

## Influence of cycle stage, age and endometrial biopsy score on oxytocin receptor distribution and gene expression in the cervix and uterus of non-pregnant mares

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### Abstract

Persistent breeding-induced endometritis (PBIE) or delayed uterine clearance (DUC) are major causes of mare subfertility. Oxytocin and its receptor are thought to play significant roles in the pathogenesis of DUC but the specific roles of oxytocin receptor (OR) distribution and gene expression remain undefined.

In this study both OR distribution and gene expression in the endometrium, myometrium and cervix during both luteal and non-luteal phases in non-pregnant mares (n=27) of differing age (young: 2 – 9 years, n=17; old:  $\geq$  10 years, n=10) and endometrial biopsy score were described using immunohistochemistry (IHC) and quantitative reverse-transcription polymerase chain reaction (RT-qPCR), respectively.

Immunohistochemistry showed a similar pattern of OR distribution in uterus and cervix, with the exception of the glandular epithelium, absent in the cervix. Uterine

ORs were localized in endometrial luminal and glandular epithelia, transmural vascular endothelium, sub-epithelial and peri-glandular stromal cells and myometrial smooth muscle cells. The OR labeling intensity was consistently greatest in the vascular endothelium. Real-time qPCR showed a higher OR gene expression in myometrium compared to cervix (P=0.001) and endometrium (P=0.009). There was no difference in OR gene expression between cervix and endometrium (P=1.0). Oxytocin receptor gene expression was significantly higher during the non-luteal phase in both combined uterine tissues (endometrium and myometrium) and myometrium. Oxytocin receptor distribution and gene expression were not influenced by a mare's age or endometrial biopsy score. As endometrial biopsy score and mare age were not predictors of OR gene expression, deficient OR gene expression is unlikely to be associated with DUC.

### Keywords

Oxytocin receptor; equine uterus; IHC; RT-qPCR; persistent breeding induced endometritis (PBIE); delayed uterine clearance (DUC)

## 1. Introduction

Oxytocin is a neuropeptide hormone that is important in parturition, milk ejection and the endocrine regulation of estrus. It is also responsible for myometrial contractility, thereby facilitating fetal expulsion during parturition and post-breeding mechanical clearance of uterine contents in mares [1-5]. Oxytocin acts by binding to oxytocin receptors (ORs) present in various target tissues both within and outside the genital tract [1, 3]. In mares, radioligand-binding assays have been used to detect ORs in endometrium [6-8] and myometrium [8]. The endometrial OR density reportedly fluctuates during the estrous cycle, peaking during late diestrus [6-8]. After binding to myometrial ORs, oxytocin causes contraction of myometrial cells through an influx of calcium via calcium channels [2, 9], whereas binding to endometrial ORs is thought to induce uterine contractions indirectly through endometrial prostaglandin release [2, 10]. Administration of oxytocin to mares in estrus stimulates prostaglandin F<sub>2-alpha</sub> (PGF<sub>2α</sub>) release and uterine contractions [11, 12].

Persistent breeding-induced endometritis (PBIE) or delayed uterine clearance (DUC) are well-recognized reproductive health concerns [13] associated with subfertility in mares [2, 14-17]. It is characterized by persistence of intraluminal fluid due to impaired physical clearance of the uterus in association with reduced uterine contractility [14, 15, 18, 19]. Mares susceptible to PBIE show reduced electrical myometrial activity in response to intrauterine bacterial challenges [20] and reduced uterine motility [21].

Several reports have suggested a role for

oxytocin and its receptors in the pathogenesis of subfertility and DUC in mares [9, 15, 19-23]. While Nikolakopoulos *et al.* [23] assumed a defective PGF<sub>2α</sub> release at the OR or post-receptor level, Rigby *et al.* [9] reported a decreased level of oxytocin-stimulated myometrial PGF<sub>2α</sub> production and a possible intrinsic contractile defect of the myometrium. Additionally, defective myoelectrical signaling and a decrease in the contractile strength of the uterine muscle was also deemed to be associated with the pathogenesis [24].

Defects at the OR level or alternatively deficient or absent receptors are hypothesized to contribute to DUC and subfertility through susceptibility to PBIE. However, neither OR distribution nor gene expression have been linked to this pathogenesis. Reports describing OR distribution and expression in the equine cervix are lacking. Furthermore, the influence of mare age and endometrial biopsy score on endo- and myometrial OR distribution and expression are unknown. Mare age is negatively correlated to: reproductive performance [14, 16, 21, 25-28], biopsy score [14, 21, 29, 30] and fertility [31]. It has also been suggested that endometrial pathologies and age are both correlated to PBIE [28, 30, 32].

This study aimed to describe variations in OR distribution and gene expression in the endometrium, myometrium and cervix during different stages of estrus in the reproductive tracts of non-pregnant mares of different ages and endometrial biopsy scores.

## 2. Materials and methods

### 2.1 Study population and design

Twenty-seven light horse mares of various breeds consigned for routine slaughter at an abattoir were included in the study. Mares were categorized (via post-mortem oral inspection of their dentition) by their estimated ages as either young (2 – 9 years, n=17) or old ( $\geq$  10 years, n=10) [33]. Mares were further categorized as either

luteal or non-luteal phase based on post-mortem macroscopic inspection of ovaries and measurement of plasma progesterone concentration (PPC). All samples were collected during the physiological breeding season in the southern hemisphere (October to April) when reproductively-

healthy mares would be expected to be cycling.

The study was approved by the Animal Ethics Committee of the Faculty of

Veterinary Science, University of Pretoria, South Africa (Study V005-12).

## 2.2 Sample Collection

### 2.2.1 Reproductive tract

The entire internal reproductive tract from each mare including the uterus, cervix, ovaries, oviducts and associated broad ligament was retrieved by en bloc dissection within an hour post-slaughter. Each tract was identified by affixing a plastic tag marked with individual mare's identity details (computer generated barcodes) and placed on ice for transport to the laboratory at the Faculty of Veterinary Science, University of Pretoria, Onderstepoort (FVS-UP). Macroscopically abnormal uteri (intra-uterine fluid, adhesion or neoplasia) were excluded from the study.

