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Full length article

Delta and kappa opioid receptors in human endometrium during the menstrual cycle: Expression and localization

Estibaliz Olabarrieta [a,b], Lide Totorikaguena [a], Roberto Matorras [c], Ekaitz Agirregoitia [a], Naiara Agirregoitia [a,*]

[a] Department of Physiology, Faculty of Medicine and Nursing, University of the Basque Country (UPV/EHU), Bizkaia
[b] Centro de Investigación Biomédica en Red de Salud Mental CIBERSAM, ISCIII, Spain
[c] Human Reproduction Unit, Cruces University Hospital, Biocruces, IVIRMA, IVI Bilbao, Leioa, University of the Basque Country (UPV/EHU), Bizkaia

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ABSTRACT

Objective: Endogenous opioid peptides were reported to be involved in the regulation of reproductive physiology and their precursors and receptors were described in many of the male and female reproductive tissues. Mu opioid receptor (MOR) was described in human endometrial cells and its expression and localization changed during the menstrual cycle. However, there is no data from the distribution of the other opioid receptors: Delta (DOR) and Kappa (KOR). The objective of the present work was to analyze the dynamics of expression and localization of DOR and KOR in human endometrium throughout the menstrual cycle.

Study design: Human endometrial samples from different menstrual cycle phases were analyzed by immunohistochemistry.

Results: DOR and KOR were present in all samples analyzed and the protein expression and localization changed throughout the menstrual cycle. Both receptor expression increased during the late proliferative phase and decreased during the late secretory-one, especially in the luminal epithelium. DOR expression was generally higher than KOR expression in all cell compartments.

Conclusions: The presence of DOR and KOR in human endometrium and their dynamic changes during the menstrual cycle join the results previously obtained in MOR suggesting a possible role of opioids in reproduction events related to the human endometrium.

Introduction

Human endometrium exhibits a number of well-known cyclic changes under the influence of estrogens and progesterone. However, there are other factors that could play an important role in this modulation, as the components of the opioid system [1]. The endogenous opioid system is composed of opioid receptors, their endogenous ligands (EOPs; endogenous opioid peptides) and enzymes involved in their synthesis and degradation. The δ-opioid receptor (DOR) and κ-opioid receptor (KOR), along with the μ-opioid receptor (MOR), are G-protein-coupled receptors that bind EOPs to exert their effects. EOPs are derived from proopiomelanocortin (POMC), proenkephalin (PENK) and prodynorphin (PDYN) precursors and are known to participate in the regulation of reproductive physiology [2,3].

The EOPs precursors mRNAs have been described in endometrium of various species [4–10]. In human, POMC and PDYN genes were detected in endometrial cells [1]. mRNA expression of PENK was reported to be higher in cells next to the implantation site in rodents [4,9]. Opioid peptides β-endorphin and met-enkephalin, are present in the uterine fluid of human and cow and their expression has an upward trend from the follicular phase to the luteal phase [11,12]. Dynorphins were described in human and Ishikawa human endometrial cells [13], where β-endorphin was also detected [14]. EOPs could be involved in the regulation of numerous endometrial processes such as cell proliferation and apoptosis, immune interactions, inhibition of uterine contraction, events related to early pregnancy and modulation of secretory activity [15]. Studies conducted in the last decade suggest that exogenous modulation of the opioid system could alter the implantation process, since the administration of morphine to mice, during the first days of gestation, caused a decrease in the occurrence of implantation sites with respect to the control group [16].

The receptors are present in the uterus of mammals [6]: KOR was

* Corresponding author.
E-mail address: naiara.aguirregoitia@ehu.es (N. Agirregoitia).

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observed in the Ishikawa human endometrial cells [17] and MOR in the nondecidualized endometrial stroma cells and myometrium from pregnant rats [18], KOR mRNA is expressed at the site of implantation and disappeared during pregnancy in mice [18] and, finally, in the only study performed in human endometrial cells, MOR was detected in all cell compartments and its expression level changed during the menstrual cycle [19].

