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Expression of immune regulatory genes in early, mid and late stages of pig (*Sus scrofa domesticus*) gestation

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ABSTRACT

Semi-allogeneic foetus and placenta exploit various mechanisms to avoid immune-mediated maternal rejection. Several factors and cytokines are attributed for production of immune tolerance during gestation and very little information on expression of these immune-regulatory genes is available in pig. Chorioallantoic membrane (CAM) from early, mid and late gestational stages (n=4) were analysed for expression of immune regulatory genes, viz. Fas ligand (FasL), transporter for antigen processing-1 (TAP-1), transforming growth factor β1 (TGF-β1) and macrophage migration inhibitory factor (MIF) whereas Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as housekeeping gene. FasL gene expression was significantly higher in mid (13.46 fold) and late (28.77 fold) gestation than the early (1 fold) stage.TAP-1 mRNA expression was enhanced by 4.95 fold and 2.69 fold during mid and late gestation respectively in comparison to the early (1 fold) stage. TGF-β1 gene expression was up regulated in mid (2.43 fold) and late (2.29 fold) gestation than the early (1 fold) stage. MIF mRNA expression was enhanced in mid (3.04 fold) and late (1.59 fold) gestation in relation to the early (1 fold) stage. Placenta of pig remains entirely epitheliochorial which may minimise immune recognition and is supposed to diminish potent immune-regulatory mechanisms. However, our present study revealed consistent expression for immune regulatory factors which suggests immune modulation does exist in pig and may impart a role in pregnancy success.

Key words: Chorioallantoic membrane, FasL, MIF, Pig, TAP-1, TGFβ1

Foetus and placenta express paternal antigens which are foreign to mother. These semi-allogenic foetal antigens are expressed by foetal trophoblast which is in contact with maternal immune-competent cells throughout pregnancy. These mediator cells include macrophages, natural killer (NK) cells, B and T lymphocytes of uterine mucosa. However, during normal gestation, foetus can evade immune-mediated maternal rejection (Nagamatsu and Schust 2010, Martínez-Varea *et al.* 2014). This implies that creation of long-term selective immune-regulatory mechanisms exists in uterus and placenta during gestation. Cytokines produced by placenta are reported to play a critical role in maternal tolerance to semi-allogenic conceptus (Saito 2001). Several factors have been proposed to underlie this immunological enigma (Thellin and Heinen 2003). Immune regulatory factors and their molecular mechanisms of action resulting in creation of maternal-tolerance to foetus are not yet fully defined.

Fas-Fas ligand (Fas-FasL) system is one of the major pathways for induction of apoptosis in cells and tissues. Fas is expressed at low levels in human trophoblast while its incidence is high in activated lymphocytes (Stenqvist *et al.* 2013). FasL is expressed in human testis and placenta (Kauma *et al.* 1999). Kauma *et al*. (1999) demonstrated marked apoptosis in activated Fas bearing lymphocytes when these cells were co-cultured *in vitro* with FasL expressing placental trophoblast cells. Moreover, functional FasL had been demonstrated in exosomes of human placenta (Stenqvist *et al.* 2013) and tumours (Andreola *et al.* 2002).

TAP-1 gene, crucial for major histocompatability complex (MHC) class I molecules assembly, is highly expressed in placenta. Superior level of TAP-1 is also an indicator of enhanced production of masked non-classical MHC 1 molecules in placenta (Kalkunte *et al.* 2009). Immunologically privileged sites avoid classical MHC class I molecules and express non-classical MHC so as to minimise tissue incompatability and thereby maternal immune attack (Tizard 2009). The predominant immune

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cells of pregnant uterus are NK cells (Sharma 2014) which cause lysis of all cells that fail to express classical MHC (Tizard 2009). This implies that placenta is liable for NK cell mediated immune rejection.

Transforming growth factor beta 1(TGF-β1) is a potent immune-regulatory cytokine and is responsible for maintenance of immunological self-tolerance and display immune modulation via affecting cytolytic activity of NK cells and T cells (Saito *et al.* 1996, Taylor *et al.* 2006) and genesis of Treg cells which are immunosuppressive (Chung *et al.* 2000).

