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Influence of the Antiandrogen Flutamide on Connexin 43 (Cx43) Gene and Protein Expression in the Porcine Placenta and Uterus During Pregnancy*

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The study focuses on the expression of connexin 43 (Cx43), a gap junctional protein in the porcine placenta and uterus. The aim was to examine Cx43 mRNA and protein expression after antiandrogen flutamide treatment. Flutamide was injected into pregnant gilts at a daily dose of 50 mg/kg body weight at different stages of pregnancy: between days 43-49 (50 dpc), 83-89 (90 dpc) and 101-107 (108 dpc) of gestation. The animals were sacrificed and tissues were collected one day after the last injection. Cx43 immunostaining was observed in epithelial and stromal cells of the fetal part of the placenta; luminal and glandular epithelial cells of maternal part of the placenta and myometrium of the uterus within placentation sites. Cx43 was also found in glandular epithelium and myometrium of non-placental uterus. Flutamide treatment caused fluctuations in Cx43 expression especially before parturition. Although significant changes in Cx43 mRNA expression were observed only in the fetal part of the placenta, Cx43 protein level was affected within the maternal part of the placenta and non-placental uterus. These results suggest the involvement of androgens in the regulation of Cx43 expression within the feto-maternal compartment in pigs. However, androgen deficiency caused pronounced changes during late pregnancy and before parturition. These results are interesting due to the functional changes in the porcine uterus during the preparturient period that is determined by Cx43 protein.

Key words: Connexin 43, flutamide, placenta, pig.

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During the past decade an increasing incidence of reproductive disorders caused by chemicals with antiandrogenic activity has been reported in several animal species (UZUMCU & ZACHOW 2007). Previously, we demonstrated that exposure to an antiandrogen flutamide during different times of gestation or neonatally affected porcine gonads functions during adulthood (DURLEJ *et al*. 2011a, b). Androgen action is mediated *via* androgen receptors (ARs). To date, ARs have been detected in the porcine uterus throughout the whole pregnancy (CARDENAS & POPE 2003; SLOMCZYNSKA *et al*. 2008), as well as in the porcine placenta and umbilical cord in late pregnancy (WIECIECH *et al.* 2013). Importantly, it has been revealed that porcine gravid uterus and placenta are able to produce androgens (SPENCER & BAZER 2004; WOJCIECHOWICZ *et al.* 2013), suggesting their role in the reproductive organs during gestation in pigs.

Gap junctions are clusters of transmembrane channels composed of connexin protein hexamers which connect adjacent cells in most mammalian tissues and facilitate exchange of small ions and metabolites between coupled cells (KUMAR &

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GILULA 1996). It has been shown that gap junctions composed of connexin 43 (Cx43) are necessary for ovarian follicular development, oocyte growth (GERSHON *et al*. 2008), corpus luteum function (GRAZUL-BILSKA *et al.* 1997) and uterine myometrial contractions (KARASINSKI *et al.* 2000). Moreover, studies by FLECHON *et al*. (2004) revealed the presence of two isoforms of Cx43 in the trophoectoderm and endoderm of pig blastocyst during blastulation and expansion. In humans, Cx43 plays a role in the development and differentiation of endometrium and placenta formation. Aberrant expression of Cx43 could result in altered physiological or pathological processes leading to early pregnancy loss (NAIR *et al.* 2011). However, later stages of pregnancy have not been investigated. Thus, we suggest that proper intercellular communication within the porcine placenta during mid- and late pregnancy seems to be crucial for efficient transport of signalling molecules determining normal fetus growth.

Tissue specific regulation of connexin expression by steroids has been demonstrated in the uterus of pregnant animals (LYE *et al*. 1993; JAHN *et al.* 1995; RISEK *et al.* 1995). Studies on rats revealed that progesterone suppressed expression of Cx43 in endometrium during the receptive phase of pregnancy as well as during pseudopregnancy (GRUMMER *et al.* 1999). Our previous study demonstrated that treatment of pregnant gilts with flutamide led to significant changes in the expression of Cx43 in the porcine ovary, corpus luteum and testis (DURLEJ *et al*. 2011a, b), indicating direct or indirect Cx43 regulation by androgens. Therefore, the objective of the present study was to examine the potential changes in the Cx43 gene and protein expression in the placenta and uterus of pregnant pigs following blocking of AR with flutamide. Flutamide is a nonsteroidal, pure antiandrogen, which binds to ARs and blocks androgen action by inhibition of AR signalling pathway (TRUSS *et al.* 1994).

