $P_4$ had beneficial effects on the embryo development and survival in pigs (Chłopek et al. 2008).

Considering the growing body of knowledge about local transfer of steroids and PGs in the porcine reproductive tract as well as their paramount role in luteal function maintenance, we decided to examine whether the administration of suppositories containing $E_2$ and $PGE_2$ would prolong the lifespan of CLs. Additionally, the local effect of hormones applied in this way on the histological properties of the cervix was examined. We assumed that the information obtained would be helpful in elaborating methods aimed at reducing embryo mortality in pigs by enhancing the luteal function.

## Materials and Methods

### Animals and experiment design

All procedures performed on animals were conducted in accordance with the rules approved by the Local Animal Ethics Committee. Crossbred gilts 6-8 months of age, after exhibiting one regular estrous cycle, were assigned to the experiment which was performed at a commercial farm. The animals were kept in individual pens and observed daily for the second estrus behavior. Considering the fact, that intramuscular injection of $E_2$ in daily doses ranging from 0.125 mg to 32 mg applied on days 12-13 of early pregnancy did not affect embryo survival on day 30 of pregnancy (Pope et al. 1986), as well as the beneficial effect of vaginal administration of suppositories containing $E_2$ (0.2 mg per day; Chłopek et al. 2008) we designed the pilot study. The vaginal suppositories contained various combination of $E_2$ (0.025; 0.05; 0.1; 0.2 and 0.4 mg per day) and $PGE_2$ (0.5; 2.0 and 2.5 mg per day) but only one combination was effective in luteal function maintenance and we used it in the present experiment. Thus, the animals after estrus detection (day 0) were randomly divided into groups (n=5 per group) administered on days 11-16 of the estrous cycle with suppositories containing: (1) placebo (Group I-Control); (2) 0.4 mg of $E_2$ (Group II); (3) 0.4 mg of $E_2$ and 2 mg of $PGE_2$ (Group III).

### Preparation of suppositories

Suppositories were prepared by a pharmacologist from the Regional Specialist Hospital in Olsztyn (Poland). Suppositories containing $E_2$ (Sigma-Aldrich, St.Louis, MO, USA) were prepared using cocoa butter (cacao oleum, Pharma Cosmetic, Poland) according to the method described previously (Price et al. 1983) and stored at +4°C. Additionally, suppositories with $PGE_2$ (Cayman Chemicals, Ann Arbor, MI, USA) were made using Witepsol H-15 base. First, adequate amounts of Witepsol H-15 were mixed with $PGE_2$ using Unguator®e/s, and then coasted to the forms. Afterwards, the suppositories were chilled to avoid sedimentation of solids and then stored at -20°C. Before application, suppositories with $PGE_2$ were kept at room temperature for 15 min and then applied simultaneously with suppositories containing $E_2$.

### Blood sample collection

A catheter was inserted into the jugular vein under general anesthesia on day 9 of the estrous cycle, according to the method of Kotwica et al. (1978). Blood samples (~5 ml) were collected twice per day (at 12 h intervals) on days 11-19 of the estrous cycle and then centrifuged for 15 min at 3000 rpm at 4°C. Plasma samples were stored at -20°C until assayed for hormone concentrations. The animals were slaugh-

tered at a local abattoir on day 20 of the estrous cycle. The stage of the estrous cycle was confirmed on the basis of uterine and ovarian morphology according to Leiser et al. (1977).

## Tissue preparation and staining

Two segments from the cervix, one adjacent to the uterus and one to the vagina, were collected for histological analysis as described previously (Eldrige-White et al. 1989) and fixed in 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS; pH 7.4).

After overnight incubation at 4°C, the tissues were rinsed with distilled water and then dehydrated using serial dilutions of ethanol (95-70%) using a Tissue Processor (Reihert-Leica, Wetzlar, Germany). Paraffin-embedded tissue sections (5 µm thick) were dried overnight on a slide warmer (40°C), then deparaffinized in xylene, and rehydrated in a series of descending ethanol baths. Hematoxylin-Eosin (HE) staining was performed according to standard procedures and used to characterize tissue components of the uterine and vaginal parts of the cervix. All measurements (*i.e.*, the number of layers and thickness of the superficial epithelium, and the number of blood vessels in both parts of the cervix) were made by analyzing a digitized optical image at 400-fold magnification using light microscopy (Axio Imager Z1, Zeiss, Germany). Six sections from each part of the cervix per gilt were analyzed.

