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Differential effects of bovine conceptus-derived signaling molecules on the uterine environment supporting embryo development while allowing maternal immune tolerance.

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ABBREVIATIONS

18S rRNA	18S ribonucleic acid
AA	amino acids
Ala	L-alanine
APAF	apoptotic protease activating factor
APC	antigen presenting cell
App.	Appendix
Arg	L-arginine
Asn	L-asparagine
Asp	L-aspartic acid
ATP	adenosine triphosphate
B2M	β2-microglobulin
BCA	bicinchoninic acid
BIRC	Baculoviral inhibitor of apoptosis protein repeat-containing protein
CASP	caspase
CD	cluster of diffentiation
cDNA	complementary desoxyribonucleic acid
CFLAR	cellular FLICE-like inhibitory protein
CL	corpus luteum
CO_2	carbon dioxide
Cq	cycle of quantification
CTL	cytotoxic T lymphocyte
DAB	3,3'-diaminobenzidine
DNA	desoxyribonucleic acid
dNTP	desoxynucleotide triphosphate
DIABLO	Direct inhibitor of apoptosis protein binding protein with low pI
DISC	death inducing signaling complex
ELISA	enzyme-linked immuno-sorbent assay
FAS	TNF receptor superfamily member 6
FASLG	FAS ligand
GE	glandular epithelium
Gln	L-glutamine
Glu	L-gutamic acid
Gly	glycine
H3F3A	Histone H3
His	L-histidine
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IDO	indoleamine 2,3-dioxygenase 1
IHC	immunohistochemistry
IFN	interferon
IFNAR	interferon-α receptor
IL	interleukin
Ile	L-isoleucine
IRF	IFN regulatory factor
ISG	IFN stimulated gene
IVF	*in vitro* fertilization
LC-MS/MS	liquid chromatography/tandem mass spectrometry
LE	luminal epithelium
Leu	L-leucine
Lys	L-lysine
MHC	major histocompabitility complex
min	minute
Met	L-methionine
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NAD	nicotinamide adenine dinucleotide
P4	progesterone
PBS	phosphate buffered saline
PG	prostaglandin
PGF2α	prostaglandin F2α
Phe	L-phenylalanine
PEtN	O-phosphoethanolamine
Pro	L-proline

RISC	RNA-induced silencing complex
RT-qPCR	quantitative real-time reverse transcription polymerase chain reaction
RLU	relative light units
SCNT	somatic cell nuclear transfer
SEM	standard error of the mean
Ser	L-serine
SLC	solute carrier
STAT	signal transducer and activation of transcription
Tau	taurine
Th	T-helper cell
Thr	L-threonine
TLR	toll like receptor
TNF	tumor necrosis factor
TNFSF10	TNF super family ligand 10
TP	total protein
Trp	L-tryptophan
Tyr	L-tyrosine
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
UBQ3	polyubiquitine
Val	L-valine
XAF1	X-linked inhibitor of apoptosis protein associated factor 1

ABSTRACT

The optimal development of an embryo prior to implantation involves coordinated embryo-maternal interactions. During this decisive phase, nutrients must be supplied by endometrial secretions to support the development of the elongating trophoblast. To provide insights into the specific requirements of a bovine embryo, we quantified the composition of 41 amino acids (AA) and derivatives in the uterine flushings by comparing pregnant and non-pregnant Simmental heifers at days 12, 15 and 18 post estrus using the highly sensitive liquid chromatography-tandem mass spectrometry. In addition, the intercaruncular endometrium ipsilateral to the corpus luteum bearing ovary was sampled for gene expression analysis of AA transporter using quantitative real-time reverse transcription PCR. Next to a pregnancy dependent increase of each essential AA analyzed, we detected elevated levels for most non-essential proteinogenic AA. As in the ewe, glycine was most abundant, but concentrations did not increase due to the pregnancy status indicating species specific exigencies. Intrauterine L-histidine concentrations and the expression of the L-histidine and peptide transporter solute carrier 15A3 (*SLC15A3)* concomitantly increased in pregnancy *in vivo* and were stimulated by interferon-tau (IFN-τ) primariliy in co-cultivated stroma cells *in vitro*. In pregnancies generated by somatic cell nuclear transfer (SCNT), diseases associated with placenta abnormalities and fetal oversize frequently occur. A defective regulation of AA supply at early pregnancy stages might contribute to failures at a later date; wherefore the intrauterine AA composition in SCNT and *in vitro* fertilization (IVF)-generated pregnancies was comparatively examined at day 18 post estrus. Oocytes were fertilized for the production of IVF-embryos, while SCNT embryos were generated from different fibroblast cells lines. Prior to the transfer of two blastocysts per recipient heifer at day 8, embryos were cultured under equal conditions. Although both SCNT and IVF-produced trophoblasts released similar levels of IFN-τ into the uterine lumen, most AA and the AA derivative O-phosphoethanolamine were lower concentrated in the presence of a SCNT conceptus with a simultaneous lower expression of the endometrial transporter *SLC7A8.* Our results indicate, that SCNT embryos fail to induce maternal transport required to optimally provide nutrients for the pre-attachment conceptus. Thus, metabolic imbalances appearantly manifest prior to implantation and might contribute to severe placenta failures. Along with rapid embryonic development, the release of trophoblast-derived IFN-τ increases. Due to the proapoptotic characteristics of IFNs, we analyzed the effects of the pregnancy recognition signal on endometrial apoptosis. The messenger ribonucleic acid (mRNA) expression of proapoptotic genes X-linked inhibitor of apoptosis protein associated factor-*1 (XAF1)*, caspase 8 (*CASP8)* and tumor necrosis factor super family ligand 10 (*TNFSF10)* were significantly increased in the endometrium of pregnant animals. An IFN-τ dependent induction was confirmed by the *in vitro* co-culture model of endometrial cells primarily in stroma cells. However, differences between pregnant and cyclic

animals were neither found for a CASP activity nor for an increase of endometrial apoptosis. These results suggest a specific regulation of IFN stimulated genes and further indicate the existence of inhibitory mechanisms preventing endometrial cells from unfavourable programmed cell death. Since paternal inherited antigens might be exposed to the maternal immune system as soon as the blastocyst hatches, a well-balanced immunological interaction is of particular importance to avoid rejection of the semi-allogenic embryo. Therefore potential tolerance inducing mechanisms were analyzed. In addition to increasing FAS ligand (FASLG) transcripts in conceptuses from day 15 to day 18, a strong cytoplasmic immunostaining was detected in trophoblast cells. Thus, FASLG could act as proapoptotic ligand to induce selective cell death on TNF receptor superfamily member 6 (FAS) receptor bearing lymphocytes. By expressing only low levels of the small subunit of major histocompatibility complex, β2-microglobulin (*B2M*), the embryo possibly avoids the detection by the maternal immune system. Indoleamine 2,3-dioxygenase 1 *(IDO*) mRNA, encoding for the initial enzyme of the kynurenine pathway, was pronouncedly more abundant in the endometrium of day 18 pregnant vs. non-pregnant animals. The significant decrease of endometrial L-tryptophan in pregnant and non-pregnant heifers detected by tandem mass spectrometry and the concomitant increase of L-kynurenine from day 12 to day 18 primarily in the endometrium of pregnant heifers caused an increased IDO activity at day 18 of pregnancy. The expression of IDO was most pronounced induced in endometrial stroma cells upon IFN-τ exposure *in vitro*, which was confirmed by *in situ* hybridization localizing *IDO* mRNA mainly in deep stroma cells surrounding the glandular ducts. The interaction of multiple tolerance inducing mechanisms during preimplantation might entail the reduced number of cluster of differentiation 45 (CD45) positive immunostained leukocytes in the zona basalis of pregnant animals providing an optimal environment and supporting embryo development. Taken together, these findings indicate the importance of an appropriate embryo-maternal signaling enabling optimal embryo development while allowing immune tolerance.

ZUSAMMENFASSUNG

Die optimale Entwicklung eines Embryos vor der Implantation geht mit koordinierten embryo-maternalen Interaktionen einher. Während dieser bedeuteden Phase, müssen die Nährstoffe für das enorme Wachstum des Trophoblasten von endometrialen Sekreten bereitgestellt werden. Um Erkenntnisse über die spezifischen Anforderungen eines bovinen Embryos zu gewinnen, wurde die Zusammensetzung von 41 Aminosäuren (AA) und deren Derivaten in uterinen Spülflüssigkeiten von trächtigen und zyklischen Fleckviehfärsen am Tag 12, 15 und 18 post Östrus mit Hilfe der hochsensitiven Flüssigkeitschromatographie/Tandemmassenspektrometrie bestimmt. Ferner wurde das interkarunkuläre Endometrium ipsilateral zum Corpus luteum tragenden Ovar für die mRNA Expressionsanalyse der AA-Transporter mit Hilfe der quantitativen real-time reverse transcription PCR beprobt. Neben einem trächtigkeitsabhängigen Anstieg aller untersuchten essentiellen AA, wurde ein Anstieg der meisten nichtessentiellen AA detektiert. Ebenso wie beim Schaf, war Glycin die am höchsten konzentrierte intrauterine AA, stieg jedoch im Gegensatz dazu nicht während der Präimplantationsphase an. Dieser Befund könnte somit auf speziesspezifische Unterschiede hinweisen. Die intrauterine L-Histidin Konzentration sowie die Expression des L-Histidin- und Peptidtransporters solute carrier 15A3 (*SLC15A3)* stieg *in vivo* während der Trächtigkeit an. Außerdem wurde die *SLC15A3* Expression *in vitro* vor allem in kokultivierten Stromazellen durch Interferon-tau (IFN-τ)-Gabe induziert. In Trächtigkeiten, generiert durch den Kerntransfer somatischer Zellen (SCNT), werden häufig Plazentaanomalien und übergroße Föten beobachtet. Um festzustellen, ob eine gestörte Regulation der intrauterinen AA-Versorgung in frühen Trächtigkeitsphasen möglicherweise diese Schäden in späteren Stadien verursacht, wurde die intrauterine AA-Zusammensetzung in SCNT-trächtigen Tieren und Tieren trächtig mit einem Embryo, der durch *in vitro* Fertilisation (IVF) erzeugt wurde am Tag 18 post Östrus miteinander verglichen. Oozyten wurden für die Generierung von IVF-Embryonen befruchtet, wohingegen Nuklei, die für die Erzeugung der SCNT Embryonen verwendet wurden, aus verschiedenen Fibroblastenzelllinien stammten. Unter identischen Bedingungen wurden die Blastozysten kultiviert und je zwei am Tag 8 pro Empfängertier transferiert. Obwohl SCNT und IVF-generierte Trophoblasten vergleichbar hohe IFN-τ Mengen in das uterine Lumen sekretierten, waren die meisten AA sowie die des AA-Derivats O-Phosphoethanolamin in der Gegenwart von SCNT-Embryonen geringer konzentriert. Gleichzeitig wurde eine verringerte Expression des endometrialen Transporters *SLC7A8* ermittelt. Unsere Ergebnisse weisen auf eine ungenügende Fähigkeit der SCNT Embryonen hin, den maternalen Transport, der für eine optimale Nährstoffversorgung nötig ist, zu regulieren. Folglich scheinen sich metabolische Imbalanzen schon vor Beginn der Implantation zu manifestieren und könnten somit für die schwerwiegenden Plazentaschäden verantwortlich sein. Die rapide Entwicklung des Embryos geht einher mit einem starken Anstieg der IFN-τ Sekretion. Aufgrund der proapoptotischen Eigenschaften der

Interferone, wurden im Folgenden die Effekte des Trächtigkeitserkennungssignals auf die endometriale Apoptose untersucht. Die messenger Ribonukleinsäure (mRNA) der proapoptotischen Gene X-linked inhibitor of apoptosis protein associated factor 1 *(XAF1)*, caspase 8 (*CASP8)* und tumor necrosis factor super family ligand 10 (*TNFSF10)* waren im Endometrium trächtiger Tiere signifikant höher exprimiert. Ein IFN-τ-abhängiger Expressionsanstieg konnte im *in vitro* Kokulturmodel endometrialer Zellen vor allem in Stromazellen bestätigt werden. Allerdings wurde weder im Endometrium trächtiger noch zyklischer Tiere eine Aktivierung der CASP-Kaskade oder eine erhöhte Anzahl apoptotischer Zellen nachgewiesen. Unsere Daten deuten auf eine sehr spezifische Regulation IFN stimulierter Gene hin, und auf möglicherweise vorhandene inhibitorische Mechanismen, um überschießenden Zelltod zu unterbinden. Um zu verhindern, dass paternal vererbte Antigene der geschlüpften Blastozyste, die dem maternalen Immunsystem präsentiert werden, eine für den Embryo schädliche Immunreaktion hervorrufen, ist eine sorgfältig balancierte immunologische Interaktion von besonderer Bedeutung. Potenzielle toleranzinduzierende Mechanismen, die die Abstoßung des semi-allogenen Embryos verhindern könnten, wurden deshalb untersucht. Neben einem Anstieg der FAS ligand (FASLG) Expression in Konzepten zwischen Tag 15 und Tag 18 wurde eine starke zytoplasmatische Immunfärbung in Trophoblastzellen beobachtet. Der proapoptotische Ligand FASLG könnte somit selektiven Zelltod in TNF receptor superfamily member 6 (FAS)-Rezeptor tragenden Immunzellen auslösen. Durch die geringe Expression der β2-Mikroglobuline (B2M) an Konzepten umgeht der Embryo möglicherweise die immunologische Detektion durch das maternale Immunsystem. Indolamin 2,3-dioxygenase 1 (*IDO*) mRNA, die für das initiale Enzym des Kynureninwegs kodiert, war deutlich höher im Endometrium Tag 18 trächtiger versus nicht-trächtiger Tieren exprimiert. Die signifikante Abnahme endometrialen Tryptophans, detektiert mittels Tandemmassen-spektrometrie, in trächtigen und nicht-trächtigen Färsen, wurde von einem gleichzeitigen Anstieg des L-kynurenines von Tag 12 bis Tag 18 begleitet, woraus sich eine erhöhte IDO Aktivität am 18. Tag der Trächtigkeit ergab. Der Befund, dass die IDO Expression *in vitro* vor allem in endometrialen Stromazellen nach IFN-τ-Gabe induziert wird, konnte mit Hilfe der *in situ* Hybridisierung bestätigt werden. IDO mRNA wurde vor allem in tiefen Stromazellen, die die glandulären Drüsenkanäle umgeben, lokalisiert. Die Interaktion einer Vielzahl toleranz-induzierender Mechanismen während der Präimplantationsphase könnte folglich für die geringere Anzahl der cluster of differentiation 45 (CD45)-positiven Leukozyten im Stroma der Zona basilis in trächtigen Tieren verantwortlich sein und dadurch eine optimale Umgebung für die Entwicklung des Embryos schaffen. Unsere Ergebnisse demonstrieren somit die Relevanz der genau abgestimmten Signalgebung, um eine optimale Entwicklung des Embryos zu ermöglichen, während gleichzeitig die Immuntoleranz aufrechterhalten werden muss.

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1. INTRODUCTION

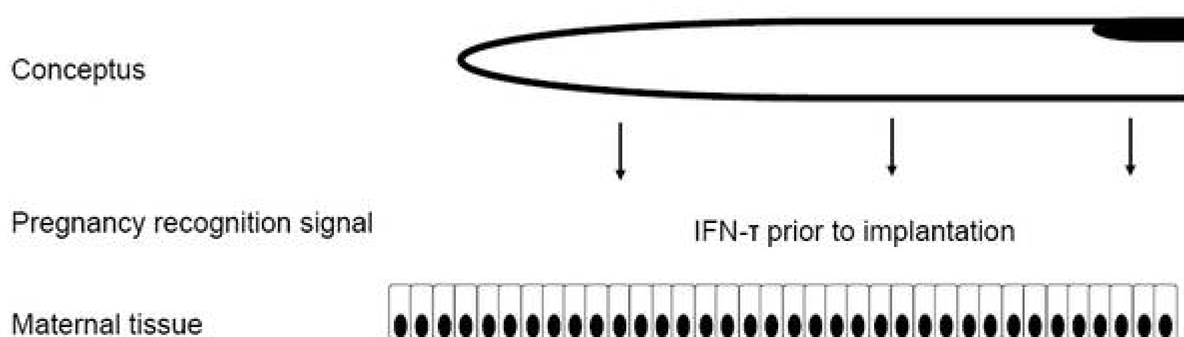
1.1 Embryo-maternal communication prior to implantation

In cattle most embryo losses occur during the preimplantation period (Diskin & Sreenan 1980). For the optimal development, a complex embryo-maternal communication is a prerequisite. Upon entering the uterus at day 3-4 post fertilization the blastocyst hatches from the zona pellucida around day 8. The trophoblast of the spherical blastocyst starts elongation at approximately day 13 most likely due to definite components in the endometrial secretions (Ruesse & Sinowatz 1998). At day 18, the exponentially elongated trophoblast of about 30 cm in length fills both uterine horns without being attached to the maternal tissue (Fig. 1). Signaling molecules including growth factors, cytokines and hormones are released into the uterine fluid also known as uterine milk or histotrophe to facilitate the complex embryo-maternal communication (Bazer 1975). In addition, the supply with nutrients such as amino acids (AA), carbohydrates and gaseous molecules must be provided via secretory products of the endometrium.

The maintenance of a functional corpus luteum (CL) is a prerequisite to sustain the secretory properties of the endometrium and consequently to establish and maintain pregnancy. The antiluteolytic signal secreted by the preimplantation trophoblast in ruminants is interferon-tau (IFN-τ) which inhibits the oxytocin induced pulsatile prostaglandin F2α (PGF2α) release from the endometrium. IFN-τ is a type I IFN, serving as primary pregnancy recognition signal in the ruminant suborder (Fig. 1). Spherical blastocysts express only low levels of IFN-τ (Hernandez-Ledezma *et al.* 1992; Yao *et al.* 2009), but the concentration increases remarkably during the elongation phase. Up to 10^5 units of antiviral IFN acitivity can be secreted per hour in culture by a day 18 trophoblast (Cross & Roberts 1991). Protein concentrations peak at day 21 and decline rapidly following attachment to the maternal tissue (Ealy & Yang 2009). Signaling of IFN-τ is mediated via the binding to the heterodimeric IFN type I receptor subunits IFN-α receptor 1 (IFNAR1) and -2 (IFNAR2), primarily expressed in the ovine luminal epithelium (LE) and the superficial glandular epithelium (GE) (Rosenfeld *et al.* 2002). Upon binding of IFN-τ to its receptor, phosphorylation of cytoplasmic tyrosine residues mediated by janus kinases allows the subsequent recruitment and phosphorylation of src-homology 2 domain containing signal transducer and activation of transcription 1/-2 (STAT1/-2). Following dimerization, STAT translocate to the nucleus, bind to distinct desoxyribonucleic acid (DNA) sites and induce along with further components the transcription of interferon stimulated genes (ISG).

With incipient apposition of the elongated blastocyst at caruncular areas at day 17 (King *et al.* 1981), the contact between mother and chorion is intensified. A separation of the trophoblast and the maternal tissue without tissue damages is no longer possible at day 20 (Fig. 1). Thereupon, invasive trophoblast binucleated cells fuse with uterine luminal epithelial cells to form syncytial giant cells wherefore the ruminant placenta is called placenta synepitheliochorialis (Wathes & Wooding 1980). However, paternal inherited antigens presented on the hatched blastocyst surface, might initiate detrimental reactions of the maternal immune system. Therefore, prior to the establishment of a placenta immunological acceptance of the semi-allogenic embryo is a precondition.

Preimplantation



Placentation

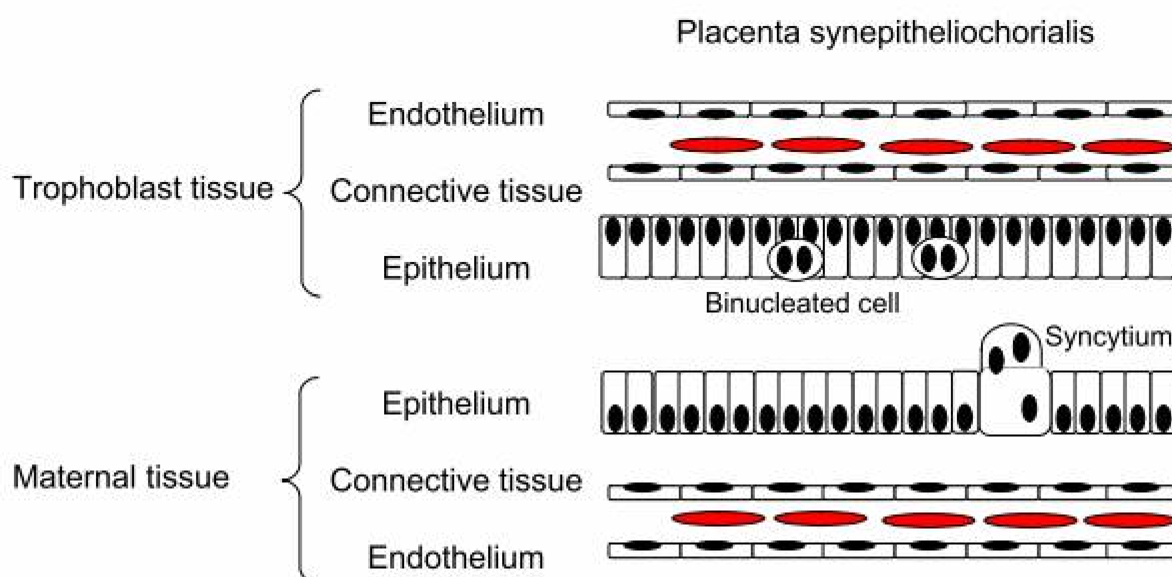


Fig. 1: Scheme showing preimplantation and placentation in *bos taurus*.
During preimplantation, the elongating trophoblast is nourished via endometrial secretions and releases increasing abundances of the pregnancy recognition signal interferon-tau (IFN-τ) to inhibit luteolysis. Bovine elongated blastocysts start first apposition at day 17 post insemination. The sparse invasive bovine placenta, established at specialized caruncular areas, is known as placenta synepitheliochorialis.

Supply with amino acids via the uterine milk is critical prior to implantation

The uterine milk is of major importance, since imbalances in the supply with nutrients during the pre- and periimplantation might severely affect the development and elongation of the ovine conceptus (Gray *et al.* 2001). In addition to common energy substrates such as glucose, lactate and pyruvate present in the ruminant uterine fluid (Gao *et al.* 2009d; Hugentobler *et al.* 2008), AA might be as well of importance to allow the development of the fast growing trophoblast. Apart from contributing to manifold cellular and metabolic functions, AA are components of proteins, enzymes, cytokines, several hormones and required for the synthesis of purines and pyrimidines. Vertebrates do not contain the sufficient enzymatic repertoire to synthesize each of the 20 proteinogenic AA. Especially, the synthesis of more complex AA such as aromatic, branched chain and some cationic AA involves complex enzymatic synthesis steps wherefore a supply via the nourishment is indispensable. During definite developmental stages a number of semi-essential AA such as L-arginine (Arg), L-tyrosine (Tyr) and L-histidine (His) may become limited when requirements exceed the synthesis rates. The directional AA transport from the maternal blood through the endometrial tissue across the epithelium includes a complex network of transport systems to provide nutrients to the uterine fluid for the free floating conceptus. Transcripts of transport systems preferring the transfer of either charged, aromatic, branched chain or aliphatic AA have been identified in different cell types of the ovine endometrium and of conceptus tissues allowing a selective accumulation (Gao *et al.* 2009b; Gao *et al.* 2009a). Depending on the orientation of cells, either the basal or apical side of the cell features heterogenous transport system for either uptake into the cytoplasm, transport through cellular compartments or release into the uterine fluid.

Membrane spanning transporters carry AA with different affinities for their substrates. Moreover, transporters of AA show overlapping substrate specificity. While most transporters act as monomers, several transporter of large AA require in addition to a catalytic active subunit covalently associated glycoproteins for their activity (Verrey 2003). A restriction of nutrients during pregnancy depletes AA in the plasma of the fetus and the mother affecting not only the development *in utero* (Kwon *et al.* 2004), but also predisposes for diseases such as diabetes in the later adult life most likely due to misdirected programming (Barker & Clark 1997). Hence, intensive research is conducted to identify the optimal composition of nutrients and signaling factors in the uterine milk to ensure the development of the pre-attachment conceptus and additionally to emulate the uterine milieu *in vitro* for assisted reproduction techniques. Manipulations of the blastocyst and inadequate *in vitro* culture conditions are supposed to cause abnormal placenta formation and fetal oversize known as large off spring syndrome (Young *et al.* 1998). For instance, culture with serum, non-protein nitrogen diet, asynchronous transfer, but in particular cloning by somatic cell nuclear transfer (SCNT) entail neonatal oversize due to

aberrations in epigenetic reprogramming of somatic cell nuclei (Dean *et al.* 2001; Niemann *et al.* 2008). To meet the requirements of bovine blastocysts, the presence of both essential and non-essential AA is crucial (Liu & Foote 1995). For instance, Glycine (Gly) and L-alanine (Ala) stimulate the *in vitro* development of bovine blastocyst (Moore & Bondioli 1993), while L-glutamine (Gln) is necessary for blastocoel formation (Rieger *et al.* 1992). During the development cationic AA Arg provides the basis for the synthesis of the signaling molecule nitric oxide as well as polyamines stimulating migration and proliferation of ovine trophoblast cells (Kim *et al.* 2011). Reduction of L-methionine (Met) decreased intracellular glutathione concentration in *in vitro* produced bovine embryos, by not affecting methylation status or cleavage rate (Bonilla *et al.* 2010). In general, non-essential AA rather support the cleavage of the zygote, blastocoel formation and blastocyst hatching, while essential AA favor the complex development and differentiation of the inner cell mass of murine embryos (Lane & Gardner 1997). Thus, an optimal uterine milieu supports the growth and development. However, how the preattachment conceptus itself contributes to the modulation of transport activity by secreted signaling molecules or whether nutrients are provided due the to the dominance of progesterone (P4) during the secretory phase as evidenced for selected nutrient accumulation is only partially understood (Satterfield *et al.* 2010).

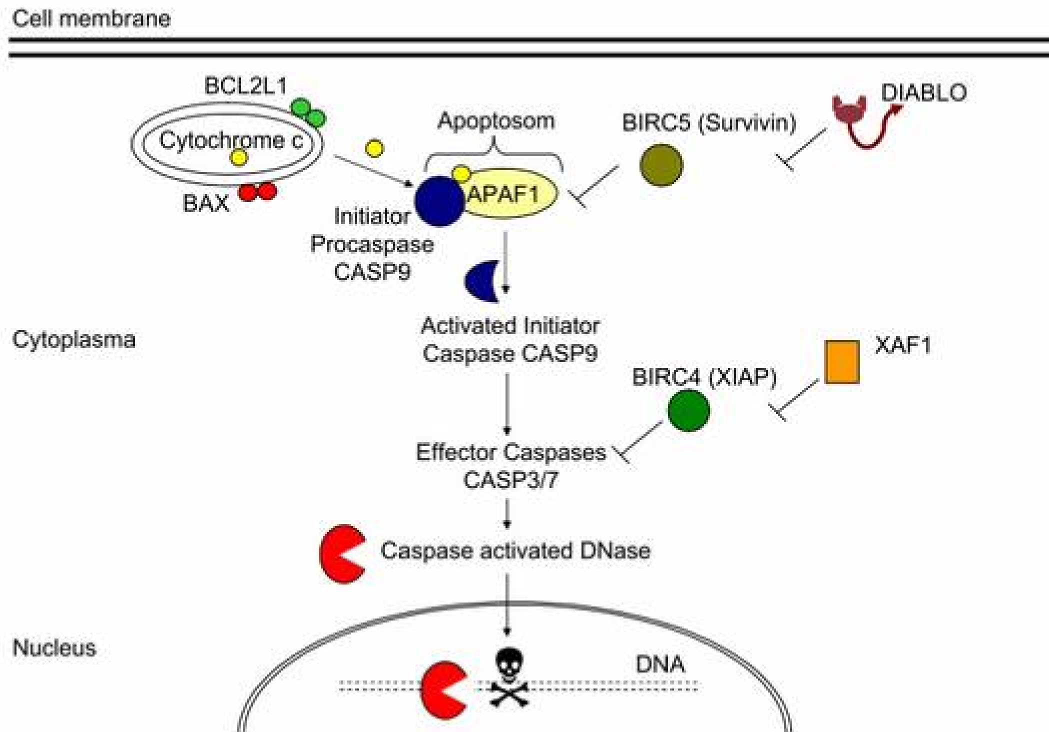
Apoptotic mechanisms at the embryo-maternal interface

Adequate maternal recognition of pregnancy involves the release of IFN-τ, a type I IFN by the trophoblast. Although IFN-τ is not associated with responses to pathogen infections as other type I IFNs, induced ISGs are associated with antiviral responses implicating immune modulatory, growth inhibitory and proapoptotic effects such as β2-microglobulin (B2M), major histocompatibility complex (MHC) class I, Myxovirus resistance protein, 2’5'-oligoadenylate synthase and X-linked inhibitor of apoptosis protein associated factor 1 (XAF1) (Bauersachs *et al.* 2006; Klein *et al.* 2006). The induction of apoptosis (also known as programmed cell death) due to type I IFN stimulation previously described (Chawla-Sarkar *et al.* 2003). Apoptosis is a well coordinated, energy dependent process occurring during normal physiological conditions. Programmed cell death plays a decisive role in differentiation processes and maintenance of homeostasis. Apoptosis can be either induced to control the proliferation of cells showing DNA damages or to inhibit the replication of cells infected with intracellular pathogens. Depending on the type of stimuli, apoptotic pathways may be activated either via the intrinsic, mitochondrial route or via the extrinsic, death receptor mediated pathway (Fig. 2). Both pathways direct the proteolytical activation of a caspase (CASP) cascade and involve the subsequent activation of DNases and further proteases finally triggering chromatin condensation, membrane inversion and formation of apoptotic bodies (Earnshaw *et al.* 1999). Subsequent clearance of apoptotic cells is mediated by phagocytes without releasing proinflammatory cytokines (Abrahams *et al.* 2004a).

The intrinsic pathway may be induced upon cellular stress or in the absence of survival factors. The interaction of proapoptotic (e.g. BAX) and antiapoptotic members (e.g. BCL2L1) of the Bcl-2 family, bound to mitochondrial membranes, control the release of cytochrome c into the cytoplasm (Fig. 2A). Upon stimulation of the intrinsic pathway, released cytochrome c binds to apoptotic protease activating factor 1 (APAF1) forming the so called apoptosome complex. This allows the recruitment of initiator procaspase 9 and its subsequent proteolytical activation. Activated CASP9 promotes apoptosis by cleaving and activating further downstream effector CASP such as CASP 3, 6 and 7. In turn, effector CASP activate a variety of proteins including CASP activated endonucleases to dissect DNA. However, the final suicide can be controlled at any level even after DNA fragmentation by components such as the cellular tumor antigen p53 that arrest the cell cycle to allow repair by DNA-polymerases and –ligases.

Antiapoptotic proteins such as inhibitors of apoptosis X-linked inhibitor of apoptosis protein (baculoviral inhibitor of apoptosis protein repeat-containing protein 4, BIRC4) or SURVIVIN (BIRC5) control the activation of the CASP cascade, but can be inactivated again by proapoptotic antagonists such as direct inhibitor of apoptosis protein binding protein with low pI (DIABLO) or XAF1. The extracellular pathway involves membrane-spanning death receptors of the tumor necrosis factor (TNF)-receptor superfamily e.g. TNF receptor superfamily member 6 (FAS) receptor, TNF receptor 1 (TNFRSF1A), TNF receptor 2 (TNFRSF1B) and TRAIL receptor 2 (TNFRSF10B), containing a cytoplasmic death domain (Fig. 2B). Upon binding of respective soluble or membrane bound ligands such as FASLG, TNF related apoptosis inducing ligand/TRAIL (TNFSF10) and TNF-α (TNF), the death receptors trimerize. The subsequent conformational change allows recruitment of adaptor proteins including the initiator procaspase 8. In turn, the formation of the death inducing signaling complex (DISC) promotes the autocatalytic cleavage of the procaspase, consequently triggering the induction of the CASP cascade. In addition, CASP8 is also able to connect the receptor mediated pathway with the intrinsic pathway, by activating proapoptotic components of the Bcl-2 family. However, the CASP8 and FAS associated death domain-like apoptosis regulator (CFLAR) shares structural similarities with CAPS 8 and compete for the cytoplasmic binding site to the death receptor, wherefore CFLAR can act as competitive inhibitor of apoptosis.

A) Intrinsic apoptosis pathway



B) Extrinsic apoptosis pathway

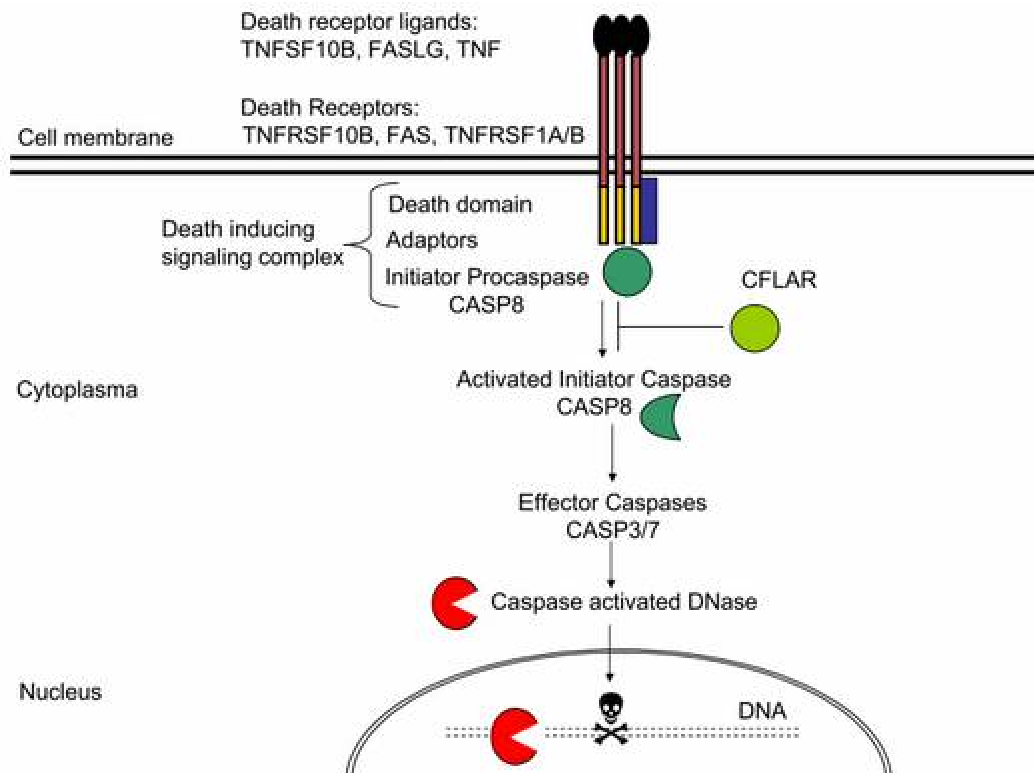


Fig. 2: Intrinsic and extrinsic apoptosis pathway.
The absence of survival factors or cellular stress may activate the induction of the intrinsic apoptosis pathway (A). The extrinsic pathway is initiated upon binding of death receptor ligands to the respective receptor (B). The induction of a caspase (CASP) cascade subsequently triggers apoptotic cell death without inducing inflammatory responses (A, B).

Apoptosis occurs at sites of trophoblast invasion. However, the frequency of programmed cell death during the implantation and placentation is highly dependent on the degree of invasiveness. In rodents and humans, numerous cell degenerative changes are required for appropriate tissue remodeling processes and invasion of the blastocyst into the endometrial stroma to form a hemochorial placenta (Straszewski-Chavez *et al.* 2005; Tassell *et al.* 2000). In contrast, programmed cell death is absent during the implantation phase in the porcine endometrium showing a non-invasive epitheliochorial type of placentation (Okano *et al.* 2007), while in cattle, cells showing apoptotic morphology appear within the luminal epithelium in the caruncular area from day 22 of pregnancy (Wathes & Wooding 1980). However, little is known whether apoptotic pathways might be induced in the intercaruncular area already during the pre- and perimplantation phase due to the presence of high concentrations of trophoblast derived IFN-τ.

Tolerance mechanisms support embryo development

An insufficient immunological acceptance of the semi-allogenic embryo and misdirected signaling between mother and the developing embryo favor diseases such as preeclampsia, intra uterine growth retardation and pre-term abortion in women (von Rango 2008b). In preparation for implantation, the initiation of excessive immune reactions towards the semi-allogenic conceptus by endometrial leukocytes and lymphocytes sensitized to paternal inherited antigens must be circumvented.

Already in 1953, Medawar noted the conflicting immunological connection between mother and the semi-allogenic embryo due to the genetic disparity of mother and conceptus (Medawar 1953). However, the following postulations that first the placenta forms an anatomical barrier, second, the antigenic immaturity of the fetus, and third, the maternal inertness would contribute to the acceptance of the fetus have been disproved. Rather, an active generation of immune tolerance involving several strategies is occurring in pregnancy of rodents and primates (von Rango 2008a). In the following section, major tolerance inducing strategies, firstly, the induction of apoptosis in leukocytes, secondly, the down-regulation of MHC to avoid immunological detection and thirdly, the regulation of the nutrient availability to maternal lymphocytes are illustrated in more detail:

The induction of programmed cell death has been shown to play also a significant role in generating immune tolerance. In particular, the death receptor mediated pathway appears to be important for the generation of human embryo tolerance by inducing cell death in deleterious immune cells (Jerzak & Bischof 2002). FAS receptor is an important mediator of apoptosis and

commonly high expressed on definite leukocytes population such as activated T and B lymphocytes as well as natural killer cells. In contrast, FASLG is not only expressed in lymphoid cells, but also on non-lymphoid tissues with high expression in immune privileged organs such as the eye, testis and brain (Griffith *et al.* 1995). Alike, sites of placentation are referred to as immune privileged site, since the semi-allogenic conceptus is not rejected in spite of the presence of foreign antigens. Therefore the induction of apoptosis by the FAS receptor-FASLG system might be a potent strategy for the establishment of immune tolerance in cattle.

Prior to the induction of tolerance “self” and “non-self” must be defined. Key molecules contributing to the discrimination between “self” and “non-self” are highly polymorphic MHC molecules expressed on the surface of every nucleated cell types. MHC class I molecules present cytosolic protein fragments to cytotoxic T lympocytes (CTLs) to induce inflammatory responses in case of infection with intracellular pathogens. On the contrary, MHC class II molecules, solely expressed on antigen presenting cells (APC) such as dendritic cells, macrophages and B lymphocytes, present lysosomal peptides. While MHC class II molecules consist of a membrane-spanning α- and β-chain, MHC class I molecules possess only a membrane spanning α-chain non-covalently associated to a smaller B2M. Subsequent to the maturation and selection processes in the thymus, only those T lymphocytes enter the periphery, which reliably recognize self MHC molecules but are not reactive to self antigens. In case of transplantation, excessive immune responses trigger the rejection of the allograft carrying non-self MHC molecules. As the conceptus represents a semi-allogenic “transplant”, overwhelming immune reaction must be avoided. MHC class I molecules are also expressed in the endometrium and increase due to IFN-τ in the bovine endometrium during preimplantation (Bauersachs *et al.* 2006; Klein *et al.* 2006). In contrast, on trophoblast cells MHC class I molecules are only marginal expressed in humans (Hunt *et al.* 1987). However, downregulation or absence of MHC class I molecules can increase the susceptibility for apoptosis caused by natural killer cells. If recognition of “self” fails, they induce programmed cell death by releasing cytotoxic granules (missing self hypothesis) (Croy *et al.* 2006). On this account, the human extravillous trophoblast expresses less polymorphic non-classical MHC class I molecules such as human leukocyte antigen-G (HLA-G). HLA-G molecules bind to killing inhibitory receptors expressed on natural killer cells, thus avoiding cell death (Roussev & Coulam 2007).

A mechanism to circumvent the activation of lymphocytes sensitized to paternal inherited antigens is the limitation of the essential AA L-tryptophan (Trp) at the sites of implantation. Obligatory for the synthesis of hormones such as serotonin and melatonin, Trp is also required for the synthesis of the cofactor nicotinamide adenine dinucleotide (NAD+). Indoleamine 2,3-dioxygenase 1 (IDO) encodes for the initial heme-containing enzyme of the kynurenine pathway, catalyzing the rate-limiting degradation of Trp and further molecules containing an indoleaminering to L-formylkynurenine. Thus, a Trp-depleted microenvironment is formed at the

site of IDO activity. Treatment of pregnant mice carrying allogenic conceptuses with the competitive IDO inhibitor 1-methyl-Trp promotes pregnancy loss due to extensive maternal T cell reactions (Munn *et al.* 1998). Through the kynurenine pathway, Trp is either completely oxidized to carbon dioxide and adenosine triphosphate (ATP), or synthesized to NAD+ via downstream catalytic steps (Fig. 3) (reviewed in Moffett & Namboodiri (2003). While IDO is expressed in many tissues including the placenta, solely hepatocytes contain the complete enzymatic possibilities to perform each metabolic intermediate synthesis step of the distinct side branches. However, divergent leukocyte populations, such as macrophages are able to produce cytotoxic quinolinic acid upon bacterial infection or induction of toll like receptor (TLR) signaling and IFN-γ (Venkateshan *et al.* 1996). IDO is therefore proposed to protect the semi-allogenic conceptus against rejection by suppressing the activity of potentially dangerous T cells at the embryo-maternal interface in species showing a hemochorial type of placentation (Mellor & Munn 1999).