In pigs, an epitheliochorial placenta type is formed. It consists of placenta stroma and epithelium (*pars fetalis*) as well as luminal and glandular epithelium of the uterus (*pars materna*). The myometrium is not included into placenta structure (WIGMORE & STIDAND 1985). We hypothesize that induced androgen deficiency may alter Cx43 expression leading to disturbances of cell-to-cell communication within the placenta. Moreover, it is of interest whether assumed changes in Cx43 expression are restricted to placentation sites or occur in the non-placental uterus. The expression of connexins has been examined in a variety of species during the critical period of implantation and early placentation while less data is available for the later stages of gestation.

Material and Methods

Animals

The experimental protocol was approved by the Local Ethics Committee at the Jagiellonian University (approval no. 122/2009) and all surgical procedures were performed by a veterinarian.

Sexually mature crossbred gilts (n=12, n – the number of animals in control and experimental groups; Large White × Polish Landrace) of similar age (10-11 months) and body weight (bw) (100-120 kg) were kept at the same farm conditions. Animals were checked daily for signs of estrous behavior. After two consecutive estrous cycles, gilts were mated with a fertile boar at the onset of estrus and again 12 and 24 h later. The gestation day was estimated from the first mating day. Pregnant pigs were assigned into three experimental groups. In the first group, animals were injected subcutaneously with flutamide (Sigma-Aldrich, St. Louis, MO, USA) suspended in corn oil and administrated daily at a dose of 50 mg/kg body weight between days 43 and 49 of gestation (50 dpc – day post coitum; n=2). In the second group animals were injected with flutamide between days 83 and 89 of gestation (90 dpc; n=2). In the third experimental group animals were injected with flutamide between days 101 and 107 of gestation (108 dpc; n=2). This schedule of flutamide administration was based on a previous study (KNAPCZYK-STWORA *et al.* 2013). For each flutamide-exposed group a respective control group was used (n=2 per each gestational period). Control animals were treated with corn oil. The days chosen for in utero flutamide exposure reflect the period of mid-pregnancy (50 dpc), late pregnancy (90 dpc) and preparturient period (108 dpc). Between days 40 and 70 of gestation, a particularly high incidence of dead and reabsorbing fetuses were observed (KNIGHT *et al.* 1977). The period from day 75 to 90 of gestation is an important stage for placental and fetal development, during which the fetuses grow rapidly and need adequate nutrition (WU *et al.* 2005). The preparturient period is important in relation to changes occurring within the gravid uterus that prepares itself for parturition.

Tissue collection

Under anesthesia induced by an injection of thiopental (10 mg/kg bw; Sandoz GmbH, Vienna, Austria) into an ear vein, the entire uterus was removed and opened by cutting along the mesenteric site to visualize the location of the fetal sacs in the uterine horns. The fetuses were then removed and placental samples and non-placental uterus were collected. Tissues were immediately fixed in 10% buffered formalin for immunohistochemistry. Other samples obtained from the placentation sites

were dissected under a magnifier (to verify the purity of samples) into the fetal part of the placenta (FP, epithelium and stroma) and maternal part of the placenta (MP, endometrium of the uterus) and snap frozen in liquid nitrogen for protein and RNA isolation. The final number of placentas excised from pregnant pigs was: on 50 dpc – control animals n=15; flutamide-treated animals n=18; on 90 dpc – control animals n=9; flutamide-treated animals n=18; on 108 dpc – control animals n=10; flutamide-treated animals n=20. Non-placental uterus was obtained from 5 different localizations from each animal. In this study, the experimental unit was an individual fetus with its placenta, therefore the samples from each placenta were considered individually (FORD *et al*. 2002; VONNAHME & FORD 2003).

Quantitative Real-time PCR

Total RNA was isolated using the NucleoSpin RNA II System (Macherey-Nagel GmbH & Co., Düren, Germany) according to the manufacturer's protocol. Reverse transcription was performed using a volume equivalent to 1 μg of total RNA and a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's protocol. Real-time PCR analyses were performed with the Step-One Real-Time PCR System (Applied Biosystems) according to the recommended cycling program. The expression level of Cx43 was quantified in each sample using TaqMan Gene Expression Assay (assay ID: Ss03374839_u1; Applied Biosystems). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an endogenous control (assay ID: Ss03373286_u1; Applied Biosystems). All qPCR experiments were performed in duplicate. The expression of Cx43 relative to GAPDH was determined using the $2^{-\Delta Ct}$ method as described elsewhere (LIVAK & SCHMITTGEN 2001).

