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Changes in nuclear factor kappa B components expression in the ovine spleen during early pregnancy

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ABSTRACT. Normal pregnancy is characterised by a systemic immunological tolerance against foetal antigens, and the spleen contributes to the adaptive immune tolerance during pregnancy. Nuclear factor kappa B (NF-κB) signallings participate in splenic immune regulation, but it is unclear whether there are changes in NF-κB components expression in the ovine spleen during early pregnancy. The objective of this study was to explore the effects of early pregnancy on the expression of NF-κB components in the maternal spleen in sheep. The spleens were sampled on day 16 of the oestrous cycle, and on days 13, 16 and 25 of gestation. The expression of *NF-κB* components, including *NF-κB1* (p50), *NF-κB2* (p52), *RelA* (p65), *RelB* and *C-Rel*, were detected by quantitative real-time PCR, Western blot analysis and immunohistochemical analysis. The results showed that *NF-κB1* and *RelB* mRNA and proteins decreased at days 13 and 16 of pregnancy, but increased at day 25 of pregnancy in comparison with that on day 16 of the oestrous cycle. Nevertheless, *NF-κB2* and *RelA* mRNA and proteins peaked at days 13 and 16 of pregnancy. In addition, early pregnancy inhibited *C-Rel* expression at days 16 to 25 of pregnancy in the maternal spleen. In conclusion, the variable expression of individual NF-κB components was found in the ovine spleen during early pregnancy, which may be related with pregnancy recognition, and essential for the embryo implantation and pregnancy maintenance.

Introduction

There is an immunological tolerance against foetal antigens, which is induced by hormonal changes during pregnancy (Fuhler, 2020). Progesterone (P4) from corpus luteum (CL), pregnancy recognition signal (interferon tau, IFNT) from the conceptus and prostaglandins secreted by the endometria affect uterine functions, and contribute to conceptus elongation, implantation and establishment of pregnancy in ruminants (Spencer et al., 2016). Conceptus signal (IFNT) and high concentrations of P4 contribute to immunological forbearance through regulating innate immune system in the uterus, peripheral immune cells and other tissues during early pregnancy in ruminants (Ott, 2020). Interferon tau, with its paracrine and endocrine actions, adjusts the maternal innate immune system and avoids conceptus rejection, and other immune regulators, such as the pattern recognition receptors, work in parallel with IFNT during early pregnancy in ruminants (Rocha et al., 2021). Pattern recognition receptors contribute to the activation of nuclear factor kappa B (NF-κB) to result in the downstream activation of innate immune responses (Heilmann et al., 2017).

Nuclear factor kappa B family consists of NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB and c-Rel that are involved in the regulation of development of the immune system, inflammation, and innate and adaptive immune responses in

mammals (Patel et al., 2017). Prolactin reduces lipopolysaccharide-induced inflammatory cytokines via suppressing NF-κB signalling in the cultured explants from human placentas, and has beneficial effects on trophoblast growth, placental angiogenesis and immunomodulation (Olmos-Ortiz et al., 2019). Lipopolysaccharide increases the expression of phosphatase and tensin homolog deleted on chromosome 10 via NF-κB pathway in trophoblasts, and reduces the ability of trophoblasts to invasion, which contributes to preeclampsia in a rat model (Xue et al., 2020). Tubulin polymerization-promoting protein 3 (TPPP3) contributes to the decidualization, and TPPP3 inhibition attenuates NF-κB transcriptional promoter activity in human endometrial stromal cells, and has adverse effects on decidualization and embryo implantation (Shukla et al., 2019). Autophagy suppression enhances the invasiveness of the trophoblastic cell lines and NF-κB activity, but NF-κB inhibitor attenuates the trophoblast invasion (Oh et al., 2020). In addition, normal morula embryos and tumour necrosis factor α (TNF-α)-treated morula embryos have differential effects on the expression of genes and proteins in uterine tissues and spleen through NF-κB pathway during preimplantation pregnancy in mice (Buska-Mach et al., 2021). It is unclear whether the expression of NF-κB components in the spleen is changed during early pregnancy in ewes.

