Albumin Fractions from Different Species Stimulate *In Vitro* Progesterone Production by Granulosa Cells in Buffalo

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ABSTRACT : The ovarian follicular fluid was found to contain steroidogenesis stimulatory protein similar to albumin from human and buffalo. Therefore, the albumins from various species, commercial and purified, were studied for their steroidogenic effect on progesterone secretion by granulosa cells from buffalo ovaries, during culture. A dose of 20 μg of bovine serum albumin was optimum to exhibit maximum progesterone secretion on day 6 of culture, in medium (350 μl) containing 10^5 cells. Among commercial albumins, chicken albumin showed highest effect on progesterone secretion, which was followed by albumins from goat, bovine, human, sheep and rat, respectively at day 6 of culture. The albumins were also purified from blood serum of buffalo, goat and rat using salt fractionation, ion-exchange chromatography, gel filtration and SDS-PAGE. The highest stimulatory effect on progesterone secretion was shown by albumin purified from buffalo blood serum and lowest by that from rat blood. Comparatively the buffalo and goat albumins were more biologically active than commercial albumins. The presence of some active molecules conjugated with freshly purified albumins may be responsible for better stimulatory effect. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 11 : 1559-1563)***

Key Words : Serum Albumin, Granulosa Cells, *In Vitro*, Steroidogenesis, Buffalo

INTRODUCTION

In mammalian ovary, as the follicles develop, there is an increase in size, number of follicular cells and constituents of follicular fluid. The follicular fluid contains large number of proteins, some of which are secreted from blood and some are synthesized from follicular cells (Shalgi et al., 1973). The various factors regulating follicular growth and steroidogenesis have been reported (Hsueh and Erickson, 1979; Bendal et al., 1988; Nayudo and Osborn, 1992; Fortune, 1994). The follicular fluid proteins play an important role in growth and development of oocyte by modulating the action of gonadotropins and some other factors (Sluss et al., 1983). The presence of a protein factor in human follicular fluid was reported (Khan et al., 1988) which stimulated testosterone production by hamster Leydig cells. The albumin fraction of rat testicular fluid stimulated the pregnenolone production by rat Leydig cells (Melsert et al., 1988). This fraction was further purified and characterized and its effect was studied on LH stimulated steroid production by immature Leydig cells in vitro. The isolation of steroidogenesis inducing protein from human follicular fluid was reported (Khan et al., 1990) and its effect on in vitro production of steroid hormone by granulosa cells, was studied. This protein was found to be modified form of albumin, as its properties resembled that of bovine serum albumin. The steroidogenesis stimulatory protein isolated from buffalo ovarian follicular fluid (Vinze, 1998) also appeared similar to albumin for its electrophoretic mobility. In view of the presence of protein similar to albumin in follicular fluid and its role in steroid hormone production by gonadal cells, the present investigation describes the effect of albumin, from various species, on progesterone production by buffalo granulosa cells.

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MATERIALS AND METHODS

The commercial albumins namely bovine albumin (heat shock precipitate), bovine albumin (alcoholic precipitate), human albumin, chicken albumin, goat albumin, rat albumin, sheep albumin and bovine serum albumin (fraction V), DEAE-Sepharose, Sephacryl S-200, sodium dodecyl sulphate (SDS), acryl amide, coomassie blue, Medium 199 and fetal calf serum (FCS) were procured from Sigma Chem. Co., U.S.A. 1,2,6,7 [^{3}H] progesterone was obtained from Amersham, U.S.A. All other chemicals used were of analytical grade.

Purification of albumin from serum

Collection of blood : The blood from buffalo, goat and rat was collected in tubes and serum was separated at room temperature and stored at 4°C.

Salt fractionation : The serum was fractionated with ammonium sulphate at 60% saturation. The supernatant collected at 10,000 g and 4°C was brought to 80% saturation with ammonium sulphate. The precipitate was collected and dissolved in Tris-HCl buffer (20 mM, pH 7.3). The content was dialyzed against the same buffer for 24 h at 4°C with frequent change of buffer.

Ion-Exchange chromatography : The column (1.5×

5.0 cm) packed with DEAE-Sepharose was equilibrated with Tris-HCl buffer (20 mM, pH 7.3). The sample containing 10 mg of protein was loaded on the column. The column was eluted first with equilibrating buffer to remove unbound protein, and then the protein was eluted with same buffer containing a gradient of 0-0.5 M NaCl. The collected fractions were monitored for protein content by measuring absorbance at 280 nm. The protein eluted in major peak was concentrated with 'Millipore' Ultrafree-15 centrifugal device fitted with a high-flux 30 kDa cut off membrane according to procedure followed by Khan et al. (1990).