Western blot analysis

Protein extraction and Western blot analysis were performed as described (WIECIECH *et al.* 2013). A primary rabbit polyclonal antibody anti-Cx43 (1:1000, 1.5h, Sigma-Aldrich), and a secondary horseradish peroxidase-conjugated, anti-rabbit IgG (1:3000, 1h, Vector Laboratories, Burlingame, CA, USA) were used. To visualize the presence of the Cx43, Western blotting Luminol Reagent (Santa Cruz) solution was used for 1 min and then images of membranes were taken by ChemiDoc Imaging System (Bio-Rad, CA, USA). The membranes were stripped and reprobed with monoclonal mouse anti-β-actin antibody (1:3000; Sigma-Aldrich) followed by horseradish peroxidase-conjugated ant-imouse IgG (1:3000; Bio-Rad Laboratories Inc.). Analysis of images was performed using the public domain ImageJ program (National Institutes of Health, Bethesda, MD, USA) using the "Gel Analysis" functions. The bands were densitometrically quantified and normalized to their corresponding β-actin bands. Analysis was performed for three separate experiments from each control and flutamide-treated group.

Cx43 immunolocalization

Immunohistochemistry was conducted routinely as previously described (WIECIECH *et al.* 2013). Nonspecific binding of IgG was prevented by incubating the sections with 5% normal goat serum (Sigma-Aldrich) prior to incubation with anti-Cx43 (1:1000, overnight incubation at 4°C in a humidified chamber), biotinylated goat anti-rabbit secondary antibody (1:400, 1.5 h, Vector Laboratories) and avidin-biotin-peroxidase complex (1:100, 40 min, StreptABComplex-HRP, Vector Laboratories) as a substrate. Sections were then counterstained with Mayer's hematoxylin. Photomicrographs were taken using a Nikon Eclipse E200 microscope (Tokyo, Japan) attached to a Coolpix 5400 digital camera system (Nikon, Tokyo, Japan) and then quantified by a densitometric analysis using ImageJ software. The intensity of Cx43 staining was expressed as a relative optical density (ROD) and was calculated using the formula: $ROD = {}^{Od}specimen / {}^{Od}background = \log(GL_{blank}/GL_{specimen}) / \log(\log GL_{blank} / GL_{background})$, where GL is the gray level for the stained area (specimen) and the unstained area (background), and blank is the gray level measured after removing the slide from the light path (SMOLEN 1990). Negative control were performed by substituting the primary antibody with non-immune rabbit IgG.

Statistical analysis

Statistical analysis was performed using Statistica v.10 program (StatSoft, Inc., Tulsa, OK, USA). Differences between experimental and control groups were estimated using nonparametric Mann-Whitney *U*-test. The data were statistically evaluated with significance at *P<0.05, **P<0.01, ***P<0.001.

Results

Effect of flutamide on Cx43 mRNA and protein expression

Real-time PCR analysis revealed expression of Cx43 in the fetal part of the placenta (FP; Fig. 1a), the maternal part of the placenta (MP; Fig. 1b) and

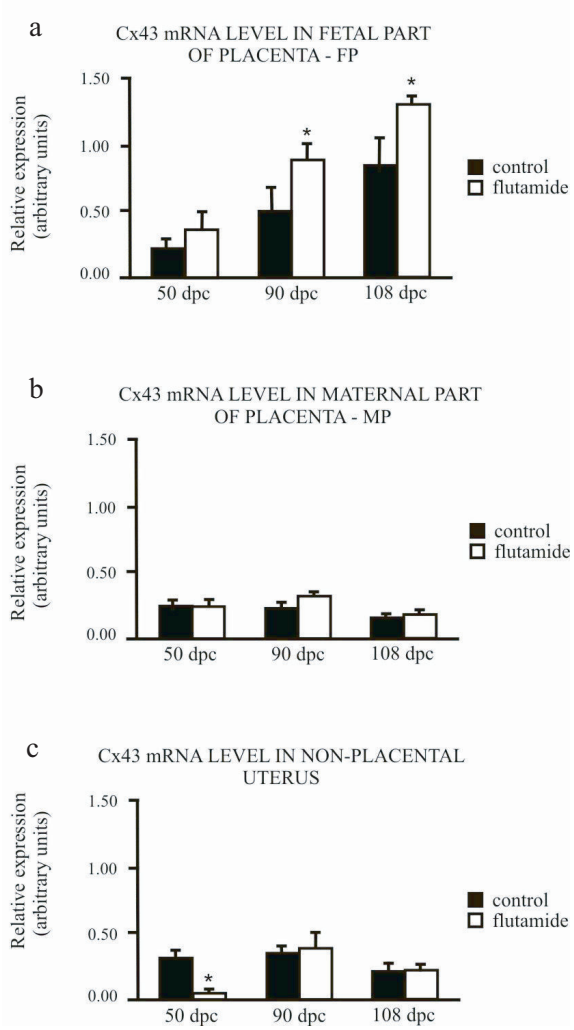

Fig. 1. Expression of mRNA for connexin 43 (Cx43) in the fetal part of the placenta (FP, epithelium and stroma) (a) and maternal part of the placenta (MP, endometrium of the uterus) (b) and in non-placental uterus (c) retrieved from control and flutamide-treated gilts on days 50, 90 and 108 of gestation using real-time PCR analysis. The expression of *Cx43* mRNA was normalized to GAPDH and the relative expression was presented as $2^{-\Delta Ct}$. Statistically significant differences were analyzed using the Mann-Whitney *U*-test (* P<0.05); dpc – day post coitum.

