The conceptus regulates tryptophanyl-tRNA synthetase and superoxide dismutase 2 in the sheep caruncular endometrium during early pregnancy

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Abstract

Conceptus-derived paracrine signals play crucial roles in the preparation of a uterine environment capable of supporting implantation and development of the conceptus. However, little is known about the regulation of endometrial tryptophanyl tRNA synthetase (WARS) and manganese superoxide dismutase (SOD2) protein expression by the implanting and post-implanting conceptus. We hypothesized that the conceptus-derived signals favourably influences uterine environment for implantation through regulation of WARS and SOD2 expression in ovine caruncular endometrium. To test this hypothesis, WARS and SOD2 protein and mRNA expression was determined in caruncular endometrial tissues of unilaterally pregnant ewes at implantation (day 16) and post-implantation (day 20) periods. WARS protein expression increased in caruncular tissues of the gravid uterine horns compared with the non-gravid uterine horns on days 16 and 20 of pregnancy. There were no changes in SOD2 protein expression between the gravid and non-gravid uterine horns, irrespective of the day of pregnancy. On day 16 of pregnancy, there were no differences in WARS and SOD2 mRNA expression between the gravid and non-gravid uterine horns but expression of both genes was higher in the gravid uterine horns when compared with the non-gravid uterine horns on day 20 of pregnancy. In conclusion, the use of the unilaterally pregnant ewe model provides for the first time firm evidence that the early implantation and post-implanting conceptus-derived signals up-regulate WARS protein expression within the caruncular endometrium. Further studies are necessary to identify these signalling molecules and to understand mechanisms whereby they exert paracrine action within the endometrium.

Keywords: Sheep; Endometrium; Tryptophanyl tRNA synthetase; Manganese superoxide dismutase; Early pregnancy

1 Introduction

The development of the conceptus (embryo and associated extraembryonic membranes) in ruminants depend upon successful attachment of the outer covering embryonic membrane, the trophectoderm, to the uterine caruncular endometrium. This leads to the formation of the placental interface between mother and offspring (Imakawa et al., 2004). The extraembryonic membranes play important roles in the establishment of pregnancy by producing paracrine signals that affect uterine function and consequent endometrial secretion of proteins into the uterine microenvironment. This facilitates attachment, invasion and survival of the conceptus (Barnes, 2000; Vigano et al., 2003). However, little is known about the mechanisms of early pregnancy signals responsible for the cross-talk between the extraembryonic membranes and the uterine endometrium during early pregnancy. This knowledge deficit is surprising given that early pregnancy failure in sheep (Dixon et al., 2007) and cattle (Diskin and Morris, 2008) reduces reproductive performance and lowers production and economic efficiency.

Several endometrial genes expressed in response to the conceptus play important roles in the establishment of pregnancy in ruminants (Walker et al., 2010). Cytoplasmic tryptophanyl tRNA synthetase (WARS) and mitochondrial manganese superoxide dismutase (SOD2) are important regulators of endometrial function. WARS catalyses the aminoacylation of tRNA(trp) with tryptophan and plays central role in protein synthesis (~~. Uncited referencesHansen et al., 1988~~

and Sallafranque et al., 1986Sallafranque et al., 1989). The expression of mRNA encoding WARS increases in the uterine decidual uterine tissue during normal human pregnancy (Kudo et al., 2004). The expression of endometrial WARS mRNA is higher in of pregnant cows on day 17 than on the matching the oestrous cycle day (Walker et al., 2010). SOD2 is the first antioxidative defence against reactive oxygen species (ROS)-induced mitochondrial oxidative damage (Orrenius et al., 2007). It has been proposed that SOD2 is important in the regulation of human (Sugino, 2007) and sheep (Al-Gubory and Garrel, 2012) endometrial function. Upregulation of WARS and SOD2 in the pregnant endometrium suggests that these genes contribute to the establishment of pregnancy. Furthermore, recent proteomic analysis of the sheep endometrium revealed that the expression of cytoplasmic WARS and SOD2 markedly increases in the caruncular endometrial tissues at the time of conceptus attachment (day 16) compared with the matching stage of the oestrous cycle (Al-Gubory et al., 2014). Upregulation of WARS and SOD2 protein expression in sheep caruncular endometrium in response to the conceptus during early pregnancy may be an important mechanism to ensure the establishment of pregnancy.

