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RESEARCH ARTICLE

Follicular fluid protein profile in buffalo

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Abstract : The present study was planned to investigate and compare the protein profile *viz*. total proteins, albumin, globulin, albumin/globulin ratio, uric acid, urea and creatinine in follicular fluid and serum in buffaloes. The electrophoretic pattern of proteins in follicular fluid of different sizes of ovarian follicles and serum were also studied. Buffalo ovaries, at random stage of oestrous cycle and with unknown reproductive status were obtained from Deonar Abattoir, Chembur, Mumbai, during their e-visceration. Pairs of ovary from each buffalo were collected in separate sterile plastic bags. They were carried to the laboratory in thermos flask containing ice packs. In the laboratory, the ovaries were cut open at the hylus using a pair of sterile scissors. These were washed with tap water, distilled water and finally with deionised water. The follicles visible on its surface were classified based on their diameter as small (<5 mm), medium (5-10 mm) and large (>10 mm) follicles using digital vernier caliper. The fluids from these follicles were aspirated using 26 gauge-2 ml syringe. Twenty four samples each from small, medium and large sized follicles along with blood samples of buffaloes belonging to respective category were collected. Besides, blood samples from 12 buffaloes during mid-oestrous cycle from a private farm (Imom Son's Dairy Farm, Thane-400 604) were collected. Blood samples were allowed to clot at room temperature. Clear serum was separated by centrifugation at 1500 rpm for 30 min. The ovarian follicular fluid samples were centrifuged at 2000 rpm for 15 minutes in order to remove the cell debris. The follicular fluid and the blood samples were analysed for total proteins, albumin, globulin, albumin/globulin ratio, urea, creatinine and uric acid using STAT FAX 2000 Autoanalyser and kits. A random sample from the three classes of follicles each and a serum sample were used for protein fractionation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The results of the present study indicated that the protein components in the follicular fluid and serum exhibited increase / decrease in accordance with follicle size. The electrophoretic pattern of follicular fluid and serum showed significant difference between two fluid compartments. The small reservoir of fluid of follicles reflects the biochemical activity of the follicle. It is, therefore, suggested to carry out further studies to elucidate the precise role of these biochemical components and separated proteins which will help in understanding of the basic changes ongoing during follicular development, so that the optimal environment could be established for the maturation of viable oocytes.

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INTRODUCTION

Follicular fluid (*Liquor folliculi*) is a serum transudate that accumulates in extracellular spaces within ovarian

follicle. It is composed partly of secretions from the follicle and partly of exudates from plasma. Its composition reflects changes in the secretory processes of the granulosa layer and theca interna and alterations in the components of plasma due to physiological or pathological processes. Follicular development and ovulatory process in mammals involve local biochemical changes as a result of substantial modifications in cellular metabolism, the best known of which is steroid variations. Follicular fluid is a viscous solution and the viscous nature is due to mucopolysaccharides. The pH of human follicular fluid is generally above 7.0 and is either similar to or lower than that of serum or plasma (Shalgi *et al*., 1972).

McNatty (1978) studied extensively the chemical composition and properties of the possible physiological role of follicular fluid. Follicular fluid contains several proteins, amino acids, sugars, enzymes, (collagenase, hylauronidase, transaminase, alkaline and acid phosphatases), salts, mucopolysaccharides, gonadotropins (L.H., F.S.H., Prolactin), vitamins and steroids (Schweigert and Zucker, 1988) and growth factors (Spicer and Geisert, 1992). The functions of follicular fluid include the following: 1) regulation of the function of granulosa cells, 2) initiation of follicular growth and steroidogenesis, 3) oocyte maturation, ovulation and egg transport to the oviduct, 4) preparation of the follicles for the formation of subsequent C.L., 5) the stimulatory and inhibitory factors in the fluid regulate the follicle cycle and the volume of fluid released at ovulation is also important along with the oviductal secretions of the environment in which sperm metabolism, capacitation and early embryonic development takes place (Hafez and Hafez, 2000). Very recently, Dhaware *et al*. (2007) showed that the biochemical parameters do change with change in size of the follicles; alkaline phosphatase, acid phosphatase, lactate dehydrogenase, triglycerides and low density lipoproteins showed a decrease in activities/concentrations with increase in size of follicles. Whereas, total cholesterol, high density lipoproteins, estradiol and progesterone showed an increase in concentrations with increase in size of follicles. The small reservoir of fluid of follicle, therefore, reflects the biochemical and especially endocrinological activity of the follicle.

