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Anti-Apoptotic Role of *p21* and *p27* Genes during Different Stages of Corpus Luteum Progression in Bovine

Research Article

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ABSTRACT

Corpus luteum is a temporary endocrine gland, secretes progesterone and estradiol crucial for establishment and maintenance of pregnancy. The cyclin dependent kinase inhibitors (CDKIs) like *p21* and *p27* plays important role in cell cycle progression and regulation. In this experiment, the expression level of *p21* and *p27* mRNA and the proliferations of immune cells in different stages of the corpus luteum (CL) were studied. As *p21* and *p27* proteins are having role in the cell cycle progression and proliferation while CL undergoes both these processes. Ovaries containing corpora lutea were collected from local abattoir. The corpus luteum were isolated and segregated based on respective stages. The excised corpus luteum were processed for the extraction of the mRNA and a portion was embedded in paraffin for the slide preparation. The results showed significantly lower expression of *p21* and *p27* in stage I and -IV, compared to stage II and III ($P<0.05$). However, there was a slight dynamism in the expression level of each individual CL in the same stage, which indicates the developmental variation within the bovine specie. Histopathological examination shows that proliferation of immune cells gradually increased in stage I, II and III; however, a quick decline was found in stage-IV. The results indicate that *p21* and *p27* plays its role in the stage I (establishment) and stage IV (regression) of the corpus luteum. Decrease in immune cell proliferation in stage IV of the corpus luteum, as evident from histopathological examination, was probably the outcome of the increased apoptosis, triggered by down-regulation of *p21* and *p27*. This indicates the reliance of apoptosis upon the expression level of *p21* and *p27*. The level of *p21* and *p27* expression is indicative of estimating quality of the corpus luteum, and subsequently the early embryonic losses, associating mal functioning of the corpus luteum.

KEY WORDS apoptosis, cattle, CDK inhibitors, corpus luteum, ovary, pregnancy.

INTRODUCTION

The consistent repetitive estrous cycle, which last for 18-21 days in bovine, have many stages, which are governs by different developmental and hormonal changes (Garcia *et al*. (2002). The estrous cycle starts on day 0, and terminated with the lysis of the corpus luteum on day 18-21 Northey and French (1980). On day 0 the follicle ruptures, freeing the egg to the in findulum Johnson (2015). Instantly after ovulation new types of cell termed as luteal cells develop inside the ovary from the granulosa cells, rather promptly up to 5-6 days these cells rise to a mature corpus luteum (Koering *et al*. 1964). Even though the term corpus luteum was lead to Marcello Malpighi in a communication to

Jacobo Spon, the major explanation and sketches of corpus luteum was completed by Catchpole (1940) who advised globular bodies in ovaries of pregnant rabbits (Jocelyn and Setchell, 1972).

Corpus luteum, a temporary endocrine body which secretes progesterone (P4), which is required for pregnancy maintenance and establishment (Devoto *et al*. 2017). In the lack of pregnancy, the CL regressed by its own called luteolysis. This process is well defined as the supervision of gonadotropin releasing hormones (GnRH) adversaries origins a quick drop in the production of progesterone., although in cattle and rodents this influence is fewer affected (Niswender *et al*. 2000). The CL development, upkeep and deterioration are distributed into four stages I, II, III, and IV (Ireland *et al*. 1979).

Regression and maintenance of corpus luteum is determined by complex interrelated events of the cell cycle (Bachelot and Binart, 2005). The cell cycle of mammals, defined as a series of procedures among two cells division is surely regulated by cyclin and cyclin dependent kinases (CDKs), which acquaintance to make heterodimeric facilities (Sherr, 1996) and adversely controlled by CDKIs i.e. *p21* and *p27* (Taguchi *et al*. 2004). CDKIs are significant adverse controllers of cell cycles headways. Once tie CDK-cyclin complexes in the cell cycles' G1 phase (Cell cycle), they chunk the CDKs activities, inhibiting phosphorylation of members of the Rb genes family and travel to S phase (Deng *et al*. 1995).

