The effects of one single dose of dexamethasone on persistent breeding induced endometritis

Trainee

Name: Smit, A.M.
Email: A.M.Smit1@students.uu.nl
Student number: 3383202

Supervisor

Name: Dr. M. de Ruijter – Villani
Email: m.villani@.uu.nl
Department: Department of Equine Science
Equine Reproduction
Faculty of Veterinary Medicine
Utrecht University

Abstract

Deposition of sperm in the reproductive tract of a mare triggers a transient inflammatory response that is normally resolved within 36h. In mares susceptible to persistent breeding-induced endometritis (PBIE), however, the inflammation is not cleared within this time span resulting in alterations in the uterine environment that can interfere with corpus luteum maintenance and/or conceptus survival and thereby compromise fertility. Recent attention has focused on suppressing the uterine inflammatory response in susceptible mares using systemic corticosteroids or non-steroidal anti-inflammatory drugs. In this respect, administration of corticosteroids at the time of insemination has been reported to improve pregnancy rates in susceptible mares (Bucca et al., 2008), and has been widely adopted in clinical practice. The aim of the current study was to investigate the effects of a single dexamethasone administration at the time of breeding on the endometrial inflammatory reaction.

In this study, five mares known to be susceptible to PBIE were monitored during two estrous cycles. During the first cycle, mares were randomly assigned to being treated or not with corticosteroids. In the second cycle, mares were crossed-over, such that each served as its own control. When the mares were in oestrus with an ovarian follicle ≥35mm, they were inseminated and simultaneously injected with hCG to induce ovulation. During the treated cycle, mares were injected with 50 mg dexamethasone intravenously 1 hour before insemination. Twenty-four hours after artificial insemination, the mares were examined by transrectal ultrasonography and the amount of oedema and intra-uterine fluid were recorded. Next, a low volume intra-uterine flush was performed. The recovered fluid was then centrifuged, the sediment was used for cytological examination and the supernatant was used for Nitric Oxide (NO) determination. After the lavage, an endometrial biopsy was recovered for rtPCR and histology and immunohistochemistry analysis. Quantitative real-time PCR analysis was used to examine mRNA expression for inflammatory cascade enzymes such as COX2, iNOS, LOX5 and LOX5 activating protein (FLAP). Histology and immunohistochemistry was used to examine the presence of PMNs and CD3 positive T cells and the expression of LOX5 and COX2.

Ultrasound finding showed no difference in uterine fluid accumulation after treatment, while the uterine edema was significant decreased by dexamethasone ($p < 0.05$). No decrease in PMN number were found after treatment. Though, the absolute PMN number was increased in the superficial layer of the endometrium after treatment. The gene expression of lipoxygenase 5 (LOX5), LOX5 activating protein (FLAP), cyclooxygenase2 (COX2) and inducible nitric oxide (iNOS) were not significant reduced after treatment. Although the LOX5 positive cell infiltration was significantly reduced in the superficial and middle layer ($p<0.05$) after treatment. There was no change in the NO concentration and the CD3 and COX2 positive cell infiltration after treatment. In conclusion, the results of this study suggest that while treating mares susceptible to PBIE with dexamethasone shortly before mating does reduce post-insemination endometrial oedema but

does not appear to prevent intra-uterine accumulation of fluid or neutrophils. Neither does dexamethasone treatment appears to suppress uterine production of various inflammatory mediators, nor the endometrial expression of mRNA for major inflammatory enzymes. Moreover, since pre-breeding dexamethasone treatment fails to suppress key elements of the major inflammatory pathways 24 h after AI, there is no clear explanation for how pre-AI corticosteroid treatment could improve pregnancy rates in susceptible mares.

1. Introduction.

An acute inflammatory response of the endometrium is a physiological event after artificial insemination and/or natural cover. The aim of this inflammation is to clear the uterus of excess spermatozoa and other components of the ejaculate and to create an optimal environment for the embryo to develop. Spermatozoa and seminal plasma have both an important role in the physiology of this inflammatory reaction; in particular the former stimulates and the latter suppresses inflammation. When spermatozoa enter the uterus they activate complement, and stimulate the cleavage of factor C5 into C5a and C5b: the latter is a potent chemotactic signal which result in an influx of polymorph nucleated neutophils (PMNs) into the uterine lumen. In the presence of complement factor C3b and others complement-independent mechanisms the activated PMNs binds and phagocitized the spermatozoa. Seminal plasma acts as an inflammatory modulator in the uterus which is important for the transient nature of breeding-induced endometritis. Although the precise mechanisms of its action is unknown, it could be the result of a stimulatory effect on myometrial contractions or of the modulation of inflammatory mediators. In vitro experiments have show a suppressive effect of seminal plasma on complement activation, PMN chemotaxis, and phagocytosis. Besides, in vivo experiments have shown a shorter duration of the induced inflammation when seminal plasma is included in the insemination dose. Moreover, seminal plasma protects spermatozoa from being phagocytized and destroyed during the physiologic inflammatory reaction. This is necessary since the PMNs afflux in the uterine lumen starts 30 minutes after insemination, while the sperm transport to the oviducts takes 3 to 4 hours. The accumulated fluid and inflammatory products are removed by uterine contractions, mediated by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) which causes contractions of the myometrium. $PGF_{2\alpha}$ is produced from the arachidonic acid via the cyclooxygenase pathway, during the activation of PMKs. Once the inflammatory products are removed the uterine environment returns to its normal state [2].

Susceptible mares fail to clear their uterus of inflammatory products and luminal contaminants and develop persistent breeding-induced endometritis (PBIE). This is a common cause of infertility and has been reported to be the third most common medical condition of horses. Predisposing factors are advanced age, poor perineal conformation, pendulous uterus, increased parity and delayed uterine clearance due to suboptimal myometrial contractility [1,3]. A recent study found that susceptible mares have an increased accumulation of nitric oxide (NO) in the uterine lumen 13 hours after insemination. Nitric oxide mediates smooth muscle relaxation and may explain the impaired uterine clearance. Also failure of the cervix to relax during estrus or insufficient lymphatic drainage may contribute to delayed clearance [2].

The treatment for persistent post breeding endometritis must reduce the inflammatory reaction and stimulate the uterine clearance. Reducing the inflammatory reaction is possible by limiting uterine exposure to semen and bacteria. Exposure to semen should be limited by reducing the number of inseminations per cycle by induction of ovulation.

Uterine clearance can be assisted by uterine lavage 6 to 24 hours after breeding or by stimulating myometrial contraction with oxytocine or cloprostenol [1].

Dexamethasone is a known immunosuppressive and anti-inflammatory drug largely used in veterinary practice to treat PMIE. Glucocorticoids interfere with the biosynthesis of many proinflammatory mediators in multiple cells involved in inflammatory reactions. During an inflammation arachidonic acid is released from membrane phospholipids by the action of the enzyme phospholipase A2 (PA2) and can be metabolized by cycloogygenase 1 and 2 to generate prostanoids like prostaglandins (PGE_2, $PGF_{2\alpha}$, PGD_2), prostacyclines (PGI_2) or tromboxanes (TXA_2). Moreover arachidonic acid can also be metabolized by FLAP (5-lipoxygenase activating protein) activated 5-lipoxygenase (LOX-5) to generate leukotrienes (LT). It has been shown that glucocorticoids reduce the expression of key enzymes of prostenoid synthesis, such as PA2 and Cyclooxygenase-2 (COX-2) which is inducible by pathologic conditions by inflammatory stimulation. There is little evidence that glucocorticoids have influence on the leukotriene biosynthese [7].

Dexamethason also affects the nitric oxide (NO) cascade. NO is known to mediate various physiologic systems including vasodilatation, inhibition of platelet aggregation, cell-mediated immune response, polypeptide and ion secretion, programmed cell death, cell growth or stimulating angiogenesis. NO is synthesized by three types of nitric oxide synthases (NOSs) that catalyses the oxidation of $_L$-arginine to $_L$- citrulline. The first NOS is the calcium-dependent endothelial NOS (eNOS) which is important for the control of vascular function and is constitutively expressed. The neuronal NOS (nNOS) is also calcium dependent and is an important neurotransmitter and neuromodulator in the central and neuronal nervous system. The third NOS is the inducible NOS (iNOS) and is expressed during inflammation in various cell types including endothelial cells, smooth muscle cells and macrophages. iNOS is calcium independent and is induced by cytokines and lipopolysaccharide (LPS). Several studies have indicated that iNOS and eNOS play an important role in the endometrium during remodeling, embryo receptivity and implantation. This NOS type can form high quantities of NO. Alghamdi A.S. *et al.* compared NO in uterine secretions and NOS expression in uterine biopsies between susceptible and resistant mares. They found an increased number of iNOS-positive inflammatory cells and an up regulation of iNOS mRNA in the endometrium of susceptible compared to resistant mares. Different studies have shown that dexamthasone reduces the translation of iNOS mRNA and increased the degradation of iNOS proteins. This could be beneficial in reducing the inflammatory reaction in susceptible mares [6, 8, 9].

