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Effects of delta-opioid receptor agonist on selected cytokine and chemokine gene expression in the endometrium of early pregnant pigs

A. Zmijewska[1,*], E. Goryszewska-Szczurek[2], W. Czelejewska[1], M. Dziekonski[1], T. Wiesak[2] and S. Okrasa[1]

[1] University of Warmia and Mazury in Olsztyn, Faculty of Biology and Biotechnology, Department of Animal Anatomy and Physiology, 10-719 Olsztyn, Poland
[2] Polish Academy of Sciences, InLife Institute of Animal Reproduction and Food Research, 10-683 Olsztyn, Poland

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* Corresponding author:
e-mail: agata.zmijewska@uwm.edu.pl

ABSTRACT. Endogenous opioid peptides (EOPs) are widely distributed in reproductive tissues and are involved in the regulation of uterine function, including immunological and endocrine processes essential for early pregnancy. Among opioid systems, enkephalins, acting via the delta-opioid receptor (δ), are considered important modulators of immune responses. This study investigated the effects of δ-opioid receptor activation on the expression of selected cytokines, chemokines, and their receptors, as well as genes associated with endometrial function in the porcine uterus during early pregnancy. Endometrial explants were collected on days 12–13 (maternal recognition of pregnancy) and 15–16 (onset of implantation) of pregnancy and incubated *in vitro* for 6 h with a δ-opioid receptor agonist (DPLPE) at concentrations of 10^{-9}–10^{-7} M. Gene expression was analysed using quantitative real-time PCR. Treatment with DPLPE at 10^{-9} M significantly increased mRNA expression of pro-inflammatory cytokines *IL1β* and *IL6* during both studied periods, and *IL15* and *IL18* during implantation. The same dose also upregulated chemokines *CXCL10, CXCL11, and XCL1, as well as the chemokine receptors CXCR3* and *CXCR4*, in a period-dependent manner. In addition, DPLPE affected the expression of genes related to endometrial activity, including *ESR1*, *PAQR9*, *TLR4*, *MAP2K2*, *CLDND1*, and *DNMT1*, while no effects were observed for *CDK1*, *PDCD10*, or *TGFα*. These results demonstrate that δ-opioid receptor activation influences gene expression associated with immune and endometrial functions in porcine endometrium, supporting a role of the opioid system in regulating the uterine environment during early pregnancy.

Introduction

Endogenous opioid peptides (EOPs) originate from three main precursor proteins: proopiomelanocortin (POMC), proenkephalin (PENK) and prodynorphin (PDYN). Peptides derived from these precursors preferentially act on distinct opioid receptor types, i.e. betaendorphins (from POMC) on *mu* receptors, enkephalins (from PENK) on delta receptors, and dynorphins (from PDYN) on *kappa* receptors (Simon, 1991).

Studies in various species have demonstrated a widespread distribution of EOPs in the central nervous system and peripheral organs (Simon, 1991), including reproductive tissues (Wahlström et al., 1985; Li et al., 1991 a,b; Okrasa et al., 2003).

Expression of genes encoding opioid precursors has been identified in the uterus of different species (Douglass et al., 1987; Jin et al., 1988; Rosen et al., 1990; Zhu and Pintar, 1998). In the porcine uterus, expression of genes coding for these opioid precursors and opioid receptors has also been confirmed (Dziekonski et al., 2015 a,b).

Generally, EOPs have been shown to influence a range of uterine processes, including cell proliferation (Vértes et al., 1996), apoptosis (Chatzaki et al., 2001), myometrial contractility (Zoumakis et al., 1997), steroidogenesis (Dziekonski et al., 2018) and immunomodulation (Dziekonski et al., 2015a; Eisenstein, 2019). Among opioid systems, enkephalins and deltaopioid receptors (δ) are considered particularly important in the regulation of immune processes (Rosen et al., 1990; Cui et al., 2021), especially by controlling local inflammatory responses in the endometrium, including suppression of pro-inflammatory mediators and NFκB signalling, as well as participation in immune adaptation at the maternal-embryonic interface during implantation.

The mechanisms involved in the regulation of early pregnancy are highly complex and involve the coordinated action of multiple factors, among others, prostaglandins, steroids, growth factors, cytokines and chemokines (Bogacki et al., 2005; Dimitriadis et al., 2005; van Mourik et al., 2009; Blitek et al., 2012; Okrasa et al., 2014). In pregnant pigs, key pro-inflammatory cytokines (e.g., interleukin-1 beta (IL-1β) and interleukin-6 (IL-6)) are important regulators of uterine peri-implantation events (Yu et al., 1998; Ross et al., 2003; Zmijewska et al., 2013). IL-1β is primarily associated with trophoblast elongation and the establishment of pregnancy, whereas IL-6 is considered to be an important mediator of embryo-uterine interactions during early gestation in pigs (Blitek et al., 2012). Moreover, these cytokines may affect the endocrine activity of the uterus, especially the synthesis of prostaglandins and steroids, as well as the expression of genes coding for opioid precursors (Franczak et al., 2010; Jana et al., 2011, Franczak et al., 2012, Franczak et al., 2014, Dziekoński et al., 2015a). Chemokines are also key regulators of endometrial tissue remodelling during early pregnancy in pigs (Lim et al., 2018; Złotkowska and Andronowska, 2019). They are produced by both the porcine endometrium and trophoblast (Złotkowska and Andronowska, 2020) and exert chemotactic effects on leucocytes, while also influencing cell proliferation, migration and apoptosis (Graves and Jiang, 1995; Dimitriadis et al., 2005). These processes are crucial during maternal recognition of pregnancy and implantation. Considering the immunomodulatory properties of enkephalins and the pivotal role of chemokines in endometrial remodelling and leucocytes migration in the uterus, it is plausible that δ-opioid receptor activation may also influence chemokine expression and thereby contribute to the regulation of immune function and other endometrial processes during early pregnancy.

