

**Research Article****Apoptosis in ovine placentome in spontaneous and induced parturition using Aglepristone****Özalp RG<sup>1\*</sup> and Zık B<sup>2</sup>**

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**Abstract**

The effects of Aglepristone treatment on placental expulsion in ewes were investigated. Fifteen crossbreed (Kivircik × Chios) pregnant does were randomly divided into three groups. Aglepristone was injected at the rate of 5 (AG5) and 10 mg/kg (AG10) once a day subcutaneously on day 140 and 141 of mating respectively. Ewes in control group (CG) were treated with 0.9% sodium chloride solution subcutaneously at the same day of pregnancy. Placentome samples were collected right after the expulsion of the last lamb. Expelled foetal membranes were weighed and expulsion period was recorded. Mean interval of expulsion of last lamb was not significant and the weight of expelled membrane was significant between AG5, AG10 and CG. In spite of non-significance of the expulsion interval between groups, the ewes in AG5 completed the placental expulsion beyond 10 hours. Therefore, tissue samples from ewes in AG5 were stained by TUNEL assay in order to assess the apoptosis. No significant difference in the number of apoptotic cells between control and Aglepristone groups was found ( $P>0.05$ ). A few binucleated trophoblast cells (BNC) were detected in AG5. The results indicate that Aglepristone treatment at the rate of 5 mg/kg could prolong the time between expulsion of the last lamb and foetal membranes with reasonable number of apoptotic cells.

**Keywords:** Aglepristone; ewe; apoptosis; foetal membrane retention

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**Introduction**

The failure of expulsion of foetal membranes within 6 hours after lambing is generally accepted as pathologic condition in ewes (Fthenakis et al., 2000; Leontides et al., 2000). The incidence of retained placenta has been reported as 6.4% in small ruminants (Franklin, 1986). The risk of retained foetal membranes (RFM) increases 4-folds higher after lambing induction

and obstetrical assistance, neonatal death and stillbirth that disrupt the separation and expulsion process (Leontides et al., 2000). These factors are associated with foetal membrane retention in ewes and the contribution in disruption of process of loosening and separation of placentome has been found (Leontides et al., 2000; Troedsson, 2008).

Artificial induction of parturition by the application of progesterone receptor blocker Aglepristone,

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glucocorticoid and/or PGF<sub>2α</sub> results in retained placenta in cows (Lewing et al., 1985; Garcia et al., 1992; Shenevai et al., 2010). However, these treatments do not cause retention of foetal membranes in ewes (Rubianes et al., 1991; Kastelic et al., 1996). Anti-progesterone, RU486, was also tested in control of lambing performance in ewes and rapid placental delivery (207 min) was recorded (Gazal et al., 1993).

Lambing was synchronized by the application of Aglepristone with two different doses in ewes by our working group (Ozalp et al., 2014; unpublished data). Placental release period was not statistically significant between groups; however, four of ten ewes showed retained foetal membranes beyond 6 hours. Recent studies in bovine placentome have shown that the incidence of retained foetal membranes has strong correlation with apoptosis (Boos et al., 2003; Hirayama et al., 2012). As no data is available about the placental delivery after Aglepristone application in ewes, this will be the first report to confirm medicinal effects of a progesterone receptor blocker by TUNEL test.

## Materials and Methods

Approval from the Ethics Committee of the Uludag University to use the animals was obtained. Fifteen healthy crossbreed (Kivircik × Chios) ewes were subjected to oestrus synchronization by inserting intravaginal sponges containing 30 mg of Cronolone Fluorogestone Acetate (Chrono-gest CR, Intervet, Turkey) for 11 days and received 400 IU pregnant mare serum gonadotrophin (Chronogest/PMSG, Intervet, Turkey) intramuscularly when the sponge was withdrawn. On day 12-14, the ewes were exposed to rams and breeding date was recorded. Pregnancy was confirmed by trans-rectal ultrasonographic examination 35 days after mating. Confirmed pregnant ewes were assigned to three groups. The ewes in group AG5 (n=5) and AG10 (n=5) were treated with Aglepristone (Alizin, Virbac, Germany) at a dose of 5 and 10 mg/kg respectively. The application was achieved subcutaneously once daily on two specific consecutive days (on days 140 and 141 post-mating). The ewes in control group (CG; n=5) received 0.9% NaCl solution (Eczacibasi, Baxter, Turkey) subcutaneously.

