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Expression of Interferon-Stimulated Gene 15 in Endometrial Explants from Early Pregnant Cows *in vitro* for 60 Days

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Abstract: Interferon-tau is generally regarded as the primary signal for maternal recognition in ruminant animals. Expression of interferon-stimulated gene 15 (ISG15) is up-regulated in bovine endometrium during early pregnancy. The experimental materials were from our primary experiment. Tissue explants of endometria from day 18 of pregnant and non-pregnant cows were cultured *in vitro* for 60 days and then the expression of ISG15 in these explants was detected by western blot. The results indicated that ISG15 and conjugated proteins were still expressed at a high level in endometrial explants from pregnant cows after cultured *in vitro* for 60 days and there was no expression in endometrial explants from non-pregnant cows after cultured *in vitro* for 60 days. In conclusion, expression of ISG15 in endometrial explants from early pregnant cows maintained *in vitro* for a long time.

Key words: Endometrium, ISG15, bovine, *in vitro*, pregnant cow, primary experiment

INTRODUCTION

During early pregnancy in bovine, expression of Interferon-τ (IFNT) is up-regulated by conceptus trophectoderm cells and IFNT is generally regarded as the primary signal for maternal recognition (Roberts *et al*., 2008). Endometrial interferon-stimulated gene 15 (ISG15) syntheses coincide with the up-regulation of IFNT in bovine (Austin *et al*., 1996). Based on western blot, endometrial ISG15 and conjugated proteins are detected on day 17 of pregnancy, peak to the highest levels from day 18-23 and then decline to low levels by day 45 in bovine. Finally, ISG15 and conjugated proteins are not presented in endometria after day 50 of pregnancy or in non-pregnant cows (Austin *et al*., 2004). Due to cross-reactivity with ubiquitin antibody, ISG15 is originally named Ubiquitin Cross-Reactive Protein (UCRP). Unlike ubiquitination, ISG15 does not conjugate to target proteins for degradation but enhanced the cellular response to IFNs by increasing Jak1 and Stat1 activity (Malakhova *et al*., 2003).

ISG15 alters the functions of a vast number of proteins through its modification which plays a critical role in many cellular processes, including transcription, DNA repair, signal transduction, autophagy and cell-cycle control (Kerscher *et al*., 2006). Bovine ISG15 conjugates to a variety of uterine cytosolic proteins during early pregnancy, suggesting that ISG15 may play a key role in establishment and maintenance of pregnancy (Johnson *et al*., 1998). It is difficult to imitate blastocyst elongation and embryonic implantation *in vitro* because this process involves in an intimate cross-talk between the embryo and the uterus.

Hatched blastocysts and trophoblastic vesicles which are transferred into the uterus can elongate normally but do not do so *in vitro* because the uterus is required for blastocyst elongation and trophectoderm outgrowth (Spencer *et al*., 2007). Bazer *et al*. (2009) also reported that the establishment of uterine receptivity for implantation is regulated by IFNT secreted by ruminant conceptus. The expression of IFNT is regulated by uterine-derived factors such as granulocyte-macrophage colony-stimulating-factor and fibroblast growth factor 2 (Ealy and Yang, 2009). Lack of uterine internal environment which IFNT induces may result in difficulties to imitate blastocyst elongation and embryonic implantation *in vitro*. If the uterine status which IFNT induces would be maintained for some time *in vitro*, this will be helpful for study on trophoblastic differentiation, gene expression and maternal-embryonic interactions in ruminant animals. In this study, we planned to explore if the endometrial status from early pregnant cows would be maintai for a long time *in vitro* based on analysis of endometrial ISG15 expression.

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MATERIALS AND METHODS

Animal and experimental design: In the primary study (Yang *et al*., 2010), we explored the expression of ISG15 in corpora lutea and endometria during bovine early pregnancy and the experimental materials were from this experiment. In the local experimental farm, Holstein cows were bred and fed a Total Mixed Ration (TMR). Animal care and experimental procedures were approved and conducted under established standards of the agricultural animal care and use committee of China Agricultural University. Controlled Internal Drug Releasing Devices (CIDR; InterAg, Hamilton, New Zealand) were used to synchronize estrous cycles. Estrous behavior was monitored three times per day. The day of Artificial Insemination (AI) was counted as day 0 of pregnancy. Cows assigned to non-pregnant group were not inseminated (control). Endometria were sampled from pregnant and non-pregnant cows on day 18 (n = 4 for each group) after slaughter. Pregnancy was confirmed by the presence of a conceptus. Some tissues were used for explants culture studies and the remains were stored at -80°C for protein analysis.