Here we have two groups or family of physically different CDKIs. The CIP/KIP family that hinder a wide-ranging CDK's by selectively tie and preventing the fully linked cyclin-CDKs complexes. The second family is the inhibitors of kinases (INK) family, that bind precisely to CDK4 or CDK6 and hinder composite development with cyclin D (Taguchi *et al*. 2004).

The *p21* and *p27* have its place in the CIP/KIP family of Cdk's inhibitors; both have effects on numerous CDKs *in vitro* (Besson *et al*. 2008). The cyclin E (protein) and CDK2 complexes can thus be deactivated by attachment of *p21* and *p27*, hat result the arrest of G1 phase resulting absence of Rb phosphorylation (Deng *et al*. 1995). Due to high degree of similarities in protein initial structure, both the *p27* and *p21* are considered to be hindering their goals by comparable machineries. Grounded on morphological study (Russo *et al*. 1996), it is now believed an alpha-helix of a CIP/KIP protein pledges its first interaction with cyclin Aparicio *et al*. (2014). Importantly CDK2 conformational variations furthermore and bolt the catalytic forked in an indolent method. Astonishingly, the massive bulk of cyclin-D and CDK's complex cover *p27* or *p21* (Sherr and Roberts, 1999).

The CL undergoes both progression and regression while *p21* and *p27* genes are mainly involved in these processes, so it is imperative to determine the role of these genes in CL. Briefly the expression of both genes in corpus luteum have been determined. As previous studies have shown, the proven role of *p21* and the *p27* proteins as anti-apoptotic, anti-cancerous and anti-proliferative genes (Murad *et al*. 2016). This study will contribute to the detect the health status of pregnancy with CL development as well as the impact of expression level of the genes.

MATERIALS AND METHODS

Indigenous cattle (n=40) were randomly selected from a local abattoir situated at Kohat road Peshawar, Pakistan. Ovaries samples were collected from all those slaughtered animals via surgical tools. Those ovaries were transported to the lab in ice box. Later on corpus luteum were gently removed from the ovary and were kept for further biological processes (Kiyma *et al*. 2016). 12 samples were excluded in the later stages.

Determination stages of CL

The stages were determined on the basis of morphology. Each stage has its own characters i.e. exterior and interior appearances, diameter, surface vasculature and follicles defined by (Ireland *et al*. 1979).

Processing of CL

The excised corpus luteum was cut into two pieces, each part was about 5mm. One part was used for biological assessments i.e., for RNA extraction, while the second part was stored in 10% formalin for histopathology. For RNA extraction, the samples were minced or grinded in liquid nitrogen. Instantly transferred into a 2ml Eppendorf tube with 1ml TRiZol reagents for instant processing or was stored in RNA later@ solution for later processing.

RNA extraction

Briefly, RNA were extracted from all 28 samples by TRiZol method through a manufacture's protocol (Chomczynski and Sacchi, 1987). The process was performed inside late fetal heart (LFH) because of the RNA sensitivity. RNA concentrations (standard: 1.7 to 1.9) were checked through Nano Drop and was diluted to obtain uniformity (Figure 1).

The cDNA synthesis

The cDNA synthesis procedure is also described by Atli *et al*. (2010). Meanwhile 2 µg of the total RNA was reverse transcribed into cDNA through RevertAid cDNA synthesis

Kit (Thermo Fisher Scientific CN: K1622 through reverse transcription PCR).