The unilateral pregnant sheep model (Moor and Rowson, 1966) enables changes in the expression of endometrial genes in the presence of conceptuses to be studied during early pregnancy (Sharif et al., 1989; Sandra et al., 2005). In sheep, cellular contact between conceptus trophectoderm and epithelium of the caruncular areas occurs on day 16 of post-mating, a dynamic process completed by day 22 of pregnancy (Spencer et al., 2004). The caruncular endometrium is vital for attachment, development and survival of the conceptus and the onset of placental formation. Therefore, understanding the regulation of key proteins in these endometrial structures is essential to enhance our knowledge about the relationship between the ovine conceptus and endometrium early in pregnancy. As WARS and SOD2 have been gaining increasing attention in developmental biology, we used the unilateral pregnant sheep model to determine the specific role of the conceptus in the regulation of endometrial WARS and SOD2 expression early in pregnancy. Gelsolin (GSN) expression is down-regulated in the sheep caruncular endometrium at the time of conceptus attachment when compared with matching day of the oestrous cycle (Al-Gubory et al., 2014). Therefore, this oestrogen-responsive endometrium gene (Punyadeeraa et al., 2005) is a good candidate to demonstrate the reliability of the model used in our study and to determine the specific role of the conceptus in the regulation of specific proteins of interest.

We hypothesized that the conceptus influences the uterine environment favourably for conceptus attachment via the regulation of WARS and SOD2 expression in ovine caruncular endometrium. To test our hypothesis, unilateral pregnant ewes, with functional ovaries, were employed to compare WARS, SOD2 and GSN transcript and protein expression in caruncular endometrial tissues collected from the gravid and non-gravid uterine horns on days 16 and 20 of pregnancy corresponding to early conceptus attachment and post-attachment periods, respectively.

2 Materials and Mmethods

2.1 Animals and surgery

All procedures relating to care and use of animals were approved by the French Ministry of Agriculture according to the French regulation for animal experimentation (authorization no., 78-34). Préalpes-du-Sud breed (18 months of age) were used in this study. Throughout the experiment, the ewes were housed under conditions of natural day-length and temperature, fed straw and hay daily and had free access to mineral licks and water. In the present study, a unilaterally pregnant model (Bazer et al., 1979) was prepared as described previously (Dunlap et al., 2008). Briefly, ewes were initially anesthetized with a mixture of pentobarbital (Sanofi, Paris, France) and thiopentone (Abbott, Aubervilliers, France). After endotracheal intubation, general anaesthesia was maintained by constant inhalation of a mixture of oxygen and halothane. Reproductive organs were exposed via midventral laparotomy, and one ovary, irrespective of the presence or absence of CL, was removed and the uterine horn at the side of removed ovary was ligatured near to the uterine horns bifurcation so that after mating the conceptus is confined to the uterine horn ipsilateral to the newly formed CL. All ewes were injected with penicillin (10 IU/day) for three consecutive days after surgery.

2.2 Animal oestrus synchronisszation and mating

All the ewes were allowed three weeks to recover from surgery. They were then treated for 14 days with intravaginal sponges containing 40 mg fluorogestone acetate (Intervet, Angers, France). Immediately after removal of the sponge, each ewe received s an intramuscular injection of 400 IU of equine chorionic gonadotropin (eCG, Intervet). Mating was performed at the time of the synchronized oestrus with fertile rams of the same breed, at an interval of 12 h.

2.3 Tissue collection

All the ewes were slaughtered at a local abattoir in accordance with protocols approved by the local institutional animal use committee at the Institut National de la Recherche Agronomique (INRA, Jouy-en-Josas, France). The reproductive tracts were collected and immediately transported to the laboratory. Pregnant ewes were randomly allocated for slaughter on days 16 (*n = 4 ewes) and 20 (n* = 4 ewes) and 20 (*n* = 4 ewes) of pregnancy corresponding to the period of implantation of the conceptus and the early post-implantation period, respectively. The stages of pregnancy were confirmed by the presence and the morphology of the conceptus in uterine flushings. Caruncular endometrial tissues were collected from the gravid horn (ipsilateral uterine horn) and non-gravid horn (contralateral uterine horn) of each ewe. Immediately after dissection, tissues were snap-frozen in liquid nitrogen and stored at −80 °C until processed.