Ovaries were measured in three dimensions (length/L, height/H and width/W) using a ruler and dissected

longitudinally using a scalpel to define ovarian size and structures. Ovaries were defined as active ovaries if they were at least: L =5cm, H =3cm, W =2cm in size and exhibiting follicles of  $\geq 2$  cm diameter and, or with corpora lutea (CLs) or corpora hemorrhagica (CHs). Macroscopic ovarian features were used in combination with PPC to categorize cyclic status as either luteal (PPC  $\geq 3.18$  nmol/l) or non-luteal phase (PPC  $\leq 3.18$  nmol/l) [34-37].

Three full-thickness tissue samples (20 x 20 x 5 mm) were obtained using a scalpel from each of three standardized areas (uterine body, base of the left uterine horn, cervix). The samples were further processed for endometrial biopsy histological evaluation, RT-qPCR and IHC.

### 2.2.2 Blood samples

A blood sample was obtained from each mare at slaughter (during exsanguination) by collection of whole blood into 5 ml heparinized vacuum tubes (BD Vacutainer®; LH Lithium Heparin 68 I.U.

blood collection tubes; Plymouth; UK). Labeled blood samples were placed on ice and transported to the FVS-UP for further processing.

## 2.3 Laboratory analyses

### 2.3.1 Plasma progesterone concentration (PPC)

Blood samples were immediately centrifuged at 1610 X g for 15 min at 25 °C and serum was stored at -18 °C in barcode-labeled 5 ml sterile plastic sample tubes until analysis. Samples were analyzed at the Endocrine Laboratory, FVS-UP by radioimmunoassay (RIA) measurement of PPC (PPC; Coat-a-count RIA kit, Code No. TKPG1 - 1593 Diagnostic Product Corporation, Los Angeles, USA) according to manufacturer's instructions. All samples were analyzed in a single batch and were read against a standard curve created by

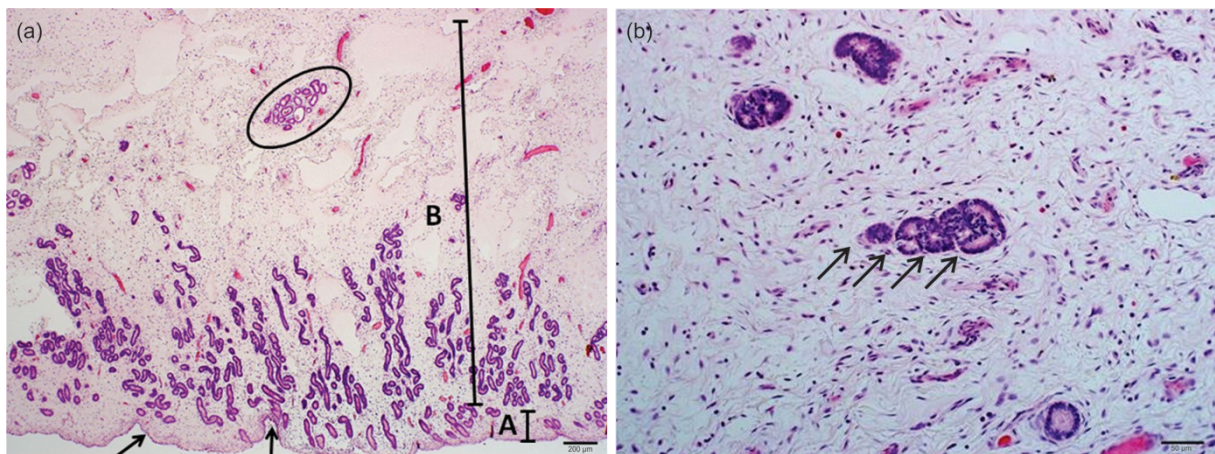
progesterone calibrators (calibrator samples A-G) included in the kit with calibrator sample C used as a standard control for assay validation purposes. The intra- and inter-assay coefficients of variation were 6.1% (2.1 nmol/l), 7.1% (2.7 nmol/l) and 5.3% (54.4nmol/l), 5.6% (57.6 nmol/l) for low, and high concentrations, respectively.

Mares with PPC  $\geq 3.18$  nmol/l were defined as having active luteal tissue [36, 37].

### 2.3.2 Endometrial biopsies

Full-thickness samples from the base of the left uterine horn were obtained for endometrial biopsy scoring. Samples were fixed in 10 % neutral buffered formalin for 24-48 h and sectioned at a thickness of 4  $\mu$ m. Sections were routinely de-waxed before staining with hematoxylin and eosin (HE) according to standard techniques [38] before examination by a single experienced operator (SJC) using an Olympus BX43 light microscope. Each biopsy was assigned a histomorphological grade (I, IIa, IIb, or III) according to histopathologic criteria (inflammation, endometrial glandular nesting and peri-glandular

fibrosis) as outlined by Kenney and Doig [39]. Score allocation did not include historical breeding data due to the nature of the study population. In particular, the extent and type of endometrial inflammation was assessed as well as the extent or severity of endometrial degeneration. The evaluation of degeneration included an assessment of glandular nesting, peri-glandular fibrosis, glandular ectasia and lymphatic lacunae (Fig. 1).



**Fig. 1.** Equine endometrial biopsy (a) assigned a Kenney-Doig grade of IIB. Hematoxylin and eosin (HE). Bar = 200  $\mu$ m. An overview of the luminal epithelium (arrows), stratum compactum (A) and stratum spongiosum with endometrial glands (B) including an endometrial glandular nest (solid circle). (b) Shows onion skin-like layering of 3-5 layers of peri-glandular connective tissue (arrows) with consequent formation of glandular "nests" within the deep stratum spongiosum. HE. Bar = 50  $\mu$ m.

### 2.3.3 Immunohistochemistry (IHC)

#### 2.3.3.1 Test samples

Three 5 mm-wide full-thickness samples per mare (from the uterine body, base of the left uterine horn and cervix) were analyzed. Tissue samples were fixed in 10

% neutral buffered formalin for 24-48 h, embedded in paraffin and sectioned at a thickness of 4  $\mu$ m.