Macrophage migration inhibitory factor (MIF) was described as a pro-inflammatory cytokine that inhibits random migration of macrophages *in vitro* (Paulesu *et al.* 2005). MIF also act as an immunomodulatory factor (Apte *et al.* 1998, Krockenberger *et al.* 2008). MIF is produced by uterine NK (uNK) cells, trophoblast and decidual cells of placenta and MIF affect uNK cell cytolytic activity (Arcuri *et al.* 2006). Super-physiological levels of MIF produced by tumours (Meyer-Siegler *et al.* 2006) inhibit T cell activation and promote T cell apoptosis (Yan *et al.* 2006).

Secretion of these cytokines which can modulate maternal immune system were better investigated in highly invasive haemochorial placenta of human and laboratory animals (Dong *et al.* 2008, Kalkunte *et al.* 2009, Stenqvist *et al.* 2013). Placenta of pig is entirely epitheliochorial wherein maternal and foetal blood streams remains entirely separated throughout gestation. It is hypothetical that this type of placenta may minimise foetal antigen presentation and maternal activation which in turn necessitate less immune-regulatory mechanisms for normal pregnancy. Very little information for immune mediators is available in non-invasive porcine placenta. Hence the present study was conducted to explore the expression of *FasL*, *TAP-1*, *TGF β1* and *MIF* genes in pig placenta and to determine existence of any variation in their expression during the three stages of gestation.

MATERIALS AND METHODS

Gestation length in pig is 112–115 days which can be grouped into three stages: early (up to 40 days), mid (41–80 days) and late (above 81 days). Since a definite placenta is established around 30 days, specimens from about 35 days, 70 days and 105 days were used in the present study to represent three stages. Placentae were collected from crossbred large white Yorkshire pigs at authorised pig slaughter houses in Bangalore. Age of placenta was determined from crown-rump length of foetus (Marrable 1971). Representative samples from foetal placenta (chorioallantoic membrane, CAM) were collected from all three gestational stages (n=4) and were employed for the study. RNA isolation and DNase treatment were carried out using RNeasy Mini Kit (74104, Germany) and TURBO DNA-*free* Kit (AM1907, USA) respectively. cDNA was synthesized using oligo-dT and reverse transcriptase (Thermo Scientific, USA).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene.Quantification of target and reference genes was performed in duplicate. Details of the primer used in the study is given in Table 1. Settings for RT-PCR reaction included 20 µl total reaction volume, 0.5 µM final primer concentration and 60 ng/reaction template concentration. After 40 cycles, uniqueness of RT-PCR product was assessed from the single band obtained on 1.8% agarose gel containing ethidium bromide. Threshold cycle value (Ct) for genes were analysed and normalized with GAPDH and ΔΔCt values were calculated by considering early gestation as control. Relative mRNA expression was calculated using formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001) and fold change in expression was compared.

RESULTS AND DISCUSSION

We obtained mRNA expression for immune regulatory genes FasL, TAP-1, TGF-β1 and MIF in all three stages of pig gestation. Representative agarose gel photograph of amplified product (Fig. 1) and relative mRNA expression (Fig. 2) in the three gestational stages are given. Present study revealed superior consistent FasL expression, a factor expressed by immune privileged site. Kauma *et al*. (1999) and Vacchio and Hodes (2005) reported FasL expression in human and mice placenta respectively. However, recorded earlier works relating FasL mRNA expression or its functional attributes in porcine placenta were not available. Stenqvist *et al.* (2013) reported superior Fas expression in activated lymphocytes and functional FasL in human placenta. Frängsmyr *et al*. (2005) and Stenqvist *et al.* (2013) demonstrated FasL in cytoplasmic microvesicles of human