On the basis of the above data, we formulated a hypothesis that stimulation of the δ opioid receptor would affect cytokine and chemokine gene expression. To test this assumption, the *in vitro* effects of δ-opioid agonist (DPLPE) on the mRNA expression of selected cytokines (*IL-1β, IL-6, IL-15* and *IL-18*) and chemokines (*CXCL10*, *CXCL11* and *XCL1*) were analysed in porcine endometrial explants collected on days 12–13 and 15–16 of pregnancy. Additionally, the influence of DPLPE on the mRNA expression of selected receptors (*CXCR3*, *CXCR4*, *ESR1*, *PAQR9* and *TRLR4*) and genes associated with cellular function (*PDCD10*, *CLDND1*, *MAP2K2*, *CDK1* and *DNMT1*) were evaluated in the same tissue. The current study has demonstrated the potential of a δ-opioid receptor ligand to affect the expression of numerous genes in the porcine endometrium during key stages of early pregnancy, namely maternal recognition of pregnancy (days 12–13) and the onset of implantation (days 15–16).

Material and methods

Animals

In accordance with the Act of 15 January 2015 on the Protection of Animals Used for Scientific or Educational Purposes and Directive 2010/63/EU of the European Parliament, ethical approval was not required for this study. A graphical overview of the experimental design is presented in Figure 1. Experiments were conducted on animal tissues collected post-mortem during routine commercial slaughter in a licensed slaughterhouse. Mature cross-bred gilts (Large White × Polish Landrace, weighting 90–110 kg) were divided into two groups (n = 5 per group) according to the stage of early pregnancy: maternal recognition of pregnancy (Group I, days 12–13) and the onset of implantation (Group II, days 15–16), respectively. Gilts were purchased from a private farm and fed a commercial diet. Oestrus behaviour was monitored and confirmed in the presence of an intact boar. The onset of the second oestrus was designated as day 0 of the oestrous cycle, and insemination was performed on day 2. Animals were transported to a local abattoir and slaughtered at the expected stage of pregnancy. Uterine horns of inseminated gilts were rinsed with 20 ml of sterile saline and the

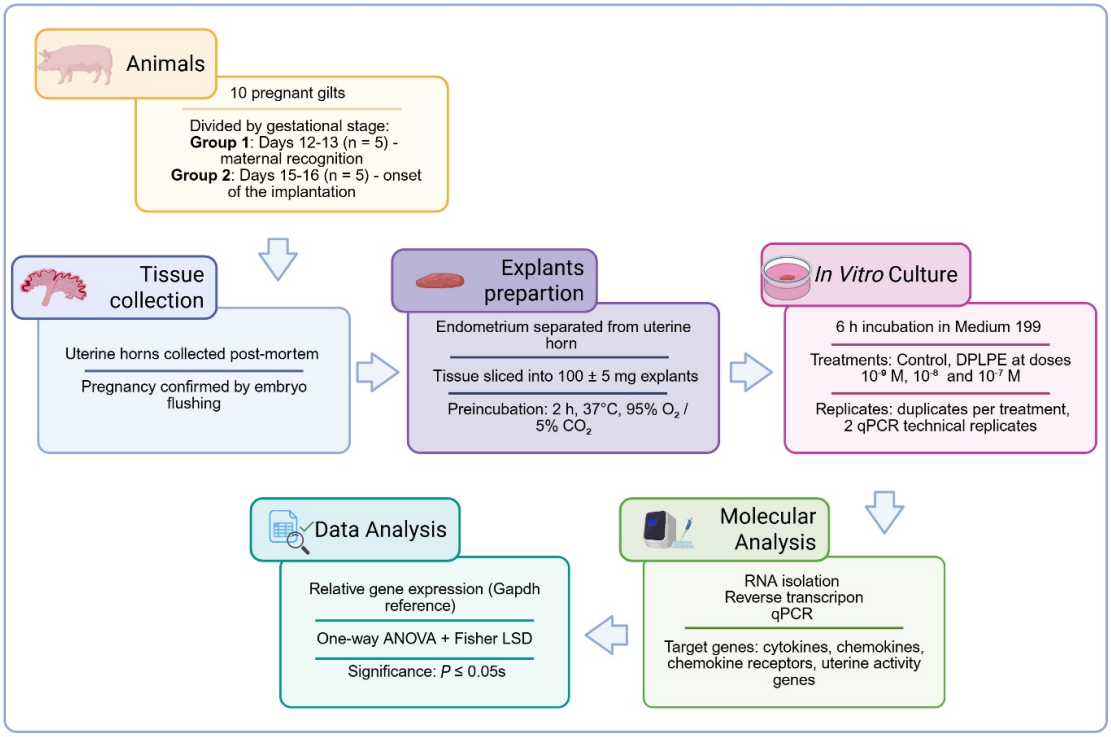

Figure 1. Graphical visualization of the experimental design created with BioRender.com (https://BioRender.com/mmeanwr)

pregnancy was confirmed by the presence of embryos in the uterine flushing. The uteri were directly placed in ice-cold phosphatebuffered saline (PBS) supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml) and transported to the laboratory for preparation of endometrial explants.