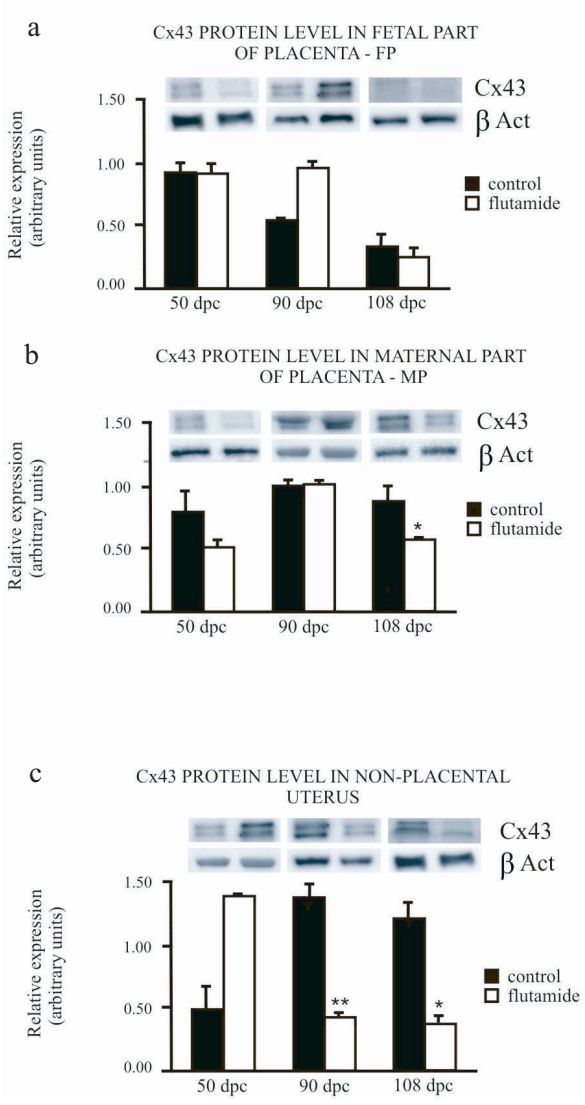

Fig. 2. Expression of protein for connexin 43 (Cx43) in the fetal part of the placenta (FP, epithelium and stroma) (a) and maternal part of the placenta (MP, endometrium of the uterus) (b) and in non-placental uterus (c) retrieved from control and flutamide-treated gilts on days 50, 90 and 108 of gestation using Western blot analysis. Representative blots are shown. The relative expression of Cx43 protein was evaluated by densitometry and expressed as the ratio to β-actin. Cx43 was detected as unphosphorylated form (43 kDa – the lower bands) and phosphorylated form (45 kDa the upper bands) in control and flutamide-treated groups. Statistically significant differences were analyzed using the Mann-Whitney *U*-test (* P<0.05, ** P<0.01); dpc – day post coitum.

in the non-placental uterus (Fig. 1c). Flutamide administration resulted in significant increase of Cx43 mRNA in FP on 90 and 108 dpc (Fig. 1a; P<0.05). On the other hand, Cx43 mRNA level was decreased after flutamide administration in the non-placental uterus on day 50 of pregnancy (Fig. 1c; P<0.05).

Cx43 protein expression was detected as a double band (lower band – unphosphorylated form of 43 kDa; upper band – phosphorylated form of 45 kDa) in both control and flutamide-treated groups at all examined stages of pregnancy (Fig. 2a, b, c). A statistically significant decrease of Cx43 protein expression was observed in MP on 108 dpc (Fig. 2b; P<0.05). In the non-placental uteri a significant decrease of Cx43 protein level was found on 90 and 108 dpc (Fig. 2c; P<0.01 and P<0.05, respectively).

