

## EXPRESSION OF ISG15 AND CONJUGATING ENZYME DURING PERI-IMPLANATION PERIOD IN SHEEP

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**Abstract:** Pregnancy recognition in ruminant affects numerous genes regulation. Interferon-stimulatory gene (*ISG15*), an ubiquitin-like protein that mediates the conjugation of different proteins through its ISGylation enzymes *UBE1L* and *UBCH8*, is also differentially expressed during early pregnancy. The purpose of this study was to investigate the role of *ISG-15* in the establishment of pregnancy and conceptus elongation during early post conception periods and to ascertain the presence of ISGylation enzymes *UBE1L* and *UBCH8*. Therefore, sheep were synchronized through cloprostenol sodium and gonadotrophin releasing hormone-1 and serviced by rams. The blood was collected on the post-mating days 6, 11, 12, 13, 14, 16, 17, 21, 23 and 25. The *ISG-15*, *UBE1L* and *UBCH8* primers were used to amplify the corresponding transcriptomic region using PCR. Recovery rate of each transcriptomic fragment was compared with the housekeeping gene *GAPDH*. *ISG-15* expression was higher on day 12, contrary to *UBE1L* were higher on day 6 and *UBCH8* on day 21. Furthermore, the *ISG-15* is ubiquitin-like protein, mediates *UBE1L* and *UBCH8* enzymes to guard the conceptus against viral pathogenicity during early pregnancy.

**Key words:** ISG-15, sheep, pregnancy, ISGylation, conception, ubiquitination.

### Introduction

In bovine and ovine, embryonic losses occur most of the times during pre-implantation stages. These embryonic losses occur due to lack of biochemical communications between the embryo and the uterus (*Li and Winuthayanon, 2017*). Interferon-tau (IFN- $\tau$ ) is from type I IFNs family, and is a basic cytokine in

establishment of ruminant's pregnancy, it is produced by the ruminant's conceptus around the time of implantation (Ka et al., 2018). IFN- $\tau$  also represents immune modulatory action towards leukocytes by changing their proliferative responses and cytokine production. This cytokine action has been extensively studied for the past ten years (Sanlorenzo et al. 2017). It has been viewed as a potential pathway in improving the performance and genetics for ruminant production. Furthermore, the high antiviral effectiveness and low cytotoxicity of IFN- $\tau$  in contrast with IFN- $\alpha$  has hired this cytokine in the development of possible therapeutic agent in humans and animals (Kiladjian et al., 2016).

ISG-15 is an IFN- $\tau$  stimulated gene, encodes for a 15 kDa ubiquitin-like protein (UBL) that was first identified in mouse and generated from IFN stimulated murine tumor cell RNA (Cella et al., 2019). It was the first ubiquitin-like modifier to be identified, initially named as a ubiquitin cross-reactive protein (UCRP) (Lin, 2017). This cross-reactivity is explained by the fact that ISG-15 consists of two domains, each domain stands high sequence homology to ubiquitin (addition of ubiquitin to a substrate protein is called ubiquitination). The main functions of ISG-15 are still unknown (Bogunovic et al., 2012). However, it is suggested that ISG-15 and the modification system have significant roles in innate immunity systems responses, interferon regulation signaling system, pregnancy, and cancer (Xiao et al. 2018).

ISG-15 role in pregnancy has been well studied in ruminant species. The establishment of early pregnancy in ruminants is due to IFN- $\tau$  activation (Chandrakar et al., 2020). The ISG-15 expression is enhanced in the endometrium of humans, baboons (Chandrakar et al., 2020), in response to IFNs activated by embryos. In addition, the placenta is accumulated with macrophages which produce IFNs when activated in embryos. The expression of bovine ISG-15 is enhanced in the endometrium during early pregnancy (Ruhmann et al., 2017). Attack on receptive uterine epithelium by the conceptus during the origination of pregnancy makes the decidual responses and this event is categorized by the initiation of angiogenesis and inflammation. The phenomenon that ISG-15 expression enhances during pregnancy is essential for embryo implantation and maintenance of early pregnancy (Yaginuma et al., 2019). The expression profile of ISG-15 was not investigated in sheep, the present study will focus on the expression and role of the ISG-15 during peri-implantation period.

## Materials and Methods

Blood was collected from sheep at the sheep resources center of The University of Agriculture, Peshawar (Pakistan), according to guidelines of the University. Twelve open Kari sheep were naturally impregnated by rams on day 0 of standing estrous cycle and four sheep were selected as control. Blood were

collected from jugular vein of all sheep at day 6, 11, 12, 13, 14, 16, 17, 21, 23 and 25 and transported to the laboratory using ice box.