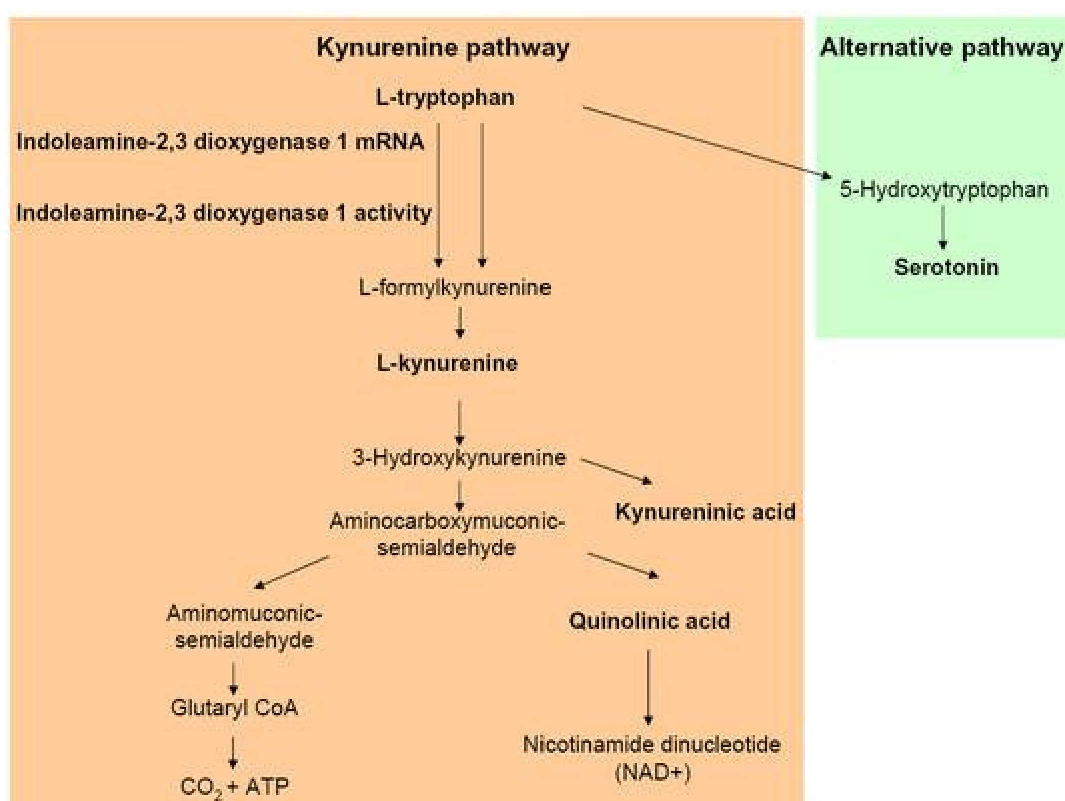


Fig. 3: The L-tryptophan degrading kynurenine pathway.
Two ways of L-tryptophan degradation are shown. L-tryptophan is either metabolized into serotonine or via the kynurenine pathway completely oxidized to carbon dioxide (CO_2) and adenosin triphosphate (ATP) or to nicotinamide dinucleotide (NAD+) (Moffett & Namboodiri 2003).

1.2 Aim of the study

In dairy cattle, high rates of pregnancy losses occur prior to implantation. The present study was carried out to decipher the complex signaling mechanisms in the embryo-maternal interface required for the establishment of pregnancy (Fig. 4). During the critical phase of elongation, the nutrition of the unattached conceptus must be conducted by endometrial secretions. The present study therefore focused on the determination of pivotal intrauterine AA and endometrial transport systems in physiological and SCNT pregnancies. Enormous trophoblast growth is accompanied by an increased proapoptotic gene expression due to the release of the ruminant pregnancy recognition signal IFN-τ. Thus, we analyzed the relevance of apoptosis in the endometrium during preimplantation. Since the establishment of pregnany presupposes a well-balanced immunological interaction between mother and the semi-allogenic embryo, potential tolerance mechanisms in cattle were as well under investigation.

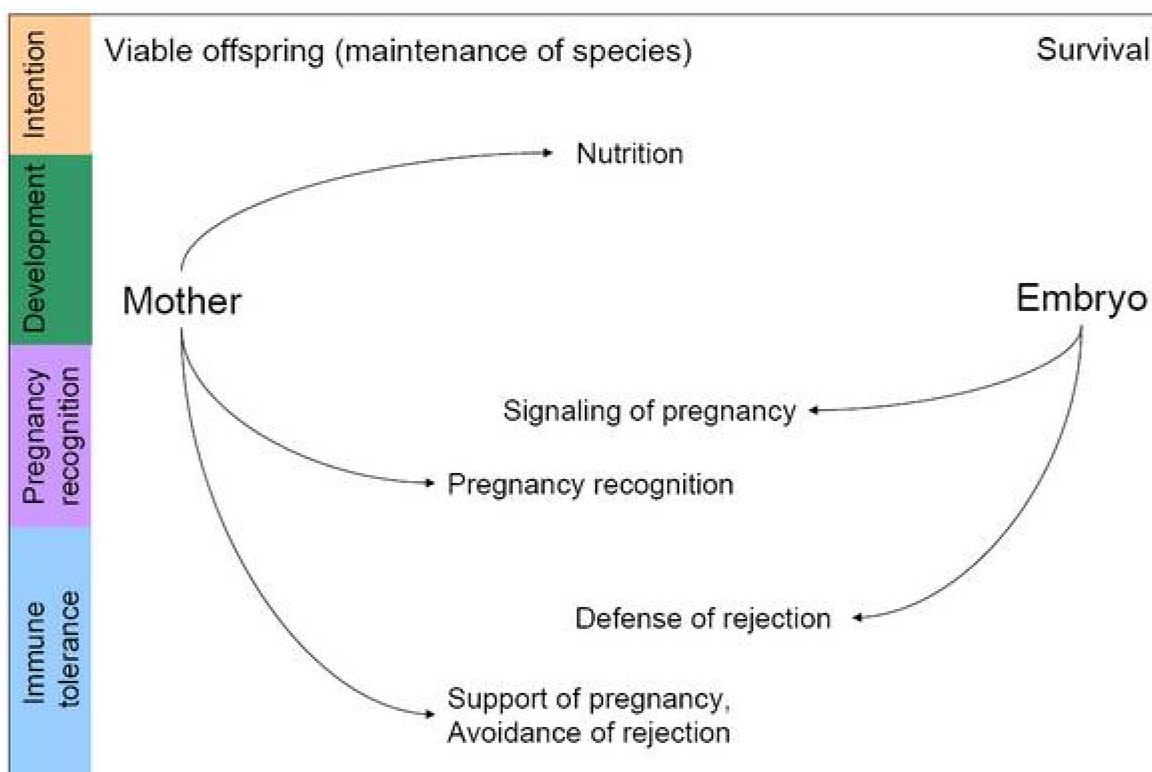


Fig. 4: Scheme showing the proposed embryo-maternal interactions.
Establishment of pregnancy is a critical process aiming the survival of the semi-allogenic embryo and the propagation of viable offspring. While the conceptus requires the supply with nutrients to ensure rapid elongation and development during preimplantation, appropriate signaling of its presence is a prerequisite for the establishment of pregnancy, while allowing immune tolerance.

2. MATERIAL AND METHODS

Animals

Cyclic Simmental heifers (*Bos taurus*, Deutsches Fleckvieh) were estrus synchronized by injecting intramuscularly 500 mg of a single dose of the PGF2α-analog Cloprostenol (Estrumate; Essex Tierarznei, Munich, Germany) at diestrus. Estrus was determined by observation for sexual behaviour. Pregnant animals were inseminated after estrus detection, whereas the cyclic control animals received the supernatant of centrifuged sperm (seminal plasma) from the same bull. Blood samples were taken to determine serum P4 levels by enzyme immunoassay according to Prakash *et al.* (1987). All animals had P4 concentration of more than 6 ng/mL at the time of slaughter. At days 12, 15, or 18 post insemination, animals were slaughtered (n=5–7 per group); the uterus was removed and flushed with 100 mL prewarmed phosphate buffered saline (PBS; pH 7.4) for the recovery of blastocysts, uterine fluids and endometrium (Fig. 5). Animals from the pregnant group were included in the study only if a conceptus was present. Total protein (TP) content and AA composition were determined in flushing fluids centrifuged at 800 *g* for 10 minutes (min). The supernatant was stored at -20° C until further processing. To obtain intercaruncular endometrium, the uterus was opened longitudinally and tissue samples ipsilateral to the CL bearing ovary were carefully cut from the lamina propria by using a scalpel. For gene expression analysis, endometrium and whole conceptuses were transferred into vials containing RNAlater (Ambion, Huntingdon, Cambridgeshire, UK). Histochemical experiments were performed on tissue samples (uteri and whole conceptuses), transferred to Bouin's fixation solution prior to serial dehydration and embedding in paraffin. Activity of CASP and cytokine protein expression were determined in endometrium samples snap-frozen in liquid nitrogen (-196° C) until further processing.

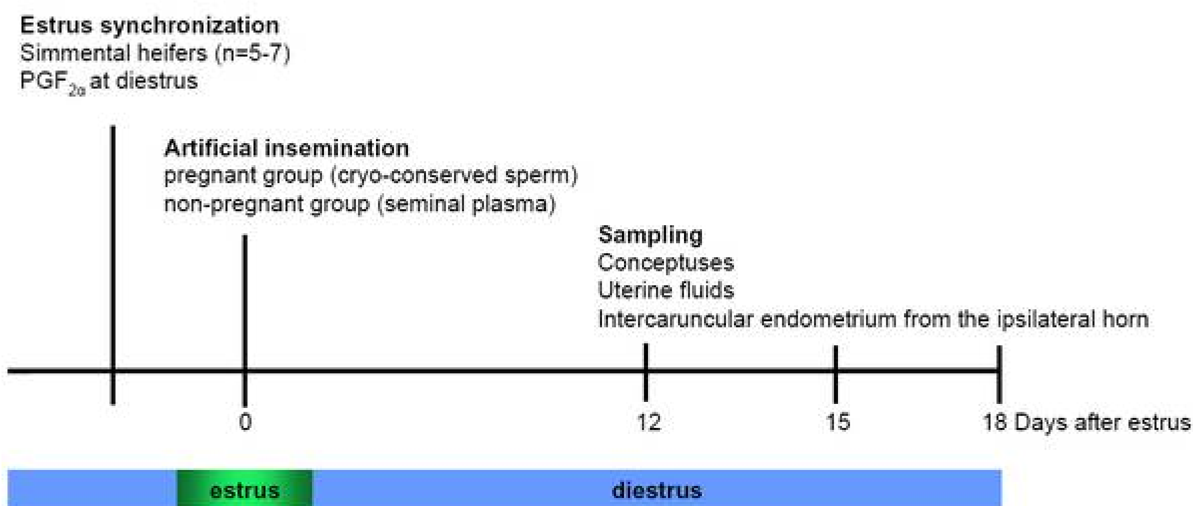


Fig. 5: Design of the study.
Estrus synchronized heifers were inseminated at estrus with either sperm or seminal plasma. Intercaruncular endometrial tissue, conceptuses and uterine flushings were sampled at days 12, 15, or 18 post insemination for the analysis of the intrauterine AA composition, apoptosis and tolerance inducing mechanisms.

Generation of in vitro fertilized embryos and embryos derived by somatic cell nuclear transfer

SCNT and IVF procedures were performed as described by Hiendleder *et al.* (2004). While IVF embryos were generated by *in vitro* fertilization of oocytes obtained from non-related heifers with sperm from the same bull, SCNT embryos were generated by transferring the nucleus of different fetal fibroblast donor cells into enucleated oocytes. Thus, the produced embryos represent half-siblings. After SCNT or IVF, embryos were cultured under identical conditions to the blastocyst stage (day 8). Briefly, presumptive embryos were cultured in 400 µl droplets of synthetic oviduct fluid culture medium enriched with 5% estrus cow serum, 40 µl/mL of 50x BME AA Solution (#B6766, Sigma-Aldrich), and 10 µl/mL of 100x MEM Non-essential AA Solution (#M7145, Sigma-Aldrich) covered with mineral oil. The culture atmosphere was 5% CO_2, 5% O_2, and 90% N_2 at 39° C and maximum humidity. Two SCNT or IVF blastocysts (grade 1) were transferred to estrus synchronized Simmental recipient heifers (day 8 of estrus cycle). Preparation of recipient animals were performed as described for artificial inseminated animals. The recipients (n=8 per group) were slaughtered at day 18 post estrus, the uteri were recovered and flushing fluids as well as endometrial samples were obtained as described above (Fig. 6). Animals were termed ‘‘pregnant’’ if filamentous trophoblast tubes and at least 1 embryonic disc were observed. A twin pregnancy was identified if two embryonic discs were detected. As both SCNT and IVF-derived blastocysts underwent an *in vitro* phase prior to transfer of synchronized heifers and as two blastocysts were transferred, the IVF situation was regarded as control for the situation in SCNT pregnancies. This tissue was kindly provided by the Institute of Molecular

Animal Breeding and Technology, Ludwig Maximilian Universität Munich, under the head of Prof. E. Wolf.

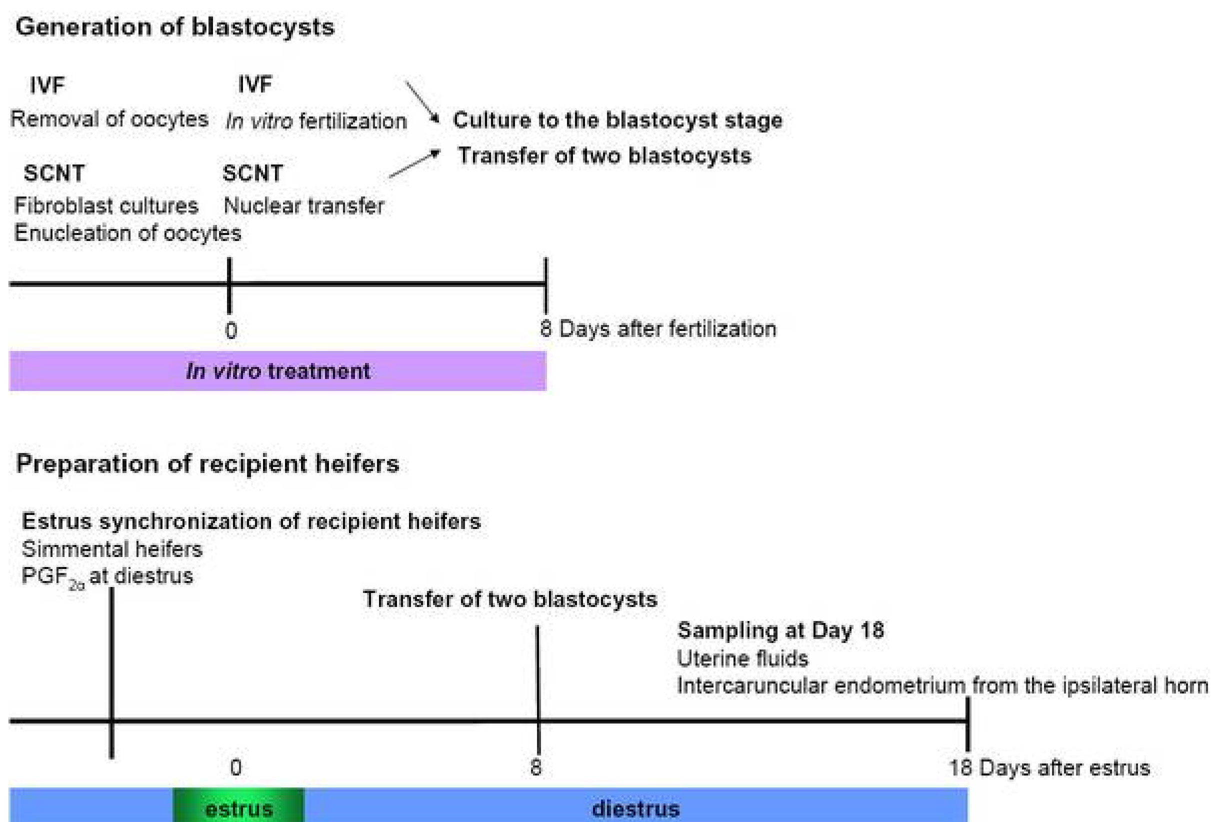


Fig. 6: Generation of IVF and SCNT embryos.
IVF-derived conceptuses were generated by *in vitro* fertilization of oocytes with sperm from a single bull. SCNT conceptuses were produced from different fibroblasts. Both IVF and SCNT conceptuses represent half-siblings. Upon cultivation under identical conditions two blastocysts were transferred into the uterine lumen of estrus synchronized heifers (n=8). Uterine flushings and intercaruncular endometrial tissue were sampled at slaughter at day 18 post estrus for the analysis of intrauterine AA and endometrial transport systems.

Extraction of total ribonucleic acid using the TRIzol-chloroform method

Total ribonucleic acid (RNA) from endometrial samples and whole conceptuses were isolated using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to manufacturer's instructions. Quality of RNA was determined by the Agilent 2100 Bioanalyzer (Eukaryote total RNA Nano Assay, Agilent Technologie, Palo Alto, CA, USA). RNA integrity numbers ranged between 7 and 10 (0 = degraded RNA, 10 = intact RNA). Quantity was spectroscopically determined at 260 nm by the Nanodrop 1000 (peqLab Biotechnologie GmbH).

Reverse transcription of total ribonucleic acid

The two step quantitative real-time reverse transcription PCR (RT-qPCR) experiments were performed in accordance with the MIQE guidelines (Bustin *et al.* 2009). One µg of total RNA was reverse transcribed in a volume of 60 µl by using random hexamer primers. To the RNA 5X Buffer (Promega, Mannheim, Germany), 10 mM desoxynucleotide triphosphate (dNTP) (Roche Diagnostics, Mannheim, Germany), 50 µM hexamer primers (Gibco BRL), 200 Units M-MLV-Superscript Reverse Transcriptase enzyme (Promega) were added and transcribed for 120 min at 48° C in a thermal cycler (Biometra, Göttingen, Germany).

Gene expression analysis by using the LightCycler platform

For each PCR reaction, 1 µl complementary desoxyribonucleic acid (cDNA) was used to amplify specific target genes. In every RT-qPCR reaction, 17 ng/µl cDNA were amplified in a 10 µl reaction mixture (3 mM $MgCl_2$, 0.4 µM per forward and reverse primer, 1 µl Light Cycler DNA Master SYBR Green I; Roche Diagnostics). Commercially synthesized PCR primer pairs were used (Eurofins MWG Operon, Ebersberg, Germany). Resulting fragments were sequenced to control amplification of designated transcripts (Sequencing Service of the Department Biology Genomics Unit, Munich, Germany). Primer sequences, references as well as product length are depicted in the respective appendices (App.): (Groebner *et al.*, 2010 App. I, Table 1, Groebner *et al.* 2011 App. II Table 1, Groebner *et al.* 2011 *in review* App. III Supplementary Table 1). The cycle number (Cq) to attain a definite fluorescence signal was calculated by the second derivative maximum method (LC software 4.05), since the Cq is inversely correlated with the logarithm of the initial template concentration. The amplification of a specific PCR product was verified by melting curve analysis following amplification. The Cq values from the target genes were normalized against the geometric mean of three reference genes (Polyubiquitine, UBQ3; Histone H3, H3F3A and ribosomal 18S RNA, 18S rRNA). In order to avoid negative digits while allowing an estimation of a relative comparison between two genes, data are presented as means ± standard error of the mean (SEM) subtracted from the arbitrary value 30 (ΔCq). Thus, a high ΔCq resembles high transcript abundance. An increase of one ΔCq represents a two-fold increase of messenger RNA (mRNA) transcripts (Livak & Schmittgen 2001).

Gene expression analysis by using the Fluidigm Dynamic Array gene expression chip

To determine gene expression of immune relavant genes, a high throughput gene expression platform was used based on Dynamic Array™ microfluidic chips (Fluidigm 96.96 Dynamic Array

IFC, San Francisco, CA, USA). This permits 9,216 simultaneous real time PCR gene expression measurements per chip. In the present experimental setup, the mRNA expression of 32 immune relevant genes in 94 preamplified endometrial and conceptus samples each was measured with three replicates on a single chip at an annealing temperature of 60° C. Before introducing the samples to the Fluidigm platform specific target amplification was undertaken lasting 14 cycles using the TaqMan® PreAmp Master Mix (Applied Biosystems, Carlsbad, California USA). For each sample 1.25 µl Assay mix consisting of a forward and reverse primer (concentration 180 nM), 2.5 µl 2xABI preamp mix and 1.25 µl cDNA (conc. 16.6 ng/µl) were subjected to the specific target amplification. Preamplified cDNA was finally 1:5 diluted and loaded onto the chip. Three chip replicates were run to determine chip to chip reproducibility. To ensure specific product amplification, a melting curve analysis was carried out and data were eliminated if unspecific products were detected. An overall standard deviation of 0.16 in addition to a chip to chip correlation of >0.99 for all three replicates was obtained. Reference genes histone H3 and polyubiquitine were chosen by using GenEx Pro Ver 4.3.4 (multiD Analyses AB, Gothenburg, Sweden). Data are presented means ± SEM substracted from the arbitrary value 20 (ΔCq).

Analysis of amino acids and respective derivatives in the uterine lumen

In 40 µl of uterine flushing fluids 41 AA and derivatives were labeled by the isobaric tagging for relative and absolute quantification (iTRAQ) methodology using the AA45/32 Starter Kit according to the manufacturer´s instructions (Applied Biosystems) and analyzed via liquid chromatography-tandem mass spectrometry (LC-MS/MS) (3200QTRAP LC/MS/MS, Applied Biosystems). The data were analyzed using the Analyst®61666; 1.5 Software as previously described (Kaspar *et al.* 2009), kindly performed at the Chair of Nutrition Weihenstephan (Prof. Hannelore Daniel). Contents of TP in uterine flushing fluids were determined using a commercial available bicinchoninic acid (BCA) Kit (Sigma-Aldrich, St. Louis, MO, USA). Content of TP and total AA concentration (calculated by the sum of each individually measured AA) correlated well (r=0.82, *p<0.0001*) (Groebner *et al.* 2011, App. III). To avoid inconsistencies due to inaccuracies in the flushing procedure and differential texture of the uterine fluid over the analyzed time points data are presented with reference to TP (mean nmol/mg TP ± SEM).

Determination of interferon-tau bioactivity in uterine fluids

IFN production was quantified in uterine flushing fluids by a bioassay based on the inhibition of the cytopathic effect of vesicular stomatitis virus (Indiana strain) on Madin–Darby bovine kidney cells (Rubinstein *et al.* 1981). The NIH recombinant human IFN-α2 reference preparation (No. Gxa01-901-535, NIH-Research Reference Reagent Note No. 31, 1984) was included in each

assay as positive control. The antiviral activity was shown to be mediated by IFN-τ, as the effects of supernatant and an appropriate control IFN-τ preparation were blocked by specific anti-IFN sera (kindly provided by Dr. R.M. Roberts, University of Missouri, Columbia, MO, USA) (Stojkovic *et al.* 1999). The bioassay was kindly undertaken at the Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Oberschleißheim (Prof. Mathias Büttner).

Caspase activity in endometrial tissue homogenates

For Caspase-Glo® 3/7 Assay and Caspase-Glo® 8 Assay (Promega), 100 mg of endometrial samples from the ipsilateral horn were homogenized in 10 mL PBS containing a tablet of the proteinase inhibitor CompleteMini (Roche Diagnostics) by using the MagNALyser (Roche Diagnostics). After a 30 min incubation time on ice, the mixture was centrifuged through a NucleoSpin Filter L (Macherey-Nagel GmbH & Co KG, Düren, Germany) and stored at –20° C until further use. Protein concentrations of homogenates were determined using a BCA standard protocol (Sigma-Aldrich). Activities of CASP8 and CASP3/7 in endometrial homogenates were determined by cleavage of a proluminescent substrate containing the selective LETD (L-Leucine-L-Glutamic acid-L-Threonine-L-Aspartic acid) and DEVD (L-Aspartic acid-L-Glutamic acid-L-Valine-L-Aspartic acid) tetrapeptide sequence, respectively. All samples and reagents were equilibrated to room temperature prior to use. Homogenates (50 µl) with a concentration of 4 µg/µl TP were introduced in duplicates to white-walled 96 well plates to 50 µl of Caspase-Glo® Reagent. Following an incubation of 40 min at room temperature luminescence was determined lasting 0.1 sec in the 96 well plate reader Victor Light (Perkin Elmer, Langen, Germany). Results shown represent mean ± SEM of RLU (relative light units) per µg TP.

Determination of cytokine expression by enzyme-linked immunosorbent assays

Cytokine concentrations of TNF-α, IFN-γ and interleukin (IL)-10 were determined in endometrial tissue homogenates by sandwich enzyme-linked immunosorbent assays (ELISA). Commercially available kits for TNF-α (BD OptEIA human TNF ELISA Set) and IFN-γ (ELISA Assay for bovine IFNG, Code 3115-1A-6) ELISA were purchased from BD Biosciences Europe (Erembodegen, Beligium) and Mabtech AB (Nacka Strand, Sweden) and performed according to the manufacturer's protocols. Results are presented as means (µmol/µg TP) ± SEM. For the detection of IL-10 protein two monoclonal antibodies were used (Serotec, Oxford, United Kingdom). In brief, microtiter plates (Nunc MaxiSorp) were coated with the cytokine antibody CC318 at 10 µg/mL in carbonate-bicarbonate coating buffer (pH 9.6) and incubated at 4° C overnight. After washing three times with PBS/Tween-20, blocking was performed with PBS

containing 0.05% Tween-20 and 1% bovine serum albumine for one hour at room temperature. Following an additional washing step, the plates were kept for two hours at room temperature with the endometrial tissue homogenates added in duplicates (diluted 1:2). After removal of the homogenates and subsequent washing, the one hour incubation time with the biotinylated cytokine antibody CC320 at a concentration of 5 µg/mL was followed by washing and addition of streptavidin-horseradish peroxidase (HRP) for 20 min at room temperature. After the final washing step, the chromogenic substrate (3,3,5,5-tetramethylbenzidine) was introduced to the wells. Color development was stopped using H_2SO_4 (2 mol/L) after 40 min. Optical density was spectrometrically measured with an ELISA-plate reader (Tecan) at 450 nm. Due to the lack of a bovine standard for IL-10, the results are presented as optical density values ± SEM (Dacal *et al.* 2009; Flynn & Mulcahy 2008).

Determination of metabolites of the kynurenine pathway by tandem mass spectrometry

Using pestle and mortar endometrial tissue was disrupted by adding liquid nitrogen and then dissolved in methanol/chloroform/H_2O 55/22/22 % (v/v/v). The samples were incubated at 4° C for 30 min and centrifuged at 20,000 *g* for 20 min. The supernatant was stored until further processing. To the extraction solution of 100 mg tissue, 10 µl trichloroacetic acid (50%) (FLUKA, Germany), 60 µl water, 100 µl methanol (JT Baker, Deventer, The Netherlands) and 10 µl deuterated standard solutions each (phenylalanine-d5, kynurenine-d6 and kynurenic acid-d5, Cambridge Isotope Laboratories, Andover, MA, USA) was added. Samples were mixed, stored at 4° C over night and centrifuged (20,000 *g*, 15 min). A Wallac MS2 tandem mass spectrometer (Perkin Elmer, Rodgau, Germany) equipped with an electrospray ion source recorded the levels of Trp, 5 times deuterated L-phenylalanine (Phe), kynurenine, 6 times deuterated kynurenine, quinolinic acid, serotonine, kynurenic acid, and 5 times deuterated kynurenic acid with a flow solvent composition of 0.2% formic acid in 50% aqueous acetonitrile and a flow rate of 50 µl/min. Respective ions were detected in a positive ion mode using multiple reaction monitoring. The two quadrupoles were set to mass-to-charge ratios of (m/z) 205/159 for Trp, 171/125 for phenylalanine-d5, 177/160 for serotonine, 209/192 for kynurenine, 215/198 for kynurenine-d6, 168/78 for quinolinic acid, 190/144 for kynurenic acid and 195/149 for kynurenic acid-d5. Nitrogen served as collision gas. Estimated IDO activity was assessed using the L-kynurenine x 100/Trp ratio as previously demonstrated (Schefold *et al.* 2010). Tandem mass spectrometry was kindly performed by Dr. Schefold.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling

For analysis of single strand DNA nicks in uterine tissue sections, the DeadEnd™ Colorimetric terminal deoxynucleotidyl transferase enzyme-mediated dUTP-biotin nick end labeling (TUNEL) System (Promega) was performed on 5 µm thick uterine tissue sections of three heifers per group according to manufacturer's protocol. In brief, after deparaffinization and rehydratation tissue sections were permeabilized with 20 µg/ml Proteinase K for 15 min at room temperature. Following washing with PBS, slides were refixed in 4% paraformaldehyde. Single strand DNA nicks were labeled by incubating the slides with recombinant terminal deoxynucleotidyl transferase enzyme (rTdT) and biotinylated nucleotides inside a humidified chamber for one hour at 37° C. Prior to labeling, DNA nicks were induced by 10 Units per mL of RQ1 RNase-Free DNase I (Promega) in the positive control for 10 min at room temperature. The negative control was kept without enzyme, but incubated with biotinylated nucleotides and equilibration buffer instead. The labeling reaction was stopped by immersing slides in 2x saline-sodium citrate for 15 min at room temperature. Subsequent washing with PBS removed unincorporated biotinylated nucleotides on the slides. After blocking of endogenous peroxidases with 0.3% H_2O_2 for 5 min at room temperature, streptavidin-HRP was introduced to the uterine tissue sections for 30 min at room temperature. The HRP-enzyme converts H_2O_2 into H_2O and protons which results in oxidation of 3,3'-diaminobenzidine (DAB) to a brown insoluble product. The tissue sections were rinsed in deinoized water and covered with Glycerol-PBS. Brown stained apoptotic cells were visualized by a light microscope.

Immunohistochemical localization of death receptor ligand FAS ligand

Protein of the death receptor ligand FASLG was localized on trophoblast and uterine tissues sections. Histological sections (5 µm) were deparaffinized and rehydrated in descending serial ethanol dilutions. Endogenous peroxidase was inactivated by treatment with 1% H_2O_2 in methanol. The slides were then washed with PBS containing 0.05% Tween-20. Following blocking with 10% normal goat serum (Dako, Glostrup, Denmark) for 30 min at room temperature, samples were incubated overnight at 4° C inside a humidified chamber with the rabbit polyclonal N-20 sc-834 antibody (4.0 µg/mL, Santa-Cruz, Biotechnology Inc. Santa-Cruz, CA, USA). Following washing, the tissue sections were incubated with secondary antibody anti-rabbit immunoglobuline G peroxidase-conjugated (2.5 µg/mL, Sigma–Aldrich) for 40 min at room temperature. After washing the slides in PBS, binding of antibody was visualized by incubating the tissue sections with DAB in the presence of 0.1% H_2O_2 for 5 min. Tissue sections were counterstained using Mayer's Haemalaun (Carl Roth GmbH, Karlsruhe, Germany), dehydrated and mounted with Eukitt quick hardening mounting medium (Sigma-Aldrich).

Immunohistochemical localization of endometrial leukocytes

The cluster of differentiation (CD) 45 protein was localized in endometrial tissue sections. This surface protein is expressed at high abundance on all cells of hematopoietic origin, except erythrocytes. Heat induced antigen retrieval was carried out on deparaffinized and rehydrated uterine tissue sections by boiling the slides in citrate buffer (pH 6.0) for 60 min. After washing with PBS containing 0.05% Tween-20, endogenous peroxidase was inactivated by treatment with 1% H_2O_2 in PBS. To avoid non-specific binding of antibodies, tissue sections were treated with 10% goat serum for 30 min. Subsequently to the washing procedure, samples were incubated with anti-CD45, CACTB51A (20 µg/mL, VMRD, Pullman, WA, USA) for 30 min at room temperature. After washing in PBS, samples were incubated with secondary antibody anti-mouse immunoglobulin G HRP-conjugated (1.3 µg/mL, Dako). Binding of antibody was detected by incubating the tissue sections with DAB in the presence of 0.01% H_2O_2 for 15 min. The reaction was stopped by washing the slides in PBS. Endometrial tissue sections were counterstained using Mayer's Haemalaun (Carl Roth GmbH). Positively immunostained leukocytes were counted by an observer unaware of the day of the cycle or the pregnancy status. CD45-positive cells were manually determined in at least 5 mm length of uterine LE and GE per tissue section by differentiating between GE in the zona functionalis and basalis (n=5-7 animals per group). In addition, leukocytes in the zona basalis and zona functionalis were also counted in a minimum area of 5 mm^2 within each uterine tissue section. The results were expressed as the mean ± SEM number of positively stained cells.

Localization of endometrial indoleamine 2,3-dioxygenase 1 messenger ribonucleic acid by in situ hybridization

In situ hybridization was performed in paraffin-embedded endometrial sections of day 18 non-pregnant vs. pregnant animals as previously described (Bauersachs *et al.* 2005). *In situ* hybridization was kindly performed at the Institute of Veterinary Anatomy, Histology and Embryology, Ludwig Maximilian University (Prof. Sinowatz). In brief, deparaffinized and rehydreated uterine sections were air dried. Subsequent to incubation in 2x saline-sodium citrate for 2x15 min at room temperature, uterine tissue sections were heated in a water bath to 80° C for ten min. After cooling to room temperature, slides were rinsed in distilled water followed by Tris-bufferded saline. Afterwards, the uterine sections were permeabilized for 20 min using 0.05% protease E (VWR, Ismaning, Germany) in Tris-buffered saline. Subsequent to washing in Tris-buffered saline and distilled water, uterine tissue sections were refixed in 4% paraformaldehyde. After washing in PBS and distilled water, slides were dehydrated in ascending ethanol dilutions and air dried. Hybridisation was performed using the following biotinylated antisense

oligonucleotide: 5'-GAG GCA GCT GCT ATT TCC AC. A complementary sense probe served as negative control. Probes (100 pm/µl) were diluted 1:20 in a *in situ* hybridization solution (Dako, Munich, Germany) and applicated on the slides for the overnight incubation at 38° C inside a humidified chamber. The following day, slides were washed in prewarmed (38° C) 2x saline-sodium citrate for 2x15 min. After washing the slides with distilled water and Tris-bufferd saline, the detection of the biotinylated probes was carried out using a HRP-labeled avidin-biotin-complex kit (DAKO) and developed using DAB.

In vitro co-culture of endometrial glandular and stroma cells

To analyze whether IFN-τ stimulates the expression of specific genes an *in vitro* co-culture model of endometrial GE and stroma cells were performed as described earlier (Ulbrich *et al.* 2009a; Ulbrich *et al.* 2010). Uteri bearing a CL approximately 8 days after ovulation were collected at the local slaughterhouse and kept on ice until further processing. Prior to the preparation of GE and stroma cells, the horn ipsilateral to the CL bearing ovary, was filled with ethylenediaminetetraacetic acid and incubated for one hour at 37° C. Luminal epithelial cells were removed from the endometrium by using a scalpel. For the separation of GE and stroma cells, the residual endometrium was digested with 0.05% collagenase in Dulbecco modified Eagle medium/F12 containing 10% fetal calf serum for three hours at 37° C. Endometrial cells were filtered through a 250 µm and a 33 µm steel mesh for the separation of stroma cells from GE cells. Filtrated stroma cells were seeded at the bottom of cell culture wells, while the remnant GE were co-cultivated in the same well on growth-factor reduced Matrigel-coated inserts (BD Biosciences). The exchange of soluble components was possible throughout the experiment. Endometrial cells were grown to confluence prior to stimulation with recombinant bovine IFN-τ (antiviral activity, 4.8 x 10^3 Units/mL medium; PBL Biomedical Laboratories, Piscataway, NJ). Co-cultivated endometrial cells, incubated with medium alone served as a control. Co-cultivated cells were harvested after four hours following stimulation and suspended in 500 µl of TRIzol reagent (Invitrogen). Total RNA extraction and gene expression analysis was carried out as detailed above.

Statistical analysis

For statistical analysis the SAS program package release 9.1.3 (2002; SAS Institute, Inc., Cary, NC, USA) was used. The data comparing endometria from cyclic and pregnant uteri were statistically evaluated using least-square analysis of variance using the General Linear Models procedure to determine effects of the day of the cycle (day), the pregnancy status (cyclic vs. pregnant, status), and their interaction (day*status). Student's *t*-test was used to compare the

transcript abundance of conceptuses at day 15 and day 18. Differences were considered significant at $p<0.05$. Differences between endometrial and embryonic tissue were estimated as fold changes, but not considered for statistical analysis. Graphs were plotted using Sigma-Plot 8.0 (SPSS software GmbH, Munich, Germany).

3. RESULTS AND DISCUSSION

3.1 Conceptus development requires an optimal nutrient supply in the uterine lumen

Increase of essential amino acids in the uterine lumen during preimplantation

Next to the increase of most non-essential AA (Groebner *et al.* 2011, App III, Fig. 2) in the uterine lumen of pregnant animals, essential AA (Groebner *et al.* 2011, App III, Fig. 4) increased most pronounced as measured by the highly sensitive LC-MS/MS method (Fig. 7). The concentrations of intrauterine AA at peri-implantation were higher than detected in the uterine fluid of the late secretory phase (days 12, 15, 18). Concordantly with results obtained from studies in the ewe, almost all AA increased in the bovine uterine fluid of pregnant animals prior to implantation (Gao *et al.* 2009d).

The most pronounced increase in the preimplantation phase was detected for the branched chain AA L-isoleucine (Ile) (Groebner *et al.* 2011, App III Fig. 4g). While Ile increased consistently with a 17.6-fold and 46-fold higher concentration in uterine flushings of days 15 and 18 pregnant vs. non-pregnant animals, levels remained almost constant in the uterine fluids of cycling cows. Further branched chain AA L-leucine (Leu) and L-valine (Val) as well as aromatic AA Phe, Tyr and Trp increased considerably during the preimplantation phase ($p<0.01$). Required not only for protein synthesis, essential AA are also necessary for rapid growth and cell diffentiation. Metabolic products of essential as well as non essential AA such as pyruvate, α-ketogluterate and acetyl coenzyme A provide energy for the tremendous embryonic development during preimplantation and may act as anaplerotic molecules for replenishing intermediates of the citrate cycle. In accordance with previous studies performed in ruminants, glycine (Gly) (Groebner *et al.* 2011, App. III Fig. 2a) was the predominant AA in the ovine and bovine uterine fluid (Gao *et al.* 2009d; Hugentobler *et al.* 2007). Conversely to the increase of intrauterine Gly in the pregnant ewe, Gly was not significantly different in pregnant vs. non-pregnant cattle at day 18 (average abundance: 236 nmol/mg TP) indicating species specific exigencies during this developmental phase. The AA Gly, interconvertible with L-serine (Ser), is assembled in proteins at sterically constricted positions, and acts as a precursor molecule for the synthesis of purines, pyrimidines, porphyrin, creatin and glutathion. Apart from glutathion, protection from reactive oxygen species can be also achieved by external anti-oxidant molecules such as hypotaurine, taurine (Tau) as well as ascorbic acid in tubal and oviducal fluids (Guerin *et al.* 2001). In the bovine uterine fluid, the non-proteinogenic sulfonic acid Tau was present with an average concentration of

163 nmol/mg TP exhibiting a significant interaction between the day of the cycle and pregnancy status. Concentrations of Tau were 30% lower in uterine flushings of pregnant animals. Antioxidant effects of Tau include the neutralization of reactive aldehydes and the inhibition of lipid peroxidation by interacting with phospholipids in biomembranes (Ogasawara *et al.* 1993). However, Tau additionally acts on calcium homeostasis and membrane stabilisation through interactions with phospholipids and serves as organic osmolyte (Schaffer *et al.* 2010). Our results did not point to an increased requirement of intrauterine Tau at peri-implantation in cattle as the concentrations were stable throughout the preimplantation phase. However, concentrations increased in non-pregnant heifers from day 15 to day 18 in the uterine lumen. A differential regulation of Tau abundance in the ovine uterine lumen might indicate species specific exigencies prior to implantation since Tau levels increased only due to pregnancy (Gao *et al.* 2009d).

Preimplantation embryos not only consume components from the surrounding medium, but also release metabolic byproducts including AA into the uterine fluid depending on the developmental stage (Booth *et al.* 2005). A differentiation whether non-essential AA and derivatives in the uterine fluid derive from either the mother or the developing conceptus was not possible within the present study, but as the conceptus considerably expands during elongation, a substantial part must necessarily be provided by the transport out of the maternal circulation into the uterine fluid.

The transcript abundance of the lysosomal His and peptide transporter *SLC15A3* also known as *PHT2* increased 6-fold in the endometrium of pregnant heifers from days 12 to 18 *(*Groebner *et al.* 2011, App. III, Table 1*),* while the transcript abundance of further AA transporter analyzed did not vary due to the pregnancy status. Expressed in lysosomal mebranes, SCL15A3 promotes the transport of peptides and free His (Daniel & Kottra 2004). In cooperation with further cationic transporters located at the apical sides of endometrial cells, His and peptides might be consequently transferred into the uterine fluid. Possible candidates for the further transport might be cationic AA transporter belonging to the cationic AA transporter family *(SLC7A1-3)* as its expression in ovine endometrial epithelia was reported only recently (Gao *et al.* 2009a). A regulation of *SLC15A3* due to the trophoblast derived IFN-τ was assumed as the transcripts of this transporter were increased due to pregnancy shown in previous holistic transcriptome analysis *in vivo* (Bauersachs *et al.* 2006). An IFN-τ dependent increase was affirmed by our *in vitro* co-culture model of endometrial GE and stroma cells where *SLC15A3* mRNA increased 177-fold most pronounced in stroma cells upon IFN-τ stimulation (Groebner *et al.* 2011, App. III, Table 2).