Endometrial explants culture: Tissue explants were cultured by the method described by Austin *et al.* (1996) with modifications. Briefly, uteri sampled from day 18 of pregnant and non-pregnant cows were placed in sterile Phosphate-Buffered Saline solution (PBS) and immediately transported to the laboratory at room temperature. About 200 mg of each endometrial tissue was minced into small (approximately 2 mm cubes) pieces in a 60 mm culture plate containing 5 mL Dulbecco's modified Eagle's medium with F12 salts (DMEM/F12; Gibco BRL; Grand Island, NY, USA). The small endometrial pieces were suspended in a new 60 mm culture plate containing 5 mL DMEM/F12 including 10% fetal bovine serum (FBS; Gibco BRL; Grand Island, NY, USA) and cultured in an atmosphere of 5% CO_2 in air at 37°C for 60 days. The medium was changed every 2 days. Finally, the endometrial explants were stored at -80°C for protein analysis.

Western blot: The endometrial explants and endometria were homogenized at a ratio of 1 g endometrial explants or endometria per 5 mL Laemmli buffer. Western blot was performed as previously described (Yang *et al*., 2010). ISG15 was detected by western blot using mouse anti-bovine ISG15 monoclonal antibody (5F10, generously provided by Dr. Thomas R. Hansen (Colorado State University, Fort Collins, CO, USA), 1:100,000). Immunoreactive proteins greater than 30 KDa were deemed conjugates (Joyce *et al*., 2005) and semi-quantified together.

Statistical analysis: The experiments were repeated at least three times and the data were examined by ANOVA procedures of the Statistical Analysis System Package version 9.1 for Windows (SAS Institute, Cary, NC). All data were expressed as least squares means. Differences were considered very statistically significant at the 99% confidence level.

RESULTS AND DISCUSSION

The results indicated that ISG15 and conjugated proteins were expressed at high levels both in endometrium from day 18 of pregnant cows and in these explants after cultured *in vitro* for 60 days and there was no significant difference between them (Fig. 1a, b).

Therefore, we speculated that the biophysical function of these explants may not change significantly, although they were cultured *in vitro* for 60 days. In addition, there was no expression of ISG15 in day 18 of non-pregnant endometrium and these explants after cultured for 60 days (Fig. 1a, b), so we suggested that the explants were not induced expression of ISG15 *in vitro* by this culture media. The endometrium does not express ISG15 and conjugated proteins after day 50 of gestation in bovine (Austin *et al*., 2004).

However, the results showed that endometrial explants from day 18 of pregnant cows could express ISG15 and conjugated proteins at high levels after cultured *in vitro* for 60 days. It is known that IFNT mRNA is expressed by bovine conceptus from day 14 of gestation, peaks on days 17-18 and declines from days 21-26 (Farin *et al*., 1990).

The expressional pattern of ISG15 mRNA is similar to that of IFNT in the bovine endometrium during early pregnancy (Hansen *et al*., 1997). Up to now, little is to know why IFNT expression ceases during embryonic implantation to the uterine lining. The placental gene expression is altered as uterine attachment begins, these genes includes a series of cell attachment factors. These cell attachment factors are involved in placentation in cattle (Burghardt *et al*., 2002; Spencer *et al*., 2004) and they may be responsible for the termination of IFNT expression (Ealy and Yang, 2009). The result also showed that these high levels of expression of ISG15 and conjugated proteins were not induce by any other factor *in vitro* but it was due to initial expression of ISG15 in these endometrial explants. Therefore, we may speculate that the loss of expression of free and ISG15 conjugated proteins in endometrium *in vivo* may be owing to the impact of uterine-derived factors and hormones and this

