INTERNATIONAL JOURNAL OF ADVANCED BIOLOGICAL RESEARCH

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ACTIVITY OF COLLAGENASE AND COLLAGENASE INHIBITOR IN RELATION TO FOLLICULOGENESIS AND FOLLICULAR ATRESIA IN BUFFALO

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ABSTRACT
Collagenase and collagenase inhibitor in buffalo ovarian follicles were measured to find out their role in follicular atresia. Granulosa cells (GC), follicular fluid (FF) and follicular wall (FW) from non-atretic and early atretic follicles of pre-ovulatory and ovulatory stages of were studied. Collagenase inhibitor was destroyed by reduction and alkylation before measuring the activity of collagenase. Inhibitor activity was estimated by incubating the samples with collagenase and determining the percent inhibition after comparing the activity with that of a control (without sample) which was considered to be 100 percent. Follicular wall had shown highest collagenase activity followed by GC. Follicular fluid from pre-ovulatory follicles had little collagenase activity but no activity could be detected in ovulatory follicles. On the other hand, FW from none of the follicles had collagenase inhibitor but GC had the highest concentration of inhibitor followed by FF. Ovulatory non-atretic follicles had the highest activity of collagenase in FW, which was significantly higher than pre-ovulatory non-atretic and ovulatory atretic follicles. No significant variation in collagenase activity in GC was observed either due to size and atresia. Collagenase inhibitor in GC also was not significantly different between atretic and non-atretic ovulatory follicles though significantly higher value was found in pre-ovulatory non-atretic follicles. It appears that these enzyme activities in FW play important physiological role in ovulation and follicular atresia.

KEYWORDS: *Collagenase, Collagenase inhibitor, Buffalo, Follicular atresia, Folliculogenesis.*

INTRODUCTION

Ovary is the dynamic female reproductive organ and 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. Follicular growth and maturation represent a series of sequential sub-cellular and molecular transformations of various components of the follicle such as oocyte, granulosa and theca. These are governed by several paracrine, autocrine and endocrine signals that regulate the recruitment, selection, differentiation, maturation and dominance of follicles (Hafez, 1987). The major challenge met by the mammalian ovary is to maintain the continuous development of small follicles and at the same time to allow species-specific number of follicles to ovulate and become corpora lutea. They also inhibit other co-existing follicles from development deviating them towards a degenerative change called atresia (Fortune, 1994). 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 (superovulation) or rate of atresia decreased.

Follicular atresia

Though many morphological and biochemical characteristics of atretic follicles have been identified (Richards, 1980; Ryan, 1981; Farookhi, 1981), 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 farm 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.

Proteolytic enzymes and their inhibitors

Role of proteolytic enzymes (proteinases) and their natural inhibitors in follicular maturation and ovulation by remodelling of extracellular matrix has been documented in many species (Beers *et al.*, 1975; Strickland *et al.*, 1976, Lipner, 1988). Among the various proteinases, the collagenase and collagenase inhibitor activity have been shown to play a key role in ovulation and follicular atresia. Hence, the present study was undertaken to study the role of collagenase and collagenase inhibitor 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

Azocoll and Collagenase were procured from M/s. Sigma Chemical Company, USA, Iodoacetamide from M/s.Merck, Germany, Casein and DTT 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.

Assay of Collagenase activity in FW, GC and FF samples

The cell-free FF stored at -20°C was used as such for the assay, while the empty, clean FW and the GC were resuspended in Tris-buffer and taken for derivation of collagenase activity.

Follicular collagenase activity was derived following the methodology described by Weeks *et al.* (1976). The empty FW and he resuspended GC were homogenised in 5.0 ml and 2.0 ml of Tris-buffer respectively using a mechanical tissue homogeniser. These homogenates were centrifuged at 5000 rpm for 20 min at 4°C in a refrigerated centrifuge (Remi, Model K 70). The FW and GC precipitates were rehomogenised in 5.0 ml and 1.0 ml of rehomogenisation buffer respectively and incubated at 60°C for 10 min to dissociate collagenase bound to collagen. Then they were recentrifuged under the conditions mentioned above. The combined supernatants from steps one and two above of both thecal and granulosa cells were dialysed overnight against a 100x excess of Tris buffer solution at 4°C. The FF was also heated at 60°C for 10 min after the addition of enough calcium chloride to make it 100mM final concentration and dialysed under the same conditions.