The ewes were controlled hourly and lambing parameters were recorded. Placental expulsion was controlled in terms of retention of foetal membranes. Expelled foetal membranes were weighed and expulsion period was recorded. Immediately after lambing, two placentomes were collected from each animal per caesarean section. Samples were fixed with a solution containing 4% paraformaldehyde for 24 h. Tissue samples were subsequently dehydrated in a graded ethanol series and finally embedded in paraffin. Five µm thick sections were cut from paraffin blocks,

mounted on slides, and dried overnight. After dewaxing and rehydration, sections were used for Crossman's triple staining to determine placenta morphology and *in situ* terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labelling (TUNEL) was used to determine the apoptotic cells. Histological examination was carried out using a light microscope (Nikon Eclipse 80i; Tokyo, Japan).

## Detection of apoptosis

To visualize apoptotic cells, sections were stained by the TUNEL method using an *in situ* Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol with slight modifications. Briefly, the deparaffinized and hydrated sections were washed in PBS, and then treated with 20 µg/ml proteinase K for 20 min at 37°C. Specimens rinsed in PBS were immersed in 3% H<sub>2</sub>O<sub>2</sub> for 10 min at room temperature to inhibit endogenous peroxidase activity. After rinsing in PBS for 10 min, the specimens were made permeable on ice with 0.1% Triton X-100 for 5 min. The sections then were incubated in the dark at 37°C for 1 h with the TUNEL reaction mixture, which contained terminal deoxynucleotidyl transferase (TdT) plus dUTP label. After labelling, samples were washed with PBS and treated with anti-fluorescein antibody TUNEL-POD (Roche Diagnostics) at 37°C for 1 h. TUNEL-POD was visualized by incubating with DAB for 10 min. Sections then were counter stained with methyl green, 0.5% in 0.1 M sodium acetate, pH 4.0, rinsed in distilled water and examined under the microscope. As negative control, sections were incubated with TUNEL label only, omitting either TdT or anti-digoxigenin antibody. Positive control sections received the same treatment, but were pre-treated with DNase I (Roche, Indianapolis, IN) for 30 min at 37°C prior to the TUNEL method. For each section, six fields of view were examined, and the number of TUNEL-positive stained nuclei was expressed as a percentage of the total number of nuclei counted. Apoptotic index (AI) was measured by calculating 100 positive cells in different areas under x400 magnification.

## Statistical analysis

Values were expressed as the mean ± SEM. Statistical analysis between the groups was performed using the Kruskal and Mann-Whitney U-test. Values were considered to be significant at P<0.05.

## Results

### Clinical findings

Mean interval between expulsion of last lamb and foetal membranes was 7.8±4.6, 6.1±2.5 and 6±2 hours in groups AG5, AG10 and CG respectively. Although

no significant difference was found in the expulsion time, yet three goats in AG5 completed expulsion process beyond 10 hours. Small parts of foetal membrane with vaginal discharge were also observed not only during the expulsion period but also 48 hours after parturition. They received no treatment and no pathologic changes were observed in general health condition.

The mean weight of expelled foetal membranes was  $458 \pm 142.1$ ,  $380 \pm 130.3$  and  $688 \pm 92.3$  g in group AG5, AG10 and CG respectively. Mean foetal membranes weight decreased significantly in AG10. This result appears as if greater amount of foetal membranes was expelled by the ewes in group AG, three of ewes in this group completed total expulsion longer than other groups as it was explained above.

### TUNEL Staining

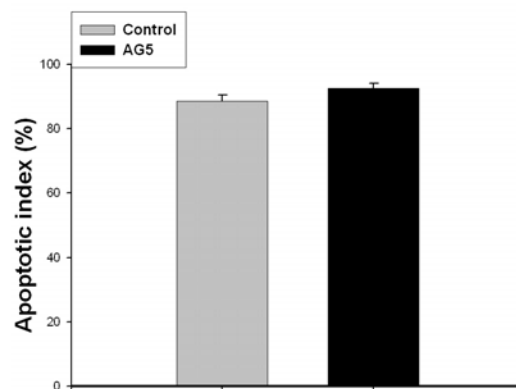
Apoptosis was assessed by TUNEL staining. Presence of apoptotic cells was high in AG5 and CG (Fig. 1). In general, apoptotic cells were smaller than neighbouring epithelial cells and were not in contact with the underlying basement membrane. TUNEL-positive nuclei or their fragments were stained brown (Fig. 2 A, B).