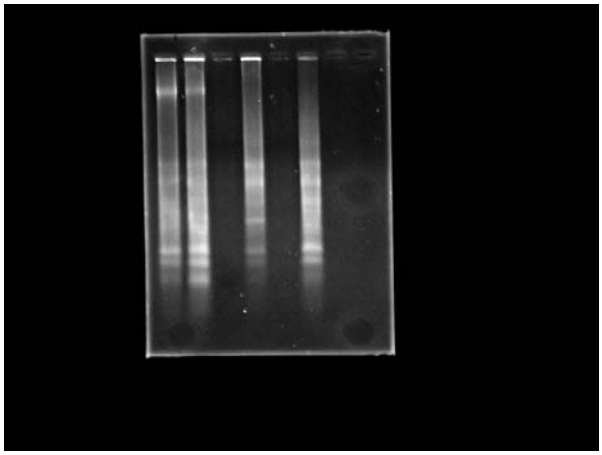

Figure 1 Extracted RNA gel electrophoresis

Amplification of the desired fragment

PCR was used to check the expression profile of *p21* and *p27*. The procedure has defined as follow: 1 µL of the cDNA was taken with 10 µL of PCR Master Mix. 5 pico-MOL of each primer were added, finally, ddWater were added to final volume (20 µL). Primers were obtained from a literature and blasted in NCBI for confirmation (Table 1). The *GAPDH* gene was spotted to be the best steady house-keeping gene, was hired to standardize the PCR figures of the studied *p21* and *p27* (Kiyma *et al*. 2016).

Gel electrophoresis

To verify the reaction specificity, each sample was separately run on 1% agarose gel. The amplification products were stained with ethidium bromide. 100 bp ladder was used for size comparison. The gel bands were captured through gel-doc (Figure 2).

Histopathology

The samples were first dehydrated with different concentration of alcohol (50, 70, 90 and 100%). After dehydration the samples were cleaned up with xylene. Samples were then cut into slices with the help of microtome, embedded with paraffin wax. The samples staining was done with H&E stain. After staining samples were fixed in buffer formalin. Slides were made and were examined with the help of various magnifications (4X, 10X and 40X).

Statistical analysis

The data thus obtained were subjected to one-way ANOVA (SPSS, 2011). The bands intensity was checked by ImageJ and the figures were made through PRISM graph-pad.

RESULTS AND DISCUSSION

Activity of p21 during different stages of the corpus luteum

In this study we checked the expression of *p21* in different stages of the corpus luteum. The relative expression of *p21* was significantly ($P \leq 0.05$) higher in stage II and III as compare to stage I and IV as shown in the Figure 3. During stage II and III, the activity of the luteal cells was higher for the production of progesterone, the expression of the p21 help in the protective mechanism of these luteal cells. During the regression stage IV and initial stage. I of luteal cells differentiation the activity of the *p21* was minimal, showing that anti-apoptotic activity of the *p21* during progression of the corpus luteum.

Relative expression of *p27* in four stages of corpus luteum

The Figure 4 shows average expressions of *p27* gene in four stages of CL. The overall results show that *p27* expression is significantly lower in stage I but increased gradually up to stage-II and-III, while a quick decline occurred in stage-IV. These results further strengthened our hypothesis that *p27* has a crucial role in CL maintenance mainly and regression as well. The results show that during progression stage of high activity of the luteal cells the higher expression of the *p27* effect the apoptosis of the luteal cells to sustain its activity.

Proliferation of the luteal cells during different stages of CL

Figure 5 shows the proliferation of luteal cells during different stages of the corpus luteum. During stage I the cells started differentiation into the immature luteal cells followed by nuclear and cytoplasm division as shown in Figure 5a. The stage-II and -III show higher number and proliferation of the luteal cells, surface vasculature and large sized vesicles with appearance of some immune cells as shown in Figures 5b and 5c. During stage IV when the expression of *p21* and *p27* become lower, the cells collapsed and the apoptosis was increased while proliferation of the cells decreased which supported the anti-apoptotic activity of the *p21* and *p27* (Figure 5d).

To confirm the role of *p27* in the regression of CL, a supportive statement was reported by Katayose *et al*. (1997). They demonstrated that high levels of *p27* expression induce apoptosis, although the effects of *p27* in promoting cell death may be more indirect. The expression of *p27* is very high in the stage-IV of CL as shown in Figures 3 and 4.

