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Lack of internucleosomal DNA fragmentation induced by cloprostenol in bovine luteal explants *in vitro*

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In cows, pulsatile release of endometrial prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) results in luteolysis at late luteal stage, which involves a complex cascade of events. The typical character of luteal regression is reduction in progesterone (P4) production and tissue degeneration through apoptosis (McCracken *et al.* 1999). In general, $PGF_{2\alpha}$ administration during mid-luteal phase (days 8–12 of the estrous cycle) decreases plasma P4 concentrations, and then induces luteal cell apoptosis in cattle (McCracken *et al.* 1999). Luteal apoptosis is induced by treatment with $PGF_{2\alpha}$ on days 8, 10, 12 and 14 in ewes (Rueda *et al.* 1995). $PGF_{2\alpha}$ causes luteolysis only after day 12 of the estrous cycle in pig (Diaz *et al.* 2011). However, $PGF_{2\alpha}$ also can reduce apoptosis in bovine luteal steroidogenic cells (LSCs) *in vitro* (Bowolaksono *et al.* 2008). Interferon-tau (IFNT) is the primary signal for maternal recognition in ruminants. When the corpus luteum (CL) is ready to undergo regression, IFNT can suppress the secretion of $PGF_{2\alpha}$ at the end of the ovarian cycle through binding to its specific receptors in the maternal uterine endometrium, thus preventing luteolysis (Bazer *et al.* 2010). It is important for understanding intracellular mechanism of inducing luteolysis for studying luteal function, and DNA fragmentation is an important event in the executionary phase of cellular apoptosis. In this study, we aimed to explore the luteal cell apoptosis with cloprostenol, a synthetic analogue of $PGF_{2\alpha}$, and using luteal explants from the mid-luteal phase *in vitro* in cattle.

Ovaries were collected from non-pregnant Holstein cows at a local slaughterhouse. All animal procedures were approved by local Institutional Animal Care and Use Committee. The physiological state of ovaries was estimated by examination of ovarian morphology. In this study, ovaries at days 10–15 of the estrous cycle were used for experiments. Luteal explants were cultured as per Ndikum-Moffor *et al.* (1997) with modifications. Briefly, About 1 g of luteal tissue was minced into small (approximately 2-mm cubes) pieces. Five, 60 mm culture plates each containing 5 ml DMEM/F12 including 0.1% BSA and about 200 mg of small luteal pieces were supplemented with 1 µg/ml of BSA (control), 2.5 nM, 25 nM, 250 nM of cloprostenol, and 25 nM cloprostenol + 10^4 IU/ml of recombinant bovine interferon-tau (rbIFNT, 1.13×10^8 IU antiviral activity/mg, provided by University of Missouri, Columbia, MO, USA, in an atmosphere of 5% CO_2 in air at 37°C for 24 h. The luteal explants were then stored at × 80°C until further analysis.

Cumulus-oocyte-complexes (COC) were aspirated from follicles (2–8 mm in diameter), and cumulus cells obtained from COC by pipetting them vigorously, and were incubated with serum free medium in an atmosphere of 5% CO_2 in air at 38.5°C for 48 h. The DNA fragments were isolated as per Rueda *et al.* (1995) with modifications. The DNA fragments of luteal explants and the cumulus cells were separated by 1.8% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light.

Luteal explants were homogenized at a ratio of 1 g explants per 5 ml 1 × Laemmli buffer, and cumulus cells were at a ratio of 2×10^7 cells / ml Laemmli buffer. Actin was detected by Western blot as per Yang *et al.* (2010). Immuno-reactive bands were inverted and semi-quantified using Adobe Photoshop 7.0 software.

All experiments were repeated at least 3- times. Statistical analysis was performed using 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. No statistical difference was declared at $P>0.05$.