Effect of flutamide on Cx43 localization

Immunohistochemistry revealed a positive reaction for Cx43 in both control and flutamide-treated animals within placenta and uterus (Fig. 3) and within non-placental uterus (Fig. 4). Cx43 immunostaining was observed in epithelial and stromal

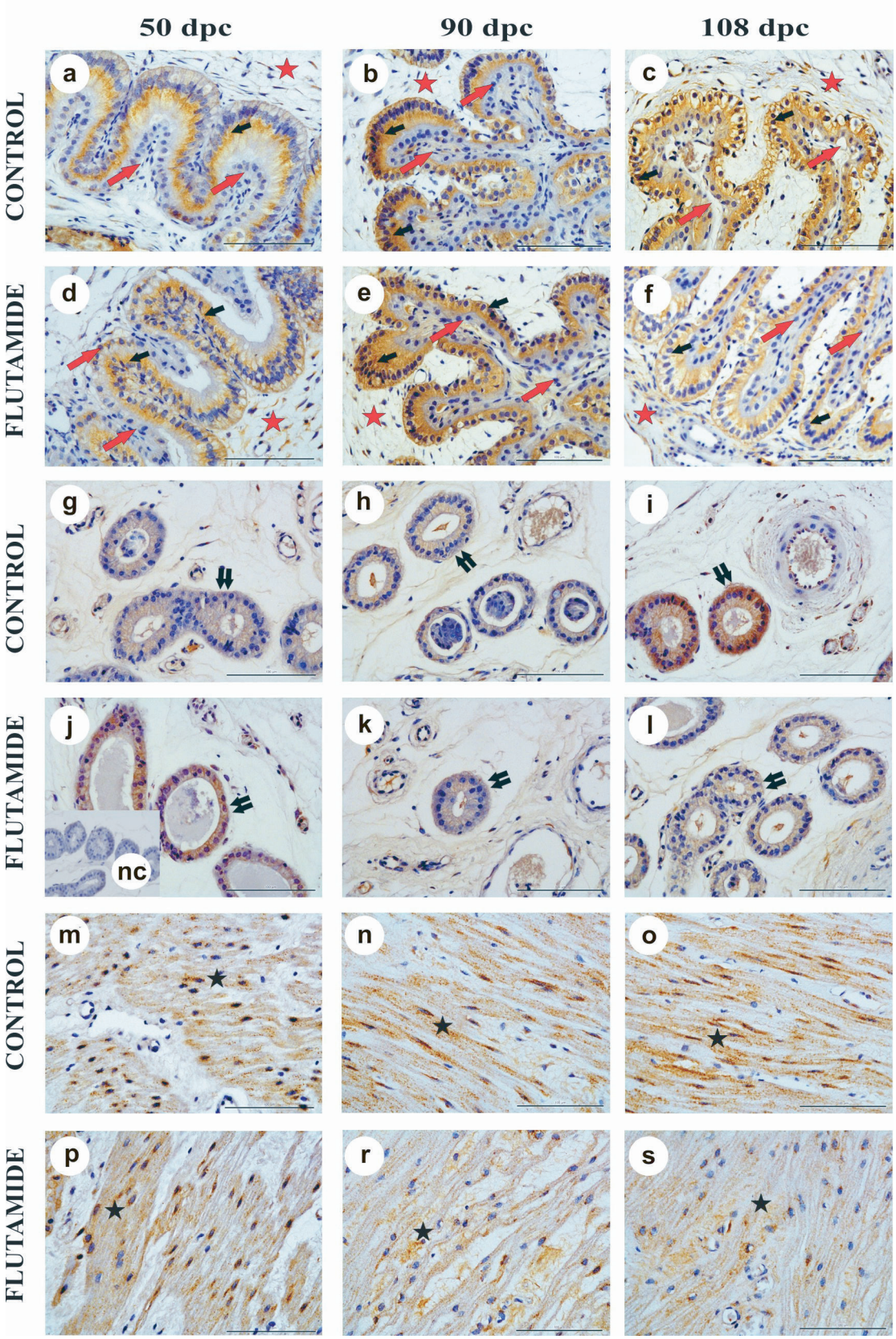

Fig. 3. Immunohistochemical localization of Cx43 in placenta and uterus retrieved from control and flutamide-treated gilts on days 50, 90 and 108 of gestation. A positive reaction was observed in both control (a-c, g-i, m-o) and flutamide-treated animals (d-f, j-l, p-s). Cx43 immunoreaction was observed in the epithelial (a-f; single black arrows) and stromal cells (a-f; red stars) of the fetal part of the placenta. The cells within luminal (a-f; single red arrows) and glandular (g-l; double black arrows) epithelium of the maternal part of the placenta also expressed Cx43. Moreover, the myometrium of the uterus showed positive Cx43 immunoexpression (m-s; black stars). Negative controls (nc) in which the primary antibody was replaced by normal rabbit IgG, displayed no immunostaining. Bar = 100 μm; dpc – day post coitum.