2.4 RNA isolation and quantification

Before quantitative real time polymerase chain reaction (qRT-PCR), total cellular RNA was isolated from frozen caruncular endometrial tissues using Trizol reagent according to the manufacture's recommendations. The concentration of RNA (ng/μl) in each sample was quantified by NanoDrop ND-1000 spectrophotometer at 260 nm. RNA 6000 Nano chip (Agilent Technologies) was employed to check the integrity of extracted RNA. In this assay, the ratio of ribosomal RNA (18S/28S) is determined, indicating

the integrity of the isolated RNA samples.

2.5 Quantitative real time polymerase chain reaction (qRT-PCR)

DNA sequences of the relevant genes were acquired from GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez) and used to design oligonucleotide primers to perform qRT-PCR using Primer 3 software (http://primer3.sourceforge.net/). Primer-Blast was carried out using (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find out specificity of primers for the desired gene. Primer sequences for qRT-PCR are indicated in Table 1. Reverse transcription (RT) was carried out using a Transcriptor First Strand cDNA Synthesis Kit with random hexamers (Roche Products Ltd, Hertfordshire, UK). A Mastermix of the reagents was made for the desired numbers of the reactions. 1 µl of each RNA samples was combined with 2 µl of random hexamer, centrifuged at 2000 rpm for 2 min and incubated in Thermocycle Eppendorf Polymerase Chain Reaction (PCR) machine for 10 min at 65 °C to ensure denaturation of RNA secondary structures. 7 µl of Mastermix and appropriate RNase free water were added to mixture of RNA and hexamer (for each reaction), centrifuged at 2000 rpm for 2 min and incubated in PCR machine at the following temperature cycles: 25 °C for 10 min (incubation), 55 °C for 30 min (reverse transcription) and 85 °C for 5 min (enzyme inactivation). Then, qRT-PCR was performed for samples with and without reverse transcriptase for each gene in duplicate. The amplified final products of qRT-PCR were electrophoresed on 2% agarose gel.

Table 1 Primer sequences used for quantitative RT-PCR analysis.

Genes	Forward primer	Reverse primer
WARS	AGCATCGCTGCTCAGGGGGA	TGGCATCCAGGCCCTCACCA
SOD2	AGCCCCAACGGTGGTGGAGA	AGCCAAGCCAACCCCAACCG
GSN	CTAATCGTGACCGGCGGAC	CAGACCAATAGTTGTCATCCCAGC
CYP	CATTCTGAAGCATACAGGTCCTG	TCCATGGCTTCCACAATATT

~~Abbreviations: WARS, C~~Abbreviations: WARS, cytoplasmic tryptophanyl tRNA synthetase; SOD2, mitochondrial manganese superoxide dismutase; GSN, Gelsolin; CYP, cyclophilin.