The pulsatile release of endometrial $PGF_{2\alpha}$ leads to luteolysis at late luteal stage, and meanwhile CL undergoes tissue degeneration through apoptosis in bovine, ovine and porcine (McCracken *et al.* 1999, Rueda *et al.* 1995, Diaz *et al.* 2011). The 24 h incubation time for luteal explants is optimal for incorporation of radiolabel *in vitro* compared with the other incubation times in bovine (Ndikum-Moffor *et al.*

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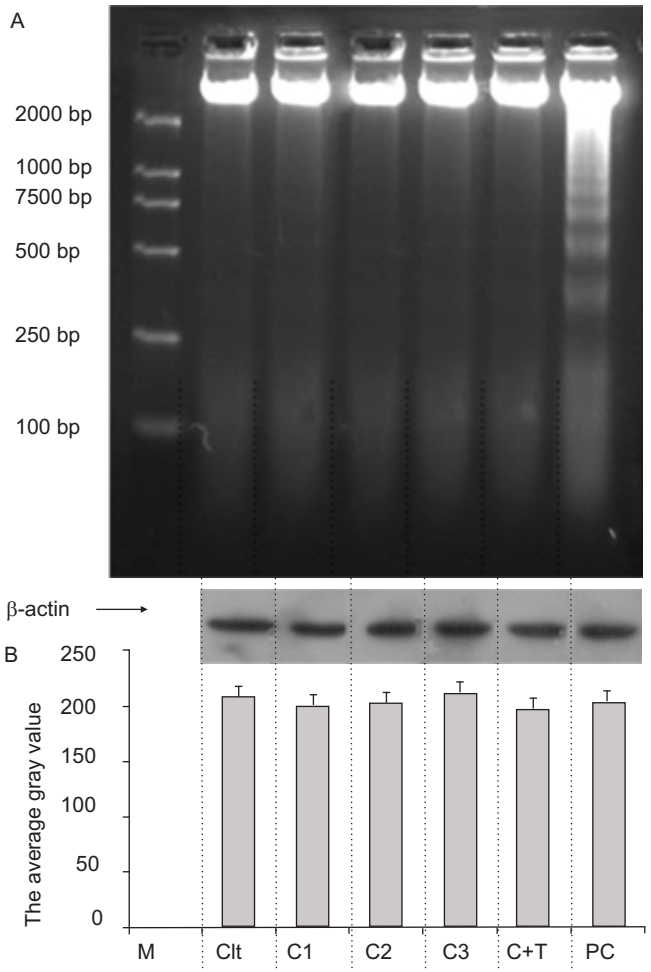

Fig. 1. Internucleosomal DNA fragmentation and expression of b-actin in bovine luteal explants and apoptotic cumulus cells. M,Maker; Clt,control; C1,2.5 nM of cloprostenol; C2,25 nM cloprostenol; C3,250 nM of cloprostenol; C+T,25 nM cloprostenol + rbIFNT; PC, The apoptotic cumulus cells (positive control).

1997). In this study, the treatment with 10^4 IU/ml of IFNT was to inhibit luteal apoptosis induced by cloprostenol, and the concentration of IFNT was coincidence with the previous study in bovine endometrial explants (Yang *et al.* 2010). This is the first attempt to induce *in vitro* luteal apoptosis using cloprostenol in bovine luteal explants, and analyze the expression of β-actin in luteal explants and cumulus cells after different treatments *in vitro* in cattle.

Our results revealed that there was lack of internucleosomal DNA fragmentation in bovine luteal explants after induced by 2.5 nM, 25 nM, 250 nM of cloprostenol *in vitro* for 24 h, and the luteal explants with cloprostenol + rbIFNT also did not produce internucleosomal DNA fragmentation (Fig. 1). However, there were evident internucleosomal DNA fragmentation in bovine cumulus cells after incubated with serum free medium *in vitro* for 48 h, which indicated that this protocol for analysis of DNA fragmentation was reliable and serum free also could induce apoptosis in bovine cumulus cells, and Goyenche *et al*. (2006) also showed that serum starvation for 24 h to 48 h can induce fragmentation of DNA in luteal cells. Rueda *et al.* (1995) mentioned that slicing (0.5 mm) of the CL alone was sufficient to initiate the apoptotic effect in luteal slices, but DNA fragmentation was not observed if CL were left intact (whether incubated or not) in ovine. In present study, the luteal explants (approximately, 2-mm cubes) were larger than luteal slices (0.5 mm), which function may be similar to intact CL, so luteal explants did not show apoptosis in present study. From these results, we confirmed that luteolysis induced by $PGF_{2\alpha}$ *in vivo* did not imitate by luteal explants with cloprostenol *in vitro*.