McNatty (1978) suggested the utilization of follicular fluid as a holding medium since this is the normal fluid bathing oocytes in an *in vivo* environment. Several studies have proved the favourable effects of supplementing IVF medium with follicular fluid in pig (Naito *et al*., 1988); sheep (Sun *et al*., 1994); cow (Kim *et al*., 1996); horse (Dell'Aquilla *et al*., 1997) and buffalo (Yadav *et al.*, 1997). Wise (1987) investigated some biochemical changes during bovine follicle development and opined that changes in biochemical components found in follicular fluid that relate to growth and atresia process may provide a more sensitive and accurate method to classify follicle status and thus, aid in understanding the complexity of events associated with maturation of bovine follicles and oocyte. The presence of bovine follicular fluid from competent follicles as a source of protein and the synthetic oviduct fluid medium supplemented with estradiol and recombinant FSH increased blastocyst numbers and improved embryonic ability to levels approaching those characteristic of *in vivo* matured oocytes. The bovine follicular fluid originating from follicles containing competent oocytes had greater developmental potential than follicular fluid derived from small follicles and that the origin of bovine follicular fluid, which is added to the maturation media as a source of protein, might be an important factor for improving *in vitro* development of bovine oocytes (Ali *et al*., 2004).

Thus, the protein content in the follicular fluid reflects the functional status of follicular cells. Detailed knowledge on protein pattern of follicular fluid would be helpful in elucidating the process of oocyte maturation. Information obtained from such studies would help in formulating the media for in vitro maturation of oocytes. The present research project was planned to evaluate the protein profile *viz.*, total proteins, albumin, globulin, albumin : globulin ratio, urea, creatinine and uric acid in follicular fluid and serum in buffalo; to study the electrophoretic pattern of proteins in follicular fluid of different sizes of ovarian follicles and serum in buffalo and to compare the follicular fluid protein profile and electrophoretic pattern of proteins studied with that in serum of buffalo.

RESEARCH METHODOLOGY

Collection of follicular fluid and serum samples :

Buffalo ovaries, at random stage of oestrous cycle and with unknown reproductive status were obtained from Deonar Abattoir, Chembur, Mumbai, during their e-visceration. Pairs of ovary from each buffalo were collected in

separate sterile plastic bags. They were carried to the laboratory in thermos flask containing ice packs. In the laboratory, the ovaries were cut open at the hylus using a pair of sterile scissors. These were washed with tap water, distilled water and finally with deionised water. The follicles visible on its surface were classified based on their diameter as small (less than 5 mm), medium (5 -10 mm) and large (more than 10 mm) follicles using digital vernier caliper. The fluids from these follicles were aspirated using 26 gauge-2 ml syringe. Twenty four samples each from small, medium and large sized follicles along with blood samples of buffaloes belonging to respective category were collected. Besides, blood samples from 12 buffaloes during mid-estrous cycle from a private farm (Imom Son's Dairy Farm, Thane-400 604) were collected. Blood samples were allowed to clot at room temperature. Clear serum was separated by centrifugation at 1500 rpm for 30 min. The ovarian follicular fluid samples were centrifuged at 2000 rpm for 15 min. in order to remove the cell debris. The supernatant ovarian follicular fluid and serum were placed in sterile vials and stored at – 20°C until used for analysis.

The follicular fluid and the serum samples were analysed for total proteins, albumin, globulin, albumin/globulin ratio, urea, creatinine and uric acid. A random sample from the three classes of follicles each and a mid-estrous cycle serum sample were used for protein fractionation by electrophoresis.

Total proteins, albumin, creatinine, uric acid and urea were estimated using STAT FAX 2000 Autoanalyser and kits [Lab-Care Diagnostics (INDIA) Pvt., Ltd and SM Diagnostics]. Globulin concentration is calculated by subtracting albumin from total proteins concentration. Albumin : Globulin ratio is obtained by dividing albumin concentration by globulin concentration. A random sample from the three classes of follicles each and a serum sample were used for protein fractionation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Statistical analysis :

Analysis of variance of the data of the concentrations of total proteins, albumin, globulin, albumin : globulin ratio, urea, creatinine and uric acid were done according to Snedecor and Cochran (1968) using Completely Randomized Design. Differences in means were tested using critical difference (CD) test.