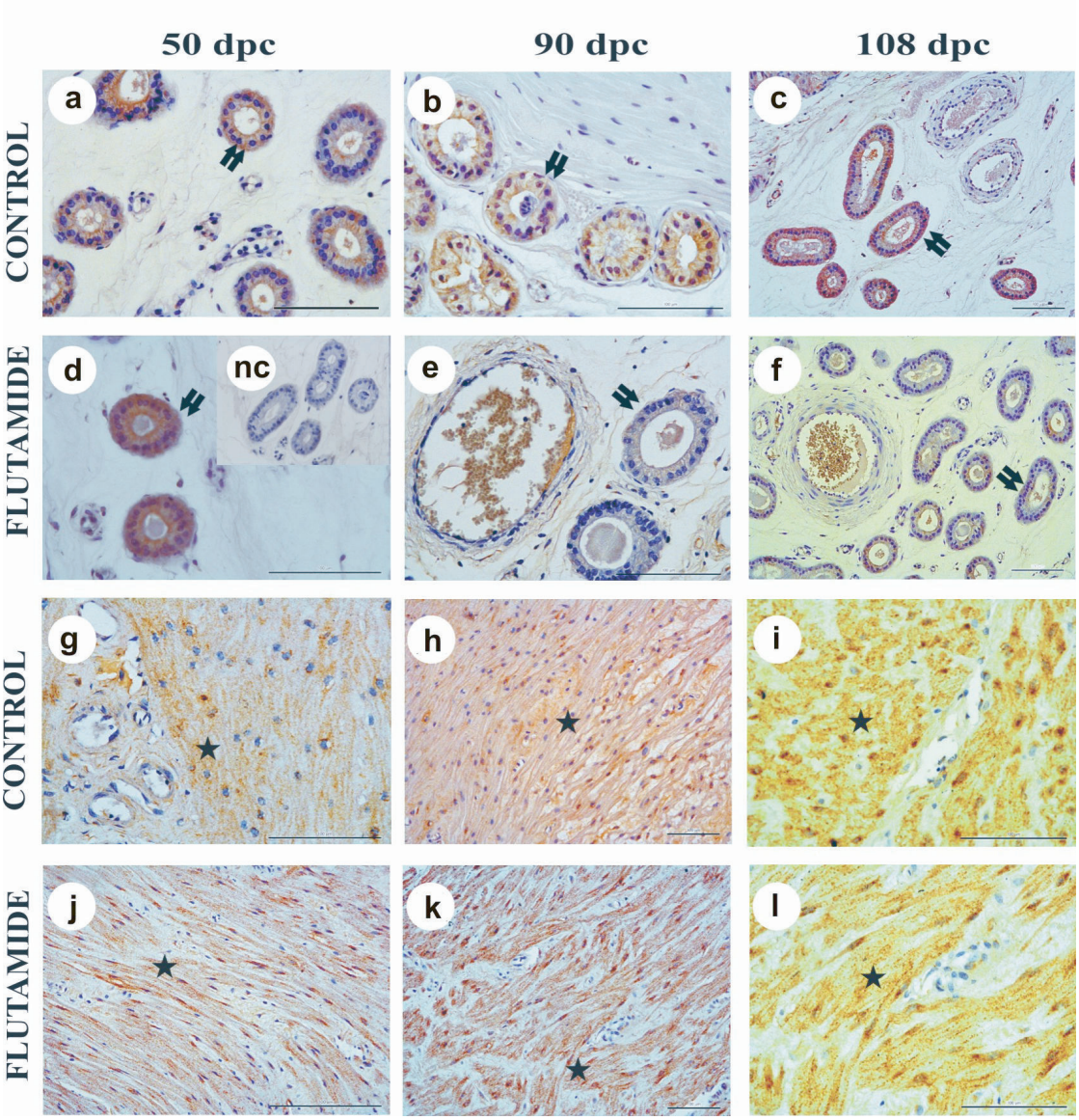

Fig. 4. Immunohistochemical localization of Cx43 in the non-placental uterus retrieved from control and flutamide-treated gilts on days 50, 90 and 108 of gestation. A positive reaction was observed in both control (a-c, g-i) and flutamide-treated animals (d-f, j-l). Cx43 immunoreaction was observed in the glandular epithelial cells (a-f; double black arrows) and myometrium (g-l; black stars). Negative controls (nc) in which the primary antibody was replaced by normal rabbit IgG, displayed no immunostaining. Bar = 100 μm; dpc – day post coitum.

cells of FP (Fig. 3a-f) as well as in luminal (Fig. 3a-f) and glandular (Fig. 3g-l) epithelial cells of MP. Moreover, myometrium (Fig. 3m-s) of the uterus within placentation sites expressed positive Cx43 staining. Cx43 was also found in glandular epithelial cells (Fig. 4a-f) and myometrium (Fig. 4g-l) of the non-placental uterus. The intensity of Cx43 staining within FP was significantly higher in stroma cells on 50 dpc (Fig. 5a; $P<0.05$), but lower on 90 and 108 dpc (Fig. 5a; $P<0.05$) following flutamide treatment. Within MP, flutamide exposure led to a decrease in Cx43 immunostaining intensity on 108 dpc in glandular epithelium (Fig. 5d; $P<0.05$). In myometrium of the uterus within placentation sites, intensity of Cx43 staining increased on 90 dpc and decreased on 108 dpc following flutamide administration (Fig. 5e; $P<0.01$). In non-placental uteri the Cx43 staining intensity was decreased in glandular epithelium on 108 dpc (Fig. 5f; $P<0.05$) and myometrium on 50 and 108 dpc (Fig. 5g; $P<0.05$) after flutamide treatment.