Moreover, the number of apoptotic cells was detected in the Aglepristone treatment groups ( $92.6 \pm 1.48$ ) compared with the control group ( $88.5 \pm 1.99$ ). No significant difference in the number of apoptotic cells between control and Aglepristone group was found ( $P > 0.05$ ) (Fig. 1).

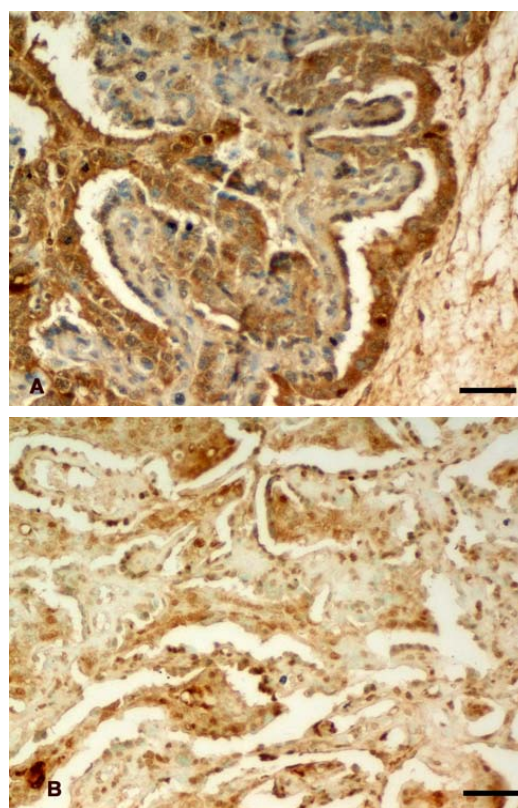
### Discussion

Retained foetal membranes are seldom observed as pathologic condition in ewes (Fthenakis et al., 2000). Dystocia, assisted parturition, abortion and some hormone application to induce parturition does not cause retained foetal membranes, but the time needed for placental expulsion can be longer than normal parturition process (Rubianes et al., 1991; Chassagne and Barnouin, 1993; Majeed et al., 1995; Kastelic et al., 1996).

Apoptotic status of foetal membrane in Aglepristone treated ewes were evaluated in this study. Apoptotic signals were detected by TUNEL assay in caruncular epithelial cells and trophoblast cells. TUNEL staining was obviously the sign of increased cell death but this result was not in accordance to the clinical findings. Similar results of retained foetal membranes in goats after the application of Aglepristone had reported. Different doses have been tested and high proportion of retention was found with lowest dose of Aglepristone application in goats (Batista et al., 2011). The high incidence of placental retention has been associated with the prolonged



**Fig. 1: Apoptotic index (%) detected in placentomes of control and Aglepristone (AG5) treated groups**



**Fig. 2: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) reaction in sheep placentomes at the end of pregnancy in control group (A). Treatment with Aglepristone (AG5; B). Apoptosis (brown stained apoptotic bodies) were observed at high rate within the foetal chorionic epithelium and the maternal crypt epithelium. Bar: 50 µm.**

induction time (Batista et al., 2011). We tested two different doses in ewes and lower dose of Aglepristone caused pathology in ewes which is in accordance with the findings found in literature (Batista et al., 2011).

Since high percentage of apoptotic cells were found in placentome, the time between expulsion of last foetus and placenta were accepted as longer expulsion process, not retained foetal membrane. This condition could be associated with insufficient myometrial activity as it was previously reported after the application of Aglepristone in cows (Shenevai et al., 2012).

Histopathologic changes such as vascular changes (oedema, hyperemia and haemorrhages) both in epithelial and connective tissues, necrosis in the maternal villi and presence of moderate to large numbers of binucleate cells (BNC) in ewes were previously reported (Majeed et al., 1995). No such histopathological changes were observed in our samples.

Dramatic decrease of binucleated trophoblast cells is important factor during normal maturation in bovine placentome. This change cannot be observed in retained foetal membranes (Williams et al. 1987; Gross et al., 1991). The involvement of BNC in the process of placental separation in ewes has also been reported (Majeed et al., 1995). We observed only few no of BNC in placentome samples in our study.

## Conclusion

The results indicate that Aglepristone treatment at the rate of 5 mg/kg could prolong the time between expulsion of the last lamb and foetal membranes with reasonable number of apoptotic cells.