RESULTS AND DISCUSSION

The study was undertaken to evaluate the protein profile *viz.*, total proteins, albumin, globulin, albumin : globulin ratio, urea, creatinine and uric acid in follicular fluid and serum of buffaloes. The electrophoretic pattern of proteins in follicular fluid and serum was also studied. The follicular fluid protein profile and electrophoretic pattern of proteins were compared to that in serum of buffalo. The results are presented in Tables 1 and 2 and electrophoretic pattern is depicted in Fig. 1.

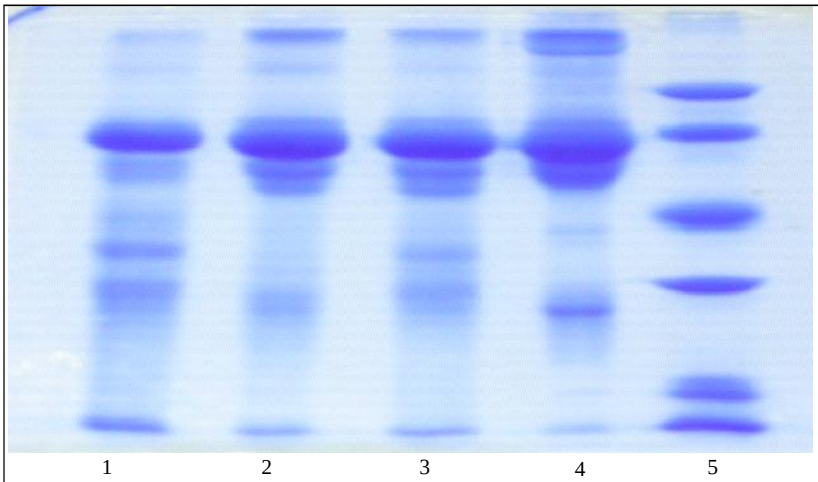

Fig. 1 : Electrophoretic pattern of proteins in fluid of follicles and serum of buffaloes

Total proteins :

The total proteins concentration in fluids of small, medium and large size follicles did not significantly differ and remained fairly constant regardless of the size of follicles. The total proteins concentration in fluid of small, medium and large size follicles and in their respective serum including the mid oestrus serum samples did not significantly differ. Further, amongst the serum samples the total protein concentration did not significantly differ. The observation of the present study that the total proteins concentration remained fairly constant regardless of the size of follicle is in agreement with the observation in bovines (Anderson *et al*., 1976). Jindal *et al*. (1997) in their study on buffalo follicular fluid found that the amount of total proteins concentration was almost similar to the values in the blood serum. The values of total proteins in fluid of follicles and serum recorded in the present study were higher than the values reported in cattle (Wise, 1987) and in buffaloes (Jindal *et al*., 1997 and Thangavel and Nayeem, 2004). Schuetz and Anisowicz (1974) in their study on cation and protein composition of ovarian follicular fluid of pig opined that the likely estrogen and water uptake relationship in growing follicles may dilute follicular protein concentrations. Thangavel and Nayeem (2004) in their study on certain biochemical profile of buffalo follicular fluid reported that the total proteins concentration did not vary between stages of oestrus cycle and the concentration decreased as the follicles grew. This, they attributed to the dilution effect of increased follicular fluid in medium and large follicles. Collins *et al*. (1997) reported lower proteins concentration in follicular fluid than serum in mares.

Albumin :

The albumin concentration showed decrease with increase in size of the follicles. The difference in albumin concentration in fluid of small and large size follicles was statistically highly ($P<0.01$) significant. The difference in albumin concentration of medium and large sized follicles did not approach statistical significance. The albumin concentrations in the fluids of small, medium and large follicles were lower than in their respective sera. The difference in albumin concentration in fluids of small and medium sized follicles and respective serum were not significant except in the fluid of large follicles and serum which was statistically highly ($P<0.01$) significant. The albumin concentration in serum showed a similar trend to that in fluids of small, medium and large size follicles. The serum albumin concentration decreased with increase in size of follicles. Amongst the serum samples, however, the small differences in the albumin concentration did not approach statistical significance. The observation of the present study that the albumin concentration decreased with increase in size of follicle is in accord with the observation of Wise (1987) in bovine heifers. Thedgar *et al*. (1983) opined that decrease in the albumin content in follicular fluid in conjunction with follicle development might not be totally from decreased protein accumulation or dilution by uptake of water by follicles. Actively developing follicles need amino acids and the ovary is one of the most active tissues in catabolizing albumin. Jindal *et al*. (1997), however, found similar albumin concentration in follicular fluid and serum. Contrasting reports that albumin concentration was higher in follicular fluid than in serum has been reported (Edwards, 1974).