Preparation and incubation of endometrial explants

Explants were collected from the middle part of uterine horn of each gilt. Uterine layers were separated using a scalpel blade, and subsequently the endometrium was cut into small slices (100 ± 5 mg). The explants were washed twice with PBS and placed in glass culture vials containing 2 ml Medium 199 (Sigma-Aldrich, Germany) with the addition of 0.1% bovine serum albumin fraction V (ICN, USA) and 20 µg gentamycin (Sigma-Aldrich, Germany). Following a 2 h preincubation in a shaking water bath at 37 °C under an atmosphere of 95% O_2 and 5% CO_2, the explants were transferred to fresh media and incubated under the same conditions for 6 h with the addition of DPLPE (10^{-9}, 10^{-8}, 10^{-7} M) or without treatments (controls) (Dziekonski et al., 2018). All samples were prepared in duplicates within each culture. After incubation, the tissue explants were collected, washed in PBS, and stored at −80 °C for further analysis.

RNA isolation and reverse transcription

Total RNA was extracted using Qiagen RNeasy columns (Qiagen, Germany) in accordance with the manufacturer's protocol. RNA concentration and purity were determined spectrophotometrically (TECAN, Switzerland) and randomly selected RNA samples were additionally assessed in 1.5% agarose gel electrophoresis. Reverse transcription (RT) was performed using the QuantiTect Reverse Transcription Kit (Qiagen, USA). Briefly, 1 µg of RNA and 2 µl of gDNA Wipeout Buffer were combined and brought to a final volume of 14 µl. Subsequently, 1 µl of Quantiscript Reverse Transcriptase, 4 µl of Quantiscript 5×RT buffer and 1 µl RT primers were added. The samples were then incubated at 42 °C for 15 min, followed by 93 °C for 3 min to inactivate the reverse transcriptase. The resulting cDNA was stored at −20 °C until PCR analysis.

Quantitative real-time RT-PCR analyses

Expression of all studied genes (Table 1) was quantified by real-time PCR (qPCR) using the TaqMan® Gene Expression Cells-to-CT™ Kit (Ambion, Life Technologies, Carsbad, CA, USA). The RT reaction products (cDNAs) were diluted 1:3 in Tris-EDTA buffer prior to analysis. The qPCR analyses were performed using a 7900 TH Real-time PCR system (Applied Biosystems, Waltham, MA, USA) in a final volume of 10 µl in 384-well plates, including non-template controls for each gene. Plates were prepared using an automatic pipetting platform (Bravo Automated Liquid Handling Platform, Agilent Technologies). Each sample was analysed in duplicate. Efficiency and cycle threshold (Ct)

Table 1. Taq Man probes used in the experiment (TaqMan™ Assays, ThermoFisher Scientific)

Gene name	Assay ID (TaqMan™ Assays)
Interleukin 1 (*IL1B*)	Ss03393804_m1
Interleukin 6 (*IL6*)	Ss03384604_u1
Interleukin 15 (*IL5*)	Ss03394853_m1
Interleukin 18 (*IL18*)	Ss03391204_m1
C-X-C motif chemokine ligand 10 (*CXCL10*)	Ss03391846_m1
C-X-C motif chemokine ligand 11 (*CXCL11*)	Ss03648934_m1
X-C Motif Chemokine Ligand 1 (*XCL1*)	Ss03649043_m1
C-X-C Motif Chemokine Receptor 3 (*CXCR3*)	Ss03375858_u1
C-X-C Motif Chemokine Receptor 4 (*CXCR4*)	Ss03392297_S1
Mitogen-Activated Protein Kinase Kinase 2 (*MAP2K2*)	Ss04327801_m1
Cyclin Dependent Kinase 1 (*CDK1*)	Ss03372912_g1
Estrogen Receptor 1 (*ESR1*)	Ss03383398_u1
Progestin And AdipoQ Receptor Family Member 9 (*PAQR9*)	Ss06882471_s1
Programmed Cell Death 10 (*PDCD10*)	Ss03820202_s1
Claudin Domain Containing 1 (*CLDND1*)	Ss04324583_m1
Toll Like Receptor 4 (*TLR4*)	Ss03389779_m1
DNA Methyltransferase 1 (*DNMT1*)	Ss03392035_m1
Transforming Growth Factor Alpha (*TGFA*)	Ss03383643_u1
Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*)	Ss03374854_g1

value for individual reactions were determined from raw fluorescence data using real-time PCR Miner software (Zhao and Fernald, 2005).

Data analysis

Statistical analyses were performed using Statistica 10.0 software (StatSoft Inc., USA). Relative mRNA expression levels of the analysed genes were calculated using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the reference gene, according to standard guidelines for gene expression analysis and the manufacturer's protocol. Statistical differences between groups were evaluated using one-way ANOVA, followed by Fisher's NIR (LSD) post hoc test. All data are presented as mean ± SEM, and differences were considered significant at $P \leq 0.05$.

Results

Effect of δ-opioid receptor agonist (DPLPE) on mRNA expression of selected cytokines in porcine endometrium

Treatment with the δ-opioid receptor agonist DPLPE at 10^{-9} M increased *IL1β* and *IL6* mRNA expression in the porcine endometrium collected on days 12–13 and 15–16 of pregnancy compared to the control (Figure 2A–D). The same dose