Although there is not much literature regarding the opioid system in the uterus, the evidences until now suggest that the opioid system could be involved in some endometrial functions. Even so, as there are not detailed studies on DOR and KOR in the human endometrium, it is interesting to continue with the description of the expression patterns of the opioid system in this tissue. Therefore, continuing with the previous work performed by our research group regarding the MOR expression [19], the aim of the present study was to analyze the dynamics of DOR and KOR expression in the human endometrium throughout the menstrual cycle.

Materials and methods

Ethics Declaration

All patients received written informed consent for this study at the time of tissue collection. This study obtained ethical approval from the Clinical Research Ethics Committee of the Basque Health System at Cruces University Hospital (CEIC EI4/36, 02/2015).

Human endometrial samples

This study involved 86 women (aged 22–39) with regular menstrual cycles (27–32 days) from the Assisted Reproduction Unit of the Cruces University Hospital. The criteria were as follows: 1) none of them were under hormonal treatment in the previous 3 months, 2) normal uterine vaginal ultrasound, 3) absence of endometriosis, polycystic ovary syndrome (PCOS), implantation failure or recurrent miscarriage and 4) no history of opioid drug use. Endometrial samples for this study were obtained by endometrial biopsy, using a Cornier pipelle (Laboratoires CCD). Prophylactic antibiotics were not used. There was no case of infection or other side effects. Endometrial dating was determined histologically by an experienced pathologist (L.A.) according to the criteria of Noyes *et al.* 1950 [20]. Only specimens with agreement between histological and chronological dating were included in the study.

These samples were categorized in groups for the different menstrual cycle phases as previously described [19]: phase I, menstrual (days 1–5, n = 4); phase II, early to midproliferative (days 6–10, n = 7); phase III, late proliferative to early secretory (day 11–19, n = 9); phase IV, midsecretory (days 20–24, n = 7); and phase V, late secretory (days 25-28±, n = 8). Collected tissues were fixed in buffered formalin (pH 7.4) and processed to paraffin wax blocks for immunohistochemistry (IHC).

Inmunohistochemical analysis (IHC)

Samples (phase I, n = 4; phase II, n = 7; phase III, n = 9; phase IV, n = 7; and phase V, n = 8) were fixed in 4 % neutral buffered formalin (pH 7,4) for 24–48 h and stored in ethanol 70 % until they were embedded in paraffin wax, and 4 μm sections were obtained with a Shandon AS 325 retraction microtome. For preliminary assessment of morphology, sections were stained with hematoxylin and eosin. On the basis on this assessment, samples were dated and grouped in one of the above described five phases.

Following deparafination and rehydration, sections were microwave heated in citrate buffer (10 mM, pH 6) during 10 min for the antigen retrieval. Afterwards sections were incubated with 0.3 % H_2O_2 in methanol (30 min) to block the endogenous peroxidase activity and with normal goat serum in PBS-0,1 % Triton X 100 (10 min) to block nonspecific binding. Sections were then incubated in anti-DOR and anti-KOR (Abcam; 1:1000, and 1:800 respectively, overnight at 4 °C) followed by the biotinylated anti-rabbit (Vector Laboratories, 1:300, 1 h) used as the secondary antibody in conjunction with the Vectastain H Elite ABC kit (Vector Laboratories). Immunostaining was visualized with Vector Nova Red (Vector Laboratories; ±1 min). Slices were dehydrated and mounted with Vectamount (Vector Laboratories). Negative controls were performed by omitting the primary antibody before addition of the secondary antibody. All images were examined and captured using Olympus BX50 optical microscopy (Olympus Optical Co.) connected to a digital color camera (Olympus XC50).

Evaluation of protein staining intensity and distribution was assessed using the semiquantitative histological digital-HSCORE, modified from Fuhrich *et al.* 2013 [21]. Briefly, the whole sample was analyzed under 100x magnification. The staining intensity was classified by 3 independent observers into four arbitrary categories: no labelling (0), weak (1), moderate (2) and strong (3). The D-HSCORE was performed using the image J software, but values of the arbitrary categories mentioned above in the range of color intensity (0–255) were established (0: 255–235, 1: 234–174, 2:113–173, 3: 112–0). Then, the following formula was used: HSCORE = $\sum$ Pi × (i + 1), where i is the intensity of staining and Pi the percentage of stained cells with this intensity. Low intraobserver ($r = 0.983$; $P < 0.0001$) and interobserver ($r = 0.994$; $P < 0.0001$) differences for HSCORE in uterine tissues had been previously reported using this technique [22].

