

DOI 10.2478/pjvs-2013-0062

Original article

Distribution of CD14+ macrophages, CD4+, CD8+ lymphocytes and mRNA expression of inducible nitric oxide synthase in the endometrium of repeat breeding cows

W. Barański¹, J. Kaleczyc², S. Zduńczyk¹, W. Podlasz²,
E. Długofęcka-Malinowska¹, T. Janowski¹

¹ Department of Animal Reproduction with Clinic,

² Department of Animal Anatomy, Faculty of Veterinary Medicine,
University of Warmia and Mazury in Olsztyn, Oczapowskiego 14, 10-719 Olsztyn, Poland

Abstract

The expression of CD14+ macrophages, CD4+, CD8+ lymphocytes and mRNA of inducible nitric oxide synthase (iNOS) was investigated in the endometrium of repeat breeders with subclinical endometritis [experimental group (EXP), n = 10] and healthy [control group (CTRL), n = 10] cows. The cows were selected on the basis of repeat breeding (3 unsuccessful inseminations), clinical and cytological examinations (> 10% polymorphonuclear neutrophils in uterine smears obtained by cytobrush). From all the cows endometrial biopsies were collected and the presence of CD14+, CD4+ and CD8+ cells in the endometrium was evaluated immunohistochemically using semi quantitative counting method. The mRNA expression of iNOS was determined using reverse transcription-PCR. In general, there were no significant differences between EXP and CTRL groups in the expression of CD4+ and CD8+ lymphocytes in all endometrial structures. In contrast, we observed a higher number of CD14+ macrophages in repeat breeding group compared to the control cows, however, this difference was slightly pronounced. CD14+ cells were detectable only in the stratum compactum and stratum spongiosum. The statistically significant ($p \leq 0.05$) higher expression of iNOS mRNA was measured in the cows with subclinical endometritis compared to the healthy animals. Our results suggest that the increased expression of CD14+ macrophages and iNOS mRNA may be associated with embryonal mortality in repeat breeding cows with subclinical endometritis.

Key words: cows, repeat breeding, macrophages, lymphocytes, iNOS

Introduction

Repeat breeding is a significant cause of reproductive wastage with great economic impact on fertility. The repeat breeder cow is a normally cyclic cow with no clinical abnormalities, which has failed to conceive after several unsuccessful inseminations (Zemjanis 1980, Gustaffsson and Emamelson 2002, Yusuf et al. 2010). Subclinical endometritis is regarded as one of the major causes of repeat breeding resulting in reduced pregnancy rate (Schmidt-Adamopolou 1978, Dogan et al. 2002, Salasel et al. 2010). This disorder is defined as a slight inflammation of the uterus that results in a significant reduction of reproductive performance with no clinical signs of endometritis (Sheldon et al. 2009). Subclinical endometritis alters the uterine environment, that is related to the enhanced embryo mortality rate (Gabler et al. 2009, Ghasemi et al. 2012). However, the exact mechanisms of early embryo loss as a consequence of subclinical endometritis are not fully understood. In this disorder the innate immune system is alerted to the presence of pathogens and the neutrophilic influx into the endometrium is the main mechanism of early response to the inflammatory agents. In addition to neutrophilic migration, other cellular components that are activated include macrophages, lymphocytes, eosinophils and mast cells (Bondurant 1999, Sheldon et al. 2009).

In embryo loss, the activation of maternal immune mechanisms plays a crucial role in determining the success or failure of a pregnancy. T lymphocytes, mainly subpopulation of CD4+ and CD8+ cells, play an important role in the development of the specific immune response during early pregnancy. Their suitable number and appropriate ratio are maintained in fertile animals, whereas their disproportion leads to embryonal mortality (Klentzeris et al. 1994, Haddad et al. 1995, 1997, Dechaud et al. 1998, Ogando et al. 2003, Oliveira and Hansen 2008). There are also a few reports considering the involvement of several cytokines such as tumor necrosis factor α (TNF α), interleukin-2 (IL-2) and interleukin-6 (IL-6) in these processes (Baines et al. 1997, Leung et al. 2000). Moreover, the detrimental role of increased macrophages and their product nitric oxide (NO) in the regulation of the early embryonal rejection have been demonstrated in the laboratory animals and humans (Haddad et al. 1995, Ogando et al. 2003, Krakowski et al. 2010). Production of NO is driven mainly by an inducible isotype of nitric oxide synthase (iNOS) related to the macrophages (Haddad et al. 1995, Bondurant 1999). The mentioned studies have been performed in rodent and human models, whereas comparable studies in the bovine have not been carried