## Determination of hormone concentrations

Concentrations of $P_4$ (DIAsource ImmunoAssays S.S, Belgium) were analyzed using commercially available RIA kits. The sensitivity of the assay was 0.19 pg/ml, whereas the intraassay coefficient of variation was 4.6%.

## Statistical analysis

The concentrations of hormones are expressed as a mean from the two blood sample collections on each experimental day. To analyze differences in the concentration of hormones between groups on particular days of the estrous cycle, two-way ANOVA followed by the Bonferroni *posthoc* test was used (GraphPad Prism v. 5.0, GraphPad Software, San Diego, CA, USA). The initial model included day, treatment and treatment by day of estrous cycle interactions. To reduce variations among $P_4$ concentrations, these data

were log-transformed. The data for superficial epithelium thickness were analyzed using one-way ANOVA (GraphPad Prism v. 5.0). In all the experiments, P<0.05 was considered to be statistically significant and numerical data are expressed as means+standard error of the mean (SEM).

## Results

The patterns of progesterone ($P_4$) concentrations following intravaginal administration of $PGE_2$ and/or $E_2$ or the placebo are presented in Fig. 1. Intravaginal suppositories containing both $E_2$ and $PGE_2$ (Group III) prolonged the luteal function in two of five gilts, therefore the gilts of Group III were subdivided into Group IIIA (n=2; presence of CLs on the ovaries) and Group IIIB (n=3; presence of follicles on the ovaries; Fig. 1C). All the gilts treated with the placebo (Fig. 1A) or $E_2$ alone (Fig. 1B) exhibited follicles on their ovaries on day 20 of the estrous cycle.

The concentration of $P_4$ (Fig. 1) was affected by day of the estrous cycle (P<0.001), treatment (P<0.05) and day by treatment interaction (P<0.01). Increased levels of plasma $P_4$ were observed in Group IIIA on days 15-19 in comparison to those found in Group IIIB and on days 16-19 when compared to those observed in Group I and Group II (P<0.05; P<0.01; P<0.001, respectively).

The cervical tissue from all three groups of the gilts showed typical structure with the superficial epithelium, lamina propria with vascular structures and tunica muscularis below (Figs. 2-3). The epithelial thickness tended to increase from the uterine (Fig. 2) to the vaginal (Fig. 3) part of the cervix. In the uterine part, the superficial epithelium was mostly mono- or double-layered, whereas in the vaginal part it was double- or multi-layered. With respect to the number of epithelial layers, differences between the two locations of the cervix were obvious.

The epithelial cells of the cervix were cylindrical and could be classified into the following types: columnar secretory, ciliated and reserve (small, undifferentiated and pluripotential) cells. The nuclei of the epithelial cells were mostly located basally, however, in the active secretory cells the nucleus was placed centrally.