Table 1. Details of primers and genes used

Gene name and ID	Accession number	Primer sequence	Product size	E-E junction
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_001206359.1	F: TGACCCCTTCATTGACCTTC R: GATCTCGCTCCTGGAAGATG	143 bp	Yes
Fas ligand (FasL)	NM_213806.1	F: AAGAGGGACCACAATGCAGG R: CCTTTGGCTGGCAGACTCTC	144 bp	Yes
Transporter 1, ATP-binding cassette, sub-family B (TAP-1)	NM_001044581.1	F: GAGATGGCCATTCCGTTCTTC R: CACCGAGCTGGCTATGATGAG	120 bp	Yes
Transforming growth factor, beta 1 (TGF-*b*1)	NM_214015.1	F: GGTGCCGGAACCTGTATTG R: TGAGGTAGCGCCAGGAATC	119 bp	No
Macrophage migration inhibitory factor (MIF)	NM_001077213.2	F: CATCATGCCGATGTTCGTGG R: TGCACCGCGATGTACTGC	126 bp	Yes

placenta and its secretion as exosomes. Kauma *et al.* (1999) demonstrated significant reduction to FasL induced apoptosis if specific neutralizing antibodies to FasL were employed. Consistent with this, presence of Fas positive leukocytes (Kauma *et al.* 1999, Aschkenazi *et al*. 2002) and their apoptosis (Mor *et al.* 1998, Aschkenazi *et al.* 2002) had been demonstrated at maternal-foetal interface in human placenta. Moreover, FasL was localized in human and mice tumor which effect cytolysis of cytotoxic T cells to produce immune tolerance (Motz *et al.* 2014). These recorded possible means of action and superior FasL expression observed in present study suggest that FasL may provide apoptotic signals for activated Fas-bearing maternal lymphocytes and prevent their entry to foetal circulation. Pig CAM being positioned at strategic interface between maternal tissues and foetus may thus contribute in acquisition of tolerance and maintenance of "immune privilege" to the foetus. Furthermore, studies are required to explore whether an exosome mediated FasL apoptotic mechanism which provide supplementary immune privilege exist in porcine.

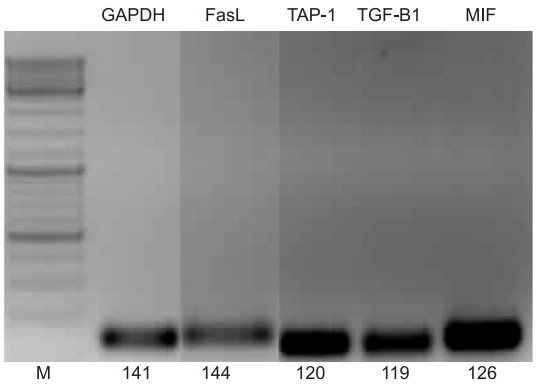

Fig. 1. Representative agarose gel photograph showing ethidium bromide-stained RT-PCR products of GAPDH, FasL, TAP1, TGF-β1 and MIF. RNA was isolated from CAM; following DNase treatment cDNA was synthesized. Immune regulatory factors were amplified with specific primers. Lane M, molecular marker; lane 2, housekeeping gene GAPDH; lane 4, FasL; lane 6, TAP1; lane 7, TGF-β1; lane 8, MIF.

FasL gene expression was significantly higher in mid (13.46 fold) and late (28.77 fold) gestation than early (1 fold) stage. This superior expression during mid and late gestation may be an adaptive mechanism to ensure adequate immune modulation during those periods necessitated by genesis of a more intimate and intricate maternal-foetal association. However, findings in the present study are contrary to Roh *et al.* (2002) and Stenqvist *et al.* (2013) in human who reported no significant difference. This discrepancy may be attributed to the wide disparity of placental structure (invasive human placenta vs. non-invasive porcine placenta) in addition to the co-existence and interaction of other potent tolerant mechanisms in both species.