Globulin :

The globulin concentration increased with increase in size of the follicles. The small differences, however, were

Table 1 : Mean ± S.E. values of biochemical parameters in fluid of follicles and serum in buffaloes

Parameter	Small size follicle (<5mm)	Serum	Medium size follicle (5 – 10mm)	Serum	Large size follicle (>10mm)	Serum	Mid estrous cycle serum	CD value at 5%
Total protein g/dl	8.12 ±0.25	8.05±0.27	8.05 ±0.21	8.50±0.27	8.03±0.15	8.74±0.29	8.96±0.33	NS
Albumin g/dl	3.63^{bc}±0.16	3.84^{c}±0.25	3.01^{ab}±0.20	3.73^{bc}±0.25	2.47^{a}±0.15	3.48^{bc}±0.21	3.46^{bc}±0.16	0.80
Globulin g/dl	4.46^{ab}±0.20	4.21^{a}±0.36	5.05^{ab}±0.27	4.77^{ab}±0.39	5.56^{b}±0.21	5.26^{ab}±0.37	5.50^{b}±0.33	1.23
A:G ratio	0.86^{ab}±0.06	1.21^{b}±0.18	0.72^{ab}±0.11	1.07^{bc}±0.18	0.48^{a}±0.05	0.81^{ab}±0.11	0.66^{ac}±0.05	0.49
Urea mg/dl	41.90^{b}±2.47	28.85^{a}±1.62	34.78^{ab}±2.36	29.73^{a}±1.61	36.69^{ab}±2.64	32.11^{a}±1.62	29.24^{a}±1.60	8.25
Creatinine mg/dl	1.65±0.07	1.79±0.05	1.72±0.05	1.80±0.05	1.59±0.07	1.78±0.06	1.59±0.08	NS
Uric acid mg/dl	7.73^{c}±0.58	1.86^{a}±0.38	5.38^{b}±0.42	1.72^{a}±0.28	6.35^{bc}±0.35	1.73^{a}±0.37	1.20^{a}±0.19	1.59

Means with atleast one common superscripts do not differ significantly ($P < 0.05$). NS= Non-significant

not statistically significant. The globulin concentration in the fluids of small, medium and large follicles was higher than in their respective serum. Amongst the serum samples, however, the globulin concentration did not exhibit a specific trend and the small differences were statistically nonsignificant. Except the report of Jindal *et al*. (1997) on globulin concentration in follicular fluid and serum, no other reports could be stressed. The values of globulin concentration recorded in the present study in the fluids of small, medium and large follicles and in their respective sera were higher than the values reported by Jindal *et al*. (1997) in buffalo follicular fluid and serum. The values of serum globulin concentration recorded in the present study are in accord with the values reported in our laboratory in lactating crossbred cows (Bharucha *et al*., 2001), earlier.

Albumin : Globulin ratio :

The albumin : globulin ratio in fluids of small, medium and large size follicles was inversely related to the follicular size. The small differences, however, were statistically non-significant. The albumin : globulin ratio recorded

Table 2 : Molecular weight of proteins in fluid of follicles and serum of buffaloes

