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ROLE OF Ca^{++} and Ca^{++}-Mg^{++} DEPENDANT ENDONUCLEASE ACTIVITY IN RELATION TO BUFFALO FOLLICILAR ATRESIA

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ABSTRACT
Majority of ovarian follicles undergo degeneration, a process called Follicular atresia. Ca^{++}-Mg^{++} dependant endonuclease activity is one of the key factors in inducing follicular atresia. In order to find out the role of Ca^{++} and Ca^{++}-Mg^{++} dependant endonuclease activity in buffalo follicular atresia, both the concentration of calcium and the endonuclease activity were measured in granulosa cells (GC) and follicular wall (FW) extracts of non-atretic and early-atretic follicles of pre-ovulatory and ovulatory sizes. In addition to above, calcium concentration was measured in the follicular fluid (FF) of the above classes of follicles. The results indicate that the endonuclease activity of GC has a definite role in atresia but in the follicular wall the activity is dependent on size of the follicles. In case of calcium the results indicated that calcium content is always more in atretic follicles of all the sizes in FF, FW and GC, than the non-atretic follicles and thus has a potent role in buffalo follicular atresia.

KEYWORDS: *Follicular atresia, Folliculogenesis, apoptosis, Buffalo, Ca^{++} and Ca^{++}-Mg^{++} dependant endonuclease activity*.

INTRODUCTION

Follicle is the ovarian compartment that enables the ovary to fulfil its dual function of gametogenesis and steroidogenesis. Folliculogenesis is a dynamic event culminating in ovulation or atresia. In mammals it has been established that a vast majority (more than 99 percent) of follicles present at birth eventually become atretic, whereas significantly less number (less than 1 percent) actually attain ovulation during the active reproductive life of an animal (Erickson,1966). Therefore, to augment reproductive performance, the number of ovulations needs to be increased (super ovulation) or rate of atresia decreased.

Follicular atresia and apoptosis

Many morphological and biochemical characteristics of atretic follicles have been identified (Richards, 1980; Ryan, 1981; Farookhi, 1981), e.g. lower estradiol / progesterone, lack of carotene cleavage activity, non-activation of plasminogen, accumulation of chondroitin sulphate in follicular fluid have all been claimed as biochemical markers of atresia. Apoptosis or programmed cell death has been evidenced as a potential mechanism underlying follicular atresia in many species ranging from avian to human beings (Hseuh *et al.,* 1994; Funayama *et al.,* 1996; Jolly *et al.,* 1994; Murdoch, 1995; Guthrie *et al.,* 1994; Nahum *et al.,* 1996; Palambo *et al.,* 1994). The best defined biochemical event in apoptosis involves cleavage of nuclear double stranded DNA at linker regions between nucleosomes, catalysed by the inducible enzyme Ca^{++}-Mg^{++} dependant endonuclease (Wylie *et al*., 1980). Upon electrophoretic fractionation of this DNA, a ladder pattern of oligo-nucleosomal DNA fragments with size multiples of 185-200 base pairs is observed, which is considered as the hallmark of apoptosis. However, neither the mechanism nor the factors initiating atresia have been established or identified. Moreover species-wise difference in many of the chemical constituents of follicular fluid indicates the need to study different far animals separately. Buffalo is an important farm animal in India and South East Asian countries. It is well known for higher milk fat percentage, better feed conversion ratio, disease resistance etc., but suffers from many reproductive problems like delayed age of maturity, longer calving interval, weak/silent oestrus, seasonality of breeding etc. and the available literature on buffalo folliculogenesis and atresia are scanty. There is also no report so far on the mechanism of apoptosis leading to follicular atresia in buffaloes.