#### 2.3.3.2 Immunoperoxidase labeling

Staining, performed by hand, followed reported protocols [40]. The standard immunoperoxidase procedure included deparaffinization and hydration of slides, incubation of sections with 3 % hydrogen peroxide in methanol for 15 min to quench endogenous peroxidase activity followed

by enzymatic epitope retrieval (Protease Type XIV, Code No. P5147-5G, Sigma-Aldrich, St. Louis, Missouri, USA). Non-specific protein binding was minimized by incubating slides with normal goat serum diluted 1:10 for 20min (Code No. G9023, Sigma-Aldrich, St. Louis, Missouri, USA).

Thereafter, the affinity purified primary polyclonal rabbit OR antibody directed against the N-terminal extracellular domain of the human OR (Code No. O4389, Sigma-Aldrich, Steinheim, Germany) was applied to the tissue sections (1:400 dilution overnight at 4°C). A polyclonal biotinylated rabbit-anti-goat IgG secondary antibody (Code No. O4389, Sigma-Aldrich, Steinheim, Germany) was applied to the tissue sections (1:500 dilution for 30 min) before application of the avidin-biotin-peroxidase complex (ABC) detection system, according to manufacturer's instructions (Vectastain Elite ABC standard kit, Code No. PK-6100, Vector Laboratories, Burlingame, CA, USA). The brownish color visualized in tissue sections was developed via incubation of sections with the liquid DAB+ Substrate Chromogen System (Code No. K3468, Dako, Denmark) for approximately 3 min, before the

sections were routinely counterstained with Mayer's hematoxylin, rinsed, dehydrated, mounted and coverslipped. The positive-tissue control was a uterine section obtained from a mare in mid to late diestrus with an anticipated increased expression of ORs according to several reports [6-8]. Positive labeling was brown in color and occurred in a finely cytoplasmic granular to cytoplasmic diffuse pattern in reported target cells, namely endometrial luminal and superficial glandular epithelium and vascular endothelium [41]. For negative reagent control purposes, two additional sections (from uterine horn or body) were cut from one randomly-selected test sample and were treated in the same way as the other tissue sections except that buffer or an irrelevant rabbit polyclonal S100 antibody (1:400 dilution overnight) was applied to each of the two designated tissue sections.

#### 2.3.4 RT-qPCR assay for OR gene expression

Full-thickness tissue samples obtained from the left uterine horn and the cervix were dissected into endo- and myometrium, cut into small blocks (0.5 cm<sup>3</sup>) and placed in 5-10 µl RNAlater (Lifetechn, Carlsbad, USA) to preserve ribonucleic acid (RNA). Samples were kept at 4 °C for 24 h before storage at -20 °C until analysis. Approximately 30 mg of frozen tissue was used for RNA extractions using RNeasy® Plus Mini kit (Qiagen, Valencia, CA, USA). The tissue material was transferred to RNase-free ceramic bead tubes and 600 µl of Buffer RLT Plus from the RNeasy® Plus Mini kit was added. Tissue samples were subsequently homogenized in a bead-beater (Precellys 24, Bertin technologies, Paris, France) at 5200 X g for 2 x 20 s with a 15 s interval. The lysate was transferred into extraction columns and RNA extraction was performed according to the RNeasy® Plus Mini kit manufacturer's instructions. To minimize adverse effects of protein contamination, only RNA samples with 260/280 ratio between 1.9 and 2.1 and 260/230 ratio > 2.0 were used for the analysis.

Oligonucleotide primers and probe sequences used for detection of OR and β-actin gene expression were obtained from

two previous studies evaluating OR expression and distribution in the conceptus, fetal membranes and endometrium obtained from pony mares at parturition [41, 42]. All primers and TaqMan probes used were synthesized by Life Technologies™ (Carlsbad, CA, USA). The SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA, United States of America) was used for the RT-qPCR reactions analysis. The 25 µl RT-qPCR reaction mixture contained 0.5 µl SuperScript III RT/Platinum® Taq Mix, 12.5 µl 2X Reaction Mix with ROX, 0.5 µl Forward primer (10 µM), 0.5 µl Reverse primer (10 µM), 0.25 µl of TaqMan probe (10 µM), 6.75 µl distilled water and 4 µl of 1 ng/µl RNA template. The PCR conditions included one cycle of complementary deoxyribose nucleic acid (cDNA) synthesis, one cycle of pre-incubation and 40 cycles of amplification consisting of denaturing and primer annealing/elongation. During each RT-qPCR run the following control reactions were included: RNA extraction control, water control, no template control and RT-qPCR reagent control. The RT-qPCR experiments were performed employing

ABI StepOnePlus™ system and software (Applied Biosystems, USA).

The endogenous control gene  $\beta$ -actin was used to normalize the input amount of RNA added to the reactions. Assays of the target and endogenous control genes (OR and  $\beta$ -actin, respectively) require similar amplification efficiency (90–110%). Thus, to determine the amplification efficiencies of the two assays, a set of 10x dilution series, ranging from 485.5 ng/ $\mu$ l to 0.0048 ng/ $\mu$ l, was prepared from RNA of an endometrial sample of uterine body and used to generate standard curves for each gene. For the RT-qPCR reaction, 4  $\mu$ l of each RNA dilution was used as a template and all dilutions were run in triplicates. The mean cycle threshold ( $C_T$ ) values generated from equivalent standard curve mass points (target gene vs endogenous control gene) were then used to calculate the  $\Delta C_T$  ( $C_T$  target gene-  $C_T$  endogenous control gene). Subsequently,  $\Delta C_T$  values were plotted against a log input amount of cDNA to create a semi-log regression line (i.e. the validation plot). The slope of the semi-log regression line was used as a general criterion for passing the validation experiment. The absolute value of the slope of the  $\Delta C_T$  vs log input plot was

## 2.4 Statistical analysis

Raw  $C_T$  values were normalized using the average  $\beta$ -actin  $C_T$  values. Additionally,  $\Delta C_T$  data were assessed for normality by calculating descriptive statistics, plotting histograms, and performing the Anderson-Darling test for normality using commercially available software (MINITAB Statistical Software, Release 13.32, Minitab Inc, State College, Pennsylvania, USA). A linear mixed model approach was used to estimate the effect of sample type (endometrium, myometrium and cervix) on the RT-qPCR results. All models included a random effect term for horse to account

## 3. Results

### 3.1 Study population

Seventeen mares (63 % of the study population) were classified as young (range: 2-9 years, mean: 3.9 years, mode: 2 years n=6). Ten mares (37 % of the study

required to be < 0.1 for the experiment to be considered valid [43, 44].

