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Expression and cellular distribution of NADPH-diaphorase and nitric oxide synthases in the porcine uterus during early pregnancy

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Abstract: Nitric oxide plays a key role in the regulation of various female reproductive processes such as ovulation, implantation and myometrial relaxation. The aim of the present study was to determine the histochemical activity and cellular localization of NADPH-d in the porcine uterus during early pregnancy, including the implantation period. Tissue samples collected from the pig uteri on days 5, 10, 12, 15 and 17 of pregnancy were stained histochemically for NADPH-d activity and immunohistochemically for NOS isoforms localization. In the luminal epithelium a significant increase of NADPH-d activity was observed on days 5 - 12 of pregnancy. On day 17 of pregnancy, two different staining patterns were observed: 1) a significant ($p<0.001$) decrease in NADPH-d activity at the site of implantation and 2) the high NADPH-d activity at inter-implantation regions. The endometrial glands showed a significant ($p<0.001$) increase in NADPH-d staining with high activity in individual glands. The arterial endothelium expressed stronger NADPH-d staining compared with venous vessels. Immunoreactivity of eNOS was similar to NADPH-d staining but no optical differences in the intensity of staining were observed. Clear iNOS immunoreactivity was detected in the luminal epithelium, endometrial stroma and individual endometrial glands. The vascular endothelium displayed weak iNOS staining.

Key words: NADPH-diaphorase - Nitric oxide synthase - Uterus - Early pregnancy - Pig

Introduction

Nitric oxide (NO) is a major mediator of numerous biological processes, including vascular functions, neurotransmission, hormone secretion and inflammation. NO is also one of the key factors regulating adaptation-related changes in uterine and placental blood vessels during pregnancy.

The establishment and maintenance of pregnancy involve conceptus-endometrial interactions to control vascular permeability, blood flow, placental attachment and immunological protection. In the pig a significant reduction in embryo survival occurs during early pregnancy. This phase is particularly critical for the establishment of pregnancy in the pig. Within that time, conceptus estrogen synthesis is initiated, spacing and final placement of conceptuses is completed, and the signal for extending the functional lifespan of corpora lutea is received by the mother. There is also a marked increase in blood flow to the uterus and the uterine endometrium produces and secretes nutrient histotrophe [1]. It is well known that nitric oxide may invoke pro-inflammatory effects, including vasodilatation, oedema and tissue remodeling.

NO is synthesized from L-arginine by nitric oxide synthases (NOSs), the family of enzymes in which three isoforms have been identified: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) [2,3].

The first published data suggest that eNOS and iNOS are present in the uteri of non-pregnant humans [4], rats [5,6], mice [7], pigs [8], sheep [9], and also in the uteri of pregnant humans [10] and laboratory animals such as rats [5,6,11] and mice [12]. Generation of NO from L-arginine requires, in addition, NOS cofactors: NADPH, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) [2]. Since NADPH-diphorase (NADPH-d) and NOS activities are caused by different properties of the same enzyme molecule, NADPH-d activity can be used as a marker for NOS [13,14]. Because all known NOS isoforms marks NADPH-d activity [15] the NADPH-d histochemical reaction is commonly used for visualization of NOS protein [8].

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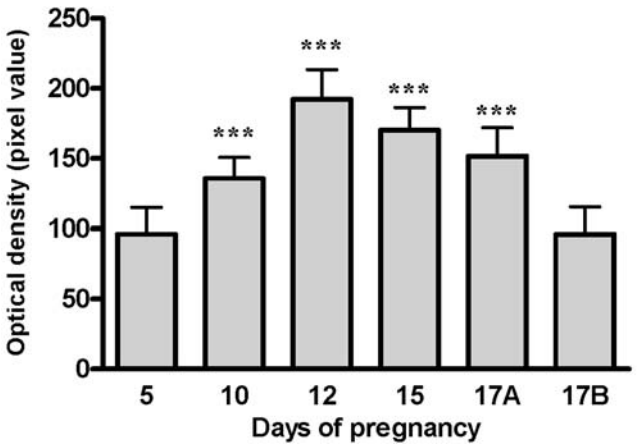

Fig. 1. Intensity of the NADPH-d histochemical reaction in the luminal epithelium of the porcine uterus from Day 5th to Day 17th of pregnancy (Day 17A - interimplantation region, Day 17B - implantation site). *** p<0.001.