As the centre of the blood defense system, the spleen works together with the liver and bone marrow to activate the defense response through innate and adaptive immunity in humans (Kashimura, 2020). Oestrogen contributes to haematopoietic stem-cell self-renewal and erythropoiesis in the spleen during pregnancy in mice (Nakada et al., 2014). Pregnancy induces changes in the erythroid lineage and the immune system, and there is increase in the expression of erythropoietin receptor and a decrease in the expression of death receptor Fas in the spleen during pregnancy (Norton et al., 2009). It has been reported that there is up-regulation of interferon stimulated genes (ISGs), P4 receptor, P4-induced blocking factor, TNF-β, interleukin (IL)-2, IL-5, IL-6, IL-10, cyclooxygenase 2, aldo-keto reductase family 1, member B1, melatonin receptor 1 (MT1), gonadotropin releasing hormone and its receptor, and down-regulation of MT2 in the ovine maternal spleen during early pregnancy (Yang et al., 2018; Li et al., 2019; Wang et al., 2019; Bai et al., 2020; Cao et al., 2021). So, it was hypothesised that there are changes in NF-κB components expression in the ovine spleen during early pregnancy. The goal of this study was to compare the expression of NF-κB1, NF-κB2, RelA, RelB and c-Rel in the maternal spleen during early pregnancy stages in sheep.

Material and methods

Animals and experimental design

The study was performed in the Department of Animal Science of the Hebei University of Engineering, Handan (China) on 24 Small-tail Han ewes. Animal procedures were approved by the Ethics Committee of the Hebei University of Engineering. After detection of oestrus (day 0) with a nepididymectomized ram, the females were randomly divided into four groups (n = 6 for each group). The ewes from the group of day 16 of the oestrous cycle were not exposed to a fertile ram. The other 18 animals were randomly divided into three groups (days 13, 16 and 25 of pregnancy), and exposed to fertile rams. The effects of early pregnancy on the expression of prostaglandin (PG) synthases in the ovine thymuses and thymic weight are mainly due to P4 and IFNT. The reasons that expression of NF-κB components in the ovine spleen is changed during early pregnancy are mainly due to P4 and IFNT. There are significantly higher concentrations of P4 on days 12–13 in plasma, and lower concentrations of P4 on days 15–16 during the ovine oestrous cycle (McNatty et al., 1973). IFNT (Protein X) and additional proteins are detected between days 14 and 21 in sheep (Godkin et al., 1982). Day 13 of the oestrous cycle is almost similar to day 13 of pregnancy according to above reasons, and at this time there are no high concentrations of P4 and IFNT on day 16 of the oestrous cycle. The spleens were collected from 24 ewes on days 13, 16 and 25 post-breeding, and from ewes on day 16 of the oestrous cycle at the time of slaughter. Pregnancy was verified by observing a conceptus in the uterus for the pregnant ewes. The splenic transverse pieces (0.5 cm^3) were fixed in fresh 4% (w/v) paraformaldehyde (Sigma-Aldrich Corp., St. Louis, MO, USA) in phosphate buffered saline (PBS) (pH 7.4), and the remaining portions were frozen in a liquid nitrogen for subsequent mRNA and protein analyses.

RNA extraction and qRT-PCR assay

Total RNA extraction was performed using TRIzol reagent (Invitrogen, California, USA) in accordance with manufacturer's instruction. Concentrations and purity of the total RNA were determined using spectrophotometry (Thermo Fisher Scientific, Wilmington, DE, USA), and absorbance ratio values (260/230) ranged between 2.0 to 2.2. Genomic DNA removal and complementary DNA synthesis

from the total RNA were performed using a FastQuant RT kit (Tiangen Biotech Co., Ltd., Beijing, China). A Bio-rad CFX96 real-time PCR system (Bio-Rad Laboratories, Inc., CA, USA) was used for quantitative real-time PCR (qRT-PCR) assay with a SuperReal PreMix Plus kit (Tiangen Biotech Co., Ltd., Beijing, China). The optimized primer pairs of *NF-κB1*, *NF-κB2*, *RelA*, *RelB*, *C-Rel* and *GAPDH* were designed and synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China) (Table 1) based on the mRNA sequence of target genes on Genbank (https://www.ncbi.nlm.nih.gov/genbank/).