Discussion

In the present study, flutamide treatment led to increased Cx43 mRNA expression on days 90 and 108 of gestation in the fetal part of the placenta,

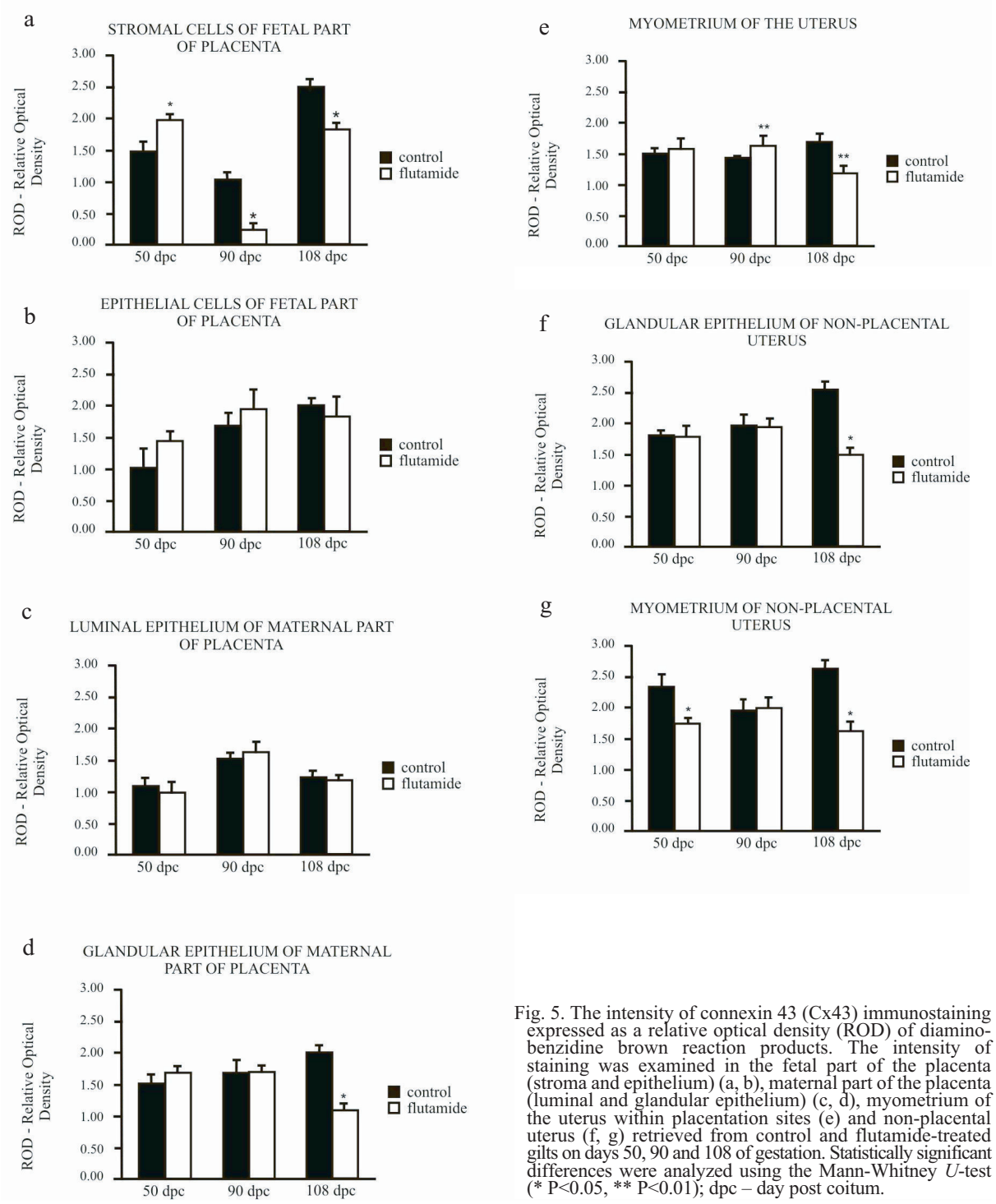

Fig. 5. The intensity of connexin 43 (Cx43) immunostaining expressed as a relative optical density (ROD) of diaminobenzidine brown reaction products. The intensity of staining was examined in the fetal part of the placenta (stroma and epithelium) (a, b), maternal part of the placenta (luminal and glandular epithelium) (c, d), myometrium of the uterus within placentation sites (e) and non-placental uterus (f, g) retrieved from control and flutamide-treated gilts on days 50, 90 and 108 of gestation. Statistically significant differences were analyzed using the Mann-Whitney *U*-test (* $P<0.05$, ** $P<0.01$); dpc – day post coitum.