Statistical analysis

To study the changes of the DOR and KOR receptors throughout the menstrual cycle, Graph Pad Prism 5 program was used. All the results were pooled by above-mentioned menstrual cycle phases (phase I, phase II, phase III, phase IV and phase V). Immunohistochemical expression of DOR and KOR was analyzed with two-way ANOVA, considering the different compartments (luminal epithelium, glands and stroma) and different phases of the menstrual cycle. Bonferroni was used as a post hoc test. Significance was set at $p < 0.05$.

Results

DOR histomorphometric analyses in human endometrium during the menstrual cycle

The presence of DOR in human endometrium was evaluated by histomorphometric analyses. We analyzed the DOR immunostaining pattern in all phases of endometrial cycle described previously. DOR protein was present in whole endometrium and each cellular compartment shared the same trend in DOR expression along the different menstrual phases: the expression was higher in phase III and IV, corresponding to late proliferative and midscretory phases (Fig. 1) and, following this increase, the presence of DOR decreased in phase V keeping in that way until the beginning of the cycle. However, analyzing the luminal epithelium, it was observed an expression peak in the menstrual phase (phase I) significantly different from the other phases ($p < 0.05$; Fig. 1D).

KOR histomorphometric analyses in human endometrium during the menstrual cycle

The analysis of KOR immunostaining pattern showed that KOR protein was present in all phases of endometrial cycle although the distribution of this protein in each cellular compartment was different (Fig. 2). KOR protein expression did not change along the cycle in stroma (Fig. 2C) but, in glands, its expression was significantly higher in phases III and IV ($p < 0.05$; late proliferative and midsecretory phases, Fig. 2B). The greatest expression changes were observed in the luminal epithelium, since KOR expression increased from the beginning of the cycle until reaching a maximum in phase IV ($p < 0.05$; midsecretory

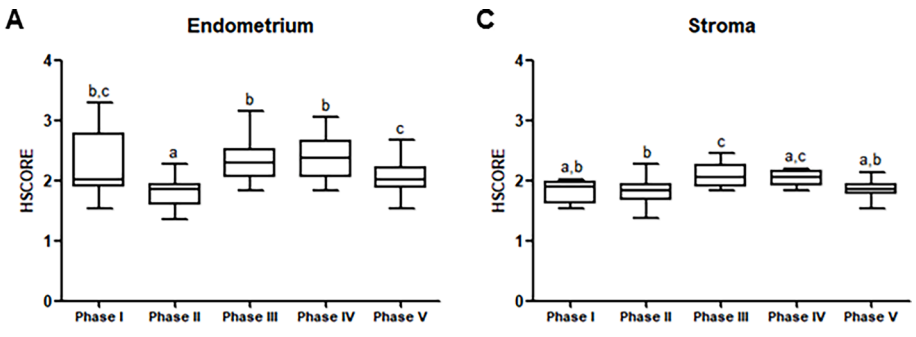

Fig. 1. HSCORE values representing the immunoreactivity intensity of DOR protein staining in the whole endometrium (A), the stromal compartment (B), the epithelial glands (C), and the luminal surface of the endometrium (D). Box and whisker plots representing median, interquartile range, and minima/maxima for phase I: menstrual phase (days 1–5; n = 4); phase II: early and midproliferative phase (days 6–11; n = 7); phase III: late proliferative and early secretory phase (days 12–17; n = 9); phase IV: midsecretory phase (days 18–23; n = 7); and phase V: late secretory phase (days 24–28; n = 8). The different combinations of letters indicate significant differences between phases; $p < 0.05$ in all cases.

