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Interferon-stimulated gene 15 (ISG15): Expression profile and role in the corpus luteum of goat (*Capra hircus*)

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ABSTRACT

The corpus luteum (CL) is a dynamic and transient endocrine gland. It secretes variety of protein hormones and have an important role in survivability of the embryo during early pregnancy. Among the interferon stimulated genes (ISGs), interferon-stimulated gene 15 (ISG15) is one of the gene having important role in maintaining corpus luteum in different ruminant species, but in goat (*Capra hircus)* its role is not yet elucidated. In the present study, temporal expression profile of cpISG15 mRNA and protein was examined by quantitative real-time PCR (qPCR) and western blot techniques, respectively, in the CL of cyclic and pregnant does. Both cpISG15 mRNA and protein were expressed maximally in the CL during early stage of pregnancy (16 to 24 days) as compared to cyclic does. But significant difference was not found in the expression of cpISG15 mRNA and protein in the CL between the later stage of pregnancy (25 to 40 days) and cyclic does. Finding of significant upregulation of ISG15 in CL during early pregnancy at mRNA as well as protein level suggest its probable role in maintaining CL and its function at this stage in caprine.

Keywords: Corpus luteum, Goat, ISG15, Pregnancy

During early pregnancy (11 to 24 days of pregnancy) in ruminant species, trophectoderm of a preimplant embryo secretes interferon tau (IFNτ) and this IFNτ binds to the type-1 IFN receptor (IFNR) localized on luminal epithelial cells of endometrium (Bazer *et al.* 2012). After binding with receptor, it exerts its anti-luteolytic activity following different mechanisms (Uze *et al.* 1995). In addition to its anti-viral and anti-luteolytic effects, IFNτ induces and/or up-regulates expression of several genes in the endometrium and interferon stimulated gene 15 (ISG15) is one of them (Johnson *et al.* 1999, Chandrakar *et al.* 2020). Interferon-stimulated gene 15 (ISG15) comes under a growing family of ubiquitin-like modifiers and its resemblance with ubiquitin is based on the point that both covalently change other proteins (Haas *et al.* 1987). It is well established that ISG15 is expressed in uterine endometrium and believed that its supports the development of an embryo by involving in vital intracellular processes (Ashley *et al.* 2010).

Apart from the uterine tissue, ISG15 expression has also been reported in uterine artery, uterine vein, uterine placental interface and CL of ovine (Joyce *et al.* 2005, Oliveira *et al.* 2008), bubaline CL (Jain and Mitra 2012) and bovine CL (Chen *et al.* 2006, Yang *et al.* 2010). In ruminants, it has been demonstrated that infusion of recombinant IFNτ into the uterine lumen or systemically, extends the life span of CL and also increases the expression of ISG15 gene in the CL (Spencer *et al.* 1994, Chen *et al.* 2006, Ealy *et al.* 2006, Oliveira *et al.* 2008). These studies suggested that IFNτ enters into the CL and increases the expression of ISG15 gene and this ISG15 gene perhaps plays an important role in maintenance of function and life span of the CL. However, exact mechanism of CL conditioning by IFNτ or pregnancy to express ISG15 remains to be established in ruminant species including caprine.

Goats, being a multi-functional animal, are popularly known as the 'poor man's cows' and show a substantial role in the economy and nourishment of landless and marginal farmers especially in India (MacHugh and Bradley 2001, Khan *et al.* 2011). Considering the significant role of ISG15 in CL, the current work was carried out to reveal the expression pattern of ISG15 gene in the CL of goats.

MATERIALS AND METHODS

Collection of samples and experimental design: All experimental processes were permitted by the Institutional Animal Ethics Committee. To study the ISG 15 expression profile in CL, freshly slaughtered whole female genitalia of goats were collected from the local slaughterhouse (Bhilai, Chhattisgarh) and carried in cooling conditions to the laboratory. On the basis of a fetus, uteri with CL were classified into two clusters i.e. gravid (n=6) and non-gravid (n=3). Pregnant uteri were further classified into two

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subcluters, early stage of pregnancy (n=3, 16 to 24 days) and later stage of pregnancy (n=3, 25 to 40 days). Single embryo pregnancies were only taken in the study. Based on crown rump length measurement (Singh *et al.* 2004), the number of days of pregnancy was decided and pieces of a corpus luteum were collected accordingly and processed immediately for mRNA and protein studies.