### **Isolation of PBMC (peripheral blood monocyte) and RNA extraction**

The blood samples were diluted with equal volume of PBS and the suspension was layered onto Ficoll solution, centrifuged at 6.000 g for 25 min at 4 °C. The samples were incubated for 5 min at 37 °C and then centrifuged at 300 g for 10 min. The supernatant was discarded, and the pellets were washed with 10 mL of PBS and centrifuged for 10 min at 300 g. After removal of the supernatant, the pellets were lysed with lysis buffer for protein extraction or TRIzol reagent (Life Technologies, Grand Island, NY, USA) for RNA extraction. The samples were stored at -80 °C until RNA extraction.

### **Real-time RT-PCR**

The cDNA samples were synthesized by annealing the oligo<sub>dT</sub>-18 primer at 72°C for 5 min followed by cooling of the samples on ice. Thereafter, 5 x reverse transcriptase (RT) buffer, 10 mM dNTPs, 20 U RNase inhibitor (Thermo Scientific) and 200 U of M-MLV reverse transcriptase (Thermo Scientific) were added to a final reaction volume of 20 µL. The samples were incubated at 42 °C for 60 min and then at 70 °C for 15 min to terminate the reaction. The amplification reactions were performed in a 20 µL reaction volume containing 1 µL of cDNA, 10 µL of dreamTaq green PCR Master Mix (2x), 7 µL of sterile water and 1 µL each of the forward and reverse gene-specific primers (10 µM). The PCR samples were then analyzed on 2 % gel. The primer sequences and GenBank accession numbers for each gene are listed in Table 1.

### **Statistical Analysis**

The relative mRNA levels of the different genes were analyzed using latin square design (LSD) and one-way ANOVA. The effects of the different treatments and endpoints (control vs natural breeding, ISG-15 treatment, pregnancy status, days) on the expression of ISG-15, UBE1L and UBCH8 were analyzed using one-way ANOVA, SPSS (SPSS16.0, Inc.).

**Table 1. The primer sequences and GenBank accession numbers for each gene  
Primer pairs (F= forward; R= reverse) used for PCR**

Gene symbol	Primer sequence 5'-3'	bp	Accession no
<i>ISG-15</i>	F: CCATGACGGTATCCGAGCTA R: GGCCTCCCTTCAAAAGACA	317bp	NM_174366.1
<i>UBE1L</i>	F:GTGTTTCATACCGCACGTGAC R:GGTTGTGGCAGGAATGTACC	109bp	NM_001012284.1
<i>UBCH8</i>	F:AGAATTCAGAAGGAACTTGCAG R:AAGGTGACCTTGGGGGGTTTA	195bp	NM_001191190.1
<i>GAPDH</i>	F:CTCCCAACGTGTCTGTTGTG R:TGAGCTTGACAAAGTGGTTCG	222bp	NM_001034034.2

## Results

### Expression of ISG-15 on successive days of pregnancy

The graph indicates the expression of ISG-15 in PBMC on different days after conception (Figure 1.1). The relative expression of ISG-15 was only seen in pregnant ewes. ISG-15 expression was observed from early days of pregnancy after conceptus elongation (starting from day 11). The highest expression was observed on day 12 after insemination and was also seen on following days (day 13 till 21) while the lower expression was found on day 6 and later days (day 23 and 25). Similarly, the expression on day 16 was also significantly higher compared to day 11. After day 21 no expression of ISG-15 gene was found in pregnant ewes. The control and non-pregnant animals exhibit minimal or no expression compared to the pregnant ones.

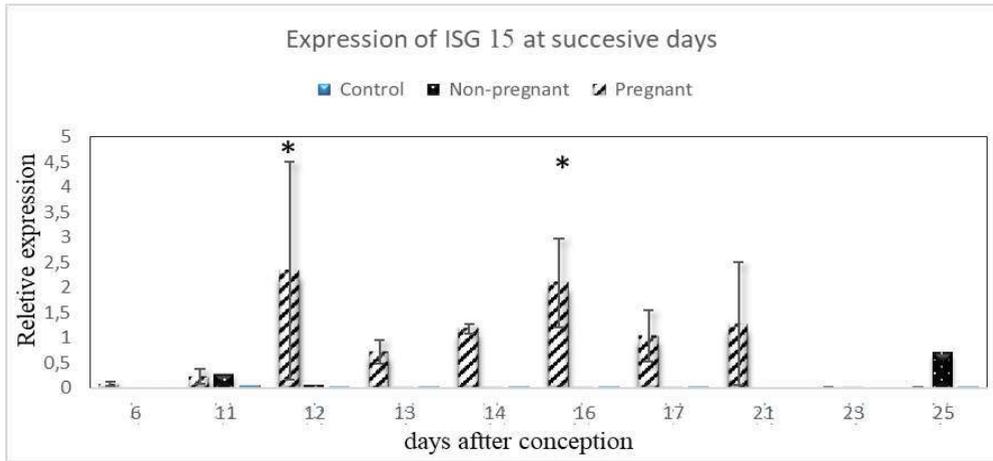


Figure 1.1 Chart of ISG-15 on important days (\* indicates P < 0.05)