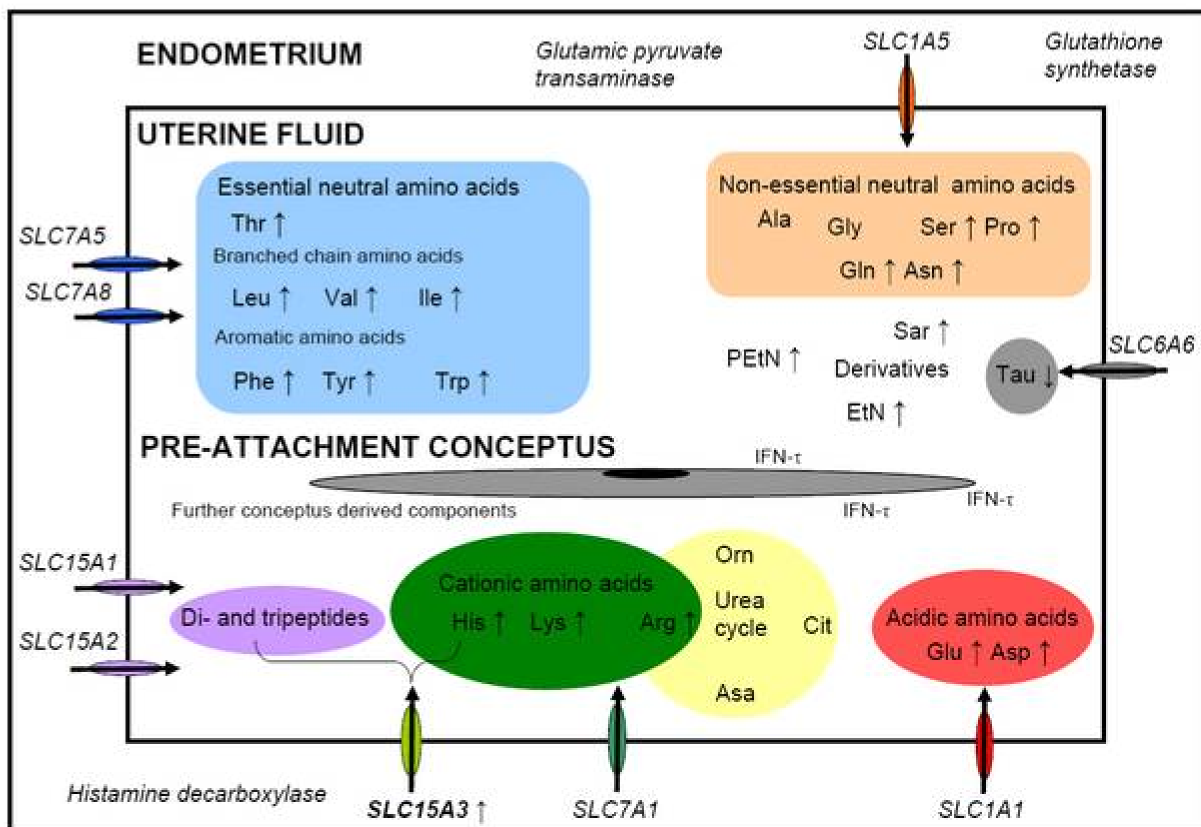


Fig. 7: Essential amino acids (AA) increased in the uterine lumen to nourish the pre-attachment conceptus.
The intrauterine concentration of most essential and non-essential AA increased in pregnancy (↑). Increasing L-histidine (His) concentrations were accompanied by increasing endometrial expression of the lysosomal His/peptide transporter solute carrier 15A3 (*SLC15A3)* due to the pregnancy recognition signal IFN-τ. Arrows indicate a significant different expression of transporters and concentrations of AA and derivatives at day 18 pregnant animals vs. non-pregnant animals (*p<0.05*).

Although the transcript abundance of most transporters was not affected by the pregnancy status, additional regulatory mechanisms must be presumed, since in particular essential AA increased in pregnant animals. The mammalian target of rapamycin (mTOR) signaling pathway actively regulates cellular proliferation and mitosis as well as migration and translation of mRNA to protein and therefore plays a key role in homeostasis. The concentration of AA is known to influence mTOR signaling (Long *et al.* 2005) wherby Leu is one of the major AA regulating protein synthesis by affecting phosphorylation of protein 4E-BP1 and S6K1 that are substrates of mTOR (Buse MG et al. Anthony, JC, 2000 et al). The steroid hormone P4 along with IFN-τ stimulate components of the mTor pathway in the ovine endometrium (Gao *et al.* 2009c) most likely by favoring enrichment of large and branched chain AA. The inhibition of the mTOR pathway in human trophoblast cells evidenced a reduced activity of large neutral AA transporter (system L) and additionally of transporter mediating the transport of small neutral AA (system A) and Tau (system β) (Roos, S, 2009). Our results point to a distinct role of the mTOR pathway regulating AA concentrations in the bovine uterine lumen, but detailed processes in regulating intrauterine

AA levels further need to be elucidated. Since the increase of most AA from day 12 to day 18 of the estrus cycle was not observed in the uterine fluid of non-pregnant cattle, a direct or indirect induction of the AA transport into the uterine lumen by components provided by the conceptus may reasonably be presumed.

Inadequate embryo-maternal signaling leads to an aberrant luminal amino acid composition

Inadequate signaling *in utero* potentially due to metabolic imbalances or unfavorable *in vitro* conditions in case of IVF and SCNT severely affect metabolic imprinting. Next to a disturbed embryonic/fetal development, the susceptibility to chronic diseases in adult life is significantly increased (Waterland & Jirtle 2004). In particular, SCNT in cattle increases the risk for abnormal placentome formation and the development of oversized fetuses in later pregnancy stages (Young *et al.* 1998). Since alterations in endometrial gene expression were detected during preimplantation (Bauersachs *et al.* 2006; Mansouri-Attia *et al.* 2009), we focused on the examination of potential imbalances in uterine AA metabolism prior to the attachment. IVF-derived embryos were used as reference to SCNT conceptuses, as both undergo a comparable *in vitro* cultivation prior to the transfer into recipient animal. Since the transferred blastocysts represent half-siblings, the genetic variability was comparable between the groups (Bauersachs et al. 2009). Pregnancy rates were 77% for the IVF and 59% for the SCNT group.

Next to a significant decrease of acidic L-glutamic (Glu) and L-aspartic acid (Asp), we detected reduced levels of L-proline (Pro) and L-alanine (Ala) (Groebner *et al.* 2011, App. IV, Table 2) in SCNT pregnancies. The concentrations of essential AA Leu, L-valine (Val), Ile and Phe were significant decreased in the presence of SCNT-embryos. Moreover, the lowered intrauterine AA concentration was accompanied by a two-fold lower expression of the large neutral AA transporter *SLC7A8 (*Groebner *et al.* 2011, *in press*, App. IV, Table 3*). SLC7A8* mRNA located in the ovine luminal epithel and subepithelial stroma is highest expressed during the elongation phase between day 16 and 20 in the ewe (Gao *et al.* 2009b) and mediates the transmembranal transfer of essential large neutral aromatic and branched chain AA (Verrey 2003). The lowered mRNA expression of *SLC7A8* in SCNT pregnancies might indicate an inappropriate endometrial response towards the SCNT embryo. However, next to the modified endometrial gene expression, the composition of the uterine fluid is also altered in SCNT pregnancies. The insufficient supply of the embryo with AA during preimplantation, possibly causes the abnormal fetal development often detected in later stages of SCNT pregnancies. Overcompensatory mechanisms of the fetus due to the early manipulations caused aberrant epigenetic reprogramming which subsequently leads to the typical pregnancy failures (Dean *et al.* 2001; Santos *et al.* 2003).

In addition to the AA composition in the uterine fluid, AA derivatives were as well under investigation. O-Phosphoethanolamine (PEtN) is a degradation product from phospholipids. As components of biological membranes, phospholipids are required for various regulatory processes and act as precursor molecules for many bioactive substances, such as eicosanoids and lysophopholipids. Distribution of distinct phospholipid species changed during murine implantation. Furhter phospholipids are involved in cell death and angiogenic events at implantation sites (Burnum *et al.* 2009). Disturbances in sphingolipid metabolism caused pregnancy loss by inadequate blood vessel formation and excessive cell death (Mizugishi *et al.* 2007). A contribution of phospholipids in establishment of bovine pregnancies might be assumed due to their increasing levels in naturally conceived pregnancies during the preimplantation phase (Groebner *et al.* 2011, App. IV, Table 2). The 11-fold lower concentration of PEtN in the presence of a SCNT conceptus might assign for a modified capacity of SCNT embryos to affect maternal prostaglandin synthesis. Prostaglandins (PG) are a central group of eicosanoid lipid molecules. Phospholipase A2 mediates the release of arachidonic acid from membranal phospholipids and the cyclooxygenase subsequently catalyzes the reaction from arachidonic acid to PGH2, the precursor molecule of PGs. Converted to various PGs by specific synthases, PGs are involved in numerous reproductive processes. Increased concentrations of PGs in the uterine lumen appear to be essential for embryonic development prior to implantation (Ulbrich *et al.* 2009b). During early murine pregnancy, arachinodate-containing phospholipids revealed a strong correlation of PEtN with the presence of cytosolic phospholipase A 2 and cyclooxygenase 2 in implantation sites (Burnum *et al.* 2009). If the abundance of PEtN cohered with the PG metabolism in bovine pregnancies, SCNT embryos would not only suffer from metabolic problems due to insufficient AA transport, but would also be affected hereof concerning regulatory disturbances.

Stojkovic *et. al.* (1999) previously reported that the size of the trophoblastic area of IVF vs. SCNT conceptuses as well as the intrauterine bioactivity of IFN-τ were not different before day 19 (Stojkovic *et al.* 1999). Alike in the present study, intrauterine IFN-τ bioactivity did not differ among the IVF and SCNT treatment groups (Groebner *et al.* 2011, App. IV). Apart from direct effects of the ruminant pregnancy recognition signal predominant in the preimplantation phase, additional conceptus derived signaling components must be hypothesized. The inadequate embryonic signaling, most likely due to epigenetic abnormalities of SCNT embryos (Dean *et al.* 2001; Santos *et al.* 2003), possibly led to the inadequate maternal transport. The optimal interactive embryo-maternal crosstalk thus appears to be indispensable for the provision of an optimal uterine environment allowing embryo development.

3.2. Expression of proapoptotic genes in response to interferon-tau is not accompanied by increased cell death in the endometrium

The ruminant pregnancy recognition signal IFN-τ is secreted by the preimplantation trophoblast to inhibit luteolysis and to sustain P4 synthesis for establishment of pregnancy. IFN-τ concentration continously increases in the bovine uterine lumen during preimplantation (Groebner *et al.* 2010, App. I, Fig. 3A). Aside from effects on the CL, IFN-τ additionally modulates endometrial gene expression and particularly favors the expression of ISGs associated with endometrial remodeling, immune function and cell adhesion (Bauersachs *et al.* 2006). We tested therefore, whether the increasing release of IFN-τ during preimplantation would affect endometrial apoptosis and the activation of the apoptotic signaling cascade *in vivo*.

Proapoptotic genes *XAF1* and *CASP8* increased in the endometrium of pregnant animals over time and were higher expressed in day 18 pregnant vs. non-pregnant animals. In addition, gene expression of *TNFSF10* increased with the preceeding preimplantation phase with 4.9-fold higher expression in pregnant vs. non-pregnant animals at day 18 (Groebner *et al.* 2010, App. I, Fig. 1B). Although the mRNA expression of proapoptotic genes was increased in response to IFN-τ in pregnant animals the activity of initiator CASP8 (Groebner *et al.* 2010, App. I, Fig. 2C) did not coincide with mRNA expression, but decreased with time irrespective of the pregnancy status. Moreover, downstream effector CASP3/7 activity (Groebner *et al.* 2010, App. I, Fig. 2D) was neither affected by pregnancy and likewise apoptotic cells death detected by the TUNEL method did not exceed the level of general cell turnover in neither group analyzed (Groebner *et al.* 2010, App. I, Fig. 4). The gene expression of each antiapoptotic component measured in the present study (*BCL2L1, CFLAR, BIRC4/-5*) was not affected by pregnancy (Fig. 8). The discrepancy of CASP8 mRNA and protein might be explained by possible antiapoptotic effects of CFLAR. Isoforms of CFLAR compete with CASP8 for the binding site on cytoplasmic death domains thereby inhibiting catalytical cleavage and activation of CASP8 (Thorburn 2004). But even at later stages of the apoptotic cascade inhibitor mechanisms might tightly control the final activation to avoid excessive cell death. Antiapoptotic component *BIRC4* inactivates CASP by mediating the transfer into the nucleus upon binding. The proapoptotic agent *XAF1* primarily expessed in stroma cells following IFN-τ treatment *in vitro*, however, antagonizes BIRC4 effects (Stehlik *et al.* 1998). The induction of proapoptotic gene expression due to IFN-τ did not lead to subsequent cell death in the endometrium of pregnant animals indicating that mechanisms exist to avoid excessive cell death prior to implanation (Fig. 8).

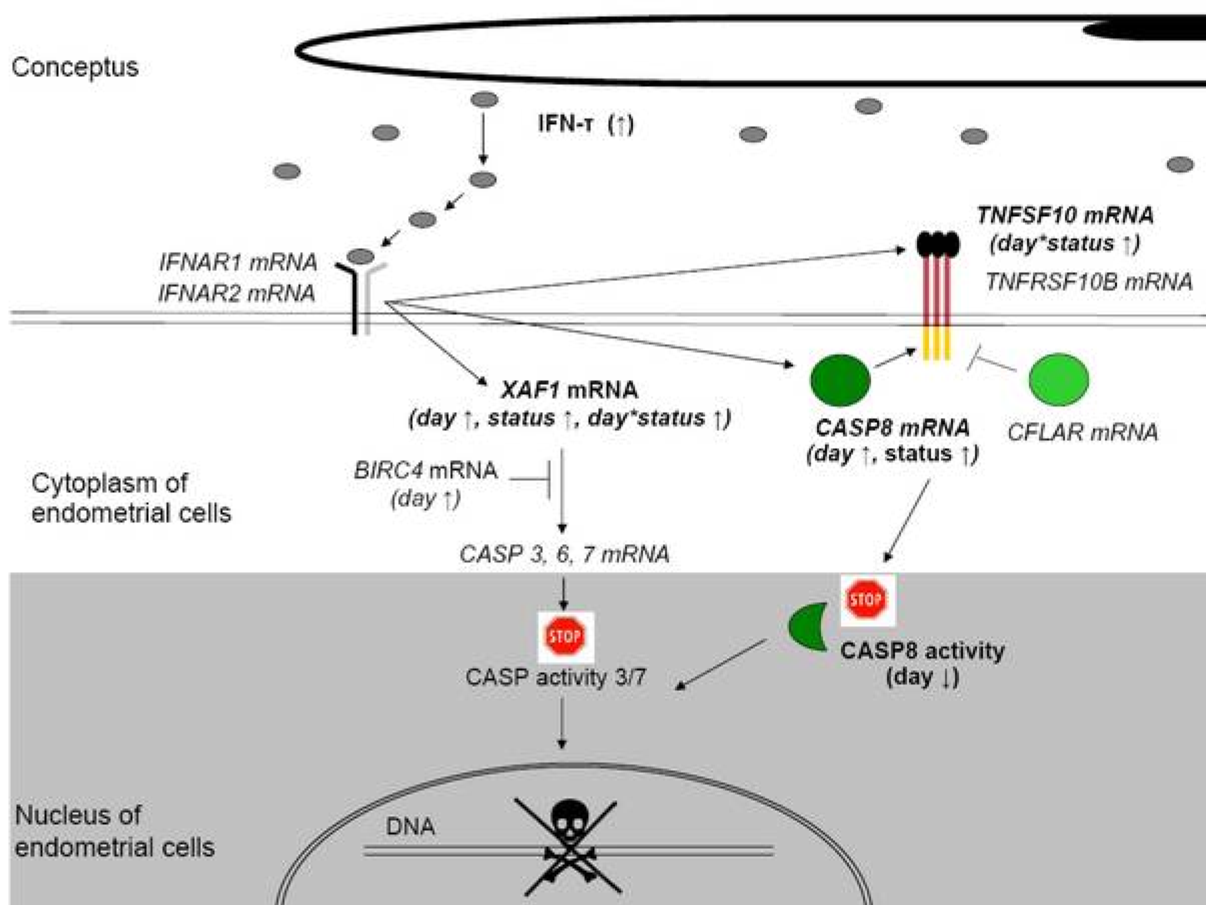


Fig. 8: Effects of trophoblast derived IFN-τ on apoptosis in the endometrium.
The gene expression of three proapoptotic components (*XAF1, CASP8* and *TNFSF10*) increased due to IFN-τ *in vivo* and *in vitro* (↑). However, the proapoptotic signal was not transduced as neither CASP acitivity nor frequency of apoptotic cells increased in case of pregnancy (↓). Anti-apoptotic mechanisms to avoid excessive cell death must be assumed as excessive apoptosis could negatively influence the secretory properties of the intercaruncular endometrium required for embryo development. Significant differences with respect to the day of the cyclus (day), the pregnancy status (cyclic vs. pregnant, status), and their interaction (day*status) are shown ($p<0.05$).

As the expression of neither anti-apoptotic gene analyzed was regulated due to pregnancy, antiapoptotic mechanisms aside from the transcriptional level might be assumed. Whether a single factor or a range of factors derived from either the conceptus or the mother entail the interruption of the apoptotic pathway remains speculative. However, attention must be drawn to microRNAs. The small non-coding RNAs regulate several cellular processes including differentiation, proliferation and apoptosis. Silencing is mediated by hybridization to expressed mRNAs and subsequent digestion through the RNA-induced silencing complex (RISC) recognizing double stranded RNAs (Garofalo & Croce 2010). Diverse microRNAs have been shown to exert anti-apoptotic effects. For instance, mi-R21 inhibits activation of effector CASP (Corsten *et al.* 2007) and more than hundreds of microRNAs are involved in regulation of

TNFSF10 induced apoptosis (Ovcharenko *et al.* 2007). Thus, microRNAs may also participate in controlling apoptosis in the bovine endometrium as excessive apoptosis might negatively influcence secretory function of the intercaruncular endometrium. Likewise, the investigation of apoptotic events in the caruncular endometrium might be of major interest as at these specialized areas attachment of the trophoblast occurs.

3.3 Establishment of immune tolerance during preimplantation avoids rejection of the semi-allogenic embryo

The dominance of P4 inhibits the activation of lymphocytes and thus promotes the acceptance of the semi-allogenic embryo for the establishment of pregnancy (Low & Hansen 1988). However, the generation of immunological tolerance at the embryo-maternal interface involves multiple strategies. Previous holistic microarray studies already demonstrated an increased expression of genes involved in immune modulating processes in the bovine endometrium (Bauersachs *et al.* 2006), but microarray technology is less sensitive than RT-qPCR. Although expression of genes associated with immunological tolerance mechanisms are often restricted to sparse immune cell populations, gene expression analysis of immune related genes by using the high-throughput Fluidigm 96.96 dynamic gene expression array revealed potential tolerance mechanisms prior to implantation expanding our knowledge about immunological interactions between mother and embryo.

Trophoblastic FAS ligand expression might induce apoptosis in maternal immune cells

A high FASLG expression is commonly attributed to immune privileged organs such as the testis, eye an brain (Griffith *et al.* 1995) and additionally FASLG expression by human placental trophoblast cells is regarded as a major mechanism in generating peripheral immune tolerance towards the semi-allogenic embryo (Kauma *et al.* 1999). Next to a significant 84-fold increasing *FASLG* expression from day 15 to day 18 in conceptuses (Groebner *et al.* 2010, App. I, Fig. 1A), an intense immunostaining of FASLG in day 18 trophoblast cells was detected (Groebner *et al.* 2010, App. I, Fig. 4). The mRNA expression of *FASLG* exceeded three-fold the levels measured in the maternal tissue at day 18. As demonstrated by Abrahams *et al.* (2004b), human first trimester trophoblast own the capacity not only to express membrane bound FASLG, but also to secrete a soluble form of the death receptor ligand via microvesicles into the uterine lumen. This appears to be a mechanism possibly to induce selective death receptor mediated killing of activated, FAS receptor bearing lymphocytes, whereas the trophoblast itself is resistant to FALSG induced apoptosis (Aschkenazi *et al.* 2002). It is currently not known, whether the bovine

trophoblast secretes FASLG into the uterine lumen prior to implantation and hence actively escapes the recognition through sensitized maternal lymphocytes, but the prominent expression of FASLG protein supports a key role for bovine FASLG in generating immune tolerance in cattle during the pre-attachment phase.

Next to to the increasing mRNA expression of endometrial FASLG during preimplantation (Groebner *et al.* 2010, App. I, Fig. 1A), an intense FASLG immune staining was detected in deep endometrial glands and endothelial cells (Groebner *et al.* 2010, App. I, Fig. 4 C-E). Our data might indicate that the mother additionally contributes to the generation of tolerance via the death receptor mediated apoptosis pathway independent of the trophoblast derived IFN-τ (Groebner *et al.* 2010, App. I, Table 2). FASLG expressing deep endometrial GE and endothelial cells might control the transition of FAS receptor bearing lymphocytes into the uterine lumen independent of trophoblast derived IFN-τ. Highest expression of FASLG was found in GE and stroma cells during the late secretory phase in the human endometrium, indicating that P4 positively influences FASLG expression (Selam *et al.* 2001). It remains unknown, whether the increase of bovine FASLG mRNA concentration in the endometrium with preceding time is modulated due to steroid hormones, but endometrial FASLG expression could support the generation of an immune suppressive environment to allow embryo development.

Marginal expression of major histocompatibility complex class I might protect the bovine preimplantation conceptus from rejection

Expression of MHC class I molecules is required for the identification of “self” and “non-self” and the presentation of foreign antigens. However, the expression of “non-self” MHC molecules leads to severe immune responses. The regulation of MHC expression on the cell surface is therefore a potent tolerance inducing mechanism to avoid rejection. In comparison to the endometrial expression, transcripts of B2M, were only marginal expressed on conceptus tissue (Groebner *et al.* 2011, App. II Table 2&3) suggesting an active down-regulation to prevent from the detection by maternal lymphocytes and subsequent rejection. Neither day 8 bovine hatched blastocysts (Low *et al.* 1990), nor ovine preimplantation trophoblasts express MHC class I molecules (Choi *et al.* 2003). However, the increased MHC class I expression of five to nine week trophoblasts derived by SCNT was accompanied by increased leukocyte numbers in the bovine endometrium (Hill *et al.* 2002). The minor expression of MHC class I molecules on conceptus tissue which persisted throughout the preimplantation in the present study and most likely ensures appropriate implantation and avoids lymphocyte mediated cell death of the developing embryo.

In the bovine endometrium, expression of the MHC class I α-chain and the B2M subunit increased in early pregnancy as shown by previous microarray experiments (Bauersachs *et al.*

2006). Moreover, an increase of B2M transcripts detected by gene expression analysis was confirmed in the present study (Groebner *et al.* 2011, App. II Table 2). In accordance with findings from the ewe, overall endometrial mRNA for MHC class I and B2M increased in the presence of trophoblast derived IFN-τ from days 14-20 of pregnancy (Choi *et al.* 2003). In contrast, the expression concomitantly decreased in the endometrial layers next to the ovine uterine lumen (Choi *et al.* 2003). The ablation of MHC molecules from the LE and superficial GE is supposed to be a protective mechanism in order to avoid the presentation of trophoblast derived antigens on endometrial MHC molecules (Choi *et al.* 2001; Choi *et al.* 2003). The restriction of ovine MHC class I molecules to the deeper GE and stroma is supposedly entailed by the expression of IFN regulatory factor 2 (IRF2), a transcriptional repressor of ISG, expressed in the LE and superficial GE (Choi *et al.* 2001). However, neither the ovine (Choi *et al.* 2001) nor the bovine IRF2 transcript abundance (Groebner *et al.* 2011, App. II, Table 2) was affected by the pregnancy status. It remains unknown; whether a similar spatial expression pattern of IRF2 and MHC class I molecules also contribute to the generation of immune tolerance in the embryo-maternal interface.

Non-classical, less polymorphic MHC class I molecules such as HLA-G molecules in human are located on placental tissue with highest expression on extravillous trophoblast cells to avoid detection by natural killer cells (Le Bouteiller P. *et al.* 1999). Binding of HLA-G to killing inhibitory receptors on natural killer cells facilitate the survival of conceptus cells. Blast search revealed a HLA-G analogue in *bos taurus* which was analyzed within the Fluidigm gene expression array (Groebner *et al.* 2011, App. II, Table 2&3). Interestingly, expression was more than 4,000-fold higher in the bovine endometrium prior to implantation. Conflicting data exist concerning the expression of HLA-G on preimplantation blastocysts. According to Hiby *et al.* (1999) HLA-G is not expressed prior to implantation on human blastocyst but continuously increases upon implantation. In contrast, novel data indicate a participation of membrane bound and soluble forms of HLA-G molecules expressed in pre- and peri-implantation conceptuses in generating immune tolerance and mediating embryo implantation in humans (Fanchin *et al.* 2007). On bovine third trimester trophoblasts, a variety of non-classical MHC class I molecules are expressed (Davies *et al.* 2006) and recently the expression of non-classical MHC class I molecules have been demonstrated from the bovine oocyte up to the spherical blastocyst stage (Doyle *et al.* 2009). Whether the survival of the bovine conceptus and the establishment of pregnancy are dependent on tolerance induction mediated by the marginal expression of non-classical MHC class I molecules prior to implantation must be elucidated. But the presence of non-classical MHC molecules strongly indicates a role in generation tolerance.

Trophoblast derived interferon-tau increased expression of endometrial indoleamine 2,3-dioxygenase 1 most likely to induce immune tolerance

The most prominent expression differences were detected for *IDO* in the endometrium of pregnant vs. non-pregnant heifers (Groebner *et al.* 2011, App. II, Table 2), while *IDO* expression of conceptuses was below the limit of detection (Groebner *et al.* 2011, App. II, Table 3). An 18-fold higher abundant gene expression in the endometrium of pregnant heifers indicated a prime importance of IDO for the establishment of pregnancy. Beside an 2,700-fold increase of IDO expression following treatment with recombinant IFN-τ in *in vitro* co-cultivated stroma cells (Groebner *et al.* 2011, App. II, Table 4), IDO mRNA was mainly localized in deep endometrial stroma cells surrounding glandular ducts in pregnant cattle demonstrated by *in situ* hybridization (Groebner *et al.* 2011, App. II, Fig. 3). The induction of IDO expression by type I (IFN-α/–β) and II IFN (IFN-γ) in addition to lipopolysaccharide and further TLR ligands has already been described (Taylor & Feng 1991), and we confirmed a stimulation by trophoblast derived IFN-τ during the pre-implantation phase both *in vivo* and *in vitro.* In species showing a hemochorial placentation, tolerance induction mediated by IDO is assumed, since IDO is present at the embryo/fetal-maternal interface at high levels (Drenzek *et al.* 2008) and an inhibition of IDO activity by 1-methyltryptophan direct the rejection of allogenic, but not syngenic murine embryos (Munn *et al.* 1998). Moreover, IDO promotes the induction of T cell anergy and generation of a less sever T-helper-2 (Th2) immune response (Xu *et al.* 2008). However, IDO transcripts in primate and rodent species are primarily located in epithelial, but not stroma cells as in *bos taurus* (Groebner *et al.* 2011, App. II, Fig. 3). Species specific differences might account for the different expression patterns in endometrial cell types, but whether the stromal IDO expression more effectively impedes the migration of potential deleterious lymphocytes into the uterine lumen during the pre-implantation phase is disputable. Next to the increased transcript abundance, an increased estimated IDO activity calculated by the L-kynurenine/Trp ratio was detected at day 18 by using tandem mass spectrometry (day *p=0.002*, day*status *p=0.03*) (Groebner *et al.* 2011, App. II, Fig. 4). While total endometrial Trp concentrations declined in endometrial tissue from day 12 to day 18 irrespective of the pregnancy status (day *p=0.008*), endometrial kynurenine abundance increased primarily in pregnant heifers (day *p=0.005,* day*status *p=0.02*). However, further downstream catabolites kynurenic acid and quinolinic acid were not influenced by pregnancy. Overall serotonine levels were reduced in the endometrium of pregnant animals throughout the analyzed time period, indicating a decreased prevalence for this metabolic pathway in pregnancy (Fig. 9). Increased IDO activity causes nutrient starvation of activated lymphocytes consequently reducing immune cell function (Mellor & Munn 1999; Mellor & Munn 2000; Mellor *et al.* 2001). Other recent reports confirmed that kynurenine pathway metabolites accumulating at the site of IDO activity suppress T cell proliferation. For instance, accumulation of cytotoxic kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid irreversibly inhibited T cell proliferation *in vitro* (Terness *et al.* 2002) and moreover quinolinic acid induced apoptosis in Th1, but not in Th2 cells

(Fallarino *et al.* 2002). To conclude, the activity of potential deleterious leukocytes can either be inhibited indirectly by the generation of a Trp-depleted microenvironment (Munn *et al.* 1998), or directly by adverse Trp-derived catabolites (Terness *et al.* 2002). Thus, both L-kynurenine which was significantly increased in the endometrium of pregnant animals at day 18 and increased IDO activity causing local Trp ablation might contribute to tolerance induction at the embryo-maternal interface necessary for implantation and placentation.

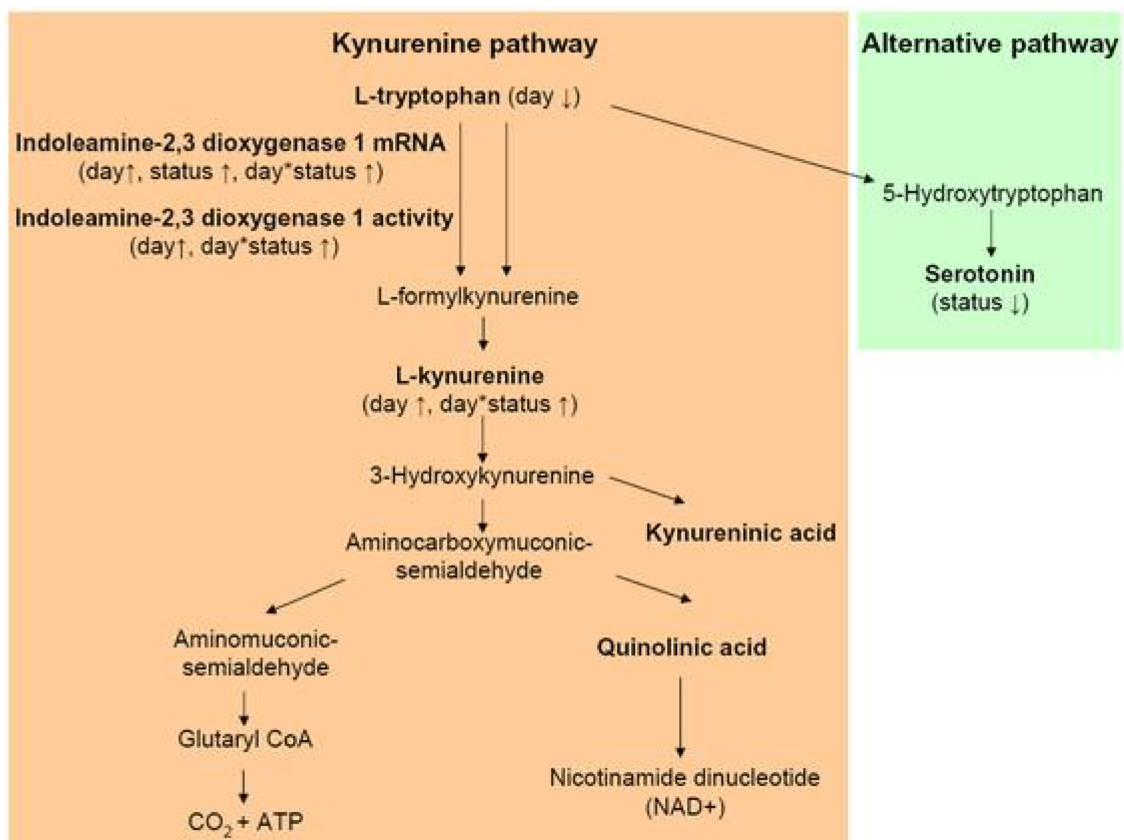


Fig. 9: Accumulation of L-kynurenine due to increased indoleamine-2,3 dioxygenase activity in the endometrium.
While endometrial Trp was decreased with time irrespective of the pregnancy status (↓), endometrial kynurenine abundance increased primarily in the endometrium of pregnant animals due to increased IDO activity at day 18 of pregnancy (↑). Components shown in bold were analyzed within the present study. Significant differences in terms of the day of the cyclus (day), the pregnancy status (cyclic vs. pregnant, status), and their interaction (day*status) are shown.

The interaction of immunological tolerance mechanisms supports embryo development

We neither detected an augmented expression of pro-inflammatory cytokines on mRNA (IL-1B, IL-6, IL-15, Groebner *et al.* 2011, App. II, Table 2 and TNF Groebner *et al.* 2010, App. I Fig. 1c) nor protein level (TNF-α, IFN-γ, Groebner *et al.* 2011, App. II, Fig. 2) in the endometrium of

pregnant animals. Moreover, cell surface markers for any lymphocyte subpopulation analyzed were not differentially expressed due to pregnancy (Groebner *et al.* 2011, App. II Table 2). The interaction of miscellaneous tolerance mechanisms during preimplantation might have caused the reduction of the number of CD45 positive leukocytes in the zona basalis of pregnant animals at day 18 (Groebner *et al.* 2011, App. II, Fig. 5) (Fig. 10). In contrast, in the zona basalis variations in leukocyte numbers were not detected. Our results are in accordance with other reports with respect to not finding changes of analyzed immune cell populations (e.g. B- and T-lymphocytes, macrophages) prior to the apposition phase in cattle (Leung *et al.* 2000; Vander Wielen & King 1984). During the subsequent attachment phase, leukocyte numbers declined in the LE of pregnant cattle (Vander Wielen & King 1984) indicating a reduced migration of maternal lymphocytes towards the placental interface. In general, total leukocyte cell counts in our analysis were fairly low when compared to a previous study (Leung *et al.* 2000), but correlated well with leukocyte numbers in the LE reported elsewhere (Vander Wielen & King 1984). The inconsistencies may arise from differences in the tissue fixation processes used. In cryo-preserved sections (Leung *et al.* 2000) a minor sufficient morphological quality may result in a higher number of false-positive staining events, whereas in formalin-fixed paraffin embedded tissue (Vander Wielen & King 1984) the antigen retrieval more likely results in false-negative cell recognition. As we counted leukocytes in a minimum area of 5 mm^2 in case of stroma cells and 5 mm length in case of luminal epithelia in at least n=4 animals per groups, our data are reliable and highly reproducible with respect to the treatment.

To conclude, tolerance mechanisms might be a prerequisite by the time the semi-allogenic embryo starts to attach to the maternal tissue, and might gain importance during the ongoing implantation and placentation.

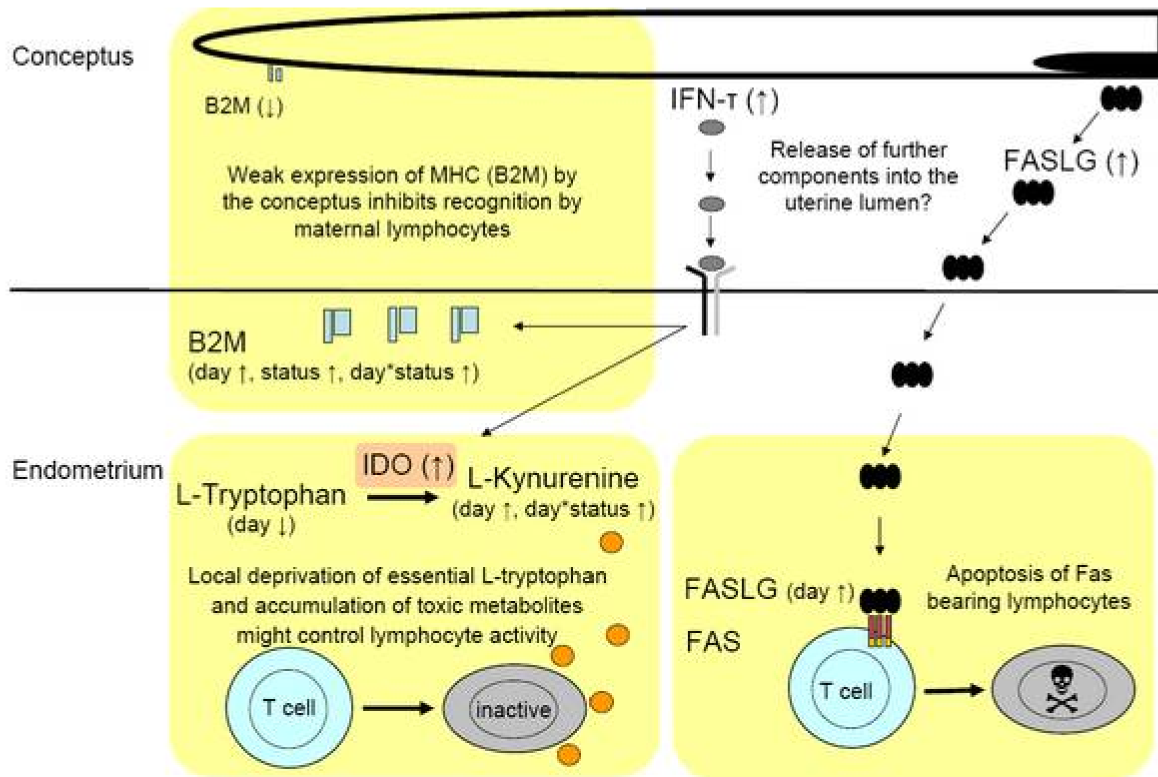


Fig. 10: The interaction of tolerance inducing strategies in early bovine pregnancy might reduce the number of maternal leukocytes.
The release of IFN-τ increased endometrial indoleamine 2,3-dioxygenase (IDO) activity (↑) thus contributing to generation of immune tolerance towards the semi-allogenic embryo. The local depriviation of L-tryptophan (↑) as well as increasing abundance of L-Kynurenine might control lymphocyte activity. Next to a weak expression of β2-microglobulin (B2M) on conceptus tissue most likely to avoid immunological detection through the maternal immune system, the release of trophoblastic TNF receptor superfamily ligand 6 FASLG into the uterine lumen might induce selective cell death of FAS receptor bearing maternal lymphocytes. Significant differences in reference to the day of the cyclus (day), the pregnancy status (cyclic vs. pregnant, status), and their interaction (day*status) are illustrated ($p<0.05$).

4. CONCLUSIONS

Establishment of pregnancy is a complex process aiming the survival of the semi-allogenic embryo and propagation of viable offspring. The appropriate nutrient supply during the prolonged preattachment phase in cattle requires the increase of essential AA to support rapid elongation of the trophoblast. The present study provides a better understanding of the necessities of the conceptus during the preimplantation phase when most pregnancy losses in cattle occur. To improve the success of *in vitro* fertilization, which is a widely accepted strategy of infertility treatment, extensive research has been performed in the last decades to allow preferably optimal development from the zygote to the blastocyst stage prior to the transfer into the recipient mother. Only recently, Robert G. Edwards received the Nobel Prize for breakthrough research in reproductive biology successfully allowing pregnancy and birth of Louise Brown, the first baby following fertilization *in vitro* in 1978. However, next to IVF, cloning by SCNT is an eligible method intended to produce animals with preferred genotypes to increase breeding efficiency in cattle. But, cloning in cattle is often associated with formation of a defective placenta and abberant fetal development at later periods of pregnancy. In contrast to IVF-derived embryos, SCNT embryos fail to induce maternal transport of most AA and AA derivatives independent of IFN-τ, wherefore additional conceptus derived signals are supposed to regulate the appropriate nutrient supply necessary for the generation of an optimal intrauterine milieu for proper embryo development. The unraveling of orchestrated embryonic signals that participate in creating a suitable environment thus might help to improve the production of viable and long-term healthy offspring. In consequence of the increasing applications of artificial reproduction technologies in human reproduction, only a detailed knowledge of specific exigiencies of early blastocysts permits a responsible handling with the incipient life. The current species-specific results add to a comparative view on embryonic development consequently providing the starting point for further analysis enlighten embryonic needs.

Appropriate embryo-maternal communication premises embryo recognition required for the establishment of pregnancy. Although the ruminant pregnancy recognition signal IFN-τ induces the expression of various proapoptotic and tolerance inducing genes, only the activity of the latter increased concomitantly (Fig. 11). Thus, our data indicate a concise regulation of the maternal response, only propagating the implementation of beneficial mechanisms required for pregnancy onset. To unravel embryo-maternal interactions during preimplantation, merely a profound view on any regulatory level would provide detailed insights into the complex cross-talk. The additional focus on the analysis of the epigenom, proteom and metabolom would thus extend our knowledge in view of the versatile interrelation during preimplantation.

Apart from tolerance inducing components derived by the mother in response to IFN-τ, the semi-allogenic conceptus provides accessory components to avoid detection through the maternal

immune system. In view of diseases associated with excessive immune responses towards the semi-allogenic embryo in women such as intrauterine growth retardation and preeclampsia, a detailed knowledge on precise processes involved in the establishment of immunological tolerance during early pregnancy is of specific importance.

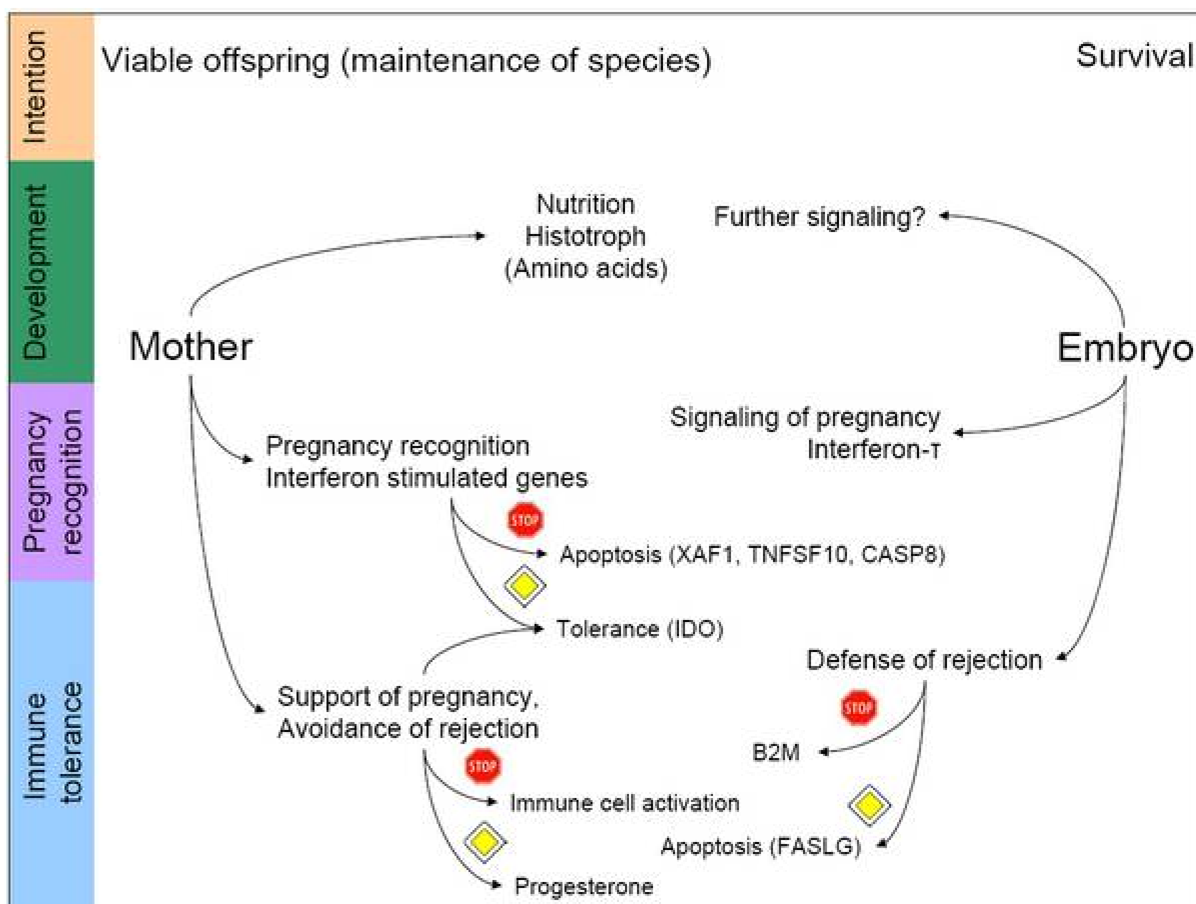


Fig. 11: Establishment of pregnancy requires a well coordinated signaling.
Pregnancy recognition upon trophoblast derived IFN-τ release maintains progesterone synthesis by the corpus luteum and promotes expression of IFN stimulated genes. Although IFN-τ the expression of proapoptotic and tolerance inducing genes provokes, only the activity of the latter increased in parallel. In addition, the conceptus also furthers immunological acceptance by avoiding immunological detection through down-regulation of the major histocompatibility complexe subunit β2-microglobulin (B2M). A concomitant induction of selective cell death in maternal lymphocytes by expressing high abundances of FAS ligand (FASLG) might additionally contribute to the formation of immunological acceptance. A misdirected interaction between mother and embryo may lead to generation of an inappropriate intrauterine environment and embryo development.