Although several studies have evaluate the effects of dexamethasone on pregnancy rates in normal mares and in mares with PBIE, it is still unclear if and how corticosteroids affect the inflammatory reaction typical of PBIE. Therefore the aim of the present study is to evaluate the effects of dexamethasone on the cell-mediated inflammatory response to PBIE and on the expression of some inflammation markers such as COX-2, LOX-5, FLAP and iNOS.

2. Materials and Methods.

2.1. Animals

Five warmblood mares between 10 to 15 years old were classified as potentially susceptible to PBIE on the basis of history of repeated persistent post-breeding endometritis, low fertility rates, poor perineal conformation, pendolous uterus and moderate-to severe pathologic changes in the uterine biopsies.

The mares were examined during two cycles. During the first cycle the mares were randomly assigned to the treated or non treated group. In the second cycle the mares were grouped in the opposing group so that the mares served as their own control. The mares were monitored regularly by transrectal palpation and ultrasonographic examination of the reproductive tract. During early estrus, mares will be examined three times a week; once the dominant follicle exceeds 35mm, the frequency of examination will be increased to daily.

2.2 Experimental procedure.

Before the beginning of the experiment a low volume flush was performed and a uterine biopsy was taken during oestrous (Control group) to evaluate cytologically, bacteriologically and histologically the endometrium and uterine content. Only mares without signs of endometritis were used in the experimental protocol.

When the mares showed estrus signs and a follicle exhibiting a diameter of 35 mm or bigger, they were inseminated with 500 x 10^6 sperm from the same fertile stallion diluted first 1:1 in a commercial skimmed milk extender and re-extended to 15ml following centrifugation to remove the bulk of the seminal plasma. At the time of insemination the mares were injected with 1500 IU human chorionic gonadotrophin (Chorulon) in order to induce ovulation. Mares in the treatment group were treated with Dexamethasone (50mg I.V. per 500kg) one hour before the time of insemination.

Uterine flushing

Twentifour hours after artificial insemination a volume of 100ml of sterile lactate Ringer solution was infused into the uterine body trough a sterile Foley catheter. The fluid was distributed to the uterine body and horns by transrectal massage. With aid of transrectal manipulation the fluid was massaged back into the 100ml bottle until at least 50% of the injected fluid was recovered.

Any cells present in the uterine secretion samples were removed by centrifugation at 1000*g* for 10 min. The sediment was used for cytological examination and the supernatant was immediately frozen at -20C until NO determination. In order to accurately compare the NO concentrations between cycles, all samples were frozen sequentially until the collection was completed then analyzed at the same time.

Endometrial biopsy

After the collection of uterine fluid one endometrial biopsy was collected from each mare. The biopsies were taken on the base of one horn using a crocodile forceps. Half of the biopsy was immediately snap frozen in liquid nitrogen for RNA extraction and rt-PCR analysis. The other half was fixed in 4% formalin overnight and then transferred in 70% ethanol prior to be embedded in paraffin and used for histology and immunohistochemistry (IHC).

RNA extraction and cDNA synthesis

Isolation of total RNA and on-column DNAse digestion was performed using the Invisorb® Spin Cell RNA Mini Kit (Invitek GmbH, Berlin, Germany) combined with the RNAse-free DNAse set (Quiagen, Valencia, CA, USA). Each sample was lysed in 700 µl lysis buffer and applied to the DNA-binding spin filter. After incubation for 2 min at room temperature and centrifugation for 2 min at 11000g, the binding filter containing DNA was discarded and the RNA containing lysate was diluted (1:1) with 70% ethanol and pipetted directly onto a RNA-binding filter. After the column had been washed twice with washing buffer, the RNA-binding filter was incubated with RNAse-free DNAse for 15 min at room temperature. After three further washes with washing buffer, the RNA was eluted from the RNA-binding filter with 33 µl RNAse free water.

Reverse transcription (RT) was performed in a total volume of 40 µl made up of 20 µl of sample RNA, 8 µl of 5 x RT buffer (Invitrogen, Breda, The Netherlands), 16 U RNAsin (Promega, Leiden, The Netherlands), 300 U Superscript II reverse transcriptase (Invitrogen), 1.2 µg of random primers (Invitrogen), 10 mM DTT (Invitrogen) and 0.5 mM of each dNTP (Promega). The mixture was incubated for 5min at 70°C, 1hr at 42°C and 5min at 80°C, before being stored at -20°C. Minus RT blanks were prepared from 10 µl of the sample RNA under the same conditions but in the absence of reverse transcriptase.

PCR primer design

The primer pairs used for rtPCR are listed in table 1. The primer pair for COX2, LOX5, LOX5 activating protein(FLAP), iNOS and GAPDH were designed using Primer Designer version 2.0 (Scientific and Educational Software, Cary, NC) on the equine coding sequence; where possible each primer of a pair was located to a separate gene exon. A standard sequencing procedure (ABI PRISM 310 Genetic analyzer, Applied Biosystem, Foster City, CA) was used to verify the specificity of the PCR products of each target gene.

Table 1: Details of primers for the genes used for rtPCR

Gene	**Sequence**	**Ta °C)**	**Amplicon size (bp)**	**Genebank Acc. N.**
GAPDH (Glyceraldehyde-3-phosphate	5' AGGCCATCACCATCTTCCAG 3'	53	112	AF 157626

dehydrogenase)	REV 5’ CCAGCCTTCTCCAAGGTAGT 3’			
COX2	5’-TACTGGAACATGGACTCACC-3’			
	REV 5’-GGTACTCATTCAGAGACTGG-3’	62	274	AF 035774
LOX5	5' GCGGTTGATTCTTATGATGTG 3'			XM_001916786.1
	REV 5' CTGTGCTGTTTGAGGATGTG 3'	60	247	
FLAP	5' CGTATCCCACTTTCCTTGTC 3'			NM_001163965.1
	REV 5' CGTTTCCCAAATATGTAGCC 3'			
		57	162	
iNOS	5'GCTATCCAATTTGTCAACCAG 3'			NM_001081769.1
	REV 5' GTTCCTGTTGTTTCTATCTCC 3'	60	106	

rtPCR

For each target gene, QRT-PCR was performed on two replicates of cDNA and a single – RT blank. Simultaneous quantification of all the samples in a 96-well plate was performed using a real-time PCR detection system (MyiQ Single-Color Real-Time PCR Detection System; Bio-Rad Laboratories, Veenendaal, The Netherlands). Standard curves were created using 10-fold serial dilutions of known amounts of target gene PCR product to quantify expression. The qtPCR reaction mixture (25µl) contained 1 µl of sample cDNA solution, 0.5 mM of each primer (Isogen Bioscience BV, Maarssen, The Netherlands) and 12.5 µl of IQ™Sybr® Green Supermix (Bio-Rad Laboratories). Initial DNA denaturation at 95°C for 5 min was followed by 40 cycles consisting of 95°C for 15 sec, the primer specific annealing temperature (see Table 1) for 30 sec, and 72°C for 45 sec. To verify the purity of the product after amplification, melting curves were plotted. Standard curves were produced by plotting the logarithm of the starting amount versus the threshold cycle for detection.

The mRNA of inflammatory mediators COX2, iNOS, LOX5 an LOX5 activating protein (FLAP) was studied in the endometrium of treated and control mares. The starting quantities obtained were normalized using GAPDH.