Table 1. Primers used for RT-qPCR

Gene	Primer	Sequence	Size, bp	Accession No.
NF-κB1	Forward	CAAGCACAAGAAGGCAGCACAAC	113	XM_027970852.2
	Reverse	CAGCCATCAGCAGCAGCAGAC		
NF-κB2	Forward	GCCTGCTGAATGCCCTGTCTG	146	XM_042238744.1
	Reverse	CTCTGTTTCCTGTTCCACCGACTG		
RelA	Forward	TGGCGAGAGGAGCACAGACAC	92	XM_027959295.2
	Reverse	TGACCAGGGAGATGCGGACTG		
RelB	Forward	CGCTGACCTCTCCTCGCTCTC	93	XM_015100238.3
	Reverse	AAGCCGAAGCCATTCTCCTTGATG		
C-Rel	Forward	TCCTCCTCTGCGTCCATCTCAAG	104	XM_004005929.4
	Reverse	GTGGGGTGGGCGATTGATGAC		
GAPDH	Forward	GGGTCATCATCTCTGCACCT	176	NM_001190390.1
	Reverse	GGTCATAAGTCCCTCCACGA		

The PCR reaction consisted of 95 °C for 10 s, 60–62 °C (60 °C for *NF-κB1* and *NF-κB2*, 61 °C for *C-Rel*, 62 °C for *RelA* and *RelB*) for 20 s, and 72 °C for 25 s, and the number of PCR cycle was 40. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as the reference gene, and the condition of *GAPDH* PCR reaction was the same as the target genes, respectively. The $2^{-\Delta\Delta Ct}$ analysis method (Livak and Schmittgen, 2001) was used to determine the relative expression values for qRT-PCR assay. The relative levels of the target genes were normalized using the mean cycle threshold (CT) values from the group of day 16 of the oestrous cycle.

Western blot analysis

Splenic samples were lysed in a RIPA buffer (BL504A, Biosharp, Hefei, China) at 4 °C, and protein concentration was determined using a bicinchoninic acid protein assay kit (Tiangen Biotech Co., Ltd., Beijing, China) The extracts were boiled in loading buffer for 5 min, and equal amounts of proteins were analysed by a SDS–polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene fluoride membranes (Millipore Corp., Billerica, MA) that were blotted with 5% nonfat milk at 4 °C overnight. The membranes were incubated with a mouse anti-NF-κB1 monoclonal antibody (sc-8414, 1:1000; Santa Cruz Biotechnology, Inc., CA, USA), a mouse anti-NF-κB2 monoclonal antibody (sc-7386, 1:1000; Santa Cruz Biotechnology, Inc., CA, USA), a mouse anti-RelA monoclonal antibody (sc-8008, 1:1000; Santa Cruz Biotechnology, Inc., CA, USA), a mouse anti-RelB monoclonal antibody (sc-166416, 1:1000; Santa Cruz Biotechnology, Inc., CA, USA), and a mouse anti-c-Rel monoclonal antibody (sc-6955, 1:1000; Santa Cruz Biotechnology, Inc., CA, USA) at 4 °C overnight, respectively. The secondary antibody was anti-mouse IgG-HRP (BL001A; Biosharp, Hefei, China) at a concentration of 0.05 μg/ml, and blots were visualized by enhanced chemiluminescence (Tiangen Biotech Co., Ltd., Beijing, China). Quantity One V452 (Bio-Rad Laboratories, Hercules, CA, USA) was used to digitally quantified the band intensities that were normalized using GAPDH with an anti-GAPDH antibody (sc-20357, 1:1000; Santa Cruz Biotechnology, Inc., CA, USA).

