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# Expression profile of interferon tau–stimulated genes in ovine peripheral blood leukocytes during embryonic death

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ABSTRACT

Early and efficient detection of embryonic death (ED) has a valuable impact as important as early pregnancy diagnosis in ruminants. Among early pregnancy diagnosis methods, detection of the expression of interferon tau–stimulated genes (ISGs) in peripheral blood leukocytes (PBLs) is well documented in cows and ewes. Therefore, we hypothesized that the expression profile of ISGs in PBLs might also be useful for detecting ED in these animals. For this purpose, pregnant ewes were used as an experimental model. Pregnancy was detected on Day 18 after mating by transrectal ultrasonography. Pregnant ewes were divided into a control group (sham injection on Day 18, n = 10) and ED group (treated with 75 μg synthetic $PGF_{2\alpha}$ on Day 18, n = 12). PBLs and plasma were collected on Days 0 (mating day), 15, 18, 19, 20, 21, 23, and 25 by jugular venipuncture. Total RNA was isolated from PBLs. ISGs expression levels were determined by real-time polymerase chain reaction in triplicate. Electrochemiluminescence immunoassay was used to measure progesterone ($P_4$) levels in plasma. In the ED group, the $P_4$ level declined to less than 1 ng/mL on Day 19 and remained at a low level until the end of the study. Compared with that on Day 0, receptor transporter protein 4 (*RTP4*) and *ISG15* expression was upregulated on Day 15 and remained high until Day 21 in both groups, and *RTP4* and *ISG15* mRNA levels were attenuated on Days 23 and 25 only in the ED group ($P < 0.001$). *Myxovirus resistance 1* expression was upregulated on Day 15 and remained high until Day 23 in both groups, but was attenuated on Day 25 in the ED group ($P < 0.05$). The *B2-microglobulin* mRNA level did not change significantly during the study in either group. These results indicate that the decline in $P_4$ concentration was an immediate response to $PGF_{2\alpha}$ and that the embryo may have survived longer than the CL on the basis of the extended period of ISGs expression. This suggests that the absence of $P_4$ could be the reason for ED rather than a direct effect of $PGF_{2\alpha}$. In conclusion, the expression of ISGs, including *ISG15*, *RTP4*, and *myxovirus resistance 1*, but not *B2-microglobulin*, in PBLs may serve as a marker of ED.



## 1. Introduction

The establishment and maintenance of pregnancy in ruminants including maternal recognition of pregnancy, implantation, and placentation depend on communication

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between the maternal system and the conceptus, and are regulated by many factors such as progesterone ($P_4$) and interferon tau (IFN-tau). Communication failures most frequently result in termination of the pregnancy [1]. Despite the high fertilization success rate in ruminants, low birth rates clearly indicate the occurrence of embryonic death (ED) and fetal losses during pregnancy [2]. Most EDs occur during the periimplantation stage of pregnancy and directly affect fertility by extending the pregnancy interval and reducing offspring number in many species, including ruminants [3]. Most of the pregnancy losses (20%–30%) occur during the embryonic stage of pregnancy in ewes [4,5]. At present, one of the most effective fertility management strategies to prevent and reduce economic losses is to identify pregnant or nonpregnant animals as soon as possible after insemination, together with application of resynchronization programs in modern commercial enterprises [6,7]. Therefore, early and efficient detection of ED tends to increase reproductive profitability. Many techniques for monitoring ED have been evaluated for ruminants. Ultrasonography is used as a detection method during earlier stages of pregnancy; however, it requires a skilled veterinarian [4]. Measurement of progesterone levels has disadvantages in terms of not being specific to pregnancy, and the occurrence of false-positive results because of extended interestrus intervals [8]. Pregnancy-associated glycoproteins are also not promising for this purpose, owing to their long half-life in blood plasma [8–11].