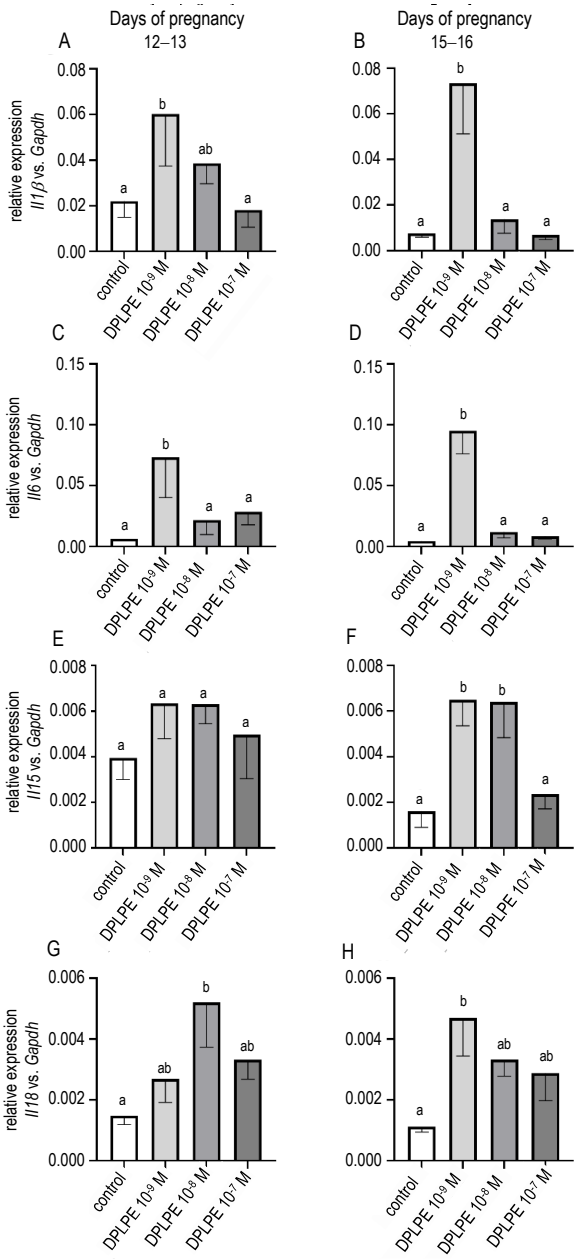

Figure 2. Relative abundance of interleukin 1 (*IL-1β*), interleukin 6 (*IL-6*), interleukin 15 (*IL-15*) and interleukin 18 (*IL-18*) mRNA transcripts in porcine endometrial slices. Endometrial tissues were collected during the maternal recognition of pregnancy (days 12–13) and peri-implantation (days 15–16) periods and incubated in vitro in the presence or absence of DPLPE (10^{-9}, 10^{-8}, 10^{-7} M) for 6 h. Data were analyzed using one-way ANOVA and are presented as means ± SEM. SEM – standard error of the mean. Different lowercase letters (a–b) indicate statistically significant differences between groups ($P \leq 0.05$). Panels A, B, etc. represent individual graphs within each figure and are provided to facilitate clear reference to specific results described in the text.

also stimulated *IL15* and *IL18* mRNA expression at the beginning of implantation period (days 15–16; Figure 2F and H). At a higher concentration (10^{-8} M) DPLPE increased *IL15* expression on days 15–16 and *IL18* expression on days 12–13 of pregnancy (Figure 2G and F). In contrast, the highest tested dose of DPLPE (10^{-7} M)

did not affect mRNA expression of any of the analysed cytokines (IL1β, IL6, IL15, IL18) in the endometrium during either period (Figure 2).

Effect of δ-opioid receptor agonist (DPLPE) on chemokine and chemokine receptor mRNA expression in the porcine endometrium

Treatment with the δ-opioid receptor agonist DPLPE at 10^{-9} M increased mRNA expression of the *CXCL10, CXCL11* and *XCL1* genes in the porcine endometrium during both pregnancy periods (Figure 3A–F). The agonist also affected the transcript abundance of chemokine receptors. Endometrial *CXCR4* mRNA expression was stimulated on days 15–16 of pregnancy following treatment with DPLPE at a dose 10^{-9} M (Figure 3J). At 10^{-7} M, DPLPE up-regulated mRNA expression of both *CXCR3* and *CXCR4* in the endometrium on days 12–13 of pregnancy (Figure 3G and I). In contrast, treatment with 10^{-8} M DPLPE did not affect the expression of the analysed chemokines (*CXCL10, CXCL11* and *XCL1*) and chemokine receptors (*CXCR3* and *CXCR4*) (Figure 3A–J).

Effect of δ-opioid receptor agonist (DPLPE) on mRNA expression of genes involved in endometrial activity

Treatment of endometrial explants collected from pigs on days 12–13 and 15–16 with DPLPE at 10^{-8} M decreased *ESR1* expression (Figure 4A and B). Conversely, a stimulatory effect of DPLPE was observed on the expression of *PAQR9* mRNA in the endometrium at 10^{-8} M and 10^{-7} M on days 12–13 and 15–16 of pregnancy (Figure 4C and D). Moreover, treatment with 10^{-9} M DPLPE increased *TLR4* mRNA epression in the endometrium on days 15–16 of pregnancy (Figure 4F).

MAP2K2 kinase expression was elevated in DPLPE-treated endometrial explants during maternal recognition of pregnancy at 10^{-7} M and during the onset of implantation at 10^{-9} M (Figure 4A and B). Similarly, *CLDND1* mRNA expression was elevated on days 15–16 of pregnancy following treatment with 10^{-9} M DPLPE (Figure 5D). *DNMT1* expression was reduced at 10^{-8} M on days 12–13, whereas it was increased at 10^{-9} M on days 15–16 of pregnancy (Figure 5E and F). No effects of DPLPE were observed on *CDK1*, *PCDC10* and *TGFα* mRNA expression in the endometrium during either period (Figure 6A–F).