### Expression of UBE1L gene on successive days of pregnancy

Figure 1.2 demonstrates the UBE1L expression on progressive long stretches of pregnancy. The UBE1L expression was observed in control, pregnant and non-pregnant sheep at day 6. Generally, the expression was checked in pregnant sheep. The most surprising expression was recorded on day 6 after mating. Then expression of UBE1L was observed at day 11 followed by day 14, afterwards no expression was observed in pregnant and non-pregnant sheep.

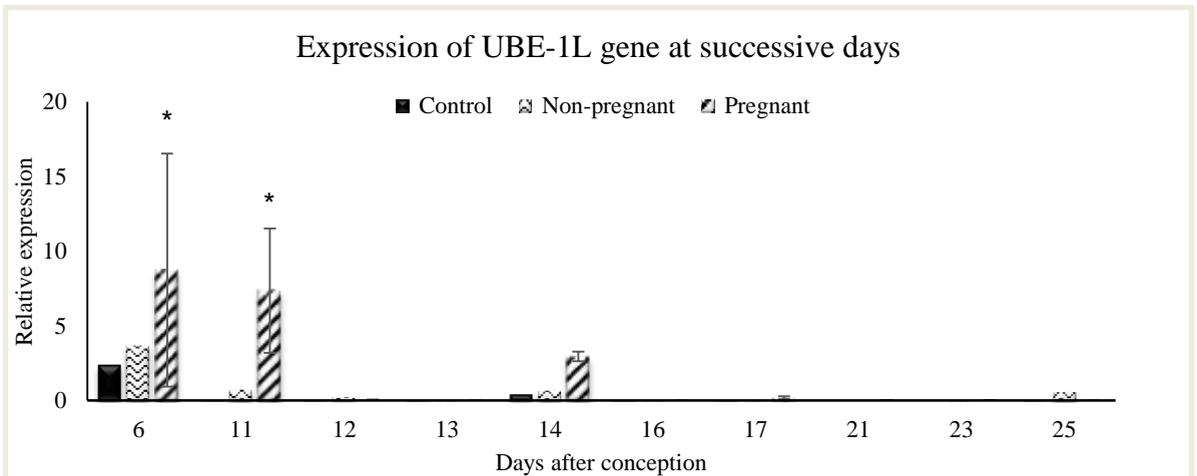
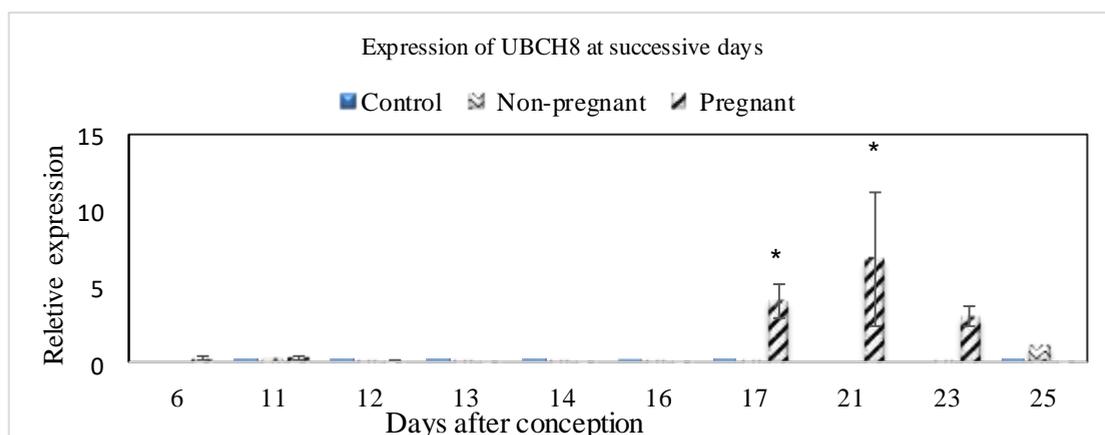


Figure 1.2 UBE1L on selected day\* indicates significance difference compared to day 6, p < 0.05, \* indicates significance difference compared to day 11, p < 0.05

## Expression of UBCH8 gene on successive days of pregnancy

The Figure 1.3 demonstrates the UBCH8 expression in PBMC on various pregnancy days. The expression was only observed in pregnant sheep. The highest expression was observed on day 21 and moderate expression was recorded on day 17 and 23. While, no expression of UBCH8 were found in control and non-pregnant ewes.