2.6 Western blot

Caruncular endometrial tissue lysates were loaded (30 µg protein/lane) onto 26-lane 1DE gels (NUPAGE Novex Midi gels, 4–12%, Invitrogen) under reducing conditions and then electroblotted onto immobilon-FL membrane (Millipore Ltd, Watford, UK) as described previously (Fowler et al., 2008). After blotting, membranes were incubated in blocking buffer, 1:1 Odyssey blocking buffer (LI-COR Biosciences UK Ltd, Cambridge, UK) and PBS, at 4 °C overnight. Primary antibodies were diluted in Odyssey blocking buffer 1:1 with 0.2 µm filtered PBST as follows: mouse anti-manganese Superoxide Dismutase (SOD2: AbCam Ltd, Cambridge, UK, ab16956), 1–1500, Rabbit anti-Tryptophanyl tRNA Synthetase (WARS: AbCam Ltd, Cambridge, UK, ab31536), Mouse anti-Gelsolin (GSN: AbCam Ltd, Cambridge, UK, ab55070), all combined with mouse anti-Alpha Tubulin (AbCam Ltd, Cambridge, UK, ab7291), 1~~–10~~–10,000 or rabbit anti-Alpha Tubulin (AbCam Ltd, Cambridge, UK, ab4074), 1 µg/ml. The membranes were incubated with primary antibodies at 4 °C overnight and then incubated with secondary antibodies for 60 min at room temperature. Secondary antibodies including anti-mouse IgG IRDYe™800 (all secondary antibodies were provided from LI-COR, Cambridge, UK, 610-732-124), 1–10,000 and anti-mouse IRDYe™700DX (610-730-124) 1~~–5,–5~~000 were diluted in Odyssey blocking buffer 1:1 with 0.2 µm filtered PBST + 0.01% SDS. Following washing the membranes, the digital images were captured using Odyssey LI-COR Infrared Imager (LI-COR, Cambridge, UK). The band volumes and molecular weights (kDa) were then obtained following a background subtraction using Phoretix-1D Advanced software (Nonlinear Dynamics).

2.7 Assay of SOD2 activity

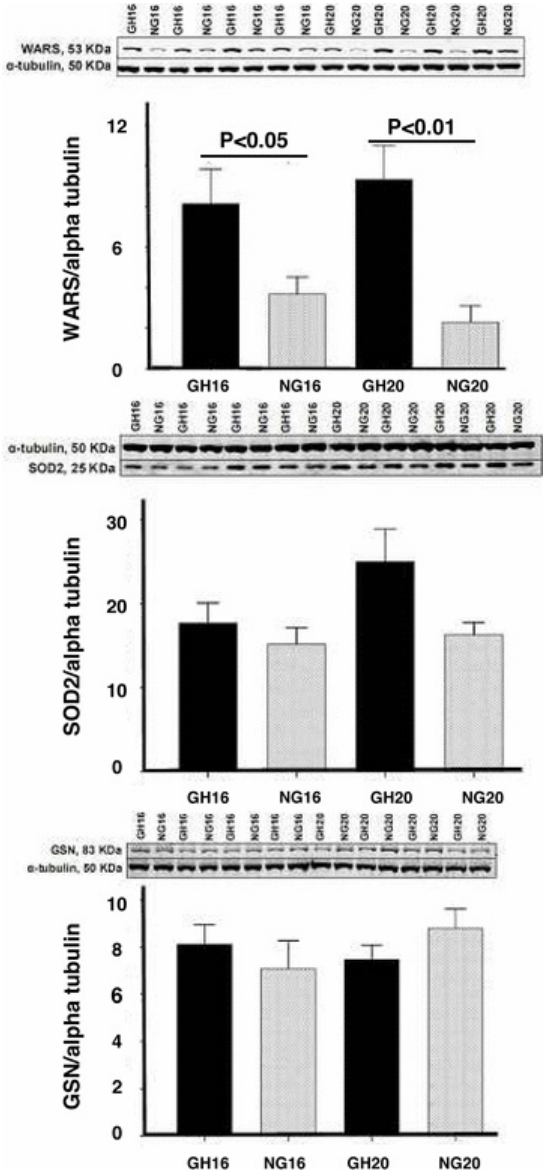
Caruncular endometrial tissues was homogenized in cold phosphate buffer (50 mM, pH 7.4) and then the homogenates were centrifuged at 15~~000 × g~~,000 × *g* for 30 min, 4 °C. The resulting supernatant was used for determination of protein concentration (Lowry et al., 1951). A standard SOD2 assay (Marklund and Marklund, 1974) that has been validated for sheep endometrium tissues (Al-Gubory et al., 2008) was used. We determined the enzymatic activity of SOD2 by assaying for SOD activity in the presence of sodium cyanide, which selectively inhibits SOD1 but not SOD2 (Jin et al., 2005). The rate of auto-oxidation is taken from the increase in the absorbance at 420 nm. One unit of SOD activity is defined as the amount of the enzyme required to inhibit the rate of pyrogallol auto-oxidation by 50%.