These results indicate that *p27* lower expression induced luteolysis in cattle.

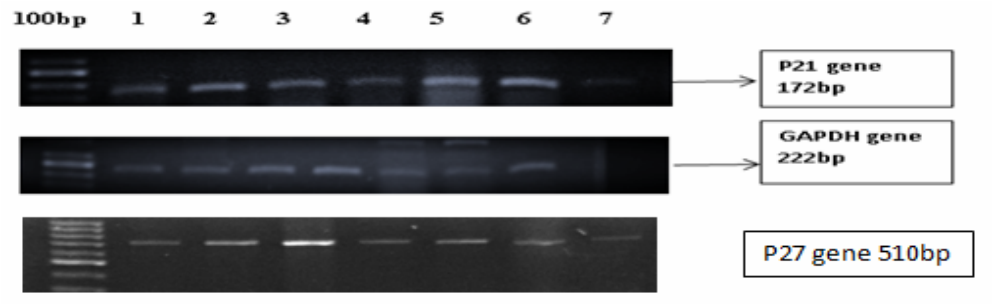

Figure 2 *P27* PCR products gel picture

Table 1 Primers*21* and *p27* NCBI results

Gene	Product size	Accession no	Primer sequence
p21	Size=164 bp	NM001098958.2	F 5´TGCTGGGTGTTACGAAGTCC 3´ R 5´CACCCTGCCCAACCTTAGAG 3´
p27	510 bp	NM001100346.1	F 5´AAACCCAGAGGACACGCATT 3´ R 5´GTTGGGGGAACCGTCTGAAA 3´