Immunohistochemical analysis

Immunohistochemistry for RelA protein in the maternal spleen was performed as described previously (Wang et al., 2019). The 5-μm thick sections from paraffin-embedded splenic tissue were incubated at room temperature with the primary antibody specific to RelA (sc-8008, 1:200; Santa Cruz Biotechnology, Inc., CA, USA) in a humidified chamber at 4 °C overnight. Specific binding sites were visualized with a DAB kit (Tiangen Biotech Co., Ltd., Beijing, China), and then counterstained with haematoxylin (Sigma-Aldrich Corp., St. Louis, MO, USA). Negative controls were performed using antiserum-specific isotype diluted at concentrations equivalent to the primary antibody. The sections were observed with a light microscope (Nikon Eclipse E800, Japan), and photographed with a digital camera DP12. The images were examined

independently by 4 observers in a blinded fashion, and the staining intensities for RelA were analyzed by assigning an immunoreactive intensity of a scale of 0 to 3, as described previously (Kandil et al., 2007).

Statistical analysis

The experimental design was completely randomized, and relative abundance levels of mRNA and protein expression were analyzed by least-squares ANOVA using a MIXED procedure of SAS software (Version 9.2; SAS Institute Inc., Cary, NC, USA). Data obtained from different spleens of ewes were analyzed for the main effects of day and status (cyclic or pregnant), and their interaction between day and status. All data were presented as the mean ± standard error of the mean (SEM). Values of $P < 0.05$ were deemed significant.

Results

Relative expression levels of *NF-κB1*, *NF-κB2*, *RelA*, *RelB* and *C-Rel* mRNA in the spleen

As shown in the Figure 1, the relative expression levels of *NF-κB1* and *RelB* mRNA were down-regulated at days 13 and 16 of pregnancy, but up-regulated at day 25 of pregnancy in comparison with that on day 16 of the oestrous cycle ($P < 0.05$). However, the relative expression levels of *NF-κB2* and *RelA* were higher at days 13 and 16 of pregnancy than that at day 25 of pregnancy and on day 16 of the oestrous cycle ($P < 0.05$). In addition, early pregnancy induced down-regulation of *C-Rel* mRNA at days 16 to 25 of pregnancy in comparison with that on day 13 of pregnancy and day 16 of the oestrous cycle, but there was no significant difference between day 13 of pregnancy and day 16 of the oestrous cycle in the maternal spleen ($P > 0.05$; Figure 1).

Expression of NF-κB1, NF-κB2, RelA, RelB and c-Rel proteins in the spleen

The expression levels of NF-κB1 and RelB proteins were higher on day 25 of pregnancy than on day 16 of the oestrous cycle ($P < 0.05$), and NF-κB1 and RelB proteins were undetected on days 13 and 16 of pregnancy (Figure 2). However, the expression levels NF-κB2 and RelA proteins were up-regulated on day 13 and 16 of pregnancy, but there was no expression of RelA protein on day 16 of the oestrous cycle. In addition, the c-Rel protein level was significantly decreased at days 16 and 25 of pregnancy in comparison with that on