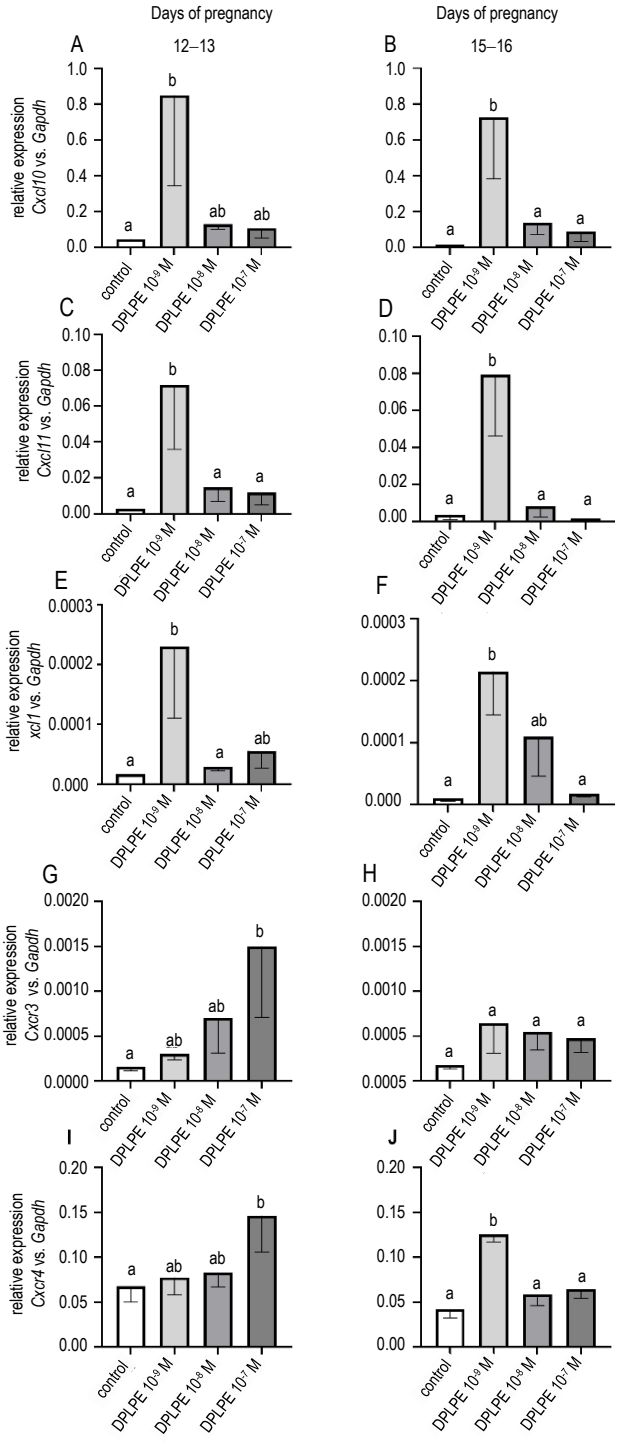

Figure 3. Relative abundance of C-X-C motif chemokine ligand 10 (*CXCL10*), C-X-C motif chemokine ligand 11 (*CXCL11*), X-C Motif Chemokine Ligand 1 (*XCL1*), C-X-C Motif Chemokine Receptor 3 (*CXCR3*) and C-X-C Motif Chemokine Receptor 4 (*CXCR4*) mRNA transcripts in porcine endometrial slices. Endometrial tissues were collected during the maternal recognition of pregnancy (days 12–13) and peri-implantation (days 15–16) periods and incubated in vitro in the presence or absence of DPLPE (10^{-9}, 10^{-8}, 10^{-7} M) for 6 h. Data were analyzed using one-way ANOVA and are presented as means ± SEM. SEM – standard error of the mean. Different lowercase letters (a–b) indicate statistically significant differences between groups ($P \leq 0.05$). Panels A, B, etc. represent individual graphs within each figure and are provided to facilitate clear reference to specific results described in the text.

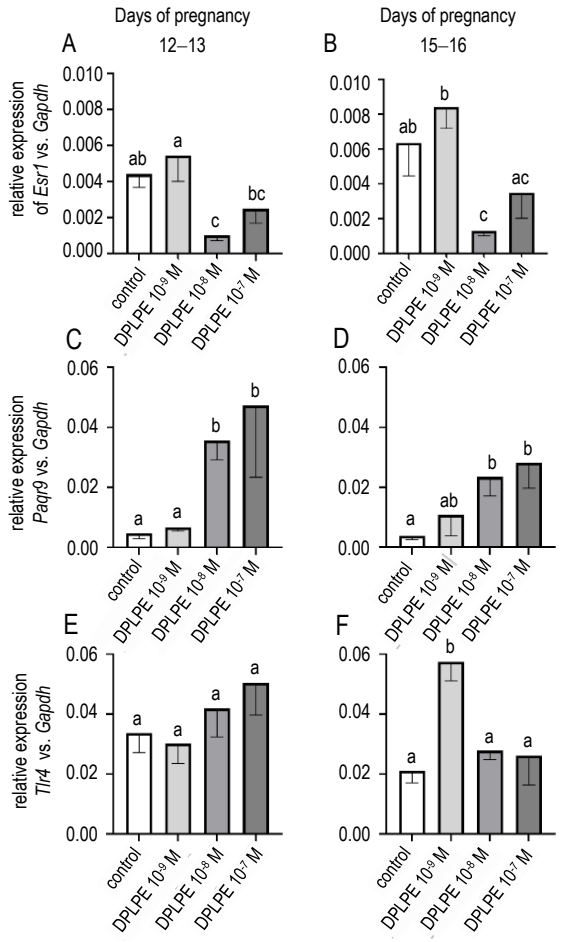

Figure 4. Relative abundance of estrogen receptor 1 (*ESR1*), progestin and adipoq receptor family member 9 (PAQR9) and toll like receptor 4 (*TLR4*) mRNA transcripts in porcine endometrial slices.