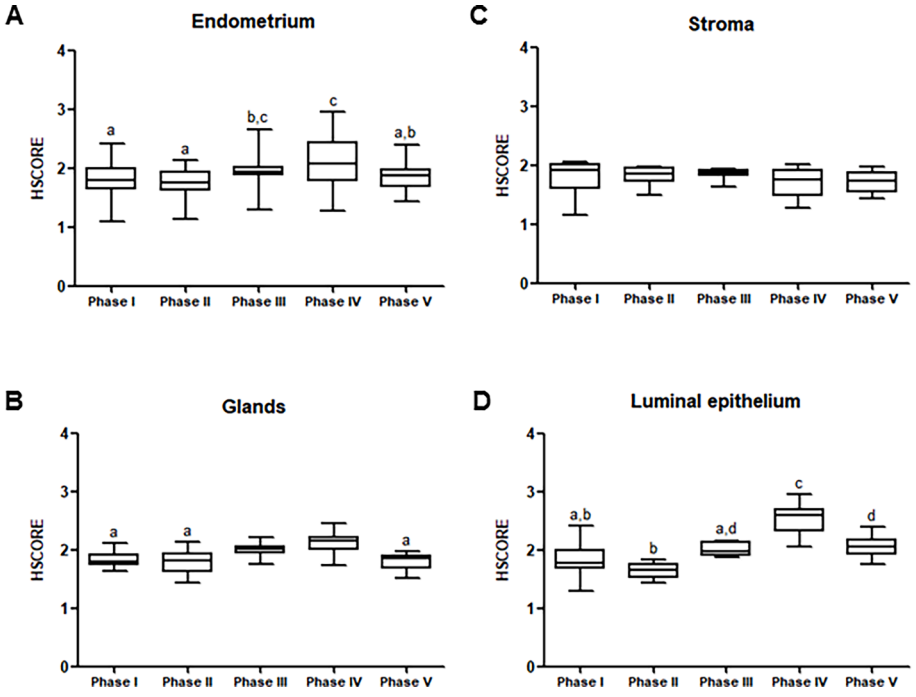

Fig. 2. HSCORE values representing the immunoreactivity intensity of KOR protein staining in the whole endometrium (A), the stromal compartment (B), the epithelial glands (C), and the luminal surface of the endometrium (D). Box and whisker plots representing median, interquartile range, and minima/maxima for phase I: menstrual phase (days 1–5; n = 4); phase II: early and midproliferative phase (days 6–11; n = 7); phase III: late proliferative and early secretory phase (days 12–17; n = 9); phase IV: midsecretory phase (days 18–23; n = 7); and phase V: late secretory phase (days 24–28; n = 8). The different combinations of letters indicate significant differences between phases; $p < 0.05$ in all cases.

phase) and then the labeling began to fade again (Fig. 2D).

DOR localization in human endometrium during the menstrual cycle

Fig. 3 shows the DOR distribution in the different phases of menstrual cycle of the human endometrium. Negative control, omitting primary antibody, is shown in Fig. 3F.

Phase I. DOR expression was weak in gland epithelium (arrowhead) and stromal cells (asterisk). However, it was more intense in the luminal epithelium (arrow) which corresponded to the staining peak mentioned above. In these cells, staining appeared to be stronger in the apical region, although the visual results are inconclusive (Fig. 3A).

Phase II. DOR expression in the early to midproliferative endometrium was weak in all compartments (Fig. 3B): in the luminal surface of the endometrium (arrows), gland epithelium (arrowhead) and stroma (asterisks).

Phase III. DOR expression was highest in this phase, coinciding with the ovulation time (Fig. 3C). The staining became more observable in the stroma (asterisk), gland epithelium (arrowhead) and luminal epithelium (arrow).

Phase IV. In this phase DOR maintained the expression level observed in the previous one (Fig. 3D) although the gland epithelial cells