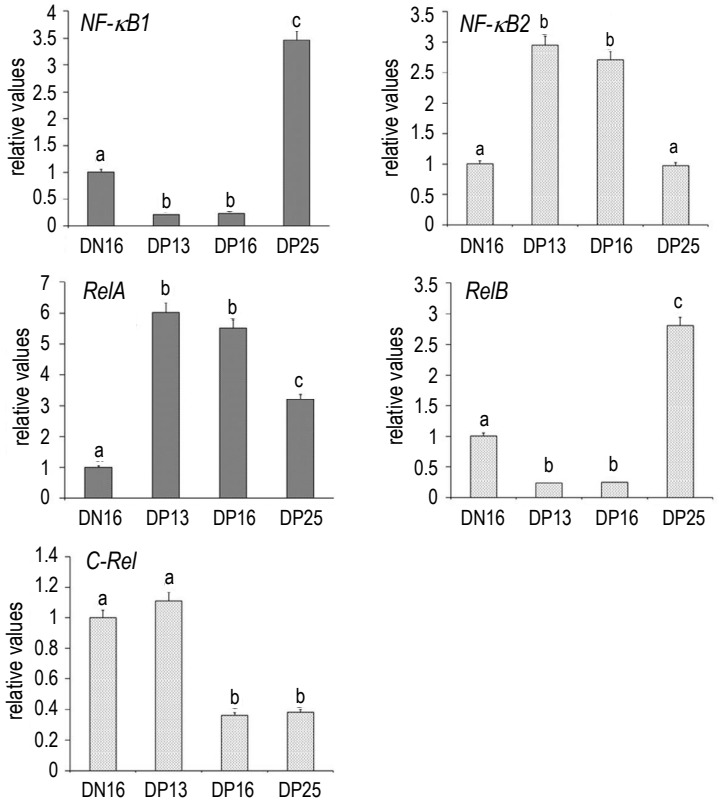

Figure 1. Relative expression values of *NF-κB1*, *NF-κB2*, *RelA*, *RelB* and *C-Rel* mRNA in the spleens of non-pregnant and pregnant ewes (n = 6 for each group) measured by quantitative real-time PCR

DN16 – day 16 of non-pregnancy, DP13 – day 13 of pregnancy, DP16 – day 16 of pregnancy, DP25 – day 25 of pregnancy; abc – bars with different superscripts are significantly different at $P < 0.05$