while decreased Cx43 protein level was observed on day 108 in the maternal part of the placenta, and on days 90 and 108 within the non-placental uterus. It seems that changes in Cx43 expression are more prominent in late pregnancy and before parturition, suggesting the role of androgens in placenta and gravid uterus function during those gestational periods.

Connexins have been shown to contribute to feto-maternal exchange within the placenta (MALASSINE & CRONIER 2005; PFARRER *et al.* 2006). In humans, Cx43 is directly involved in trophoblast cell fusion and villi formation (DUNK *et al.* 2012). Reduced expression of the Cx43 transcript and protein in human fetal chorionic villi and maternal decidua indicate a key role of Cx43 in the risk of early pregnancy loss (NAIR *et al.* 2011). However, this gap junctional response and decidualization is present in the hemochorial type of placenta, but absent in non-invasive epitheliochorial placenta of pigs (DAY *et al.* 1998). Our data showed for the

first time the presence of transcript and protein in the porcine placenta starting from day 50 of gestation. Although the expression and role of Cx43 at early stages of pregnancy in pigs are elusive, we assume that Cx43 during mid- and late pregnancy might be involved in placental cell-to cell communication.

Previously, AR has been localized in the stromal and epithelial cells of the fetal part of the placenta during late gestation in pigs (WIECIECH *et al.* 2013) that corresponds with Cx43 immunolocalization in the same structures presented herein. In the fetal part of the placenta, Cx43 mRNA upregulation was found only on days 90 and 108 of gestation, while protein expression was unchanged. Although our mRNA data might suggest increased cell-to-cell communication within the fetal part of the placenta following flutamide treatment, unaffected protein level does not allow us to make that conclusion. The current results are in line with recent findings indicating Cx43 regulation by androgens in pigs (DURLEJ *et al.* 2011a).

The expression of Cx43 in the porcine uterus during pregnancy has been clearly shown (LENHART *et al.* 1999), and is consistent with the current results demonstrating the presence of Cx43 mRNA and protein within the maternal part of the placenta. It was suggested that differences in the sensitivity of the uterus between subsequent days of pregnancy may be dependent on the hormonal status of pregnant swine (LENHART *et al.* 1999). Importantly, porcine uterus of pregnancy was shown to be able to produce steroid hormones, including androgens (FRANCZAK 2008). Moreover, ARs have been found in luminal and glandular epithelium within the maternal part of the placenta in pigs (WIECIECH *et al.* 2013). Because flutamide treatment resulted in decreased Cx43 protein level on day 108 of pregnancy, it appears that androgen deficiency affects cellular communication within the maternal part of the placenta. Importantly, disrupted cell-to-cell interactions in luminal epithelium might influence proper feto-maternal exchange, while in glandular epithelium it might alter the function of glands.

The level of maternal progesterone decreases before delivery, therefore the progesterone block in the myometrium induces parturition (LYE *et al.* 1993). The importance of gap junctional communication in controlling myometrial contraction at the time of parturition is well documented (RISEK *et al.* 1995). It was reported that progesterone decreases Cx43 expression and maintains the smooth muscle cells in a quiescent state (RISEK *et al.* 1995). Androgen deficiency caused diminished intensity of Cx43 immunoreaction in the myometrium of gravid uterus, which may indicate weaker myometrial contractions and delayed parturition event.

The further aim of the present study was to investigate whether changes in Cx43 expression following flutamide exposure are restricted to placentation sites. Interestingly, we have found a decreased Cx43 protein level on days 90 and 108 of gestation within the non-placental uterus. This indicates that androgens act similarly within the whole gravid uterus and the interplacentation sites are as important as placentation sites in pigs. The changes in Cx43 expression might also result in disrupted cell-to cell communication in the uterus and its impaired function as a consequence.

In summary, we suggest that androgens are important factors regulating Cx43 expression within porcine placenta and non-placental uterus especially during late pregnancy and the preparturient period. It seems that changes in placental Cx43 expression may result in improper cell-to-cell communication and feto-maternal exchange. Moreover, a diminished Cx43 level in the myometrium of gravid uterus may indicate delayed parturition in pigs under androgen deficiency.

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