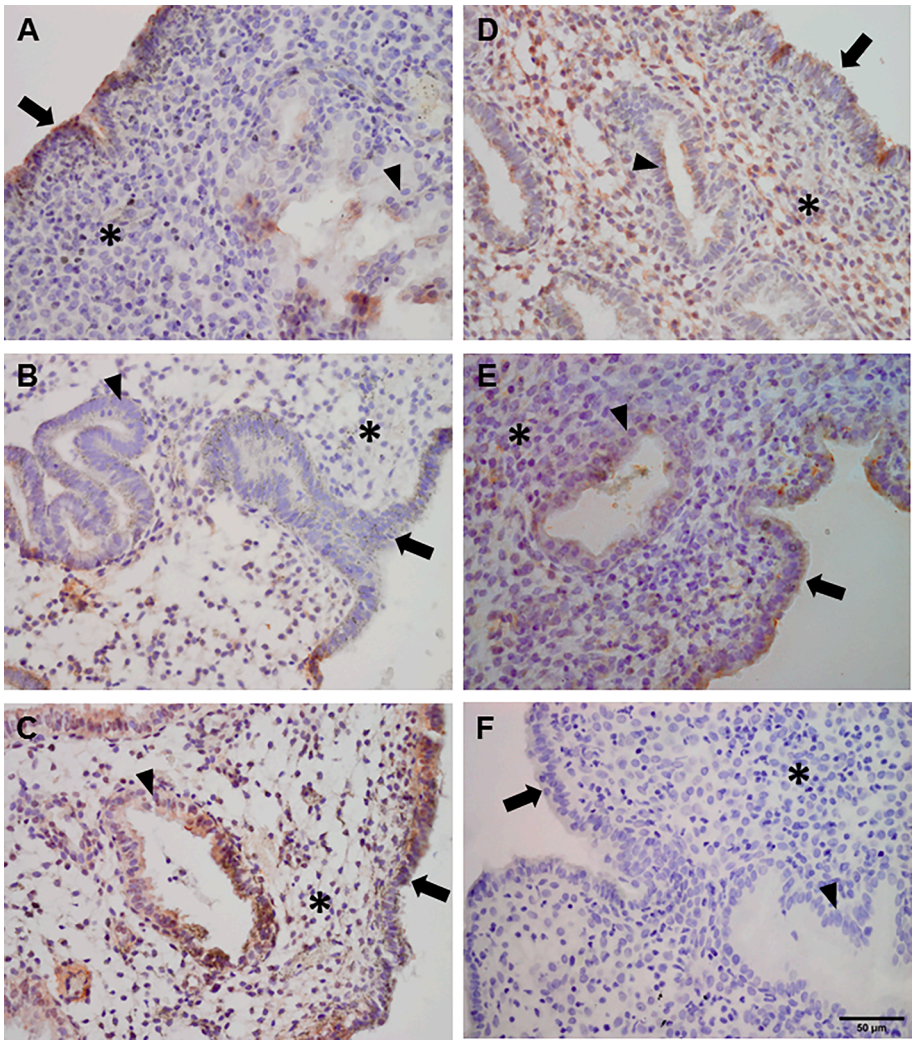

Fig. 3. DOR localization in human endometrium during the menstrual cycle. Representative pictures of immunostaining with DOR antibody at menstrual phase, phase I (day 1, A); early to midproliferative phase, phase II (day 8, B); late proliferative to early secretory phase, phase III (day 14, C); midsecretory phase, phase IV (day 18, D); and late secretory phase, phase V (day 30, E). DOR immunoreactivity varies during the cycle in all endometrium compartments: epithelial gland cells (arrows), luminal epithelium (arrowheads) and stroma (asterisk). Negative control by omitting the primary antibody before addition of the secondary antibody (Day 1, F). Scale bar, 50 µm.

labelling was stronger in the apical region, which is in contact with the glandular lumen (arrowhead).

Phase V. DOR immunoreactivity was less intense in the late secretory phase. It was still possible to observe DOR staining in all compartments (Fig. 3E), especially in glands and luminal epithelium (arrow and arrowhead).

KOR localization in human endometrium during the menstrual cycle

Fig. 4 shows KOR distribution in the different menstrual cycle phases of the human endometrium. Negative control, omitting primary antibody, is shown in Fig. 4F.

Phase I. KOR expression was weak in all cell compartments in the menstrual phase (Fig. 4A).

Phase II. The expression from the early to midproliferative endometrium was weak in all compartments (Fig. 4B): in the luminal surface of the endometrium (arrows), gland epithelium (arrowhead) and stroma (asterisks).

Phase III. Although KOR maintained a similar labeling pattern (Fig. 4C), in this phase it is observed a slight increase of KOR expression in glands and luminal epithelium (arrow and arrowhead).