Small size	Medium size	Large size	Mid size
Follicle	follicle	follicle	estrous cycle
(<5 mm)	(5-10 mm)	(>10 mm)	serum
	123.0	123.0	123.0
114.8 - 112.2	120.2 - 112.2	120.2 - 112.2	120.2 - 112.2
-	-	-	109.6
107.2	-	107.2	-
-	-	-	102.3
97.7 - 95.5	97.7 - 95.5	97.7 - 95.5	97.7 - 91.2
-	-	-	87.1
-	81.3	81.3	81.3
-	79.4	79.4	-
-	77.6	77.6	-
-	-	-	75.9
-	70.8	70.8	70.8
70.8 - 61.7	77.6 - 58.9	77.6 - 58.9	77.6 - 58.9
60.3 - 53.7	56.2 - 53.7	56.2 - 52.5	56.2 - 51.3
-	-	51.3	-
-	52.5 - 47.9	49.0 - 47.9	47.9
45.7	-	-	-
-	-	-	43.7
42.7	42.7	42.7	-
-	-	-	40.7
-	-	-	39.8
38.0 - 34.7	-	37.2 - 33.9	-
-	32.4	32.4	-
31.6 - 28.2	28.5 - 27.5	30.2 - 28.2	29.5 - 26.3
-	-	26.3	-
-	-	-	25.7
23.4	-	-	-
22.4	-	-	-
-	-	-	19.9
19.5	19.5	19.5	-
17.4	-	-	-
-	-	-	17
-	16.2	-	-
15.1 - 13.8	14.8 - 13.8	14.8 - 13.8	14.1 - 13.8

in the fluid of small, medium and large size follicles were lower than that recorded in their respective serum. Amongst the serum samples, the albumin : globulin ratio exhibited the similar trend to the observation in fluid of follicles. The ratio decreased with increase in size of follicles. The albumin : globulin ratio recorded in mid-oestrous cycle serum was lower than that in the remaining sera. The albumin : globulin ratio in fluid of follicles in the present study are in agreement with the only reported findings of Jindal *et al*. (1997) in buffaloes. The values of albumin : globulin ratio in serum are in agreement with the values reported in lactating crossbred cows (Bharucha *et al*., 2001) and in Gir and crossbred calves (Patil *et al*., 2000).

Urea :

The urea concentration recorded in fluid of small size follicle was higher, than that in medium and large sized follicles. The small difference in the fluids was not statistically significant. Urea concentration in the fluid of small size follicle was significantly higher ($P<0.01$) than in its respective serum and mid oestrous cycle serum. The values of urea concentration in follicular fluid and serum obtained in the present study are in agreement with reported observations in Holstein Freisan cows (Leroy *et al*., 2004).

Creatinine :

The creatinine concentration in fluid of follicles and serum did not significantly differ and remained almost same. The values of serum creatinine concentration obtained in the present study are in agreement with Yeh *et al*. (1993) in water buffaloes and with reference values reported for cow (Kaneko *et al*., 1997). No work on creatinine values in follicular fluid could be traced. However, Gerard *et al*. (2002) in their study on follicular growth and maturation in the mare using proton nuclear magnetic resonance reported no difference between follicular fluid and serum with regard to creatine values.

Uric acid :

The uric acid concentration in fluids of small, medium and large size follicle was highest in small followed by large and medium size follicles. The difference in uric acid concentration in small and medium size follicle was statistically highly ($P<0.01$) significant. Small differences in uric acid concentration between small and large follicles and between medium and large sized follicles did not approach statistical significance. The uric acid concentration recorded in fluids of small, medium and large size follicles were significantly higher ($P<0.01$) than in their respective sera. Amongst the serum samples, uric acid concentration however, did not significantly differ. The results of uric acid concentration recorded in present study in fluid of follicles and serum could not be compared as no report could be stressed. The values of uric acid concentration in serum recorded in the present study are in accordance with the values reported in crossbred heifers and cows (Deepak and Singh, 2006).

SDS-PAGE :

Comparative SDS-PAGE patterns of fluid of small, medium and large follicles with mid-oestrous cycle serum proteins are presented in Fig. 1 and their molecular weights in Table 2. 14 (lane1), 16 (lane2), 19 (lane3) and 20 (lane 4) bands of varying intensity in the molecular weight ranging from 123.0 to 13.8 kDa were observed in the fluid of small, medium, large size follicles and serum of buffaloes, respectively. Some of these bands were common to specific protein fractions of different categories of follicles. After deducting the number of common bands, the total number of bands observed in the follicular fluid of three sizes of follicles and serum were 34. Proteins of molecular weight 114.8-112.2, 97.7-95.5, 70.8-61.7, 56.2-53.7, 28.5-28.2 and 13.8-15.1 kDa were present in the fluid of all the classes of follicles and serum. Two peptide bands of molecular weight 42.7 and 19.5 kDa were present in the fluid of small, medium and large sized follicles and absent in serum. In the fluid of small and large size follicles, a lighter peptide band of 107.2 kDa and an intense band in the molecular weight range of 37.2-34.7 kDa was observed, but not in the fluid of medium size follicles and serum. Three faint bands of 79.4, 77.6 and 32.4 kDa were expressed in the fluids of medium and large sized follicles only and absent in the fluid of small follicles and serum. Fluids of medium and large size follicles and mid-oestrous serum shared four proteins of molecular weight 123.0, 81.3, 70.8