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7. SCIENTIFIC COMMUNICATIONS

Original peer reviewed scientific publications

Groebner, A.E.; Schulke, K.; Unterseer, S.; Reichenbach, H.D.; Reichenbach, M.; Büttner, M.; Wolf, E.; Meyer, H.H.D.; Ulbrich, S.E.: Enhanced proapoptotic gene expression of XAF1, CASP8 and TNFSF10 in the bovine endometrium during early pregnancy is not correlated with augmented apoptosis. *Placenta* 31 (2010) 168-177.

Groebner, A.E.; Schulke, K.; Schefold, J.C.; Fusch, G.; Sinowatz, F.; Reichenbach, H.D.; Wolf, E.; Meyer, H.H.D.; Ulbrich, S.E.: Immunological mechanisms to establish embryo tolerance in early bovine pregnancy. *Reproduction, Fertility and Development* 23 (2011) 619-32.

Groebner, A.E.; Rubio-Aliaga, I.; Schulke, K.; Reichenbach, H.D.; Daniel, D.; Wolf, E.; Meyer, H.H.D.; Ulbrich, S.E.: Increase of essential amino acids in the bovine uterine lumen during preimplantation. *Reproduction* 141 (2011) 685-95.

Groebner, A.E.; Zakhartchenko, V.; Bauersachs, S.; Büttner, M.; Rubio-Aliaga, I.; Daniel, D.; Reichenbach, H.D.; Wolf, E.; Meyer, H.H.D.; Ulbrich, S.E.: Reduced amino acids in the bovine uterine lumen of cloned vs. *in vitro* fertilized pregnancies prior to implantation. *Cellular Reprogramming* 13 (2011)*,* 403-10*.*

Ulbrich, S.E.; Schulke, K.; **Groebner, A.E.**; Reichenbach, H.D.; Angioni, C.; Geisslinger, G.: Quantitative characterization of prostaglandins in the uterus of early pregnant cattle. Reproduction 138 (2009) 371-382.

Oral scientific presentations

Groebner, A.E.; Schulke, K.; Bauersachs, S.; Wolf, E.; Meyer, H.H.D.; Ulbrich, S.E.: Expression of inducible and endothelial nitric oxide synthase in the bovine endometrium during estrous cycle and early pregnancy. Reproduction in Domestic Animals, Suppl. 1, 43 (2008) page 12, Abstract 29, presented at 41. Jahrestagung Physiologie und Pathologie der Fortpflanzung gleichzeitig 33. Veterinär-Humanmedizinische Gemeinschaftstagung, February, 28th 2008, Gießen, Germany

Groebner, A.E.; Schulke, K.; Reichenbach, H.D.; Meyer, H.H.D.; Ulbrich, S.E.: The Fluidigm dynamic gene expression array revealed embryo-maternal tolerance mechanisms in the bovine endometrium prior to implantation. (2010), presented at European Fluidigm Roadshow, November, 5th 2010, Innovations- und Gründerzentrum Biotechnologie Martinsried, Munich, Germany

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Schulke, K.; **Groebner, A.E**.; Waldschmitt, N.; Weppert, M.; Reichenbach, H.D.; Schams, D.; Meyer, H.H.D.; Ulbrich, S.E.: Increased fibroblast growth factor 2 protein in the bovine endometrium coincides with beginning trophoblast elongation during preimplantation development. Reproduction in Domestic Animals, Supplement 1, 44 (2009) page 33, Abstract 95. presented at 43. Jahrestagung Physiologie und Pathologie der Fortpflanzung gleichzeitig 35. Veterinär-Humanmedizinische Gemeinschaftstagung, February, 26th-27th 2009, Leipzig, Germany

Schulke, K.; Zitta, K.; **Groebner, A.E.**; Reichenbach, H.D.; Weppert, M.; Meyer, H.H.D.; Ulbrich, S.: Bovine uterine flushings reveal an increase of prostaglandins E2 and F2 alpha during the luteal phase and in early pregnancy. Reproduction in Domestic Animals, Supplement 1, 43 (2008) page 30, Abstract 87, presented at 41. Jahrestagung Physiologie und Pathologie der Fortpflanzung gleichzeitig 33. Veterinär-Humanmedizinische Gemeinschaftstagung, February, 28th 2008, Gießen, Germany

Abstracts presented at scientific meetings

Groebner, A.E.; Rubio-Aliaga, I.; Schulke, K.; Reichenbach, H.D.; Daniel, H.; Wolf, E.; Meyer, H.H.D.; Ulbrich, S.E.: Increase of essential amino acids in the bovine uterine lumen during preimplantation. Proceeding of the 3rd General Meeting of GEMINI, page 167, Abstract 57, October, 1st-2nd 2010, Soustons, France

Groebner, A.E.; Schulke, K.; Reichenbach, H.D.; Meyer, H.H.D.; Ulbrich, S.E.: Expression of endothelin and angiotensin family members in the bovine endometrium during the estrous cycle and early pregnancy. First international symposium of the Journal “Hormone Molecular Biology and Clinical Investigation”, Poster 126-P, September, 11th–14th 2010, Seefeld, Tyrol, Austria

Groebner, A.E.; Schulke, K.; Reichenbach, H.D.; Meyer, H.H.D.; Ulbrich S.E.: The Fluidigm® dynamic gene expression array revealed embryo-maternal tolerance mechanisms in the bovine endometrium prior to implantation. Advances in Genomics Symposium 2010, January 28th, 2010 Ghent, Belgium

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Groebner, A.E.; Schulke, K.; Reichenbach, H.D.; Meyer, H.H.D.; Ulbrich, S.E.: Differential expression of apoptotic factors in bovine endometrium and embryos during the preimplantation period. Proceedings of the 4th International qPCR Event 2009, Symposium & Exhibition & Workshops, page 60, P085, Freising-Weihenstephan, March, 9th-13th 2009, Technische Universität München, Munich, Germany

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Ulbrich S.E, Schulke K. **Groebner A.E**., Reichenbach H.-D., Angioni C., Geisslinger G., Meyer H.H.D.: Quantitative characterization of the most relevant prostaglandins in the bovine uterus: embryonic impact generates an intrauterine environment of prostacyclin and prostaglandin PGF2alpha outranging PGE2 by far. Proceedings of the 4th International qPCR Event 2009, Symposium & Exhibition & Workshops, page 62, P092, Freising-Weihenstephan, March, 9th-13th 2009, Technische Universität München, Munich, Germany

Ulbrich S.E, Schulke K. **Groebner A.E**., Reichenbach H.-D., Angioni C., Geisslinger G., Meyer H.H.D.: Prostaglandins in the bovine uterine lumen during the preimplantation period: Principal actors, temporal sequential role and direction of action. Abstractband 52. Symposium der Deutschen Gesellschaft für Endokrinologie PS1-01-5, March, 4th-7th 2009, Kongresshalle Gießen

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APPENDIX

Appendix I

Groebner, A.E.; Schulke, K.; Unterseer, S.; Reichenbach, H.D.; Reichenbach, M.; Büttner, M.; Wolf, E.; Meyer, H.H.D.; Ulbrich, S.E.: Enhanced proapoptotic gene expression of XAF1, CASP8 and TNFSF10 in the bovine endometrium during early pregnancy is not correlated with augmented apoptosis. *Placenta* 31 (2010) 168-177.

Appendix II

Groebner, A.E.; Schulke, K.; Schefold, J.C.; Fusch, G.; Sinowatz, F.; Reichenbach, H.D.; Wolf, E.; Meyer, H.H.D.; Ulbrich, S.E.: Immunological mechanisms to establish embryo tolerance in early bovine pregnancy. *Reproduction, Fertility and Development 23* (2011), 619-32.

Appendix III

Groebner, A.E.; Rubio-Aliaga, I.; Schulke, K.; Reichenbach, H.D.; Daniel, D.; Wolf, E.; Meyer, H.H.D.; Ulbrich, S.E.: Increase of essential amino acids in the bovine uterine lumen during preimplantation. *Reproduction* 141 (2011), 685-95.

Appendix IV

Groebner, A.E.; Zakhartchenko, V.; Bauersachs, S.; Büttner, M.; Rubio-Aliaga, I.; Daniel, D.; Reichenbach, H.D.; Wolf, E.; Meyer, H.H.D.; Ulbrich, S.E.: Reduced amino acids in the bovine uterine lumen of cloned vs. *in vitro* fertilized pregnancies prior to implantation. *Cellular Reprogramming* 13 (2011), 403-10.

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Enhanced proapoptotic gene expression of *XAF1*, *CASP8* and *TNFSF10* in the bovine endometrium during early pregnancy is not correlated with augmented apoptosis

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ABSTRACT

Bovine trophoblast cells release interferon-τ (IFNT), a type I IFN, as the pregnancy recognition signal. Since type I IFNs exert growth inhibitory and proapoptotic actions, the effect of the conceptus on components of the apoptosis pathways was determined in the bovine endometrium during the periimplantation period. Uteri of Simmental heifers were flushed post mortem at days 12, 15, and 18 of cycle or pregnancy for the recovery of conceptuses and the sampling of ipsilateral endometrial tissue at slaughter for quantitative RT-PCR, immunohistochemistry, caspase activity and TUNEL assays. Endometrium samples of pregnant animals revealed increased transcript levels for the proapoptotic genes *XAF1* (day 15: 2.9-fold; day 18: 15.1-fold; $p = 0.005$) and *CASP8* (day 18: 2.4-fold; $p = 0.007$). The mRNA expression increased significantly with the day of the cycle for the proapoptotic genes *FASLG*, *TNFSF10*, *TNF* and *TNFSF1A* ($p = 0.004$, $p = 0.006$, $p = 0.001$ and $p = 0.007$) and the antiapoptotic gene *BIRC4* ($p = 0.03$). We detected high amounts of *FASLG* transcripts in day 18 conceptuses (16-fold higher than day 18 endometria). This finding was validated at the protein level by immunohistochemistry. To further analyse the endometrial activation of the caspase cascade, the activities of initiator caspase 8 and effector caspases 3/7 were determined luminometrically. No difference between pregnant and cyclic animals was found for either caspase activity. Additionally, a TUNEL assay showed no increase of apoptotic cells in the pregnant endometrium. In conclusion, although the bovine conceptus induces the expression of proapoptotic genes, neither an activation of a caspase cascade nor an increase of apoptotic cells was noticed. These results suggest inhibitory mechanisms preventing endometrial cells from programmed cell death.
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1. Introduction

Interferons (IFNs) are a family of cytokines that exert immune regulatory and inflammatory actions [1]. Initially described as trophoblast protein-1, interferon-τ (IFNT), a type I IFN, serves as the primary maternal pregnancy recognition signal by preventing the release of luteolytic $PGF_{2\alpha}$ pulses by the endometrium [2]. The release of IFNT appears to occur from day 12 to at least day 25 of pregnancy in cattle [3] with a secretion of up to 10^5 units per hour in culture at day 18 [4], although mRNA can be observed as early as the 16-cell and blastocyst stages. Upon binding of IFNT to the common type I IFN receptor subunits IFNAR1 and IFNAR2 [5], the JAK-STAT signalling pathway is activated and in further downstream reactions the transcription of a variety of IFN stimulated genes (ISGs) is upregulated. Common to all type I IFNs including IFNT are their antiviral, growth inhibitory and proapoptotic activities [6,7]. Induction of apoptosis by interferon-α, the most extensively studied type I IFN, is initiated by diverse mechanisms and involves both up-regulation of proapoptotic factors [8] as well as suppression of antiapoptotic actions [9].

Apoptosis is a tightly controlled process by which tissue eliminates unwanted cells. Either activation of the intrinsic or the extrinsic pathway finally results in formation of characteristic vesicles also known as apoptotic bodies [10], which are phagocytosed by macrophages without inducing an inflammatory response [11]. The intrinsic or mitochondrial pathway may be induced via multiple stimuli like cellular stress or absence of survival factors. The ratio of pro- and antiapoptotic Bcl-2-family members alters the permeability of mitochondria which results in release of cytochrome c and

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formation of the apoptosome complex [12]. Extracellular stimuli and the cytokines FAS ligand (FASLG), TNF related apoptosis inducing ligand/TRAIL (TNFSF10) and TNF-α (TNF) initiate the extrinsic, receptor mediated pathway. Through ligation of cell surface death receptors such as the members of the TNF-ligand superfamily FAS, TNF receptor 1 (TNFRSF1A), TNF receptor 2 (TNFRSF1B) and TRAIL receptor 2 (TNFRSF10B) trimerization can occur [13] which causes recruitment of adaptor proteins to the cytoplasmic death domain followed by activation and subsequent autocatalytic cleavage of initiator procaspase 8 (CASP8).

Both apoptosis pathways activate further downstream effector caspases that cleave numerous substrates such as ICAD (DFFA) [14]. The activation can be prevented by inhibitors of apoptosis (IAPs). Otherwise, IAPs e.g. XIAP (BIRC4) or SURVIVIN (BIRC5) can be inactivated again by proapoptotic antagonists such as DIABLO (SMAC) or XIAP associated factor 1 (XAF1). Cell death may be impeded at every level even after DNA fragmentation; there are factors e.g. p53 (TP53) that stop the cell cycle in order to allow mending by DNA-polymerases and -ligases.

In our recent transcriptome studies on endometrial gene expression during the periimplantation period, the increased transcription of specific ISGs related to apoptosis has been shown [15,16]. The programmed cell death might act as an additional mechanism to generate immune privilege for the protection of the semi-allogenic conceptus from the maternal immune system [17]. Death receptor ligands provided by the conceptus mediate receptor induced killing of death receptor bearing leukocytes [18]. In primates and rodents showing a hemochorial placentation, the regulation of apoptosis has been studied extensively [19]. Luminal epithelial and stromal cells expressing apoptosis markers and exhibiting DNA damage along with an apoptotic appearance were predominantly located at the implantation sites to allow invasion of the trophoblast [20].

Ruminants exhibit a late implantation with a synepitheliochorial type of placentation. An epitheliochorial placenta is also present in pig, and cell death was absent throughout the window of implantation [21]. As the bovine endometrium is exposed to high amounts of IFNT, mechanisms of apoptosis may as well be important for generating immune privilege allowing early embryonic development. Therefore, we analysed whether the induction of proapoptotic factors in the bovine endometrium leads to an activation of initiator and effector caspases eventually triggering cell death events during the periimplantation period. Inhibitor mechanisms preventing apoptosis derived from either endometrium or conceptuses were as well under investigation.

2. Material and methods

2.1. Pretreatment of animals

All experiments were performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as promulgated by the Society for the Study of Reproduction and with the European Convention on Animal Experimentation. Cyclic Simmental heifers (*Bos taurus*, Deutsches Fleckvieh) were estrus-synchronized by injecting intramuscularly 500 µg of a single dose of the $PGF_{2\alpha}$-analog Cloprostenol (Estrumate; Essex Tierarznei, Munich, Germany) at diestrus as described previously [22]. The pregnant groups were inseminated after estrus detection, whereas the cyclic control groups received supernatant of centrifuged sperm from the same bull. At day 12, 15, or 18 after insemination, animals were slaughtered ($n = 4$–7 per group); the uterus was removed and flushed with 100 ml phosphate buffered saline (PBS; pH 7.4) for the recovery of embryos as described previously [22]. The flushing fluid was centrifuged at 800*g* for 10 min and the supernatant was stored at $-20\,^{\circ}$C until further usage.

2.2. Tissue sampling

Intercaruncular endometrial samples were taken from the uterine horn ipsilateral to the corpus luteum bearing ovary as previously described [15]. Whole conceptuses were transferred into vials containing 4 ml RNAlater (Ambion, Huntingdon, Cambridgeshire, UK) and further processed as the endometrial samples. For TUNEL assays and immunohistochemistry, tissue samples (uteri and whole conceptuses) were transferred to Bouin's fixation solution prior to embedding in paraffin.

2.3. Extraction of total RNA

Total RNA from endometrial samples and whole conceptuses were isolated using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to manufacturer's instructions. Quality of RNA was controlled by the Agilent 2100 Bioanalyzer (RNA 6000 Nano Assay Kit). RNA integrity numbers ranged between 7 and 10. Quantity was spectroscopically determined at 260 nm by the Nanodrop 1000.

2.4. Two step quantitative real-time RT-PCR

The quantitative real-time PCR experiments were performed in accordance with the MIQE guidelines [23]. In the present study, quantitative real-time PCRs using the LightCycler DNA Master SYBR Green I protocol (Roche Diagnostics, Mannheim, Germany) were undertaken as described earlier [22]. Sequences of commercially synthesized PCR primer pairs (Eurofins MWG Operon, Ebersberg, Germany), references, mean Cq, annealing temperature (AT), fluorescence acquisition points (FA), melting points (MP) as well as product length [bp] are depicted in Table 1.

2.5. Data analysis and statistics

The cycle number (Cq) to attain a definite fluorescence signal was calculated by the second derivative maximum method (LC software 4.05), as the Cq is inversely correlated with the logarithm of the initial template concentration. The Cq values from the target genes were normalized against the geometric mean of three reference genes (*UBQ3*, *H3F3A* and *18S rRNA*) according to the bestkeeper method [24]. In order to avoid negative digits while allowing an estimation of a relative comparison between two genes, data are presented as means $\pm$ SEM subtracted from the arbitrary value 30 (ΔCq). Thus, a high ΔCq resembles high transcript abundance [25]. An increase of one ΔCq represents a two-fold increase of mRNA transcripts. For statistical analysis the SAS program package release 9.1.3 (2002; SAS Institute, Inc., Cary, NC, USA) was used. The data comparing endometria from cyclic and pregnant uteri were subjected to least-square analysis of variance using the General Linear Models procedure to determine effects of the day, the status (cyclic vs. pregnant), and their interaction. Significant different days within each status as well as significant different groups at each time point were localized by the differences of least-square means. Graphs were plotted using Sigma-Plot 8.0 (SPSS software).

2.6. IFNT bioactivity

IFN production was quantified in flushing fluids by a bioassay based on the inhibition of the cytopathic effect of vesicular stomatitis virus (Indiana strain) on Madin–Darby bovine kidney (MDBK) cells [26]. The NIH recombinant human IFN-α_2 reference preparation (No. Gxa01-901-535, NIH-Research Reference Reagent Note No. 31, 1984) was included in each assay. The antiviral activity was shown to be mediated by IFNT, as the effects of supernatant and an appropriate control IFNT preparation were blocked by specific anti-IFN sera (kindly provided by Dr. R.M. Roberts, University of Missouri, Columbia, MO) [27].

2.7. Caspase activity

For Caspase-Glo® 3/7 Assay and Caspase-Glo® 8 Assay (Promega GmbH, Mannheim, Germany) endometrial samples were homogenized and centrifuged through a NucleoSpin Filter L (Macherey-Nagel GmbH & Co KG, Düren, Germany). Protein concentrations were determined using a BCA standard protocol (Sigma–Aldrich Chemie GmbH, Steinheim, Germany). The activity assay was performed according to manufacturer's instructions. Luminescence measurement, lasting 0.1 s, was carried out in the Victor Light Luminescence Counter. Results are shown as mean $\pm$ SEM of RLU (relative light units) per µg total protein (TP).

2.8. TdT-mediated dUTP-biotin nick end labelling (TUNEL)

For analysis of single strand DNA nicks in uterine tissue sections, the DeadEnd™ Colorimetric TUNEL System (Promega) was performed on sections of three animals per group according to manufacturer's protocol. The negative control was incubated without rTdT enzyme and DNA nicks were induced in the positive control by 10 units per ml of RQ1 RNase-free DNase I (Promega). After blocking of endogenous peroxidases in the presence of 0.3% H_2O_2, streptavidin-horseradish peroxidase (HRP) was introduced. HRP converts H_2O_2 into H_2O and protons which results in oxidation of 3,3′-diaminobenzidine (DAB) to a brown insoluble product. Apoptotic cells in uterine tissue sections were determined by light microscopy using Olympus Cell-F software.

2.9. Immunohistochemistry

FASLG protein was localized in trophoblasts and endometrial tissue. Endogenous peroxidase activity was blocked with 1% H_2O_2 in methanol. Following blocking with 10% goat serum, samples were incubated overnight at $4\,^{\circ}$C with the rabbit polyclonal

Table 1
Forward and reverse primer sequences. Sequences of primer pairs and corresponding references as well as annealing temperatures (AT), fluorescence acquisition (FA), melting points (MP) and the resulting fragment length are shown.

Gene	reference		mean Cq	primer (5′ → 3′)		fragment size [bp]	AT [°C]	FA [°C]	MP [°C]
UBQ3	Z18245		16.3	for rev	AGA TCC AGG ATA AGG AAG GCA T GCT CCA CCT CCA GGG TGA T	198	60	83	89
H3F3A	BT020962		18.0	for rev	ACT GGC TAC AAA AGC CGC TC ACT TGC CTC CTG CAA AGC AC	233	60	80	87
18S rRNA	AF176811		8.5	for rev	AAG TCT TTG GGT TCC GGG GGA CAT CTA AGG GCA TCA CA	488	60	82	89
BAX	U92569	[50]	24.2	for rev	TCT GAC GGC AAC TTC AAC TG GGT GTC CCA AAG TAG GAG AGG	203	62	85	92
BCL2L1	AF245487	[50]	21.3	for rev	GGC ATT CAG CGA CCT GAC CCA TCC AAG TTG CGA TCC	203	60	80	90
FAS	NM_000043	[50]	23.9	for rev	AGA AGG GAA GGA GTA CAY MGA TGC ACT TGT ATT CTG GGT CC	124	60	78	84
FASLG	X89102	[50]	28.6	for rev	CAT CTT TGG AGA AGC AAA TAG GGA ATA CAC AAA ATA CAG CCC	205	62	80	87
TNFSF10	XM_583785		20.7	for rev	CAA TCC CTG CTG GGA ACT AA CCT GGA ACT GGC AAC GTA CT	195	60	77	81
TNFRSF10B	XM_001790072		26.6	for rev	TTC AGT GTT GGC TTG CTC TG CAA AGC TTA CAG AGG GGC TG	120	68	80	86
TNF	AF348421		29.5	for rev	CCA CGT TGT AGC CGA CAT C ACC ACC AGC TGG TTG TCT TC	108	60	83	87
TNFRSF1A	U90937	[50]	27.9	for rev	CAC CAC CAC CAT CTG CTT TCT GAA CTG GGG TGC AGA	257	70	91	96
TNFRSF1B	AF031589	[50]	27.2	for rev	AGC AGC ACG GAC AAG AGG CTG TGT CCC TCG TGG AGC	220	60	80	95
CFLAR	AY882619		20.2	for rev	GGG TGC TGA TGA TGG AGA TT GCT CCT TGA GCT GAC TGC TT	253	60	77	82
CASP8	NM_001045970		21.6	for rev	TGT CAC AAT CGC TTC CAG AG GAA GTT CAG GCA CCT GCT TC	183	60	80	86
CASP3	NM_214131	[50]	26.5	for rev	AAC CTC CGT GGA TTC AAA ATC TTC AGG RTA ATC CAT TTT GTA AC	114	60	75	81
CASP7	NM_033338	[50]	32.3	for rev	CTC TTC AAG TGC TTC RAA RC TTC TCT ARC AGG GTT TTG CAT C	241	60	80	86
CASP6	NM_001226	[50]	> 40	for rev	TGT TCA AAG GAG ACA AGT GTC AG CAG AGT AGC ACA TGA GGA AGT C	206	60	80	92
XAF1	BT021626		20.6	for rev	GAG GAG GCT CTG AGC TTG C GCA GAG AAA GAT GTC CGT CC	143	64	80	85
BIRC4	NW_001501790		21.1	for rev	AAC ACA GGC GAC ACT TTC CT CTG CCA TGG CTG GAT TTA TT	135	71	77	82
DIABLO	AF203914	[50]	23.2	for rev	AGG AAG ATG AGG TGT GGC AG AAC TGG ATG TGA TTC CTG GC	184	62	83	87
BIRC5	AY606044	[50]	27.8	for rev	AGC TCT ACC TCA AGG ACC ACC CTT CTA TGG GGT CGT CAT CTG	195	64	80	93
IFNAR1	X68443		20.5	for rev	CTC CAG TCA TCA GCG TGA AA GTG CTC TGG CTT TGA CAC AA	228	60	77	83
IFNAR2	U75304		22.6	for rev	GAA AAA GTG GCT ACC GTG GA CTG GGT GAA GTG GTG GAA GT	176	60	77	85

N-20 sc-834 antibody (4.0 μg/mL, Santa-Cruz, Biotechnology Inc. Santa-Cruz, CA, USA). Samples were then incubated with secondary antibody anti-rabbit IgG peroxidase-conjugated (2.5 μg/mL, Sigma–Aldrich). Binding of antibody was detected by incubating with DAB in the presence of 0.1% H_2O_2 for 5 min. Tissues were counterstained using Mayer's Haemalaun (Carl Roth GmbH, Karlsruhe, Germany).

2.10. In vitro co-culture of glandular and stroma cells

Co-culture of glandular and stroma cells was performed as described earlier [28]. Cells were stimulated with recombinant bovine IFNT (antiviral activity, 4.8×10^3 U/ml medium; PBL Biomedical Laboratories, Piscataway, NJ) for four hours. RNA extraction and qPCR experiments were carried out as detailed for the endometrial samples.

3. Results

3.1. Expression of intrinsic and extrinsic pathway factors

The death receptor ligands investigated increased significantly over time most prominently during pregnancy as the mRNA expression of *FASLG* (day $p = 0.004$), *TNFSF10* (day $p = 0.006$) and *TNF* (day $p = 0.001$) was 3.6-fold (Fig. 1A), 11.9-fold (Fig. 1B), and 4.8-fold (Fig. 1C) higher in endometria of day 18 than of day 12 pregnant animals, respectively. In addition, we detected a significant day * status interaction for TNFSF10 ($p = 0.048$). The mRNA

levels of analysed death receptors FAS (Fig. 1D), TNFRSF10B (Fig. 1E), and TNFRSF1B (not shown) exhibited no variation. Solely for TNFRSF1A (Fig. 1F) we detected an increase in expression over time (day $p = 0.007$) in endometria. The expression of *FASLG* strikingly increased 82-fold in conceptuses between days 15 and 18 ($p = 0.0001$). The mRNA of conceptus-derived *TNFSF10* was more than 2,000-fold lower than the endometrial expression, and the transcript abundance of *TNF* in conceptuses was below the detection limit (Cq > 40). Transcript abundance revealed no variation for either endometrial *BAX* or *BCL2L1* (not shown) subjected to the day and to the status. While the expression of *BAX* was augmented in conceptuses of day 18 vs. 15 (20.4 ± 0.2 ΔCq vs 21.2 ± 0.3 ΔCq, $p = 0.049$), the expression of *BCL2L1* dropped more than 2.1-fold (19.9 ± 0.2 ΔCq vs 18.8 ± 0.2 ΔCq, $p = 0.02$).

3.2. Expression and activity of initiator and effector caspases as well as expression of inhibitor of apoptosis (IAPs) and their antagonists

Transcripts of initiator *caspase 8* (*CASP8*) constantly rose from day 12 to day 18 in control and pregnant animals (1.6-fold and 2.9-fold, for control or pregnant animals (day $p = 0.007$), respectively) (Fig. 2A). Day 18 pregnant animals exhibited (2.4-fold) increased transcript abundance compared to the respective control animals at day 18. *CASP8* transcripts in pregnant animals were status dependent enhanced (status $p = 0.007$). We could not detect changes in transcript abundance of pregnant animals of the *CASP8* binding site competitor *FLIP* (*CFLAR*) (data not shown). The measurement of luminescence in endometrial homogenates revealed a 30% decrease of CASP8 activity from day 12 to day 18 (day $p = 0.0001$) (Fig. 2C) in both pregnant and non-pregnant animals. The mRNA levels of effector caspases 3 (*CASP3*) (Fig. 2B) and 7 (*CASP7*) (not shown) were not influenced. *CASP7* was only marginally expressed (mean Cq 32.3) and caspase 6 (*CASP6*) expression was below the detection limit of the assay (Cq > 40) (data not shown). *CASP8* was expressed to a lesser extent in conceptuses than in endometria (Fig. 2C) whereas effector *CASP3* (Fig. 2D) exhibited a more pronounced expression in day 18 conceptuses.

BIRC4 transcripts (Fig. 2E) rose 2.3-fold and 2.8-fold in cyclic and pregnant animals over the analysed time points (day $p = 0.02$), while *BIRC5* (Fig. 2F) did not show marked variation. Strikingly, the transcript abundance of *XAF1* (Fig. 2G) was 2.9-fold increased in day 15 pregnant animals when compared to their cyclic counterparts, and 15.1-fold increased at day 18 (day $p = 0.0008$, status $p = 0.0005$, day * status $p = 0.003$). No differences were observed

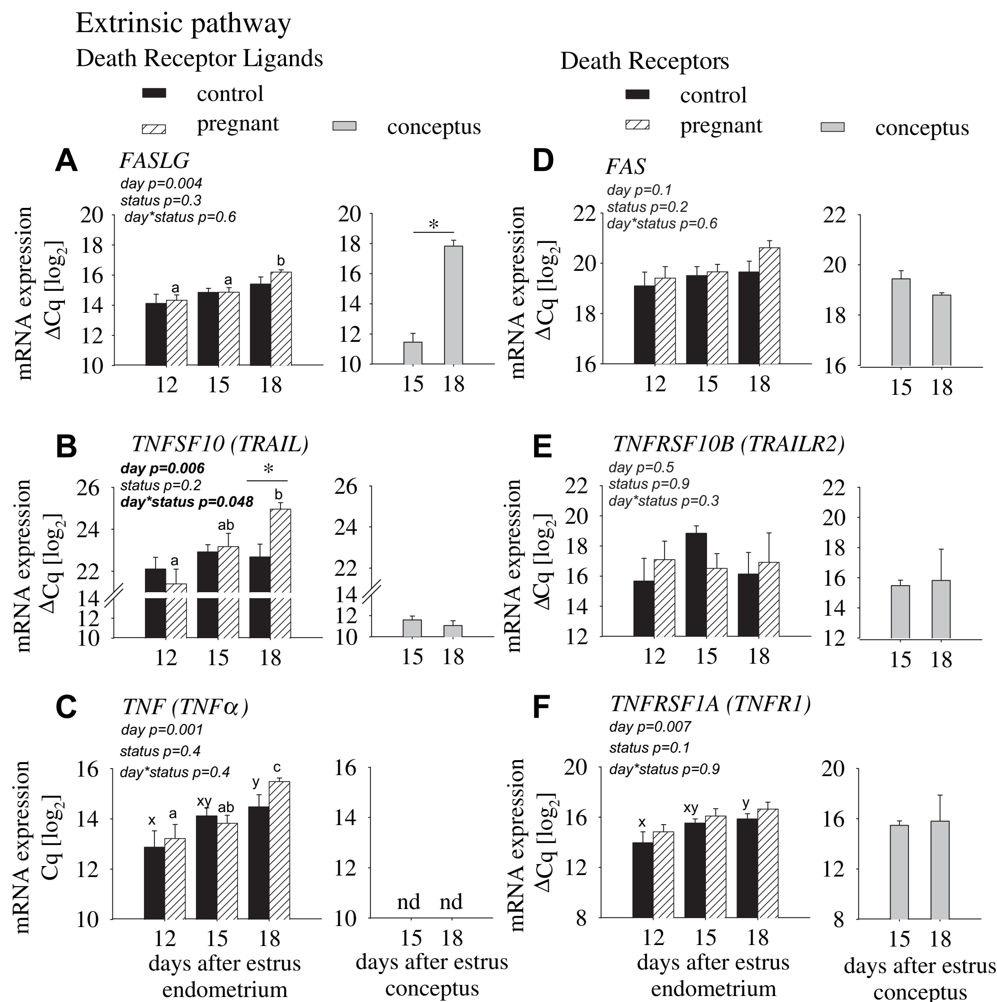

Fig. 1. Transcript abundances of extrinsic apoptosis factors. Messenger RNA expression of extrinsic TNF family members *FASLG* (C), *TNFSF10* (D) and *TNF* (E) and their death receptors *FAS* (F), *TNFRSF10B* (G) and *TNFRSF1B* (H) in endometria and conceptuses are shown as means ΔCq ± SEM. Normalized Cq (ΔCq) values were subtracted from the arbitrary value 30. The effect of the day within the status is shown by different superscript letters (x–z in cyclic animals and a–c in pregnant animals). Asterisks indicate significant differences ($p < 0.05$). nd indicates non-determinable values.

for mRNA expression of *DIABLO* (Fig. 2H). In contrast to pregnant endometria *XAF1* was expressed at very low abundance in conceptuses, whereas *BIRC5* mRNA was 76-fold more abundant in conceptuses than in endometria of day 18 pregnant animals.

3.3. Bioactivity of IFNT in uterine flushing fluids and expression of IFN type I receptor

Bioactivity of IFNT increased significantly ($p < 0.001$) with proceeding pregnancy in uterine flushing fluids (Fig. 3A), whereas flushing fluids of non-pregnant control animals showed no antiviral activity (not shown). Gene expression analysis of IFNAR receptor subunits revealed no variation for *IFNAR1* and *IFNAR2* over the analysed time points (Fig. 3B, C). *IFNAR1* and *IFNAR2* showed a 5.8-fold and more than 1000-fold lower expression in day 18 conceptuses when compared to endometria of day 18 pregnancy.

3.4. Detection of apoptotic cells by using the TUNEL technique and immunohistochemical localisation of FASLG in bovine endometria and day 18 trophoblasts

DNA fragmentation indicated by TUNEL was barely detected in bovine endometria of cyclic and early pregnant cows. Only a few positive cells were located in the subepithelial stroma and the luminal epithelium close to the uterine lumen (Fig. 4B). The number was slightly enhanced in day 15 control animals. Additionally, most TUNEL-positive cells also showed apoptotic morphology and apoptotic bodies.

Deep endometrial glands showed intense FASLG immune labelling (Fig. 4D), whereas the staining in the luminal epithelial cells was only weak (Fig. 4C). Endothelial cells also showed intense FASLG labelling (Fig. 4E). In uterine sections there were no obvious differences in immunostaining pattern of FASLG protein among cyclic or pregnant animals irrespective of the day. In day 18 trophoblast cells, we found a strong immune staining (Fig. 4F).

3.5. Expression of XAF1, CASP8 and FASLG in an in vitro co-culture system of glandular and stroma cells

Expression of *XAF1* (7.9-fold) and *CASP8* (6.2-fold) was significantly elevated following IFNT treatment ($p < 0.0001$) in glandular epithelial cells (Table 2). The increase in transcript abundance was even higher in stroma cells (32.2-fold and 13.4-fold for *XAF1* and *CASP8*, $p < 0.0001$) (Table 2). We did not observe an IFNT dependent increase for FASLG in either cell type analysed (Table 2).

4. Discussion

Extensive apoptosis due to the process of implantation has been demonstrated in rodent and primate species as the attachment of the conceptus induces vigorous tissue remodeling during the invasive hemochorial placentation [19]. Implantation in ruminants is sparsely invasive, since only binucleate trophoblast cells invade the maternal epithelium from day 20 onward when IFNT secretion has already reached its maximum [3,29]. Degenerative changes within the uterine epithelium are described in the caruncular area not earlier than day 22 of pregnancy [29]. A recently published gene expression analysis revealed 96 differentially expressed genes related to cell death in the caruncular areas of bovine day 20 endometria, but 245 genes were as well differentially expressed in intercaruncular tissue [30]. As little is known about apoptotic events in the intercaruncular endometrium when conceptus-derived IFNT acts on maternal tissue, we focused in our analysis on the functional secretory endometrium of the luteal phase.

In ruminants, IFNT is massively released during the peri-implantation period [3], and the elevation of proapoptotic gene transcripts in bovine endometrium prior to implantation has been described [7,15,16,31]. Elevated transcript levels of ISGs showing proapoptotic function, including *XAF1*, *CASP8*, *TNFSF10*, and *FASLG*, are well-known from humans following type I IFN treatment (reviewed in [6]). In the present study, *in vivo* during preimplantation as well as in glandular epithelial and stroma cells *in vitro* following IFNT treatment the expression of *XAF1* and *CASP8*, but not *FASLG*, was enhanced, indicating species-specific differences. The IFN-signalling suppressor interferon regulatory factor 2 (IRF2) has been shown to be present in the luminal and superficial glandular epithelium in the ewe during pregnancy restricting the expression of ISGs to the zona basalis of the endometrium [32–34]. Interestingly, an *in situ* hybridization of *XAF1* performed earlier [16] showed that *XAF1* in bovine (referred to as *HSXIAPAF1*) was present in the luminal epithelium as well as in superficial and deep endometrial glands in both day 18 cyclic and pregnant animals indicating another species-specific peculiarity. Whether IRF2 in bovine might in general be restricting IFNT related gene expression to the endometrial zona basalis during pregnancy *in vivo* as found in the ewe needs to be proven. Nonetheless, most probably the presence of IFNT in the bovine uterus during early pregnancy led to enhanced endometrial *XAF1* and *CASP8* transcripts without concomitant apoptotic events in any region of the endometrium.

The most intense increase of a proapoptotic gene expression was observed for *XAF1* in line with rising IFNT during the peri-implantation period. The results obtained are in accordance with earlier transcriptome analyses [15,16]. Therein, four percent of the genes upregulated in endometria of day 18 pregnant animals were associated with apoptotic function of which *XAF1* was the most pronounced [15]. The main function of XAF1 is the direct binding of the BIRC4-caspase complex and the subsequent transfer to the nucleus for effective degradation [35] (Fig. 5). XAF1 causes further sensitization to TNF and TNFSF10-induced cell death and additionally is able to promote cytochrome c release from mitochondria through blocking Bcl-2-family proteins [36]. These multiple functions suggest a fundamental role in the endometrium at the window of implantation. Following IFNT stimulation, XAF1 may primarily act on endometrial cells or may be able to elicit selective cell death in lymphocytes in order to protect the semi-allogenic embryo. In the present study, the observed apoptotic cells were primarily located close to the uterine lumen in both pregnant and cyclic animals which might either indicate endometrial cells in natural cell turnover or leukocytes in a stage of apoptosis [37].