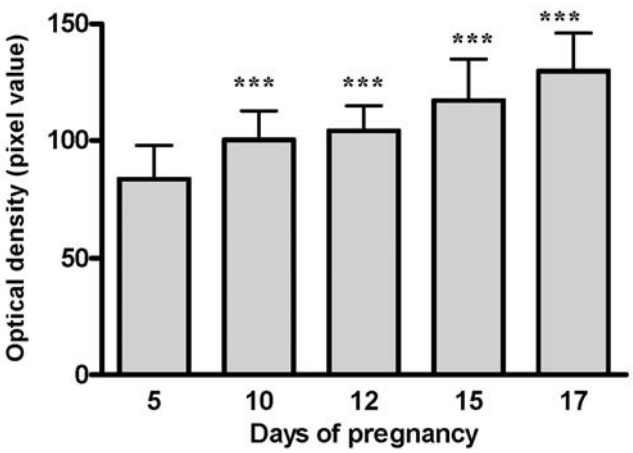

Fig 2. Intensity of the NADPH-d histochemical reaction in the glandular epithelium of the porcine uterus from Day 5th to Day 17th of pregnancy. *** p<0.001

The available reports on the distribution of NOS in the reproductive organs indicate that NO may play an important role in the modulation of uterine smooth muscle contractility [16,17], as well as in control of endometrium function during the estrus cycle, at the time of implantation [10,12,18] and pregnancy [11,19].

Because the contribution of NO in the control of implantation is not fully understood, in the present study we assessed the expression and cellular localization of the of NADPH-d, eNOS and iNOS in the porcine uterus during early pregnancy and implantation.

Materials and Methods

Tissue Collection. Gilts (Large White × Landrace) randomly assigned to a pregnant group, after exhibiting two estrous cycle of normal length, were bred at the onset of estrous (day 0), and then 12 h and 24h later. The animals were housed on a farm. Three days before slaughtering the animals were transported to the local animal house and kept in individual stalls under natural light and temperature. They were fed a commercial grain mixture and tap water *ad libitum*. All procedures involving animals were approved by the Local Research Ethics Committee and conducted in accordance with the national guidelines for agricultural animal care.

On days 5 (n=5), 10 (n=5), 15 (n=5), 17 (n=3) of pregnancy, the gilts were euthanized by electrical shock, exsanguined and the uteri were dissected out. Afterwards, the uteri were transported on ice to the laboratory within 3 min. Pregnancy was confirmed by the presence at least four of conceptuses in the uterine horns. From each animal two tissue samples from the middle part of each uterine horn were collected. On day 17 of pregnancy two uterine samples with visible trophoblast and two samples without trophoblast from each horn were collected.

Cross sections of uterine samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH=7.4), washed in 0.1M PB and cryoprotected in 18% sucrose. Afterwards, 10 m cryostat sections were stained histochemically for NADPH-d activity and immunohistochemically for NOS isoforms detection.

Immunocytochemistry for eNOS and iNOS protein. Immunostaining was carried out on consecutive sections. To block endogenous peroxidase, the sections were treated with hydrogen peroxide in methanol and washed in 0.1M PBS (phosphate-buffered saline, pH=7.4). Then they were blocked with 10% normal goat serum (NGS) for 1 hour at room temperature, incubated overnight at room temperature with primary mouse monoclonal antibody raised against eNOS or iNOS, both diluted 1:200 (#N30020 and #N32020, Transduction Laboratories, UK, respectively), washed in PBS, incubated for 1 hour at room temperature with 1:200 biotynylated anti-mouse antibody in 10% NGS (Vectastain ABC Kit), washed, incubated for 45 min with ABC Reagent in PBS, and washed again. Immunoreactive NOS was visualized by incubating the sections in 0.3 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co. Ltd.) in 0.01% hydrogen peroxide in TBS (Tris-buffer saline, pH=7.2) for 2-3 min. Finally, sections were dehydrated and cover-slipped with DPX mounting medium (Park Scientific Ltd., Northampton, UK).

To test the specificity of immunohistochemical staining two types of controls were performed: (1) the primary antibody was omitted during the immunostaining procedure; (2) the primary antibody was substituted with nonspecific IgG. The observations and photographs were made using a light microscope (NIKON FXA, Japan).