The mucosal folds of the vaginal part of the cervix in control, $E_2$ and $PGE_2+E_2$ treated gilts were covered by the stratified squamous epithelium (Figs. 4, 6, 8, respectively). There were no significant differences in the thickness of multi-layered epithelium between the control and experimental gilts. The basal layer consisted of one or two layers of small, oval cells with large and dark-stained nuclei. The parabasal
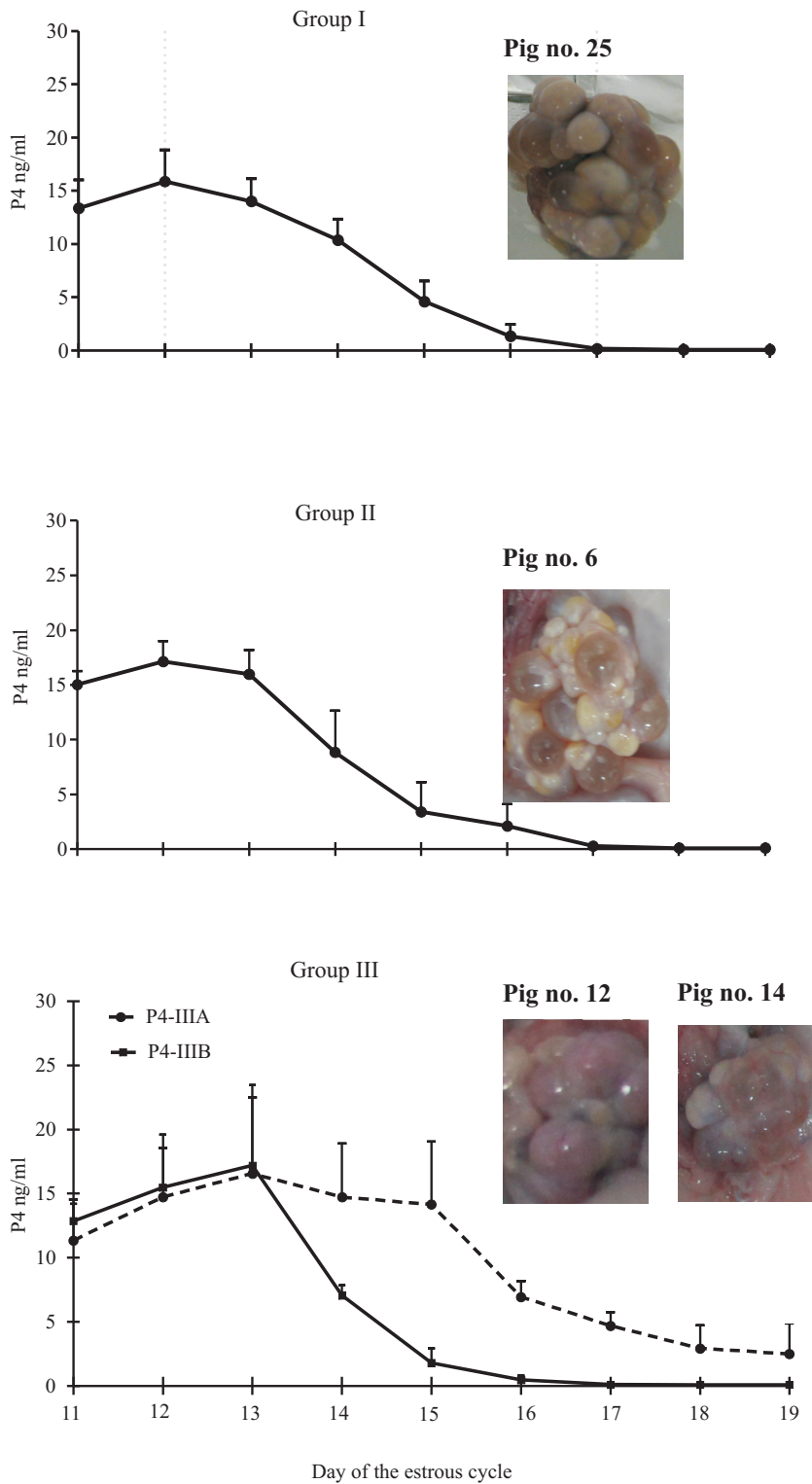
Fig. 1. Mean plasma $P_4$ concentrations following intravaginal administration of placebo (Group I), $E_2$ (Group II), $PGE_2$ and $E_2$ (Group III). Values are expressed as mean ± SEM (n=5). Due to the different response to the treatment among gilts of Group III, it was subdivided into Group IIIA and IIIB (see: Results). Representative photography of the ovaries of each group examined is presented in the right narrow. For Group III photography of one gilt ovary of Group III A and Group III B was shown.
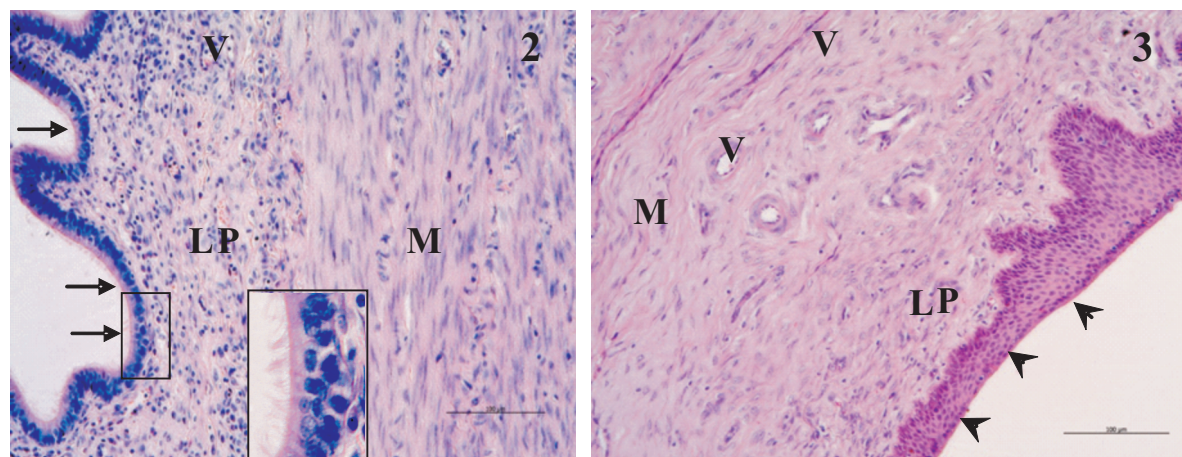
Fig. 2-3. Histological morphology of the porcine cervix showing differences between the uterine (Fig. 2) and vaginal (Fig. 3) part. The superficial epithelium of the uterine part was composed mostly of one or two layers (Fig. 2 insert), whereas in the vaginal part (Fig. 3) was multi layered. The lamina propria (LP) consisted mainly of the connective tissue with collagen fibers, fibroblasts and blood vessels. The lamina muscularis was composed of smooth muscle fibers circularly oriented. **LP** – lamina propria, **M** – lamina muscularis, **V** – blood vessels. *Bar* 100 μm.