out until now. Previous studies on the number and distribution of T lymphocytes and macrophages across the bovine uterus were focused on the estrous cycle, early pregnancy and periparturient period (Cobb et al. 1995, Leung et al. 2000, Miyoshi et al. 2002). Cobb et al. (1995) described an increase in the frequency of CD4+ and CD8+ cell populations, but not macrophages, during the late luteal and follicular phases. In another report, the differences between endometrial macrophage populations of cows with or without retained membranes have been detected (Miyoshi et al. 2002), whereas there was no such a difference between the pregnant and non-pregnant cows on day 16 after artificial insemination (AI) (Leung et al. 2000).

Therefore, the investigation on the distribution of macrophages and main subtypes of lymphocytes in endometrial layers of cows with repeat breeding and subclinical endometritis is still lacking.

In order to better understanding the mechanisms of repeat breeding in dairy cows, the aims of this study were: a) evaluation of distribution and frequency of CD14+ macrophages and CD4+, CD8+ lymphocytes in the endometrium, b) assessment of mRNA expression of inducible iNOS in repeat breeders with and without subclinical endometritis.

Material and Methods

Animals and study design

The study was carried out in one dairy herd on 120 Polish Holstein-Friesion cows, two-six years old, with an average milk yield of 7500 l. The cows were kept in a loose barn, and fed grass and maize silage, concentrates, vitamin and mineral supplements. Total mixed rotation (TMR) feeding system was used. Standard practice for the reproductive management of a herd was followed with all cows during the postpartum and breeding period.

Twenty animals with clinically healthy uterus and at least 3 unsuccessful inseminations were included. They were at 170 +/- 32 days in milk. The examination procedure included inspection of the vulva, tail and perineum, vaginoscopy, transrectal palpation and ultrasonography of the uterus and ovaries. Two groups of animals with the corpus luteum were selected: cows with subclinical endometritis (experimental [EXP], n=10) and cows without this disorder (control [CTRL], n=10). The animals were assigned to the experimental or control group on the basis of cytological examination of uterine smears (threshold >10% of polymorphonuclear neutrophils) obtained by the cytobrush method as described previously (Barański et al. 2012).

In turn, from the cows in the luteal phase of the estrous cycle two endometrial tissue biopsies were collected: the first for the immunohistochemical evaluation of CD14+ macrophages and CD4+, CD8+ lymphocytes, the second one for the analysis of mRNA of the iNOS.

Samples collection

Endometrial biopsies were collected using an endometrial biopsy instrument (Hauptner, Solingen, Germany) as described previously (Bonnet et al. 1991). After cleaning the perineum and external genitalia, the biopsy instrument was introduced into the uterus guided by trans-rectal palpation. Two biopsies were taken from the larger diameter horn. One sample was placed immediately into a 1.5 ml tube containing solution of 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The second sample was placed into a 1.5 ml tube containing preservative to maintain mRNA integrity, and transported to the laboratory.