Apoptosis can be either elicited by the intrinsic pathway through proapoptotic Bcl-2-family factors (BAX) or the extrinsic pathway via death receptor ligands (FASLG, TNFSF10 and TNF) by binding to respective receptors (FAS, TNFRSF10B, TNFRSF1A,-B). We did not detect changes in gene expression of the intrinsic apoptosis pathway, but a conspicuous increase of *FASLG* expression concomitant to an intense immune labelling of trophoblast cells suggests a role for FASLG in ruminants during the periimplantation phase. Studies in rodents and primates showed that immune privilege is generated via the FAS/FASLG system in order to protect the semi-allogenic embryo from activated lymphocytes [38]. The human trophoblast itself produces low levels of FAS and is usually resistant to FASLG mediated apoptosis [39]. In our study, transcripts of *FAS* were comparably abundant in both the endometrium and the conceptus. Aside from membrane-bound and soluble FASLG, a form secreted via microvesicles also exists in trophoblast cells which may act on maternal FAS-bearing immune cells [17]. Whether this secretion of FASLG also occurs in ruminants is currently unknown. If released, trophoblast-derived FASLG could act on FAS-bearing

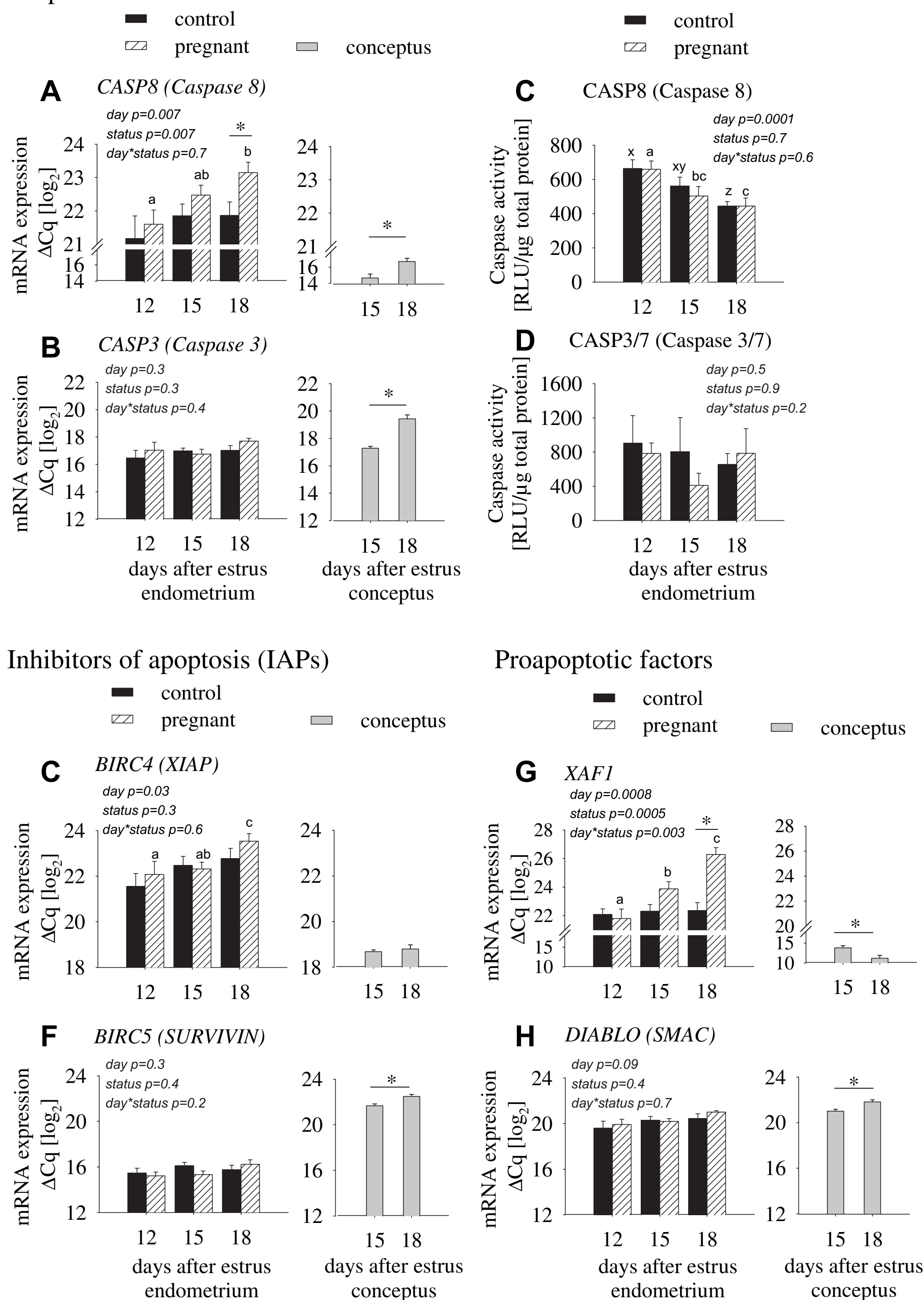

Fig. 2. Messenger RNA expression of caspases as well as inhibitor of apoptosis (IAPs) and their antagonists. Messenger RNA expression of initiator *CASP8* (A) and effector *CASP3* (B) as well as inhibitor of apoptosis (IAPs) *BIRC4* (E) and *BIRC5* (F) in addition to their antagonists *XAF1* (G) and *DIABLO* (H) in endometria and corresponding conceptuses are shown. Messenger RNA expression is shown as means ΔCq ± SEM. Caspase activity of CASP8 (D) and CASP3/7 (E) in endometrial homogenates is presented as means ± SEM relative light units per µg total protein (RLU/µg TP). The effect of the day within the status is shown by different superscript letters (x–z in cyclic animals and a–c in pregnant animals). Asterisks indicate significant differences ($p < 0.05$). nd indicates non-determinable values.

leukocytes in the luminal epithelium (Fig. 5). In the present study, FASLG protein was found in the deep endometrium which is in contrast to the human endometrium where FASLG protein was primarily localized to glandular epithelial cells of the zona functionalis [40]. Only the deep maternal endometrium expresses ISGs as MHC class I and beta-2 microglobulin in response to IFNT [15,33] which impedes recognition of conceptus antigens by immune cells. Expression of FASLG in the zona basalis might inhibit the entrance of leukocytes to the endometrium as suggested in human [40] and therefore could act as additional mechanism to generate immune privilege by possibly preventing the maternal immune cells from attacking the conceptus.

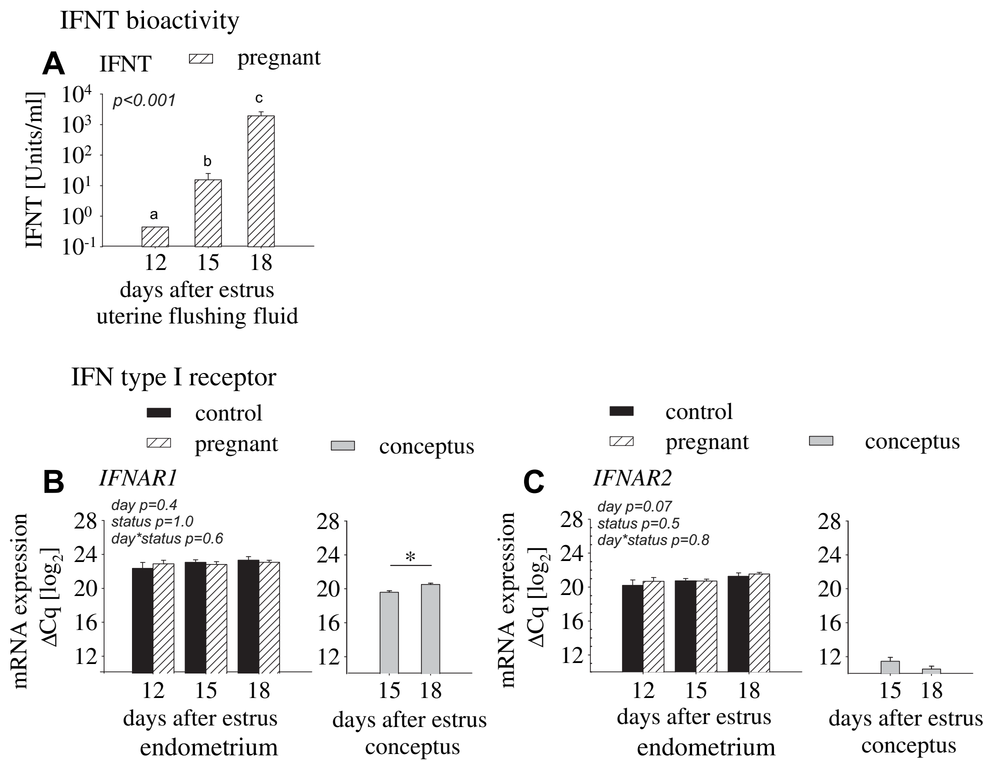

Fig. 3. Bioactivity of IFNT in uterine flushing fluids (A) and messenger RNA expression of IFN type I receptor subunits *IFNAR1* (B) and *IFNAR2* (C). Results are shown as means ± SEM. Asterisks indicate significant differences ($p < 0.05$).

A role in developing immune privilege during early pregnancy is also attributed to the death receptor ligand TNFSF10, since TNFSF10 has prominently been found in human syncytiotrophoblast and in Hofbauer cells [41]. *TNFSF10* and its receptor *TNFRSF10B* are also expressed in rodent trophectoderm cells during the periimplantation phase, whereas the expression in bovine conceptus cells was only minor. In cattle, a more important role for maternal TNFSF10 in enabling immune privilege might be suggested. Four receptor subtypes exist of which two (TNFRSF10C/TRAILR3 and TNFRSF10D/TRAILR4) lack a functional death domain and therefore act as decoy receptors [42]. Activation of apoptosis by TNFSF10 depends on the binding of the receptor, and as we could not observe a remarkable increase of apoptosis, decoy receptors could prevent the endometrium from detrimental TNFSF10 effects. TNF represents a pleiotropic cytokine with both proinflammatory and proapoptotic properties. Transcripts rose steadily in cyclic and foremost in pregnant animals. Apart from proapoptotic action transduced by TNFRSF1A and TNFRSF1B, antiapoptotic genes can be activated via the NF-κB pathway [43]. Despite an increased *TNF* expression we could not demonstrate a pronounced expression of antiapoptotic genes. Our data indicate an increase of death receptor ligand transcripts, whereas the analysed death receptors (apart from TNFRSF1A) appear to be constitutively expressed in the endometrium. Although we found a marked increase of *CASP8* transcripts due to the progressing periimplantation phase, the activity decreased over time in both cyclic and pregnant animals to the same extent (Fig. 5). As *CASP3* but not *CASP7* showed abundant expression, we assumed that *CASP3* contributed primarily to the activity in endometrial homogenates. These data are contrary to findings in the human endometrium where activity of CASP8 and CASP3 significantly increased within the secretory phase [44]. We therefore suggest that the proapoptotic signals caused by IFNT are not transduced.

Resistance to IFNT induced cell death might derive from the competition with CFLAR (FLIP) which shares sequence homologies to CASP8 and competes for binding to the death inducing signalling complex. CFLAR exists in multiple splice variants, which differ in their antiapoptotic abilities (reviewed in [13]). We could not demonstrate changes in *CFLAR* expression; however ongoing investigations will include the differentiation of splice variants possibly involved as well as the analysis of transcript and protein stability which is important for adequate availability of this factor.

Bovine periimplantation conceptuses showed differential expression of apoptosis related factors. The proapoptotic *FASLG* along with the antiapoptotic factor *BIRC5* exhibited marked expression in conceptuses compared to the maternal endometrium, whereas *BCL2L1*, *TNFSF10*, *TNF*, *CASP8*, *CASP7* as well as *XAF1* were only marginally expressed in conceptuses. In order to explain whether the differential expression of apoptosis related genes between the endometrium and conceptuses was due to the action of IFNT, the expression of the heterodimeric type I IFN receptor was analysed. A very faint expression in particular for *IFNAR2* in conceptuses could be demonstrated. Thus, our data indicate a reduced susceptibility of the conceptus to its own IFN-signalling as described in the ewe [45] and hence a lack of proapoptotic gene expression induction through IFNT.

Conceptus *BIRC5* exceeded by far the expression in the endometrium. BIRC5 is not a target of XAF1, it is rather inhibited by degradation, since the XAF1–BIRC4 complex causes its ubiquitination [46]. BIRC5 represents the smallest IAP and exhibits a bifunctional role in the regulation of cell division and in the suppression of effector caspases. In conceptuses, this factor is localized to microtubules and/or kinetochores in order to ensure an accurate distribution of chromosomes [47]. A contribution to the development of bovine embryos has also been described, since BIRC5 was expressed throughout all stages of oocyte development and in *in vitro* produced bovine conceptuses. Moreover, suppression of BIRC5 resulted in increased apoptosis [48]. Our data indicate

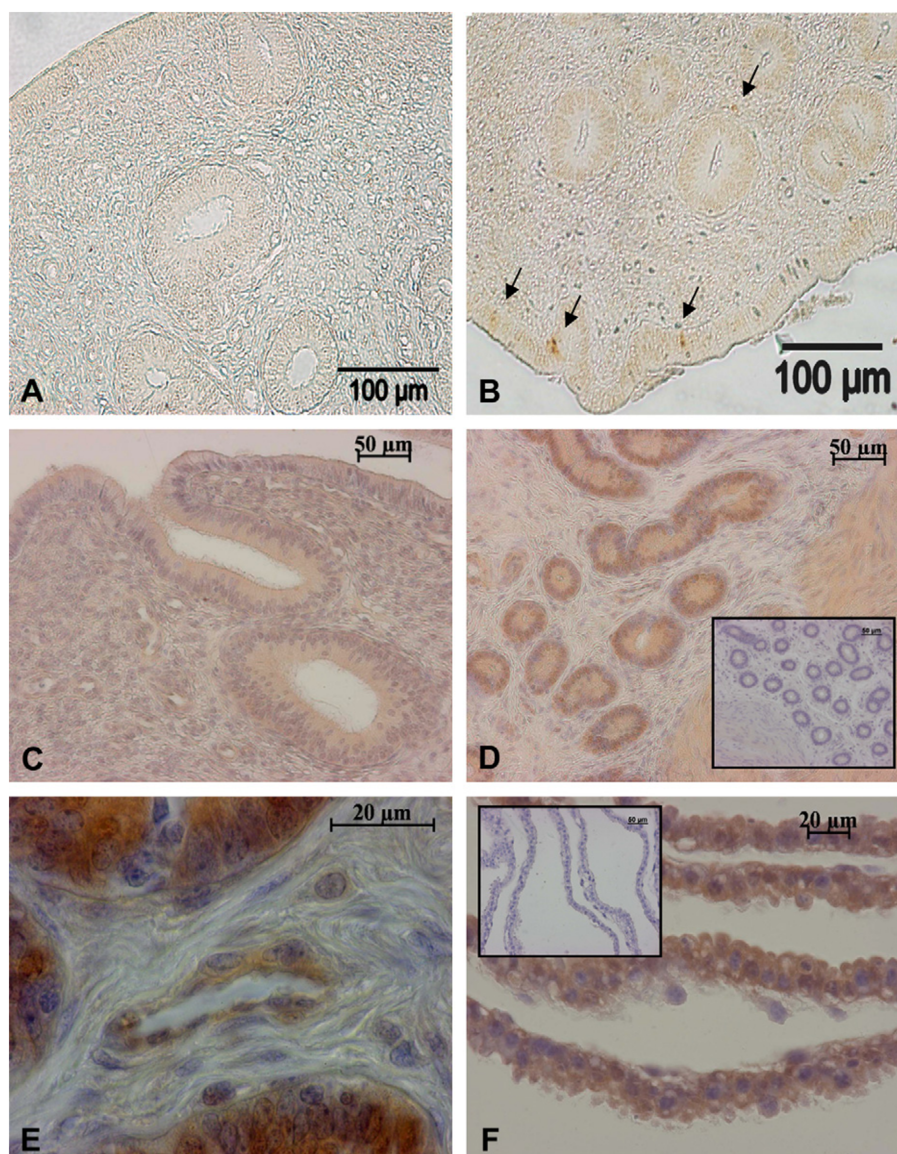

Fig. 4. Localisation of apoptotic cells by TUNEL in bovine endometrium and immunohistochemical localisation of FASLG in bovine tissue sections. TUNEL experiments: The negative control was incubated without rTdT enzyme (A) and the positive control with 10 Units DNase/ml (insert). Light microphotograph of an endometrial section showing TUNEL-positive cells (B). Arrows point to apoptotic cells. FASLG immunohistochemistry: Pictures show representative sections of the superficial endometrium (C), the deep endometrium (D), endothelial cells (E) and day 18 trophoblasts (F). Inserts represent negative controls.

a particular relevance for the intrinsic pathway, because *BAX* along with *CASP3* was markedly expressed in conceptuses. On the other hand neither factors from the extrinsic pathway (except for *FASLG*) nor *CASP8* was appreciably present. Apoptosis is a tightly regulated process which also occurs in early bovine conceptuses [49]. Apoptotic events as part of regular development could be suppressed as long as the formation of the conceptuses is untainted, however if cell division failures or insufficient supply of maternal growth factors prevail, the balance could be immediately shifted towards apoptotic cell death.

In the present study the messenger RNA transcripts of proapoptotic factors *XAF1*, *CASP8* and *TNFSF10* were increased close to the beginning of trophoblast attachment in pregnant animals, but not concomitantly accompanied by an elevation of apoptosis. The lack of an increased activity of the caspase cascade and the presence of only few apoptotic cells in the early pregnant endometrial

Table 2
Messenger RNA expression of *XAF1*, *CASP8* and *FASLG* in IFNT treated and untreated glandular and stroma cells in an *in vitro* co-culture system. Messenger RNA expression is shown as means ΔCq ± SEM. The relative increase of transcript abundance following IFNT treatment is also shown.

	glandular epithelial cells mRNA expression				stroma cells mRNA expression			
	ΔCq [log_2] mean ± SEM		fold increase	*p*-value	ΔCq [log_2] mean ± SEM		fold increase	*p*-value
	control	IFNT treatment			control	IFNT treatment		
XAF1	25.4 ± 0.2	28.4 ± 0.2	7.9	<0.001	22.1 ± 0.5	27.1 ± 0.2	32.2	<0.001
CASP8	25.7 ± 0.3	28.3 ± 0.4	6.2	<0.001	23.8 ± 0.4	27.5 ± 0.2	13.4	<0.001
FASLG	13.6 ± 0.5	14.1 ± 0.5	1.4	0.5	13.9 ± 0.2	14.3 ± 0.6	1.3	0.9

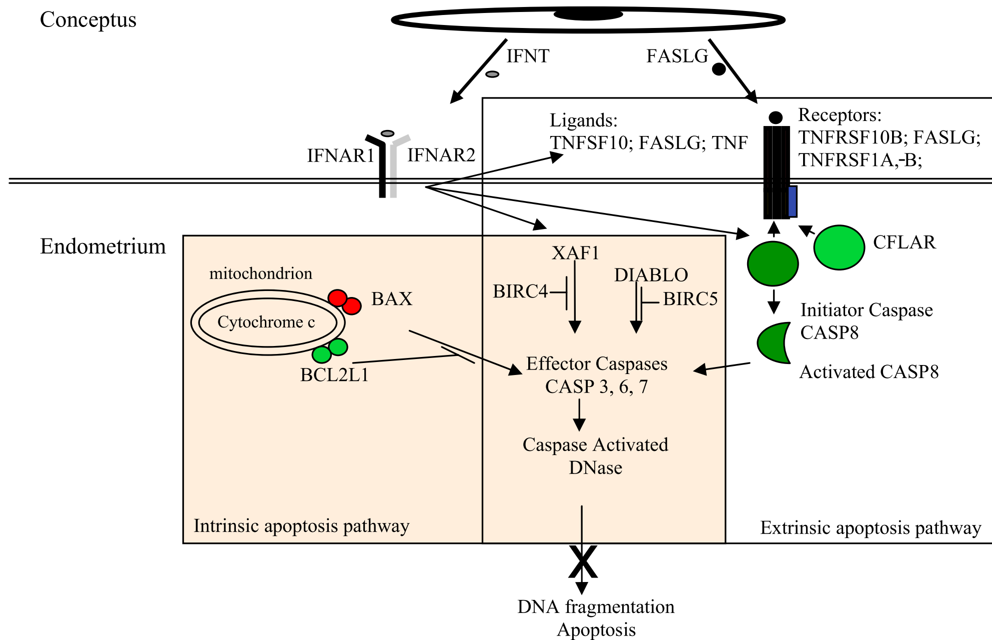

Fig. 5. Apoptosis can be either elicited by the intrinsic pathway through proapoptotic Bcl-2-family factors (BAX) or the extrinsic pathway via death receptor ligands (FASLG, TNFSF10 and TNF) by binding to respective receptors (FAS, TNFRSF10B, TNFRSF1A,-B). Both pathways cause activation of initiator caspases (CASP8) and effector caspases (CASP3, 6, 7). Subsequent activation of caspase activated DNases result in DNA fragmentation and apoptosis. Proapoptotic components (XAF1, DIABLO) may diminish the effects of anti-apoptotic factors (BCL2L1, CFLAR, BIRC4, 5). Although expression of proapoptotic genes *XAF1*, *CASP8* and *TNFSF10* was elevated in pregnant animals in the presence of trophoblast IFNT the activity of caspases and the incidence of apoptotic cells did not increase. Inhibitory mechanisms preventing endometrial cells from apoptosis are presumed.

tissue may suggest that inhibitory mechanisms exist to protect the bovine endometrium from tissue damage potentially caused by IFNT. In addition, FASLG release by the trophoblast might prevent from the attack of immune cells sensitized to conceptus antigens to support implantation and conceptus development.

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Immunological mechanisms to establish embryo tolerance in early bovine pregnancy

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Abstract. A well-balanced immunological interaction between mother and the semi-allogenic embryo is of particular importance. The objective of the present study was to analyse mechanisms of immune tolerance in bovine pregnancy during peri-implantation. Simmental heifers inseminated with either cryopreserved spermatozoa or seminal plasma were killed 12, 15 or 18 days after oestrus. Uteri were flushed for the recovery of conceptuses and the ipsilateral intercaruncular endometrium was sampled for gene expression analysis. Indoleamine 2,3-dioxygenase (IDO) mRNA, coding for the initial enzyme of the kynurenine pathway, was 18-fold ($P < 0.001$) more abundant in the endometrium of Day 18 pregnant *v.* non-pregnant animals. Tandem mass spectrometry revealed a decrease of endometrial L-tryptophan ($P = 0.0008$), but an increase of L-kynurenine concentration ($P = 0.005$) from Day 12 to Day 18, suggesting increasing IDO activity ($P < 0.03$). An *in vitro* coculture model of endometrial cells showed an induction of *IDO* expression following interferon-τ exposure primarily in stroma cells, which was confirmed by *in situ* hybridisation localising IDO mRNA mainly in deep stroma cells. Immunohistochemical analysis revealed fewer CD45-positive leucocytes in the zona basalis of pregnant animals. Elevated IDO activity may reduce the presence of leucocytes in the pregnant endometrium, providing a possible mechanism for protecting the semi-allogenic conceptus from maternal rejection.

Additional keywords: *Bos taurus*, endometrium, indoleamine 2,3-dioxygenase, peri-implantation.

Introduction

In the reproductive tract, efficient immune defence mechanisms are essential to control the invasion of potential pathogens, especially at oestrus (Bondurant 1999). During early pregnancy, immune mechanisms towards deleterious pathogens and the immune tolerance of the semi-allogenic conceptus need to be thoroughly balanced to ensure a successful pregnancy outcome. As soon as the blastocyst hatches, antigens and major and minor histocompatibility complexes of the semi-allogenic preattachment conceptus are exposed to the innate and adaptive maternal immune system *in utero*. Ruminants exhibit a synepitheliochorial type of placentation with trophectodermal binucleate cells invading and fusing with caruncular epithelial cells to form syncytial giant cells from Days 18–20 onwards (Wooding and Wathes 1980). Despite this slight invasiveness, pregnancy can be established without triggering an immune response towards the semi-allogenic embryo. A central mechanism to protect the conceptus before implantation is attributed to the immune-suppressive actions of progesterone (P_4). For example, P_4 delays rejection of an allograft transplanted in the uterus of ovariectomised P_4-treated ewes (Padua *et al*. 2005).

Major histocompatibility complex (MHC) Class I molecules, consisting of an α chain and a smaller β_2-microglobulin (B2M) unit, discriminate between self and non-self by presenting cytosolic protein fragments on somatic cells mainly to cytotoxic T cells (CTL; Gogolin-Ewens *et al*. 1989). The MHC antigens

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expressed on transplants are commonly responsible for severe lymphocyte responses towards the allograft. As shown for Day 8 bovine blastocysts, semi-allogenic conceptuses escape from recognition by expressing only marginal MHC Class I antigens (Low *et al*. 1990). However, the overall expression of classical MHC Class I molecules increases due to pregnancy in the ovine and bovine endometrium (Choi *et al*. 2003; Bauersachs *et al*. 2006; Klein *et al*. 2006), but primarily increases in the deep ovine endometrial stroma and glandular epithelium (GE; Choi *et al*. 2003). The MHC Class I α chain and B2M are expressed primarily in ovine endometrial luminal epithelium (LE) and superficial GE on Days 10 and 12 of the oestrous cycle and pregnancy (Choi *et al*. 2003). However, on Days 14–20 of pregnancy, the increase in MHC Class I and B2M expression is restricted to the stroma and GE resulting from interferon regulatory factor 2 (IRF2) expression in ovine LE and superficial GE (Choi *et al*. 2001). The risk of presentation of inherited paternal antigens on maternal MHC Class I molecules may therefore require the reduction of classical MHC molecules in the uterine LE and superficial GE during peri-implantation to establish maternal tolerance (Choi *et al*. 2003). This may be critical in preventing immune rejection of the conceptus semi-allograft (Bazer *et al*. 2008).

An abnormal expression of MHC Class I molecules in the trophoblast is concomitantly accompanied by elevated T cell numbers in the maternal bovine endometrium (Hill *et al*. 2002). The downregulation of classical high polymorphic MHC Class I molecules on the blastocyst may highly increase the vulnerability to natural killer cells because these large granular lymphocytes elicit apoptosis by releasing cytotoxic granules in case self-recognition fails (missing self-hypothesis; for a review, see Croy *et al*. 2006). Human trophoblasts express less polymorphic non-classical MHC Class I molecules, such as human leucocyte antigen-G (HLA-G), which is an important strategy for the maintenance of pregnancy and tolerance of the conceptus (Roussev and Coulam 2007). Recently, Doyle *et al*. (2009) provided evidence for the expression of non-classical MHC Class I molecules in early bovine blastocyst; in addition, non-classical MHC Class I are expressed with a high abundance in third trimester bovine interplacentomal trophoblasts (Davies *et al*. 2006).

A supplementary mechanism to prevent maternal T cell activation at the embryo–maternal interface may be the withdrawal of the essential amino acid L-tryptophan (Trp). The first enzyme of the kynurenine pathway, namely indoleamine 2,3-dioxygenase (IDO), catalyses the rate-limiting step of Trp degradation and is proposed to control Trp availability at implantation sites (Mellor and Munn 1999, 2000). In this metabolic pathway, IDO activity results in the formation of kynurenine, kynurenic acid and quinolinic acid. Binding of lipopolysaccharide to Toll-like receptors (TLR) and the induction of proinflammatory T helper 1 (Th1) cytokines, such as interferon (IFN)-γ, increase IDO (Puccetti and Grohmann 2007). From murine models, IDO is known to be essential during pregnancy to protect the semi-allogenic fetus from maternal T cell attack (Mellor *et al*. 2001).

Immunological tolerance at the embryo–maternal interface in the bovine may involve multiple mechanisms and involve a complex cross-talk of immunological mechanisms (Wolf *et al*. 2003). Previous studies of endometrial gene expression in pregnant *v*. non-pregnant animals revealed an increased transcript abundance for a large number of genes related to immune functions (Bauersachs *et al*. 2006; Klein *et al*. 2006). The objective of the present study was to analyse embryo–maternal immune-modulating mechanisms during preparation of the endometrium for embryo attachment and implantation in cattle.

Materials and methods

Pretreatment of animals

All experiments were performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals (http://www.en.gen.vetmed.uni-muenchen.de/index.html, accessed 21 March 2011), as promulgated by the Society for the Study of Reproduction and with the European Convention on Animal Experimentation. Cyclic Simmental heifers (*Bos taurus*, Deutsches Fleckvieh) with an average age of 22 months were oestrus synchronised by intramuscular injection of a single 500-μg dose of the prostaglandin $F_{2\alpha}$ analogue cloprostenol (Estrumate; Essex Tierarznei, Munich, Germany) at dioestrus, as described previously (Bauersachs *et al*. 2005). Approximately 60 h after injection, standing heat was determined by observation of sexual behaviour. The pregnant group was inseminated with cryopreserved spermatozoa (ejaculate : diluter, 1 : 10) at oestrus, whereas the non-pregnant control group was inseminated with a supernatant of centrifuged spermatozoa (seminal plasma) from the same bull. Serum P_4 concentrations were determined according to Prakash *et al*. (1987). On Days 12, 15 and 18 after insemination, animals were killed, the uterus removed immediately and flushed for the recovery of blastocysts using phosphate-buffered saline (PBS; pH 7.4). If artificially inseminated with cryopreserved spermatozoa, animals were termed pregnant in case an intact conceptus was found, whereas artificially inseminated non-pregnant animals were excluded from the study. Experiments were performed in four to seven animals per group.

Tissue sampling

Intercaruncular endometrial tissue samples from the ipsilateral uterine horn were obtained as described previously (Bauersachs *et al*. 2005). For gene expression analysis, whole recovered embryos and intercaruncular endometrium were immediately transferred into vials containing RNAlater (Ambion-Applied Biosystems, Darmstadt, Germany) and incubated overnight at 4°C. The following day, samples were stored at −80°C until further processing. For ELISA experiments, endometrial tissue was homogenised in PBS, pH 7.4, containing a Complete-Mini protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Total protein was determined using the Bicinchoninic Acid Kit (Sigma-Aldrich, St Louis, MO, USA). For *in situ* hybridisation and immunohistochemistry, endometrial tissue samples were transferred into 3.7% formaldehyde. Water was removed in serial ascending ethanol dilutions. Following a 1-h incubation in the intermedial xylene substituent Rothihistol (Carl Roth, Karlsruhe, Germany), the tissue was finally embedded in paraffin.

Extraction of total RNA and reverse transcription

Total RNA of endometrial tissue and whole conceptuses was isolated using TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The quality of the RNA was controlled by the Agilent 2100 Bioanalyzer (RNA 6000 Nano Assay Kit; Agilent Technologies, Palo Alto, CA, USA). RNA integrity ranged between 7.0 and 10.0. The quantity of RNA was determined spectroscopically at 260 nm using the Nanodrop 1000 (peQLAB, Erlangen, Germany). Extracted RNA (1 μg) was reverse transcribed in a total volume of 60 μL in the presence of 50 μM Random Hexamer Primer (Invitrogen), 10 mM dNTPs (Fermentas, St Leon-Rot, Germany), 5× First Strand Buffer (Promega, Madison, WI, USA) and 200 U M-MLV RT H(–) Enzyme (Promega, Madison, WI, USA) at 48°C for 120 min.

Gene expression analysis using the Fluidigm Dynamic Array gene expression chip

To determine the gene expression of immune-related genes, which were selected on the basis of a literature search, a high-throughput gene expression platform was used based on Dynamic Array microfluidic chips (Fluidigm 96.96 Dynamic Array IFC; Fluidigm, San Francisco, CA, USA). This allows 9216 simultaneous real-time polymerase chain reaction (PCR) gene expression measurements per chip. The sequences of forward and reverse primers, references and fragment lengths are given in Table 1. In the present experimental set-up, the mRNA expression of 32 immune-relevant genes in 94 preamplified endometrial and conceptus samples was measured with three replicates on a single chip at an annealing temperature of 60°C. Before introducing the samples to the Fluidigm platform, specific target amplification was undertaken lasting 14 cycles. For each sample, 1.25 μL Assay mix, consisting of forward and reverse primers (180 nM), 2.5 μL 2× ABI preamp mix and 1.25 μL cDNA (16.6 ng μL^{-1}) were subjected to specific target amplification. Preamplified cDNA was finally diluted 1 : 5 with DNA suspension buffer containing 10 mM Tris, pH 8.0 and 0.1 mM EDTA and loaded onto the chip. Three chip replicates were run to determine chip-to-chip reproducibility. To ensure specific product amplification, melting curve analysis was performed and data were eliminated if unspecific products were detected. An overall standard deviation of 0.16 in addition to a chip-to-chip correlation of >0.99 for all three replicates was obtained.

Data analysis and statistics

The cycle number (Cq) to obtain a definite fluorescence signal was calculated by the second derivative maximum method, because the Cq is inversely correlated with the logarithm of the initial template concentration. Reference genes histone H3 and polyubiquitine were chosen using GenEx Pro Ver 4.3.4 (multiD Analyses, Gothenburg, Sweden). To avoid negative digits while allowing an estimation of a relative comparison between two genes, data are presented as the mean ± s.e.m. subtracted from the arbitrary value 20 (ΔCq; Groebner *et al*. 2010). Thus, a high ΔCq indicates high transcript abundance and an increase of 1 ΔCq represents a twofold increase in mRNA transcripts. Endometrial gene expression data were analysed using SAS release 9.1.3 (2002; SAS Institute, Cary, NC, USA). Data were subjected to least-square analysis of variance using the General Linear Models procedure to analyse effects of day of the cycle (day), pregnancy status (pregnant *v*. non-pregnant; status) and their interaction (day × status). Student's *t*-tests were used to evaluate the significance of differences in expression in conceptuses. $P < 0.05$ was regarded as significant. The limit of detection was Cq >35.

In vitro *coculture of endometrial glandular and stroma cells*

A coculture of primary endometrial glandular and stroma cells was performed as described previously (Ulbrich *et al*. 2009). Glandular epithelial cells were cultured in matrigel-coated inserts, whereas stroma cells were cultured in the bottom of cell culture wells. Cells were stimulated with recombinant bovine IFN-τ (antiviral activity 4.8×10^3 U mL^{-1} medium; PBL Biomedical Laboratories, Piscataway, NJ, USA) for 4 h. RNA extraction and quantitative polymerase chain reaction (qPCR) experiments were performed as described previously (Ulbrich *et al*. 2009).

Cytokine ELISA

Cytokine concentrations were determined in endometrial tissue homogenates using commercially available sandwich ELISA kits for tumour necrosis factor (TNF; BD OptEIA human TNF ELISA Set; BD Biosciences Europe, Erembodegen, Belgium) and IFN-γ (ELISA Assay for bovine IFN-γ, Code 3115-1A-6; Mabtech AB, Nacka Strand, Sweden) according to the manufacturers' instructions. Results are presented as the mean ± s.e.m. (pg mg^{-1} total protein). Interleukin (IL)-10 protein was detected using a monoclonal antibody (Serotec, Oxford, UK). Briefly, microtitre plates (Nunc MaxiSorp, Roskilde, Denmark) were coated with anti-cytokine antibodies (CC318 for IL-10) at a concentration of 10 μg mL^{-1} in carbonate-bicarbonate coating buffer (pH 9.6) and incubated overnight at 4°C. After a blocking step with PBS, 0.05% Tween 20 and 1% bovine serum albumin (BSA) for 1 h at room temperature, plates were washed three times with PBS–Tween-20 (PBST) and incubated for 1 h at room temperature with endometrial tissue homogenates, added in duplicate and diluted 1 : 2 with PBS. The addition of the secondary biotinylated antibody (CC320; 5 μg mL^{-1}) was followed by washing with PBS containing Tween 20 (Fluka, Taufkirchen, Germany) and the addition of streptavidin–peroxidase (1 : 1000) for 20 min at room temperature. After the final washing step, the chromogenic substrate (3,3,5,5-tetramethylbenzidine) was added, colour development was stopped using H_2SO_4 after 40 min and optical density was measured spectrophotometrically using an ELISA plate reader (Tecan, Maennerdorf, Switzerland) at 450 nm. Owing to the lack of a standard for IL-10, the results are presented as mean optical density values ± s.e.m. at 450 nm (Flynn and Mulcahy 2008; Dacal *et al*. 2009).

Localisation of endometrial IDO *mRNA by* in situ *hybridisation*

In situ hybridisation was performed in endometrium sections of Day 18 control and pregnant animals, as described previously (Bauersachs *et al*. 2005). The sequence of the antisense

Table 1. Sequences of primer pairs, corresponding reference genes and resulting fragment lengths

B2M, β_2-microglobulin; MHC, major histocompatibility complex; BoLA, bovine leucocyte antigen; HLA-G, human leucocyte antigen G; IL, interleukin; LIF, leukaemia inhibitory factor; TGFB1/2, transforming growth factor-β1/2; SOCS3, suppressor of cytokine signalling; NCR1, natural cytotoxicity; DQA, bovine leukocyte antigen MHC class II locus region DQA; TLR, Toll-like receptor; IRF2/3/9, interferon regulatory factor 2/3/9; IFITM3, interferon-induced transmembrane protein-3

Gene	Reference	Forward primer (5′→3′)	Reverse primer (5′→3′)	Fragment size (bp)
Reference genes				
Polyubiquitin	Z18245	AGATCCAGGATAAGGAAGGCAT	GCTCCACCTCCAGGGTGAT	198
Histone H3	BT020962	ACTGGCTACAAAAGCCGCTC	ACTTGCCTCCTGCAAAGCAC	233
Indoleamine 2,3 dioxygenase (IDO)				
IDO	ENSBTAT00000027449	GGGCCCATGACTTATGAGAA	GAGGCAGCTGCTATTTCCAC	107
MHC Class I molecules				
B2M, Lactollin MHC Class I	AY911322	GCGTCCTCCAAAGATTCAAG	TCCCCATTCTTCAGCAAATC	126
BoLA similar to HLA-G	DQ121134	ATGGGAACCTCCTCAGACCT	CGCTTCTTCCTCCAGATCAC	105
Cytokines				
IL1B	M37211	TTCTCTCCAGCCAACCTTCATT	ATCTGCAGCTGGATGTTTCCAT	198
IL6	NM_173923	TGAGTGTGAAAGCAGCAAGG	AGCAAATCGCCTGATTGAAC	107
IL15	U42433	TTGGGCTGTATCAGTGCAAG	ACTTTGCAATTGGGATGAGC	149
LIF	U65394	CAGCAACCTCATGAACCAGA	CTGCACAGCTTGTCCAGGT	126
TGFB1	ENSBTAT00000027261	TGACCCGCAGAGAGGAAATA	CCGTTAATGTCCACTTGAAGC	91
TGFB2	NM_001113252	AAGCACGCTTTGCAGGTATT	TAGCAGGAGATGTGGGCTCT	110
IL10	ENSBTAT00000008793	AGCTCCAAGAGAGGGGTGTC	TCACCATCCTGGAGGTCTTC	129
SOCS3	AY026859	CCCCCAGGAGAGCCTATTAC	ACGGTCTTCCGACAGAGATG	112
Lymphocytes				
CD8B	XM585436	ACTGTGTATGGCAAGGAGGTG	GGGTATCCCAATGATCATGCAG	127
CD69	BC142335	CACGCTTGCTGTCATTGATT	CTTGGCCATTTGACCATTTC	125
NCR1 (CD335)	AF422181	CGCAGAAAGACCATCTCCTC	GCCAGTCTTCAAGCAGGAAC	108
CD3D	BC102857	CGAATGTGCCAGAACTGTGT	GTCTCATGTCCAGCAAAGCA	119
CD3G	BC103010	CCTCATCCTGGCTGTCTTTC	AGCCATGTGATCGTTGTGTC	129
Antigen-presenting cells				
CD14	AF141313	GCAGCCTGGAACAGTTTCTC	ACCAGAAGCTGAGCAGGAAC	124
BoLA-DQA (MHC Class II)	AY730727	TGAAGACATTGTGGCTGACC	CAGTCTCCCTCTTTTCCAGGT	134
CD86	BT025428	GCAACTTGAGCCAACGAAG	AGCTGCAATCCAGAGGATGT	106
LY75 (Dec205)	AY264845	AAAGCGGGCTGTAACCTTTT	GCCCTGAAACTTCTGCAGTC	126
TLR system				
TLR2	NM_174197	CCATGTGGAGAGGGTGTT	GGGGACACAAAACAGCACTT	140
TLR3	NM_001008664	CGATCCCTTTAAAAACCTGAAG	GGTTCTCCAGTTGGAGCTGA	98
TLR4	NM_174198	GACCCTTGCGTACAGGTTGT	GGTCCAGCATCTTGGTTGAT	103
TLR7	DQ333225	AGGCTCTGTGGCAGTTCTGT	GGTCACGTGATTGTCTGTGG	105
TLR8	NM_001033937	TCACACGGGTAACGAATGAA	GAGAAATGCCCGTCTGTAA	134
MYD88	BC102851	CTGCAAAGCAAGGAATGTGA	AGGATGCTGGGGAACTCTTT	122
IRF3	AJ879589	GGCTTGTGATGGTCAAGGTT	TGCAGGTCGACAGTGTTCTC	103
Interferon signalling				
IRF2	Q8MII2	AAACTGGGCCATCCATACAG	TTAGAAGGCCGCTCAGACAT	169
IRF9	Q58DC4	GAGAACTGCTGTCCCTGGAG	AAGATCACCTGCTCCATGCT	192
IFITM3	XM_598998.1	CGTGTGGTCCCTGTTCAAC	CCATCTTCCGGTCCCTAGAC	95

oligonucleotide was as follows: 5′-GAG GCA GCT GCT ATT TCC AC-3′. A complementary sense probe was used as a negative control.

Determination of metabolites of the kynurenine pathway

Using a mortar and pestle, endometrial tissues were disrupted by the addition of liquid nitrogen dissolved in methanol : chloroform : H_2O (55% : 22%: 22%, v/v/v). Samples were mixed, incubated at 4°C for 30 min and centrifuged (20 000*g*, 20 min). The supernatant was collected stored at −80°C until further use. For extraction, 100 mg tissue was added to a mixture of 10 μL trichloroacetic acid (50%; FLUKA, Taufkirchen, Germany), 60 μL water, 100 μL methanol (JT Baker, Deventer, The Netherlands) and 10 μL each deuterated standard solution (phenylalanine-d_5, kynurenine-d_6 and kynurenic acid-d_5; Cambridge Isotope Laboratories, Andover, MA, USA). Samples were mixed, stored at 4°C overnight and then centrifuged (20 000*g*, 15 min). A Wallac MS2 tandem mass spectrometer (Perkin Elmer, Rodgau, Germany) equipped with an electrospray ion source recorded the levels of tryptophan, phenylalanine-d_5, kynurenine, kynurenine-d_6, quinolinic acid, serotonin, kynurenic acid and kynurenic acid-d_5 with a flow solvent composition of 0.2% formic acid in 50% aqueous acetonitrile and a flow rate of 50 μL min^{-1}. The ions were detected in positive ion mode using multiple reaction monitoring. The two quadrupoles were set to mass-to-charge ratios of (*m/z*) 205/159 for tryptophan, 171/125 for phenylalanine-d_5, 177/160 for serotonin, 209/192 for kynurenine, 215/198 for kynurenine-d_6, 168/78 for quinolinic acid, 190/144 for kynurenic acid and 195/149 for kynurenic acid-d_5. Nitrogen served as the collision gas. Estimated IDO activity was assessed using the Kyn × 100/Trp ratio, as described previously (Schefold *et al.* 2010).

CD45 immunohistochemistry

The CD45 protein was localised in endometrial tissue. These surface proteins are highly abundant on all cells of haematopoietic origin, except for erythrocytes. Uterine sections (5 μm) were deparaffinised and rehydrated in descending serial ethanol dilutions. For heat-induced antigen retrieval, tissue sections were boiled for 60 min in citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 1% H_2O_2 in PBS. Following blocking with 10% goat serum, samples were incubated with anti-CD45 (CACTB51A; VMRD, Pullman, WA, USA) for 30 min at room temperature. Samples were then incubated with a secondary antibody, namely peroxidase-conjugated anti-mouse IgG (Dako, Glostrup, Denmark). Binding of antibody was detected by incubating sections with 3,3′-diaminobenzidine in the presence of 0.01% H_2O_2 for 15 min. Tissues were counterstained using Mayer's Haemalaun (Carl Roth). Positively immunostained leucocytes were counted by an observer blinded to the day of the cycle or pregnancy status. CD45-positive cells were manually determined in a ≥5 mm length of uterine luminal and glandular epithelium per tissue section by differentiating between glandular epithelia in the zona functionalis and basalis ($n = 4$–7 animals per group). In addition, leucocytes in the zona basalis and zona functionalis were counted in a minimum area of 5 mm^2 within each uterine tissue section. Results are expressed as the mean ± s.e.m. number of positively stained cells.