Histochemistry for NADPH-diaphorase. Frozen sections were warmed at room temperature for 30 min. After washing in PB the sections were stained for NADPH-d activity with application of nitro-blue tetrazolium (NBT), a salt that yields an insoluble blue formazan visible by the light microscopy. Staining was performed by incubating the sections in PB containing 0.1 mg/ml of NBT, 4 µl/ml of Triton-X 100 and 1 mg/ml of β-NADPH for 1 hour at 38°C. Control sections were exposed to the same solutions without β-NADPH. No staining were observed

Statistical analysis. The intensity of the NADPH-d histochemical reaction in the luminal and glandular epithelium was estimated by measurement of optical density (0-255) using Olympus DP SOFT Software (OLYMPUS). Three slides of each samples were stained and examined using the light microscope (NIKON Microphot FXA). The data were analyzed with one-way analysis of variance ANOVA and differences between day 5 of pregnancy and other groups were evaluated by the Bonferroni-adjusted t value (Graph

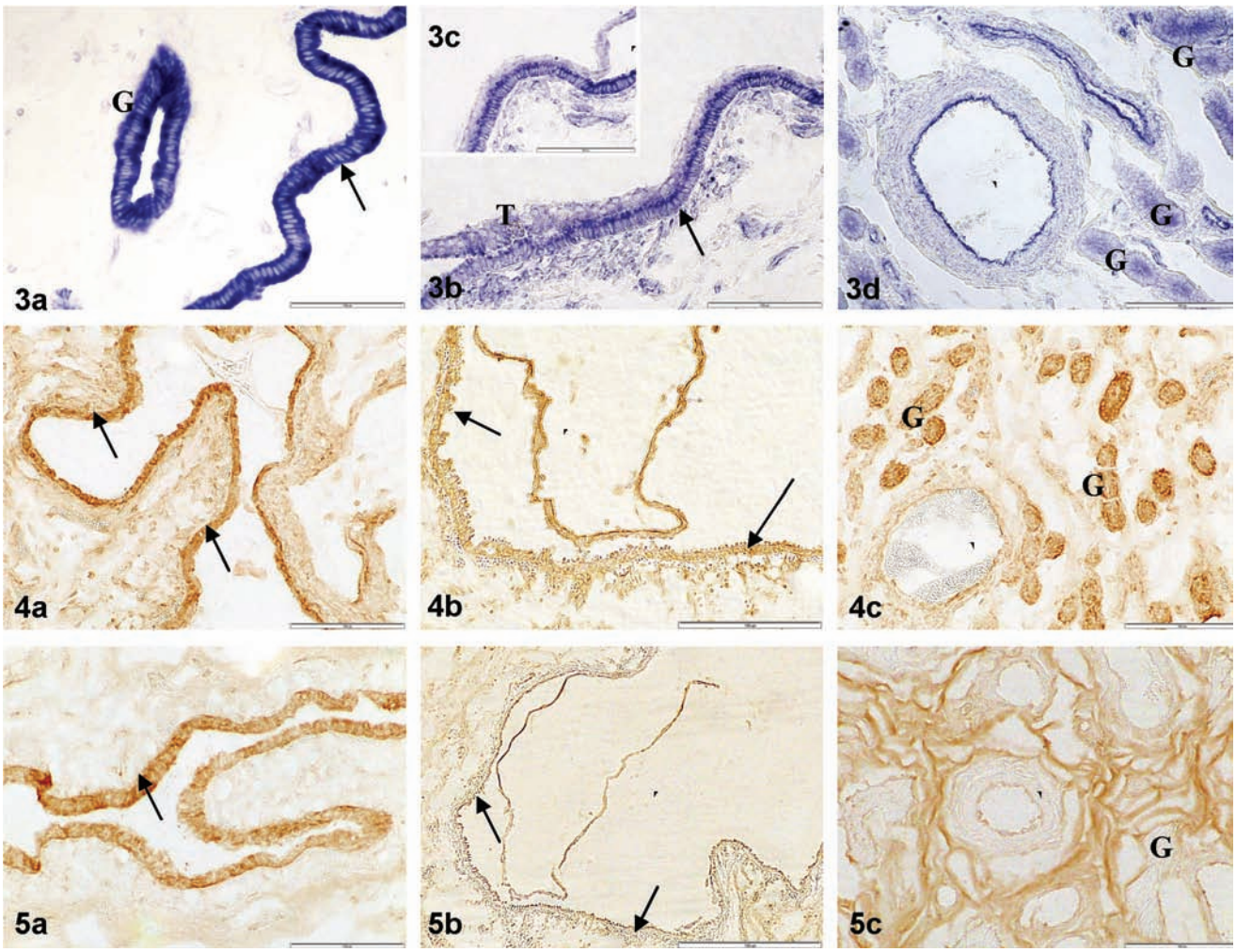

Fig. 3a. NADPH-diaphorase activity in the luminal (↑) and glandular (G) epithelium on Day 5-th of pregnancy in the swine. **Fig. 3b**. NADPH-diaphorase activity in the luminal epithelium (↑) at the site of implantation; Day 17-th of pregnancy. **Fig. 3c**. NADPH-diaphorase histochemical activity is also visible in the trophoblast (T). **Fig. 3d**. The activity of NADPH-diaphorase in the endometrial blood vessels (V) and uterine glands (G), Day 15-th of pregnancy in the swine. **Fig. 4a**. Immunoreactivity of eNOS in the porcine endometrium on Day 5-th of pregnancy; (↑) - luminal epithelium. **Fig. 4b**. Immunoreactivity of eNOS at the site of implantation; Day 17-th of pregnancy; (↑) - luminal epithelium, (T) - trophoblast. **Fig. 4c**. eNOS immunoreactivity in the glandular epithelium (G) and endometrial blood vessels (V). **Fig. 5a**. Immunoreactivity of iNOS in the porcine endometrium on Day 5-th of pregnancy; (↑) - luminal epithelium. **Fig. 5b**. Immunoreactivity of iNOS at the site of implantation; Day 17-th of pregnancy; (↑) - luminal epithelium, T - trophoblast. **Fig. 5c**. iNOS immunoreactivity in the glandular epithelium (G) and endometrial blood vessels (V).

Pad Prism, USA). The level of significance was taken as $p<0.05$. All values are expressed as mean SD.

Results

NADPH-diaphorase

Light microscopic observations revealed a differential expression of NADPH-diaphorase in the luminal (Fig. 1) and glandular (Fig. 2) epithelium. During pregnancy NADPH-d is present in the luminal (Fig. 3a and 3b) and glandular epithelium, vascular endothelium (Fig. 3d) and myometrium itself. The positive NADPH-diaphorase staining was also visible in the trophoblast (Fig. 3b and 3c). The differences in histochemical staining were found in the luminal and glandular epithelium. In the luminal epithelium (Fig. 1) a significant increase in NADPH-d activity was observed