Immunohistochemistry

The samples were washed in PB and cryo-protected in 18% sucrose in 0.1 M PB with 0.1% sodium azide, and kept for further 48h at 4°C. They were then embedded in Tissue-Tek (Miles, Elkhart, USA) and cut with a cryostat into 16 µm-thick sections. The sections were mounted (SuperFrost Plus, Menzel-Glaser, Braunschweig, Germany) and air dried at RT for 15 min. Then they were washed with phosphate buffered saline (PBS, pH 7.4), preincubated with a blocking mixture containing 10% normal goat serum, 0.1% Triton X-100, 0.1% sodium azide and 0.05 thimerosal in PBS (1 h; RT), and incubated overnight (18-20 h) at room temperature (RT) in a humid chamber with the monoclonal mouse primary antisera (anti-CD4, LifeSpan Biosciences, 1:500; anti-CD8, LifeSpan Biosciences, 1:500; anti-CD14, BioLegend, 1:200) in blocking mixture. After incubation with primary antibody, the sections were washed 3x in PBS and incubated with goat anti mouse secondary antibody (Alexa Fluor® 488, Invitrogen, 1:1000) diluted 1:500 in blocking mixture for 1 hour at RT. After 3x washing in PBS, they were counterstained with DAPI and Evans blue. Finally, the sections were washed with PBS and mounted with a mixture of glycerol and PBS (1:1).

The sections were examined with a Axio Imager.Z2 epifluorescence microscope (Zeiss, Germany)

and a LSM 700 confocal microscopy system (Zeiss, Germany). The fluorophores were excited by pig-tail-coupled solid-state lasers at 405 nm for DAPI, 488 nm for Alexa488 and 555 nm for Evans blue. Stacks of images were compiled to produce maximum-intensity projection images with ZEN 2009 software (Zeiss, Germany).

To estimate the size of the immune cell population 5 stained sections comprising studied layers of the endometrium from each biopsy were analyzed. The frequency of the cells was assessed in areas covering 0.09 mm² for each picture, and the size of the population of the immunoreactive (IR) cells were defined as: (-) absence of IR cells, (+) 1-5 IR cells, (++) 6-20 IR cells, (+++) 21-50 IR cells, (+++++) more than 50 IR cells.

RNA extraction

The tissues were homogenized in tubes containing Lysing Matrix D beads using MP FastPrep homogenizer. RNA was extracted using TRI Reagent® (T9424, Sigma-Aldrich) according to the manufacturer's instructions. Evaluation of RNA concentration was done spectrophotometrically (NANO Drop 2000, OD 260/280). One microgram of total RNA from each sample was reverse transcribed using a QuantiTest Reverse Transcription Kit (205311, Qiagen, Hilden, Germany) as described in the supplier's protocol. The generated cDNA was stored at -20°C until use.

Real-time RT-PCR

Real-time RT-PCR assays were performed in a 7900HT Real Time PCR System (Applied Biosystems™, Warrington, UK), using the default thermocycler program for all genes: 10 min pre-incubation at 95°C was followed by 40 cycles for 15 sec at 95°C and 1 min at 60°C. A further dissociation step (15 sec at 95°C, 30 sec at 60°C and 15 sec at 95°C) ensured the presence of a single product. In each real time assay, target gene and HKG (housekeeping gene, GAPDH) were run simultaneously. All reactions were carried out in duplicate wells on a 96 well optical reaction plate (Applied Biosystems, ref. 4306737, UK) in 20 µl reaction volume: 9 fl water with forward primer (160 nM) and reverse primer (160 nM); 10 fl Power SYBER Green Master Mix (Applied Biosystems, Ref. 4367659, UK) and 1 fl of 4 time diluted cDNA. The primer pairs are shown in Table 1.

Table 1. Gene transcripts, primer sequences, amplicon length and GenBank accession number.

Gene	Primer sequence	Amplicon length (bp)	GenBank accession no.
GAPDH (Bos taurus)	CACCCTCAAGATTGTCAGCA/ GGTCATAAGTCCCTCCACGA	103	BC102589
iNOS	GGTGGAAGCAGTAACAAAGGA/ GACCTGATGTTGCCGTTGTTG	230	AF340236

Table 2. Distribution and relative frequency of populations of CD4+, CD8+ and CD14+ cells in different layers of the endometrium in the CTRL and EXP groups. LE – luminal epithelium, GE – glandular epithelium, SC – stratum compactum, SS – stratum spongiosum.

	LE		GE		SC		SS	
	CTRL	EXP	CTRL	EXP	CTRL	EXP	CTRL	EXP
CD4	+	+	+	+	++++	++++	++	++
CD8	++	++	++	++	+++	+++	++	++
CD14	-	-	-	-	+	++	+	++

For the relative quantification of mRNA expression levels (target gene versus housekeeping gene), miner software was used <http://www.miner.ewindup.info/version>.

