Selected Proteins and Enzymatic Analysis of Follicular Fluid of Indigenous Pig at Different Developmental Stages of Ovarian Follicle: a Quantitative Study

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Abstract

In the present study, characteristics of porcine follicular fluid (FF) harvested from different sized ovarian follicles and developmental competences of enclosed oocytes in relation to their sizes were investigated. It has been observed that some protein and enzyme components of FF like lipase, amylase and CPK increased in concentration as the follicles increased in size, while some other components like AFP, ACP and ALP were found to be decreasing order with increase in follicular size. A few unexplored components like AFP and Li have been studied here in the present study. Moreover, presence of amylase, lipase and CPK in FF and their concentrations have also been detected in the porcine FF for the first time in this study.

1. Introduction

Follicular fluid (FF) where oocytes grow and mature is a mixture of serum exudates and locally produced metabolites of follicular cells (Gerard et al., 2002) and is found in an avascular compartment within the ovarian follicle. Not only that, now a days intensive studies involving incorporation of FF into medium showed encouraging results in *in-vitro* maturation and fertilization of oocytes (Bordoloi et al.,1999; Sangha et al., 1999). Although biochemical profiles of FF is easily available in different domesticated animals, but information on analysis of FF is scanty in case of indigenous breed of pig in India. Moreover, some of the new information like presence of α-fetoprotein, amylase, lipase and creatinine kinase in FF has not been reported so far in porcine. Hence, the aim of the present work was quantitative biochemical estimation of different proteins like total protein, albumin, globulin and α-fetoprotein and enzymes like alkaline phosphatase, acid phosphatase, lipase, amylase, creatinine kinase concentrations of Porcine FF harvested from different sized ovarian follicles, collected from slaughtered sows, in an attempt to correlate possible changes in the compositions of FF with follicular dynamics and also having an objective to enhance more success in reproduction in this species, which may improve the culture conditions of oocytes.

2. Materials and Methods

In total 200 number ovaries, free from gross pathological lesions, were collected from slaughter house at Kolkata, India during the months of March and May, 2011 from clinically healthy sows with good reproductive history. Average body weight of the animals varied from 95 to 110 kg. After collection, ovaries were washed thoroughly with normal saline solution and then ovaries were wrapped in plastic sheets, placed in a thermos flask and taken to the laboratory within 3 h after slaughter.

Ovaries were placed into Petri dishes and washed thoroughly with normal saline solution to make them free from cellular debris and foreign materials. Bursa and fatty tissues were dissected carefully from the ovaries. The diameter of various follicles (1-12 mm) present on each ovary was measured with the help of a geometrical divider and scale. According to the follicular diameter, the follicles were divided into three

groups, i.e. small (1-2 mm), medium (3-5 mm) and large (6-12 mm), respectively.

FF was aspirated by sterile insulin syringes from all the three categories of follicles individually, by holding them in forceps and giving negative pressure on the follicles. Separate pools were made for each size of follicles, and kept into properly coded sterilized graduated vials. Antiproteolytic agent, phenyl methyl sulfonyl fluoride [PMSF) and anti-clotting agent Heparin were added to each vial at the rate of 20 mg ml^{-1} and 25 IU ml^{-1}, respectively (Nandi et al., 2007b). To aspirate FF from small, medium, and large sized follicles, 170, 65 and 20 number follicles were selected, respectively. A total number of 10 pooled samples of 2 ml from each of the groups were collected. All the procedures were completed within 2 h after collection and processed at 4°C.

Samples of aspirated FF was centrifuged at 5000 g for 30 minutes to remove the cells and cellular debris as per Maniwa et al. (2005), and filtered through 0.2 mm filter paper. The aliquots of FF, 2 ml each were kept individually into three sterilized coded tubes. Except other biochemical analysis, enzymatic estimations were performed with only 1 ml freshly processed FF from each category of follicle and the rest 1 ml of FF in each of the three tubes were frozen quickly and stored at -20°C until used. The frozen samples were analysed within 7 days. Biochemical analysis of FF was performed using commercial kits. For biochemical estimation of following parameters, frozen samples were thawed quickly to minimize protein denaturation.

Total protein and albumin were estimated as per Doumas et al. (1975) and Doumas et al. (1972), respectively using standard kit. Globulin content was calculated from the difference between FF total protein and albumin. α-fetoprotein was estimated as per Engall (1980) from FF by standard kit. Analysis of enzymes from freshly collected FF was performed for acid phosphatase (ACP) and alkaline phosphatase (ALP) as described by Tietz (1995) by standard kit. Estimation of lipase as per Lott et al. (1986) and creatinine kinase (CK) as per Szasz et al. (1976) was performed with the kits, respectively. Estimation of amylase was performed by chromogenic method as described by Klein et al. (1970) using standard kit.