Gel filtration : The concentrated protein sample was loaded on a column (1.6×85 cm) packed with Sephacryl S-200 and equilibrated with Tris-HCl buffer (20 mM, pH 7.2). The protein was eluted with equilibrating buffer containing 0.15 M NaCl with a flow rate of 1 ml/min and 2 ml of fraction volume. The protein content in the fractions was monitored by measuring absorbance at 280 nm.

Polyacrylamide gel electrophoresis : The purity of purified protein (albumin) was checked using SDS-PAGE according to modified method of Laemmli et al. (1970).

Protein estimation : The protein content at various stages of purification was estimated by dye binding method (Bradford, 1976).

Estimation of bilirubin : The bilirubin content of purified albumin fraction was estimated using standard commercial kit provided by Bayer Diagnostics India.

Isolation and culturing of granulosa cells

The granulosa cells (GC) were isolated from buffalo ovarian follicles by aspiration with the help of syringe and collected in plain Medium 199.The pooled granulosa cells were pelleted at 1,000 rpm for 6 min. The cell pellet after washing with Medium 199 supplemented with FCS, was treated with sterile 0.1% hyaluronidase in PBS containing 0.1% BSA, at 37°C for 5 min. The GC were then collected by centrifugation. The viability of cells was determined by trypan blue exclusion method. The equal volumes of trypan blue (0.4% in normal saline) and cell suspension were mixed and counting of cells was done with hemocytometer. The cells, which did not take up the stain, were viable and those taking bluish tinge were considered dead. The viability of cells ranged as 80-90%. The viable cells (1×10^5/well) were cultured in CO_2 incubator using method of Wickings (1986) in 48 well culture plate containing Medium199 (350 µl/well) supplemented with 10% FCS and antibiotics, for a total period of 6 days. Initially the pooled granulosa cells were cultured in Medium 199 supplemented with FCS and antibiotics. After 48 h, the spent medium was removed and GC were washed with plain medium and cultured without FCS. At this stage (day 2), appropriate concentrations of albumin from various species were added into culture medium. At day 4, the spent medium was collected for progesterone assay, and replaced with fresh medium containing optimum dose of albumin and culture continued for another 48 h. At day 6 of culture, again the spent medium was collected for progesterone assay.

Progesterone assay

The progesterone content in culture medium was estimated by modified technique of Radio-immunoassay (Kamboj and Prakash, 1993) using 1,2,6,7 [^{3}H] progesterone as tracer. The interassay and intraassay variation of progesterone was 10.5 % and 9.3 %, respectively.

Statistical analysis

The data for the effect of different doses of albumin on progesterone secretion were analyzed by 't' test (Snedecor and Cochran, 1967). A 20 µg dose of BSA (fraction V) significantly stimulated progesterone secretion at 1% level. The effect of albumin (20 µg) from different species, on progesterone secretion by 't' test was significant at 1% level on day 6 of culture. The effect of purified albumins was also analyzed by 't' test and was significant at 1% level on day 4 and 6 of culture. One-way ANOVA was used to test the effect of different doses of BSA (fraction V) on day 4 and 6 of culture.

RESULTS AND DISCUSSION

The present study describes the purification of albumin from blood serum of buffalo, goat and rat and the effect of purified and commercial albumins on progesterone secretion by buffalo granulosa cells, during culture. During salt fractionation the majority of albumin was obtained at 60-80% saturation of ammonium sulphate. Melsert et al. (1988) also got the albumin fraction at 60-80% saturation from rat testicular fluid in a sequential fractionation process. Similar results were found when albumin like major protein was isolated from human and buffalo follicular fluid (Vinze, 1998), using salt fractionation. The albumin fraction (60-80%) were subjected to ion-exchange chromatography (Figure 1). The buffalo, goat and rat albumins got eluted

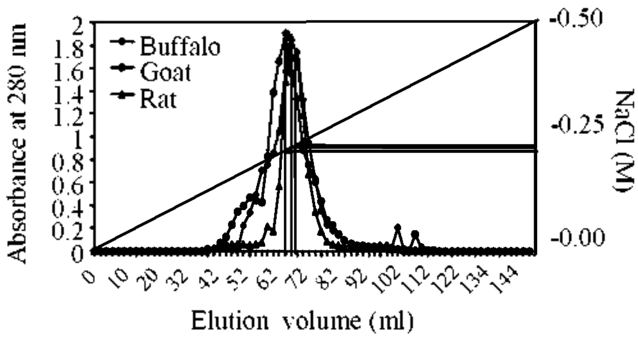

Figure 1. Ion-exchange chromatography of ammonium sulphate (60-80%) pellet of buffalo, goat and rat serum on DEAE-Sepharose.