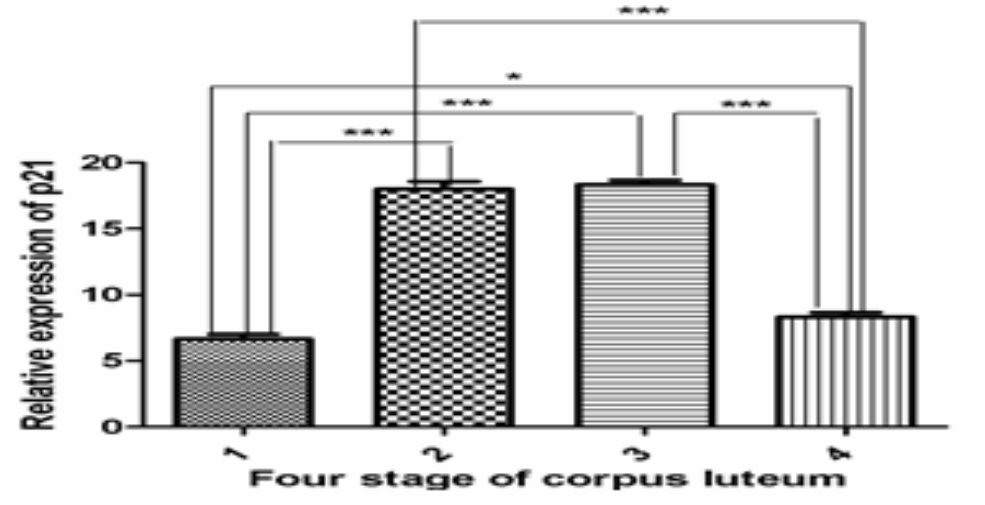

Figure 3 Relative expression of p21 in different stages of corpus luteum

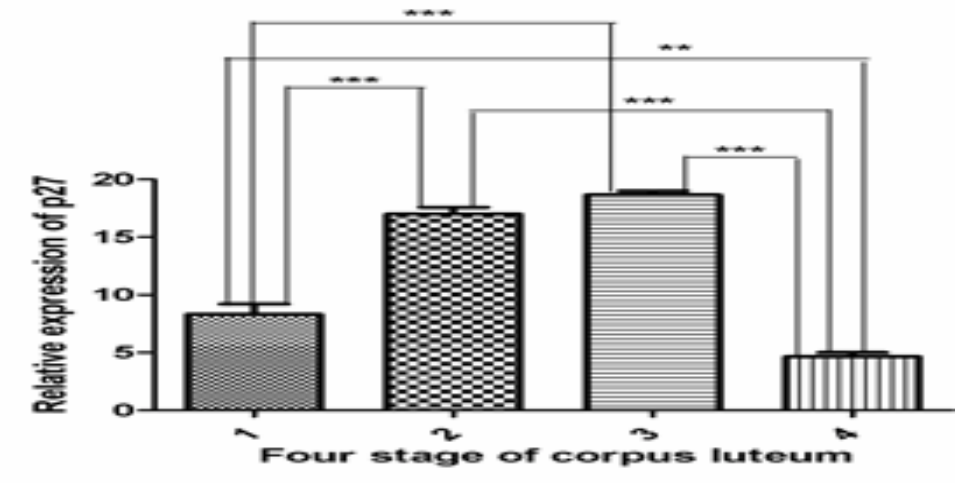

Figure 4 Relative expression of p27 in different stages of corpus luteum

The expression of *p27* is lower in CL as compared to *p21* because *p27* is expressed at high levels in lymphocytes and in normal non-proliferating mammary epithelial cells serving as internal control cells in the immunohistochemically evaluation of *p27* expression (Porter *et al*. 1997).

Our results are in the line with Ghanem and Steinman (2005). Interestingly, *p21* has conflicting roles in apoptosis, having been demonstrated to both promote and inhibit programmed cell death. The pro-apoptotic activity of *p21* has been attributed to both *p53*-dependent and *p53*-independent regulation of the apoptotic effector protein bax. Their hypothesis clearly suggested that p21 and p27 has dual role in nature like they are apoptotic as well as anti-apoptotic. This nature can be distinguished on the basis of their expression level.

Dramatic changes in expression of two cell cycle regulators, p21, p27 occur during the first 48 h of luteinization *in vivo* (Robker and Richards, 1998). The data of Robker and Richards (1998) and Richards *et al*. (1998) and our results show that p27 accumulates early in the development of luteinized tissue.

It is shown here that p27 is maintained at this high level until down-regulation that occurs after about day 18 of pregnancy. Moreover, changes in cyclin D3, CDK4, and CDK6, and in their associations with p27, occur with dynamics similar to that of p27. Taken together, fully luteinized cells are devoid of any major fluctuations in cell cycle regulatory machinery until all the molecules studied here change together late in luteal life (Richards *et al*. 1998).