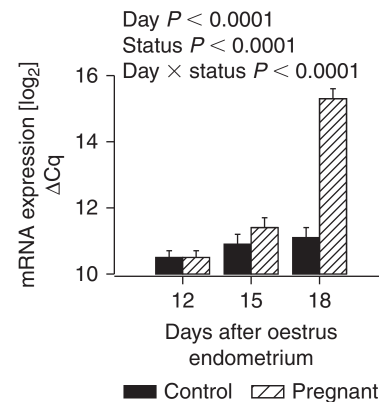

Fig. 1. Transcript abundance of indoleamine 2,3-dioxygenase (*IDO*) is shown as the mean ± s.e.m. ΔCq in the endometrium of pregnant and non-pregnant cattle. Normalised cycle number (ΔCq) values were subtracted from the arbitrary value 20.

Results

Gene expression of immune-relevant components in the endometrium and conceptuses

Gene expression of IDO

The mRNA expression of IDO, the initial enzyme in the kynurenine pathway, increased significantly with time in the endometrium of pregnant animals and was 18-fold higher in pregnant *v.* non-pregnant endometrium at Day 18 (day, $P < 0.0001$; status, $P < 0.0001$; day × status, $P < 0.0001$; Fig. 1). The expression of *IDO* mRNA in conceptuses was below the detection limit at all time points (data not shown).

Gene expression of classical and non-classical MHC Class I molecules

Endometrial gene expression data are summarised in Table 2 and expression data from conceptuses are given in Table 3. For the small subunit of the MHC Class I complex B2M transcript, we detected a 3.4-fold increase in endometrial expression on Day 18 compared with Day 12 in pregnant animals. In addition, on Day 18, B2M expression was significantly (2.3-fold) increased in the endometrium of pregnant *v.* control animals (day, $P < 0.0001$; status, $P < 0.\ 0001$; day × status, $P < 0.03$). Expression of mRNA transcripts for the possible non-classical MHC Class I molecule bovine leucocyte antigen (BoLA) increased significantly (2.1-fold) over time, most strikingly in the endometrium of pregnant animals (day, $P < 0.0001$). Interestingly, mRNA expression in bovine conceptuses was reduced for both MHC Class I molecules tested, specifically 37-fold for B2M and 4100-fold for BoLA, in Day 18 conceptuses compared with expression in the endometrium and was 3.1-fold decreased for B2M in Day 15 conceptuses compared with Day 18 conceptuses.

Gene expression of cytokines and cell surface molecules of leucocytes

The mRNA expression of cytokines IL-1B, IL-15, leukaemia inhibitory factor (LIF), transforming growth factor-β1

Table 2. Messenger RNA expression of immune-relevant genes in the endometrium of cyclic and pregnant animals

Data are presented as the mean ± s.e.m. ΔCq. Normalised cycle number (ΔCq) values were subtracted from the arbitrary value 20. Effects of the day of the cycle (day), pregnancy status (status) and their interaction on mRNA expression were regarded significant if $P < 0.05$. B2M, β2-microglobulin; MHC, major histocompatibility complex; BoLA, bovine leucocyte antigen; HLA-G, human leucocyte antigen G; IL, interleukin; LIF, leukaemia inhibitory factor; TGFB1/2, transforming growth factor-β1/2; SOCS3, suppressor of cytokine signalling; NCR1, natural cytotoxicity triggering receptor 1; TLR, Toll-like receptor; IRF2/3/9, interferon regulatory factor 2/3/9; IFITM3, interferon-induced transmembrane protein-3

	$\log_2$ mRNA expression						*P* value		
	ΔCq non-pregnant			ΔCq pregnant			Day	Status	Day × status
	12	15	18	12	15	18			
MHC Class I molecules									
B2M	20.1 ± 0.2	20.6 ± 0.2	20.9 ± 0.2	20.3 ± 0.1	21.0 ± 0.2	22.0 ± 0.2	<0.0001	<0.0001	0.03
BoLA (sequence similarity to HLA-G)	20.2 ± 0.2	20.5 ± 0.3	20.8 ± 0.1	20.5 ± 0.2	20.3 ± 0.2	21.5 ± 0.2	0.0007	0.2	0.1
Cytokines									
IL-1B	8.2 ± 0.3	9.8 ± 0.3	9.6 ± 0.2	8.1 ± 0.3	9.1 ± 0.3	9.4 ± 0.2	<0.0001	0.1	0.5
IL-6	5.3 ± 0.2	5.6 ± 0.3	5.5 ± 0.4	5.0 ± 0.4	5.5 ± 0.3	6.0 ± 0.3	0.3	0.9	0.6
IL-15	11.9 ± 0.1	13.1 ± 0.2	13.1 ± 0.2	11.9 ± 0.1	13.1 ± 0.2	13.4 ± 0.1	<0.0001	0.4	0.5
LIF	9.3 ± 0.2	9.9 ± 0.3	10.0 ± 0.2	9.3 ± 0.1	9.9 ± 0.1	10.2 ± 0.2	0.0006	0.6	0.8
TGFB1	16.8 ± 0.4	15.0 ± 0.5	16.4 ± 0.1	16.4 ± 0.4	16.9 ± 0.2	17.1 ± 0.3	0.04	0.01	0.04
TGFB2	12.1 ± 0.1	12.3 ± 0.2	11.9 ± 0.2	12.3 ± 0.1	11.8 ± 0.2	10.9 ± 0.2	<0.0001	0.005	0.07
IL-10	9.7 ± 0.3	8.7 ± 0.3	9.1 ± 0.3	8.8 ± 0.6	9.5 ± 0.2	12.4 ± 1.1	0.004	0.02	0.001
SOCS3	8.6 ± 0.2	9.2 ± 0.2	9.2 ± 0.3	8.7 ± 0.2	9.1 ± 0.2	9.9 ± 0.2	0.002	0.2	0.3
Lymphocytes									
CD8b	12.3 ± 0.2	13.0 ± 0.3	12.8 ± 0.2	12.4 ± 0.2	12.3 ± 0.1	12.5 ± 0.3	0.4	0.09	0.2
CD69	3.7 ± 1.5	4.6 ± 0.9	4.2 ± 0.2	0.8 ± 1.6	4.7 ± 0.6	5.3 ± 0.3	0.01	0.4	0.7
NCR1 (CD335)	10.3 ± 0.1	11.1 ± 0.2	11.1 ± 0.2	10.4 ± 0.2	10.8 ± 0.2	11.0 ± 0.1	0.006	0.7	0.8
CD3D	11.8 ± 0.1	12.8 ± 0.3	12.5 ± 0.2	11.6 ± 0.2	12.5 ± 0.2	12.6 ± 0.2	<0.0001	0.5	0.5
CD3G	10.8 ± 0.1	11.5 ± 0.2	11.7 ± 0.2	10.6 ± 0.1	11.5 ± 0.2	11.6 ± 0.2	<0.0001	0.5	0.8
Antigen-presenting cells									
CD14	14.9 ± 0.2	15.3 ± 0.2	15.5 ± 0.2	15.0 ± 0.2	15.2 ± 0.1	15.1 ± 0.2	0.06	0.3	0.3
MHC Class II	17.1 ± 0.2	17.2 ± 0.4	17.1 ± 0.4	16.0 ± 0.8	15.8 ± 1.0	16.4 ± 1.0	0.9	0.06	0.9
CD86	7.3 ± 0.1	8.6 ± 0.3	7.7 ± 0.2	7.6 ± 0.2	7.8 ± 0.3	8.3 ± 0.2	0.01	0.9	0.01
LY75 (Dec205)	9.3 ± 0.2	9.6 ± 0.2	9.6 ± 0.2	9.3 ± 0.2	9.2 ± 0.3	9.3 ± 0.2	0.7	0.3	0.7
TLR system									
TLR2	9.5 ± 0.2	11.1 ± 0.5	10.7 ± 0.2	9.8 ± 0.1	10.6 ± 0.2	10.4 ± 0.2	0.0005	0.5	0.4
TLR3	9.4 ± 0.2	11.4 ± 0.6	10.1 ± 0.2	9.5 ± 0.2	9.9 ± 0.3	10.2 ± 0.2	0.002	0.1	0.02
TLR4	13.9 ± 0.1	14.4 ± 0.2	14.4 ± 0.2	13.9 ± 0.1	14.2 ± 0.1	14.4 ± 0.1	0.01	0.6	0.8
TLR7	5.3 ± 0.4	8.3 ± 1.1	5.6 ± 0.2	5.2 ± 0.2	5.9 ± 0.4	6.4 ± 0.4	0.007	0.2	0.02
TLR8	6.3 ± 0.4	9.3 ± 0.9	7.0 ± 0.3	6.4 ± 0.4	6.6 ± 0.5	7.7 ± 0.4	0.01	0.2	0.005
MYD88	17.4 ± 0.1	17.5 ± 0.2	17.4 ± 0.2	17.3 ± 0.1	17.6 ± 0.1	17.6 ± 0.1	0.3	0.4	0.7
IRF3	14.8 ± 0.1	15.2 ± 0.2	15.0 ± 0.2	15.0 ± 0.1	15.6 ± 0.2	17.7 ± 1.5	0.03	0.02	0.049
Interferon signalling									
IRF2	17.4 ± 0.1	17.5 ± 0.2	17.5 ± 0.2	17.4 ± 0.1	17.5 ± 0.1	17.6 ± 0.1	0.7	1	0.9
IRF9	16.4 ± 0.1	16.7 ± 0.2	16.8 ± 0.2	16.6 ± 0.1	18.2 ± 0.3	19.5 ± 0.1	<0.0001	<0.0001	<0.0001
IFITM3	19.0 ± 0.1	19.3 ± 0.2	19.3 ± 0.2	18.9 ± 0.2	20.2 ± 0.3	22.1 ± 0.0	<0.0001	<0.0001	<0.0001

(TGFB1), TGF-β2 (TGFB2), IL-10 and suppressor of cytokine signalling (SOCS3) increased in the endometrium from Day 12 to Day 18 (day, $P < 0.0001$, $P < 0.0001$, $P = 0.0006$, $P = 0.04$, $P < 0.0001$, $P = 0.004$ and $P = 0.002$, respectively; Table 2). Pregnancy status-dependent differences were found for the anti-inflammatory cytokines TGFB1 and TGFB2 in addition to IL-10 (status, $P = 0.01$, $P = 0.005$ and $P = 0.02$, respectively). The expression of TGFB1 was reduced 3.6-fold in the endometrium of Day 15 control animals compared with non-pregnant controls, but increased again on Day 18. However, TGFB2 expression on Day 18 was 1.9-fold lower in the endometrium of pregnant animals. In contrast, the expression of the anti-inflammatory cytokine IL-10 was augmented in the endometrium of pregnant animals on Day 18 (9.9-fold). The day × status interaction was significant for TGFB1 and IL-10 (day × status, $P = 0.04$ and $P = 0.001$, respectively). The expression of early activation marker CD69 and the natural killer (NK) cell marker natural cytotoxicity triggering receptor 1 (NCR1, CD335) increased significantly over the time points analysed (day, $P = 0.01$ and $P = 0.006$, respectively; Table 2). The transcript abundance of both the γ- and δ-subunits of the T cell receptor (*CD3G* and *CD3D*, respectively) in the endometrium increased 2.1- and 2.0-fold, respectively ($P = 0.0001$) from Day 12 to Day 18 in pregnant animals, with respect to the day in

Table 3. Messenger RNA expression of major histocompatibility complex molecules and the Toll-like receptor system in conceptus tissue

Data are presented as the mean ± s.e.m. ΔCq. Normalised cycle number (ΔCq) values were subtracted from the arbitrary value 20. ND, non-determinable values; B2M, β_2-microglobulin; MHC, major histocompatibility complex; BoLA, bovine leucocyte antigen; HLA-G, human leucocyte antigen G; TLR, Toll-like receptor; IRF3, interferon regulatory factor 3

	mRNA expression [$\log_2$]		*P* value
	ΔCq Day 15	ΔCq Day 18	
MHC molecules			
B2M	7.4 ± 0.3	4.8 ± 0.3	0.008
BoLA (sequence similarity to HLA-G)	10.0 ± 0.6	9.4 ± 0.6	0.5
MHC Class II	9.4 ± 0.5	8.3 ± 0.1	0.1
TLR system			
TLR2	5.1 ± 0.1	ND	0.004
TLR3	4.1 ± 0.3	4.9 ± 0.5	0.3
TLR4	6.4 ± 0.6	7.2 ± 0.5	0.4
TLR7	2.4 ± 0.9	3.9 ± 0.5	0.3
TLR8	5.3 ± 0.7	4.2 ± 0	0.5
MYD88	15.8 ± 0.3	16.5 ± 0.2	0.1
IRF3	14.5 ± 0.2	13.7 ± 0.2	0.01

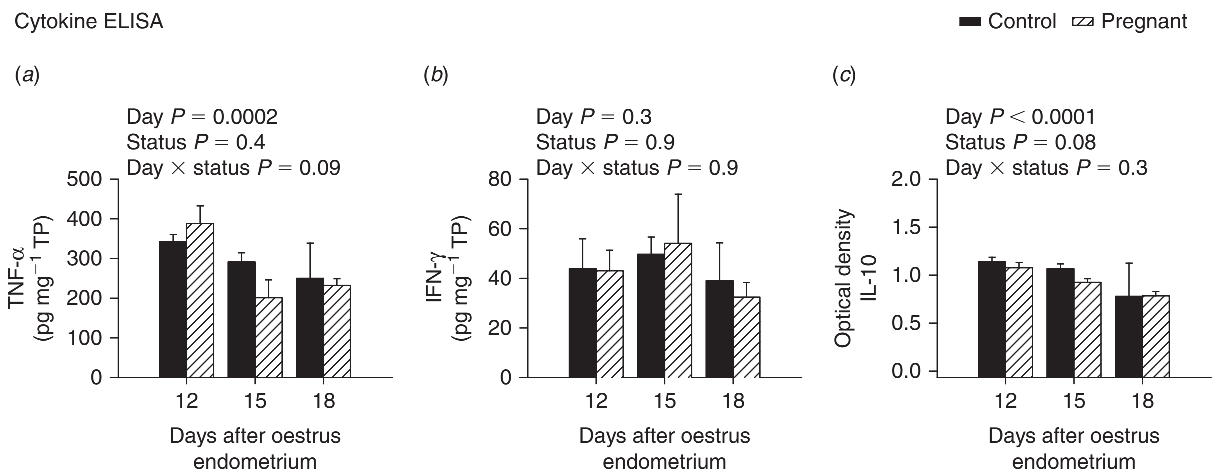

Fig. 2. Pro- and anti-inflammatory cytokines in endometrial tissue homogenates from cyclic and pregnant cows for: (*a*) tumour necrosis factor (TNF)-α; (*b*) interferon (IFN)-γ; and (*c*) interleukin (IL)-10.

pregnant animals. The expression of CD86, which is located on the surface of antigen-presenting cells (APC), increased with time in both pregnant and control groups (day, $P = 0.01$). Moreover, the day × status interaction was significant for CD86 expression ($P = 0.01$). With the exception of IRF2, the transcript levels of the genes related to IFN signalling, namely IRF9 and IFN-induced transmembrane protein-3 (IFITM3), increased significantly (day, $P < 0.0001$; status, $P < 0.0001$; day × status, $P < 0.0001$) in the endometrium of pregnant animals.

Gene expression of TLR

Transcripts for TLR2, TLR3, TLR4, TLR7 and TLR8 all increased significantly over time (day, $P = 0.0005$, $P = 0.002$, $P = 0.01$, $P = 0.007$ and $P = 0.01$, respectively; Table 2). The transcript abundance of TLR recognising nucleic acids (i.e. TLR3, TLR7 and TLR8) was higher in control, but not in pregnant, animals on Day 15 after insemination and therefore exhibited a significant interaction (day × status, $P = 0.02$, $P = 0.02$ and $P = 0.005$, respectively). Expression of TLR2 transcripts in conceptuses decreased and was below the detection limit on Day 18. The abundance of IRF3 transcripts was significantly increased in the endometrium of pregnant animals (day, $P = 0.03$; status, $P = 0.02$; day × status, $P = 0.049$), but mRNA expression in conceptuses decreased 1.7-fold from Day 15 to Day 18 ($P = 0.01$; Table 3).

Protein expression of cytokines

The protein concentration of the proinflammatory cytokine TNF-α exhibited a day-dependent variation ($P = 0.0002$), but did not differ with pregnancy status (Fig. 2*a*). The concentration of the Th1-specific cytokine IFN-γ did not change with day or status ($P > 0.05$; Fig. 2*b*). Interestingly, in contrast to IL-10 mRNA expression in the endometrium, IL-10 protein declined over the time points analysed (day, $P < 0.0001$) and there was a

Table 4. Messenger RNA expression of indoleamine 2,3-dioxygenase and interleukin-10 in interferon-τ-treated and -untreated glandular and stroma cells in an *in vitro* coculture system

Transcript expression is shown as the mean ± s.e.m. ΔCq ± s.e.m. Normalised cycle number (ΔCq) values were subtracted from the arbitrary value 20. The relative increase of transcript abundance following interferon (IFN)-τ treatment is indicated. IDO, indoleamine 2,3-dioxygenase; IL-10, interleukin-10

Cell type	$\log_2$ mRNA expression		Fold increase with IFN-τ treatment	*P* value
	ΔCq control	ΔCq IFN-τ treatment		
IDO				
Glandular epithelial cells	13.3 ± 0.5	20.9 ± 0.9	194	<0.001
Stroma cells	14.5 ± 0.5	25.9 ± 0.4	2702	<0.001
IL-10				
Glandular epithelial cells	16.0 ± 0.9	17.8 ± 0.7	3.4	0.1
Stroma cells	16.1 ± 0.9	18.5 ± 1.0	4.3	0.1

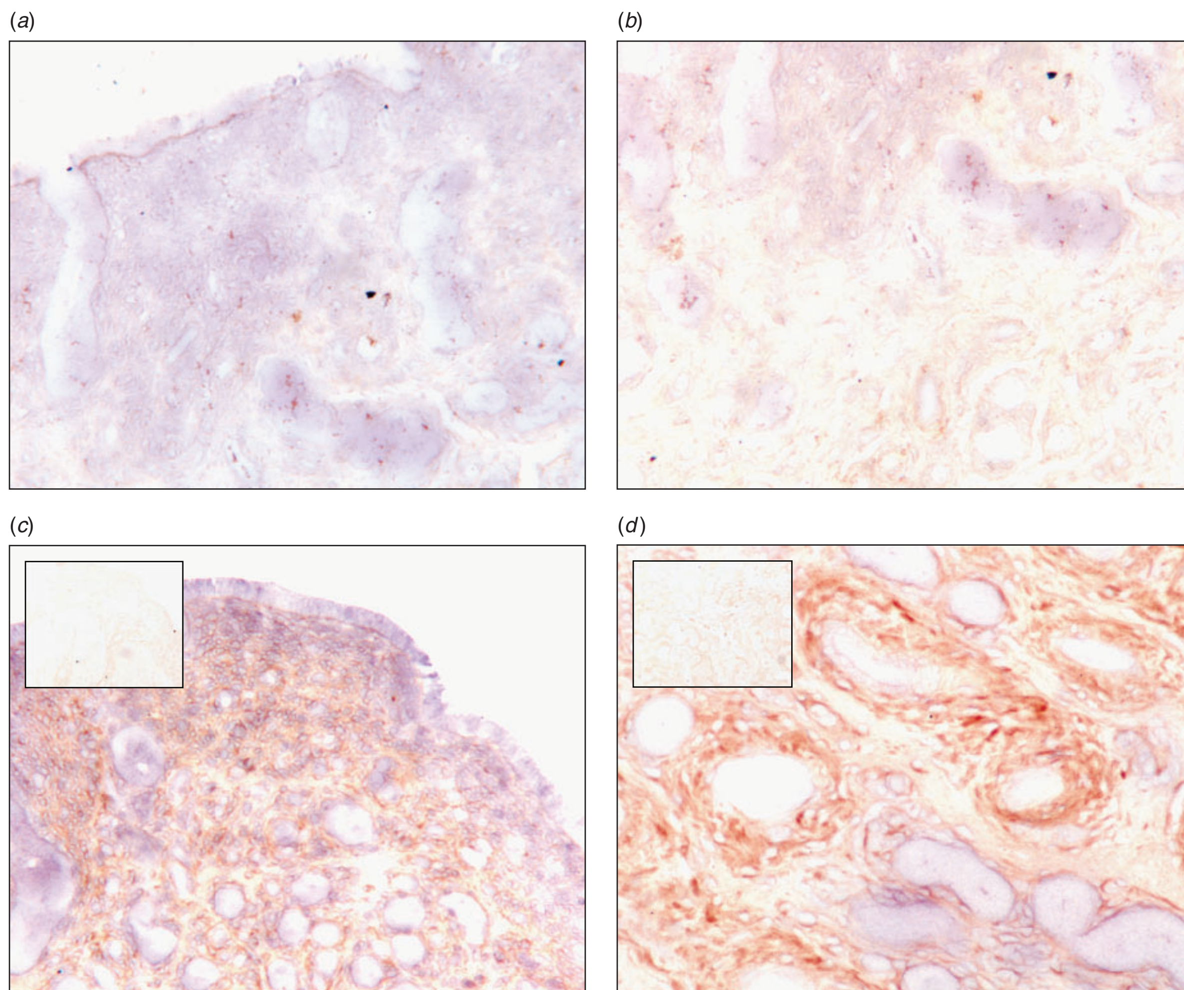

Fig. 3. *In situ* localisation of indoleamine 2,3-dioxygenase (*IDO*) mRNA in the endometrium of non-pregnant and early pregnant cows. Cross-sections of the endometrial tissue were hybridised with biotinylated antisense *IDO* probes and corresponding antisense control (inserts). Endometrial sections from the (*a*) zona functionalis on Day 18 of the cycle, (*b*) zona basalis on Day 18 of the oestrous cycle, (*c*) zona functionalis on Day 18 of pregnancy and (*d*) zona basalis on Day 18 of pregnancy are shown.

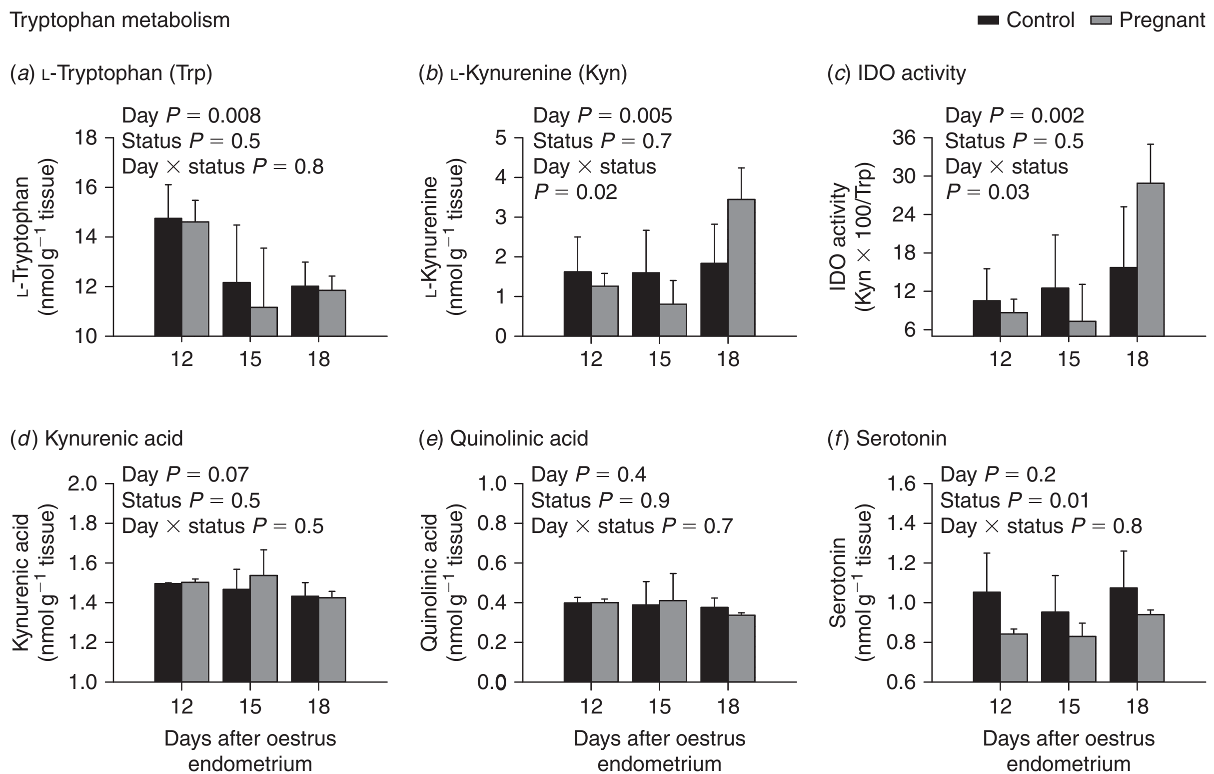

Fig. 4. Metabolites of the kynurenine pathway were analysed using tandem mass spectrometry in the endometrium of cyclic and pregnant animals. (*a*) L-Tryptophan, (*b*) L-kynurenine, (*c*) estimated indoleamine 2,3-dioxygenase (IDO) activity (Kyn × 100/Trp), (*d*) kynurenic acid, (*e*) quinolinic acid and (*f*) serotonin.

tendency for decreased endometrial expression in pregnant *v.* non-pregnant animals (status, $P = 0.08$; Fig. 2*c*).

Gene expression of IDO *and* IL-10 *in an* in vitro *coculture system of glandular and stroma cells*

Following IFN-τ treatment, IDO mRNA expression increased significantly in glandular and foremost in stroma cells compared with untreated cells (194-fold and 2702-fold, respectively; $P < 0.0001$). In contrast, there were no differences in IL-10 expression between IFN-τ-treated and untreated cells (Table 4).

Localisation of IDO mRNA by in situ *hybridisation*

The IDO mRNA transcripts were distributed in stroma cells, with gradually more intensive staining in the zona basalis (Fig. 3). In particular, intense staining was observed in stroma cells surrounding the glandular ducts. Luminal or glandular epithelia were devoid of IDO mRNA. regardless of pregnancy status or the day of the cycle. The staining in the endometrium of gravid animals (Fig. 3*c*, *d*) was more intense than in non-pregnant animals (Fig. 3*a*, *b*).

Tryptophan metabolism

Although levels of Trp, a substrate for IDO, decreased over the time points analysed in the endometrium of control and pregnant animals (day, $P = 0.008$; Fig. 4*a*), levels of the product L-kynurenine (Kyn) increased from Day 12 to Day 18 (day, $P = 0.005$), in particular in the endometrium of Day 18 pregnant animals (day × status, $P = 0.02$; Fig. 4*b*). IDO activity, estimated as the ratio of Kyn × 100/Trp, increased with day (day, $P = 0.002$) and was greatest in the endometrium of Day 18 pregnant animals (day × status, $P = 0.03$; Fig. 4*c*). Levels of the metabolites kynurenic acid (Fig. 4*d*) and quinolinic acid (Fig. 4*e*), which are formed downstream of the pathway, did not change with the day of the cycle or pregnancy status. Concentrations of serotonin, an alternative product of Trp catabolism, were decreased in the endometrium of pregnant cattle at all time points investigated (status, $P = 0.01$; Fig. 4*f*).

CD45-positive immune cells in the endometrium of cyclic and pregnant animals

In the stroma of the zona basalis of Day 18 animals, the number of leucocytes decreased significantly as a result of pregnancy (31 ± 5 *v.* 6.5 ± 2 cells/mm^2 in the basal endometrium of control and pregnancy animals, respectively; Fig. 5*b*, *c*; day, $P = 0.08$; status, $P = 0.03$; day × status, $P = 0.02$; Table 5). No significant differences were found in the number of leucocytes within the zona functionalis among any of the control or pregnant groups (Fig. 5). Lymph nodes, used as a positive control, exhibited intense CD45-positive staining cells (Fig. 5*a*).

Discussion

Several studies have investigated endometrial gene expression using microarray technology during the peri-implantation

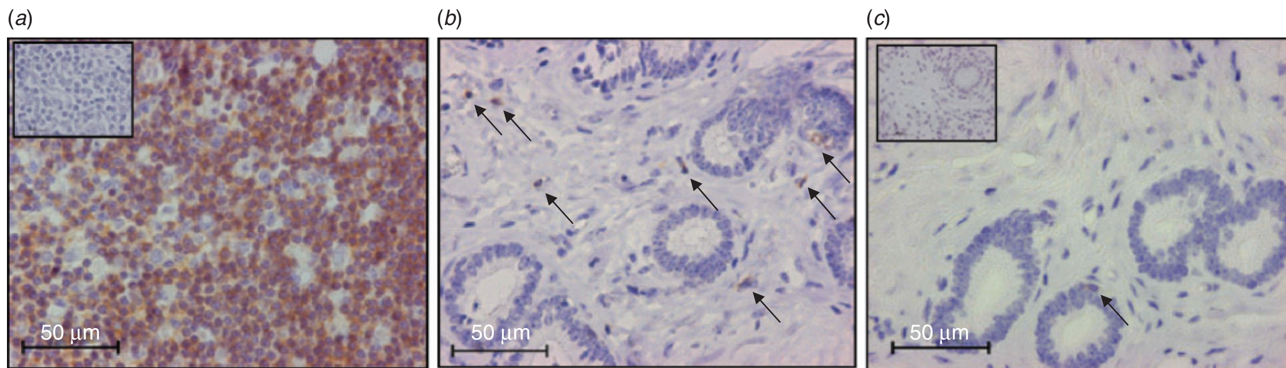

Fig. 5. Immunohistochemical localisation of leucocytes in the endometrium of non-pregnant and early pregnant animals. Light microscopy revealed CD45-positive cells (arrows) in the (*a*) lymph nodes (positive control), as well as in the zona basalis of Day 18 non-pregnant (*b*) and pregnant animals (*c*). Inserts, negative controls incubated without primary antibody.

Table 5. Cell counts of CD45-positive leucocytes in endometrial epithelia and stroma of non-pregnant and early pregnant cattle
Cells were counted in the luminal and glandular epithelia (cells/mm) in addition to the stroma (cells/mm^2) by distinguishing leucocytes in the zona functionalis and those in the zona basalis. Day, day of oestrous cycle; status, pregnancy status

Day of cycle	Treatment	CD45-positive leucocytes in the endometrium				
		Luminal epithelium (cells/mm)	Glandular epithelium (cells/mm)		Stroma (cells/mm^2)	
			Zona functionalis	Zona basalis	Zona functionalis	Zona basalis
12	Control	6.8 ± 1.8	6.2 ± 1.4	13.4 ± 2.7	23.1 ± 4.2	9.2 ± 3.2
	Pregnant	8.3 ± 2.3	4.9 ± 1.6	11.4 ± 3.8	37.0 ± 8.5	7.2 ± 2.5
15	Control	7.7 ± 2.7	3.6 ± 1.2	13.0 ± 3.0	36.1 ± 19.1	15.1 ± 6.2
	Pregnant	8.5 ± 2.7	4.5 ± 1.5	11.3 ± 2.7	51.4 ± 18.2	15.6 ± 4.7
18	Control	8.4 ± 1.5	7.0 ± 1.4	9.5 ± 3.6	63.9 ± 19.0	31.0 ± 4.5
	Pregnant	6.0 ± 1.6	4.0 ± 1.8	6.1 ± 2.8	27.4 ± 14.7	6.5 ± 2.4
Statistics						
Day effect (*P* value)		>0.05	>0.05	>0.05	>0.05	0.08
Status effect (*P* value)		>0.05	>0.05	>0.05	>0.05	0.03
Day × status interaction (*P* value)		>0.05	>0.05	>0.05	>0.05	0.02

period in the bovine (Bauersachs *et al*. 2006; Klein *et al*. 2006) and specific attention needs to be drawn to the in-depth characterisation of the candidate genes and mechanism of gene regulation discovered. The present study provides a detailed insight into the tolerance mechanisms during the preimplantation period in cattle.

It is know that IDO is present in the placenta and endometrial glandular epithelium of mice and primates (rhesus monkey and common marmoset; Drenzek *et al*. 2008; Jeddi-Tehrani *et al*. 2009), where it has a role in generating an immune-suppressive environment. Owing to deleterious T cell responses, suppression of IDO leads to fetal rejection of allogenic, but not syngenic, murine embryos (von Rango *et al*. 2007).

Herein, we provide evidence that the increase in IDO mRNA expression in the bovine endometrium due to trophoblast-derived IFN-τ is accompanied by elevated enzyme activity in pregnant animals. Coculture of endometrial cells revealed an induction of IDO primarily in stroma cells, which was confirmed by *in situ* hybridisation of *ex vivo*-derived samples, in which IDO was localised to the deeper stroma cells surrounding the glandular ducts. Type I IFNs are generally involved in viral defence. Interestingly, IFN-τ has evolved as a pregnancy-recognition signal in ruminants, possibly through duplication of IFN-ω 36 million years ago (for a review, see Roberts *et al*. 1998). Genes typically upregulated in response to pathogen infection are commonly expressed with IFN-τ (e.g. 2′-5′-oligoadenylate synthetase, MX protein; Bauersachs *et al*. 2006; Klein *et al*. 2006).

The findings of the present study indicate a relevance of IDO activity, as summarised in Fig. 6, especially at the beginning attachment phase in the bovine, a species showing a less invasive synepitheliochorial type of placentation. Serotonin, formed in an alternative Trp-degrading pathway, was found to be reduced in the endometrium of pregnant animals, suggesting a particular relevance of the kynurenine pathway in early pregnancy. The activity of potential deleterious leucocytes can either be inhibited indirectly by the generation of a Trp-depleted microenvironment or directly by adverse Trp-derived catabolites, such as L-kynurenine (Della Chiesa *et al*. 2006; Fallarino *et al*. 2006). Thus, both L-kynurenine, which was significantly increased in the endometrium of pregnant animals on Day 18, and increased IDO activity, resulting in local Trp ablation, may

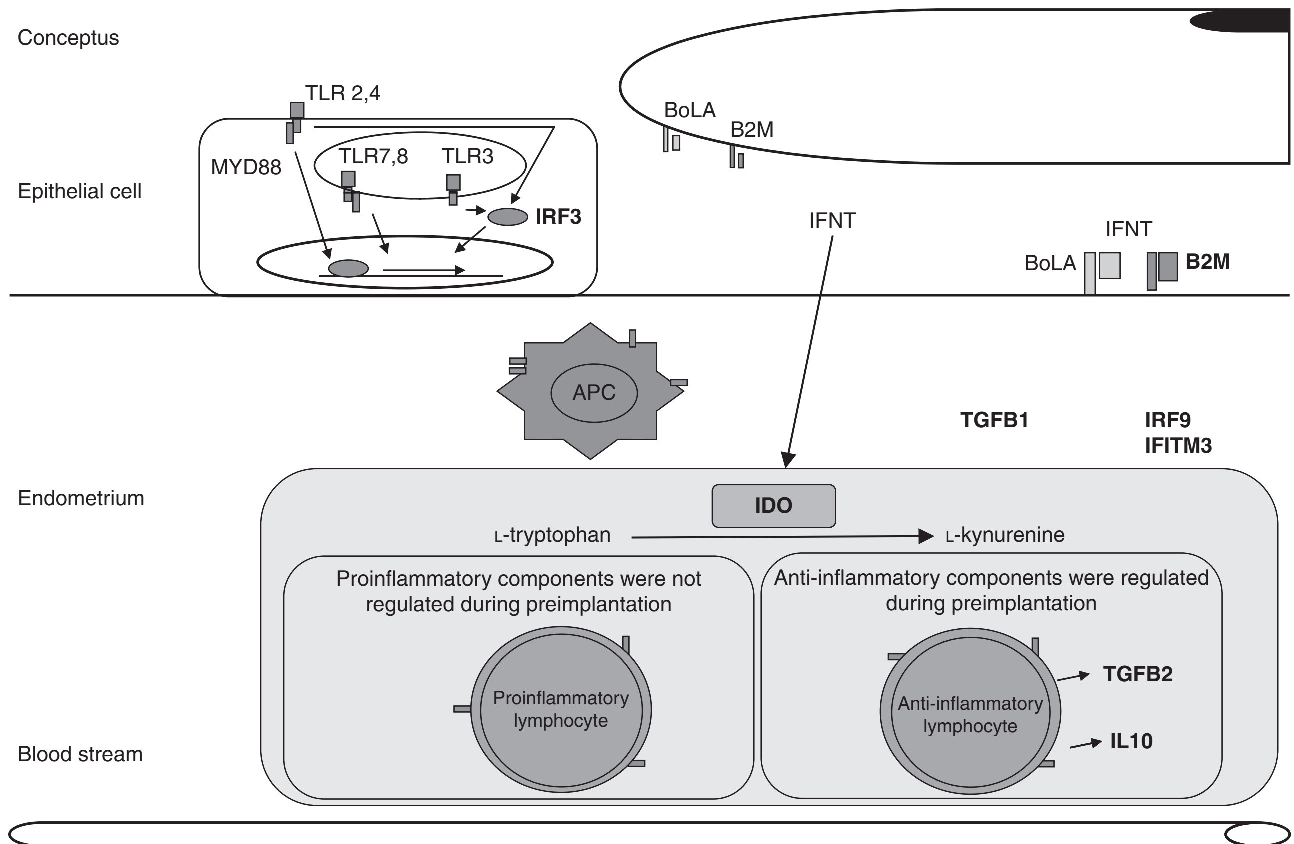

Fig. 6. Local L-tryptophan ablation and formation of L-kynurenine is likely mediated by the initial enzyme of the kynurenine pathway, indoleamine 2,3-dioxygenase (IDO), in the endometrium of pregnant animals in order to avoid detrimental lymphocyte activation towards the semi-allogenic conceptus. Increased expression of IDO due to trophoblast-derived interferon (IFN)-τ resulting in local L-tryptophan deprivation may lead to a lower number of leucocytes in the zona basalis necessary for trophoblast attachment. The diagram presents the presumed effect of IDO on diverse subsets of endometrial leucocytes, such as antigen-presenting cells (APC), as well as lymphocyte populations. We did not find any regulation of proinflammatory components analysed (data not shown), but anti-inflammatory cytokines, such as transforming growth factor-β1/2 (TGFB1/2) and interleukin (IL)-10, were significantly regulated during pregnancy. Further components of the Toll-like receptor (TLR) system and major histocompatibility complex (MHC) Class I molecules were investigated. Immunological components, such as β_2-microglobulin (B2M), interferon regulatory factor (IRF) 3, IRF9 and interferon-induced transmembrane protein-3 (IFITM3), shown in bold, represent cytokines or immunological components regulated by pregnancy status.

have reduced the number of leucocytes in the zona basalis of pregnant animals on Day 18. To our knowledge, this is the first report indicating that IDO expression may not be an evolutionary remnant due to IFN-τ secretion by the trophoblast but, rather, may contribute to immunological tolerance of the semi-allogenic bovine embryo.

In the present study, the expression of mRNAs for MHC molecules in conceptuses was low. It has been shown that bovine trophoblasts express MHC molecules in the interplacentomal region during the second half of pregnancy and it has been hypothesised that the expression of MHC molecules on trophoblasts may initiate a destructive, although beneficial, immune response at term (Davies *et al*. 2000). Potentially, the expression of MHC molecules in peri-implantation conceptuses may contribute to a limited immune reaction controlling trophoblast invasion (von Rango 2008).

We did not detect increased expression of either proinflammatory cytokines or cell surface markers due to pregnancy for any lymphocyte subpopulation analysed. Most immunological factors analysed in the present study in addition to TNF (Groebner *et al*. 2010) exhibited increased expression with respect to the day of the cycle when the endometrium is controlled by P_4, possibly in order to prepare the secretory endometrium for a potential pregnancy. In many cases, this effect may be amplified during pregnancy by signals from the embryo (Gao *et al*. 2009).

We assume that, to a great extent, anti-inflammatory effects of P_4 may contribute to embryo tolerance (Lewis 2003), because only anti-inflammatory cytokines were regulated in pregnancy. Tolerance mechanisms may be a prerequisite by the time the semi-allogenic embryo starts to attach to the maternal tissue and gain importance during the ongoing implantation, as well as placentation (Zenclussen *et al*. 2007). Fluctuations in intra-epithelial lymphocytes have been reported during mid- and late pregnancy in the ovine endometrium (Lee *et al*. 1992; Meeusen *et al*. 1993, 2001), but we did not detect major spatial or temporal changes in immune cell quantity in the zona functionalis during the preimplantation period. Our results are in

accordance with other reports with respect to not finding any changes in the immune cell populations analysed (e.g. B and T lymphocytes, macrophages) before the apposition phase in cattle (Vander Wielen and King 1984; Leung *et al*. 2000). During the subsequent attachment phase, leucocyte numbers declined in the LE of pregnant animals (Vander Wielen and King 1984), indicating a reduced migration of maternal lymphocytes towards the placental interface. In general, total leucocyte cell counts in our analysis were fairly low compared with those reported in a previous study (Leung *et al*. 2000), but correlated well with leucocyte numbers in the LE reported elsewhere (Vander Wielen and King 1984). Due to the cryofixation, the structure of the tissue section is less intact when compared to formalin-fixed paraffin embedded tissue sections possibly resulting in a higher number of false-positive cells. However, in formalin-fixed paraffin-embedded tissue (Vander Wielen and King 1984) antigen retrieval more likely may result in false-negative cell recognition. Because we counted leucocytes in a minimum area of 5 mm^2 in the case of stroma cells and over a $\geq$5 mm length in the case of LE in at least four animals per group, our data are reliable and highly reproducible with respect to treatment.

The TGFB family represents a group of pleiotropic cytokines that control cell migration, differentiation and growth, as well as regulation of the extracellular matrix. Growth- and differentiation-promoting effects are mainly attributed to TGFB1, whereas TGFB2 is an anti-inflammatory cytokine. In the present study, the expression of TGFB1 was decreased in Day 15 non-pregnant control animals, whereas the transcript abundance was remained unchanged in pregnant animals. However, the expression of TGFB2 decreased significantly over time, particular in pregnant animals. In contrast with these findings, a significantly lower transcript abundance for TGFB1, but not TGFB2, at the beginning adhesion phase has been described in the ewe (Dore Jr *et al*. 1996). Our data indicate that although TGFB transcript abundance was decreased in pregnant animals, the expression of the anti-inflammatory cytokine *IL-10* was significantly higher in the endometrium of pregnant animals on Day 18, which was not caused by IFN-τ, as demonstrated at the protein level and *in vitro*. In early caprine pregnancy, elevated *IL-10* expression is hypothesised to contribute to an environment innocuous for the developing embryo (Imakawa *et al*. 2005). Our ELISA results do not point towards a specific relevance for anti-inflammatory IL-10 during preimplantation in the bovine, although IL-10 may be necessary at later stages when the contact between mother and conceptus is more intimate.