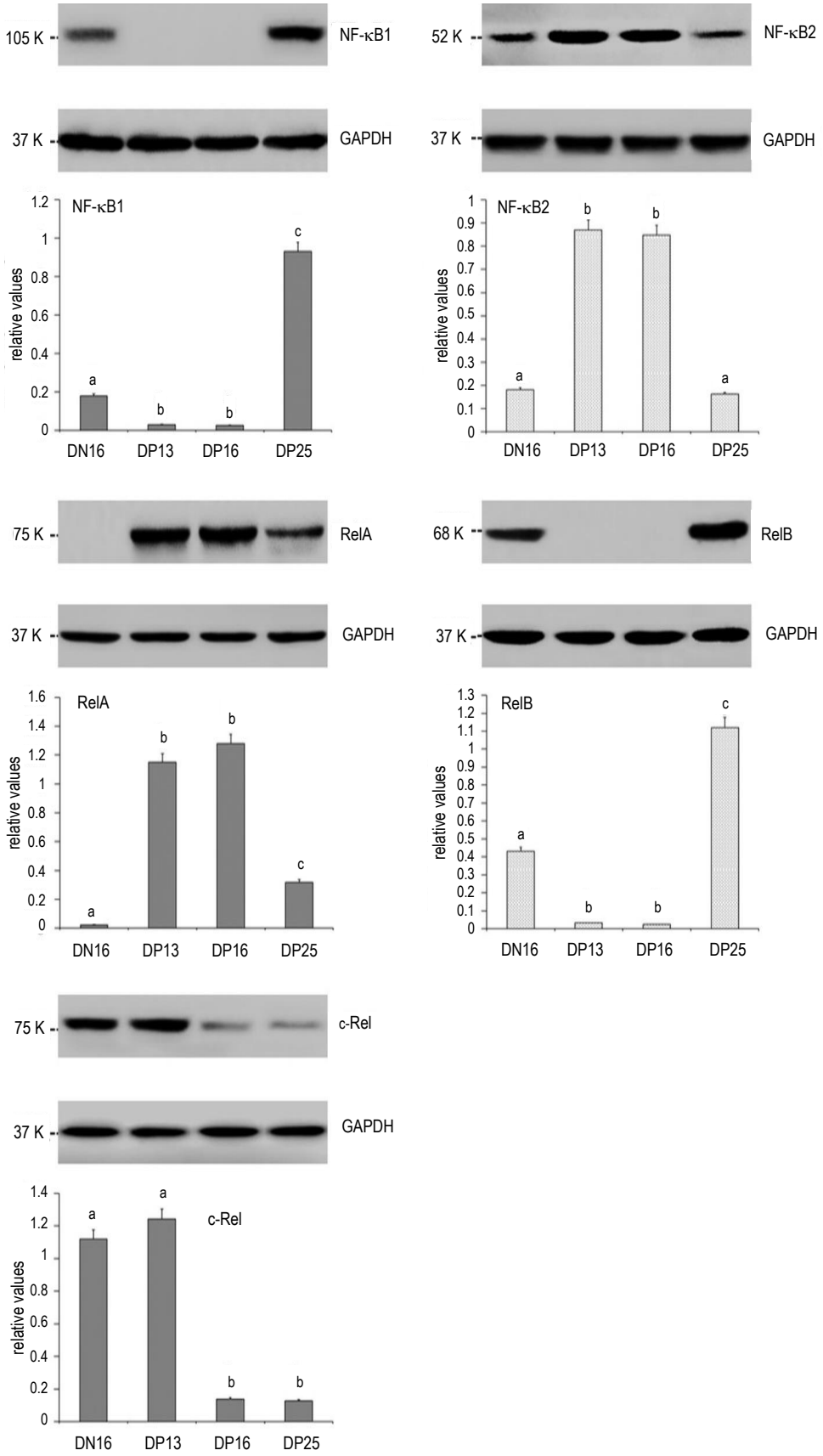

Figure 2. Expression of NF-κB1, NF-κB2, RelA, RelB and c-Rel proteins in the spleens of non-pregnant and pregnant ewes (n = 6 for each group) analyzed with Western blot

DN16 – day 16 of non-pregnancy, DP13 – day 13 of pregnancy, DP16 – day 16 of pregnancy, DP25 – day 25 of pregnancy; abc – bars of the same colour with different superscripts are significantly different at $P < 0.05$

day 13 of pregnancy and day 16 of the oestrous cycle ($P > 0.05$; Figure 2), and there was no significant difference between that at day 13 of pregnancy and on day 16 of the oestrous cycle, as well as between that at day 16 of pregnancy and at day 25 of pregnancy in the maternal spleen ($P > 0.05$; Figure 2).

Immunohistochemistry for RelA protein in the spleen

The RelA protein was mainly limited to the capsule, trabeculae and splenic cords (Figure 3). The staining intensities for RelA protein were 0, 0, 2+, 2+, and 1+ for the negative control, the spleens from day 16 of the oestrous cycle, and spleens from days 13, 16, and 25 of pregnancy, respectively (Figure 3). The staining intensity was as follows: 0 = negative; 1+ = weak; 2+ = strong.