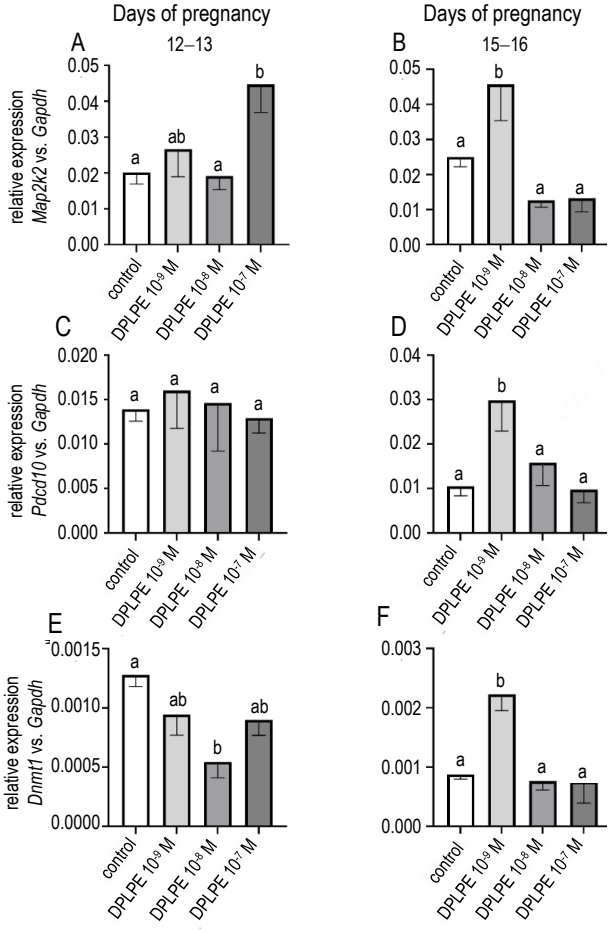

Figure 5. Relative abundance of mitogen-activated protein kinase kinase 2 (*MAP2K2*), claudin domain containing 1 (*CLDND1*) and DNA methyltransferase 1 (DNMT1) mRNA transcripts in porcine endometrial slices.

Concerning Figures 4, 5 and 6: Endometrial tissues were collected during the maternal recognition of pregnancy (days 12–13) and peri-implantation (days 15–16) periods and incubated *in vitro* in the presence or absence of DPLPE (10^{-9}, 10^{-8}, 10^{-7} M) for 6 h. Data were analyzed using one-way ANOVA and are presented as means ± SEM. SEM – standard error of the mean. Different lowercase letters (a–c) indicate statistically significant differences between groups ($P \leq 0.05$). Panels A, B, etc. represent individual graphs within each figure and are provided to facilitate clear reference to specific results described in the text.

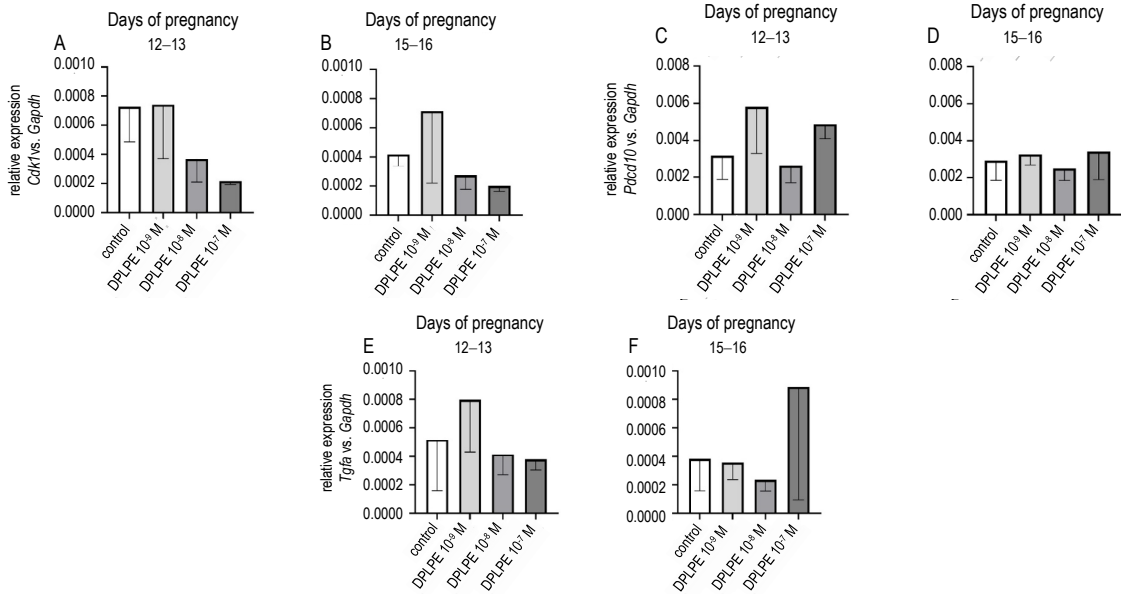

Figure 6. Relative abundance of Cyclin Dependent Kinase 1 (*CDK1*), Programmed Cell Death 10 (*PDCD10*) and Transforming Growth Factor Alpha (*TGFα*) mRNA transcripts in porcine endometrial slices.

