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Expression of indoleamine 2,3-dioxygenase 1 as transcript and protein in the healthy and diseased equine endometrium

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ABSTRACT

The enzyme indoleamine 2,3-dioxygenase 1 (IDO1) acts immunomodulatory and restricts bacterial growth. In the uterus of women and mice, it likely contributes to tissue homeostasis and disease pathogenesis. Pregnancy failure in mares is often caused by endometritis and endometrosis. The pathogenesis of nonsuppurative endometritis and endometrosis is still uncertain. To the authors' knowledge, no information on IDO1 expression in the equine endometrium is published. Aim of this study was to examine the presence of IDO1 as transcripts and proteins in the healthy and diseased endometrium of 25 mares and to determine its cellular expression. By PCR, IDO1 transcripts were detected in healthy (3 mares) and diseased endometria (22 mares). Western blot on 15 samples showed the concurrent presence of IDO1 proteins. Immunohistochemistry revealed its expression in macrophages and epithelial cells. Endometria of 21 mares showed an intense staining of glandular epithelia, whereas glands of the remaining 4 mares were negative or contained only few positive cells. Tissue samples of all mares showed a minimal to mild IDO1 expression in the surface epithelium and glandular ducts. Quantification of immunohistochemistry on biopsies of 6 mares collected at different stages of the same endometrial cycle indicated that the IDO1 expression is not influenced by the endometrial cycle. This study confirmed IDO1 expression also in the equine endometrium and suggests an immunomodulatory role of uterine macrophages and epithelial cells. A markedly reduced glandular IDO1 expression as detected in 4 mares may be associated with alterations of uterine immune defenses.

1. Introduction

Dysregulation of uterine immune mechanisms is an important cause of pregnancy failure (Girling and Hedger, 2007; Kannaki et al., 2011). In mares, endometrial diseases are the most important cause of subfertility and have therefore an economic significance for the horse breeding industry. These include different forms of endometritis, periglandular fibrosis (endometrosis) and angiosclerosis (Schoon et al., 1997). It has been hypothesized that immunological mechanisms are involved in the pathogenesis of nonsuppurative endometritis and endometrosis.

The equine endometrium is equipped with the main components of the innate and adaptive immune system including Toll-like receptors (Schöniger et al., 2017), antimicrobial peptides such as beta-defensin (Schöniger et al., 2013) and different lymphocyte and macrophage subsets (Rudolph et al., 2017).

Most tissues also harbor immunomodulatory mechanisms, e.g. the enzyme indolamine 2,3-dioxygenase 1 (IDO1). This enzyme degrades tryptophan and downregulates immune activation and inflammation (Curti et al., 2009). Tryptophan depletion induces immune tolerance by inhibiting effector T cells and activating regulatory T cells (Curti et al., 2009). Since tryptophan is required for bacterial proliferation, its depletion inhibits bacterial growth (Curti et al., 2009). In the pregnant uterus, IDO1 is involved in the maintenance of materno-fetal tolerance (Mellor et al., 2001). IDO1 has been detected in the pregnant and non-pregnant uterus of women (Sedlmayr et al., 2002) and mice (Hemmati et al., 2009; Jeddi-Tehrani et al., 2009).

The designations IDO and IDO1 are used as synonyms, since after the discovery of IDO a closely related enzyme was detected and named as IDO-like protein or IDO2 (Ball et al., 2007).

To the authors' knowledge, no data about the expression of IDO1 in the equine uterus are published.

The aim of the present study was to examine whether IDO1 as transcript and protein is expressed in the equine endometrium and in case of its detection, to reveal its cellular expression pattern. To receive first insights into IDO1 function in the equine endometrium we aimed to investigate if its expression is influenced by the stage of the endometrial cycle and/or by the presence of endometrial diseases. Results

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of this investigation will provide further information on immune mechanisms of the equine endometrium as well as their possible involvement in reproductive failure. This may also lead to the development of new therapeutic strategies.

2. Material and methods

2.1. Animals and tissue samples

From 25 mares, uterine tissue samples were collected 1–3 h after their death. These mares were euthanized or had died spontaneously because of different causes. Samples of the entire uterine wall measuring 1.5 × 0.5 × 0.5 cm and endometrial specimens with a size of 0.5 × 0.5 × 0.5 cm were obtained from each mare. The former samples were fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin wax and used for histology and immunohistochemistry. The latter samples were immediately snap frozen in fluid nitrogen and stored at −80 °C for PCR investigations and Western blot.

From 6 mares, endometrial biopsies were collected repeatedly over the course of the same endometrial cycle (days 0, 5, 10, 13, 16, 19 and 21; day 0 is defined as the ovulation day). These were fixed in 10% buffered formalin, embedded in paraffin wax and routinely processed for histology and immunohistochemistry.

2.2. Histology

The functional morphology of endometrial glands as well as the presence of endometrial alterations, i.e. endometritis, endometrosis, angiosclerosis and/or glandular maldifferentiation were determined in all samples or biopsies as described (Schöniger et al., 2013; Schoon et al., 1997). In regard to the biopsies of the 6 mares that were collected repeatedly over the course of the same endometrial cycle it was confirmed that their histological features corresponded to those that are diagnostic for the examined days of the endometrial cycle (Kenney, 1978).