The OR gene expression was determined from three tissue types (endometrium, myometrium and cervix) with each tissue group consisting of 27 samples. The  $\beta$ -actin  $C_T$  values were similarly determined for all three tissues using RNA of sample RS 042. For normalization of OR RT-qPCR data, a single sample was run in triplicate. To standardize the results for accurate comparison of OR gene expression between tissues, the same RNA concentration (1 ng/ $\mu$ l) was used for all samples. For  $\Delta\Delta C_T$  analysis, the  $\Delta C_T$  values for each tissue type were calculated from average  $C_T$  values of specific samples. The resulting  $\Delta C_T$  values were used to calculate  $\Delta\Delta C_T$  values determined from  $\Delta C_T$  (control) -  $\Delta C_T$  (target) [43].

For this study, the luteal phase, young mares and biopsy score I were selected as controls and the fold change in target (i.e. non-luteal phase, old mares and biopsy scores IIA and IIB) relative to the control was determined using the  $2^{-\Delta\Delta C_T}$  method. The fold change was used as a descriptive method to quantify different gene expressions between tissues [43].

for the repeated measurements. Bonferroni correction was used to adjust P values for multiple post-hoc comparisons. Biopsy scores were compared between groups using Kruskal-Wallis and Mann-Whitney U test for three and two group analyses, respectively. Statistical analyses were performed using commercially available software (IBM SPSS Statistics Version 22, International Business Machines Corp., Armonk, New York, USA) and results were interpreted at the 5% level of significance.

population) were categorized as old (range: 10-20 years, mean: 14.6 years, mode: 15 years n=5).

All 27 mares showed evidence of cyclic activity as confirmed by a combination of macroscopic ovarian evaluation and PPC measurements. Fourteen (52 %; six old

and eight young) mares were in the luteal phase and 13 in the non-luteal phase (48 %; three old and nine young).

### **3.2 Endometrial biopsies**

Biopsies from five mares (19 %, 1 old and 4 young) were designated as grade I; 16 mares (59 %, 6 old and 10 young mares) as grade IIA; and six mares (22 %, 3 old and 3 young mares) as grade IIB, respectively. None of the mares in this study population were categorized as grade III. There was

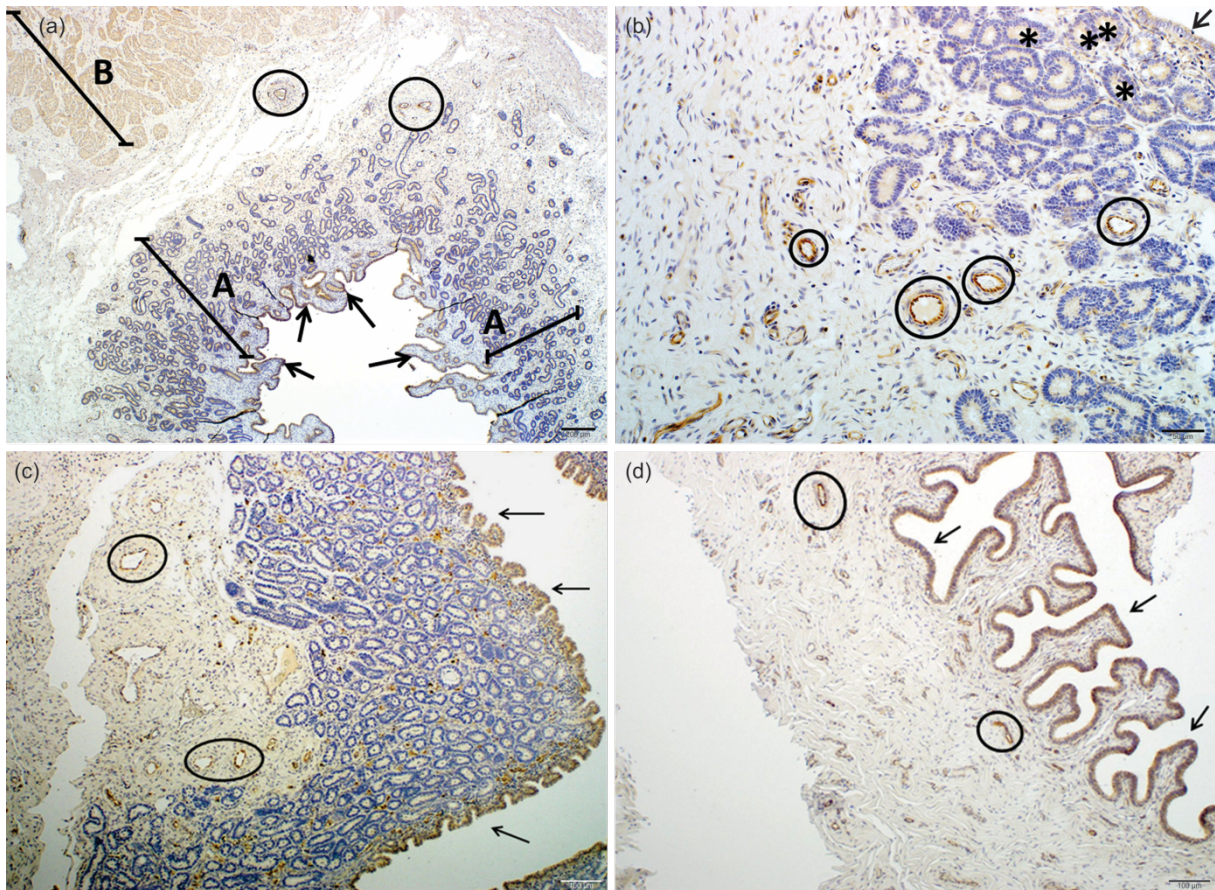
no significant difference in biopsy score between the old and young mares ( $P=0.386$ ). Mares in the non-luteal phase had significantly lower biopsy scores compared to mares during the luteal phase ( $P = 0.003$ ).