Phase IV. KOR reached its maximum level of expression in this stage (Fig. 4D), being more prominent in the luminal epithelium (arrow), although this expression increase was also observed in stromal cells (asterisk).

Phase V. KOR inmunoreactivity was less intense in the late secretory phase, comparing with the previous stage. The expression became almost undetectable, except in some areas of the luminal epithelium, where the signal remained (Fig. 4E).

Discussion

We analyzed and evaluated the presence of delta and kappa opioid receptors in each region of the human endometrium along the menstrual cycle using histological techniques. Our data showed the presence of DOR and KOR protein in human endometrium, coinciding with a previous study performed in Ishikawa human endometrial cells [17]. The novelty of the present study is that we have used human endometrium samples coming directly from human patients instead of cell cultures. This gives more reliability to the results and allows analysis of the distribution and changes of the receptors that occurs in the different cell compartments of this tissue during the phases of menstrual cycle.

DOR and KOR receptors were differentially expressed throughout the menstrual cycle in the human endometrium. Although both receptors maintained their presence in all cycle stages, our results showed an expression variation during the menstrual cycle. DOR increased its expression from the beginning of the cycle, it reached a peak in the late proliferative and midsecretory phases and, finally, the expression decreased back, similar to what was previously observed in the distribution of MOR during the menstrual cycle [19]. Analyzing the different cell compartments, we observed a peak of both DOR and KOR expression in the menstrual phase in luminal epithelium. Menstrual phase is