NO evaluation

Thawed uterine flushing samples were used to measure NO concentrations using a commercially available assay kit (Bioxytech, Oxis International Inc., Portland, OR, USA). Briefly, nitrate in the samples was reduced to nitrite by the cadmium method as described by Davison and Woof (1978). After nitrate reduction, total nitrite was

determined spectrophotometrically after the addition of Griess reagent to the samples and reading the color absorbance at 540 nm. The concentration was calculated using a standard curve. In order to accurately compare the NO concentration between groups, all samples were frozen sequentially until the collection was completed then analyzed at the same time.

Histology

The 4% fixed formaline samples were sectioned at 5µm and stained with Haematoxylin-Eosin (H&E) to assess each biopsy and visualize the PMKs.

Immunohistochemistry for CD3, LOX5 and COX2

Endometrium tissue sections of 5 µm were mounted on silan-coated microscopic slides and dried overnight at 55°C. The paraffin sections were deparaffinized and rehydrated in consecutive 2x xylene (2x5 min), 2x alcohol 100%, 2x alcohol 96%, 2x alcohol 70% and 2x in distilled water (2x3 min). Subsequently the slides were incubated in pretreated citrate buffer PH 6.0 in the microwave to retrieve the antigens and cooled for 30 min at room temperature.

The endogenous peroxidase activity was blocked by incubating the slides in 35% H_2O_2 in methanol (30 min) and subsequently washed with PBS/Tween (3x5 min). The slides were pre-incubated for 15 min with goat serum diluted in 1:10 PBS (Phosphate-buffered-saline). After that the tissues were incubated with the primary antibodies (rabbit anti CD3 (Cell Marque (103A-76)), diluted in PBS 1:200; rabbit anti LOX5 ((pAb), Novus Biologicals, NB-100-92138, lot 360703, Bio- Connect), diluted in PBS 1:100; Polyclonal Rabbit Anti-human COX-2 (kindly provided by Dr. G. O'Neill,Merck Frosst Canada Ltd., Canada (Kargman S et al., 1996)), diluted in PBS 1:2500) at 4°C overnight. The negative control sections were incubated in PBS.

The slides were washed with PBS/Tween (3x5 min) then incubated at room temperature for 30 min with the secondary antibody (goat anti rabbit biotinylated; Vector BA-1000, diluted in 1:250 PBS).

The tissues were rinsed with PBS/Tween (3x5 min) and incubated with avidin-biotin-complex solution (VECTASTAIN® ABC Kit (standard); Vector PK-4000) in PBS for 30 min. The ABC/PO-complex was prepared 30-60 min before use. After rinsing with PBS (3x5 min) the CD3 slides were developed in AEC (3-amino-9-ethylcarbazole) (Dako K3469, lot 10052681) and the COX5 and LOX2 in DAB (diaminobenzidinetetrahydrochloride) (Sigma life science, D5637-10G, 40496BK) for 10 min. The nuclei were stained with Haematoxylin (30-60 sec) and rinsed in running tap water for 20 min. All the slides, with the exception for the CD3 coloured, were dehydrated in consecutive 2x alcohol 70%, 2x alcohol 96%, 2x alcohol 100% (3 min) and 2x xyleen (5 min). The CD3-slides were covered with Aquatex and the COX2 and LOX5 with Eukitt.

Analysis of total PMKs, CD3-, LOX5- and COX2 positive cells

For each endometrial biopsy the cell count for PMNs, CD3 positive T-cells and LOX5 positive plasmacells was performed at 40x magnification in ten different fields and three layers to assess the grade of cellular infiltration present in the endometrium. The layers which were analysed are the superficial layer or stratus compactum (the sublayer of the stratum functionale facing the interior of the uterus and containing the necks of the uterine glands); the middle layer or stratus spongiosus (the sublayer of the stratum functionale underlying the stratum compactum containing the tortuous portions of the uterine glands); and the deep layer or basal layer consisting of stroma and the lowest layer of the glandular tissue.
In the endometrial biopsies stained for COX2 the color intensity were graded (1-4) in the 3 different endothelial layer (1: negative, 2:mild, 3:moderate, 4:marked).

Statistical analysis

The statistic analysis was performed with the statistical software SPSS 16. When not all data were normally distributed the data were transformed in logarithm 10 values. Difference between groups were analysed by Anova-one way test. If there was a significant difference between groups, the Turkey post hoc test was used to evaluate which group differed significantly from the others. Values of $p<0.05$ were considered statistically significant.

3. Results

3.1 Ultrasound findings and cytology

Post-insemination intra-uterine fluid depths was significantly higher ($p<0.05$) than pre-insemination depths in the inseminated non treated group (mean±SD: 38.7 ± 14.3mm versus 12.8 ± 15.2) (Picture 1); while in the inseminated treated group there was no significant difference between pre- and post insemination intra-uterine fluid (mean±SD: 29.7 ± 19.9 mm versus 19.7 ± 20.4 mm). There was no significant difference between post insemination fluid in the treated and non-treated groups. Therefore uterine fluid accumulation was not inhibited by dexamethasone treatment.

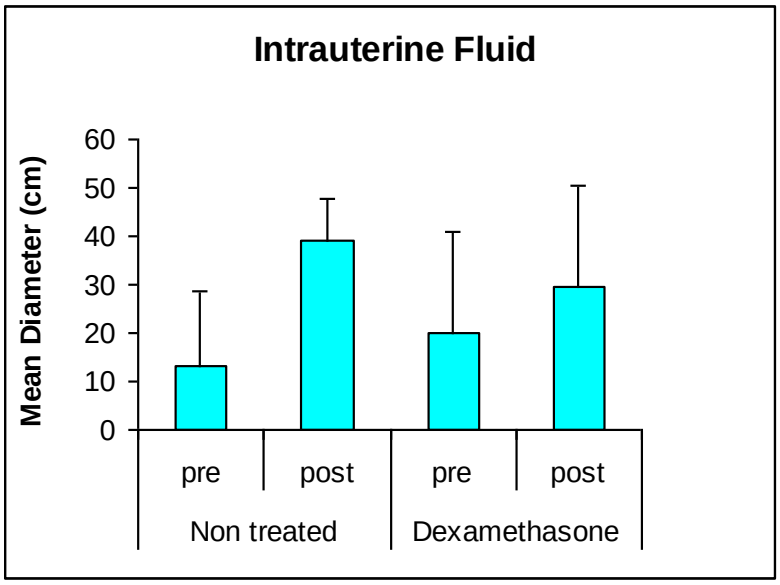

Figure 1: Mean diameter intrauterine fluid (cm) in non treated and treated mares, before and after insemination.

Similarly, whereas the percentage of total cells classified as polymorphonuclear neutrophils was much lower ($p<0.01$) in the non-inseminated cycle (mean±SD: 3 ± 3.5 %) than in either of the inseminated cycles (mean±SD: treated-AI, 96.5 ± 0.5 %; non treated-AI, 94.4 ± 4.3 %), there was no apparent effect of dexamethasone treatment (Figure 2).

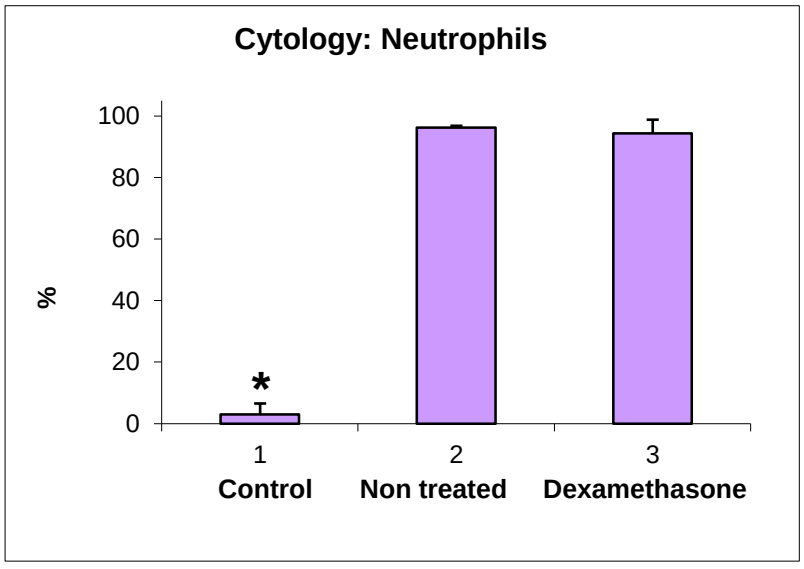

Figure 2: Percentage neutrophils in control, inseminated non-treated and inseminated treated groups.