Discussion

Successful implantation and maintenance of pregnancy depend on a balanced cytokine and chemokine secretion in the endometrium, while disturbances in the uterine microenvironment leading to embryonic loss represent a serious economic concern in pig production. In the present study, the δ-opioid receptor agonist (DPLPE) increased mRNA expression of selected cytokines, chemokines, and genes associated with endometrial function in a dose-dependent manner. The most pronounced stimulatory effects of DPLPE on cytokines (*IL-1B, IL-6, IL-15* and *IL-18*), chemokines (*CXCL10, CXCL11, XCL1*) and their receptors (*CXCR3, CXCR4*), as well as *TLR4* were observed at a concentration of 10^{-9} M during the peri-implantation period.

Expression of IL-1β mRNA in the porcine endometrium during maternal recognition of pregnancy and the implantation period has been previously confirmed (Ashworth et al., 2010; Ka et al., 2018). In the present study, the δ-opioid receptor agonist acted as a stimulatory regulator of IL-1β expression in the porcine endometrium during early pregnancy. Interleukin-1β is considered an initial stimulus for conceptus trophoblast elongation and attachment to the uterine epithelium in pigs (Ross et al., 2003; Mathew et al., 2016). It also modulates the synthesis and/or secretion of steroid hormones (Franczak et al., 2013) and prostaglandins (Franczak et al., 2010, 2012) by the endometrium during early pregnancy. An increased concentration of IL1β in the uterine lumen during trophoblast development and conceptus attachment coincides with a transient release of oestrogen between days 12 and 15 of pregnancy (Ross et al., 2003; Mathew et al., 2016), indicating its role in establishing an appropriate hormonal environment for embryo development.

The present study also showed that the δ-opioid receptor agonist (10^{-8} M) suppressed the transcription of oestrogen receptor (*ESR1*), which may alter the responsiveness of endometrial tissue to this steroid. In contrast, δ-opioid receptor activation increased mRNA expression of the G-protein-coupled progesterone receptor (PAQ9R), suggesting a potential effect on endometrial sensitivity to progesterone (P4). Both receptor types have been identified in porcine endometrial epithelial cells during early pregnancy (Ka et al., 2018). Reduced ESR1 expression following δ-opioid receptor stimulation may exert a protective effect against excessive oestrogen (E2) levels in the uterus, which can be cytotoxic for developing embryos. Earlier studies demonstrated that δ-opioid receptor stimulation affected endometrial steroidogenesis during the oestrous cycle in pigs, but not during early pregnancy (Dziekonski et al., 2018). Overall, these findings suggest that in early pregnant gilts, δ-opioid receptor activation is associated with endometrial responsiveness to steroid hormones rather than their synthesis.

In the current study, DPLPE increased *IL6* mRNA expression in the endometrium, similarly to *IL1β*. *IL6* is a pleiotropic cytokine with well-established pro-inflammatory activity (Scheller et al., 2011). It also supports maternal-embryonic interactions by stimulating trophoblast migration, invasion, and integrin expression. Its stage- and cell-specific regulation (including enkephalins) at the foeto-maternal interface indicates an important role of this cytokine in maintaining early pregnancy in pigs (Jovanović and Vićovac, 2009; Blitek et al., 2012; Yoo et al., 2017). One of the basic downstream signalling cascades mediating *IL6* action is the mitogen-activated protein kinases (MAPKs) pathway (Scheller et al., 2011).The present study has demonstrated increased expression of *MAP2K2* as a result of DPLPE (10^{-9} M) treatment. This suggests that δ-opioid receptor ligands may influence not only the endometrial capacity for IL6 synthesis, but also the activity of IL6-dependent intracellular transduction pathways. This interpretation is supported by previous findings showing that met-enkephalin increased *IL6* concentration in mouse serum *in vivo*, as well as *IL6* gene expression in murine splenocytes and *IL6* secretion by peritoneal macrophages (Zhong et al., 1998).

The present study also confirmed the involvement of δ-opioid receptor ligands in the regulation of the uterine immune system by influencing the capacity of the porcine endometrium to synthesise IL15 and IL18. IL15 is a pleiotropic cytokine that promotes activation and migration of natural killer (NK) cells (Gordon, 2021; Kanter et al., 2021). In the present study, *IL15* mRNA expression was increased by DPLPE (10^{-8} M and 10^{-9} M) in endometrial explants representing the onset of implantation. In pigs, early pregnancy is associated with increased activity of endometrial NK cells (Yu et al., 1993). Therefore, δ-opioid receptors and IL15 may be at least partially responsible for the recruitment of uterine NK (uNK) cells. The presence of uNK is critical for establishing the embryo-maternal interactions in humans and subsequent placentation, including stromal cell decidualisation, spiral artery remodelling and extravillous trophoblast invasion (Kanter et al., 2021). In pigs, IL15 and uNK cells are also involved in changes in

mucosal immunity across reproductive stages (Han et al., 2021). These findings indicate that enkephalins may influence uterine immune function, at least in part, by increasing IL-15 expression in the endometrium.

In the present study, DPLPE treatment also increased endometrial expression of *IL18* on days 12–13 of pregnancy (10^{-8} M), and on days 15–16 (10^{-9} M). Similarly, Ashworth et al. (2010) reported elevated uterine IL18 levels between days 12 and 18 of pregnancy in pigs. These authors implied that type I cytokines (IL1β and IL18) in the uterine lumen are necessary for conceptuses attachment to the uterine surface and maintenance of pregnancy in pigs (Ashworth et al., 2010). In addition, IL18 is also recognised as an interferon-inducing factor that enhances interferon synthesis and secretion by porcine conceptuses (Ashworth et al., 2010). Thus, δ-opioid receptor ligands may contribute to the regulation of early immunological interactions at the conceptus-uterine interface.