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## References

- Batista M, Reyes R, Santana M, Alamo D, Vilar J, González F, Cabrera F, Gracia A (2011) Induction of parturition with aglepristone in the Majorera goat. *Reprod Domest Anim* 46: 882-888.
- Boos A, Janssen V, Mulling C (2003) Proliferation and apoptosis in bovine placentomes during pregnancy and around induced and spontaneous parturition as well as in cows retaining the fetal membranes. *Reproduction* 126: 469-480.
- Chassagne M, Barnouin J (1993) The effect of inhibition of prostaglandin f<sub>2</sub>, synthesis on placental expulsion in the ewe. *Can J Vet Res* 57: 95-98.
- Franklin JS (1986) Retained placenta, metritis and pyometra. In: Morrow DA (Ed), *Current therapy in theriogenology; diagnosis, treatment, and prevention of reproductive diseases in small and large animals*, 2<sup>nd</sup> edition. WB Saunders Philadelphia, London, p: 595.
- Fthenakis GC, Leontidis LS, Amiridis GS, Saratsis P (2000) Incidence risk and clinical features of retention of foetal membranes in ewes in 28 flocks in southern Greece. *Prev Vet Med* 43: 85-90.
- Garcia A, Barth AD, Mapletoft RJ (1992) The effects of treatment with cloprostenol or dinoprost within one hour of induced parturition on the incidence of retained placenta in cattle. *Can Vet J* 33: 175-183.
- Gazal OS, Li Y, Schwabe C, Anderson LL (1993) Attenuation of antepartum relaxin surge and induction of parturition by antiprogesterone RU 486 in sheep. *J Reprod Fertil* 97: 233-240.
- Gross TS, Williams WF, Russek-Cohen E (1991) Cellular changes in the peripartum bovine fetal placenta related to placental separation. *Placenta* 12: 27-35.
- Hirayama H, Ushizawa K, Takahashi T, Sawai K, Moriyasu S, Kageyama S, Miura R, Matsui M, Fukuda S, Naito A, Fujii T, Minamihashi, A (2012) Differences in apoptotic status in the bovine placentome between spontaneous and induced parturition. *J Reprod Dev* 58: 585-591.
- Kastelic JP, Cook RB, McMahon LR, McAllister TA, McClelland LA, Cheng KJ (1996) Induction of parturition in ewes with dexamethasone or dexamethasone and cloprostenol. *Can Vet J* 37: 101-102.
- Leontidis L, Fthenakis GC, Amiridis GS, Saratsis P (2000) A matched case-control study of factors associated with retention of fetal membranes in dairy ewes in Southern Greece. *Prev Vet Med* 44: 113-120.
- Lewing FJ, Proulx J, Mapletoft RJ (1958) Induction of parturition in the cow using cloprostenol and dexamethasone in combination. *Can Vet J* 26: 317-322.
- Majeed AF, Al-Sadi HI, Ridha AM (1995) Ovine placenta related to binucleated cells in fetal membrane retention. *Small Rum Res* 17: 97-99.
- Özalp RG, Yavuz A, Udum-Küçükşen D, Orman A, Demiral ÖO, Rışvanlı A, Wehrend A (2014) Clinical findings following parturition induction in ewes by a progesterone receptor blocker, Aglepristone, and subsequent neonatal survival. *Repro Domest Anim* (Submitted)
- Rubianes E, Rodas E, Benech A, Carrau A, Ferreira A (1991) Lambing and placental expulsion time after dexamethasone-induced parturition in Corriedale and Polwarth ewes. *Theriogenology* 36: 329-334.
- Shenavai S, Hoffmann B, Dilly M, Pfarrer C, Ozalp GR, Caliskan C, Seyrek-Intas K, Schuler G (2010) Use of the progesterone (P4) receptor antagonist aglepristone to characterize the role of P4

- withdrawal for parturition and placental release in cows. *Reproduction* 140: 623-632.
- Shenavai S, Preissing S, Hoffmann B, Dilly M, Pfarrer C, Ozalp GR, Caliskan C, Seyrek-Intas K, Schuler G (2012) Investigations into the mechanisms controlling parturition in cattle. *Reproduction* 144: 279–292.
- Troedsson MHT (2008) Alterations in Sexual Function. In Smith BP (Ed), *Large Animal Internal Medicine*, 4<sup>th</sup> edition. Elsevier, USA, p: 193.
- Williams WF, Margolis MJ, Manspeaker J, Douglass LW, Davidson JP (1987) Peripartum changes in the bovine placenta related to fetal membrane retention. *Theriogenology* 28: 213–223.