with buffer containing 0.20-0.25 M NaCl, in a total volume of 150 ml. The major protein peaks obtained for buffalo, goat and rat samples from ion-exchange chromatography were concentrated with 'Millipore' Ultrafree-15 centrifugal device as explained at 1(iii) in material and methods. During concentration a yellowish zone of colour on protein was observed. This colour was suspected to be of bilirubin. Therefore, the bilirubin content of albumin fraction was estimated as described elsewhere, and in all three samples was found in range of 6.8-7.2 μg/ml. The gel filtration pattern of albumin sample, obtained with ion-exchange chromatography, has been shown for the three species in Figure 2. In all cases a single protein peak was eluted indicating that protein has been purified to a great extent. The presence of minor proteins observed in SDS-PAGE (Figure 3) indicated the heterogeneous nature of albumin. The possible causes for this heterogeneity were reported (Peters, 1985) as binding of certain ligands like fatty acids, bilirubin and glycans with albumin. Darcel (1987) also suggested that serum albumin is actually a population of heterogeneous molecules.

The effect of commercial albumins from different sources (bovine, human, chicken, goat, rat and sheep) was studied on progesterone secretion by buffalo GC during culture. The results are presented in Table 1 and Table 2. In a dose response study, 20 μg of BSA in 350 μl culture medium containing 1×10^5 granulosa cells was most effective in stimulating progesterone secretion on the day 6 of culture as compared to that shown at day 4. This suggested that buffalo granulosa cells were highly differentiated and became more steroidogenic in nature at day 6 of culture. BSA (fraction V) at a concentration of 0.25 and 1.0% had no or minor effects (Melsert et al., 1988) on LH stimulated steroid production by rat granulosa cells. However, similar dose of BSA showed stimulatory effect on pregnanolone production by Leydig cells in-vitro. At day 6 of culture, chicken albumin (Ovalbumin) showed highest stimulation of progesterone secretion (1443.05±63.00 pg/ml) and rat albumin showed lowest stimulation (1184.08±43.32 pg/ml) over a control value of 1012.08±43.36 pg/ml. Melsert et al. (1988) reported that hemoglobin and ovalbumin were not effective in stimulation of steroid production by rat Leydig cells. The difference in response of albumins from various species towards progesterone secretion may be due to difference in these albumins at molecular levels.

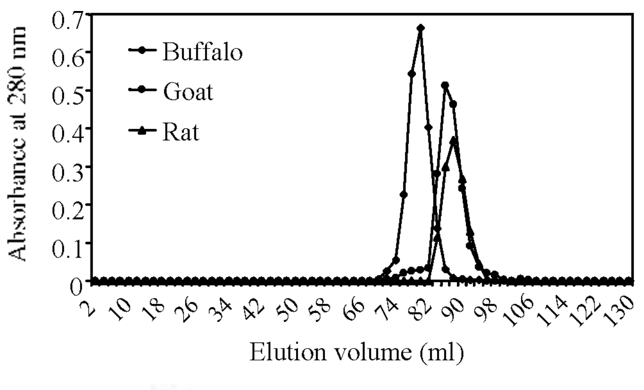

Figure 2. Gel filtration pattern (Sephacryl S-200) of albumin fractions of buffalo, Goat and rat serum obtained by ion-exchange chromatography.