layer was formed of two or three layers of polygonal cells with a central nucleus. Additionally, the superficial layer was composed of elongated, flattened cells with pyknotic nuclei.

In the uterine part of the cervix, the mucosal folds were elongated and branched. Primary branches as well as the bottom of the their cavity were covered by the columnar epithelium with numerous cilia (Figs. 5, 7, 9). Beneath the columnar cells, round or polygonal reserve cells with round nuclei (subcylindrical) were observed in the control and $E_2$ treated gilts (Fig. 5, 7, respectively). In the gilts of Group II, the epithelial cells were higher (P<0.001) compared to those observed in the control animals. The lamina propria was composed of a stroma rich in elastic fibers and small blood vessels were observed in the control Group I. However, in both experimental groups (Groups II and III) enlarged blood vessels were found in the uterine (Figs. 7, 9) as well as in the vaginal (Figs. 6, 8) parts of the cervix.

## Discussion

The discovery of local hormone transfer and its numerous benefits became a milestone in human medicine. A wealth of vaginal drug delivery systems have been developed, but it remains a challenge to design similar formulations for this application route in domestic animals. Currently, intravaginal sponges are routinely applied to synchronize estrus and improve pregnancy rates in sheep. Increasing knowledge about the local transfer of steroids and prostaglandins (PGs) in the porcine reproductive system (Stefan-

czyk-Krzymowska et al. 2006), as well as the paramount role of prostaglandin $E_2$ (PGE$_2$) and 17β-estradiol ($E_2$) during early pregnancy in the pig, led us to design the present study. The potential value of the intravaginal hormone delivery route is enhanced by the observation that prolongation of CL function after multiple injections of milligram amounts of estrogen could not mimic the hormonal status of natural pregnancy in the pig (Ziecik et al. 1986).