from days 5-12 of pregnancy. On day 17, two different patterns of staining were observed: 1) a significant ($p<0.001$) decrease in NADPH-d activity at the site of implantation compared with 2) NADPH-d activity in the regions where trophoblast was not present. The endometrial glands showed a significant increase (Fig. 2) in NADPH-d staining with very high activity in individual glands. The vascular endothelium displayed positive NADPH-d staining, but no significant differences were observed. However, arterial endothelium expressed stronger staining compared with venous vessels.

eNOS and iNOS

Light microscopic observations revealed that during pregnancy eNOS is present in the luminal (Fig. 4a, 4b) and glandular epithelium, vascular endothelium (Fig. 4c) and myometrium itself. No optical differences in immunostaining of eNOS were observed during the studied days of pregnancy. Clear iNOS immunoreactivity was detected in the luminal epithelium (Fig. 5a, 5b), endometrial stroma (Fig. 5c) and individual endometrial glands, whereas other glands were devoid of the reaction product. The vascular endothelium displayed slight iNOS staining (Fig. 5c). The expression of iNOS in the luminal epithelium decreased from days 5 to 17 of pregnancy.

The trophoblast cells, observed on day 17 of pregnancy, displayed clear eNOS and slight iNOS immunoactivity.

Discussion

This is the first study demonstrating the cellular localization of NADPH-d and expression of eNOS and iNOS in the porcine uterine tissues on days 5-17 of pregnancy.

The NADPH-d, histochemical marker for NOS, was observed mainly in the luminal and glandular epithelium, vascular endothelium and myometrium itself during all the studied days of pregnancy and also in the trophoblast on day 17 of pregnancy. To localize the particular izoforms of NOS the specific monoclonal antibodies were used.

The highest histochemical activity of NADPH-d in the luminal epithelium was found on day 12 of pregnancy, but then a gradual decrease in NADPH-d reaction was observed. On day 17 of pregnancy significant differences in NADPH-d activity, dependent on the presence or absence of conteptuses, were found. In the implantation region the optical density of NADPH-d histochemical reaction was very slight and similar to that observed on day 5 of pregnancy. However, in the interimplantation region NADPH-d activity was still very high. In the glandular epithelium the NADPH-d activity increased from day 5 and reached the highest level on day 17 of pregnancy. The vascular endothelium displayed almost the same NADPH-d histochemical activity during all the studied days of pregnancy. The monoclonal antibodies used in this study confirmed the presence of eNOS and iNOS in the analyzed tissues. Expression of eNOS did not vary and was on the same level until day 17 of pregnancy. The optically slight iNOS immunoreactivity compared to eNOS immunoreactivity, gradually increased from days 5 to 17 of pregnancy.