Expression analysis of the APC surface markers CD14, lymphocyte antigen-75 (Ly75, Dec205) and MHC Class II revealed neither status- nor day-dependent regulation, but for CD86 we found expression differences with respect to the day and to the day × status interaction. Moreover, TLRs, which also participate in antigen recognition, were found to exhibit day-dependent increased expression. In the bovine endometrium, TLR1, TLR7 and TLR9 are located in epithelial cells, whereas TLR1, TLR4, TLR6, TLR7, TLR9 and TLR10 have been demonstrated in stroma cells (Davies *et al*. 2008). Interestingly, the transcript abundance of TLRs recognising nucleic acids was increased significantly in the endometrium of non-pregnant animals on Day 15, correlating with increased endometrial prostaglandin pulses (Schallenberger *et al*. 1989). Thus, in the bovine, augmented expression of TLR is possibly induced by prostaglandins, as has been already demonstrated in mice (Yoon *et al*. 2008).

The present study provides novel findings concerning the regulation of pro- and anti-inflammatory cytokine expression in addition to lymphocyte and APC markers in the bovine endometrium during the preimplantation period. Proinflammatory cytokines were not augmented close to the beginning of trophoblast attachment in pregnant animals. Because the activity of deleterious T cells is highly dependent on Trp availability, the distinct increase in transcripts and elevated enzymatic activity of the Trp-degrading enzyme IDO at the beginning implantation may indicate a particular relevance for the tolerance of the semi-allogenic conceptus at the embryo–maternal interface. As placentation progresses, the risk of non-self recognition may increase concomitantly. Thus, beyond placentation, further tolerance mechanisms, especially in the caruncular area, are likely to enable pregnancy maintenance.

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REPRODUCTION
RESEARCH

Increase of essential amino acids in the bovine uterine lumen during preimplantation development

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Abstract

Amino acids (AAs) are crucial for the developing conceptus prior to implantation. To provide insights into the requirements of the bovine embryo, we determined the AA composition of the uterine fluid. At days 12, 15, and 18 post-estrus, the uteri of synchronized pregnant and non-pregnant Simmental heifers were flushed for the analysis of 41 AAs and their derivatives by liquid chromatography–tandem mass spectrometry. The ipsilateral endometrium was sampled for quantitative PCR. In addition to a pregnancy-dependent increase of the essential AAs ($P<0.01$), we detected elevated concentrations for most non-essential proteinogenic AAs. Histidine (His) and the expression of the His/peptide transporter solute carrier 15A3 (*SLC15A3*) were significantly increased at day 18 of pregnancy *in vivo*. In addition, *SLC15A3* was predominantly stimulated by trophoblast-derived interferon-τ in stroma cells of an *in vitro* co-culture model of endometrial cells. Our results show an increased concentration of AAs most likely to optimally provide the elongating pre-attachment conceptus with nutrients.

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Introduction

Amino acids (AAs) are components of enzymes, cytokines, and several hormones that further contribute to multiple cellular and metabolic functions (Supplementary Table 1, see section on supplementary data given at the end of this article). They further play important roles in catalytic functions, synthesis of purines, and pyrimidines as well as organic osmolytes. Mammals rely on essential AAs for proper growth and development, but even semi-essential AAs such as L-arginine (Arg), L-histidine (His), and L-tyrosine (Tyr) may become limited during a number of developmental stages such as during rapid growth and development or stressful conditions. Various transporter systems that carry cationic, anionic, aromatic, or aliphatic AAs with different affinities for their substrates and with overlapping substrate specificity have been identified as AA delivery systems (reviewed in Van Winkle (2001)) and allow selective accumulation of nutrients and further components in the uterine lumen as shown previously for cycling and early pregnant ewes (Gao *et al*. 2009*a*, 2009*b*).

Cattle exhibit a late implantation starting with the initial apposition at day 17 post-insemination (King *et al*. 1981). During the prolonged preimplantation phase, the growing conceptus is solely dependent on the nutrient supply from secretions of the uterine glands and selective transport of nutrients into the uterine lumen. A specific temporal supply with essential and non-essential AAs is vital to promote blastocyst formation and hatching both *in vivo* and *in vitro* (Liu & Foote 1995, Partridge & Leese 1996, Steeves & Gardner 1999). Depletion or an insufficient supply of essential as well as non-essential AAs resulted in altered development of bovine embryos *in vitro* (Steeves & Gardner 1999). For instance, a reduction of L-methionine (Met) decreased intracellular concentrations of glutathione in *in vitro* produced bovine embryos, although this did not affect apoptosis, methylation status, or cleavage rate (Bonilla *et al*. 2010). Whereas non-essential AAs seem to be crucial for cleavage of the zygote, blastocoel formation, and blastocyst hatching, essential AAs are particularly important for the complex development of the inner cell mass of murine embryos (Lane & Gardner 1997).

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In ruminants, the developing trophoblast undergoes enormous growth prior to tight attachment to the maternal endometrium with the transport of nutrients governed by the endometrium into the uterine lumen. The expression of AA transporter systems in the trophoectoderm and primitive endoderm of the conceptus permits the AA uptake from the uterine lumen and adequate proliferation and differentiation (Gao *et al.* 2009*a*, 2009*b*). In cattle, the composition of AAs in the uterine fluid during the secretory phase has recently been investigated (Hugentobler *et al.* 2007); however, physiological concentrations of AAs during the bovine preimplantation phase have not been determined yet. Thus, we analyzed whether the presence of the rapidly elongating bovine conceptus would lead to a modulation of the composition of intrauterine AAs prior to implantation. To inquire the participation of endometrial transport mechanisms, the expression levels of AA transporters and metabolic enzymes were additionally investigated.

Results

Total protein (TP) content (Fig. 1A) and total free AAs (Fig. 1B; calculated by the sum of all measured AAs) varied in uterine flushings of pregnant and non-pregnant heifers. The overall abundance of TP was affected by the status ($P=0.04$) as well as by the day of the cycle ($P=0.04$) and declined from days 15 to 18 in pregnant and non-pregnant animals. Overall total AA amounts were affected by the day of the cycle ($P=0.03$) and were greatest at days 12 and 15 and declined thereafter from days 15 to 18. The amounts of TP and total AAs significantly correlated (Fig. 1C; $r=0.82$, $P<0.0001$).

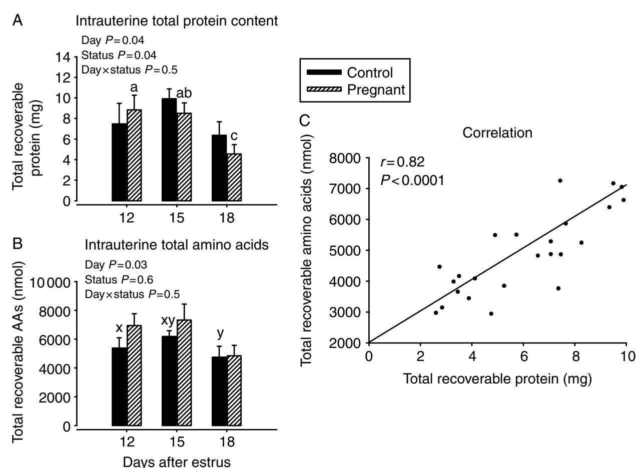

Figure 1 Total recoverable intrauterine protein content (mg) (A) and total recoverable AAs (calculated by the sum of individual AAs) (nmol) (B) concomitantly vary in the uterine flushings of pregnant and non-pregnant heifers at days 12, 15, and 18 post-estrus ($n=4$–7 animals per group). AAs were measured by the highly sensitive LC–MS/MS. The correlation of TP over total AAs is depicted in (C). As total protein content correlated well with intrauterine AAs, the respective AA data are presented as nmol AAs related to TP (mean nmol/mg TP±S.E.M.). The effect of the day of the estrous cycle within the pregnancy status is shown by different superscript letters (x, y, and z in cyclic animals and a, b, and c in pregnant animals) and was regarded as significantly different if $P<0.05$. Thus, the same superscript letters indicate that there was no statistical difference between groups. Treatment effects at the respective time points are indicated (*) if $P<0.05$.

Non-essential neutral AAs in the uterine lumen and expression of the respective transporters in the endometrium

The small neutral AA glycine (Gly; Fig. 2A) was most abundant in the uterine lumen (236 nmol/mg TP ±32), followed by taurine (Tau; Fig. 3A; 163 nmol/mg TP ±19), L-serine (Ser; Fig. 2B; 100 nmol/mg TP ±22), and L-glutamine (Gln; Fig. 2E; 85 nmol/mg TP ±11). Pregnant animals had greater levels of Gly at day 15 ($P<0.05$) than the respective controls (Fig. 2A). Ser (Fig. 2B) increased continuously from days 12 to 18 in uterine flushings of the pregnant animals (day $P=0.01$) and were 2.2- and 2.4-fold greater in pregnant than in non-pregnant animals at days 15 and 18 respectively (status $P=0.0008$). For L-proline (Pro; Fig. 2C), greater abundances were detected at days 15 and 18 in pregnant animals (status $P=0.001$). L-alanine (Ala; Fig. 2D) had overall greater abundances in uterine flushings from pregnant than non-pregnant animals (status $P=0.004$). In addition, Ala amounts were influenced by the day of the cycle ($P=0.02$). For both Gln and L-asparagine (Asn; Fig. 2F), the amounts were greater at day 18 in uterine flushings of pregnant animals (Gln: day $P=0.0001$, status $P=0.02$, and Asn: $P=0.002$, status $P=0.02$). The expression of the solute carrier 1A5 (*SLC1A5*), which mainly transports small neutral AAs such as Ala, Ser, and L-cysteine, increased from days 12 to 18 (day $P=0.0001$; Table 1).

Although L-glutamine (Glu; Fig. 2G) and L-aspartate (Asp; Fig. 2H) amounts in the uterine flushings were larger in pregnant animals at day 18 (status $P=0.03$ and $P=0.04$ respectively), similar levels for both the groups were detected at day 15. Moreover, amounts of Asp were affected by the day of the estrous cycle (day $P=0.008$). The acidic AA transporter *SLC1A1* and the glutamic-pyruvate-transaminase (*GPT*; Table 1) mRNA levels were influenced neither by the day of the cycle nor by the pregnancy status in the endometrium. In conceptuses, the *GPT* mRNA levels were not different between days 15 and 18 ($P>0.05$) (mean ΔCq±S.E.M.: 23.0±0.0 and 23.0±0.1 respectively; data not shown). However, *GPT* mRNA was expressed slightly greater in conceptuses (mean Cq 26.0) than in the corresponding endometrium samples (mean Cq 26.8) (data not shown). The cytosolic enzyme glutathione synthetase (GSS) catalyzes the formation of glutathione as a primary intracellular antioxidant. A pregnancy-dependent difference in mRNA expression levels was not observed (Table 1).

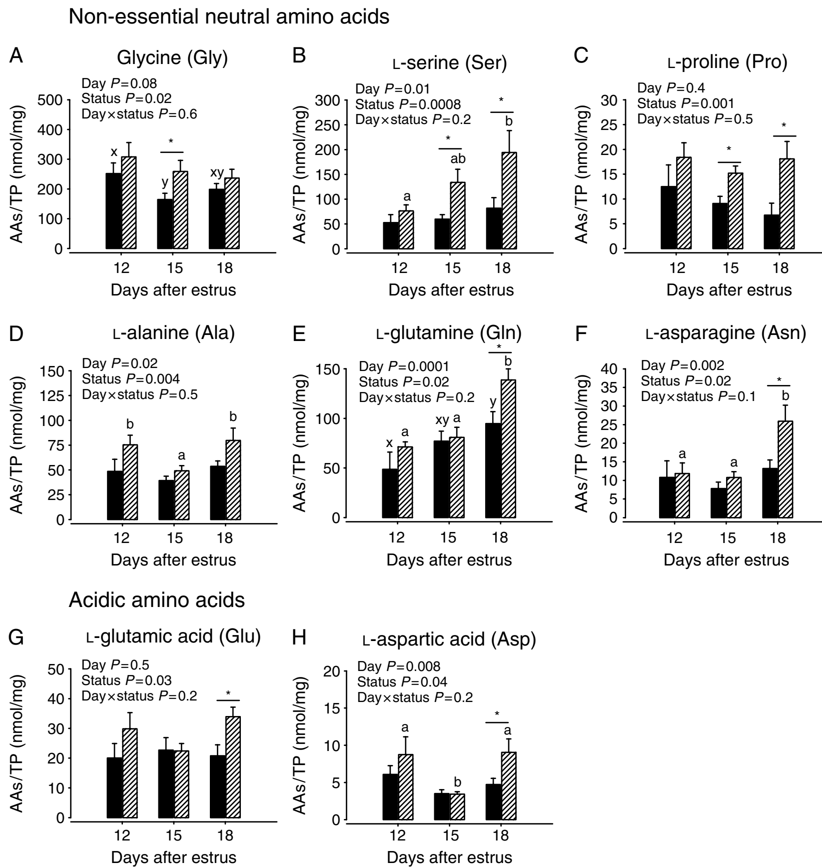

Figure 2 Small neutral and acidic AAs: Gly (A), Ser (B), Pro (C), Ala (D), Gln (E), Asn (F) Glu (G), and Asp (H) in uterine flushings of cycling and pregnant heifers at days 12, 15, and 18 post-estrus are presented as (nmol/mg TP)±S.E.M. The effect of the day of the estrous cycle within the pregnancy status is shown by different superscript letters (x, y, and z in cyclic animals and a, b, and c in pregnant animals) and was regarded as significantly different if $P<0.05$. Treatment effects at the respective time points are indicated (*) if $P<0.05$.

Essential neutral AAs in the uterine lumen and expression of the respective transporters in the endometrium

Although L-threonine (Thr) levels (Fig. 4A) declined in the uterine lumen of non-pregnant animals over time, increasing amounts were detected in pregnant animals, reaching 2.1- and 3.9-fold greater amounts than in non-pregnant animals at days 15 and 18 respectively (status $P<0.0001$, day×status $P=0.001$). In the uterine flushings of non-pregnant animals, the aromatic AAs L-phenylalanine (Phe; Fig. 4B), Tyr (Fig. 4C), and L-tryptophan (Trp; Fig. 4D) remained almost constant over time. The Phe levels increased 3.7-fold in pregnant animals from days 12 to 18 and were 2.1- and 3.8-fold higher in pregnant versus non-pregnant animals at days 15 and 18 respectively (day $P<0.0001$, status $P<0.0001$, day×status $P<0.0001$). The Tyr and Trp amounts also increased in pregnant animals during the preimplantation period (5.3- and 4.0-fold respectively; Tyr: day $P<0.0001$, status $P<0.0001$, day×status $P<0.0001$; and Trp: day $P=0.001$, status $P=0.0007$, day×status $P=0.004$). Whereas Tyr levels were 2.0- and 4.5-fold greater in pregnant versus non-pregnant animals at days 15 and 18, Trp abundances were 2.0- and 2.8-fold at days 15 and 18 in pregnant versus non-pregnant animals. All branched chain AAs (BCAAs) L-leucine (Leu; Fig. 4E), L-valine (Val; Fig. 4F), and L-isoleucine (Ile; Fig. 4G) increased consistently and were 1.8-, 1.9-, and 17.6-fold greater at day 15 and 2.3-, 3.0-, and 46-fold greater in uterine flushings of day 18 in pregnant versus non-pregnant animals. In contrast, the expression of mRNAs for both large neutral AA transporters, *SLC7A5* (Table 1) and *SLC7A8*, were not affected by day, status, or their interaction.

Basic AAs and respective AA derivatives related to the urea cycle

The basic AAs L-lysine (Lys; Fig. 5A) and Arg (Fig. 5C) were more abundant in uterine flushings from pregnant heifers. At day 18, the Lys and Arg levels were increased 5.8- and 13.5-fold, respectively, compared with day 12 due to effects of day (Lys $P<0.0001$, Arg $P<0.0001$), status (Lys $P=0.002$, Arg $P=0.01$), and their interaction (Lys $P=0.002$, Arg $P=0.02$). His levels (Fig. 5B) were not affected by day in cyclic heifers, but increased 4.8-fold between days 12 and 18 of pregnancy and were 1.7- and 3.9-fold greater in uterine flushings of pregnant versus non-pregnant animals at days 15 and 18 (day $P<0.0001$, status $P<0.0001$, day×status, $P=0.0001$). Ornithine (Orn) resulting from the metabolism of Arg by arginase was not affected by day or status (data not shown). However, L-citrulline (Cit; Fig. 5D) formed from Orn

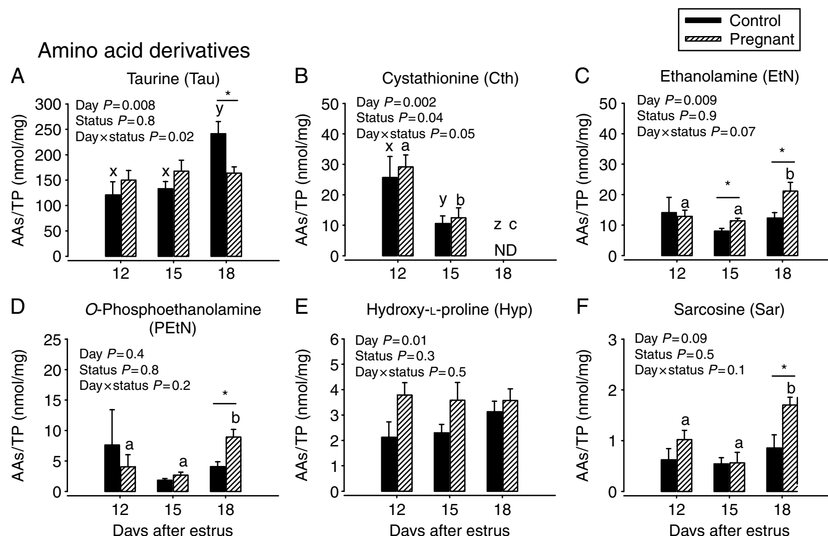

Figure 3 AA derivatives in uterine flushings of pregnant and non-pregnant heifers at days 12, 15, and 18 post-estrus. Amounts of Tau (A), Cth (B), EtN (C), PEtN (D), Hyp (E), and Sar (F) are shown as nmol/mg TP±S.E.M. The effect of the day of the estrous cycle within the pregnancy status is shown by different superscript letters (x, y, and z in cyclic animals and a, b, and c in pregnant animals) and was regarded as significantly different if $P<0.05$. Treatment effects at the respective time points are indicated (*) if $P<0.05$.

within the urea cycle was more abundant in uterine flushings from pregnant heifers on day 15 (day $P=0.04$). The abundance of the cationic transporter *SLC7A1* mRNA was not affected by day or status (Table 1). However, expression of the putative His/peptide transporter, *SLC15A3*, increased in pregnant animals from days 12 to 18 and was 5.5-fold greater in pregnant than in cyclic heifers by day 18 (day $P=0.0003$, status $P=0.002$, day×status $P<0.008$; Table 1). Conceptuses showed a similar expression of *SLC15A3* (mean Cq 22.6) compared with the maternal endometrium (mean Cq 22.9), and the transcript abundance did not differ between days 15 and 18 ($P>0.05$; mean ΔCq±S.E.M.: 26.6±0.3 and 26.1±0.1 respectively; data not shown).

Non-proteinogenic AAs in the uterine lumen

In the uterine lumen of non-pregnant animals, Tau (Fig. 3A) increased between days 15 and 18 of the cycle. At day 18, the amount of Tau was 1.5-fold greater in the uterine flushings of non-pregnant versus pregnant animals (day $P=0.008$, day×status $P=0.02$). The expression of the β-AA transporter *SLC6A6* (Table 1) in the endometrium increased in animals between days 12 and 18 (day $P=0.002$). Cystathionine (Cth; Fig. 3B), which is an intermediate product in Met metabolism, decreased in uterine flushings from days 12 to 18 post-estrus in both the treatment groups (day $P=0.002$; Table 1). Ethanolamine (EtN; Fig. 3C) originates from Ser and is a precursor molecule for *O*-phosphoethanolamine (PEtN; Fig. 3D), which is a component of phospholipids in biological membranes. Intrauterine EtN varied with the day of the cycle ($P=0.009$) with 1.4- and 1.7-fold increased amounts in the uterine flushings of pregnant heifers at days 15 and 18. The amount of PEtN was 2.2-fold greater in the uterine flushings of pregnant heifers at day 18. Hydroxy-L-proline (Hyp) amounts were different with the day of the cycle (day $P=0.01$; Fig. 3E). The co-product of Gly synthesis sarcosine (Sar) was 2.0-fold increased in uterine flushings of pregnant heifers ($P<0.05$; Fig. 3F). AA derivatives that were not depicted in Fig. 3: 1-methyl-L-histidine (M1His), 3-methyl-L-histidine (M3His), L-α-amino-*n*-butyric acid (Abu), L-α-aminoadipic acid (Aad), argininosuccinic acid (Asa), β-alanine (bAla), L-carnosine (Car), *O*-phospho-L-serine (PSer), homocitrulline (Hcit), delta-hydroxylysine

Table 1 mRNA expression of amino acid transporter and metabolic enzymes in days 12, 15, and 18 of pregnant and non-pregnant heifers. Data shown represent means ΔCq±S.E.M. Different superscript letters indicate significant differences over time in control and pregnant animals respectively.

Treatment	Day	*SLC1A1*	*SLC1A5*	*SLC6A6*	*SLC7A1*	*SLC7A5*	*SLC7A8*	*SLC15A3*	*GPT*	*GSS*
Control	12	21.7±0.7	17.6 ± 0.5^{x}	17.7 ± 0.6^{x}	21.5±0.2	16.6±0.9	19.3±0.7	20.0±0.5	16.2±0.6	15.4±1.0
	15	22.5±0.4	18.4 ± 0.3^{xy}	19.2 ± 0.3^{y}	21.6±0.2	16.9±0.2	19.6±0.3	20.2±0.2	16.9±0.4	16.9±0.3
	18	22.6±0.4	19.0 ± 0.4^{y}	19.5 ± 0.4^{y}	21.8±0.3	17.3±0.5	19.8±0.3	20.5±0.4	17.0±0.4	16.0±0.4
Pregnant	12	22.1±0.5	17.8 ± 0.3^{a}	18.2 ± 0.3^{a}	21.5 ± 0.2^{a}	16.7±0.7	19.4±0.3	20.0 ± 0.4^{a}	16.5±0.5	16.1±0.6
	15	22.0±0.2	18.8 ± 0.3^{b}	18.9 ± 0.2^{b}	$22.0\pm0.1^{b*}$	16.6±0.3	19.2±0.2	20.7 ± 0.3^{a}	16.7±0.2	17.1±0.3
	18	22.1±0.2	19.7 ± 0.4^{b}	19.6 ± 0.2^{b}	21.9 ± 0.1^{b}	17.7±0.5	19.8±0.2	$23.0\pm0.3^{b*}$	16.8±0.2	17.4±0.4
P value										
Day		0.7	0.001	0.001	0.3	0.2	0.5	0.0003	0.5	0.07
Status		0.7	0.2	0.7	0.3	0.9	0.8	0.002	1	0.1
Day×status		0.6	0.8	0.7	0.7	0.8	0.9	0.008	0.8	0.6

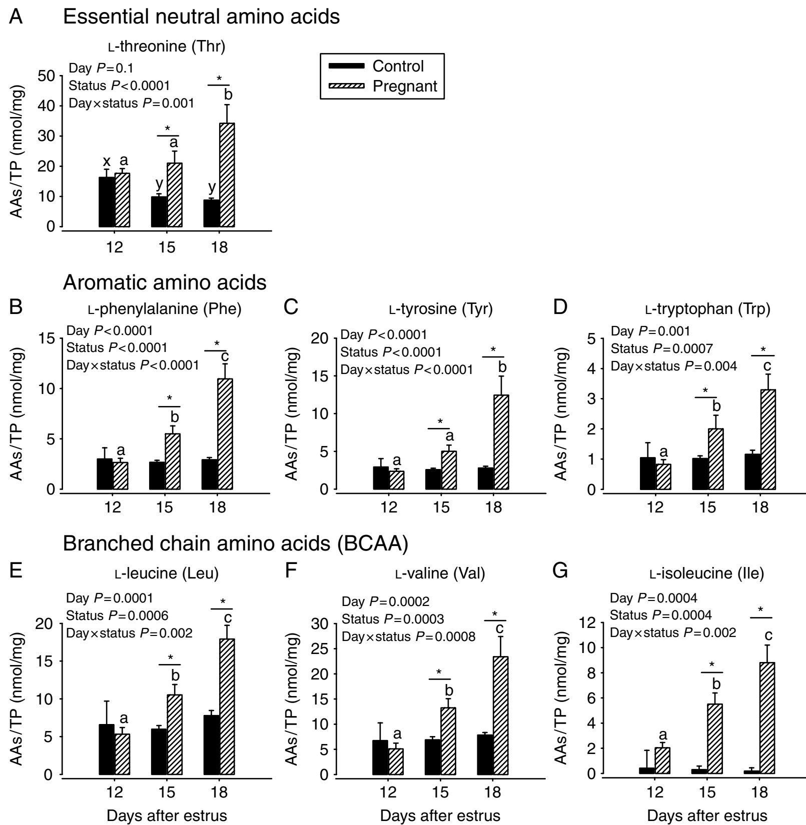

Figure 4 Large neutral AAs in uterine flushings of pregnant and non-pregnant heifers at days 12, 15, and 18 post-estrus. Amounts of AAs Thr (A), Phe (B), Tyr (C), Trp (D), Leu (E), Val (F), and Ile (G) are shown as nmol/mg TP±S.E.M. The effect of the day of the estrous cycle within the pregnancy status is shown by different superscript letters (x, y, and z in cyclic animals and a, b, and c in pregnant animals) and was regarded as significantly different if $P<0.05$. Treatment effects at the respective time points are indicated (*) if $P<0.05$.

(Hyl), γ-amino-*n*-butyric acid, D,L-β-aminoisobutyric acid (bAib), L-anserine (Ans), L-homocysteine (Hcy) and L-cystine (Cys), were not detectable or only detectable at low levels in the assay system.

Gene expression following interferon-τ treatment in an in vitro *co-culture model of endometrial cells*

To determine whether the trophoblast-derived interferon-τ (IFNT) stimulates *SLC15A3* expression as observed *in vivo*, the transcript abundance was determined following recombinant IFNT stimulation within an *in vitro* co-culture model of endometrial glandular and stromal cells. Interestingly, *SLC15A3* mRNA expression was increased in co-cultivated glandular epithelium (36-fold, $P=0.001$) and foremost in co-cultivated stroma cells (177-fold, $P<0.0001$) compared with the respective untreated co-cultured cells (Table 2). The *SLC15A3* mRNA was expressed 6.1-fold greater in stroma cells (mean ΔCq 17.9) than in glandular epithelial cells (mean ΔCq 15.3) following IFNT treatment in the *in vitro* co-culture model.

Discussion

Malnutrition during pregnancy can severely affect embryonic/fetal development and may have negative effects on metabolic imprinting, thereby affecting the susceptibility to chronic diseases during adult life (Waterland & Jirtle 2004). An optimal supply of nutrients is thus critical for the pre-attachment conceptus and successful pregnancy outcomes. This study investigated changes for the first time in profiles of essential and non-essential AAs in bovine uterine flushings during early gravidity prior to conceptus attachment.

In accordance with Schultz *et al.* (1971), TP content varied in the uterine flushings of pregnant and non-pregnant animals and decreased over the analyzed time points. Interestingly, total free AAs varied concomitantly, as total AA variation correlated well with the TP content ($r=0.82$, $P<0.001$). Thus, to circumvent possible inaccuracies resulting from the flushing procedure due to differential consistency of the bovine uterine fluid or due to differential solubility of particular components over the analyzed time points, we normalized the AA data with respect to the respective TP.

Significant variation in abundances of Gly, Ser, Thr, Met, His, Tau, and of the aromatic AAs Phe and Tyr in addition to BCAAs Val, Ile, and Leu has been demonstrated in uterine fluids of cycling cows, comparing days 6, 8, and 14 after estrous (Hugentobler *et al.* 2007). In comparison, our data show that the differences on days 12, 15, and 18 of the estrous cycle were less than the more remarkable increases in these AAs in the uterine lumen of pregnant heifers. In concordance with recent results from studies with sheep (Hugentobler *et al.* 2007,

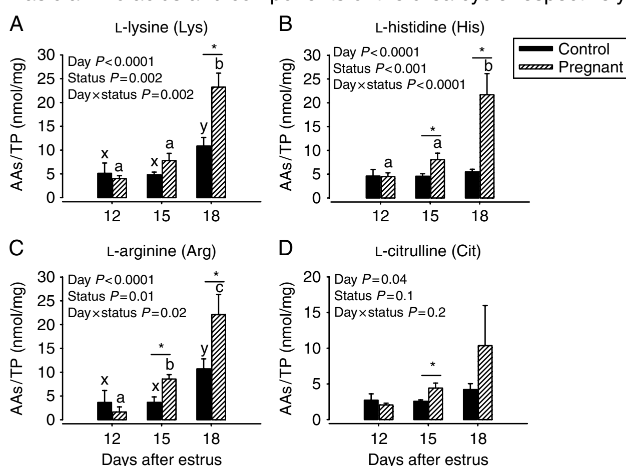

Figure 5 Basic AAs and components of the urea cycle in bovine uterine flushings are shown. L-lysine (A), His (B), Arg (C), and Cit (D) in uterine flushings of cycling and pregnant heifers at days 12, 15, and 18 post-estrus are presented as nmol/mg TP±S.E.M. The effect of the day of the estrous cycle within the pregnancy status is shown by different superscript letters (x, y, and z in cyclic animals and a, b, and c in pregnant animals) and was regarded as significantly different if $P<0.05$. Treatment effects at the respective time points are indicated (*) if $P<0.05$.

Gao *et al*. 2009*e*), Gly was the predominant AA in the uterine lumen. Gly is inter-convertible to Ser and Ala and is furthermore necessary for protein and DNA synthesis. It most prominently serves as an energy source within the citrate cycle (Hobbs & Kaye 1985). However, in contrast to the ovine uterus (Gao *et al*. 2009*e*), a pregnancy-dependent increase did not occur possibly due to species-specific exigencies of Gly during trophoblast elongation. As ruminants share a number of regulatory pathways but obviously differ in definite components during this important phase, it is of prime importance to understand individual species differences and specific requirements with respect to the AA composition (Spencer *et al*. 2008).

The mammalian target of rapamycin (MTOR) signaling pathway actively regulates cell proliferation and migrations, as well as translation of mRNA to protein. Concentrations of energy substrates and AAs and other components such as progesterone and IFNT affect MTOR signaling (Long *et al*. 2005, Gao *et al*. 2009*d*). Among the BCAAs, leucine is the major AA regulating protein synthesis (Buse & Reid 1975) by affecting phosphorylation of proteins 4E-BP1 and S6K1 that are substrates of MTOR (Anthony *et al*. 2000). We detected elevated concentrations of all BCAAs and aromatic AAs due to pregnancy. These AA might enter the uterine lumen via the sodium-independent L-System transporter (preference for Leu) expressed in the microvillous and basal membrane of the ovine endometrium in order to support the development of the conceptus as shown in sheep (Gao *et al*. 2009*b*). The MTOR signaling influences the activity of members of several transport systems (e.g. System L, A, and Taut), modulates translation, and influences in this manner AA uptake in the placenta (Roos *et al*. 2009). We suggest a regulatory effect of MTOR on AA transport in maternal and conceptus tissues during the preimplantation period. The L-system is regarded as the main route for the transport of BCAAs and aromatic neutral AAs across plasma membranes, each displaying a different affinity (Grillo *et al*. 2008). The L-system transporters are heterodimeric and covalently associated with the glycoprotein 4F2hc/CD98. They act as obligatory exchangers and induce an overall change in relative composition of distinct AAs. An accumulation of distinct AAs is achieved by unidirectional transport systems (Verrey 2003). For both transporters analyzed in this study (*SLC7A5*: LAT1, and *SLC7A8*: LAT2), neither day nor status significantly affected expression levels. *SLC7A5*, located in the epithelia and stroma cells in sheep endometrium, exceeded the expression of *SLC7A8* of pregnant ewes (Gao *et al*. 2009*b*); however, in the endometrium of cattle, *SLC7A8* revealed a more pronounced expression without exhibiting a pregnancy-dependent regulation. Generally, *SLC7A8* is abundantly located at the basal membrane of epithelia and acts in concert with cationic transporters to mediate the reabsorption of cationic AAs, whereas *SLC7A5* is mainly expressed in apical membranes of epithelia. Possibly, reabsorption of cationic AAs might play a more prominent role in cattle than in the ewe prior to implantation (Gao *et al*. 2009*b*).

During early pregnancy, we found an increased presence of almost all AAs, foremost the essential AAs in the uterine lumen (Fig. 6). These essential AAs within the uterine lumen originate from blood and are transported through the vascular wall and the endometrial tissue to nourish the fast growing conceptus. We assume that the developing conceptus induces the increase in AA transport processes via several signaling molecules, primarily and most likely through the effects of progesterone and IFNT (Farin *et al*. 1990, Godkin *et al*.

Table 2 mRNA expression of interferon-τ (IFNT)-treated and -untreated co-cultured bovine glandular and stroma cells analyzed separately are depicted. mRNA expression of *SLC15A3* is shown as means ΔCq±S.E.M. The relative increase of transcript abundance following IFNT treatment is depicted.

Cell type	mRNA expression (log_2)			
	ΔCq mean ±S.E.M. control	ΔCq mean ±S.E.M. IFNT treatment	Fold increase IFNT treatment	*P* value
SLC15A3 expression *in vitro* in a co-culture model of glandular epithelial and stromal cells				
Glandular epithelial cells	15.3±0.8	20.0±0.8	36	0.001
Stroma cells	17.9±0.4	25.0±0.4	177	<0.0001

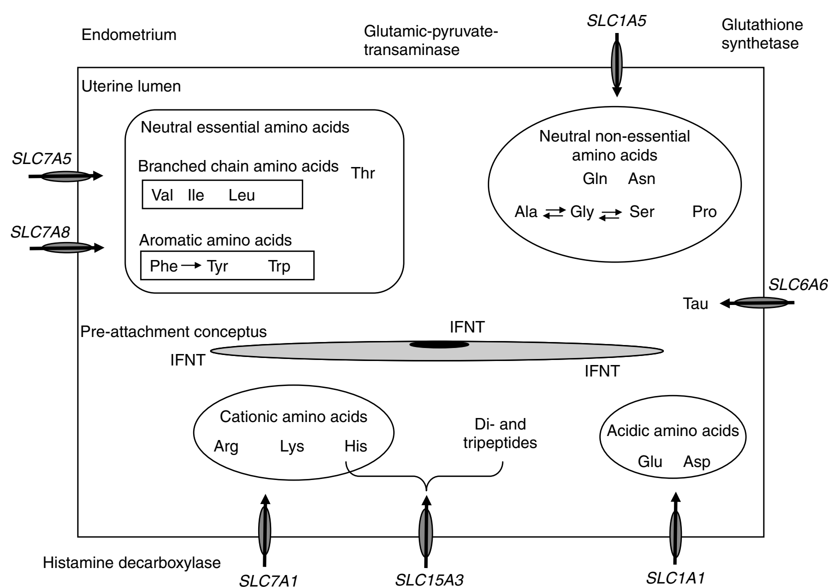

Figure 6 Schematic picture of amino acids (AAs) and AA transporters probably involved in embryo nutrition during the preimplantation phase in the bovine uterus. Amino acids, respective endometrial AA transporters (solute carrier, SLC), as well as metabolic enzymes are indicated in the uterine lumen and endometrium at day 18 of pregnancy. Components showing augmented concentration during pregnancy are presented in bold. Primarily essential AAs increased in the uterine lumen of pregnant animals pointing to a precise adjustment of the supply for developing conceptus prior to implantation.

1997). *SLC15A3* (*PHT2*) is located in lysosomal membranes in which it mediates the electrogenic co-transport of short chain peptides in addition to free His out of lysosomes (Daniel & Kottra 2004). In cooperation with other cationic transporters located at membranes directed toward the uterine lumen, His might be transported into the uterine fluid. Increased *SLC15A3* expression has already been demonstrated in the endometrium of day 18 pregnant cows (Klein *et al.* 2006). We affirmed that IFNT increased the expression mainly in stroma cells *in vitro*, which may relate to the elevated His concentrations found *in vivo*. As histidine decarboxylase was not expressed at detectable levels in endometria of any group (data not shown), we presume that His is not involved in histamine biosynthesis, but rather contributes to protein synthesis for the elongating conceptus. Ongoing studies will provide evidence as to whether other conceptus-derived signaling molecules affect enrichment of AAs in the lumen.

AA transport occurs via a variety of rheogenic co-transporters and exchangers with overlapping substrate specificity and differences in the affinity of their substrates. Although some transport systems are mainly expressed at basolateral membranes to perform the transport from the maternal blood (System L: SLC7A8) into the cell, others are rather located at the apical side (System ASC: SLC1A5; Castagna *et al.* 1997). The majority of transporters analyzed in this study revealed no pregnancy-dependent regulation. For *SLC1A5* and *SLC6A6* transcripts, we observed an increase over time, possibly induced by progesterone exposure as previously demonstrated in the ewe for *SLC1A5* (Gao *et al.* 2009*b*). Blood flow increases from days 14 to 18 in the uterine artery supplying the gravid uterine horn with sufficient nutrients (Ford *et al.* 1979, Silva & Ginther 2010). This may enable an accumulation of nutrients even in the absence of increases in expression of genes for most solute carriers.

Cationic AAs utilize distinct carrier systems (y^+, y^+L, $b^{(0+)}$, N) for transport. Na^+-independent transport of cationic AAs (Arg, Lys, Orn, and protonated His) by the y^+ transport system occurs with high affinity and low capacity in various tissues (MacLeod 1996). Endometrial *SLC7A1* transcript analysis failed to detect an effect of pregnancy, but it may become a subject of regulation following implantation as demonstrated for the ewe (Gao *et al.* 2009*a*). Arg represents a semi-essential AA since it is formed via the urea cycle to a certain extent, although the requirements must be complemented through dietary supplements. The gaseous signaling molecule, nitric oxide (NO), arises from Arg and is the major component contributing to vasodilatation. It is further hypothesized to play a role in implantation events by regulating blood flow, tissue remodeling, and immune suppression in species exhibiting invasive placentation (Purcell *et al.* 2009). In addition, polyamines are synthesized from Arg via Orn and control DNA as well as protein synthesis. They are key regulators of angiogenesis that stimulate vascular functions, which has been shown during the first half of pregnancy in the ewe (Kwon *et al.* 2003). Inhibition of polyamine synthesis is involved in the development of intrauterine growth restriction in rat (Ishida *et al.* 2002). In this study, Arg increased in the uterine lumen of early gravid cows and exceeded Orn, but as demonstrated earlier (Gao *et al.* 2009*c*), NOS amounts declined. Thus, as shown in the ewe, our results suggest an intensified requirement for polyamines during the preimplantation period in cattle (Kwon *et al.* 2004).

Cth abundance declined significantly in both cyclic and pregnant heifers to undetectable levels on day 18. An increase in Tau in the uterine fluid was observed at day 18 only in cycling heifers. Tau is a metabolic product of sulfurous AAs. Although it is not a component of proteins, it fulfills many regulatory functions such as osmoregulation, membrane stabilization, antioxidation, and modulation of Ca^{2+}-flux. In the uterine fluid, concentration of Tau is fairly high in contrast to the bovine blood plasma or oviduct fluid (Hugentobler *et al*. 2007). Lowered Tau amounts in rat uterine epithelial cells were confirmed during the estrous phase as well as during pregnancy and epithelial cells contain large amounts of Tau at diestrus (Lobo *et al*. 2001). We demonstrated an increase in the expression of transcripts for AA transporter for β-AA (which include Tau and bAla) *SLC6A6* (*TAUT*) over time, which could account for elevated Tau in the uterine lumen. However, the transcript abundance did not differ from the endometrium of pregnant animals. Thus, mechanisms to inhibit Tau uptake into the uterine lumen must be implicit. The recession of Tau in the uterine luminal fluid during pregnancy might be a consequence from a loss of osmotic force to antagonize the effects of steroid hormones that encourage water retention (Phoenix & Wray 1994).

In animal husbandry, metabolic imbalances may not only negatively affect health and productivity of dairy cattle during early lactation but also alter the nutrient composition of the uterine fluid. In this study, we present a comprehensive and quantitative analysis of the physiological AA composition in the uterine lumen of early pregnant cattle. These results are critical to our understanding of specific requirements of the bovine pre-attachment conceptus during this critical period. Most notably, all essential AAs accumulated with advancing stages of pregnancy when the trophoblast undergoes continuous elongation. Apart from the His/peptide transporter *SLC15A3*, increasing amounts of AAs in the uterine fluid were not accompanied by increases in the expression of transcripts for specific AA transporters in endometria of pregnant heifers. This may indicate a relevance for an adjusted blood flow, allowing an adequate delivery of nutrients. The results of this study provide the basis for further studies to determine whether local deprivation or imbalances in nutrients occurs with possible negative consequences on the development of bovine conceptuses during the pre- and peri-implantation periods of pregnancy.

Materials and Methods

Pretreatment of animals

All experiments were performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as promulgated by the Society for the Study of Reproduction and with the European Convention on Animal Experimentation. Cyclic Simmental heifers (*Bos taurus*, Deutsches Fleckvieh) were synchronized to estrus by injecting i.m. 500 μg of a single dose of the prostaglandin $F_{2\alpha}$-analog cloprostenol (Estrumate; Essex Tierarznei, Munich, Germany) at diestrus as described previously (Ulbrich *et al*. 2009*b*). Pregnant groups were inseminated after estrus detection, whereas the cyclic control groups received supernatant of centrifuged sperm from the same bull. Blood samples were taken to determine serum progesterone by RIA (Prakash *et al*. 1987). All animals had concentration of progesterone >6 ng/ml at the time of slaughter, thus assuring that luteolysis had not commenced. At day 12, 15, or 18 post-insemination, animals were slaughtered (n=4–7 per group); the uterus was removed and flushed with 100 ml PBS (pH 7.4) for the recovery of proteins, AAs, and other components in uterine secretions, as well as conceptuses in pregnant heifers. Animals from the pregnant group were included in the study only if a conceptus was present. The flushing fluid was centrifuged at 800 ***g*** for 10 min and the supernatant was stored at −20 °C until further usage (Ulbrich *et al*. 2009*b*). Intercaruncular endometrium from the middle part of the ipsilateral uterine horn was sampled for gene expression analysis as described previously (Bauersachs *et al*. 2005).