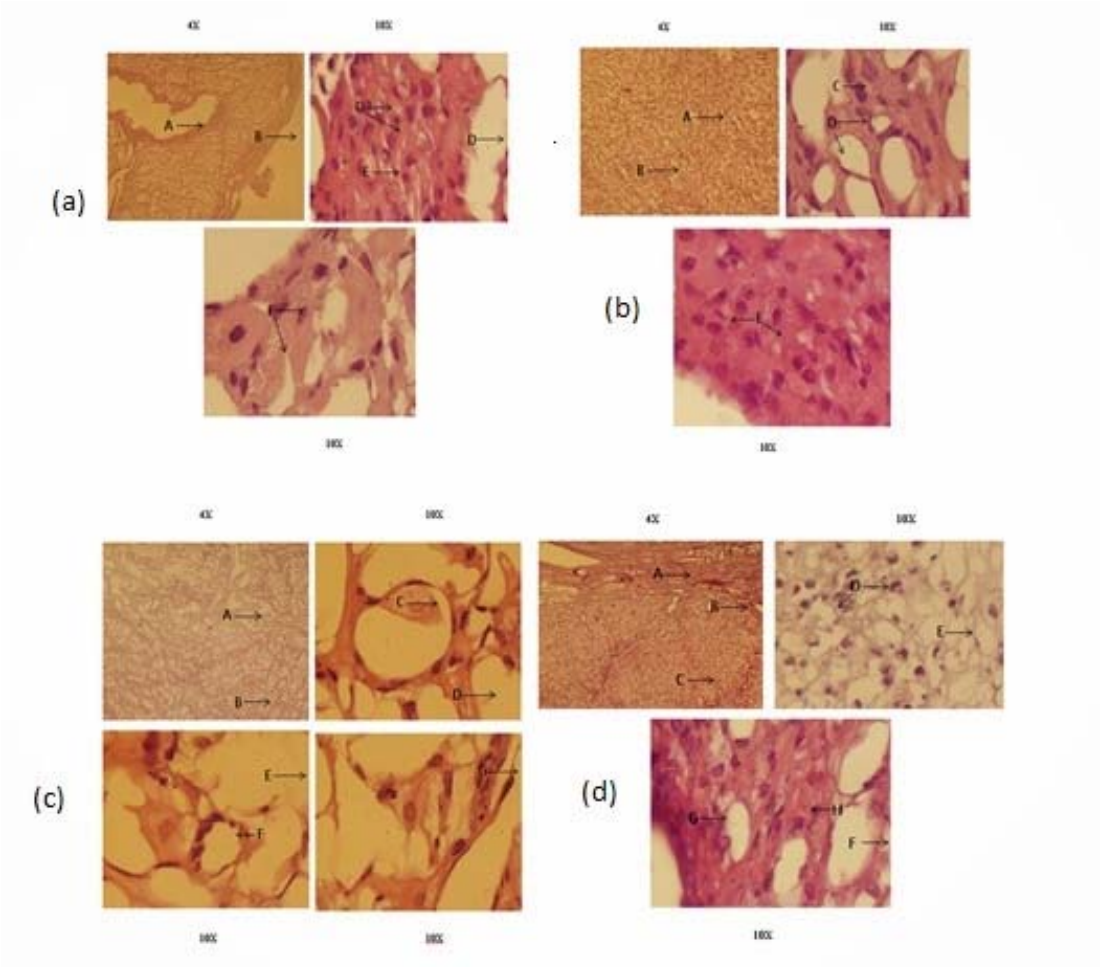

Figure 5 Histopathology of corpus luteum
a) A: follicle secreted area; B: theca cells; C: nuclear division; D: large vesicle; E: fresh cells injury and F: proliferation of luteal cells
b) A: surface vasculature; B: proliferation; C: vesicles; D: Immune cells and E: typical nuclei
c) A: connective cells proliferation; B: theca cells; C: cell collapse; D: regressed cells; E: vesicles and F: apoptosis
d) A: suppressed granulosa cells; B: cell structure destroyed the vasculature; C: apoptosis; D: chromatin condensation; E: unequal nuclear and cytoplasmic ratio and F: tissue and vesicles enlargement

Considering the expression level of *p21* and *p27* gene, the results showed a dynamic pattern in four stages of CL, as *p21* expression in stage I and-IV is significantly lower while it higher in both stage-III and-IV. These results showed that the role of *p21* expression in the luteinization and regression is lower than the maintenance of CL. The difference in the expression level of *p21* and *p27* gene in four stages may be due to the fact that experimental animals might have age differences or probably their genetic makeup might be different. Or some of the animals could be pregnant. On the other hand as *p21* in an anti-apoptotic gene as well Gomez-Manzano *et al*. (1997), so it could have higher expression in the regression of CL but these results showed a slight contradiction with the dual nature of *p21* as an apoptosis and anti-apoptosis (Ghanem and Steinman, 2005).

CONCLUSION

Overall, this study showed the expression pattern of *p21* and *p27* genes in different stages of corpus luteum. The lower expression in initial stages indicated the higher level of maintenance and recruitment, while the higher expression of both genes was found in the regression stages of corpus luteum. The results and comparative analysis showed that their functions maybe significantly related to apoptosis.

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