The mean values (±SE) for concentrations of various biochemical constituents of FF from different sized follicles were computed. Differences between the biochemical constituents among the different sized follicles were analyzed by the linear regression model. Data were analyzed using SPSS package following standard procedures as described by Snedecor and Cochran (1994) for ANOVA and Duncan's multiple range tests.

3. Results and Discussion

The mean values (±SE) for concentrations of various biochemical constituents of FF from different sized follicles were computed (Table 1). Differences between the biochemical constituents among the different sized follicles were analyzed by the linear regression model.

The albumin content had no significant difference between small and medium follicles but differed significantly ($p \leq 0.05$) in large follicles (Table 1). This indicated that follicular growth does not seem to have any effect on albumin content in small and medium follicle. Globulin plays a significant role in the body due to its immunity producing activity. In the present study, globulin was found non-significant in follicular fluid. The small quantity of globulin might be necessary for protecting the follicles from external environments.

The injected rat α-fetoprotein was localized to the zona pellucida of follicles undergoing atresia. Although, physiological role of AFP remains an enigma, its presence was found in FF and this study demonstrated that AFP was not only present

Table 1: Concentration of different protein and enzyme components in porcine FF

Parameter	Small sized follicles (1-2 mm)	Medium sized follicles (3-5 mm)	Large sized follicles (6-12 mm)
Total protein (g dl^{-1})	6.055 ±.112	5.804 ±0.059	5.53 ±0.09
Albumin (g dl^{-1})	3.931 ±0.02	3.796 ±0.055	3.536 ±.08
Globulin (g dl^{-1})	2.164 ±0.1	2.008 ±0.042	1.994 ±0.1
A:G	1.8546 ±.0.1	1.8975 ±0.05	1.927 ±0.16
AFP (ng ml^{-1})	1.816 ±.167[a]	0.88 ±.019[b]	0.691 ±.086[c]
ACP (μ l^{-1})	15.28 ±0.120[a]	6.775 ±0.0941[b]	6.55 ±0.0833[b]
ALP (μ l^{-1})	11.788 ±.06119[a]	8.267 ±.06314[b]	6.801 ±.03542[c]
Lipase (μ l^{-1})	129.5 ±1.0672[c]	178.4 ±.8969[b]	253.2 ±1.5406[a]
Amylase (μ l^{-1})	1114.7 ±3.7949[c]	1946.10 ±4.5472[b]	3128.50 ±2.0344[a]
CPK (μ l^{-1})	1140.1 ±2.6602[c]	7604.7 ±23.2795[b]	10191.1 ±3.8914[a]

Values were expressed as mean±SE; Values with different superscripts in a row differed significantly ($p<0.05$)

in porcine FF but its concentration decreased significantly ($p<0.01$) with the development of follicles. However, the functions of AFP in FF remain to be investigated.

Lipase activity in this study found to be highest in large follicular fluid and lowest in small follicular fluid. Amylase activity was found to be highest in large follicular fluid and lowest in small follicular fluid. In the present study, CK concentration was found significantly higher in large follicles than in the medium follicles. Proteins in the living cells are intimately associated with various phases of activity that constitute the life of the cells, McDonald et al. (1984). Total protein concentration of different sized follicular fluid in the present study was comparable with earlier reports of Schuetz and Anisowicz (1974) in pig. However, the present study was not comparable with Chang et al. (1976) in pig. Albumin concentration of different sized follicular fluid in the present study was corroborated well with earlier works of Bardoloi et al. (2003) in goat, however differed with Arshad et al. (2005). The present finding on globulin was similar with the studies of Arshad et al. (2005) in buffalo. Result of A:G ratio in this study was found to be similar with the findings of Madhan Mohan et al. (1997) in buffalo.

The gradual decreases in the concentration of total protein, albumin and globulin in the different sized follicles might have relation with estrogen and water uptake in growing follicles and due to this water uptake there is dilution of follicular protein concentration (Schuetz and Anisowicz, 1974). Sidhu et al. (1985) observed that total protein concentrations in small, medium, and large sized goat ovarian follicular fluid were 84.24%, 69.16%, and 71.02% of total protein of serum, respectively. A number of proteins reside in follicular fluid and surrounding granulosa cells, therefore, a small change in hydration could produce large effects on protein concentrations as follicles were growing (Rupley et al., 1983).