2.8 Statistical analysis

Normality of data was tested with the Shapiro–Wilk test. Normally distributed data were subjected to one- and two-way ANOVA and Bonferroni-Dun post-hoc test using SPSS 17.0 software to assess significance of differences. Differences were considered significant at ~~P~~*P* < 0.05.

3 Results

WARS, SOD2 and GSN proteins were detected at the expected molecular weights of 53, 25 and 83 ~~K~~kDa respectively on the immunoblotted membranes (Fig~~ure.~~ 1). WARS band volumes increased significantly in gravid uterine horns compared to non-gravid uterine horns on days 16 (~~*P < 0.05) and 20 (P*~~ ≤ 0.05) and 20 (*P* < 0.01) of pregnancy (Fig~~ure.~~ 1). While there were no significant changes in WARS mRNA expression between the gravid and non-gravid uterine horns on day 16 of pregnancy (Fig~~ure.~~ 2), WARS mRNA expression significantly increased (~~*P*~~*P* < 0.05) in gravid uterine horns on day 20 of pregnancy when compared to non-gravid uterine horns (Fig~~ure.~~ 2). WARS mRNA expression significantly decreased (~~*P*~~*P* < 0.05) from day 16 to day 20 of pregnancy in non-gravid uterine horns (Fig~~ure.~~ 2).



Fig~~ure.~~ 1 Expression of mRNA transcripts coding for tryptophanyl tRNA synthetase (WARS); manganese superoxide dismutase (SOD2) and gelsolin (GSN) in sheep caruncular endometrial tissues collected from the uterine gravid horn (GH) and non-gravid horn (NG) on days 16 and 20 of pregnancy. Data was normal~~is~~ed against the expression levels of cyclophilin gene. Data are shown as means ± SEM for four ewes per group. The acceptable level of significance was set at ~~*P < 0.05. Figure*~~*P* ≤ 0.05.

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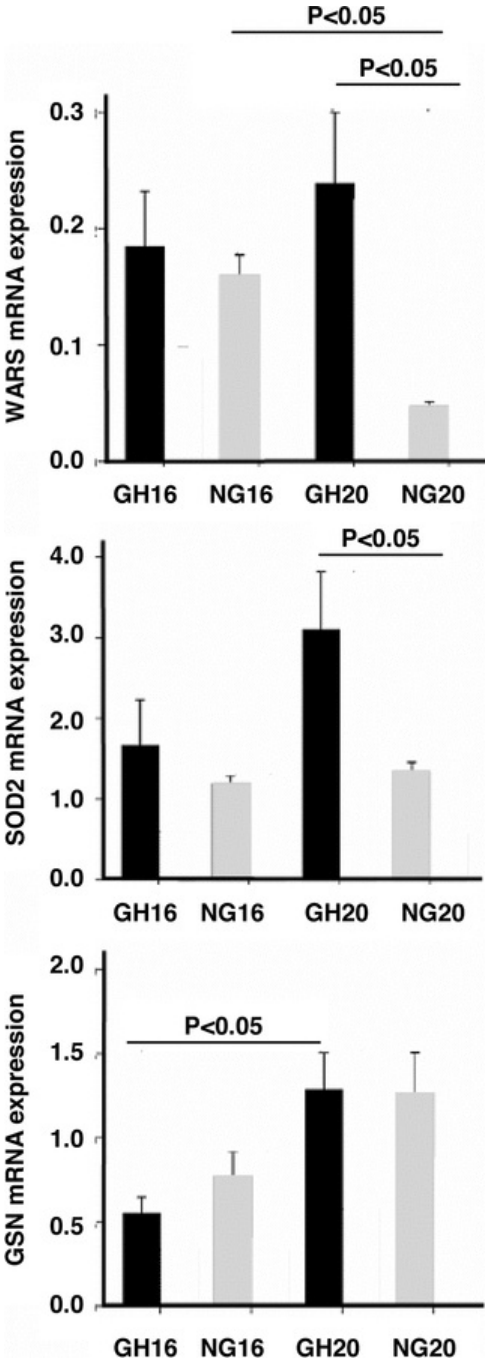

Fig. 2 Western blot (WB) analysis of tryptophanyl tRNA Synthetase (WARS); manganese superoxide dismutase (SOD2) and gelsolin (GSN) expression in sheep caruncular endometrial tissues collected from the uterine gravid horn (GH) and non-gravid horn (NG) of ewes on days 16 (~~*n = 4) and 20*~~ ~~*n*~~ = 4) and 20 (*n* = 4) of pregnancy. In all WB, there were no significant changes in alpha tubulin band volumes between the samples and all groups, indicating its validity as a load control in this experiment. Normalized band volumes are shown as means ± SEM for four ewes per group. The

acceptable level of significance was set at ~~P < 0.05.Figure~~P ≤ 0.05.