Statistical analysis

Expressions of mRNA are shown as a mean \pm SEM. The statistical significance of differences in iNOS mRNA expression between the both groups was analyzed by one-tailed nonparametric Mann-Whitney test (GraphPAD PRISM, Version 4.00, GraphPad Software, San Diego, CA, USA). The level of significance was set at $p < 0.05$.

Results

Distribution of macrophages and lymphocytes

The results of immunohistochemical investigations dealing with populations of macrophages and lymphocytes are depicted in Table 2.

CD4

There were no significant differences in the number of CD4+ cells in different layers of the endometrium between the studied groups. Single CD4+ cells were observed in the luminal and glandular epithelium (Fig. 1A). Numerous CD4+ cells were distributed in the stratum compactum of

the endometrium (Fig. 1A, B) while in the stratum spongiosum they were slightly less frequent (Fig. 1C, D). CD4+ cells were quite often observed to occur near the glandular epithelium, surrounding lumen of the uterine glands (Fig. 1C). They often formed large clusters located mainly in the cortical layer of the endometrium. These clusters were found in both the experimental as well as the control group (Fig. 1B).

CD8

There were no significant differences in the number of CD8+ cells in particular layers of the endometrium between the studied groups. CD8+ cells were much more often encountered in the luminal (Fig. 2A, B) and glandular epithelium than CD4+ cells (Fig. 2C, D). CD8+ cells were quite numerous in the stratum compactum (Fig. 2A, B) while in the stratum spongiosum they were less frequent (Fig. 2C, D).

CD14

The luminal and glandular epithelium in the CTRL and EXP groups contained no CD14+ cells. Small numbers of these cells were distributed in the stratum compactum (Fig. 3A, B) and stratum spongiosum (Fig. 3C, D). It should be noted that in these structures the CD14+ cells were slightly more numerous in the experimental than in the control animals.