The inactive metalloproteinase inhibitors, the dialysed supernatants from FW and GC and the dialysed FF were treated with 2mM DTT at 37°C for 30 min and then with 5 mM iodoacetamide at the same temperature during another 30 min and dialysed overnight against Tris buffer solution under the conditions mentioned above. These samples were taken for assay of collagenase activity.

Collagenase Assay Proper

Collalgenolytic activity was determined in the above samples by the method of Chavira *et al.* (1984) using Azocoll, an insoluble protein-dye conjugate. The reaction mixture in the total volume of 1.0 ml contained an uniform suspension of Azocoll in Tris buffer (pre-incubated at 37°C for 15 min) and a suitable aliquot of the samples. These were incubated at 37°C for 1 h under constant agitation in a rotary water bath shaker. Assays were stopped by placing tubes on ice for 3 min and centrifuging at 3000 rpm for 15 min. The rate of the reaction was followed by measuring the increase in absorbance of the supernatants at 520 nm in a PC based UV-Vis Spectrophotometer (GS 5703 AT, ECIL, India). The enzyme activity was expressed as units per mg protein, where one unit was defined as equivalent to 0.01 absorbance.

Assay of Collagenase inhibitor activity in FW, GC and FF samples

Inhibitory activity towards the collagenase was estimated by percent inhibition after incubating pure enzyme (Collagenase) with samples (FW/GC/ FF) and measuring residual activity. The activity of collagenase in a control tube was considered as 100 percent. The enzyme activity was measured by increase in absorbance at 280 nm of a casein solution (Rakhee, Ph.D. Thesis, 1999).

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.

Collagenase Inhibitor Assay Proper

The cell-free FF and GC supernatants were suitably diluted with assay buffer while FW supernatant was used as such for this assay. The experimental tubes contained 0.1 ml sample with 0.4 ml enzyme and were incubated at 37°C for 30 min. Then 2.0 ml of substrate (casein

solution) was added and further incubated for 30 min at 37°C. The control tubes had 2.0 ml of substrate and 0.4 ml of enzyme in a total volume of 2.5 ml made with assay buffer, while the blank tube contained 0.5 ml assay buffer and 2.0 ml substrate solution. Both the control and blank tubes were incubated at 37°C for 30 min. The reaction was stopped by adding 4.0 ml of 5% TCA solution. All these tubes (the experimental, control and blank) were centrifuged at 3000 rpm for 10 min at 4°C and the absorbance of these supernatants were measured at 280 nm. The enzyme activity was expressed as Inhibitory units/mg protein (IU/mg protein), where one IU was defined as equivalent to 5% inhibition.

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.

Statistical analysis

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

RESULTS

Collagenase activity

The results of collagenase activity are presented in Table I and IA and are expressed as Mean ± SE. The collagenase activity in FW differs significantly (P< 0.01) among all the four classes of follicles, with the activity being highest in ON, followed by PN, OA and PA. Similarly the activity was significantly different (P< 0.01) among all the four classes of GC samples, but with the activity being highest in PN, followed by PA, ON and OA. In case of FF, the PN class showed a significantly (P< 0.05) higher activity than PA and the activity was not detected in other two classes (ON and OA).

TABLE I : Collagenase activity in different components of buffalo ovarian follicles

Sl.No.	Class of Follicles	Follicular wall U/mg protein	Granulosa cells U/mg protein	Follicular fluid U/mg protein
1.	PN	513.30±0.578^{b}	313.20±0.309^{a}	2.83±0.04^{a}
2.	PA	386.73±0.896^{d}	284.73±0.308^{b}	2.63±0.04^{b}
3.	ON	644.18±0.486^{a}	234.06±0.491^{c}	ND
4.	OA	415.76±0.870^{c}	216.24±0.580^{d}	ND

TABLE IA : Comparative level of significance between different class of follicles with respect to collagenase activity

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 = <0.05
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	-

(-) indicate statistical analysis not done as enzyme activity was ND

Collagenase inhibitor activity

The results of collagenase inhibitor activity are presented in Table II and IIA and are expressed as Mean ± SE. It is interesting to note that the activity was not detected in any of the FW samples, while it was present in all the classes of GC and FF samples. Like the collagenase activity, the collagenase inhibitor activity was also highest in the PN class of GC samples and it showed a significant variation (P< 0.01) from the other three classes. But no significant variation was observed between ON and OA classes of GC samples though a progressive decline in the activity from ON, OA and PA was observed. The FF collagenase inhibitor activity showed a significant difference (P< 0.01) among all the four classes of follicles, with the activity being highest in PN, followed by ON, PA and OA.