β-actin protein was normally expressed (Fig. 1) both in bovine luteal explants, after induced by cloprostenol and in cumulus cells (the positive control of apoptosis), with no significant difference between them ($P>0.05$). As a component of cytoskeleton, β-actin is involved in many cellular activities, but the potential linkages between cytoskeletal β-actin and apoptosis is still not well understood (Gourlay and Ayscough 2005). In this experiment, β-actin protein was normally expressed both in luteal explants and apoptotic cumulus cells, which indicated that β-actin did not degrade in bovine-cumulus cell during apoptosis.

Calf serum (CS) can suppress apoptosis of cultured luteal cells, suggesting that CS contains anti-apoptotic substances (such as growth factors) which amplify cell survival pathways in the bovine luteal cell *in vitro* (Skarzynski *et al.* 2007). However, in this study, the culture media were supplemented with 0.1% BSA instead of CS or fetal bovine serum (FBS), and we also used luteal explants instead of bovine luteal cell, because luteal explants can imitate luteal function *in vivo* (Ndikum-Moffor *et al.* 1997). Nevertheless, internucleosomal DNA fragmentation was not induced by cloprostenol in bovine luteal explants, so we speculated that luteal functions were not maintained completely *in vitro*.

Functional CL requires the interaction between several tropic hormones secreted by pituitary, decidua and placenta (Stocco *et al.* 2007). Shirasuna (2010) also reported that luteal blood flow regulates luteal function in the cow. Immune cells are recruited into CL, which plays a role in regulating luteal homeostasis or demise. As leukocytes are recruited into CL, leukocytes and luteal cells communicate in different ways to maintain homeostasis within the functional CL (Pate *et al.* 2010). We also reported that IFNT does not induce expression of interferon stimulated gene 15 (ISG15) in luteal explants, but CL expresses ISG15 owing to IFNT during early pregnancy in the cow (Yang *et al.* 2010). Therefore, we speculated that luteal apoptosis induced by $PGF_{2\alpha}$ *in vivo* was involved in many cytokines besides $PGF_{2\alpha}$.

In conclusion, our results revealed that there was no internucleosomal DNA fragmentation in bovine luteal explants after induced by cloprostenol *in vitro* for 24 h. Meanwhile, β-actin protein was also normally expressed in

bovine luteal explants and apoptotic cumulus cells. Therefore, we suggested that the *in vitro* culture system of luteal explants did not imitate the luteal function *in vivo*, and luteolysis *in vivo* induced by $PGF_{2\alpha}$ may be involved in numerous hormones and cytokines, and β-actin did not degrade in bovine cumulus cell during apoptosis.

SUMMARY

Luteolysis can be induced by treatment with exogenous $PGF_{2\alpha}$ in bovine. In this study, we explored the effect on luteal cell apoptosis in bovine luteal explants *in vitro* with cloprostenol, a synthetic analogue of $PGF_{2\alpha}$. Our results revealed that there was no internucleosomal DNA fragmentation in bovine luteal explants induced by cloprostenol *in vitro* for 24 h. However, β-actin protein was normally expressed both in luteal explants and apoptotic cumulus cells. In conclusion, the *in vitro* culture system of luteal explants did not imitate luteal function *in vivo*, and β-actin did not degrade in bovine apoptotic cumulus cell.

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