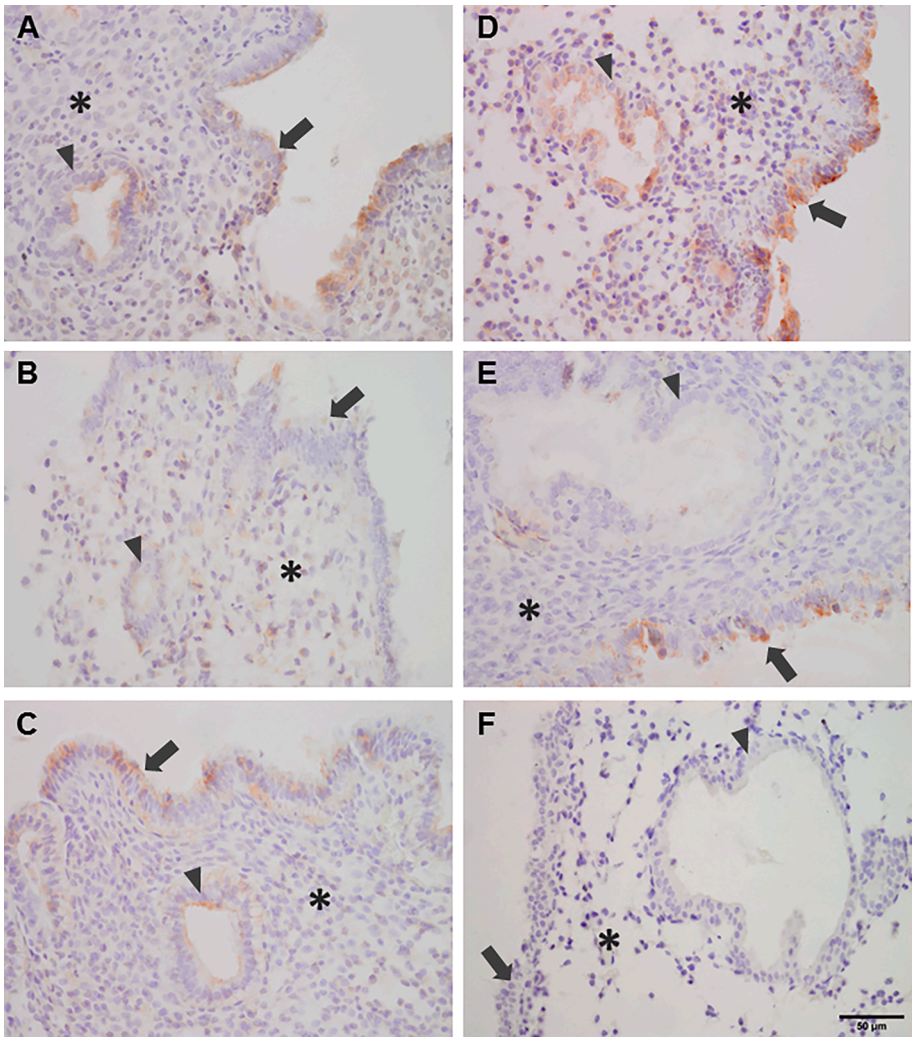

Fig. 4. KOR localization in human endometrium during the menstrual cycle. Representative pictures of immunostaining with KOR antibody at menstrual phase, phase I (day 1, A); early to midproliferative phase, phase II (day 10, B); late proliferative to early secretory phase, phase III (day 15, C); midsecretory phase, phase IV (day 23, D); and late secretory phase, phase V (day 26, E). KOR immunoreactivity varies during the cycle in all endometrium compartments: epithelial gland cells (arrows), luminal epithelium (arrowheads) and stroma (asterisk). Negative control by omitting the primary antibody before addition of the secondary antibody (Day 23, F). Scale bar, 50 µm.

characterized by an inflammatory response, and, as DOR has been linked to an anti-inflammatory effect in bovine endometrium [23], the expression peak observed in our results during this phase could be a tool to fight inflammation. Although in stroma KOR maintained stable during all menstrual phases, in the other cell compartments its expression increased during the cycle until reaching a peak in the midsecretory phase. These results provide evidence of a possible involvement of both opioid receptors in the endometrial cycle progression. It is also remarkable that DOR and KOR increased their expression in late proliferative and midsecretory phases, which correspond to ovulation and decidualization process respectively [24]. There has been found that some opioid augmented its concentration during the ovulation time in the uterus [25,26], which could be the reason why we detected DOR and KOR expression increase during this phase. On the other hand, decidualization, which begins in the midsecretory phase, is the process by which endometrial cells prepare to receive the embryo by differentiating stromal cells into decidua cells [27]. cAMP accumulation in stromal cells leads to growth factors, cytokines and other molecules synthesis necessary to decidualization and implantation [28]. As we know, EOPs act inhibiting adenylate cyclase and thus reducing cAMP [29], so DOR and KOR expression could start declining and being almost nonexistent in stromal cells after midsecretory phase because cAMP accumulation is crucial to prevent any alteration in stroma transformation. This hypothesis is supported by studies on MOR, in which implantation was altered following morphine administration [16]. Furthermore, in the endocannabinoid system, which its cell signaling is similar to that of the opioid system, its receptors presence decreased during this time of the cycle [30].

Finally, the presence of DOR and KOR was more evident in epithelial cells (from glands and luminal epithelium). There is increasing evidence that EOPs are able to modulate the secretion of steroid hormones [15], which could explain the presence of opioid receptors in endometrial glands. On the other hand, during the proliferative phase, a process of ciliogenesis occurs in the luminal epithelium enhanced by estrogen secretion [31]. MOR is expressed in the isthmus of mare oviduct and its presence is related with ciliogenesis and the contraction of musculature to facilitate gamete transport [32]. In our previous work on MOR, we also observed its expression in endometrium luminal epithelium during the post ovulation time, suggesting again a function in gamete transport [19]. In that sense, it may be possible that DOR and KOR were related to these functions as well.

It is also important to take these results into account because after the administration of opiates, it has been proved the presence of drug residues in the follicular fluid and in the uterine cavity of humans, both areas involved in reproduction [33,34]. Although MOR is the most studied receptor because of its implication in the clinic, DOR and KOR are gaining relevance due to new therapies developed to avoid the side effects of currently used MOR agonist drugs [35–38].

Conclusions

In conclusion, our data lead us to hypothesize that DOR and KOR,

modulating their presence and absence in endometrial cells, could take part in the regulation of the endometrial cycle, which is essential for a successful embryo implantation. It should be noted that these results are from women participating in an assisted reproduction program and therefore may not always reflect the general population. Therefore, we consider that it would be interesting to transfer this study to an animal model to assess whether opioid receptor modulation affects the estrous cycle and could thus be related to cases of infertility or the onset of some reproductive pathologies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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