### **3.3 IHC to describe OR distribution**

Uterine ORs were distributed in order of decreasing labeling intensity in the cytoplasm of vascular endothelial cells throughout the uterine wall, endometrial luminal and glandular epithelia, and throughout the smooth muscle of the myometrium. The greatest intensity of labeling occurred consistently in the transmural vascular endothelium. In the cervix, a similar pattern of distribution and

labeling intensity of ORs was observed with the obvious exception of the glandular epithelium, which is absent in the cervix (Fig. 2).

There was no apparent difference in OR distribution associated with a mare's luteal phase status, her age or endometrial biopsy score.



**Fig. 2. a-d: Immunolocalization of ORs in the equine uterus and cervix. IHC with DAB substrate-chromogen and Mayer's hematoxylin counterstain.**

- (a) In equine uterine tissue (200  $\mu\text{m}$ ): **A** delineates the endometrium with intense positive cytoplasmic labeling of the superficial luminal epithelium (arrows) and vascular endothelium (solid circles); **B** delineates far less intense labeling of myometrial smooth muscle.
- (b) In the equine endometrium (50  $\mu\text{m}$ ): There was pale/weak staining of the superficial luminal (arrow) and glandular epithelium (\*) and stronger labeling of vascular endothelium (solid circles). The strongest labeling intensity was consistently found in association with vascular endothelium throughout the uterine wall.
- (c) In the equine endometrium during the non-luteal phase (100  $\mu\text{m}$ ): There was intense positive staining of the superficial luminal epithelium (arrows) and vascular endothelium (solid circles).
- (d) In the cervix during the non-luteal phase (RS 57; 100  $\mu\text{m}$ ): labeling of the apical cytoplasmic membrane of the luminal epithelium (arrows) and of vascular endothelium (solid circles).