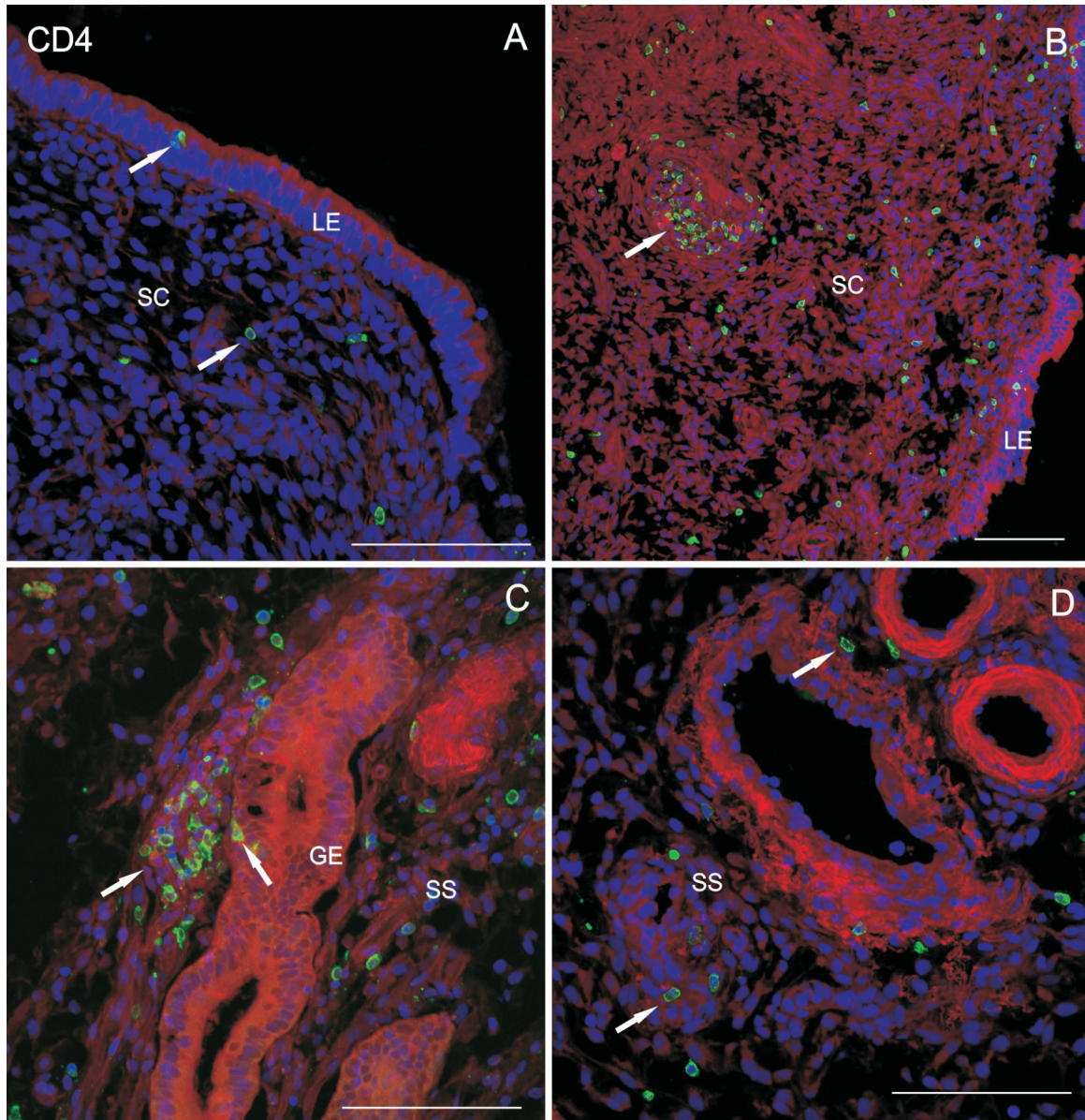


Fig. 1. Immunohistochemical staining of the endometrial biopsies with CD4 antibody (green), counterstained with DAPI (blue) and Evans blue (red). A, C – CTRL group, B, D – EXP group. Arrows show CD4+ cells. LE – luminal epithelium; GE – glandular epithelium; SC- stratum compactum; SS – stratum spongiosum. Scale bar 100 µm.