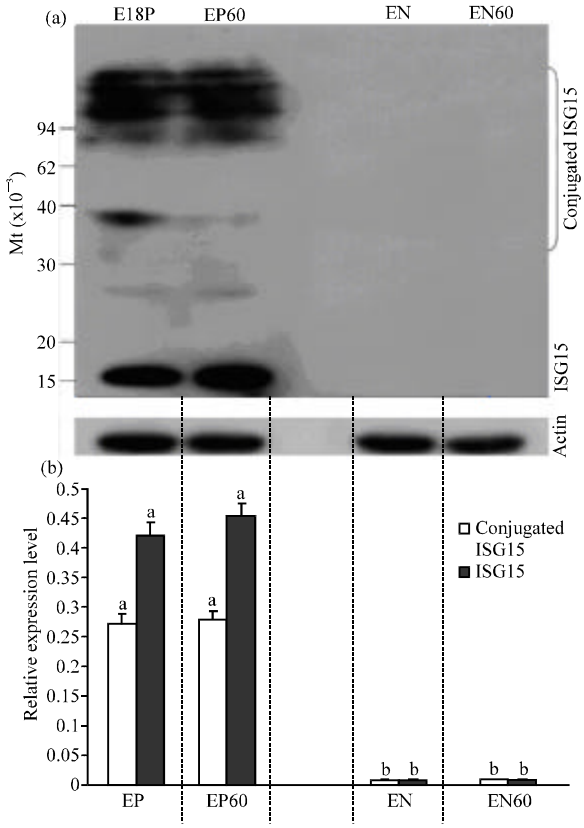

Fig. 1: Expression of interferon-stimulated gene 15 (ISG15) and its conjugated proteins in bovine endometrium from day 18 of pregnancy and non-pregnancy and these explants after cultured for 60 days. Protein extracts (100 μg/lane) were analyzed by Western blotting with a specific anti-ISG15 antibody and an anti-actin antibody was used to monitor variation in loading of samples (a). Data (means, n = 4) were shown in (b). All data represent relative values of expression of ISG15 and conjugated proteins compared with actin. Immunoreactive proteins greater than 30 kDa were deemed conjugates and semi-quantified together. Very significant differences ($p<0.01$) are indicated by different superscript letters within the same color column. Note: E18P = Endometrium from day 18 of pregnancy; EP60 = The endometrial explants from day 18 of pregnancy cultured for 60 days; EN = Endometrium from day 18 of non-pregnancy; EN60 = The endometrial explants from day 18 of non-pregnancy cultured for 60 days

loss of expression of ISG15 may be helpful for embryonic development of post-implantation. It is known that intracellular ISG15 and its conjugates is present in bovine endometrium from day 17 of gestation, peaks from day 18-23 and then declines to low but detectable levels by day 45. ISG15 and its conjugates are not detected on day 50 of pregnancy or during the estrous cycle (Austin *et al*., 2004). The results showed that expression of ISG 15 and conjugated proteins in endometrial explants from early pregnant cows maintained *in vitro* for a certain time. ISG15, as an UCRP, contains two domains with structural homology similar to ubiquitin (Narasimhan *et al*., 2005). It can bind covalently its target proteins to regulate their function as well as affect their half-life, subcellular localization, enzymatic activity and ability to interact with protein or DNA partners (Dye and Schulman, 2007). Austin *et al.* (2004) postulated that unlike ubiquitination, protein ISG15 modification (ISGylation) did not target proteins for degradation but stabilized proteins in endometrium. Klein *et al.* (2006) also reported that, along with the increase of ISG15 and E1-like enzyme (UBE1L), four potential components of the ISG15ylation system were found at increased levels which are probably involved in the regulation of the response of the endometrium to the signaling of the embryo. In addition, IFNT, one of two families of immunoregulatory molecules in ruminants (Hansen and Tekin, 2005) may regulate the immunosuppressive effects on uterine immune function through ISG15ylation which may be helpful for embryonic development.

The pre-implantation trophoblast grows robustly in size and particularly in length at the peak production of IFNT while the conceptuses remain free within the uterine lumen (Roberts *et al*., 2008). The growth and differentiation of the inner cell mass to an embryonic disc is coincident with the elongation of the trophoblast in ungulates (Blomberg *et al*., 2008).

The endometrial intracellular ISG15 and its conjugates also peak at this time (Austin *et al*., 2004), so we speculated that the high level of ISG15 may be necessary for embryonic growth and implantation in bovine. It is known that ruminant elongated embryos provide a unique sthece of information about trophoblastic differentiation, gene expression and maternal-embryonic interactions but they are difficult and costly to obtain, especially bovine elongated embryos. Failure of ungulate conceptus elongation *in vitro* indicates that cross-talk between fetal and maternal compartments is critical for this process (Blomberg *et al*., 2008).

Therefore, it was possible to culture ruminant elongating embryos *in vitro* together with the endometrial explants from day 18 of pregnant cows which may be helpful for the culture of ruminant elongating embryos. The current *in vitro* culture systems are not suitable for the bovine embryos of post-hatching and elongating stages. Helige *et al.* (2008) reported that tissue fragments from human first trimester decidua parietalis were used as a model of trophoblastic invasion.

We also found that the *in vitro* endometrial explants were not present significant change at 1st week. After that

the stromal cells grew from the explants and then the glandular epithelial cells predominantly grew from the explants. In addition, high levels of ISG15 were expressed by endometrial explants from pregnant cows after cultured *in vitro* for 7 days through western blot.

Therefore, we speculated that the endometrial explants from early pregnant period may be used to construct *in vitro* culture model and the endometrial explants cultured *in vitro* at 1st week may be suitable for co-culture with the embryos at post-hatching and elongating stages. This *in vitro* model will be helpful for research on trophoblastic differentiation, gene expression and maternal-embryonic interactions which may be useful for decreasing pregnant loss and increasing pregnant rate.

CONCLUSION

The results of this study indicated that endometrial explants from day 18 of pregnant cows maintained expression of ISG15 and conjugated proteins at high levels *in vitro* for a long time. Therefore, it suggested that the loss of expression of free and ISG15 conjugated proteins in endometrium *in vivo* may be owing to the impact of uterine-derived factors and hormones or other reasons but not due to the lack of expression of IFNT. In addition, we speculated that the endometrial explants from early pregnant period may be used to construct *in vitro* culture model for the culture of embryos at post-hatching and elongating stages.

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