On the other hand, the dexamethasone treated AI cycles were characterized by a significantly lower ($p<0.05$) mean endometrial oedema score 24 h after AI than control-AI cycles (mean±SD: 0.6 ± 0.55 versus 0.8 ± 0.84) (Figure 3).

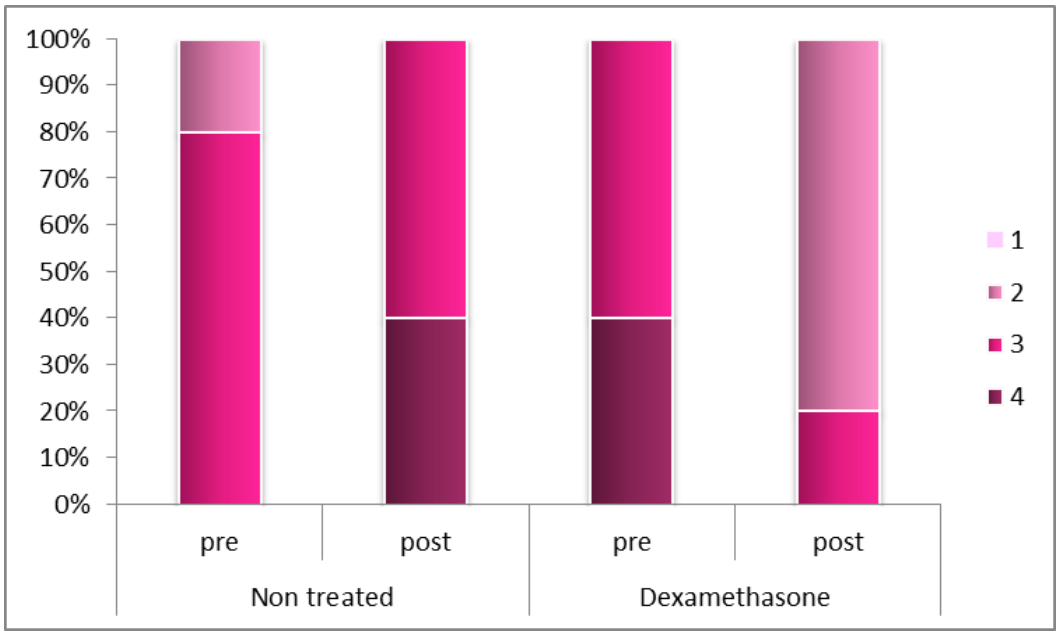

Figure 3: Endometrial oedema score in non-treated and treated mares before and after insemination; Grading 1-4, 1: negative; 2: mild; 3: moderate; 4: marked.

3.2 Rt-PCR

Relative gene expression for COX-2 was significantly lower in non-inseminated cycles (mean±SD: 12.8 ± 12.0 REL) than in either treated-AI (mean±SD: 54.6 ± 23.0 REL) or control-AI (mean±SD: 70.7 ± 65.9 REL) cycles, but was not significantly influenced by dexamethasone treatment (figure 4). Relative LOX5 and FLAP mRNA expression were not affected by either insemination or dexamethasone treatment (figures 5, 6); mean expression levels for non-inseminated, treated-AI and control-AI cycles were respectively mean±SD: 8.6 ± 4.6, 8.8 ± 3.5 and 4.4 ± 1.5 for LOX5, and mean±SD: 7.3 ± 3.5, 9.9 ± 5.4 and 6.0 ± 2.7 for FLAP. Similarly, there was no difference in iNOS mRNA expression between the non-inseminated (mean±SD: 13.6 ± 5.3), the treated-AI (mean±SD: 14.3 ± 14.6) and the control-AI (mean±SD: 16.5 ± 16.3) cycles (figure 7).

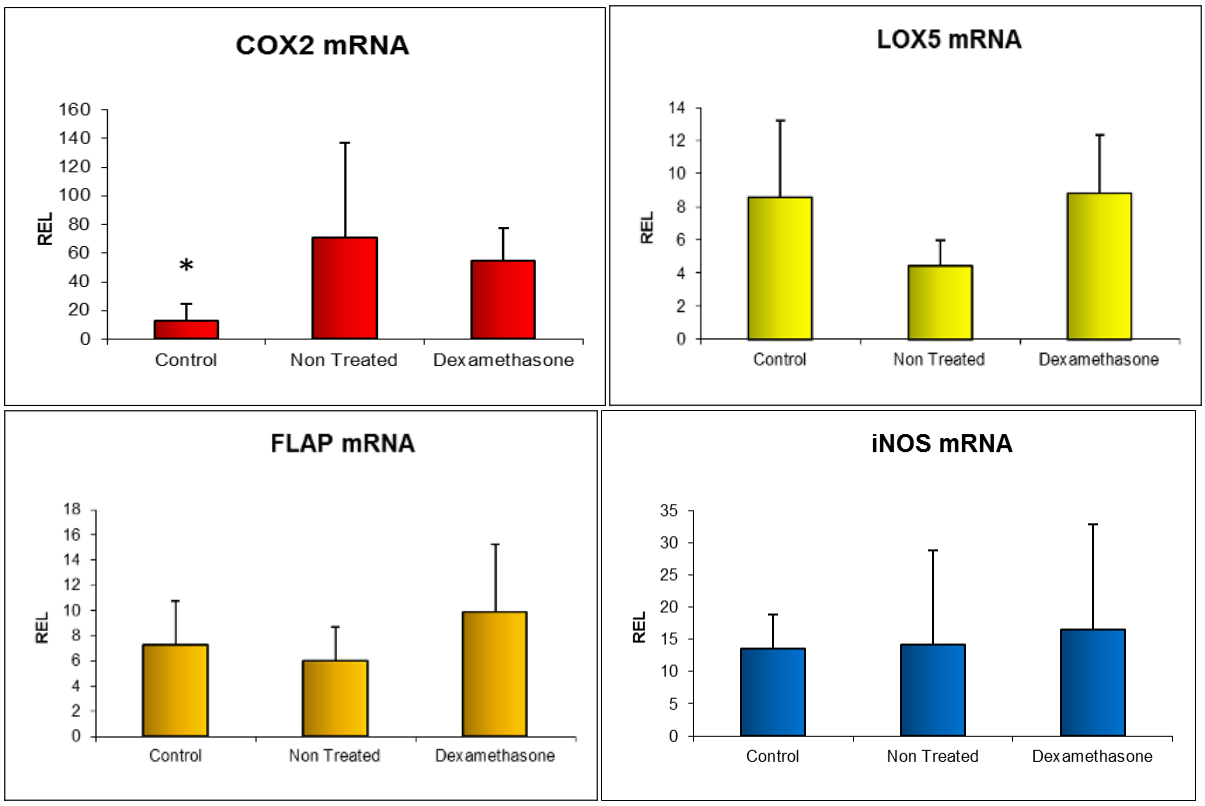

Figure 4-7: The relative expression of COX2, LOX5, FLAP, iNOS mRNA in the non-inseminated (Control), inseminated non treated (Non Treated) and inseminated treated with dexamethasone (Dexamethasone) groups.

3.3 NO concentration

The concentrations of NO in uterine lavage samples ranged between 6.6 and 132.8 mM, but did not differ significantly different between treated-AI (mean±SD: 15.8 ± 13.3 mM) and non treated-AI cycles (mean±SD: 20.7 ± 8. 5 mM) (figure 8).

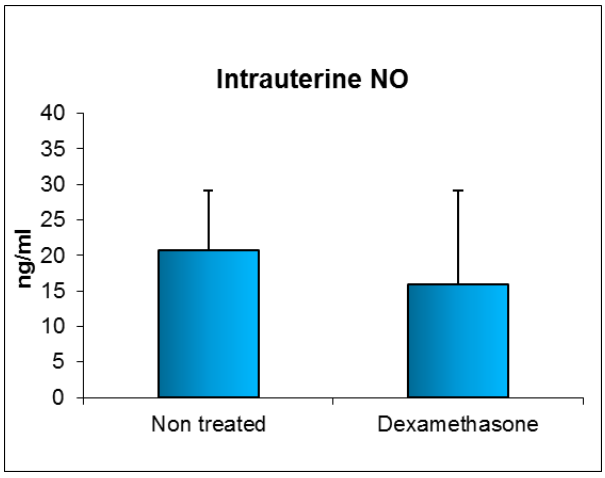

Figure 8: The intrauterine NO concentration in the non treated and treated cycles.