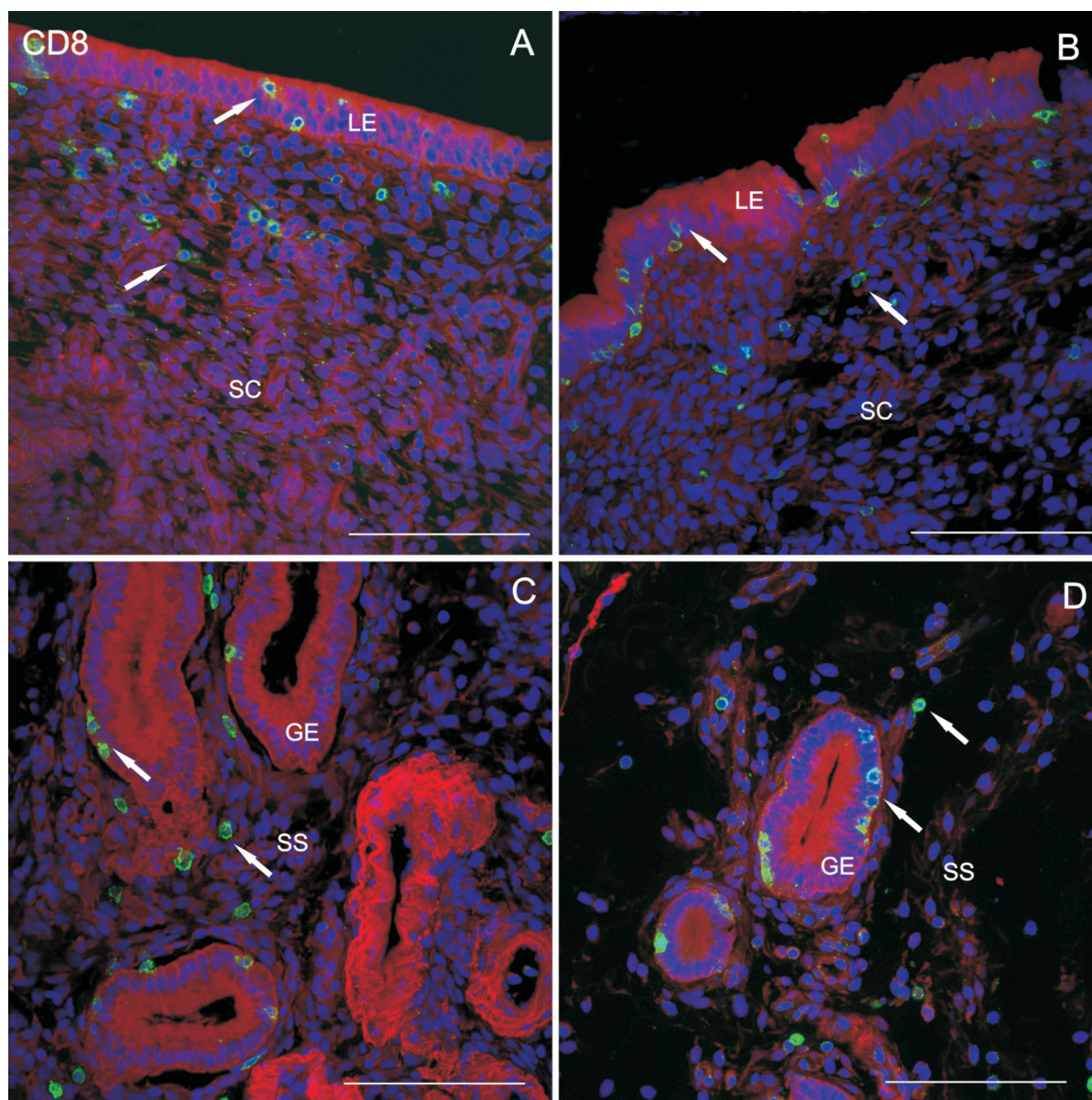


Fig. 2. Immunohistochemical staining of the endometrial biopsies with anti-CD8 mAb (green), counterstained with DAPI (blue) and Evans blue (red). A, C- CTRL group, B, D – EXP group. Arrows show CD8+ cells. LE – luminal epithelium; GE – glandular epithelium; SC- stratum compactum; SS – stratum spongiosum. Scale bar 100 μ m.