**Figure 1.3 Chart of UBCH8 on important days\* indicates higher expression in pregnant sheep  $P<0.05$  while minimal expression in non-pregnant and control indicates  $P<0.005$**

## Discussion

The expression of ISG-15 was observed in early day (from day 12 till 21) of the pregnancy after mating only in pregnant ewes. The expression of ISG-15 ISGylation enzyme UBE1L and UBCH8 in PBMC was observed at early days of pregnancy, in which the higher expression of UBE1L were found on day 6. However, the expression of UBE1L was observed in pregnant, non-pregnant and control sheep. The expression of UBCH8 was also found significantly different from UBE1L, which was found only in pregnant ewes. There was no expression of UBCH8 in non-pregnant and control ewes. Similar to our results, *Haq et al. (2016)* investigated this work in bovine in which they found the high expression of ISG-15 and its ISGylation enzymes UBE1L and UBCH8 in early pregnant cows. They found these genes have role in the establishment of early pregnancy in cow, and also investigated that ISG-15 conjugates to target protein through UBE1L and UBCH8 while further E3 enzyme to control pathogenicity. *Kiyama et al. (2016)* likewise explored the outflow of ISG-15 is exceptionally up-regulated in the

endometrium of early pregnant ewes and they found the high accumulation of ISG-15 on day 13 after developing life implantation. In the current study, the most noteworthy ISG-15 expression was observed on day 12 of the pregnancy, which may due to the exceptional shorter gestation length of Kari sheep (*Reagan-Shaw et al., 2008*). *Ling et al. (2017)* examined this work in bone marrow of ewes during early pregnancy. They investigated that ISG-15 is highly expressed in early pregnant ewes during embryo implantation. They have also shown that ISG-15 ISGylation enzymes UBE1L and UBCH8 were expressed during early pregnancy and were involved in the establishment of early pregnancy, basically they are anti-pathogenic. Similarly, this work shows the expression of ISG-15, UBE1L and UBCH8 during early days of pregnancy in Kari sheep. *Yang et al. (2010)* worked on finding ISG-15 and related proteins in bovine endometrium during early pregnancy. ISG-15 was highly expressed in early pregnant cows and the expression of ISG-15 mediates the conjugation of some genes that helped ISG-15 conjugate to target protein during embryo implantation. They also investigated that the ISGylation gene UBE1L are expressed in all cyclic and pregnant cows.

## Conclusion

In the light of above findings, we concluded and suggest that ISG-15 was highly expressed in PBMC in early pregnant ewes and the ISG-15 ISGylation enzyme UBE1L and UBCH8 are involved in the establishment of early pregnancy. Furthermore, ISG-15 is ubiquitin-like protein (*Zhang et al., 2005*) stimulates its ISGylation enzyme UBE1L and UBCH8 to control pathogenicity during early implantation of embryo.

## Ekspresija ISG15 i enzima za konjugaciju tokom perioda peri- implantacije kod ovaca

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## Rezime

Uspostavljanje graviditeta kod preživara utiče na regulaciju brojnih gena. Interferonski stimulativni gen (ISG15), ubikvitinu-sličan protein koji posreduje u konjugaciji različitih proteina preko ISGilacionih enzima UBE1L i UBCH8, takođe se različito izražava tokom ranog graviditeta. Svrha ove studije bila je istražiti ulogu ISG-15 u uspostavljanju graviditeta i elongacije zametka tokom ranih perioda nakon začeća i utvrditi prisustvo ISG enzima UBE1L i UBCH8. Zbog toga

su ovce sinhronizovane korišćenjem kloprostenol natrijuma i gonadotropin oslobađajućeg hormona-1 i pripuštene ovnovima. Krv je sakupljana u danima nakon parenja 6, 11, 12, 13, 14, 16, 17, 21, 23 i 25. Prajermeri ISG-15, UBE1L i UBCH8 korišćeni su za amplifikaciju odgovarajućeg transkriptomskog regiona korišćenjem PCR. Brzina oporavka svakog transkriptomskog fragmenta je upoređena sa GAPDH. Ekspresija ISG-15 bila je veća 12. dana, suprotno od UBE1L gde je bila viša 6., a UBCH8 21. dana. Štaviše, ISG-15 je protein koji je sličan ubikvitinu, posreduje enzimima UBE1L i UBCH8 za zaštitu zametka od virusne patogenosti tokom rane trudnoće.

**Ključne reči:** ISG-15, ovce, graviditet, ISG, koncepcija, ubikvitinacija

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