3.4 Histology

The numbers of PMNs in the superficial and middle layer were significantly higher in the treated-AI (mean±SD: superficial: 21.1 ± 13.2 cells/field; middle: 5.1 ± 5.1 cells/field; deep: 2.9 ± 5.0 cells/field) and control-AI cycles (mean±SD: superficial: 15.2 ± 3.6 cells/field; middle: 10,0 ± 56 cells/field; deep: 8.4 ± 7.2 cells/field) than in the non-inseminated cycles (Control) (mean±SD: superficial: 0.9 ± 1.0 cells/field; middle: 0.6 ± 0.3 cells/field; deep: 0.1 ± 0.2 cells/field). The observed PMNs number in the superficial layer of the treated cycle was higher than the inseminated non treated cycle, while the observed PMNs number in de middle and deep layer was lower. But there was no significant influence of dexamethasone. (figure 9). Figure 10 and 11 shows representative H&E stained endometrium biopsies.

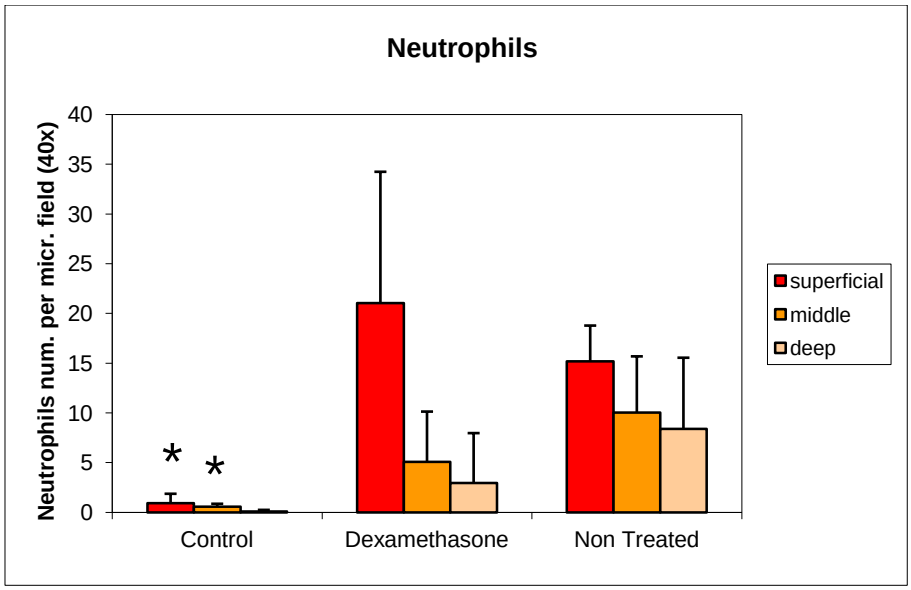

Figure 9: Number of Neutrophils per microscopic field (40X) in the superficial, middle and deep layer of endometrium in the control, non treated and treated groups. * significantly different ($p<0,01$).

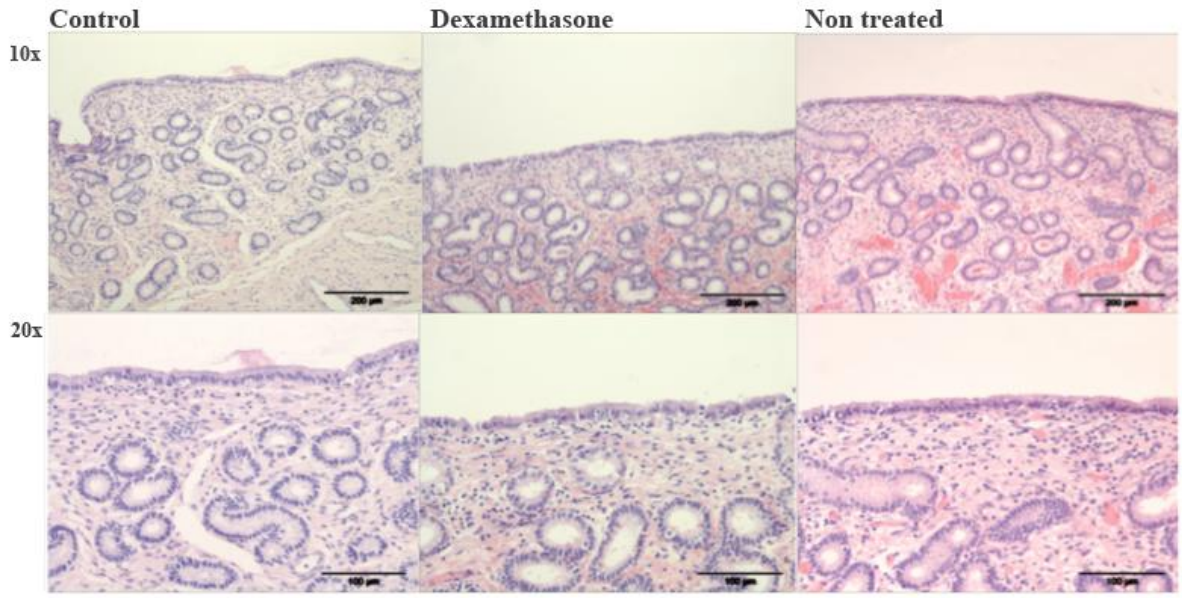

Figure 10: H&E stained endometrium from a control, a treated and a non treated mare. PMNs displayed in superficial, middle and deep layer.

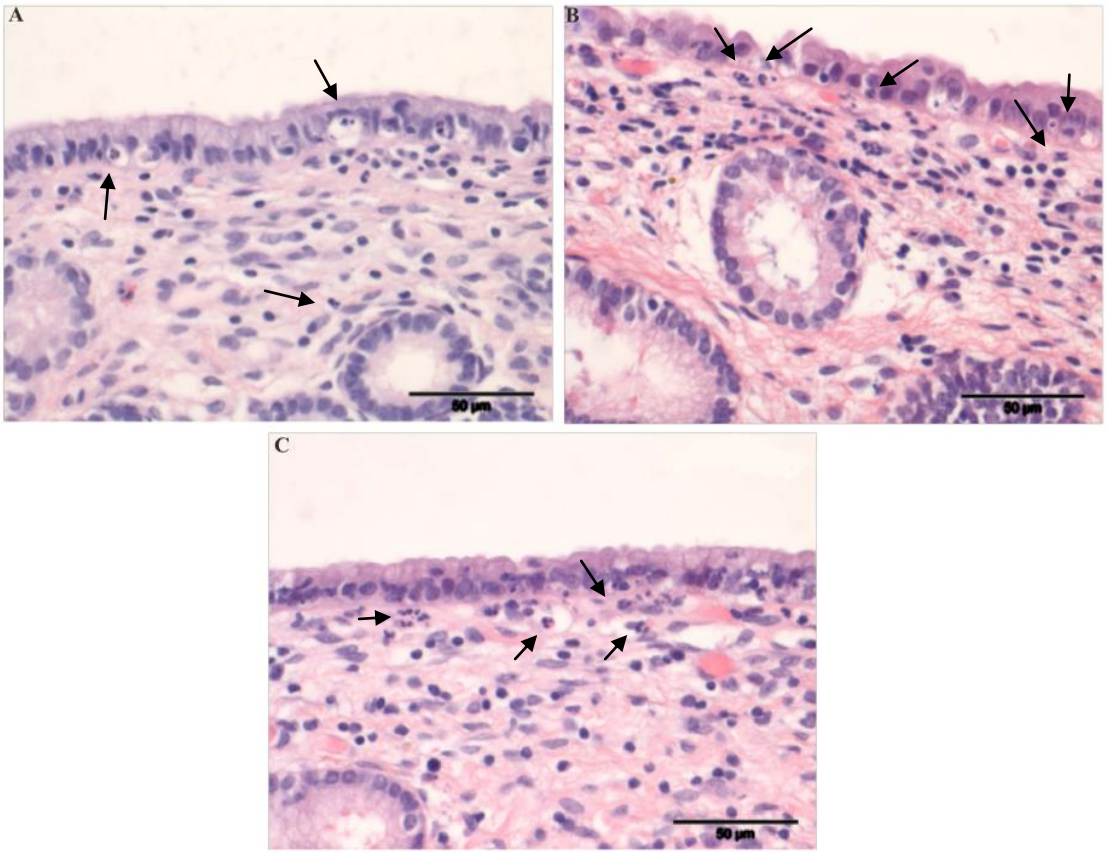

Figure 11: PMNs in superficial layer of the endometrium marked by arrows in a control (A), a treated (B) and a non treated (C) mare (40x magnification).