RNA isolation and cDNA synthesis: Corpus luteum tissue of goat (approximately 50 mg) was collected and homogenized using electric homogenizer (BioSpec Products) directly in Tri reagent solution (Sigma, USA). RNA was isolated from colourless upper aqueous phase as per manufacturer's instructions. Quality of isolated RNA was checked by agarose gel electrophoresis and quantity was determined by Qubit 3 Fluorometer (Invitrogen, USA). Isolated RNA (2 µg) was used for complementary DNA (cDNA) synthesis using the reverse transcription system (Thermo Scientific, USA) and prepared cDNA was diluted four times with proteinase and nuclease-free water and put at -24˚C till further use.

Expression of cpISG15 mRNA: Expression profile of cpISG15 mRNA was carried out by quantitative real-time PCR (qPCR) in a CFX-96 Real-Time PCR System (BioRad, USA) as described earlier (Chandrakar *et al.* 2020). Briefly, published primers of ISG15 (ISG15F: 5'-TGATGGTATCYGAGCTGAAG-3'; ISG15R: 5'-CTTGAGCACAGCCACAGTCT-3') and beta-actin (ACTBF: 5'-TCGGCAATGAGCGGTTCC-3'; ACTBR: 5'-ACYGTGTTGGCGTAGAGGTC-3'), as endogenous control, genes were used. Primer matrix experiments were performed to optimize the primer concentration and all qPCR reactions were performed in duplicates in 20 µl volume containing 1× SYBRGreen PCR master mix (Sigma, USA), 10 pM of each primer, 2 µl of diluted cDNA template and nuclease-free water. Cycling conditions of PCR were optimized according to RT PCR system (initial denaturation at 95˚C for 10 min, repeated 40 cycles of denaturation at 95˚C for 30 sec; annealing as well as extension at 60˚C for 60 sec). Negative control and melting curve were also run to exclude any background amplifications and assess the specificity of the final PCR product. Delta-delta-C_T formula was used to calculate the relative mRNA abundance for target gene (Livak and Schmittgen 2001).

Western blotting: Western blotting was carried out to quantify the protein as described earlier (Chandrakar *et al.* 2020). Briefly, total protein was collected from goat's corpus luteum (pregnancy and cyclic stages) and approximately, 50 mg of tissue was homogenized in PBS using electric homogenizer (BioSpec Products). After centrifugation, the supernatant was collected and lysis was done in the same volume of lysate and Laemmli buffer (Amresco, USA). The above mixture was denatured by boiling at 95˚C for 7 min and snap chilling was carried out immediately after boiling. Samples were dispersed by using 12% SDS–PAGE. After separation, the proteins were transferred from the gel onto the PVDF membrane (PALL Corporation, India) following standard procedure using mini vertical electrophoretic apparatus (Hoefer, USA) in pre-cool buffer at low temperature for 2 h. The PVDF membrane was blocked to reduce the background staining. After blocking, membrane was incubated with rabbit ISG15 polyclonal antibody (Proteintech, USA) in 1:500 dilutions at 4˚C for 16 h with slow movement on shaker. After two-three washings, the blot was incubated to an HRP conjugated secondary antibody (anti-rabbit secondary antibody, Santa Cruz, CA, USA) in 1:1500 dilution for 1.5 h at room temperature. DAB substrate buffer system (GeNei, India) was used to detect the presence of the secondary antibody. Intensity of the protein bands was calculated by densitometric analysis using National Institutes of Health ImageJ 1.44p software (Bethesda, Maryland, USA). The band's relative intensity of ISG15 protein was regularized and quantified with ACTB as an endogenous control (Santa Cruz, CA, USA).