There were no significant differences in SOD2 band volumes between the gravid and non-gravid uterine horns on day 16 of pregnancy (Figure 1). SOD2 band volumes tended to be increased in gravid horns compared to non-gravid uterine horns on days 20 of pregnancy, although not attaining statistical significance (Figure 1). Similarly, there were no significant differences in SOD2 mRNA expression between the gravid and non-gravid uterine horns on day 16 of pregnancy (Figure 2) although there was a significant increase (~~P~~P < 0.05) in SOD2 mRNA expression in gravid uterine horns on day 20 of pregnancy when compared to non-gravid uterine horns (Figure 2). When SOD2 enzymatic activity was determined, there were no differences between the gravid and non-gravid uterine horns and irrespective of the day of pregnancy studies (Figure 3). In the gravid uterine horn, the activity of SOD2 increased significantly (~~P~~P < 0.01) from day 16 to day 20 of pregnancy (Figure 3).

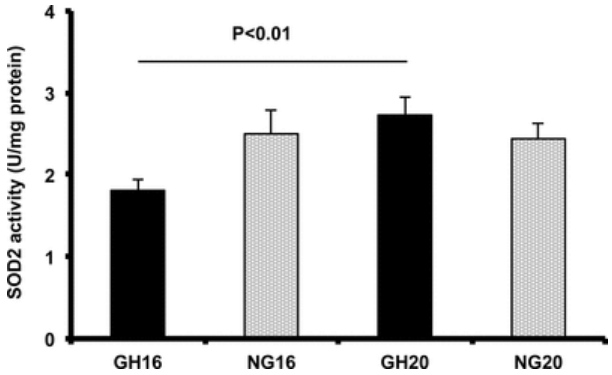

Fig. 3 Manganese superoxide dismutase (SOD2) activity in sheep caruncular endometrial tissues collected from the uterine gravid horn (GH) and non-gravid horn (NG) on days 16 and 20 of pregnancy. Data are shown as means ± SEM for four ewes per group. The acceptable level of significance was set at ~~P < 0.05.Figure~~P ≤ 0.05.

There were no significant differences in GSN band volumes between the gravid and non-gravid uterine horns and irrespective of the day of pregnancy studies (Figure 1). However, while there were no significant differences in GSN mRNA expression between the gravid and non-gravid uterine horns on both days 16 and 20 (Figure 2), GSN transcript expression was significantly higher (~~P~~P < 0.05) in gravid uterine horns of on day 20 than day 16 of pregnancy.

4 Discussion

The mechanisms involving in the maternal recognition of pregnancy in domestic animals, including ruminants, are not fully understood. Therefore, a major strength of the present study is the assessment of the expression of three functional proteins (WARS, SOD2 and GSN) identified in sheep caruncular endometrium (Al-Gubory et al., 2014) at both the protein and mRNA level in the ipsilateral gravid and the contralateral non-gravid uterine horns of unilaterally pregnant ewes. We have demonstrated that the expression of WARS and SOD2 protein increases in the caruncular endometrium of pregnant ewes at the time of implantation compared with the matching stage of the oestrous cycle (Al-Gubory et al., 2014). Although such a difference may be due to factors produced by the conceptus that act in a paracrine manner on the endometrium to elicit upregulation in WARS and SOD2 gene and protein expression, the effect of endocrine factors can not be excluded. Indeed, the endometrium is a steroid-responsive tissue and hormonal changes that occur during early pregnancy compared those at the end of the oestrous cycle may influence WARS and SOD2 expression. In the present study we determined, using unilaterally pregnant ewes with active and functional ovaries, that conceptus-derived paracrine signalling molecules up-regulate the expression of WARS and SOD2 in the caruncular endometrium of the gravid horn at the time of conceptus implantation and post-implantation. Upregulation of WARS protein expression by conceptus-derived signals observed here had not been reported previously in endometrium of any mammalian species.