3.5 Immunohistochemistry

There was a significance difference ($p<0.01$) in the number of CD3 positive T-cells in the superficial layer of the control cycle (mean±SD: 1.2 ± 0.9 cells/field) if compared to both the inseminated cycles (mean±SD: inseminated treated: 6.3 ± 1.5 cells/field; inseminated non treated: 7.3 ± 4.9 cells/field). But no significant difference was found between the inseminated treated and inseminated non treated group (figure 13). Figure 14 shows representative CD3 stained endometria.

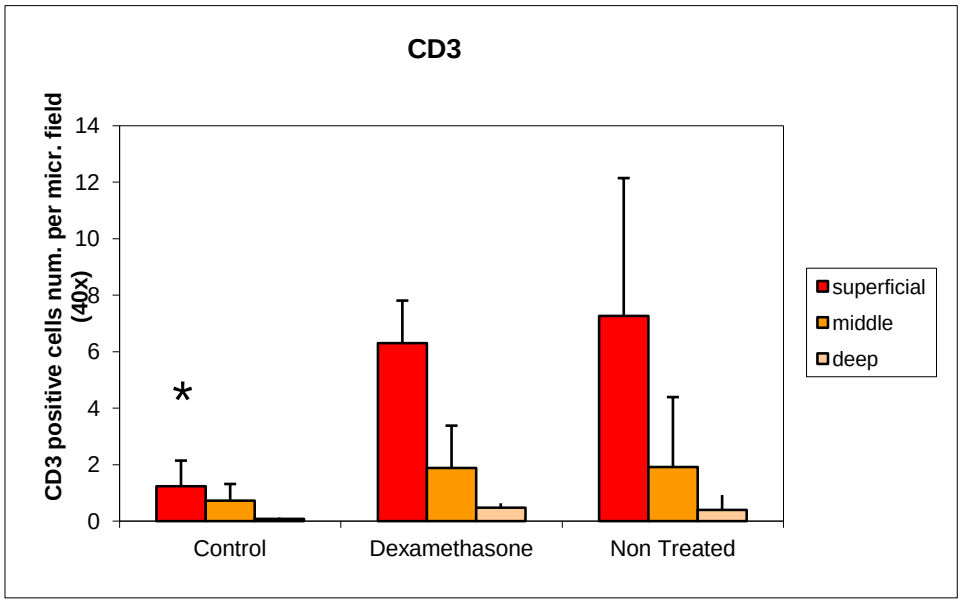

Figure 13: Number of CD3 positive T-cells per microscopic field (40X) in the superficial, middle and deep layers of the endometrium in the control, non treated and treated groups. * significantly different ($p<0,01$).

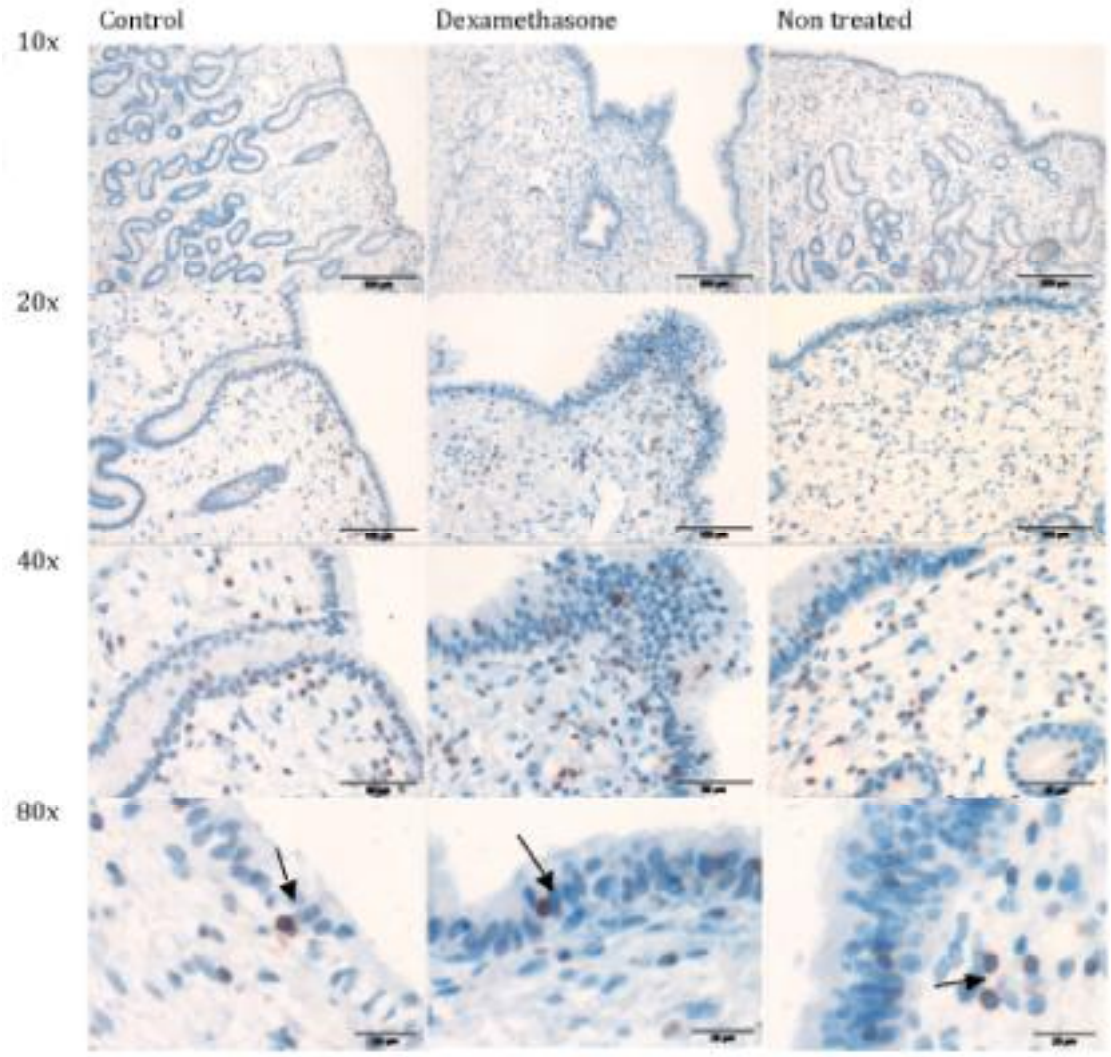

Figure 14: CD3 stained endometrium from a control mare, a treated mare and a non treated mare. CD3 positive T-cells displayed in superficial, middle and deep layers.

The number of LOX-5 positive cells in the superficial and middle layers of the treated-AI cycle (mean±SD: superficial: 1.95 ± 1.64 cells/field; middle: 1.15 ± 0.60 cells/field; deep: 0.53 ± 0.25 cells/field) was significantly lower than the inseminated non treated group (mean±SD: superficial: 6.04 ± 3.43 cells/field; middle: 3.86 ± 2.56 cells/field; deep: 2.63 ± 1.05 cells/field) (figure 15). Figure 16 and 17 shows LOX-5 stained endometrium biopsies.