Analysis of AAs and TP in the uterine lumen

In 40 μl of uterine flushing fluids, 41 AAs and their derivatives (Supplementary Table 1) were labeled by the isobaric tagging for relative and absolute quantification methodology using the AA45/32 Starter Kit according to the manufacturer's instructions (Applied Biosystems, Carlsbad, CA, USA) and analyzed via liquid chromatography–tandem mass spectrometry (LC–MS/MS; 3200QTRAP LC/MS/MS, Applied Biosystems) as described previously (Kaspar *et al*. 2009). The data were analyzed using the Analyst 61666; 1.5 Software. TP content in uterine flushing fluids was determined by a conventional bicinchoninic acid assay (Sigma–Aldrich). As TP content and total AA concentration (calculated by the sum of each individually measured AA) correlated well (r=0.82, P<0.0001), data are presented as mean nmol/mg TP±s.e.m.

Endometrial expression of genes for relevant transporters and metabolic enzymes

Total RNA from ipsilateral intercaruncular endometrial samples was isolated using TRIzol reagent (Invitrogen Corporation) according to the manufacturer's instructions. Quality of RNA was monitored by the Agilent 2100 Bioanalyzer (RNA 6000 Nano Assay Kit, Agilent Technologies, Böblingen, Germany). RNA integrity numbers ranged between 7 and 10 (10=intact RNA). The quantitative real-time PCR (qPCR) experiments were performed in accordance with the MIQE guidelines (Bustin *et al*. 2009). Quantitative PCR using the LightCycler DNA Master SYBR Green I protocol (Roche Diagnostics) was performed as described earlier (Ulbrich *et al*. 2009*b*). The sequences of commercially synthesized PCR primer pairs (Eurofins MWG Operon, Ebersberg, Germany), the sequence

accession IDs, as well as product lengths (bp) and the mean Cq-values are provided in Supplementary Table 2, see section on supplementary data given at the end of this article. The cycle number (Cq) to attain a definite fluorescence signal was calculated by the second-derivative maximum method (LC software 4.05), as the Cq is inversely correlated with the logarithm of the initial template concentration. The Cq values from the target genes were normalized against the geometric mean of three reference genes (polyubiquitin 3 (*UBQ3*), H3 histone (*H3F3A*), and 18S ribosomal RNA (*18S* rRNA)) according to the bestkeeper method (Pfaffl *et al*. 2004). In order to avoid negative digits while allowing an estimation of a relative comparison between two genes, data are presented as means ± s.e.m. subtracted from the arbitrary value 30 (ΔCq). Thus, a high ΔCq implies high transcript abundance. An increase of one ΔCq represents a twofold increase of mRNA transcripts.

Statistical analysis

For statistical analysis, the SAS program package release 9.1.3 (2002; SAS Institute, Inc., Cary, NC, 75 USA) was used. The data from uterine flushing fluids and endometria from cyclic and pregnant uteri were compared using the least-square ANOVA general linear models procedure to determine the effects of the day (day) of the estrous cycle and pregnancy, pregnancy status (cyclic or pregnant; status), and day by status interaction. The differences between the days (within cyclic and pregnant animals respectively) are shown by using different superscript letters (x, y, and z in cyclic animals and a, b, and c in pregnant animals, whereby same superscript letters indicate that there is no statistical difference between groups) and treatment effects at the respective significant time points are marked with asterisks (*). Values of $P<0.05$ were regarded as significantly different. Graphs were plotted using Sigma-Plot 8.0 (SPSS, Inc., Chicago, IL, USA).

In vitro *co-culture of glandular and stroma cells*

To test the effect of IFNT on glandular epithelial and stroma cells *in vitro*, co-culture experiments of these cell types were performed as described earlier (Ulbrich *et al*. 2009*a*). Approximately 10^6 stromal cells were seeded at the bottom of a 6-well cell culture plate (Nunclon Δ Surface, Nunc, Wiesbaden, Germany) and 10^6 glandular epithelial cells in culture inserts (Anopore membrane, 0.2 µm, Nunc) freshly coated with Growth Factor Reduced Matrigel (BD Biosciences, San Jose, CA, USA). Cells were cultured in 400 µl medium (DMEM/F12 with 10% FCS) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Immunocytochemistry using specific antibodies against cytokeratin (for glandular epithelial cells) and vimentin (for the stromal cells) showed that the purity of each cell population was >90% (data not shown). When the cells reached 90% confluence, they were cultured serum free for 24 h after which the IFNT stimulation of the cells was performed. A native phenotype of *in vitro* co-cultivated cells populations was previously confirmed by transmission electron microscopy (Ulbrich *et al*. 2010). Epithelial cells showed distinct characteristics, including microvilli on the apical surface and interdigitations in the lateral part between cell as well as desmosomes and tight junctions. Glandular epithelial and stroma cells in co-culture were stimulated with recombinant bovine IFNT (antiviral activity, 4.8×10^3 U/ml medium; PBL Biomedical Laboratories, Piscataway, NJ, USA) for 4 h. The medium with the respective diluent served as a control. Following stimulation, the medium supernatant in the wells and inserts was discarded and the glandular epithelial cells were washed with PBS. TRIzol (500 µl; Invitrogen) was added, shortly incubated, and mixed vigorously using a pipette. The cell lysates were kept at −80 °C until processing. RNA extraction and qPCR experiments were carried out as detailed for the endometrial samples.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-10-0533.

Declaration 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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Supplementary table 1

Forty-one proteinogenic and non-proteinogenic AAs were measured in bovine uterine flushings of Days 12, 15 and 18 of the estrous cyclus and pregnancy, respectively. Abbreviations used in the text are indicated in addition to the measurement range [µmol/L].

PROTEINOGENIC AMINO ACIDS

amino acid	abbreviation	essential	measurement range [µmol/L]
Small neutral amino acids			
glycine	Gly	no	0.5-5000
L-alanine	Ala	no	1-10000
L-glutamine	Gln	no	0.5-5000
L-asparagine	Asn	no	5-10000
L-proline	Pro	no	5-10000
L-serine	Ser	no	0.5-5000
Large neutral amino acids			
L-threonine	Thr	yes	1-5000
branched chain amino acids			
L-leucine	Leu	yes	0.5-5000
L-isoleucine	Ile	yes	0.5-10000
L-valine	Val	yes	1-5000
aromatic amino acids			
L-phenylalanine	Phe	yes	0.5-5000
L-tyrosine	Tyr	no*	0.5-10000
L-tryptophan	Trp	yes	0.5-5000
Charged amino acids			
anionic amino acids			
L-aspartic acid	Asp	no	0.5-10000
L-glutamic acid	Glu	no	0.5-5000
cationic amino acids			
L-arginine	Arg	no*	5-5000
L-histidine	His	no*	0.5-5000
L-lysine	Lys	yes	0.5-1000

NON-PROTEINOGENIC AMINO ACIDS

amino acid	abbreviation	measurement range [µmol/L]
Urea cycle		
L-ornithine	Orn	5-10000
L-citrulline	Cit	0.5-10000
argininosuccinic acid	Asa	5-5000
β-amino acids		
taurine	Tau	1-5000
b-alanine	bAla	1-10000
Further amino acids and derivatives		
O-phospho-L-serine	PSer	1-1000
O-phosphoethanolamine	PEtN	0.5-10000
hydroxy-L-proline	Hyp	0.5-10000
ethanolamine	EtN	0.5-10000
sarcosine	Sar	0.5-10000
1-methyl-L-histidine	1MHis	1-5000
3-methyl-L-histidine	3MHis	0.5-10000
homocitrulline	Hcit	5-1000
g-amino-n-butyric acid	GABA	1-10000
D,L-b-aminoisobutyric acid	bAib	0.5-10000
L-a-amino-n-butyric acid	Abu	0.5-10000
L-a-aminoadipic acid	Aad	0.5-5000
L-anserine	Ans	1-10000
L-carnosine	Car	1-1000
d-hydroxylysine	Hyl	1-5000
cystathionine	Cth	10-10000
L-homocysteine	Hcy	50-5000
L-cystine	Cys	10-5000

* Essential at some developmental stages (eg. growth, stress)

Supplementary table 2

Primer sequences used for endometrial and conceptus mRNA expression of AA transporter (solute carrier, SLC) and metabolic enzymes. Alternative names, average Cq-value, length of PCR product [bp] as well as sequence accession ID are indicated.

Transporter/Enzyme (alternative names)	High affine substrate	Primer sequence	Sequence accession ID	mean Cq	Product length [bp]
SLC1A1(EAAT-3)	acidic amino acids (Glu/Asp)	AGT GTC ACT GCC ACC GCC G ACC GGT CCA GGA GCC AGT CA	U72534	21.3	145
SLC1A5 (ASCT-2)	small neutral amino acids (Ala, Ser, Cys)	CTG GGG GCG AGG TTG AGG GT TCC CAC AGG GGC GTA CCA CA	AY039236, BC123803, EF551339	25.0	176
SLC6A6 (TAUT)	β-amino acids (including Tau, bAla)	CCT GGC CTG GCC TTC ATC GC GCA GGT CAA CCA AGG ACG TGA TCT G	AF260239	24.6	121
SLC7A1 (CAT-1)	basic amino acids (Arg, Lys)	AAC CTC GGG TGC CAT TGC CG TGG GCT GCT CGG GCT GGT AT	DQ399522	23.0	143
SLC7A5 (LAT-1)	branched chain, aromatic, large neutral amino acids	CCC GTG TTC GTG GGC CTG TC TGA GGG TAC GGG CGT CAG CA	AF174615, BC126651	26.5	147
SLC7A8 (LAT-2)	branched chain, aromatic, large neutral amino acids	GGC CAC CCG GGT TCA AGA CG AAG CCA GTG CGA TGA GGC CG	EU118967	24.0	167
SLC15A1(PEPT1)	di- and tripeptides	CAT CGC CGA CTC CTG GCT GG ATG GAG AGC GCC ACG TGC AC	AB252574, ENSBTAT00000005822	22.7	162
SLC15A2 (PEPT2)	di- and tripeptides	GCC ATC TCC GAA AAT CTG TGG CTC C GGC TGC TGA AGG CGT GGT ACA	BC122641, ENSBTAT00000001891	25.5	179
SLC15A3 (PHT2)	di- and tripeptides, His	CCC GAC TGT GGC ACC GAC AC GGC GAT GTC CTC TTG CGG GG	BC149465	22.9	104
GPT (glutamic pyruvate transaminase)		TGC CGA GAG CTC GCA GTT CC CGC ACT CGC CCA TGT AGC CC	BC134583	26.8	122
GSS (glutathione synthetase)		CCG CCT CCG TGC CAC CTT T GCC GCT CCA GGG CTT GTA CC	BT020647	27.0	170
HDC (Histidine decarboxylase)		GCT GGG ACT CCC GGA GCA CT GGC ATA GGC CAC AAG CCG GG	BT020686	>35	190

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Original Article

Reduced Amino Acids in the Bovine Uterine Lumen of Cloned versus *In Vitro* Fertilized Pregnancies Prior to Implantation

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Abstract

Fetal overgrowth and placental abnormalities frequently occur in pregnancies following somatic cell nuclear transfer (SCNT). An optimal intrauterine supply of amino acids (AA) is of specific importance for the development of the bovine preimplantation embryo, and a defective regulation of AA supply might contribute to pregnancy failures. Thus, we analyzed 41 AA and derivatives by liquid chromatography-tandem mass spectrometry in uterine flushings of day 18 pregnant heifers carrying *in vitro* fertilized (IVF) or SCNT embryos, which were cultured under identical conditions until transfer to recipients. The concentrations of several AA were reduced in samples from SCNT pregnancies: L-leucine (1.8-fold), L-valine (1.6-fold), L-isoleucine (1.9-fold), L-phenylalanine (1.5-fold), L-glutamic acid (3.9-fold), L-aspartic acid (4.0-fold), L-proline (2.6-fold), L-alanine (2.0-fold), L-arginine (2.5-fold), and L-lysine (1.9-fold). The endometrial transcript abundance for the AA transporter *solute carrier family 7 (amino acid transporter, L-type), member 8 (SLC7A8)* was also 2.4-fold lower in SCNT pregnancies. O-phosphoethanolamine (PetN) was 11-fold ($p = 0.0001$) reduced in the uterine fluid of animals carrying an SCNT conceptus, pointing toward changes of the phospholipid metabolism. We provide evidence for disturbed embryo–maternal interactions in the preimplantation period after transfer of SCNT embryos, which may contribute to developmental abnormalities. These are unlikely related to the major embryonic pregnancy recognition signal interferon-tau, because similar activities were detected in uterine flushings of the SCNT and IVF groups.

Introduction

Since the first cloning of a ruminant from cultured cells in the late nineties of the last century (Campbell et al., 1996; Wilmut et al., 1997), embryos produced by somatic cell nuclear transfer (SCNT) have frequently sustained a number of abnormalities leading to embryonic losses from early embryonic stages. During pregnancy, placental abnormalities occur, comprising hydroallantois and placental dysfunction, enlarged and fewer placentomes, enlarged umbilical vessels, and preterm abortion (Young et al., 1998). Newborns frequently suffer from respiratory failures, reluctance to suckle, and perinatal death (Constant et al., 2006; Young et al., 1998). Manipulations of the early blastocyst and inadequate *in vitro* culture conditions are supposed to cause primarily neonatal oversize. For instance, culture with serum, nonprotein nitrogen diet, asynchronous transfer, but in particular cloning by SCNT, entail the large offspring syndrome (Young et al., 1998) most likely due to aberrations in epigenetic reprogramming of somatic nuclei (Dean et al., 2001; Niemann et al., 2008). Anomalies in endometrial gene expression can be detected as early as day 18 of pregnancy by microarray approaches, suggesting aberrant signaling of the SCNT conceptus in the embryo–maternal interface (Bauersachs et al., 2009).

Bovine trophoblasts initiate the first apposition to the endometrium at day 17 postinsemination (Wathes and Wooding, 1980). During the prolonged preimplantation phase, the "uterine milk" is of importance for the supply of nutrients to permit the tremendous trophoblast elongation prior to attachment to the endometrium and subsequent formation of a placenta. Amino acids (AA) in the uterine fluid are of utmost importance for nutrient supply and protein synthesis

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(Gao et al., 2009c). As components of enzymes, cytokines, and hormones, AA contribute to multiple functions in biosynthesis, metabolism, and development. AA are required for the synthesis of nucleotides and may act as antioxidant agents preventing cellular damage caused by reactive oxygen species. The presence of both essential and nonessential AA is crucial to meet the requirements of a developing bovine blastocyst *in vitro* (Liu and Foote, 1995). Further, the amounts of AA increase during the preimplantation phase in the ewe (Gao et al., 2009c) and the cow *in vivo* (Groebner et al., 2011).

Ruminant preimplantation blastocysts fail to elongate *in vitro.* A lack of knowledge exists regarding the basic needs of a developing blastocyst after day 7. Transporter systems expressed in the membranes of diverse cell types of the ovine endometrium (Gao et al., 2000a, 2009b) mediate the uptake from the maternal blood and the directional transfer through the endometrial tissue into the uterine lumen. The selective accumulation of cationic, anionic, aromatic, or aliphatic AA is tightly regulated by either sodium-dependent or -independent membrane spanning transporter systems with different affinities for their substrates and with overlapping substrate specificity.

As altered trophoblast differentiation has been found in day 17 bovine SCNT conceptuses in addition to abnormal placental development present at early stages postimplantation (Arnold et al., 2006), we hypothesized that abnormalities in bovine cloned pregnancies may originate from disturbed embryo–maternal communication during the preimplantation period (Bauersachs et al., 2009). IVF-derived embryos were used as reference to SCNT conceptuses because both IVF and cloned embryos underwent a comparable *in vitro* cultivation prior to the transfer ($n=2$ per recipient) into the surrogate dam. Pregnancy rates were 77% for the IVF and 59% for the SCNT group. In both groups, about 40% twin pregnancies were obtained. The genetic variability was comparable in both groups as the donor fibroblast cell cultures were generated from different fetuses as previously described (Bauersachs et al., 2009). In the present study, we asked if AA abundances in the uterine lumen differ between pregnant heifers carrying IVF versus SCNT conceptuses. AA concentrations were determined by highly sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS). Furthermore, the mRNA levels of endometrial AA transporters were determined as a first step to clarify if the presence of cloned embryos may affect maternal AA transfer into the uterine lumen.

Material and Methods

Production of IVF and SCNT embryos, embryo transfer, and collection of samples

SCNT and IVF procedures were performed as previously described (Hiendleder et al., 2004). Embryos were cultured under identical conditions to the blastocyst stage (day 7). Briefly, putative embryos were cultured in 400-μL droplets of synthetic oviduct fluid medium supplemented with 5% estrous cow serum, 40 μL/mL of 50×BME Amino Acids Solution (#B6766, Sigma Aldrich, St. Louis, MO), and 10 μL/mL of 100×MEM Nonessential Amino Acids Solution (#M7145, Sigma Aldrich) covered with mineral oil. The culture atmosphere was 5% CO_2, 5% O_2, and 90% N_2 at 39°C and maximum humidity. Two SCNT or IVF blastocysts (grade 1) were transferred per estrus-synchronized Simmental recipient heifer (day 7 of the estrous cycle). Preparation of recipient animals and embryo transfer were done as described by Klein et al. (2006). The recipients ($n=8$ per group) were slaughtered 11 days later, the uteri were recovered, and flushed with 100 mL phosphate-buffered saline (PBS; pH 7.4) for the recovery of uterine fluids and conceptuses (Bauersachs et al., 2005). The flushing fluid was centrifuged at $800 \times g$ for 10 min and the supernatant was stored at −20°C until further usage (Ulbrich et al., 2009). Animals were termed pregnant if filamentous trophoblast tubes and at least 1 embryonic disc were observed. A twin pregnancy was diagnosed if two embryonic discs were detected. Endometrial tissue samples were collected, preserved, and processed for isolation of RNA as described previously (Bauersachs et al., 2009).

Antiviral activity of interferon-tau (IFNT) in uterine flushings

IFN production was measured in uterine flushings by using a bioassay based on the inhibition of the cytopathic effect of vesicular stomatitis virus (Indiana strain) on Madin–Darby bovine kidney (MDBK) cells (1.5×10^6 cells/mL) (Rubinstein et al., 1981; Stojkovic et al., 1999). The NIH recombinant human IFN-alpha2 preparation (No. Gxa 01-901-535, NIH-Research Reference Reagent Note No. 31, 1984) was included for standardization in each assay. The standard dilution series covered a range from 1.7×10^2 pg/mL to 6.6×10^{-1} pg/mL IFN-alpha2 with a 50% inhibition of the cytopathic effects at a concentration of 2.4 pg/mL IFN-alpha2. The antiviral activity was shown to be mediated by IFNT, as the effects of supernatant and an appropriate control IFNT preparation were blocked by specific anti-IFN sera (kindly provided by Dr. R.M. Roberts, University of Missouri, Columbia, MO) (Klemann et al., 1990). Recombinant bovine IFNT (PBL Biomedical Laboratories, Surrey, BC, Canada) inhibited the cytophathic effects to 50% at a concentration of 0.44 U/mL (9.4 pg/mL).

Analysis of AA in the uterine lumen

In 40 μL of uterine flushings 41 AA and derivatives were labeled by the isobaric tagging for relative and absolute quantification (iTRAQ) methodology using the AA45/32 Starter Kit according to the manufacturer's instructions (Applied Biosystems, Carlsbad, CA) and examined via LC-MS/MS (3200QTRAP LC/MS/MS, Applied Biosystems) as previously described (Kaspar et al., 2009). The data were analyzed using the Analyst®61666; 1.5 Software. Total protein (TP) content in uterine flushings was determined by a commercially available Bicinchoninic Acid Assay (Sigma-Aldrich). To circumvent discrepancies with flushing procedures, data are shown as mean nmol AA/mg TP ± SEM.

Reverse transcriptase-quantitative PCR (RT-qPCR)

Total RNA from endometrial intercaruncular tissue samples was isolated using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA) according to manufacturer's instructions. Quality of RNA was determined by the Agilent 2100 Bioanalyzer (Eukaryote total RNA Nano Assay, Agilent Technology, Palo Alto, CA). RNA integrity numbers were between 7 and 10. Quantity was spectroscopically determined at 260 nm by the Nanodrop 1000 ND-1000 (peqLab

Biotechnologie GmbH). A two-step real-time RT-qPCR was conducted as described recently (Ulbrich et al., 2009). The RT-qPCR experiments were performed in agreement with the MIQE guidelines (Bustin et al., 2009). One microgram of total RNA was reverse transcribed in a volume of 60 μL by using random hexamer primers. Quantitative PCR reactions using the LightCycler DNA Master SYBR Green I protocol (Roche Diagnostics, Indianapolis, IN) were carried out. The primers listed in Table 1 were used to amplify specific fragments referring to the selected transcripts. The cycle number required to achieve a definite SYBR green fluorescence signal (Cq) was calculated by the second derivative maximum method (LightCycler software version 4.05, Roche). The Cq is correlated inversely with the logarithm of the initial template. Because polyubiquitine was not differentially expressed between the experimental groups, the transcript abundances of target genes were normalized against this reference gene. To avoid negative values while allowing an evaluation of a relative comparison between two genes, data are presented as means ± SEM subtracted from the arbitrary value 30 (ΔCq). Thus, a high ΔCq resembles high gene expression and an increase of one ΔCq corresponds to a twofold increase of mRNA transcripts.

Statistical analysis

AA concentration and AA transporter expression data were statistically evaluated using the general linear models (GLM) procedure (SAS program package release 9.1.3; SAS Institute, Inc., Cary, NC). The model included the Group (IVF vs. SCNT) as fixed effect and IFNT concentration in the flushing medium as covariate. The effect of Group was considered significant at $p < 0.05$. Graphs were plotted using Sigma-Plot 8.0 (SPSS Inc., Chicago, IL).

Results

Antiviral activity of IFNT in uterine flushings of heifers carrying in vitro *fertilized versus cloned conceptuses*

The IFNT bioactivity was similar in the uterine flushings of both groups and corresponded to $1.1 \times 10^4 \pm 0.2 \times 10^4$ and $1.5 \times 10^4 \pm 0.3 \times 10^4$ U/mL in IVF and SCNT pregnancies, respectively ($p > 0.05$).

Neutral nonessential amino acids in the uterine fluid

Glycine (Gly) was the most abundant AA in uterine flushings of both groups with 232.3 versus 371.0 nmol Gly/mg TP in the uterine flushings of animals carrying SCNT versus IVF-derived conceptuses (group $p = 0.01$) (Table 2). Luminal L-serine (Ser) was not different between the groups ($p > 0.05$). The concentration of L-proline (Pro) was 2.6-fold less abundant in the uterine lumen of heifers pregnant with a cloned conceptuses (26.2 vs. 66.8 nmol Pro/mg TP) (group $p = 0.003$). L-alanine (Ala) was 2.0-fold (group $p = 0.005$) reduced in the uterine flushings of animals carrying conceptuses generated by SCNT (Ala: 93.1 vs. 184.9 nmol Ala/mg TP). L-glutamine (Gln) and L-asparagine (Asn) concentrations were 141.0 vs. 283.4 nmol Gln/mg TP (group $p = 0.003$) and 17.1 versus 27.8 nmol Asn/mg TP (group $p = 0.01$) in the uterine flushings of heifers carrying conceptuses generated by SCNT versus IVF.

Table 1. Sequences of Primer Pairs, Corresponding References, as Well as the Resulting Fragment Lengths Are Shown

Transporter/enzyme (alternative names)	*High-affinity substrate*	*Primer sequence*	*Sequence accession ID*	*Product length (bp)*
SLC1A1 (EAAT-3)	acidic amino acids (Glu/Asp)	AGT GTC ACT GCC ACC GCC G ACC GGT CCA GGA GCC AGT CA	U72534	145
SLC1A5 (ASCT-2)	small neutral amino acids (Ala /Ser/Cys)	CTG GGG GCG AGG TTG AGG GT TCC CAC AGG GGC GTA CCA CA	AY039236, BC123803, EF551339	176
SLC6A6 (TAUT)	β-amino acids (Tau/bAla)	CCT GGC CTG GCC TTC ATC GC GCA GGT CAA CCA AGG ACG TGA TCT G	AF260239	121
SLC7A1 (CAT-1)	basic amino acids (Arg/Lys)	AAC CTC GGG TGC CAT TGC CG TGG GCT GCT CGG GCT GGT AT	DQ399522	143
SLC7A5 (LAT-1)	branched chain, aromatic, large neutral amino acids	CCC GTG TTC GTG GGC CTG TC TGA GGG TAC GGG CGT CAG CA	AF174615, BC126651	147
SLC7A8 (LAT-2)	branched chain, aromatic, large neutral amino acids	GGC CAC CCG GGT TCA AGA CG AAG CCA GTG CGA TGA GGC CG	EU118967	167
SLC15A3 (PHT2)	di- and tripeptides, His	CCC GAC TGT GGC ACC GAC AC GGC GAT GTC CTC TTG CGG GG	BC149465	104

Table 2. Intrauterine Amino Acid (AA) Abundances of Neutral and Acidic and Cationic AA as Well as AA Derivatives Presented as Mean [nmol aa/mg Total Protein (TP)] ± SEM

Amino acid	IVF mean ± SEM (nmol/mg TP)	SCNT mean ± SEM (nmol/mg TP)	Statistical Analysis	
			Group (p-value)	IFNT (p-value)
Neutral non-essential AA				
Gly	371 ± 45.7	232.3 ± 25.3	0.01	0.3
Ser	131.4 ± 26.6	141.9 ± 19.6	1.0	0.07
Pro	66.7 ± 6.5	26.2 ± 5.2	0.003	0.4
Ala	184.9 ± 20.1	93.1 ± 18.3	0.005	0.5
Gln	283.4 ± 35.0	141 ± 23.2	0.003	0.3
Asn	27.8 ± 2.7	17.1 ± 2.6	0.01	0.4
Neutral essential AA				
Thr	48.7 ± 7.2	39.3 ± 8.1	0.2	0.06
Phe	26.8 ± 2.9	17.3 ± 3.2	0.03	0.2
Tyr	21 ± 2.5	16.9 ± 3.5	0.3	0.2
Trp	5.7 ± 0.6	4.7 ± 0.9	0.3	0.1
Leu	41.5 ± 4.3	23.4 ± 4.2	0.007	0.3
Val	49.2 ± 4.5	29.9 ± 4.8	0.008	0.3
Ile	22.8 ± 2.4	11.8 ± 2.0	0.003	0.4
Acidic AA				
Glu	247.9 ± 28.0	62.8 ± 14.8	< 0.0001	0.6
Asp	46 ± 8.2	11.2 ± 2.7	0.007	0.8
Cationic AA				
Lys	77 ± 6.9	40.6 ± 10.5	0.008	0.006
His	31.3 ± 3.5	25.5 ± 4.9	0.2	0.09
Arg	74.2 ± 6.0	30.2 ± 6.2	0.001	0.3
Components of the urea cycle				
Orn	13.1 ± 0.9	7.5 ± 2.0	0.004	0.006
Cit	4.1 ± 0.4	3 ± 0.4	0.1	0.7
Asa	2.4 ± 0.8	nd	nd	nd
AA derivatives				
Tau	79.2 ± 4.4	100 ± 9.2	0.03	0.1
PEtN	62.7 ± 9.7	5.7 ± 1.2	0.0001	0.4
Hyp	4.5 ± 0.4	3.1 ± 0.4	0.02	0.6
Car	1.6 ± 0.2	0.4 ± 0.1	0.04	0.1
M3His	3.3 ± 0.3	1.3 ± 0.2	0.0003	1.0

The statistic model included the Group (IVF vs. SCNT) as fixed effect and IFNT concentration in the uterine flushings as a covariate. The effects were considered significant at $p < 0.05$.

Acidic amino acids in uterine fluid

Both L-glutamic acid (Glu) and L-aspartic acid (Asp) (Table 2) were 3.9-fold (group $p < 0.0001$) and 4.1-fold (group $p = 0.007$) less abundant in the uterine flushing of SCNT pregnancies (Glu: 62.8 vs. 247.9 nmol Glu/mg TP; Asp: 11.2 vs. 46.0 nmol Asp/mg TP).

Neutral essential amino acids in the uterine fluid

The essential AA L-threonine (Thr) (Table 2) did not differ between the two groups (44.0 nmol Thr/mg TP on average). Although the aromatic AA L-phenylalanine (Phe) was lower (group $p = 0.03$) in the uterine flushings of heifers carrying a cloned animal (17.3 vs. 26.8 nmol Phe/mg TP), L-tyrosine (Tyr) and L-tryptophan (Trp) were not different between the groups (Tyr: 19.0 nmol Tyr/mg TP and Trp: 5.2 nmol Trp/mg TP on average). The branched chain AA L-leucine (Leu), L-valine (Val) and L-isoleucine (Ile) were 1.8-fold (23.4 vs. 41.5 nmol Leu/mg TP; group $p = 0.007$), 1.6-fold (29.9 vs. 49.2 nmol Val/mg TP; group $p = 0.008$) and 1.9-fold (11.8 vs. 22.8 nmol Ile/mg TP; $p = 0.003$) less abundant in the uterine fluid derived from SCNT vs. IVF pregnancies.

Cationic amino acids in the uterine fluid

The cationic AA L-lysine (Lys) and L-arginine (Arg) in uterine flushings from SCNT pregnancies were 1.9-fold (40.6 vs. 77.1 nmol Lys/mg TP; group $p = 0.008$, IFNT $p = 0.006$) and 2.5-fold (30.2 vs. 74.2 nmol Arg/mg TP; group $p = 0.001$) less abundant than in flushings from IVF pregnancies (Table 2). In contrast, the concentration of L-histidine (His) did not differ between the two groups (28.4 nmol His/mg TP on average).

Intrauterine components of the urea cycle

L-ornithine (Orn) (Table 2) was 1.8-fold less abundant in the uterine flushings of animals pregnant with a SCNT conceptus (7.5 vs. 13.1 nmol Orn/mg TP, group $p = 0.004$, IFNT $p = 0.006$). However, L-citrulline (Cit) (3.5 nmol Cit/mg TP on average) was not significantly different between the two groups. Argininosuccinic acid (Asa) was only detectable in the uterine lumen of heifers carrying an IVF-derived

conceptus (2.4 nmol Asa/mg TP), but was below the detection limit in SCNT pregnancies.

Further AA derivatives in the uterine fluid

The uterine luminal concentration of the nonproteinogenic sulfonic acid taurine (Tau) was higher in the SCNT than IVF group (100.0 vs. 79.2 nmol Tau/mg TP, group $p=0.03$; Table 2). The strongest reduction in SCNT pregnancies was found for O-phosphoethanolamine (PEtN) (11-fold, 5.7 vs. 62.7 nmol PEtN/mg TP, group $p=0.0001$). Concentrations of hydroxyproline (Hyp) were lower in the SCNT group (3.1 vs. 4.5 nmol Hyp/mg TP on average, group $p=0.02$). L-carnosine (Car) and 3-methyl-L-histidine (M3His) were reduced [0.4 vs. 1.6 nmol Car/mg (group $p=0.04$), and 1.3 vs. 3.2 nmol M3His/mg TP (group $p=0.0003$), respectively] in uterine flushings from SCNT vs. IVF pregnancies. Ethanolamine (EtN average, 22.7 nmol EtN/mg TP), sarcosine (Sar average, 1.4 nmol Sar/mg TP) 1-methyl-L-histidine (M1His average, 3.0 nmol M1His/mg TP), L-alpha-amino-*n*-butyricacid (Abu average, 3.0 nmol Abu/mg TP) L-alpha-aminoadipicacid (Aad average, 6.3 nmol Aad/mg TP) delta-hydroxylysin (Hyl average, 2.2 nmol Hyl/mg TP), cystathione (Cth average, 0.5 nmol Cth/mg TP), and L-cystine (Cys average, 6.3 nmol Cys/mg TP) in the uterine flushings of pregnant heifers at day 18 (data not shown) were not different between the two groups ($p>0.05$).

Gene expression of endometrial AA transporter

The expression of the large neutral AA transporter *SLC7A8* (also known as *LAT2*) was 2.4-fold lower in the endometrium of heifers carrying a cloned conceptus vs. an IVF-derived (mean ΔCq 23.4 vs. 24.6 SCNT; group $p=0.004$). The mRNA expression of further endometrial transporters was not affected by the type of conceptus (Table 3).

Discussion

Inadequate composition of culture media unveil the early environmental sensitivity of *in vitro*-derived blastocysts, frequently leading to placental abnormalities in later stages of pregnancy (Sinclair et al., 1999). Specifically, the overgrowth phenotype and the disproportionate placentomes indicate a severe metabolic issue in clone pregnancies in ruminants. An optimal temporal and spatial supply with AA is essential for the development of the conceptus (Gardner, 1998). During the prolonged ruminant preimplantation phase, AA increase considerably in the ovine (Gao et al., 2009c) and bovine (Groebner et al., 2011) uterine fluid to nourish the rapidly elongating conceptus. Insufficient supply of AA during early stages of ruminant pregnancy might cause or at least contribute to developmental deficiencies.

Alterations in endometrial gene expression during preimplantation demonstrated that the maternal endometrium responds inappropriately in case of inadequate embryonic signaling (Bauersachs et al., 2009; Mansouri-Attia et al., 2009). In the present report we demonstrate for the first time that next to a peculiar endometrial transcriptome, the uterine luminal histotroph encompassing the embryo is altered in SCNT pregnancies as well. The histotroph is particularly important for the preimplantation phase of the embryo, and alterations thereof may imply metabolic challenges affecting the development at later stages of pregnancy and adult life (Waterland and Jirtle, 2004).

The finding of altered AA concentrations in uterine flushings from clone pregnancies, specifically essential AA which cannot be synthesized by the embryo, confirm the importance of the reciprocal embryo–maternal interactions prior to implantation required for an adequate embryonic development. The histotroph consists of endometrial and embryonic secretions as well as maternal transudate, and for many signaling substances, either contribution is difficult to disentangle. But clearly, essential AA must be derived by maternal supply. Thus in our model, inadequate embryonic signaling, likely being caused by epigenetic abnormalities of SCNT embryos (Bauersachs et al., 2009; Dean et al., 2001; Santos et al., 2003; Shi et al., 2003), must have led to the inadequate maternal transport of essential AA into the uterine histotroph, which may in turn impact embryonic development. Interestingly, neither the size of the trophoblast nor the intrauterine bioactivity of IFNT were found to differ between cloned and IVF-derived conceptuses (Stojkovic et al., 1999), which is in line with our observations. A significant effect of intrauterine IFNT concentrations was only found for the cationic AA Lys and Orn. Thus, the differences in intrauterine AA concentrations are mainly independent of the predominant ruminant pregnancy recognition signal IFNT. Further investigation is needed to elucidate other specific signals involved.

Apart from the concentrations of essential AA such as Phe and Arg and branched chain AA, nonessential neutral and acidic AA were additionally reduced in the uterine fluid of SCNT pregnancies. The small neutral AA Pro and Ala were 1.6- to 2.6-fold less abundant in the uterine fluids of heifers carrying an SCNT conceptus, while intrauterine acidic AA Glu and Asp were found 75% less abundant. Enzymatic facilities for the synthesis of nonessential AA are entirely available in mammals, wherefore these AA can be easily converted and therefore serve as additional energy substrate as well as anaplerotic molecules for replenishing intermediates of the citrate cycle, allowing the development of the fast growing conceptus. In particular, the acidic AA Asp can be converted into oxalacetate, whereas Glu enters the citrate cycle upon conversion into alpha-ketogluterate. Nonessential AA such as Ala, and less likely Gln and Arg, are additionally required for the fixation of excessive amounts of ammonium to avoid an accumulation of free ions due to their toxic effects

Table 3. Gene Expression of Endometrial AA Transporter

	mRNA expression (log_2)		*Statistical analysis*	
AA transporter	*IVF mean ΔCq ± SEM*	*SCNT mean ΔCq ± SEM*	*Group (p-value)*	*IFNT (p-value)*
SLC1A1	25.0 ± 0.1	25.1 ± 0.2	0.6	0.7
SLC1A5	21.7 ± 0.1	22.2 ± 0.3	0.3	0.7
SLC6A6	23.5 ± 0.1	23.2 ± 0.4	1.0	0.6
SLC7A1	24.4 ± 0.1	24.5 ± 0.3	0.8	0.2
SLC7A5	22.5 ± 0.2	21.5 ± 0.5	0.09	0.2
SLC7A8	24.6 ± 0.2	23.4 ± 0.2	0.003	0.8
SLC15A3	25.8 ± 0.1	26.0 ± 0.3	0.7	0.7

Values are shown as mean ΔCq ± SEM and were regarded as significantly different if $p<0.05$. The statistic model included the Group (IVF vs. SCNT) as fixed effect and IFNT concentration in the uterine flushings as a covariate. The effects were considered significant at $p<0.05$.

(Orsi and Leese, 2004). The reduced intrauterine concentrations of small neutral and acidic AA in the presence of a conceptus derived by SCNT may thus implicate difficulties in mobilization of adequate amounts of nutrients for the rapid elongation process.

Cationic AA regulate a number of metabolic processes, and the reduced availability of the latter may imply developmental failures of the early embryo. In particular, Arg is required for the synthesis of multiple signaling molecules such as nitric oxide acting on vasodilation, angiogenesis, tissue remodeling, and regulation of apoptosis. Low Arg bioavailability in human placentas causes severe damages due to insufficient nitric oxide dependent vasodilatation and excess formation of reactive oxygen species associated with preeclampsia (Noris et al., 2004). Ornithine, derived from Arg, is a substrate for the synthesis of polyamines regulating protein synthesis and angiogenesis during ovine placentation (Kwon et al., 2003). Lys in its posttranslational modified form hydroxylysine (Hyl) is a main component of collagen in the extracellular matrix. In preparation of implantation and placentation, impaired induction of tissue remodeling and vasculogenesis due to insufficient supply of required AA might be involved in abnormal placentome formation, cuboidal chorionic epithelium structure, and decreased allantoic blood vessel development often occurring in pregnancies with SCNT-derived embryos (Hill et al. 2000, 2001). Increased placentomes and fetal oversize in later pregnancy stages might be entailed by compensatory mechanisms due to the reduced availability of numerous proteinogenic AA at preimplantation.

Endometrial *SLC7A8* mRNA was less expressed in the endometrium of SCNT pregnancies, while the expression of *SLC7A5* (also known as LAT1) was not affected. Covalently associated with the noncatalytic subunit 4F2hc/CD98, both transporters mediate the transmembrane transfer of essential large neutral aromatic and branched chain AA independent of sodium ions (Pineda et al., 1999). SLC7A5 and SLC7A8 act as obligatory exchanger with a 1:1 stoichiometry. In contrast to SLC7A5, mainly expressed in growing cells, SLC7A8 rather mediates the directed basolateral transport in differentiated cells (Verrey, 2003). However, due to its broader substrate range, SLC7A8 also transports smaller neutral AA. In the endometrium of pregnant ewes, SLC7A8 is most abundant in the luminal epithelial and subepithelial stroma with highest expression during the elongation phase between days 16 and 20 (Gao et al., 2009b). The decreased supply of intrauterine essential AA in the presence of a SCNT conceptus might lead to reduced protein synthesis of the elongating trophoblast. As each branched chain AA was approximately twofold less abundant, the SCNT conceptuses possibly displayed a reduced ability to direct the maternal transport into the uterine fluid, potentially mediated via SLC7A8. Defective signaling mechanisms provided by the conceptus might account for decreased activity of the maternal transport systems independent of IFNT resulting in a defective implantation and placentation with increased placentomes in order to compensate early nutrient deficiency during the early stages.

As components of biological membranes, phospholipids are required for various regulatory processes and act as precursor molecules for many bioactive substances, such as eicosanoids and lysophospholipids. In the mouse, the distribution of distinct phospholipids is known to change during implantation and phospholipids were shown to be involved in cell death and angiogenic events at implantation sites (Burnum et al., 2009). Disturbances in sphingolipid metabolism were demonstrated to cause pregnancy loss by inadequate blood vessel formation and excessive cell death (Mizugishi et al., 2007). O-phosphoethanolamine (PEtN), dramatically reduced in SCNT pregnancies, is a degradation product of phospholipids. Due to the differences in implantation and placentation between species it is of question whether phospholipids act in the same manner during implantation in ruminants, but a contribution of phospholipids in bovine pregnancy might be assumed. Prostaglandins (PG) are central eicosanoid lipid molecules of major importance during numerous reproductive events. Phospholipase A2 mediates the release of arachidonic acid from membranal phospholipids and cyclooxygenase subsequently catalyzes the reaction from arachidonic acid to PGH2, the precursor molecule of functional PG. Increases of PG in the uterine lumen appear to be essential for successful embryonic development in the uterine fluid prior to implantation (Ulbrich et al., 2009). If PEtN were a byproduct of PG synthesis, further clarification would thus be needed to evidence whether the 11-fold reduced PEtN in SCNT pregnancies might be a sign for a modified capacity of cloned conceptuses to affect maternal PG synthesis.

Due to the termination of pregnancy at peri-implantation, it is not possible to further predict pregnancy outcome and offspring viability of the embryos studied herein. However, severe failures are known to be associated with pregnancies of cloned embryos (Dean et al., 2001). Our results suggest that SCNT embryos appear to be less capable of inducing the endometrial transport of AA into the uterine lumen. This not only emphasizes the importance of a well-orchestrated embryo–maternal crosstalk prior to implantation, but specifically draws attention to the signaling capacity of the embryo to drive the maternal environment allocating optimal growth conditions. The decreased supply of numerous AA and derivatives in SCNT pregnancies might affect the development of the fast growing preattachment conceptus and entail placental abnormalities in later stages of pregnancy. As a consequence, overcompensation in later stages of implantation and placentation may result in enlarged placentomes and fetal oversize. This hypothesis is in line with observations of repeated ultrasound monitoring of pregnancies, that is, initially reduced growth of cloned bovine fetuses compared to fetuses derived by fertilization (Chavatte-Palmer et al., 2006). The data evidence the orchestrated signaling beyond the predominant pregnancy recognition signal IFNT required for the adequate accommodation of the preimplantation embryo. Our animal model thus may likely be used to further identify signaling mechanisms possibly disturbed during epigenetic reprogramming in SCNT embryos, but of principal importance during early preimplantation development.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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