Developing follicles need amino acids for their activity. Ovary is one of the most active tissues in catabolizing albumin (Yedgar et al., 1983). There is active inward transport of albumin from blood into follicles which may be required for binding some chemicals as well as minerals inside the follicular fluid for various physiological functions including growth and maturation of follicles (Arshad et al., 2005).

AFP is present in high level in foetal fluids, certain neoplasm and in regenerating liver. In human AFP, Carcino Embryonic Antigen (CEA), and CA-125 have been found in FF after ovarian stimulation (Jimena et al., 1993). Presence of AFP in serum, bile, CSF and faeces suggests that AFP plays a similar role in body homeostasis. Presence of AFP in the developing follicles of the ovary, in human ovarian follicle, and in the developing and newborn brain, suggests it may somehow be involved in the maturation and programming of the positive feedback exerted by ovary-derived estrogen on brain LH and FSH levels (Germain et al., 1978). Physiological studies on AFP-estrogen interaction revealed rat α-fetoprotein was able to inhibit the formation of water-soluble metabolites of E_2 and E_1, when microsomes from rat liver were incubated in the presence of NADPH, and to regulate the activity of 17β-hydroxy steroid dehydrogenase *in-vitro* (Aussel and Masseyeff, 1976). AFP has also been associated with the delay in the onset of puberty in postnatal rat pups (Greenstein, 1992). The injection of AFP during the pre-pubertal period in rats has resulted in a decreased number of primordial and primary follicles in the ovary (Stora et al., 1987).

The changes in enzymatic activity of ACP have an important role in ovulation, and it seems that there is relationship between its presence and oocyte fertilization by spermatozoon (Gonzalez et al., 1989). However, ACP may be important for steroidogenesis. Phosphatase enzymes were implicated in both growth and atresia of follicles. A progressive decrease in the ACP and ALP activity with follicular maturation was noted. Phosphatase activities found in the present study were similar with the findings of Chang et al. (1976) in pig. ALP and ACP are lysosomal enzymes which catalyze various reactions in the body and are involved in the active transport of protein and DNA turnover in nucleus (Mishra et al., 2003). The higher alkaline phosphatase activity in the initial stages of follicular development might be due to a progesterone and androgen dominant environment that exits in the small follicle, in that a higher concentration of progesterone and androgen could be conducive to phosphatase activity (Kalmath, 2000). The decreased FF alkaline phosphatase activity with the development of the follicle in the present study could be due to the shift in the follicular hormonal milieu from androgen to estrogen dominance, with the development of the follicle. Thus, the changes in the phosphatase activity could reflect changes in metabolic activity on tissue synthesis mechanisms induced by the different hormones.

The role of the endothelial-bound lipases in ovarian physiology is largely unknown. The ovary is metabolically very active organ that needs fatty acids as energy substrate for synthesis of lipid mediators and cholesterol for hormone synthesis. The rat ovary has been shown to be highly dependent on oxidation of fatty acids for the generation of adenosine 5'-triphosphate for energy metabolism, growth, and steroidogenesis (Flint and Denton, 1970; Tan and Robinson, 1981).The lipase would facilitate the supply of fatty acids from lipoproteins to cells of several parts of the ovary, including the corpus luteum and the growing follicle with its enclosed oocyte and the avascular granulosa layer. Lipases bound to the luminal side of the vascular endothelium may thus be the first in line among several mechanisms facilitating the uptake of fatty acids and

cholesterol by ovarian cells (Camps el al., 1990). According to Kasperczyk et al. (2001), α-amylase activity in ovarian tissue was more during pro-estrus when follicular size was large and low in diestrus when follicular size was small, because FF is partially derived from ovarian epithelium. Findings of the present study corroborated well with this observation. Kasperczyk et al. (2001) also suggested that change of amylase concentration might be due to action of sex hormones on ovarian tissues. According to Malnick et al. (1983), brain-type isozyme of creatinine kinase (CKBB) responds to estrogen in female reproductive tract. Chang et al. (1976) reported that estrogen concentration increased as follicular size increased in pig. Therefore, from the present study, we can presume that there might be a positive correlation between concentration of CK and estrogen in all three sizes of follicles in pig and it could be used as an excellent biochemical marker for maturation process of follicles.

4. Conclusion

The present research may throw new light in understanding the process of follicular maturation and successful ovulation leading to augmentation of reproductive efficiency in farm animals.

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