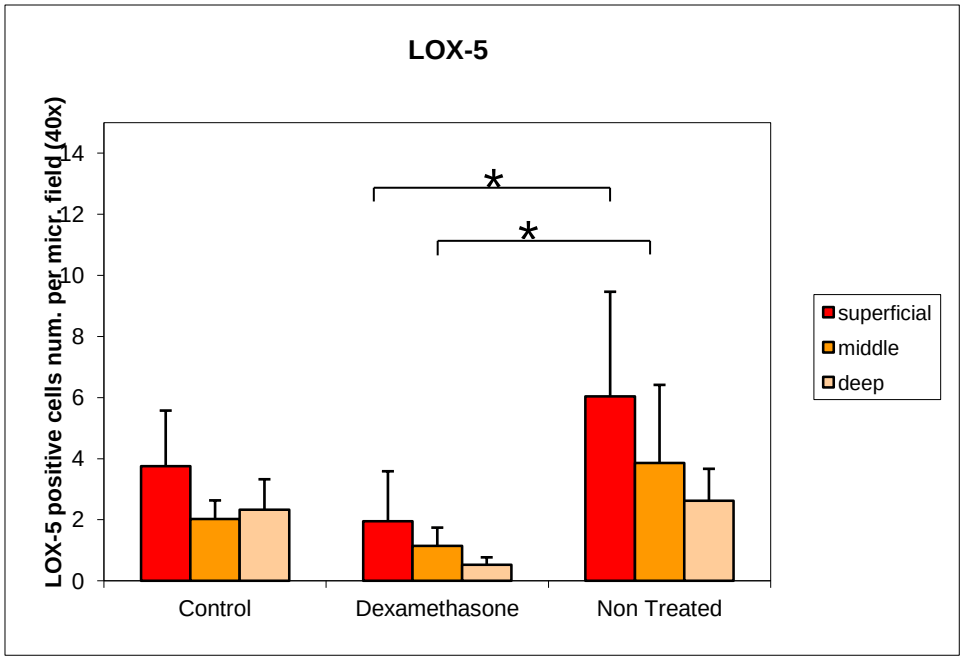

Figure 15: Number of LOX5 positive cells per microscopic field (40X) in the superficial, middle and deep layers of the endometrium in the control, non treated and treated groups. * significantly different (p<0.0.5).

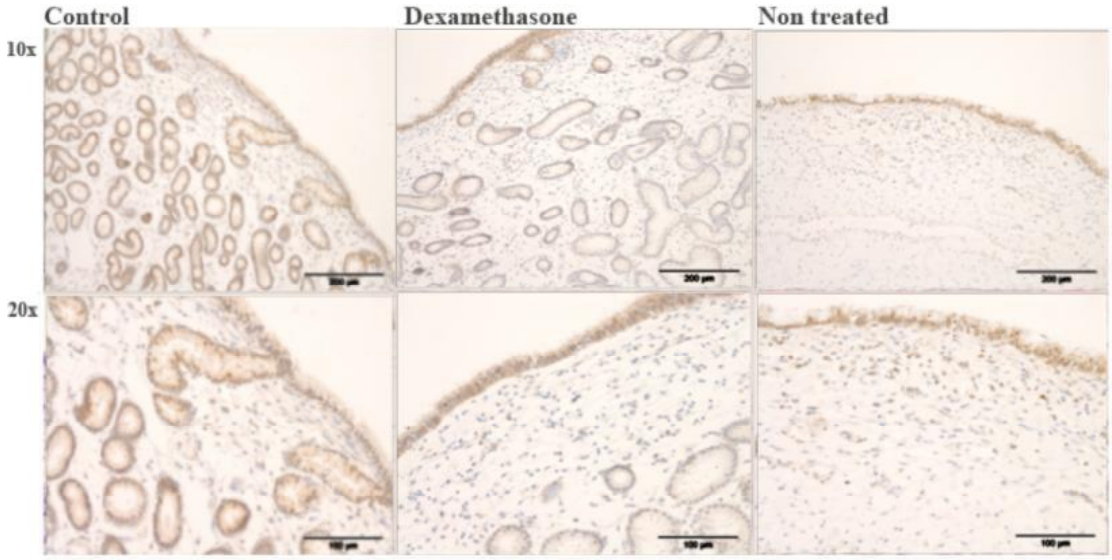

Figure 16: DAB-stained endometrium from a control, a treated and a non treated mare. LOX-5 positive cells displayed in superficial, middle and deep layer.

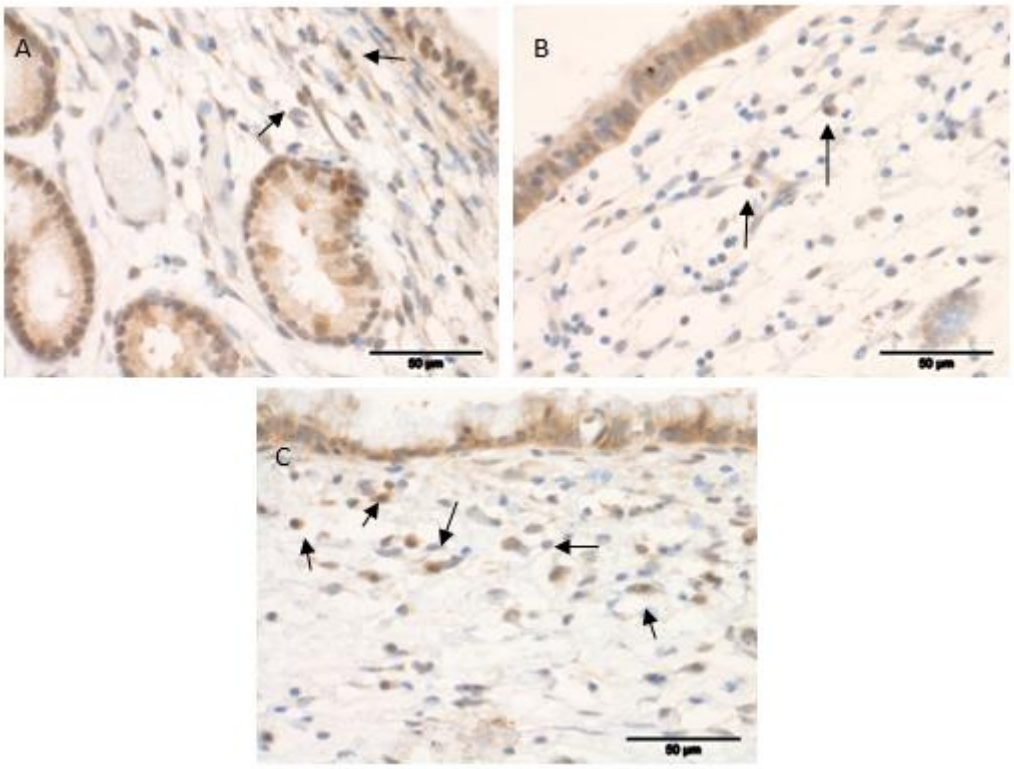

Figure 17: CD3 positive T cells are marked by arrows in a control (A), a treated (B) and a non treated (C) mare (40x magnification).

No significant difference was seen in the COX-2 expression between the control and both inseminated cycles. However, the treated group was the only one to show a grade 4 staining (Figure 18). Figure 19 shows COX-2 stained endometrium biopsies.

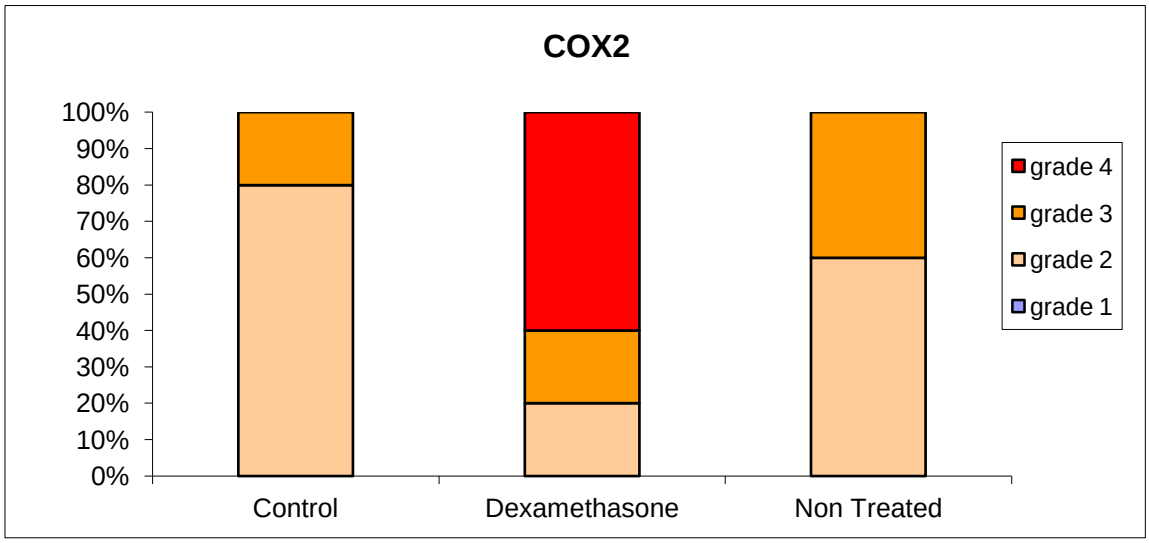

Figure 18: percentages of COX2 positive cells per microscopic field (40x) in the control, non treated and treated groups. Grades 1-4 (1: negative, 2:mild, 3:moderate, 4:marked).