Expression of TAP-1 gene observed in present study may relate to improved expression of non-classical MHC 1

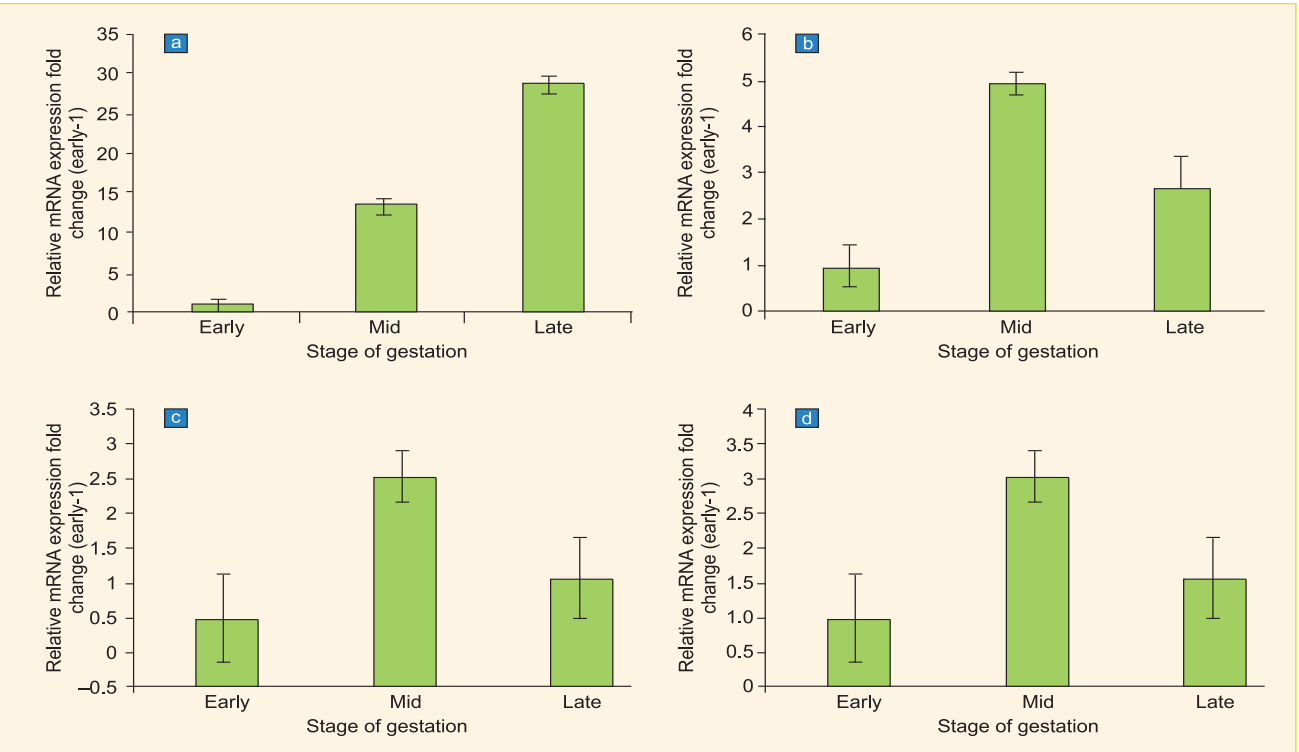

Fig. 2. FasL mRNA, TAP1 mRNA, TGF-α1 mRNA and MIF mRNA expression using RT-PCR. (a) FasL, (b) TAP1, (c) TGF-β1 and (d) MIF. mRNA levels of early, mid and late stages were measured by RT-PCR and were normalised with internal control gene, GAPDH. Fold change of expression levels of mid and late in comparison to early (early=1) is shown. The values (Mean±SEM) are from four independent experiments, each conducted in duplicate. RT-PCR results revealed high up-regulation for fasL and TGFβ1 in mid and late stages, and high up-regulation for TAP1and MIF at mid stage, and a moderate elevation in late stage.

molecules in pig CAM. Chorionic cells employ this pathway to evade routine immune recognition, but such cells are liable for NK cells mediated rejection (Tizard 2009). Hackmon *et al.* (2017) demonstrated expression of non-classical MHC molecules (HLA-F, HLA-E, HLAC, and HLA-G) in human extravillous trophoblast. Decidual NK cells represent the second largest sub-population in the decidua. Further, Dimova *et al.* (2008) localised NK cells in porcine endometrium. Moreau *et al.* (1998) demonstrated that non-classical MHC molecules will bind to inhibitory receptors of NK cells in order to achieve immune tolerance for foetus. These reports suggest that TAP-1 mRNA expression recorded in the present study may play a role in immune modulation. Kalkunte *et al.* (2009) postulated that extent of inhibition is positively correlated to amount of MHC molecules assembly which has parallel association to TAP-1 expression.