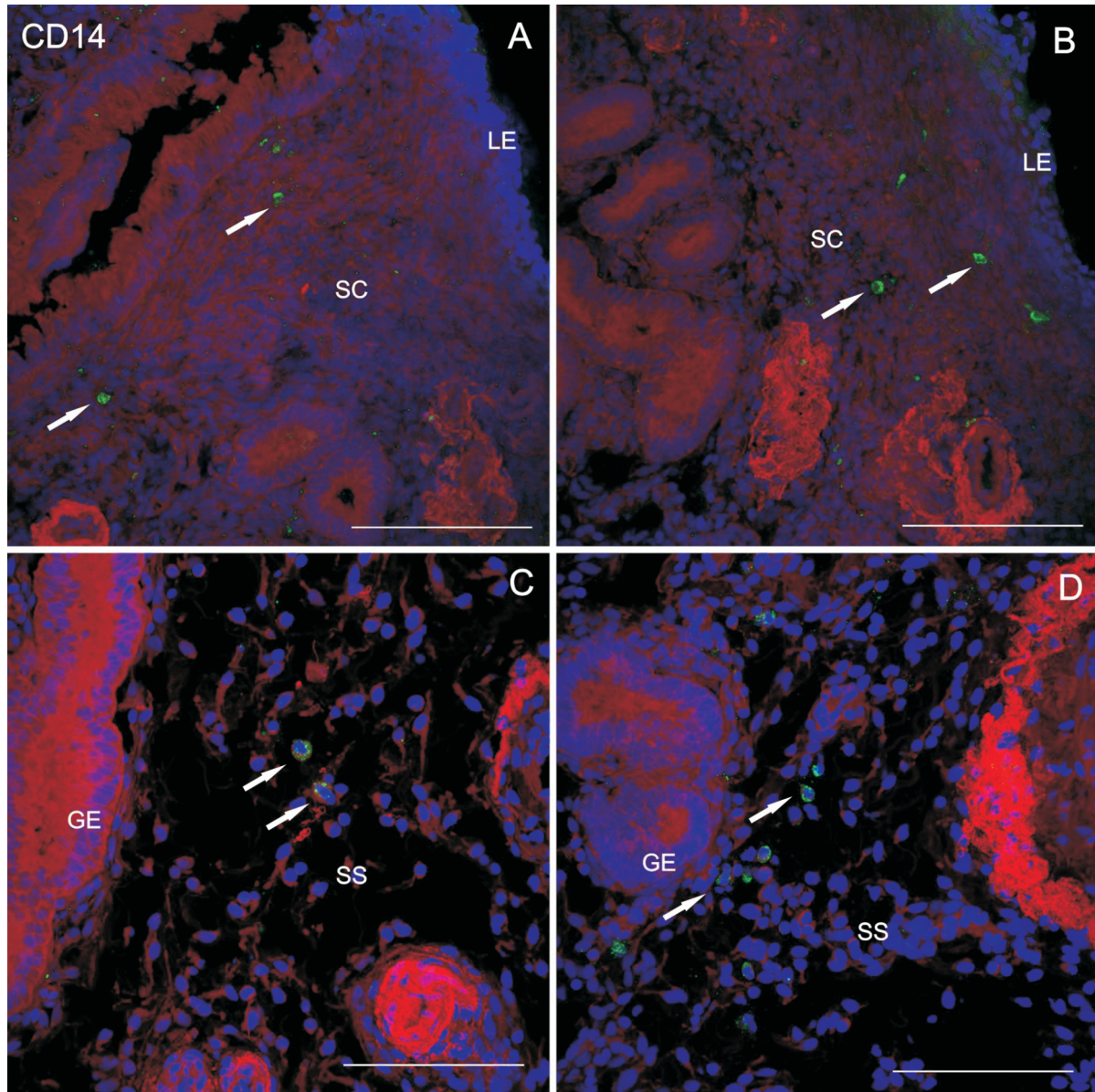


Fig. 3. Immunohistochemical staining of the endometrial biopsies with anti-CD14 mAb (green), counterstained with DAPI (blue) and Evans blue (red). A, C – CTRL group, B, D – EXP group. Arrows show CD14+ cells. LE – luminal epithelium; GE – glandular epithelium; SC – stratum compactum; SS – stratum spongiosum. Scale bar 100 μ m.

Expression of iNOS mRNA

The mRNA expression of iNOS was significantly higher ($p < 0.05$) in the repeat breeding cows with subclinical endometritis compared to samples from healthy cows (Fig. 4).

Discussion

Our study revealed a slightly higher density of macrophages in the experimental than in the control cows. The present study is one of a few reports (Cobb et al. 1995, Leung et al. 2000, Oliveira and Hansen

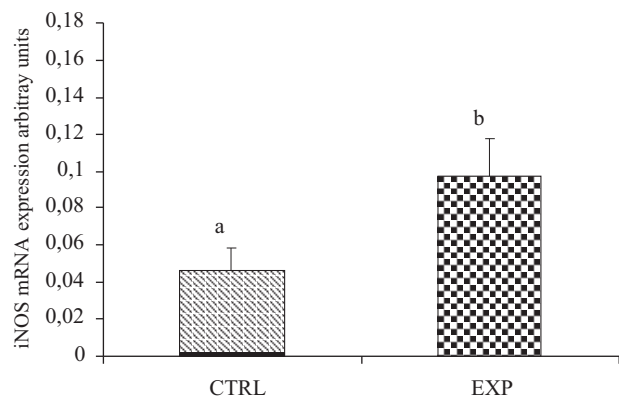


Fig. 4. Expression of iNOS mRNA in the endometrium of repeat breeding cows in CTRL and EXP groups. a-b difference statistically significant at $p < 0.05$