Early pregnancy factors stimulate the expression of mRNA encoding WARS gene within the endometrium (Kudo et al., 2004; Walker et al., 2010). However, the nature of conceptus signalling molecules that regulate WARS expression within the endometrium remains unknown. Interferon τ (IFN-τ) is major signal produced by the trophoblast of the peri-implanting ruminant conceptus between days 10 and 21 of pregnancy with maximal production on days 14~~ to ~~16 (Bazer et al., 1997). WARS is an IFN-inducible gene (Fleckner et al., 1991; Rubin et al., 1991; Kisselev et al., 1993). In addition, WARS expression is stimulated by IFN-γ (type II IFN) and IFN-α (type I IFN) in different cell types (Tolstrup et al., 1995). The increase in WARS protein expression both in the caruncular endometrial tissue of pregnant ewes on day 16 of pregnancy compared with the matching stage of the oestrous cycle (Al-Gubory et al., 2014) and in the caruncular endometrial tissue of the gravid uterine horns when compared with the non-gravid uterine horns on day 16 of pregnancy (present study) may be due to local production of IFN-τ by the conceptus at the time of implantation.

WARS protein expression was markedly up-regulated in the caruncular endometrium of the gravid uterine horns when compared with the non-gravid horns on day 20 of pregnancy (present study) although the production of IFN-τ declines between days 16 and 20 of pregnancy (~~Hansen et al., 2004~~Hansen et al., 1988). Therefore, it is likely that conceptus-derived factors other than IFN-τ up-regulate WARS protein expression in caruncular endometrium during the post-implantation period. Indeed, many molecules secreted by the conceptus are involved in concepts-endometrium communication during implantation and early post-implantation periods. Prostaglandins (PGs) produced by the conceptus (Lewis and Waterman,

1985) regulate the expression of many endometrium genes important for conceptus development during the peri-implantation period (Dorniak et al., 2011). The role of IFN-τ and/or PGs in the regulation of WARS protein expression required further properly designed in vivo and in vitro experiments.

Uncontrolled ROS production can result in oxidative damage to cellular macromolecules and consequently leads to lipid peroxidation, protein degradation and DNA lesions (Jezek and Hlavatá, 2005) that adversely affect prenatal developmental outcome (Al-Gubory et al., 2010). The antioxidative defence mechanism requires a tight control of mitochondrial superoxide radicals ($^{\bullet}O_2^-$) before their transformation to toxic ROS. The toxicity of $^{\bullet}O_2^-$ is based on generation of so-called downstream products of $^{\bullet}O_2^-$, mainly hydroxyl radical ($^{\bullet}OH$) and peroxynitrite ($ONOO^-$). In the presence of free unbound iron and/or copper ions, $^{\bullet}O_2^-$ tends to interact with H_2O_2 in a Haber–Weiss reaction, which is a common reaction source of $^{\bullet}OH$ (Halliwell and Gutteridge, 2007). Mitochondria are endowed with nitric oxide ($NO^{\bullet}$) synthase (mtNOS) and are a source of cellular $NO^{\bullet}$ and $ONOO^-$ (Valdez and Boveris, 2007). Mitochondrial $^{\bullet}O_2^-$ that escapes dismutation by SOD2 may react with $NO^{\bullet}$ to form $ONOO^-$ which is a highly reactive oxidant (Alvarez and Radi, 2003). In the present study, SOD2 mRNA expression was markedly increased in the gravid uterine horns when compared with the non-gravid uterine horns on day 20 of pregnancy. At that time, SOD2 protein expression and activity tended to be increased in the gravid uterine horns when compared to non-gravid uterine horns. It has been reported that the enzymatic activity of SOD2 in sheep caruncular endometrial markedly increases from day 16 to day 21 of pregnancy (Al-Gubory and Garrel, 2012). Therefore, the elevated levels of SOD2 reported here in the sheep caruncular endometrium of the gravid uterine horns may prevent mitochondrial oxidative damage induced by locally produced ROS during the early post-implantation period. To the best of our knowledge, the up-regulation of SOD2 expression in the caruncular endometrial tissue of pregnant ewes by conceptus-derived factors observed here has not been previously described in the literature.