The maintenance and establishment of pregnancy in the pig requires a biphasic pattern of estrogen secretion, predominantly $E_2$, on days 11-12 and again between days 15 and 25-30 of pregnancy (Geisert et al. 1990). Additionally, a supporting role is played by increased amounts of PGE$_2$ originating from conceptuses and the endometrium prior to implantation (Waclawik and Ziecik 2007). Many studies indicate a direct luteotrophic action of PGE$_2$ and $E_2$ (Conley and Ford 1989, Ford and Christenson 1991). Concomitantly, antiluteolytic actions of both PGE$_2$ and $E_2$ are mediated through redirection of luteolytic prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$) from the uterine venous drainage (Bazer and Thatcher 1977), and/or the venous blood and uterine lymph (Krzymowski and Stefanczyk-Krzymowska 2004) into the uterine lumen. Interestingly, other mechanisms indicate a change of PGs synthesis in favor of PGE$_2$ on days 10-13 post-mating in the pig (Waclawik and Ziecik 2007). The importance of PGE$_2$ and $E_2$ in the luteal function maintenance during early pregnancy in pigs was also raised in our latest report (Bołzan et al. 2013). Therefore, it appears that the prolonged luteal function in two of the gilts receiving PGE$_2$ and $E_2$ simultaneously confirmed the luteotrophic and antyluleolytic action of
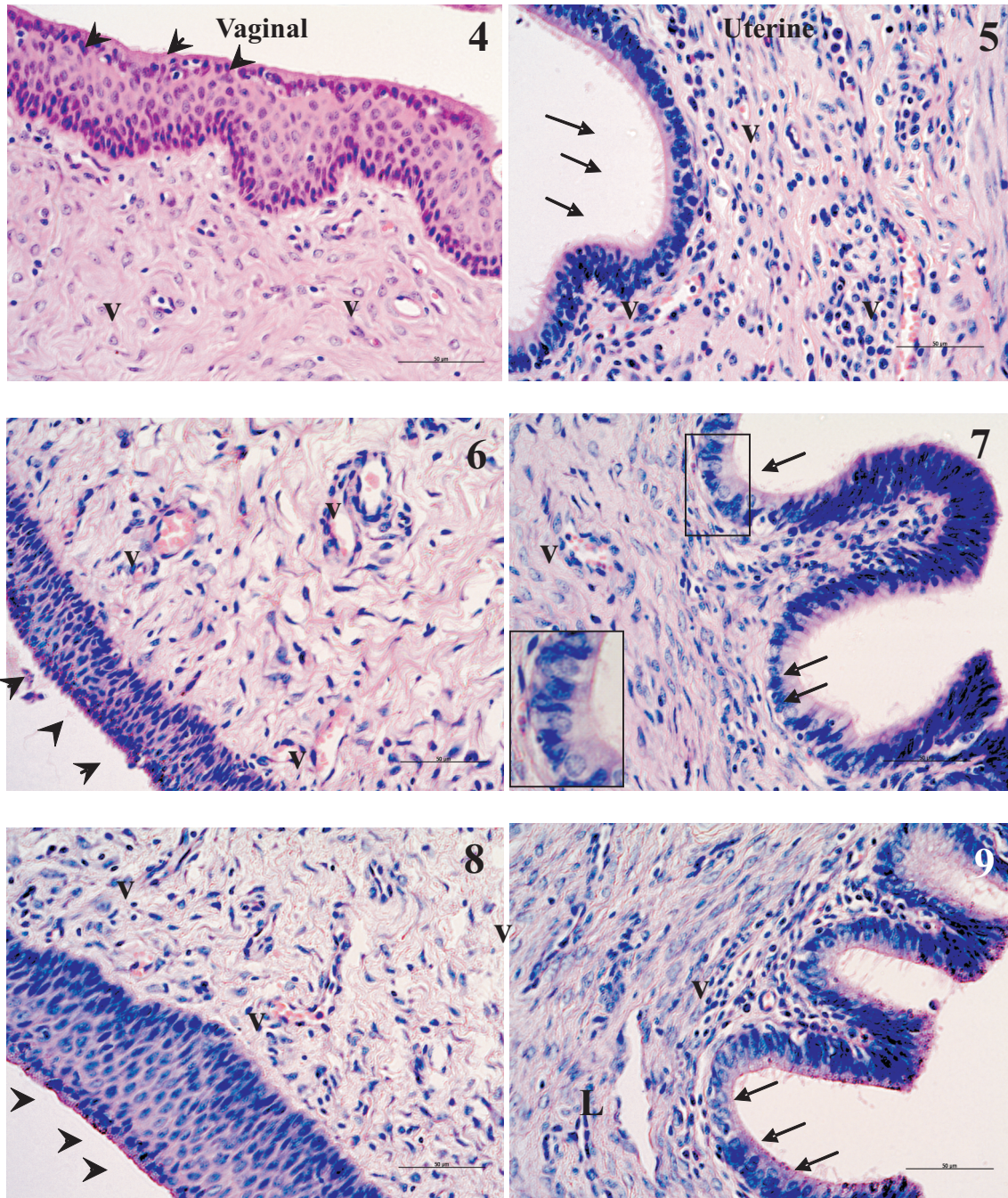
Fig. 4-9 Representative photomicrographs of the vaginal (4, 6, 8) and uterine (5, 7, 9) part of porcine cervix stained with hematoxylin and eosin (H&E) from the control (Figs. 4, 5), and estradiol (Figs. 6, 7) and estradiol and prostaglandin $E_2$ (Figs. 8, 9) treated pigs. Note secretory columnar epithelial cells with bright round nucleus and ciliated cells with dark stained nucleus (Fig. 7, insert). Arrowheads – multilayered epithelium, arrows – simple columnar epithelium with cilia, **L** – lymphatic capillaries, **v** – blood vessels. *Bar* 50 μm.