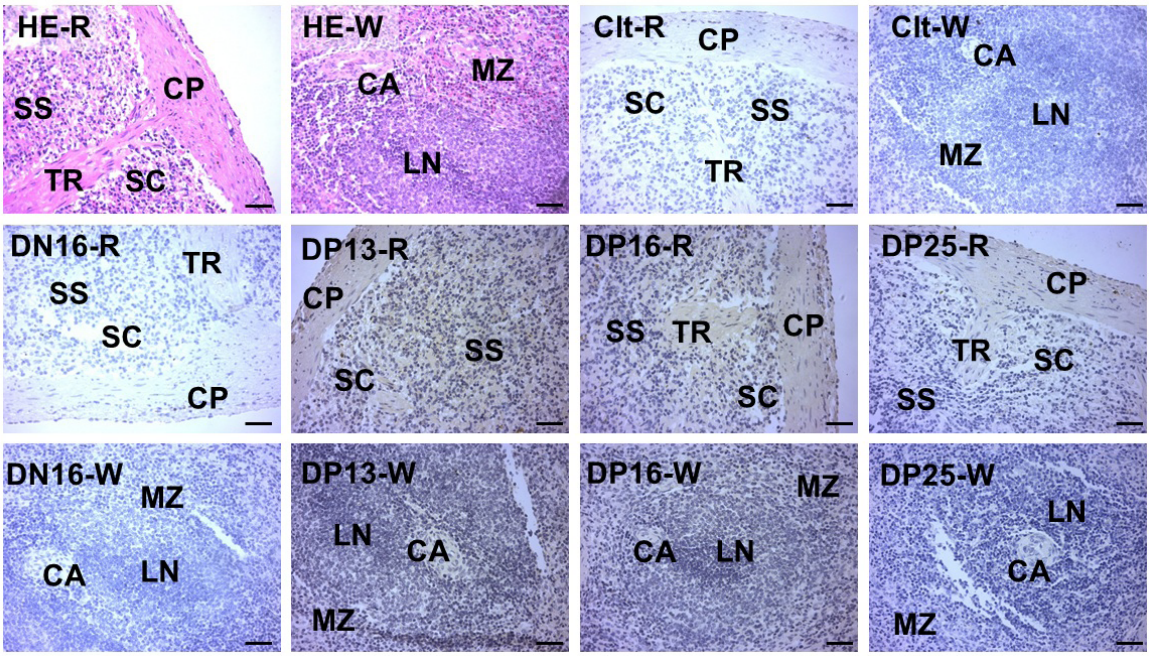

Figure 3. Representative immunohistochemical localization of RelA protein in the spleens of non-pregnant and pregnant ewes (n = 6 for each group). The spleen is divided into red pulp (R) and white pulp (W), and surrounded by a thickened capsule (CP). Capsule (CP) with several trabeculae (TR) projects into the substance of the spleen.
HE – stained by hematoxylin and eosin, Ctl – negative control, SS – splenic sinuse, SC – splenic cords, MZ – marginal zone, LN – lymphoid nodule, CA – central arteriole, DN16 – day 16 of non-pregnancy, DP13 – day 13 of pregnancy, DP16 – day 16 of pregnancy, DP25 – day 25 of pregnancy; bar – 50 μm

Discussion

In this study, NF-κB1 decreased in the maternal spleen at days 13 and 16 of pregnancy, but increased at day 25 of pregnancy. NF-κB1 plays a key role in controlling lymphocyte and macrophage functions in immune and inflammatory responses, and NF-κB1 knockout displays multifocal defects in immune system function in mice (Beinke and Ley, 2004). The expression level of *NFKB1* gene in the endometrium at day 0 of the oestrous cycle was high in comparison with that during early pregnancy, but increased during early pregnancy, which contributes to the establishment and maintenance of pregnancy in the pig (Ross et al., 2010). Endometrial polyp is a factor for sub-fertility with a higher level of NF-κB1 in the endometrium, but hysteroscopic polypectomy leads to a significant decrease in endometrium NF-κB1 activity in humans (Bozkurt et al., 2015). NF-κB1 is mainly localized in the endometrial epithelium, and increases during the implantation period, which is essential for embryo implantation in mice (Nakamura et al., 2004). Thus, the expression of NF-κB1 in the maternal spleen is changed during early pregnancy, and the up-regulation of NF-κB1 at day 25 of pregnancy may be related to the embryo implantation.