Apart from endometrium, increased expression profiles of genes, called *interferon stimulated genes* (*ISGs*; *myxovirus resistance 1* (*MX1*), *MX2*, *ISG15*, *receptor transporter protein 4* (*RTP-4*), the *2′-5′-oligoadenylate synthase 1*, and so forth), are also found outside of uterine tissues, such as in the CL and peripheral blood leukocytes (PBLs), during pregnancy [12,13]. The question is whether extrauterine presence of ISGs can be used for the early, noninvasive detection of pregnancy. Initially, Yankey et al. [14] reported significant increases in *MX1* and *MX2* mRNA levels on Day 15 after insemination in the pregnant ewes. Subsequent studies have confirmed these results in dairy cows [15–17]. Moreover, exogenous administration of IFN-tau upregulates *ISGs* expression in the endometrium, CL, and PBLs *in vivo* [18,19]. The idea is that expression pattern of ISGs in PBLs could be a gold indicator of embryonic development as emphasized by many scientists [19,20]. A dying embryo would not produce sufficient amounts of IFN-tau to affect PBLs; therefore, monitoring ISG levels in those animals could facilitate detection of ED versus healthy pregnancies. Matsuyama et al. [19] reported that nonpregnant cows with an extended interestrus interval had variable *ISG15* levels in their PBLs after embryo transfer. This variability was explained by ED. Owing to the impossibility of knowing the time of ED after embryo transfer; the *ISGs* expression profiles during ED are unknown. Therefore, in this study, we investigated the *ISGs* expression profiles in PBLs during ED. Pregnant ewes were chosen as an experimental model and were induced to undergo ED by injection of $PGF_{2\alpha}$. The mRNA levels of ISGs, including *RTP4*, *interferon tau–stimulated gene 15* (*ISG15*), *B2-microglobulin (B2M)*, and *MX1*, were investigated in their PBLs.

## 2. Materials and methods

### 2.1. Animal materials

All animal experimental procedures were approved by the Local Ethics Committee of Dicle University (2011/66). The field study was completed during the breeding season in southeastern Turkey (August–September 2013). Ewes were kept on the pasture during the day and were placed in pens during the night. Ewes with at least one birth in the records and no health problems (n = 22, aged 3–5 years) were used in this experiment. Estrous cycles were synchronized by two intramuscular injections of $PGF_{2\alpha}$ (75 -μg d-cloprostenol,Dalmazin, Vetaş Istanbul, Turkey) at 10-day intervals. Estrus was detected by a teaser ram. Ewes were mated with fertile rams on Day 0. Pregnancy was determined as the presence of an embryo and embryonic vesicle by transrectal ultrasonography on Day 18 after mating. Then, pregnant ewes were divided into a control group (sham injection on Day 18, n = 10) and ED group (ED; treated with 75 -μg synthetic $PGF_{2\alpha}$ on Day 18, n = 12). Effect of the treatment on pregnancy was also evaluated by transrectal ultrasonography on Day 25. Blood samples were collected serially (on Days 0, 15, 18, 19, 20, 21, 23, and 25 after mating) from both groups. All blood samples were transferred to the laboratory on ice within 1 hour after collection.

### 2.2. PBLs isolation and total RNA extraction

The PBLs were isolated using a 10 -mL tube containing Na-ethylenediaminetetraacetic acid according to Kurar et al. [21]. Briefly, a 10 -mL blood sample was centrifuged at 300 × g for 20 minutes at 4 °C. The buffy coat was harvested and resuspended in 1:5 (v:v) 0.87% Tris-$NH_4Cl$ lysis buffer. Samples were incubated at 37 °C for 10 minutes and then centrifuged at 300 × g for 10 minutes. The PBL pellet was washed with 10 -mL ice-cold PBS buffer and subjected to total RNA extraction. The RNA isolation, quality control, genomic DNA removal by DNase-I, and complementary DNA (cDNA) synthesis procedures were conducted as described by Atli et al. [22]. Briefly, total RNA isolation was performed using TRIzol reagent (Invitrogen, USA). RNA samples (2 μg) were cleaned of possible genomic DNA contamination by DNase-I treatment and then subjected to reverse transcription to synthesize first strand cDNA using the RevertAid First-Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions.

### 2.3. Quantification of expression by real-time PCR

Primers for *ISGs* (*MX1*, *RTP4*, *ISG15*, and *B2M*) were derived from published studies [12,15] or known ovine sequences using Primer3 and the NCBI database (http://www.ncbi.nlm.nih.gov/). Gene expression profiles were evaluated on blood sampling days (i.e., Days 0, 15, 18, 19, 20, 21, 23, and 25 after mating) in both the control and ED groups. The reaction was set up as follows: 5 -μL SYBR Green Master Mix (2 × ), 5 pmol of each primer, 1 -μL cDNA, and $ddH_2O$ to a final volume of 10 μL. Thermal