Calcium and Ca^{++}-Mg^{++} dependant endonuclease

Calcium content in the follicular fluid is reported to be almost similar to that of serum. Calcium content increased in large size follicles of buffalo but no increase is reported in small / medium sized follicles (Jagjit *et al.,* 1997). The role of calcium in the atretic follicles of rats (Luciano *et al.,* 1994) has been studied and is found to be regulated by EGF, which prevents granulosa cell apoptosis by stimulating progesterone synthesis. Calcium is essential for the activation of the key apoptotic enzyme Ca^{++}-Mg^{++} dependant endonuclease, which cleaves the DNA at internucleosomal regions of granulosa and luteal cells (Zeleznik *et al.,* 1989). Calcium is also essential for the activation of proteolytic enzymes involved in degrading IGF binding proteins during folliculogenesis and atresia along with zinc ions (Besnard *et al.,* 1996). The down regulation of N-catherin (Ca dependant cell adhesion molecule) or the absence of a functional extra cellular domain of the molecules prevent cell aggregation and is associated with granulosa cell apoptosis. Moreover, N-catherin modulated cell signalling via cAMP, playing a significant role in in follicular and luteal cell survival (Makrigiannakis, 1999). Hence, the present study was undertaken to study the role of calcium and Ca^{++}-Mg^{++}

dependant endonuclease activity in buffalo folliculogenesis and follicular atresia by examining the pre-ovulatory and ovulatory stages of non-atretic and early-atretic follicles.

MATERIALS AND METHODS

Chemicals and Biologicals

EGTA, Nonidet P-40 and DNA molecular weight marker (λ double digest) were procured from M/s. Sigma Chemical Company, USA; Proteinase K was from Bangalore Genei, India; Agarose, Ethidium bromide, Phenol, BSA and Standard calf thymus DNA were procured from M/s. SRL, India and all other routine chemicals used were of AR grade.

Collection of Ovaries, pre-ovulatory and ovulatory follicles

Buffalo ovaries were collected from local abattoir immediately after slaughter and transported to the laboratory in chilled saline. They were kept cool throughout the collection of experimental materials. After thorough washing in chilled normal saline, the diameters of each follicle protruding on the surface of the ovaries were measured at two perpendicular points (short and long axes). Based on the average of the two measurements follicles were either categorised as pre-ovulatory (5-8 mm) or ovulatory (>10 mm) follicles. These follicles were dissected free of stromal tissue using scissors and micro-dissection forceps under stereo-zoom microscope.

Microscopic evaluation of follicles

All the isolated follicles of both the above categories were examined under stereo-zoom microscope (Trycon 100z) for the following characters:

a. Degree of translucency
b. Degree of thecal vascularisation
c. Continuity of membrana granulosa
d. Presence or absence of free floating particles inside follicular cavities.

Follicles with a uniformly bright, translucent appearance, extensive vascularisation, a regular continuous granulosa layer and absence of free floating particles in the follicular cavity were graded non-atretic, while the follicles characterized by loss of translucency, slight greyish appearance, observable discontinuities of granulosa layer with acceptable vascularisation were graded as early-atretic as described by Moor *et.al.* (1978) and Kruip and Dieleman (1982).

Thus the following four classes of follicles were taken for study:

1. Preovulatory non-atretic (PN)
2. Preovulatory early-atretic (PA)
3. Ovulatory non-atretic (ON)
4. Ovulatory atretic (OA)

Collection of experimental materials

The experimental materials *viz.* follicular fluid (FF), granulosa cells (GC) and follicular walls / sacs (FW) were collected from each class of the above follicles and pooled separately as follows: Follicular fluid was aspirated from each follicle using a 5 ml syringe with 22G needle. Then the follicular wall was slit open and the inner surface was scraped thoroughly to collect the remnant GC. These cell scrapings were added to the centrifuge tubes containing FF of respective classes and were centrifuged at 3000 rpm for 10 min. The cell-free supernatant, which is FF sample was collected and stored at -20°C till further analysis. The pellet containing GC was suspended in known volume of 0.25 M sucrose solution and stored at -20°C till further analysis. Likewise the empty follicular walls representing the thecal cells were weighed, suspended in 0.25 M sucrose solution and stored at -20°C till further analysis.

Estimation of Calcium in FF, GC and FW samples

The cell-free FF stored at -20°C was used as such for the assay, while the FW and GC were homogenised in 0.25 M sucrose solution and the homogenates were centrifuged at 3000 rpm for 15 min at 4°C. The supernatants thus obtained were taken up for the calcium assay. All the glassware used in the assay were washed thoroughly with dilute Hydrochloric acid (30% v/v) and rinsed well with deionised water before use. Calcium was estimated by OCPC method using a diagnostic kit (Qualigen Diagnostics, India), wherein the calcium reacts with O-cresolphthalein complex one in alkaline medium to form a purpled colored complex. The increase in absorbance was measured at 570 nm in a PC based UV-Vis Spectrophotometer (GS 5703 AT, ECIL, India) and the results were expressed as µg calcium per mg protein in all the samples.