Our data showed that RelB declined in the maternal spleen at days 13 and 16 of pregnancy, but was up-regulated at day 25 of pregnancy. RelB is essential for secondary lymphoid tissue organization and immune tolerance to inflammation, and RelB-deficient leads to premature mortality in mice

(Baud and Collares, 2016). The pregnancy recognition signal IFNT (type I interferon) participates in the implantation and establishment of pregnancy in ruminants (Spencer et al., 2016), and also induces expression of ISGs in the ovine maternal spleen during early pregnancy (Yang et al., 2018; Wang et al., 2019). RelB is a negative regulator of the type I interferon signalling pathway in dendritic cells (Saha et al., 2020), and up-regulation of ISGs in the ovine maternal spleen may be related to the down-regulation of RelB in the spleen. RelB plays a key role in silencing or inhibiting the expression of the pro-inflammatory cytokines, and in a strong constitutive activation of RelB in decidual endothelial cells is beneficial for both avoiding pregnancy failure and immune tolerance to microorganisms during pregnancy (Masat et al., 2015). Therefore, it is suggested that the low level of RelB in the maternal spleen at days 13 and 16 of pregnancy may be related to the pregnancy recognition, and the up-regulation of RelB at day 25 of pregnancy may be important for the embryo implantation and avoidance of pregnancy failure.

Our data indicated that early pregnancy induced expression of *NF-κB2* at days 13 and 16 of pregnancy. NF-κB2 is an inhibitor of κB protein, and heterozygous NFKB2 mutations lead to a syndrome of immunodeficiency and autoimmunity in humans (Wirasinha et al., 2021). There was a marked decrease in the B cell compartment in the spleen in NF-κB2-deficient mice, suggesting that NF-κB2 is essential for the maintenance of the peripheral B cell population, humoral responses, and normal spleen architecture (Caamaño et al., 1998). There were increases in NF-κB2 protein in the CL on days 12, 14, and 16 of pregnancy, which is necessary for the survival of CL, secretion of P4 and the establishment of pregnancy in sheep (Lee et al., 2016). Therefore, the up-regulation of NF-κB2 in the maternal spleen at days 13 and 16 of pregnancy may be related to the establishment of pregnancy.

Our results revealed that early pregnancy enhanced the expression of *RelA* in the spleen, and *RelA* peaked at days 13 and 16 of pregnancy. In addition, RelA protein was mainly limited to the capsule, trabeculae and splenic cords. There is an absence of RelA in the NF-kB complex in peripheral blood mononuclear cells and liver of pregnant fulminant hepatic failure patients, suggesting that RelA suppression leads to deregulated immunity, as well as the death of the patient (Prusty et al., 2007). RelA protein increases in CL on days 12, 14, and 16 of pregnancy, which is related to CL survival, P4 secretion and the establishment of pregnancy in sheep (Lee et al., 2016). *In vivo* oestrogen treatment suppresses the expression of RelA protein in mouse splenocytes, which contributes to the regulation of the immune system (Dai et al., 2007). It is has been reported that there is significantly lower plasma concentration of oestradiol-17β at days 10 and 20 of gestation in sheep (Hamon and Heap, 1990). Therefore, the up-regulation of RelA in the capsule, trabeculae and splenic cords of the maternal spleen during early pregnancy may be related to a lower concentration of plasma oestradiol-17β, and so it could be beneficial for the establishment of pregnancy.

In this study, there was a decrease in the c-Rel expression at days 16 and 25 of pregnancy in the maternal spleen. NF-κB subunit c-Rel is predominantly expressed in B cells that mediate humoral immune response and participate in the regulation of cellular immune response (Basavarajappa and Ramakrishnan, 2020), and is also involved in the maintenance of B cell mature in the spleen (Yamazaki and Kurosaki, 2003). The c-Rel level down-regulates the myometrium of pregnant women in comparison with nonpregnant controls, which is beneficial for pregnancy maintenance (Chapman et al., 2004). The expression of c-Rel protein in villi of the normal placenta is weak, which is related to the invasion and migration of choriocarcinoma cells (Sekiya et al., 2017). Thus, the decline of c-Rel at days 16 and 25 of pregnancy may be related to B cell mature in the spleen, and it contributes to the pregnancy maintenance.

Conclusions

A variable expression of individual NF-κB components in the maternal spleen during early pregnancy may be related to pregnancy recognition, embryo implantation, and pregnancy maintenance in sheep.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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