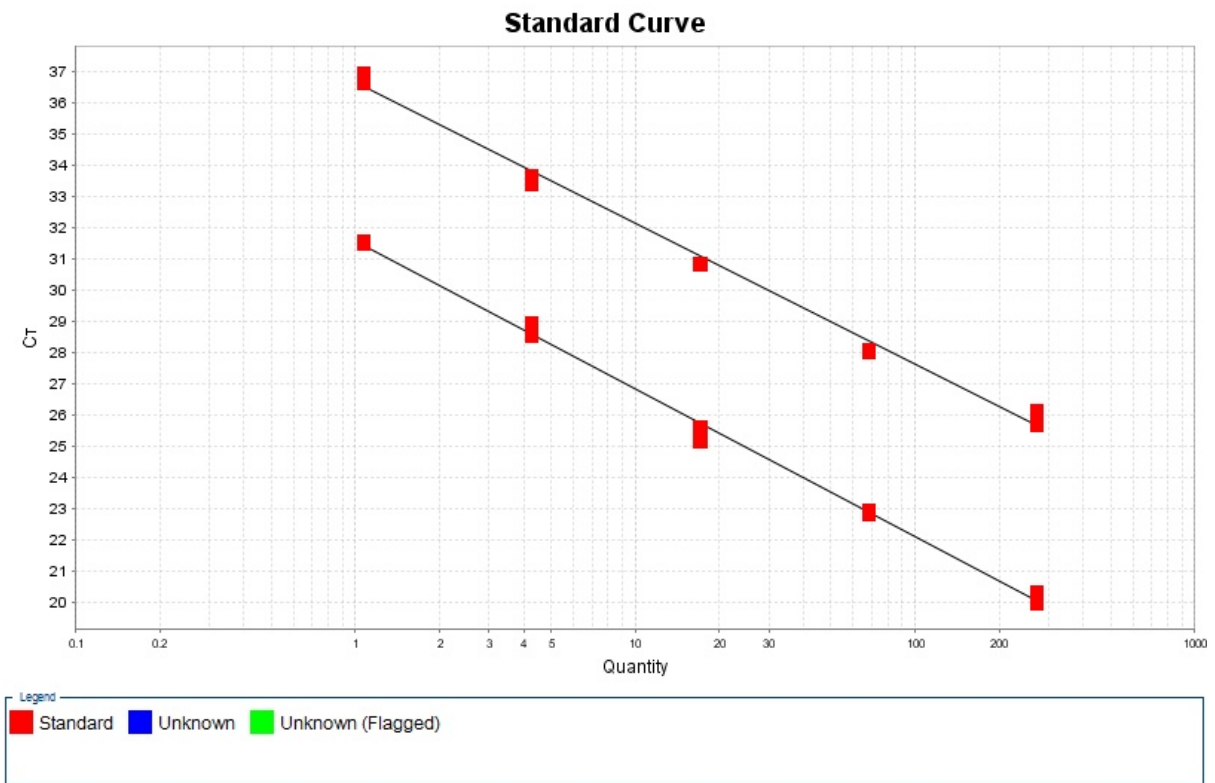
### 3.4 RT-qPCR

#### 3.4.1 Validation experiment

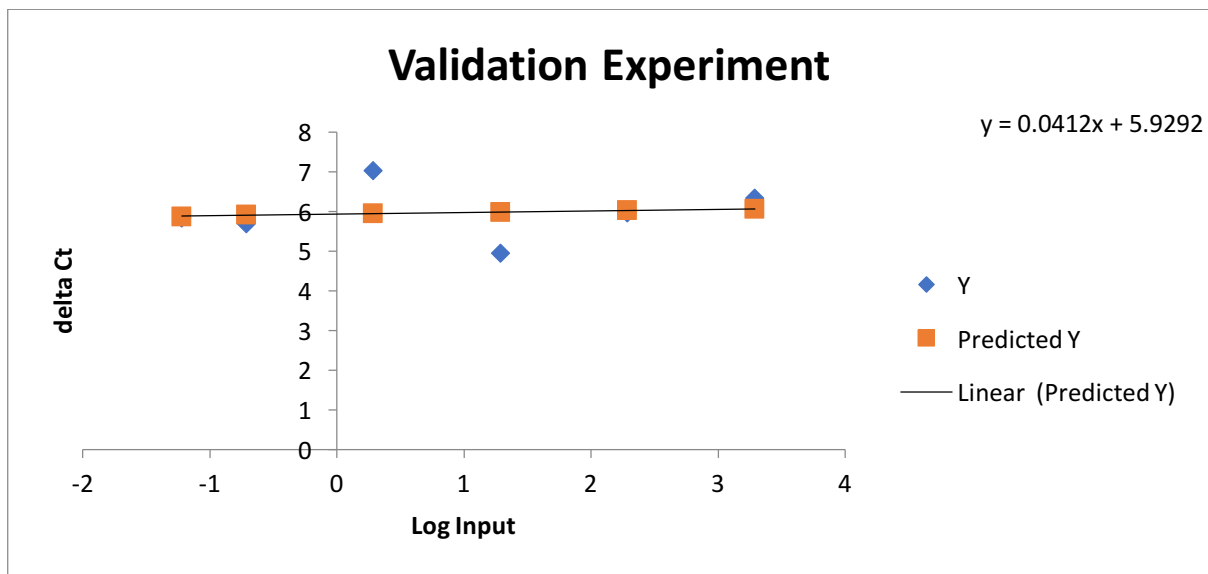
The amplification efficiency for the  $\beta$ -actin and OR assay was 94.5 % (slope = -3.459;  $r^2 = 0.98$ ; amplification = 1.95) and 93% (slope = -3.501;  $r^2 = 0.97$ ; amplification 1.93), respectively (Fig. 3). Slopes obtained

for the two standard curves were between the acceptable range of -3.1 and -3.6, establishing reaction efficiencies of between 90-110 % (Fig. 4).





**Fig. 3.** The RT-qPCR standard curves of OR and  $\beta$ -actin assays: Generated for construction of the validation plot used for evaluation of the amplification efficiencies of the two assays.



**Fig. 4.** The validation plot generated from standard curves of the OR and  $\beta$ -actin assays (**Fig. 3**). The validation experiment is conducted to evaluate the compatibility of the amplification efficiencies of two assays to allow analysis of RT-qPCR data using the comparative  $C_T$  method.

### 3.4.2 Oxytocin receptor gene expression

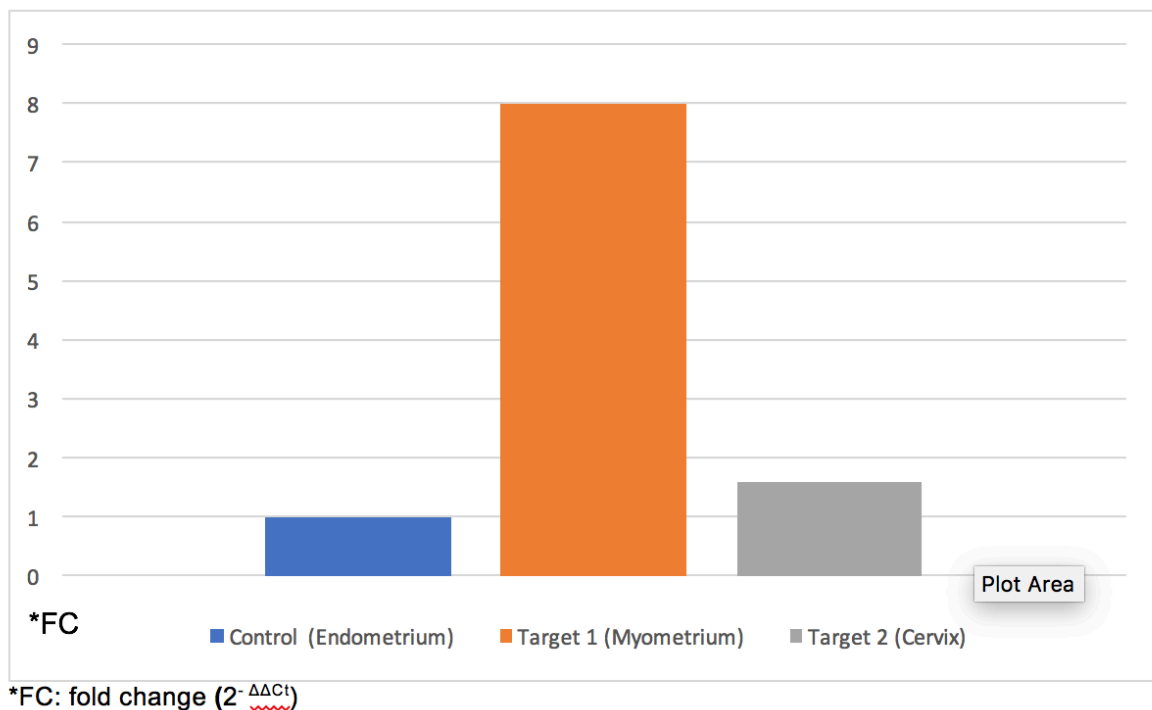
The expression of OR gene was significantly up-regulated in the myometrium when compared to cervix ( $P=0.001$ ) and endometrium ( $P=0.009$ ). There was no significant difference when comparing cervix and endometrium ( $P=1.0$ ). Relative to the endometrium, the fold change of eight and two was observed for the up-regulation of OR gene expression in the myometrium and cervix, respectively (Fig. 5).

There was also a significant difference in the expression of OR gene in the non-luteal phase of uterine tissues (endo- and myometrium combined) compared to luteal phase samples ( $P=0.003$ ).