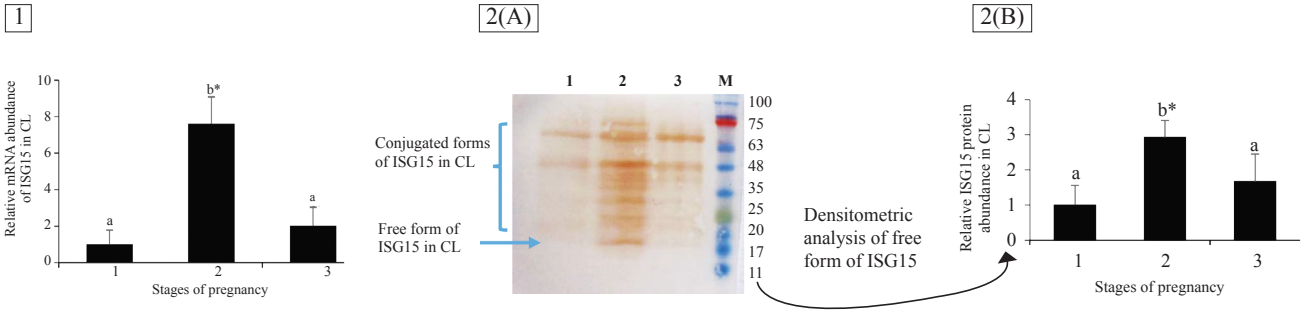
Statistical analysis: Values of cpISG15 mRNA and protein were analyzed using SPSS software (version 2015). Mean differences of cyclic and across all the stages of the pregnancy were analyzed using one-way ANOVA followed by the least square mean (LSD). Data for mRNA expression were analyzed based on $\Delta\Delta C_T$ values. Results were analyzed at 5% level of significance ($P<0.05$) and expressed as mean±standard error of mean.

RESULTS AND DISCUSSION

ISG15 gene's expression profile has already been studied in CL of various species but has not been explored in caprine CL. To the best knowledge of authors, current study is the first report revealing expression profile of ISG15 gene in the CL at different stages of the pregnancy in local goats (Anjori goats).

In the present study, expression of cpISG15 mRNA and protein was detected in the CL across all the three stages of does. The free (15 kDa) and conjugated (>30kDa) cpISG15 proteins were noticed in the CL of pregnant does **(**Fig. 2A). Only significant ($P<0.05$) difference in mRNA and protein abundance of cpISG15 gene was found between early stage of pregnancy (16 to 24 days) and cyclic does or later stage of pregnancy (25 to 40 days). Caprine ISG15 mRNA and protein expression was 7.6-fold and 2.93-fold higher ($P<0.05$), respectively, in the CL of early stage of pregnancy (16 to 24 days) as compared to cyclic does (Figs 1 and 2B). Significant difference was not found between cyclic stage and later stage of pregnancy both at mRNA and protein (Figs 1 and 2B). Single peak was observed in the dissociation curve indicating specificity of qPCR reaction and it also signified that primer was highly specific to the target and had no primer dimer formation. ACTB was used as housekeeping control and it revealed the same intensity of 43 kDa protein band among the three groups (data not shown).

Significantly higher ISG15 expression gene has been reported in the endometrium of different ruminant species (Johnson *et al.* 1999, Joyce *et al.* 2005, Jain *et al.* 2012



Figs 1-2. **1**. Relative quantitative PCR analysis of cpISG15 mRNA in the corpus luteum of pregnant and non-pregnant does. 1, Non-pregnant does; 2, 16 to 24 days pregnant does; 3, 25 to 40 days pregnant does. Bars indicate mean±SEM values (n=3). Different alphabets indicate significant difference (P<0.05). **2**. Detection of cpISG15 gene by western blotting **(A)** and relative expression of the cpISG15 protein **(B)** in the corpus luteum of pregnant and non-pregnant does. M, Molecular weight marker (Himedia); Lane 1, Non-pregnant does; Lane 2, 16 to 24 days pregnant does; Lane 3, 25 to 40 days pregnant does. Bars indicate mean±SEM values (n=3). Different alphabets indicate significant difference (P<0.05).