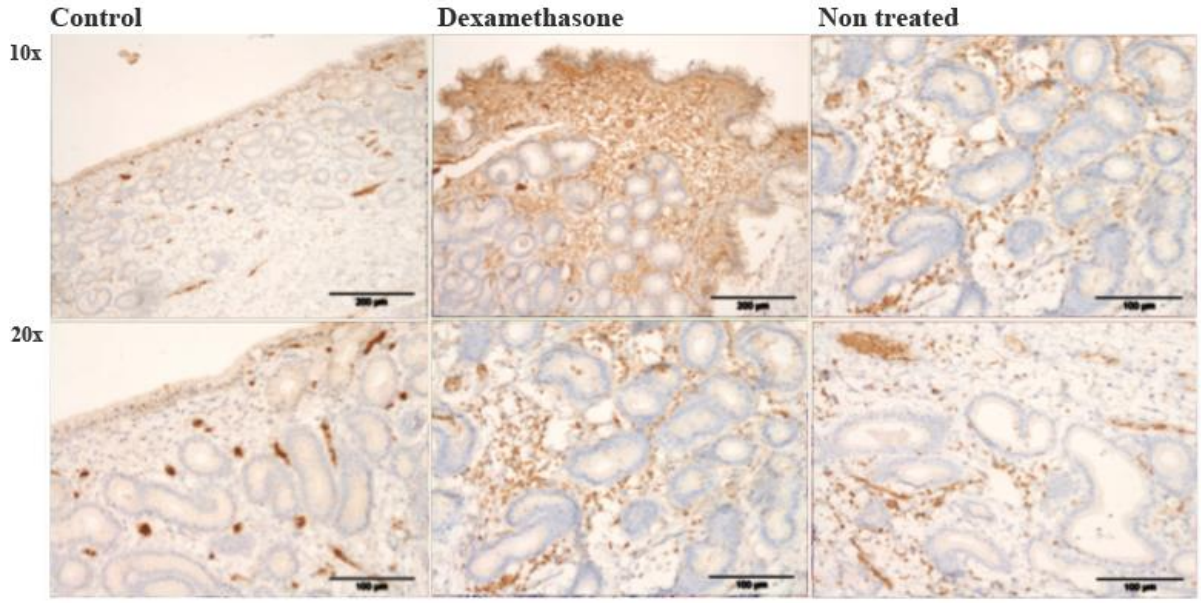

Figure 19: DAB-stained endometrium from a control, a treated and a non treated mare. COX-2 positive cells displayed in superficial, middle and deep layer.

4. Discussion and Conclusions

In this study, the effect of dexamethasone on the persistent post-breeding endometritis was analyzed. This glucocorticoid is largely used as treatment of PMIE.

Previous studies analyzed the effect of dexamethasone on the fertility with conflicting results. Bucca *et al.* (2008)demonstrated that dexamethasone decreased endometrial oedema, fluid accumulation, quality of efflux post-breeding and increased pregnancy rate in mares with 3 or more predisposing factors for PMIE. McDonnell and Watson compared the effects of dexamethasone-ampiciline and the combination of the two drugs in mares with and induced bacterial endometritis. They showed that mares treated alone with dexamethasone had a more pronounced neutrophil and plasma cell infiltration of the stratum compactum and stratum spongiosum and concluded that dexamethasone did not have any beneficial therapeutic effect in reducing the inflammation associated with endometritis compared to the antibiotic treatment alone [4]. In according with the results of Bucca *et al.* (2008) the present study showed a marked decrease of endometrial edema post insemination in the dexamethasone treated group. Samper (2007) correlated endometrial edema score and pregnancy rates and showed a significant decline in pregnancy rates, when elevated endometrial edema scores were detected at breeding time [10]. Therefore, a corticosteroid treatment may be proposed as beneficial when excessive uterine edema is present at breeding time.

However the present study failed to show an effect of dexamethasone on post-breeding intra-uterine fluid accumulation. This could be explained by the fact that in the study by Bucca *et al.* (2008) besides Dexamethasone also uterine lavage and treatment with oxytocine was used to stimulate uterine clearance. Instead in the present study no additional treatments were performed.

In the present study cytological and histological examination showed a significant increase of PMNs and T-cells (CD3+) after insemination. Therefore treatment with dexamethasone did not show to reduce the PMNs and T-cells infiltration.

Moreover dexamethasone had no effect on the LOX 5 and FLAP gene expression which mean that the synthesis of leukotriene is not modulated. However the LOX-5 positive cells seemed to be reduced in the superficial and middle layer after treatment with dexamethasone.

COX2 gene and protein expressions were not significantly influenced by dexamethasone. COX2 protein expression in dexamethasone treated mares showed a even higher, although not significant, grading of COX2 positive tissue in de the superficial and middle layer than the inseminated non treated mares. Therefore a single dose of dexamethasone at breeding time does not seems to affect the ability of the endometrium to synthesize prostaglandins.

Furthermore dexamethasone treatment did not show any effect on the expression of endometrial iNOS mRNA nor on the intra-uterine NO concentration.

In this respect, it is possible that the absence of any difference in mRNA expression for LOX5, FLAP and iNOS between the 3 groups (control, inseminated treated and

inseminated not treated) was partially due to the timing of sampling. All 3 mediators are primarily active during the acute stage of the inflammatory reaction, and it is therefore possible that sampling at 24 hours missed differences in expression levels in the immediate post-AI period. A further study with shorter AI to sampling intervals would be necessary to confirm or rule out this possibility.

In conclusions a single intravenous injection of 50 mg dexamethasone one hour before artificial insemination reduces post-insemination oedema but it does not measurably modulate other important components of the inflammatory reaction such as neutrophil influx and fluid accumulation. Since pre-breeding dexamethasone treatment also fails to suppress key elements of the major inflammatory pathways at 24 h after AI, there is still no clear explanation for how pre-AI corticosteroid treatment could improve pregnancy rates in susceptible mares.

References

1. Bucca S. *et al*, The use of dexamethasone administered to mares at breeding time in the modulation of persistent mating induced endometritis. *Theriogenology* 70 (2008) 1093-1100
2. Troesson M.H.T., Breeding-Induced Endometritis in Mares. *Vet. Clin. Equine* (2006), 22, 705-712
3. Card C., Post-breeding inflammation and endometrial cytology in mares. *Theriogenology* (2005), 64, 580-588
4. McDonnell A.M., Watson E.D. The effects of dexamethasone sodium phosphate on mares with experimentally-induced endometritis. *Journal of Equine Veterinary Science* (1993), 13, 202-206
5. Fioratt E.G *et al.* Embryo Recovery in Mares Treated with systemic Dexamethasone. *Bioloy of Reproduction* (2010) 83, poster
6. Davison W, Woof C: Comparison of different forms of cadmium as reducing agents for the batch determination of nitrate. *Analyst* (1978), 103, 403–406.
7. M. Goppelt-Struebe, D. Schaefer en A.J.R. Habenicht: Differential regulation of cyclo-oxygenase-2 and 5-lipoxygense activating protein (FLAP) expression by glucortoids in monocytic cells. *Britisch journal of Pharmacology* (1997), 122, 619-624
8. H. Welter *et al.* Expression of endothelial and inducible nitric oxide synthases is modulated in the endometrium of cyclic and early pregnant mares. *Reproduction, Fertility and Development*, (2004), 16, 689-698
9. G. Walker *et al.* Nitric-oxide Synthase (iNOS) Expression in Interferon (IFN)-γ-stimulated RAW 264.7 Cells by dexamethasone. *The Journal of Biological Chemistry*, (1997), 26, 16679-16687
10. J.C. Samper How to Interpret Endometrial Edema in Brood Mares. *Reproduction* (2007)