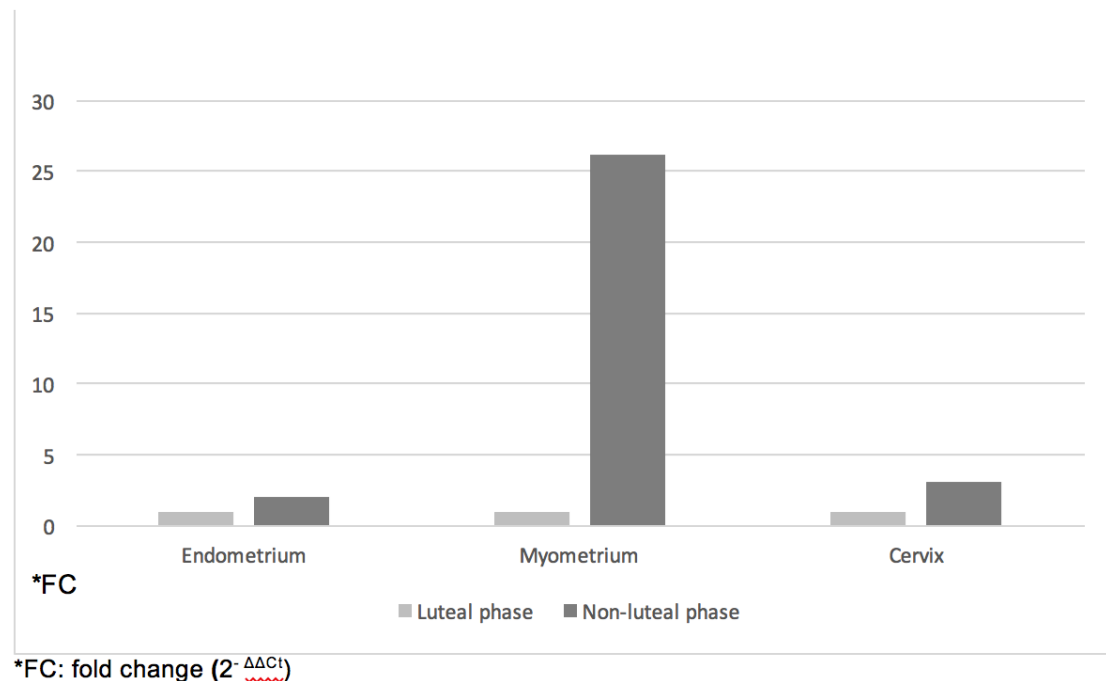
The OR gene expression was up-regulated during the non-luteal phase in all three tissues, compared to the luteal phase. Apparent was the significant up-regulation of OR gene expression in the myometrium (26 fold) during this phase ( $P = 0.004$ ), compared to the non-significant two and three-fold up-regulation observed in the endometrium ( $P = 0.574$ ) and cervix ( $P = 0.339$ ), respectively (Fig. 6).

Neither age ( $P=0.846$ ) nor endometrial biopsy score ( $P=1.0$ ) were associated with OR gene expression.

Fold changes ( $2^{-\Delta\Delta Ct}$ ) evaluated relative to OR gene expression in mares with endometrial biopsy score I, were lower in those with endometrial biopsy score IIA (1/6) and higher in those with a score of IIB (4 times). Neither of these differences using  $\Delta C_T$  values were statistically significant.



**Fig. 5.** Oxytocin receptor gene expression in different tissues relative to endometrium (control) shown as fold changes ( $2^{-\Delta\Delta Ct}$ ). Relative to the endometrium (FC=1), the fold change of eight and two showed the up-regulation of OR gene expression in the myometrium and cervix, respectively.



**Fig. 6.** Oxytocin receptor (OR) gene expression at different cycle stages (luteal versus non-luteal phase) for the three different tissues. Displayed is the two, 26 and three-fold up-regulation of OR gene expression during the non-luteal phase in the endometrium, myometrium and cervix, respectively, as compared to the luteal phase.

#### 4. Discussion

Oxytocin receptors were successfully identified by both IHC and RT-qPCR in the endometrium, myometrium and cervix of this mare population. The demonstration of ORs by RT-qPCR in myometrium and cervix has not been previously reported.

In the endometrium, IHC identified ORs in luminal and glandular epithelia, endothelium of blood vessels and sub-epithelial and peri-glandular stromal cells, supporting the findings of Palm et al. [41]. This distribution suggests that not only is oxytocin being produced locally in the endometrium's secretory luminal and glandular epithelial cells [10], but furthermore that ORs are also present in these cells. The detection of ORs in myometrial smooth muscle cells and vascular endothelia was similar to reports in the cow [45]. In particular, OR distribution in smooth muscle cells supports the physiological association of oxytocin with myometrial contractions during post-mating mechanical drainage of cellular debris and

uterine fluid [18]. The greatest intensity of IHC labeling occurred consistently in microvascular endothelial cells throughout the uterine wall, implying a regulatory role for oxytocin in the uterine vasculature. Oxytocin receptors were previously reported in bovine and human myometrial vascular endothelium [45, 46] and oxytocin was hypothesized to cause necrosis and ischemia of placental caruncles via vasoconstriction in post-parturient cows thus playing an important role in uterine involution. The peptide hormone, vasopressin, structurally similar to, and able to cross react with oxytocin, causes vasoconstriction and may function in uterine involution [45]. Additional studies may better define the physiological role of ORs in equine microvascular endothelial cells.

A physiological role of oxytocin in cervical function during the estrous cycle is proposed by the demonstration of ORs in the cervix of non-pregnant mares. Cervical

ORs have previously been demonstrated in cows and ewes [47, 48]. In cows, a high OR expression in the cervical luminal epithelium around estrus was followed by a rapid down-regulation for the duration of the luteal phase [47]. Similar findings in ewes showed lower OR levels during diestrus [48]. Oxytocin stimulates prostaglandin E2 (PGE<sub>2</sub>) release in the cervix of non-pregnant cows [47, 49]. Intracervical PGE<sub>2</sub> application softens the cervical collagenous framework and has been shown to cause pre-partum cervical dilatation in mares [50]. It can be hypothesized that oxytocin-induced PGE<sub>2</sub> release in estrus promotes cervical relaxation and dilatation in mares, supporting the hypothesis that a relaxed cervix plays an important role in uterine clearance [15]. Although not statistically significant, in this study, the 3-fold up-regulation of cervical OR gene expression in the non-luteal compared to the luteal phase, seemingly supports this hypothesis.