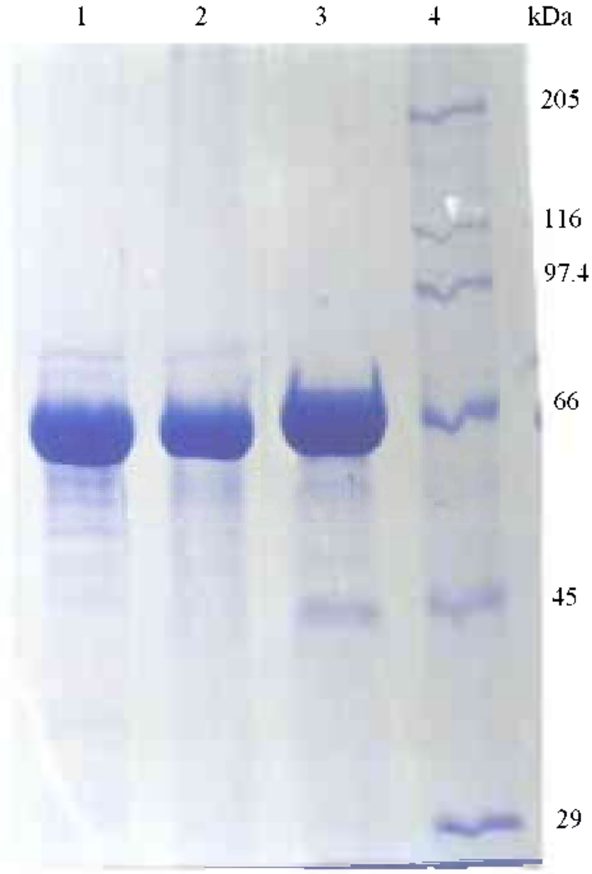

Figure 3. SDS-PAGE profile of protein peaks obtained after gel filtration. Lane 1, buffalo; Lane 2, goat; Lane 3, rat; Lane 4, mol. wt. markers

The albumins purified from blood serum of buffalo, goat and rat stimulated progesterone secretion significantly at day 4 as well as on day 6 of culture (Table 3). The albumin preparations from three species were almost equally competent for stimulatory effect. The progesterone values on day 4 were in range (pg/ml) of 777.95±32.56 to 820.02±22.99, over a control of 551±20.66 (pg/ml). On day 6 these values were in range of 1898.00±19.67 to 2099.75±36.76, over control of 1003.22±54.76.The comparatively better effect shown by purified albumins, on progesterone secretion, may be due to higher biological activities of these albumins as these were prepared fresh. Additionally these albumins were also found rich in bilirubin content as observed while concentrating them

Table 1. Effect of different concentrations of BSA (fraction V) on progesterone secretion by granulosa cells during culture (Mean±S.E., n=4)

BSA	Progesterone (pg/ml)	
dose (μg)	Day 4	Day 6
Control	585.16±21.06	854.36±37.28
20	553.79±46.58	1127.55±42.61**
40	511.73±32.10	931.3±46.41
60	478.88±32.52	641.72±35.39
80	460.06±25.57	702.95±25.01
100	491.41±17.91	720.48±31.27

**Significant over control (p<0.01).

Table 2. Effect of commercial albumin (20 μg) from different species on progesterone secretion by granulosa cells during culture (Mean±S.E., n=4)

Serum albumin	Progesterone (pg/ml)	
source	Day 4	Day 6
Control	760.89±34.01	1,012.08±43.36
Bovine albumin (Heat shock ppt.)	635.10±5.68	1,294.49±54.76**
Bovine albumin (Alcohol ppt.)	714.57±11.87	1,332.08±26.17**
Human albumin	689.21±30.67	1,329.17±47.77**
Chicken albumin	800.90±38.02	1,443.05±63.00**
Goat albumin	861.00±62.10	1,345.15±37.21**
Rat albumin	866.73±34.30*	1,184.08±43.32**
Sheep albumin	915.70±63.00*	1,252.13±77.92**

Ppt=Precipitate.
* Significant over control (p<0.05).
** Significant over control (p<0.01).

Table 3. Effect of purified albumin (20 μg) from blood serum on progesterone secretion by granulosa cells during culture (Mean±S.E., n=4)

Serum albumin	Progesterone (pg/ml)	
source	Day 4	Day 6
Control	551.00±20.66	1,003.22±54.76
Buffalo albumin	820.02±22.99**	2,099.75±36.76**
Goat albumin	777.95±32.56**	1,989.25±74.74**
Rat albumin	800.30±47.26**	1,898.00±19.67**

**Significant over control (p<0.01).

during purification steps. The bilirubin has been reported (Stroker et al.. 1990) as biological antioxidants of potential importance. since it scavenges peroxyl radicals with high efficiency. Other free radical scavenging systems were found (Yuji et al., 1993) in ovarian tissues. which have important role in steroidogenesis in follicular cells. Therefore. an indirect role of bilirubin in steroid hormone production is not ruled out.

It is concluded that albumin purified from buffalo. goat and rat blood serum were found to be heterogeneous molecules. These albumins and commercial albumins stimulated progesterone secretion by pooled granulosa cells from buffalo ovary during culture. The better effect of purified albumins was suspected due to presence of bilirubin in these albumin preparations. However it can further be ascertained by studying the effect of bilirubin as such on pooled granulosa cells isolated from follicles of different stages of development. This may reflect the importance of bilirubin and albumin as carrier of bilirubin. in reproductive process of female animals.

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