2005) evaluating the number and distribution of macrophages and T lymphocytes across the bovine endometrial structures. A novel aspect of our research was the use of cows with or without subclinical endometritis affected by repeat breeding. In contrast to macrophages we did not find any difference in the endometrial expression of both lymphocyte subtypes between the cows with endometritis and healthy animals. This finding is in line with the only available data in pregnant and non-pregnant cows on day 16 after AI (Leung et al. 2000). Nevertheless, the distribution of these immune cells across the endometrial structures agrees with earlier reports originating from studies in rodents, humans and cows (Cobb et al. 1995, Dechaud et al. 1998, Ogando et al. 2003). The fluctuation of CD4+ and CD8+, but not CD14+ cells numbers, has been documented in cyclic cows. The significant increase of the number of these cells was found during late luteal and follicular phase (Cobb et al. 1995). In the present study, all experimental and control cows were in the luteal phase, thus this factor did not influence the results obtained. Therefore, our observation suggests an involvement of enhanced macrophages in the repeat breeding in cows with subclinical endometritis.

The altered uterine environment as a consequence of subclinical endometritis has been suggested to be an important cause of embryo loss in cows (Gabler et al. 2009, Ghasemi et al. 2012). Moreover, earlier reports performed on rodents and humans confirmed significant relationship between the activation of macrophages and enhanced embryo mortality (Haddad et al. 1995, Baines et al. 1997, Dechaud et al. 1998). Thus, our results are in line with these findings and seem to suggest a potential role of macrophages in embryo rejection leading to repeat breeding in cows.

However, the functional link between the presence of these immune cells in the bovine endometrium and early embryonic mortality is not clear until now. In the rat and mouse, the association of maternal macrophages with embryo loss was mediated by NO (Haddad et al. 1995, 1997, Ogando et al. 2003). These authors found enhanced mRNA expression of inducible iNOS in the endometrium of animals with early embryonic loss, whereas a selective inhibitor of iNOS, such as aminoguanidine, increased an average litter size (Haddad et al. 1995, 1997, Ogando et al. 2003). The inducible iNOS is the main isoenzyme governing the production of NO, which is considered to be an important mediator of cellular interactions and a potent regulator of many local processes (Haddad et al. 1995). iNOS is typically expressed at sites of inflammation, produces large amounts of NO for a prolonged time (Ekerhovd et al. 2000) and is related to

the activation of macrophages (Dechaud et al. 1998). In this context, our findings regarding a significantly higher expression of iNOS in repeat breeders with subclinical endometritis correlate with those dealing with enhanced macrophages density in the same animals and strongly suggest a possible involvement of a macrophage-NO complex in the rejection of an embryo during repeat-breeding in cows.

Interestingly, we recorded the macrophages only in the subepithelial structures of the endometrium. This finding is in line with earlier reports regarding the distribution of these cells across the bovine endometrium showing their presence only in the stroma (Cobb et al. 1995, Leung et al. 2000). However, it has been suggested that the stromal cells, similarly to the epithelial cells, have an important immune function and fulfill an important physiopathological role (Sheldon et al. 2009).

Summarizing, our results prove a macrophage-NO pathway in the regulation of early embryonic loss in the repeat-breeding cows with subclinical endometritis. It is difficult to interpret, why in these cows we recorded only a slight increase in macrophages frequency, while at the same time a significantly higher expression of iNOS was found. Moreover, in contrast to our study, Leung et al. (2000) did not record any difference in the endometrial expression of macrophages in pregnant and non-pregnant cows on day 16 after AI. However, it has been suggested that a threshold of significance regarding the number of these immune cells to define fertility and infertility is difficult to set up (Dechaud et al. 1998, Leung et al. 2000, Alghamdi et al. 2005). It should be also stressed, that the general pathogenesis of repeat-breeding is a very complex phenomenon, whereas, our study considers only a part of those mechanisms.

In conclusion, our study suggests a possible role of macrophages and iNOS in the repeat-breeding in cows with subclinical endometritis.

Acknowledgements

This research was supported by grant MNiSW 544/N-COST/2009/0

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