Assay of Ca^{++}-Mg^{++} dependant endonuclease activity in GC and FW samples

The Ca^{++}-Mg^{++} dependant endonuclease activity was assayed by the method of Kyprianou and Isaacs (1988). The harvested and stored GC and FW samples were homogenised in Tris buffer, pH 7.4 with 0.25M Sucrose, 3mM $MgCl_2$ and 3mM EGTA called buffer A (10ml/g) and centrifuged at 800 x g for 15 min at 4°C. Nuclear pellet thus obtained was resuspended in buffer ANP containing 0.5% Nonidet P-40 in buffer A(10ml/g), incubated at 4°C for 10 min and centrifuged at 800 x g for 15 min at 4°C. The pellet was resuspended in buffer A (10ml/g), vortexed well to form a uniform suspension and the total DNA content was estimated by Diphenylamine method. The suspension was then recentrifuged at 800 x g for 15 min at 4°C and then the pellet was suspended in buffer B (10 mM Tris buffer, pH 7.4, containing 25 mM NaCl and 0.34 M Sucrose) and centrifuged at 800 x g for 15 min at 4°C. The pellet was suspended in 0.5 ml of buffer BCM (1 mM $CaCl_2$ and 5 mM $MgCl_2$ in buffer B) and incubated at 37°C for 1h. Incubation was terminated by addition of EGTA to 5 mM and EDTA to 2 mM final concentration followed by rapid chilling on ice. Then the material was centrifuged at 800 x g for 15 min at 4°C and the supernatant stored. The nuclear pellet was suspended in a solution containing 1.5 mM EDTA and 3 mM EGTA to extract additional low molecular weight DNA and was incubated on ice for 20 min, followed by centrifugation at 9000 x g for 20 min at 4°C. This supernatant was combined with that from the earlier step and the low molecular weight DNA was estimated in the pooled supernatant by Diphenylamine method. The results of the Ca^{++}-Mg^{++} dependant endonuclease activity were expressed in terms of the percent DNA degraded in each incubation.

Agarose gel electrophoresis of genomic DNA from GC and FW samples

The genomic DNA was extracted by the classical phenol/chloroform/ isoamylalcohol protocol from the above samples and subjected to agarose gel electrophoresis to find out the difference in DNA fragmentation pattern between non-atretic and early-atretic follicles. The tissue / cells were placed in a solution of proteinase K and sodium dodecyl sulphate and incubated until most of the cellular protein was degraded. The digest was deproteinised by successive phenol/chloroform/ isoamylalcohol extractions, recovered by ethanol precipitation, dried and resuspended in TE buffer. The DNA samples were electrophorosed in 1% agarose gels containing 1.0 µg/ml ethidium bromide. The gels were run in Genei Mini- Submarine electrophoresis unit at 50 V for 3h with a running buffer. Gels were photographed under UV transillumination.

Estimation of Protein

Protein content in the samples was estimated according to the method of Lowry *et al.,* (1951) using bovine serum albumin as standard.

Estimation of DNA

DNA content in the samples was estimated according to the modified diphenylamine method of Burton (1956), using calf thymus DNA as standard.

Statistical Analysis

Data were analysed statistically using One-Way Analysis of Variance (Snedcor and Cochram, 1994).

RESULTS

Calcium

The results of concentration of calcium are presented in Table I and IA and are expressed as Mean ± SE. The concentration of calcium showed significant variation ($P<0.01$) among all the four classes of FW samples, with highest value in OA, followed by PA, ON and PN. In case of GC, the early atretic classes (PA and OA) showed a significantly ($P<0.01$) higher concentration of calcium than non-atretic classes (PN and ON). Though no significant variation was observed between PA and OA classes in GC, PN class showed a significantly higher calcium concentration than ON. The concentration of calcium in FF showed a significantly ($P<0.01$) higher value in early-atretic classes (PA and OA) than the non-atretic classes (PN and ON) and there was no significant difference within them i.e. within PA and OA and PN and ON.