Additional evidence supporting the involvement of δ-opioid receptor activation in the uterine immune response during early pregnancy is the increased *TLR4* expression in response to DPLPE treatment. Toll-like receptors (TLRs) present in mucosal tissues are required for innate immune responses against various pathogens with TLR4 being one of the best-characterised members of this family (Turner et al., 2012). Expression of TLR4 mRNA and protein has previously been identified in the endometrium of pregnant pigs (Yoo et al., 2019) and mares (Atli and Kose, 2019). In humans, activation of TLR4 in uterine epithelial and stromal cells increases the secretion of inflammatory cytokines IL6, IL-8, and tumour necrosis factor alpha (TNFα) (Yoo et al., 2019). Moreover, some endogenous opioid peptides have been reported to act as TLR4 ligands (Meng et al., 2017; Zhang et al., 2020). Overall, these findings suggest that enkephalins indirectly participate in immunotolerance mechanisms at the maternal-conceptus interface in pigs by regulating *TLR4* mRNA expression. This study is the first to demonstrate the effects of a δ-opioid receptor ligand on the expression of selected chemokines and their receptors in the endometrium of pregnant pigs. Specifically, DPLPE at 10^{-9} M increased endometrial mRNA expression of *CXCL10* and *CXCL11*, as well as their receptor *CXCR3*, on days 12–13 and 15–16 of pregnancy. Previous studies have shown that CXCL10, CXCL11 and receptor CXCR3 are expressed in the endometrium of several species, including pigs (Han et al., 2017; McLendon et al., 2020). In the latter animals, *CXCR3*, *CXCL10* and *CXCL11* expression is elevated on day 15 of pregnancy compared with the oestrous cycle or earlier stages of gestation, with corresponding increases in protein levels in response to IFN-γ (Han et al., 2017; McLendon et al., 2020). These chemokines have been implicated in the recruitment of T and NK cells to uterine tissues, particularly during this stage of pregnancy (McLendon et al., 2020). In addition, DPLPE increased the expression of *XCL1* mRNA at 10^{-9} M and *CXCR4* mRNA at multiple doses in the porcine endometrium during the analysed stages of pregnancy. Earlier studies have demonstrated the presence of XCL1 in the human endometrium and rat uterus, as well as CXCR4 and its ligand CXCL12 in the human and porcine uterus (Hanna et al., 2003; Wu et al., 2005; Bellelis et al., 2013; Han et al., 2018; Menzies et al., 2019). The XCL1-dependent pathway has been reported to promote trophoblast invasion into the endometrium (Zhang et al., 2018). Similar effects were observed for CXCR4 stimulation, which also induced migration of trophectoderm and T cells at the maternal-conceptus interface during implantation in pigs (Han et al., 2018). Collectively, δ-receptor opioid ligands, through their influence on the expression of *CXCL10, CXCL11, XCL1* and chemokine receptors *CXCR3* and *CXCR4* may play a key role in intrauterine immune cell migration, a process essential for the establishment of maternal-conceptuses interactions during early pregnancy.

In the current study, the effects of δ-opioid receptor activation on intracellular processes related to apoptosis (*CDK*1, *PCDCD10* and *TGFα*), DNA methylation (*DNMT1*) and tight junctions formation (*CLDND1*) were evaluated. No effects of DPLPE on *CDK*1, *PCDC10* or *TGFα* transcript abundance were observed. However, DPLPE was found to differentially regulate *DNMT1* expression in the porcine endometrium depending on dose and stage of early pregnancy. DNMT1, which is essential for maintaining DNA methylation patterns and regulating gene expression, may play an important role during the establishment of pregnancy and development of uterine receptivity (Franczak et al., 2017; Zhang et al., 2019; Peral-Sanchez et al., 2021; Wydorski et al., 2024). In addition, DPLPE upregulated *CLDND1* expression in endometrial explants collected during the peri-implantation period. *CLDND1* encodes the claudin domain containing 1 protein, a transmembrane protein involved tight junction formation that regulates cellular permeability and epithelial cell transformation (Ohnishi et al., 2017). The present findings suggest that δ-opioid receptor activation may strengthen tight junctions

and contribute to restructuring of the uterine epithelium during early pregnancy. Importantly, recent studies demonstrated that claudin 1 (CLDN1) expression in the porcine endometrium was increased on day 12 of pregnancy (Jalali et al., 2019) and is upregulated by oestradiol *in vivo* (Kaczyński et al., 2020).

Conclusions

In summary, the present study is the first which allows to indicate pleiotropic effects of δ-opioid receptor activation on processes occurring in the porcine endometrium during maternal recognition of pregnancy (days 12–13) and the onset of implantation (days 15–16). DPLPE increased mRNA expression of selected cytokines (*IL1β*, *IL6*, *IL15*, *IL18*), chemokines (*CXCL10*, *CXCL11*, *XCL1*) and their receptors (*CXCR3* and *CXCR4*). This implies that endogenous opioid peptides acting through δ-opioid receptor may contribute to the establishment of a proper immunological environment and the maternal-foetal interface required for conceptus implantation and development. In addition, the observed effects of DPLPE on *CLDN1* and *DNMT1* expression indicate a potential role of δ-opioid receptor signalling in the regulation of tight junctions and epigenetic modifications in the porcine endometrium during early pregnancy. Further studies are required to clarify the effects of δopioid receptor activation in the porcine endometrium during early pregnancy and the oestrous cycle. These should include protein-level validation, intracellular signalling analyses, and comparative studies with non-pregnant gilts.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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