2.3. RT-PCR for the detection of IDO1 transcripts

The predicted mRNA sequences were received from NCBI Genbank (www.ncbi.nlm.nih.gov/Genbank). For the detection of IDO1, three different primer sets were used that were designed with the program primer 3 (http://bioinfo.ut.ee/primer3) (Table 1). In parallel, the housekeeping gene glycerinaldehyde-3-phosphat dehydrogenase (GAPDH) was amplified; the primer pair was obtained from the literature (Klein et al., 2011). For isolation of the RNA from endometrial samples of 17 mares (Nos. 2–4, 6, 8–10, 13–16, 18, 20–22, 23–24) the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) was used. The RNA of the remaining endometrial tissue samples was extracted with Peq-Gold RNA Pure™ according to the manufacturer's instructions (Peqlab Ltd., Sarisbury Green, UK). The reverse transcription of mRNA to first-strand cDNA was performed with the Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT) (Promega, Madison, WI, USA) and random primer (Invitrogen, Carlsbad, CA, USA) by a standardized method. For cDNA amplification of IDO1 and GAPDH 25 µl of the reaction mixture and the PTC 200 Thermocycler (MJ Research, St. Bruno, Canada) were used. The reaction mixture contained 1 µl of the generated cDNA, 0.2 mM dNTP mix (Roche Diagnostics GmbH, Mannheim, Germany), 0.4 µM sense and antisense primers (Eurofins MWG Operon, Ebersberg, Germany), PCR buffer with 1.5 mM MgCl (Roche Diagnostics GmbH) and 0.5 U Taq DNA polymerase (Roche Diagnostics GmbH). In the negative controls, the cDNA was substituted by diethyldicarbonate treated water. For all primer pairs the following thermocycling conditions were applied: an initial denaturation (4 min, 94 °C), 40 cycles of denaturation (30 s, 94 °C), annealing (60 s, 56 °C) and extension (30 s, 72 °C) and final elongation (10 min, 72 °C) followed by cooling to 4 °C. The amplified products were separated in a 1.5% agarose gel using Gene Ruler TM 50 bp/100 bp DNA ladder (Fermentas, St. Leon-Rot, Germany) as molecular markers. They were stained with Gel Red Nucleic Acid Stain (Biotium, Brussels, Belgium) and detected in a Bio-Rad Detection System (ChemiDocXRS, UV application).

In the RNA samples obtained by the Qiagen RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) the genomic DNA was removed by the genomic DNA eliminator column of this kit. To rule out contamination with genomic DNA in RNA isolates obtained by the application of Peq-Gold RNA Pure™ a mock reverse transcription containing all reagents except the reverse transcriptase was performed on 5 representative RNA isolates (Nos. 5, 7, 11, 12, 17). No amplification products were obtained. The mRNA quality of representative RNA isolates from 8 endometrial tissue samples (Nos. 4, 8, 10, 13, 19, 20, 21, 23) was measured with a bioanalyzer (Agilent 2100 Bioanalyzer). The obtained RNA integrity numbers ranged from 8.1–10.

2.4. Western blot for the detection of IDO1 proteins

Tissue lysates were prepared from native tissue samples of 15 representative mares (1–3, 6, 8, 12, 13, 15, 16, 18, 19, 21, 22, 24, 25); as positive controls, mouse uterus and epididymis (Dai and Zhu, 2010) were used. For protein extraction, pulverized frozen native tissue samples were dispensed in 1000 µl homogenisation buffer by the use of a highly efficient disperser (Ultra-thurrax, IKA T10 basic homogenizer workcenter) and centrifugated (2 × 15 min, 13,200 rpm) at 4 °C. The protein content of the supernatant was determined using a commercial kit based on the Bradford assay (Coomassie Plus Protein Assay Reagent, Thermo Scientific, Rockford, USA).

For protein denaturation, per sample 10 µg protein were mixed with modified Laemmli buffer (Laemmli, 1970) to obtain a sample size of 15 µl and incubated for 5 min at 95 °C.

Samples were subjected to electrophoresis using a ready gel (Mini Protean TGX Stain Free Gel 12%; Bio Rad) and a molecular marker (Roti Marker Western Set, Carl Roth GmbH + Co. KG, Karlsruhe, Germany).

Proteins were transferred onto nitrocellulose membranes with a pore size of 0.2 µm (Bio-Rad Laboratories, Munich, Germany). After a blocking step with 5% non-fat-dry milk for 1 h at room temperature

Table 1
Primer pairs used for the detection of indoleamine 2,3-dioxygenase 1 (IDO1) and glycerinaldehyde 3-phosphat dehydrogenase (GAPDH) in the equine endometrium.

Gene	Primer designation	Primer sequence	Amplicon size (bp)	Anneal-ing temp.	Genbank accession
IDO1	IDO1–1	F: TGCTGGTGAAAAATCTGCTG R: GGTGTGCAGGAATCACAATG	233	56 °C	XM_014736538.1
IDO1	IDO1-2	F: TGCTGGTGAAAAATCTGCTG R: TGAGGGGCCTGACTCTAATG	100	56 °C	XM_014736538.1
IDO1	IDO1-3	F: CATTGTGATTCCTGCACACC R: CATGACATCAGTGCCTCCAG	97	56 °C	XM_014736538.1
GAPDH	GAPDH	F: AGAAGGAGAAAGGCCCTCAG R: GGAAACTGTGGAGGTCAGGA	88	54 °C	NM_001163856.1

F: forward primer; R: reverse primer; bp: base pairs; temp: temperature.