TABLE I: Concentration of calcium in different components of buffalo ovarian follicles

Sl.No.	Class of Follicles	Follicular wall µg/mg protein	Granulosa cells µg/mg protein	Follicular fluid µg/mg protein
1.	PN	34.39 ± 0.767^{d}	4.81 ± 0.075^{b}	1.37 ± 0.015^{b}
2.	PA	70.23 ± 0.274^{b}	5.63 ± 0.058^{a}	1.71 ± 0.012^{a}
3.	ON	57.83 ± 0.809^{c}	3.07 ± 0.055^{c}	1.44 ± 0.030^{b}
4.	OA	86.46 ± 0.706^{a}	5.75 ± 0.056^{a}	1.69 ± 0.051^{a}

Different superscripts in each column indicate significant difference

TABLE I A: Comparative level of significance between different class of follicles with respect to calcium concentration

Sl.No.	Class of Follicles	Level of significance		
		Follicular wall	Granulosa cells	Follicular fluid
1.	Between PN and PA	P = <0.01	P = <0.01	P = <0.01
2.	Between ON and OA	P = <0.01	P = <0.01	P = <0.01
3.	Between PN and ON	P = <0.01	P = <0.01	NS
4.	Between PA and OA	P = <0.01	NS	NS

NS = Not significant

Ca^{++}-Mg^{++} dependant endonuclease activity

The results are presented in Table II and IIA and are expressed as Mean ± SE. A significant difference ($P<0.01$) was observed among all the four classes of FW and GC samples. In case of FW the activity was highest in OA followed by ON, PA and PN, while in GC the highest activity was seen in OA, followed by PA, PN and ON.

TABLE II : Ca^{++}-Mg^{++} dependant endonuclease activity in different components of buffalo ovarian follicles

Sl.No.	Class of Follicles	Follicular wall % DNA degraded	Granulosa cells % DNA degraded
1.	PN	27.51 ± 0.418^{d}	36.52 ± 0.382^{c}
2.	PA	33.79 ± 0.497^{c}	40.27 ± 0.363^{b}
3.	ON	36.04 ± 0.098^{b}	33.75 ± 0.191^{d}
4.	OA	39.44 ± 0.244^{a}	43.63 ± 0.244^{a}

Different superscripts in each column indicate significant difference

TABLE II A: Comparative level of significance between different class of follicles with respect to Ca^{++}-Mg^{++} dependant endonuclease activity

Sl.No.	Class of Follicles	Level of significance	
		Follicular wall	Granulosa cells
1.	Between PN and PA	P = <0.01	P = <0.01
2.	Between ON and OA	P = <0.01	P = <0.01
3.	Between PN and ON	P = <0.01	P = <0.01
4.	Between PA and OA	P = <0.01	P = <0.01

Agarose gel electrophoretic pattern of genomic DNA

The genomic DNA from FW and GC samples were subjected to agarose gel electrophoresis. The FW samples showed a single distinct well defined band which corresponded with the 21.22 Kb molecular weight markers in all the four classes (Fig 1). In case of GC samples, the PN and ON classes showed the same distinct single band like FW samples, while the PA and OA samples showed a smear pattern extending towards the low molecular weight region (Fig. 2).

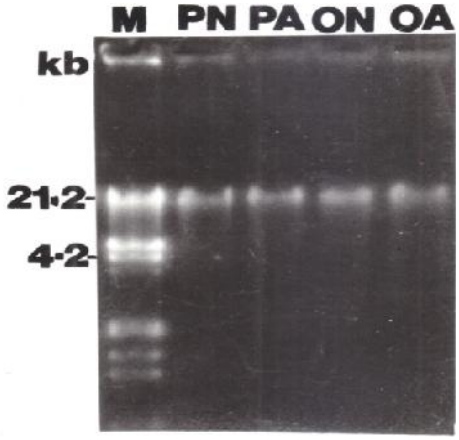

FIGURE 1: Agarose gel electrophoretic pattern of genomic DNA from follicular wall (FW)

Lane M : DNA molecular weight marker (λ DNA – EcoRI and Hind III double digest)
Lane PN: Genomic DNA extracted from pre-ovulatory non-atretic FW
Lane PA: Genomic DNA extracted from pre-ovulatory early-atretic FW
Lane ON: Genomic DNA extracted from ovulatory non-atretic FW
Lane OA: Genomic DNA extracted from ovulatory early-atretic FW