and 47.9 kDa. Proteins of molecular weight 45.7, 23.4, 22.4 and 17.4 were present in the fluid of small follicles only and not in the fluid of medium and large size follicles and serum. Similarly, peptide of molecular weight 16.2 was found in the medium and peptides of molecular weight 26.3 in large follicles only. As many as 10 peptides of molecular weight 109.6, 102.3, 87.1, 75.9, 43.7, 40.7, 39.8, 25.7, 19.9 and 17.0 were present in serum only.

The major protein bands in the range of 61.7-70.8 and 28.2-28.5 noticed in all the three classes of follicular fluid in the present study is in agreement with Gupta *et al*. (2006[b]). The present study has revealed a total of 34 bands in follicular fluid and serum which is higher than Kulkarni (1988) who reported 26 protein bands in buffalo follicular fluid and plasma and Kumaresan *et al*. (2003) who reported a total number of 28 protein bands in bovine follicular fluid. However, the present result is considerably lower than 72 bands reported by Gupta *et al*. (2006[b]) who suggested that electrophoresis of whole follicular fluid and serum results in the overlapping of peptide bands. Kulkarni (1988) reported identical spectrum of follicular fluid proteins in small, medium and large follicles and blood plasma proteins which is not the result in the present study. Prealbumin and albumin of 55 and 68 kDa, respectively were present in the fluid of three categories of follicles including the serum. This observation is in accordance with Anderson *et al*. (1976) in bovine follicular fluid by rocket line immunoelectrophoresis. Kulkarni *et al*. (1998) in their study on SDS-PAGE on cauda epididymal fluid, vas deferens fluid and seminal plasma of buffalo bulls indicated that the heavy and broad protein bands around 66 kDa in the fluids could be partly due to transudation of this protein in the lumen of epididymis and/or it could be due to contamination of blood plasma albumin.

Protein bands of 59, 34 and <30 kDa follicular fluid and serum has been shown to posses anti-alpha inhibin antibody (Driancourt *et al*., 2001), though in the present study, the band of 59 kDa was absent in fluid of small follicle and of 34 kDa absent in medium follicles and serum. Also, peptide band of 70 kDa detected in all the fluid in the present study has been shown to be Mullerian inhibiting substance (Driancourt *et al*., 2001). The observation that 30 kDa protein was present in fluids of small and large follicles only and not in medium follicles could be due to decreasing amount of the relative synthesis of 30 kDa after the LH surge (Rabahi *et al*., 1991). Rabahi *et al*. (1991) reported predominant peak of 56 kDa secreted by the granulosa cells in to the medium throughout the experimental period (before as well as after LH surge). This protein of 56 kDa was present in all the follicular fluid in the present study.

An intense band in the range of 28 kDa in the follicular fluids of small, medium and large follicle and serum has been shown to have a restricting effect of sperm-egg interaction *in vitro* (Ramsoondar *et al*., 1995). Gupta *et al*. (2006[a]) reported that peptides of 30, 52 and 65 when incorporated in the basic *in vitro* maturation medium stimulated the maturation rate of bubaline oocytes in a dose dependent manner.

Various studies on protein bands in porcine follicular fluid of molecular weight 80 kDa, 85 kDa (Kimura *et al*., 2000) and 25 kDa (Baratta *et al*., 2000) could not be traced in buffalo follicular fluid.

In conclusion, the results of the present study indicated that the protein components in the follicular fluid and serum exhibited increase / decrease in accordance with follicle size. The electrophoretic pattern of follicular fluid and serum showed significant difference between two fluid compartments. The small reservoir of fluid of follicles reflects the biochemical activity of the follicle. It is, therefore, suggested to carry out further studies to elucidate the precise role of these biochemical components and separated proteins which will help in understanding of the basic changes ongoing during follicular development, so that the optimal environment could be established for the maturation of viable oocytes.

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