In conclusion, this study provides firm evidence that the early implantation and post-implanting conceptus-derived signals up-regulate the expression of WARS protein within the caruncular endometrium via paracrine mechanisms (Figure. 4). Further in vivo and in vitro studies are necessary to identify these signalling molecules, to understand the mechanisms whereby they exert paracrine action within the uterine endometrium and to determine the role of WARS in the establishment of pregnancy. These findings establish a new reference database and will open the avenue for such studies.

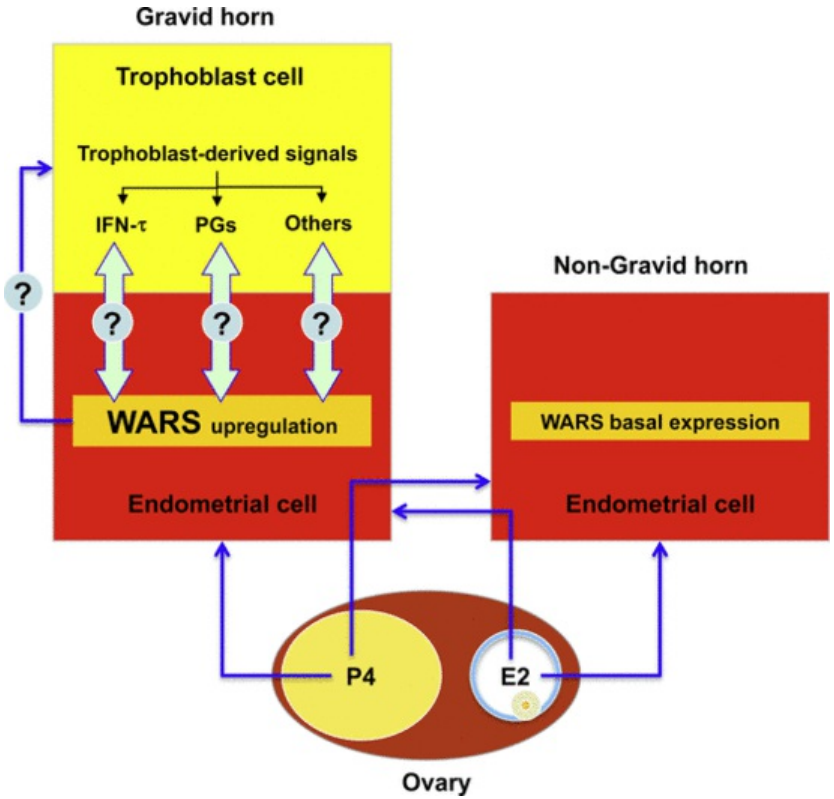

Fig. 4 A schematic overview of the regulation of endometrial tryptophanyl tRNA synthetase (WARS) expression by the conceptus during the peri-implantation period in unilaterally pregnant ewes subjected to the same systemic maternal hormone environment, including corpus luteum progesterone (P4) and follicular estradiol (E2). P4 acts directly on the E2-primed uterine endometrium to regulate cell differentiation, growth and function required for conceptus implantation and development. Interferon τ (IFN-τ) and prostaglandins (PGs), among others, are the potential conceptus-derived

signals that may be involved in the regulation of WARS in the gravid uterine horn.

~~Declaration~~Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributions

KHA jointly conceived and designed the study with PAF. KHA prepared the animal model, performed surgery and tissue collection, wrote the manuscript and acted as corresponding author. MA carried out the experiment, performed production, acquisition and statistical analysis of data. CG was responsible for analysis of antioxidant enzyme activity. PAF supervised development of work, helped in data interpretation and manuscript evaluation. All authors approved the final version of manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.12.013.

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Appendix A. Supplementary data

The following are the supplementary data to this article:

Multimedia Component 1

Queries and Answers

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