TABLE II : Collagenase inhibitor activity in different components of buffalo ovarian follicles

Sl.No.	Class of Follicles	Follicular wall U/mg protein	Granulosa cells U/mg protein	Follicular fluid U/mg protein
1.	PN	ND	9.92±0.170^{a}	12.30±0.571^{a}
2.	PA	ND	6.75±0.207^{c}	8.47±0.205^{c}
3.	ON	ND	7.27±0.130^{b}	10.93±0.021^{b}
4.	OA	ND	6.99±0.153^{bc}	3.78±0.151^{d}

TABLE IIA : Comparative level of significance between different class of follicles with respect to collagenase inhibitor activity

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
2.	Between ON and OA	-	NS	P = <0.01
3.	Between PN and ON	-	P = <0.01	P = <0.01
4.	Between PA and OA	-	NS	P = <0.01

(-) indicate statistical analysis not done as enzyme activity was ND
NS = Not significant

DISCUSSION

Collagenase activity

The above results show that the collagenase activity is always higher in non-atretic follicles (which are destined to ovulate) than the early atretic follicles in all the three components of the follicle. *i.e.* FW, GC and FF. Further the maximum activity was present in FW followed by GC. Very low activity was detected in FF of pre-ovulatory while no activity was detected in FF of ovulatory follicles. All these findings are in close agreement with those of Garcia *et al.* (1997) who has reported similar pattern of collagenase activity in all these components of the goat follicles. The finding implicates that the prime importance of collagenase is to rupture the follicular wall at the time of ovulation and it plays other probable roles in GC and FF like the degradation of the regulatory peptides (inhibin, activin, EGF, bradykinin, angiotensin II, substance P, vasoactive intestinal peptide, etc.) or proteoglycans (Garcia *et al.,* 1997). A pre-ovulatory increase of collagenase activity has been observed in GC and FF in this study and this observation also is in agreement with those by Garcia *et al.* (1997) in the GC and FF of goats, FF of rats (Curry, Jr. *et al.*, 1986) and humans (Fukumoto *et al.*, 1981, Puistolo *et al.*, 1986 and Reich *et al.*, 1986).

It was demonstrated that the pre-ovulatory increase of collagenase activity is gonadotropin dependent (Curry, Jr. *et al.*, 1986) and gonadotropins increase tissue collagenase production presumably in fibroblasts, germinal epithelial cells, granulosa cells or blood borne accumulation in leukocytes. This pre-ovulatory increase in the collagenase activity in GC and FF, together with the presence of higher activity in ovulatory FW and a lower activity in atretic follicles suggests:

1. The granulosa cells synthesise collagenases and export them extracellularly to FF and FW.
2. The synthesis is maximal during pre-ovulatory stage of development as it gets induced by LH (pre-ovulatory LH surge).
3. The FW may also acquire collagenases from serum besides GC, particularly during the ovulatory stage of development because of increased vascularisation.
4. The major role of collagenases is to help in the process of ovulating healthy non-atretic follicles.

Collagenase inhibitor activity

Thus the maximum activity of Collagenase inhibitor was present in pre-ovulatory non-atretic categories of GC and FF. Rakhee (1999) has also reported that the inhibitor activity was more in non-atretic buffalo FF than the atretic classes and a follicle specific low molecular weight collagenase inhibitor is secreted by the buffalo granulosa cells. Further no activity was detected in any of the FW categories in this study. Hence all these observations suggest that

1. The granulosa cells synthesise collagenase inhibitors maximally during pre-ovulatory stage and export them to FF.
2. Their role may be limited in regulating the collagenase activity primarily in FF and secondarily in GC. Moreover, their effect on FW is minimal so that collagenases can exert their full potential in the degradation of FW resulting in ovulation.

From the reports of Ny *et al.* (1985) and Reich *et al.* (1986) it is evident that the tissue plasminogen activator (a serine protease inhibitor) converts the zymogen plasminogen into plasmin which in turn converts the latent collagenase (procollagenase) to active enzyme. Granulosa cells were found to contribute to more than 80-90% plasminogen activator activity. Further, the findings of present study and those of Rakhee (1999) indicates the role of specific collagenase inhibitors in finely tuning the collagenase activity with respect to time or stage of follicular development and maturation, all of which culminate in ovulation or atresia.

CONCLUSION

Hence, it may be concluded that the modification of the steady state of this system (collagenase and collagenase inhibitor interactions) is essential in regulating the processes by which normal maturation and atresia of the follicles take place leading to the appropriate number of ovulatory follicles in each animal species including buffalo.

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