In this study, the highest OR gene expression as quantified by RT-qPCR was in the myometrium. This is in accordance with a previous observation by Stull [8] who used radioligand-binding assays. While the present study showed a difference in OR gene expression between myometrium and cervix, OR gene expression was similar for endometrium and cervix. This was unexpected as the histomorphological structure of the cervix (largely smooth muscle without glandular tissue) would imply a greater similarity with myometrium than endometrium.

Combined uterine tissue samples (both endo- and myometrium) in the non-luteal phase had greater OR gene expression than those of mares in the luteal phase. For individual tissues, however, myometrium had significantly higher OR gene expression during the non-luteal compared to the luteal phase. These findings seem to contradict earlier studies in the mare reporting the highest concentrations of ORs in the endometrium and myometrium at luteolysis (late diestrus) and the lowest receptor density during estrus and mid-diestrus [6-8]. This disparity may be associated with the current study's inability to determine the exact day of the estrous

cycle due to a lack of serial clinical monitoring data, with mares being consigned more broadly into luteal and non-luteal phases. The possible inclusion of both the first 24-48 h post-ovulation and the last 2-3 days of diestrus into the non-luteal phase of the current study may explain the observed disparity with previously reported higher OR gene expression in late diestrus [6-8].

Oxytocin and its receptors play an important role in stimulating PGF<sub>2α</sub> release from the endometrium which, in turn, causes luteolysis [5, 10, 11, 51, 52]. In agreement with previous studies [7-9], our findings confirmed the expected increased OR gene expression in late diestrus, which would facilitate this luteolytic effect. During estrus, oxytocin-stimulated uterine motility enhances luminal clearance [53, 54]. Previous studies [6-8] have postulated increased OR expression in myometrium during estrus, and this was confirmed for the non-luteal phase in our study. This contradicts a recent *in vitro* study by Steckler *et al.* [55] who postulated that low OR concentrations in estrogenized myometrial tissue was the reason for higher oxytocin concentrations required to induce contractions. Currently, there is no reported association between OR expression and degree of tissue responsiveness to oxytocin. Further studies are required to investigate these findings.

Mares in the non-luteal phase had significantly lower biopsy scores than those in the luteal phase. These lower non-luteal phase scores were possibly biased by normal cyclic endometrial morphological changes especially interstitial edema, a characteristic of estrus [56, 57].

Mare age did not influence biopsy scoring in either the luteal or non-luteal phase, with both low and high scores being distributed relatively evenly amongst young and old mares. This corresponds to a study by Shideler *et al.* [58]. In contrast, several other studies reported an association between increasing age and both endometrial fibrosis and fertility [14, 21, 29-31, 59]. Endometrial biopsies scored IIB had the greatest OR gene expression compared to those scored either IIA or I based on fold difference. Overall however,

there was no significant difference in OR gene expression between sections with different biopsy scores. Similarly, no appreciable difference in the IHC distribution of ORs in samples with different biopsy scores was seen. The highest relative OR gene expression was observed in the IIB sections. This was an unexpected outcome and may be postulated as an association with a compensatory up-regulation of ORs for pathological endometrial changes or a decreased responsiveness to oxytocin.

A previous study by Gray [60] did not identify significant differences in endometrial OR gene expression during estrus between groups of mares of varying ages and susceptibility to DUC and concluded that instead of a change in OR gene expression, oxytocin secretion or binding was impaired with increasing age or uterine disease [60].

Nikolakopoulos *et al.* [61] identified reduced circulating levels of PGF<sub>2 $\alpha$</sub>  in DUC mares following endogenous oxytocin release and also after exogenous oxytocin administration. The current findings suggest that reduced PGF<sub>2 $\alpha$</sub>  levels are not associated with decreased OR gene expression but possibly with an impaired oxytocin binding capacity which could decrease PGF<sub>2 $\alpha$</sub>  release.

The current study was limited by the finding that no mares were categorized as biopsy score III. Analyzing samples with a greater degree of endometrial pathology may have influenced results with a possibility of altered OR gene expression. Severe histopathological endometrial changes were previously associated with susceptibility to chronic uterine infections (CUI), while mild to moderate endometrial changes were not consistently correlated to susceptibility or resistance to CUI [30]. Arguably, however, endometrial and myometrial OR gene expression cannot be related to endometrial biopsy score, supporting previously observed endometrial OR gene expression in estrus being similar for mares both resistant and susceptible to PBIE [60]. This suggested that DUC was not due to a decrease in OR gene expression but rather dysfunction at

other proposed levels within the endo- and myometrium [9, 23, 24]. This may include defective PGF<sub>2 $\alpha$</sub>  release at the OR or post-receptor level [23], a decreased level of oxytocin-stimulated myometrial PGF<sub>2 $\alpha$</sub>  production and a possible intrinsic contractile defect of the myometrium [9], or defective myoelectrical signaling and a decrease in the contractile strength of the uterine muscle [24]. A further study limitation was the inclusion of more young mares (n=17) than old mares (n=10). However, given that the mean age for the young mare group was 3.9 years while it was 14.6 years for the old mare group, ensured a clear age difference between the two groups. The existing literature is ambiguous regarding a consistent definition for reproductively young and old mares. Carnevale *et al.* [21] classified young and old as 5-7 years and >15 years, respectively, while for other publications mares of <9 [26], <11 [31], <15 years [30] were considered young. Another study used an age grouping of 2-8, 9-16 and >17 years to categorize mares [32].

The findings of this study are applicable to a representative mare population, which contrasts with most previous reports where mares specifically susceptible to PBIE were allocated to the study populations [19, 20, 32].

In conclusion, this study demonstrated the presence of ORs in several cell- and tissue-types in the endometrium, myometrium and cervix of the mare. Their distribution was consistent across the different tissue types. A significantly higher myometrial OR gene expression, peaking during the non-luteal phase was observed. It seems likely that the described tissues and cells are targets for oxytocin action including stimulation of PGF<sub>2 $\alpha$</sub>  release during luteolysis and uterine contraction as well as possible softening of the cervix through oxytocin-induced PGE<sub>2</sub> release. It was also concluded that endometrial biopsy score and age were not predictors of OR gene expression and furthermore that DUC was unlikely related to deficient OR gene expression. Further studies including mares with grade III biopsy scores, confirmed DUC and known historical breeding data, might add value to the current data.

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