Though enhanced TAP-1 mRNA expression was expected as gestation advanced for better immune competence, we obtained an up-regulation of 4.95 fold during mid-gestation and 2.69 fold at late stage as compared to early (1 fold) stage. This pattern in time may be attributed to distribution of NK cells in endometrium which re-orient from superficial subepithelial position to deeper position as gestation advanced (Dimova *et al.* 2008). Thus an elevated expression obtained during mid-gestation may be related to better immune suppression necessitated by abundant sub-epithelially positioned NK cells so as to ensure sufficient immune protection.

We obtained consistent TGF-β1expression in all stages of gestation and higher relative abundance was observed in mid (2.43 fold) and late (2.29 fold) stages than early (1 fold) stage. TGF-β1 is a potent anti-inflammatory cytokine that was localised in pig placenta (Massuto 2010) and in human placenta (Hsiao *et al.* 2004). The suggested pathways of TGF-β1, viz. generation of immune suppressive T_{reg} cells (Chung *et al.* 2000), lymphokine activated killer activity (Hsiao *et al.* 2004), inhibition of proliferation of T cells (Taylor *et al*. 2006) and inhibition of cytolysis by NK cells (Saito *et al.* 1996) point that its presence in pig may play crucial role in immune modulation.Though statistically non-significant, superior expression was obtained in mid and late gestation than early stage.

A steady MIF gene expression was obtained in all three stages and intensity was up regulated in mid (3.04 fold) and late (1.59 fold) gestation in relation to the early (1 fold) stage. Our results were in accordance with MIF protein localisation in placenta of pig (Paulesu *et al.* 2005) and mice (Faria *et al.* 2010). MIF is produced by uNK cells, trophoblast and decidual cells of placenta and MIF affect uNK cell cytolytic activity (Arcuri *et al.* 2006). MIF had been recorded to inhibit non-specific cytolysis of NK cells in aqueous humour of eye, an immune-privileged site like placenta without MHC expression (Apte *et al.* 1998). Further, Krockenberger *et al.* (2008) pointed out that NK cell activating receptor, NKG2D is down-regulated by MIF. Moreover, super-physiological levels of MIF production by tumours (Meyer-Siegler *et al.* 2006, Yaddanapudi *et al.* 2016) will inhibit T-cell activation and proliferation (Yan *et al.* 2006, Yaddanapudi *et al.* 2016). The authors also proposed that MIF might induce T cell death and eliminate activated T cells from tumour micro-environment. Foetus along with placenta simulates tumour in that both express allo-antigens and can evade host immune surveillance. All the findings suggested MIF pathways along with consistent expression noticed in present study might imply an immune protective function in pig placenta.We obtained up regulation during mid and late gestation though these values were not statistically significant.

Our study revealed FasL, TAP-1, TGF-β1and MIF gene expression in foetal CAM during all the three stages of gestation. Relative FasL gene expression was significantly higher in mid and late gestations than early stage. TAP-1 and MIF mRNA expression was up-regulated in mid-gestation. TGF-β1 had enhanced expression in mid and late gestation. Recognition of mRNA for immune regulatory factors FasL, TAP-1, TGF-β1 and MIF in porcine CAM of the present study suggested that multiple potent immune regulatory mechanisms operate via the said immune modulators and co-exist in placenta which may ensure successful pregnancy. Selective immune modulation strategies of these genes may provide novel therapeutic protocol in tumours, graft transplantation and auto-immune diseases.

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