Chandrakar *et al.* 2020) during early stage of pregnancy (13 to 21 days). Its role in endometrial receptivity and embryo survivability is well recognized. Extra uterine ISG15 expression in CL and peripheral blood leukocytes (PBL) of bovine (Yang *et al.* 2010, Cheng *et al.* 2019), bubaline (Jain *et al.* 2012, Nag *et al.* 2018) and ovine (Oliveira *et al.* 2008) had also been reported. Findings of the current study are comparable with other studies in bovine (Yang *et al.* 2010), bubaline (Jain and Mitra 2012) and ewes (Chen *et al.* 2006, Oliveira *et al.* 2008) in which ISG15 level was upregulated in CL during early stage of pregnancy (13 to 24 days). This significantly higher level of ISG15 mRNA in CL corresponds with the release of IFNτ from the embryo during early pregnancy in between 16-21 days in goat (Bazer *et al.* 2011). Inoculation of exogenous IFNτ systemically or into the uterine lumen has been found to extend CL life span in non-pregnant ewes (Spencer *et al.* 1999, Bott *et al.* 2010) and cows (Ealy *et al.* 2013), and also increase the expression of ISG15 mRNA and protein in CL of ovine (Spencer *et al.* 1999, Chen *et al.* 2006, Oliveira *et al.* 2008). It has also been proven that IFNτ directly or indirectly boosts trophectoderm cell propagation and supports early embryo development in ruminants (Wang *et al.* 2013, Bao *et al.* 2014). Embryonic mortality rates are high during early pregnancy (up to 24 days) and then decreased in later stage of pregnancy in goats and cows (Lucy 2001, Samir *et al.* 2016). Further, knockdown and knockout reports have also shown the embroilment of ISG15 in embryos development. ISG15 gene deletion ensued in foetal loss in mice (Dey *et al*. 2004) and ISG15 knockdown by siRNA decreases the hatching percentage and diameter of the blastocysts in bovine embryos (Zao *et al.* 2017).

Population of different immune cells is present in bovine CL chiefly due to high blood flow to CL (Niswender *et al.* 1997) and may have an active role in controlling the life span and function of the CL with the assistance of their cytokine products during pregnancy (Pate *et al.* 2003). It has been reported that IFNτ stimulates gene expression in components of the circulating immune system during early pregnancy in ewes (Yankey *et al.* 2001). Among several ISGs during early pregnancy, ISG15 controls the dynamic balance of cytokines to accommodate cell-mediated immune reaction to elude the rejection (Robertson *et al.* 1994).

Low level of ISG15 has been found in later stage of pregnancy and cyclic does, which is congruent with other studies in bovine (Yang *et al.* 2010) and bubaline (Jain and Mitra 2012). Constitutive expression of ISSG15 gene in PBMC in human (D'Cunha *et al.* 1996) and presence of immune cells population in CL could explain the existence of low level of ISG15 expression in CL of cyclic does and later stage of pregnancy. ISG15 expression has also been found at uterine-placental junction in entire pregnancy in ewes suggesting it as a key component of microenvironment (Joyce *et al.* 2005). In ovine, release of IFNτ into the uterine vein and localization of ISG15 in large luteal cells suggested that conceptus-derived IFNτ act on CL through an endocrine pathway (Oliveira *et al.* 2008, Bott *et al.* 2010). Findings from the current study together with other reports hint engrossment of ISG15 in maintenance of corpus luteum life span during early pregnancy but precisely how IFNτ enters in the CL and acts remains to be elucidated.

In conclusion, the present study reports expression profile in the CL during first trimester of pregnancy in cyclic does. Findings of the enhanced level of cpISG15 and its conjugated proteins in the CL indicate its probable role in maintaining luteal function or luteotrophic action during early pregnancy in goat. Further, research works are needed to detect the ISG15 gene and its protein in luteal cells and to disclose the precise mechanism of ISG15 gene in CL during pregnancy in does.

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