The high NADPH-d activity in the luminal epithelium, particularly on day 12 of pregnancy, suggest that NO can participate in the preparation of endometrium for implantation. Increased NO production until day 12 of pregnancy may facilitate the migration process. On the other hand, decreased NO production after day 12 of pregnancy can be caused by embryo signals, which are necessary for maternal recognition and pregnancy maintenance. Considering the type of pig placenta, a high concentration of NO can be toxic for the endometrial luminal epithelial and trophoblast cells, by activation of apoptosis and thus preventing later embryo development. Increased expression of NADPH-d in the luminal epithelium, especially in the peri-implantation period, might indicate a nutritional function which endometrial gland and endometrium perform themselves until implantation is finished successfully. Constant expression of eNOS in all the analyzed days of pregnancy suggest, that this isoform is probably the main source of NO during early pregnancy in the pig.

Studies performed on cyclic pigs [8] revealed differential expression of NADPH-d and iNOS in the luminal and glandular epithelium. The highest expression of NADPH-d/iNOS was found in the late luteal phase. High activity of NADPH-d/iNOS was still present during the follicular phase. The individual glands also displayed high NADPH-d/iNOS activity. No significant differences in eNOS immunoreactivity during the estrous cycle in the uterine tissues were detected, but optically stronger eNOS immunoreactivity was observed in the vascular endothelium.

In contrast to our current observations in the pig, studies in mice showed high NADPH-d activity after day 5 of pregnancy within the primary decidual zone with a slight reaction within interimplantation region. No NADPH-d activity was detected in the trophoblast cells and in embryonic tissue, except on day 8. This suggests the presence of embryos and decidualization caused increased NADPH-d activity in the luminal epithelium at the site of implantation [12]. In the first few days (1-4) of pregnancy in mice, the intensity of NADPH-d was low and comparable to NADPH-d activity during the estrous cycle . The low NADPH-d activity in mouse morula and blastula phase was observed [20]. Rapid growth of NADPH-d activity occurred in the trophoblast cells during the postimplantation period. Immunohistochemical localization of eNOS and iNOS, with the use of polyclonal antibodies, displayed a lack of NOS immunoreactivity in the early stages of blastocyst development. However, on day 7.5 of pregnancy both eNOS and iNOS protein and NADPH-d activity was detected in the trophoblast cells.

Pregnancy is associated with high concentrations of estrogens and progesterone in circulation. Between days 10 and 15 of pregnancy pig conceptuses secrete estrogens, which are essential for establishing pregnancy [21]. Placental estrogens affect the endometrial epithelium by increasing expression of specific growth

factors, including IGF-1 and IGF-7. These factors act on trophoectoderm to stimulate cell proliferation and development [22]. Estrogens act on the endometrium after preconditioning with progesterone and both are responsible for the characteristic pattern of endometrial sensitivity during implantation. The earliest endometrial response to estrogens during implantation includes a localized increase in vascular permeability leading to the development of stromal oedema. There are reports that some vasodilatory substances produced and released under the influence of estrogens, induce or modulate endogenous NO [23,24].

Burnett *et al.* [25] suggest that iNOS is not heavily involved in implantation or early developmental processes. However, other studies revealed that iNOS is a required component of uterine natural killer cell-dependent vascular and decidual remodeling that occurs during pregnancy [25,26].

Western blot analysis conducted by Farina *et al.* [27] displayed the presence of eNOS and iNOS isoforms in the rat gravid uterus. The authors revealed that eNOS protein was present on all studied stages with a significant increase on day 13 of gestation and decreased thereafter. The weakest intensity of eNOS reaction was observed after parturition. Monoclonal antibody for iNOS reacted with the band corresponding to 130 kDa. Expression of iNOS was found in the non-pregnant uterus and during pregnancy on days 5, 13 and 21. The protein of iNOS was not detected on day 22 of pregnancy and in uteri collected after labor.

In conclusion, this study clearly demonstrates the differential expression of NADPH-d and iNOS in the endometrium of early pregnant pig uterus. The expression of NADPH-d within the luminal epithelium decreased at the implantation site contrary to that described in mice. Furthermore, NOS expression reached the maximum level between days 12 and 15 of pregnancy, maternal recognition of pregnancy. These observations indicate the important role of NO during early pregnancy.

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