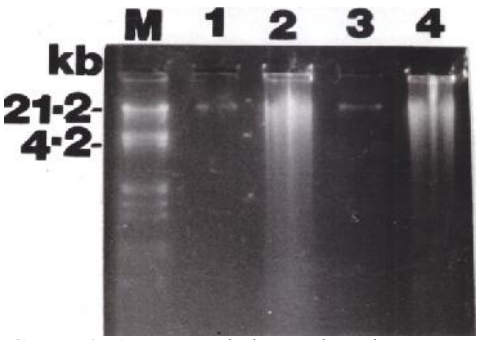

FIGURE 2: Agarose gel electrophoretic pattern of genomic DNA from granulosa cell (GC)

Lane M : DNA molecular weight marker (λ DNA – EcoRI and Hind III double digest)
Lane 1: Genomic DNA extracted from pre-ovulatory non-atretic GC
Lane 2: Genomic DNA extracted from pre-ovulatory early-atretic GC
Lane 3: Genomic DNA extracted from ovulatory non-atretic GC
Lane 4: Genomic DNA extracted from ovulatory early-atretic GC

DISCUSSION

Calcium

The role of calcium is diverse in that they play a key role both in folliculogenesis and atresia. For instance, N-Catherin, a follicle survival factor (Makrigiannakis, 1999) and Collagenases which aid folliculogenesis (Garcia *et al*., 1997) need calcium. On the other hand, the Ca^{++}-Mg^{++} dependant endonuclease, the potent enzyme underlying apoptosis leading to follicular atresia requires calcium (Zeleznik *et al.,* 1989) and calcium is kept under control during folliculogenesis by EGF, which would otherwise induce atresia (Luciano et al., 1994) suggests the active role of calcium in inducing follicular atresia. The result of the present study indicates that there is progressive decrease in the concentration of calcium from FW to GC and into FF. Further their concentration was not related to the stage of development but was related to atresia i.e. a higher concentration of calcium was observed in all the components of early atretic follicles. All these observations suggest that calcium is derived from serum and plays an important role in inducing atresia.

Ca^{++}-Mg^{++} dependant endonuclease activity

The results show that this inducible enzyme is present in both thecal and granulosa cells. However, the activity in FW (thecal cells) increases with the growth of the follicle and in GC, they show a direct positive correlation with the concentration of calcium i.e. higher activity in early-atretic follicles. Therefore it is possible that calcium induces the activity of these enzymes in GC where initiation of follicular atresia takes place via apoptosis. Further the highest activity present in the OA granulosa cells was 43% only which means that this enzyme activity alone cannot initiate atresia and may do so by acting together with other atretogenic factors. Likewise the least activity present in ON granulosa cells itself was 33% which signifies that other folliculogenic factors (like EGF) must be involved in controlling the enzyme in non-atretic follicles. The present results of the enzyme activity in GC are in close agreement with that reported in rat by Zeleznik *et al.* (1989).

Agarose gel electrophoretic pattern of genomic DNA

The presence of an intact single band in the high molecular weight region (21.2 Kb) in all the lanes of FW sample and in the non-atretic classes of GC sample show that there is no cleavage of DNA and hence no apoptosis. On the other hand, presence of a DNA smear which is extending towards the low molecular weight region (<0.83 kb) in the pre-ovulatory and ovulatory categories of GC collected from atretic follicles indicates extensive cleavage of DNA by apoptosis. Resolution of DNA ladders was not clear which may be due to the limitation in the sensitivity of ethidium bromide staining procedure (50 g of DNA is needed for visualization of DNA ladders by ethidium

bromide staining) as described by Bosu *et al.* (1996). Nevertheless it was clear that mechanism linked to apoptosis and not necrosis was responsible for GC death in atretic follicles. Thus, this result not only served as a tool for judging the microscopic evaluation of follicles but also proved that buffalo GC also undergo follicular atresia through apoptosis like other species (rats, cows, sheep, human, etc). Moreover, it also confirmed the reports of Bilig *et al*. (1993) and Manabe *et al.* (1996) that only GC undergo apoptosis and not thecal cells of a follicle.

CONCLUSION

The study revealed that apoptosis is the potential mechanism underlying buffalo follicular atresia and calcium induces the activity of the enzyme Ca^{++}-Mg^{++} dependant endonuclease which does play an important role in initiating apoptosis in the early-atretic follicles of buffalo, leading to follicular atresia.

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