both hormones. Whereas the intramuscular injection of 5 mg of $E_2$ daily on days 11-16 of the estrous cycle was applied to induce pseudopregnancy (Geisert et al. 1987), it led to 3-4 folds higher concentration of plasma $E_2$ than that normally found during the estrous cycle (Ziecik et al. 1986). Thus the results of the present study are significant considering the very low

doses (0.4 mg) of $E_2$ used compared to previous attempts to prolong CL function by multiple $E_2$ injections (Geisert et al. 1987).

On the other hand, in the present study the discrepancies among gilts treated with suppositories containing both $PGE_2$ and $E_2$ showed the weakness of the vaginal administration route. Interestingly, Einer-Jen-

sen (1993) revealed a rapid absorption of infused progesterone ($P_4$) from the vaginal lumen and its transfer into arterial blood supplying the uterus and ovary in the pig. Furthermore, vaginal administration of suppositories containing $E_2$ and $P_4$ proved to be beneficial for the embryo development and survival in pigs (Chłopek et al. 2008). However, concerning the observations described here, it may be supposed that this disparity results also from the length of the porcine vagina that hindered a proper application of suppositories (Smith and Nalbadov 1985). Additionally, among pigs lacking prolonged luteal function, in some cases possibly the suppositories were lost before they melt within the vagina.

Although local and systemic effects of $E_2$ on luteal $P_4$ secretion were revealed in cyclic gilts receiving $E_2$ unilaterally into the isolated uterine horn on days 11-15 (Ford et al. 1982), in the present study, unexpectedly, vaginal administration of $E_2$ alone was generally ineffective. However, this might be associated with some problems with the application of the suppositories or absorption of the hormones. Numerous factors affecting the absorption of drugs (*i.e.*, the volume, viscosity and pH of vaginal fluid, physicochemical features of the drug) are well known.

The majority of the histological features of the porcine cervix observed in the present report are compatible with results described previously (Edstrom 2009). Since the experiment was performed on intact gilts, it is possible that the changes observed are driven by $E_2$ or $P_4$, rather than directly by exogenous $PGE_2$ and/or $E_2$. However, some particular changes (*e.g.* enlarged blood vessels or thickness of the vaginal epithelium) demonstrated local effects of the hormones applied.

Studying factors affecting drug absorption from the vaginal lumen, Carlstrom et al. (1988) have revealed that the vaginal absorption of steroids is affected by the thickness of the vaginal epithelium. Although the stimulatory action of $E_2$ on cell proliferation is well known, induction of the thicker epithelium by $E_2$ in gilts receiving this hormone might impede its own absorption.

Due to the antagonistic action of estrogens and progesterone on the uterine blood supply, a high level of $P_4$ and low level of $E_2$ during the mid-luteal phase coincide with reduced uterine blood supply (Ford and Christenson 1979). Conversely, a greater $E_2$:$P_4$ ratio leads to increased blood flow through this organ. Additionally, enhanced uterine blood flow in pregnant pigs is accompanied by the appearance of conceptus signals (Ford et al. 1982). $PGE_2$ also has a vasodilatory effect (Bell et al. 1990). Thus, corresponding outcomes obtained in the present investigations are consistent with previous studies reporting enlarge-

ment of blood vessels in gilts treated with $PGE_2$ and/or $E_2$.

In conclusion, we confirmed the synergistic luteotrophic/antiluteolytic action of $E_2$ and $PGE_2$ in two of five gilts after intravaginal application of these factors. To our knowledge, this is the first report demonstrating luteotrophic and antiluteolytic effects after vaginal administration of $PGE_2$ and $E_2$ in vivo in the pig. The present study demonstrates the feasibility of the luteal function maintenance using much lower doses of $E_2$ than those generally applied in intramuscular injections for the induction of pseudopregnancy. However, the inadequate response in some gilts and local effects on the histological properties of the porcine cervix after exposure to $PGE_2$ and/or $E_2$ indicate the necessity to improve the vaginal administration route in order to enhance their applications in the pig.

## Acknowledgements

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