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Effects of Different Concentrations of Bovine Follicular Fluid and Estrous Cow Serum on Development of Murine 2-Cell Embryos

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Abstract: Murine 2-cells embryos were isolated from murine oviducts at laboratory and transferred into Ham's F-10 medium containing 0.1 mg mL^{-1} streptomycin and 100 IU mL^{-1} penicillin G and supplemented with 3 mg mL^{-1} bovine serum albumin (BSA) or different concentrations of bovine follicular fluid (bFF) and estrous cow serum (ECS). Significantly higher ($p<0.05$) ≥4-cell embryos were developed when embryos were cultured 20% bFF (84.33%) comparing to 10 and 15% bFF (48.33 and 69.33%) as well as 3 mg mL^{-1} BSA (65.66%). Morula rates were also lower in 10% bFF (22.33%) comparing to the other groups and were similar in 15 and 20% bFF (62.66 and 72.33% morula rates) as well as BSA containing media (55.33%). The highest ($p<0.05$) blastocyst rates were obtained in medium containing 20% bFF (64.33%) and the lowest belonged to 10% bFF (15%) comparing to 15% bFF (33.66%) or 3 mg mL^{-1} BSA. When embryos were cultured in ECS, no significant different was observed in different culture media (76.66, 72.33, 82.5 and 65.66% ≥4-cell embryos in 10, 15 and 20% bFF and 3 mg mL^{-1} BSA, respectively). Morula and blastocyst rates were also similar in all groups (32.33, 41.66 and 66.25 and 55.33% morula rates and 15.33, 27, 44.50 and 29.66% blastocyst rates for 10, 15 and 20% bFF and 3 mg mL^{-1} BSA, respectively). The results of the present study demonstrated that 20% bFF could be substituted for BSA when *in vitro* culture of murine embryos is carried.

Key words: Murine embryos, IVC, bFF, ECS, morula, blastocyst

INTRODUCTION

It is now well understood that the development of preimplantation mammalian embryos *in vitro* is less than optimal. Early attempts to develop fertilized mouse ova *in vitro* demonstrated that murine morula developed into blastocysts in complex medium or in simple medium consisting mainly of Kreb's-Ringer bicarbonate (Loutradis *et al*., 1987). Studies on culture of preimplantation embryos advanced considerably after the development of biological medium containing egg white egg yolk and a chemically semi-defined medium with BSA for mouse embryos (Han and Niwa, 2003).

Mammalian embryos are generally cultured in medium supplemented with serum as protein supplement (Babaei *et al*., 2006; De Santis *et al*., 2007; Banwell *et al*., 2007). Any use of serum involves the addition of wide range of proteins, hormones and other elements which may vary widely from batch to batch (Barnes and Sato, 1980). Serum is an extremely complex fluid containing a variety of energy substrates, amino acids, vitamins and growth factors that may support survival and growth of mammalian cells in culture (Han and Niwa, 2003). However, many reports indicate that exposure of 2- to 8-cell embryos to FBS is detrimental to their development to blastocyst *in vitro* (Bavister, 1995; Maurer, 1992), indicating that sera contain toxic factors. In our previous study (Tajik, 2006) bovine fertilized eggs could reach to blastocyst stages in protein-free medium. On the other hands, Pinyopummintr and Bavister (1991) reported that serum had a biphasic effect on bovine embryo development, inhibiting the first cleavage of 1-cell embryos and having no beneficial effect from the 2-cell to the morula stage, but subsequently enhancing development of morula to blastocyst. The beneficial effect of serum on advanced-stage embryos also been reported in pigs (Pollard *et al*., 1995) and mice (Torensi and Archer, 1996).

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It has been hypothesized that protein in embryo culture medium may function as a fixed nitrogen source (Fissore *et al*., 1989). Fetal cord serum (FCoS) has been used as protein source for embryo culture media since 1988 because it was easily available and was considered a good substitute for maternal serum (Lavarge *et al*., 1997).

Serum albumin, on the other hand, is a relatively pure fraction, although its content can also be very variable (Bavister, 1995). The varying conditions present current embryo culture systems may contribute to the poor cultured embryos (Racowsky, 2002). On the other hand, it is well documented that the follicular fluid plays an important biological role in folliculogenesis, oocyte maturation, granulosa cells luteinization and ovulation (Gotting *et al*., 2002).

Follicular fluid is primarily the transudation of plasma that contains specific constituents such as steroids, glycosaminoglycans and many other metabolites synthesized by the cells of the follicle wall (Choi *et al*., 1998). Moreover, it has been reported that the concentrations of vitamins (Schweigert and Kucker, 1988), insulin-like growth factors (Einspanier *et al*., 1993) and other factors vary with follicular size and degree of atresia (Kruip *et al*., 2000).

Bovine Serum Albumin (BSA) is the most common protein added to culture media as a fixed nitrogen source for embryos but it is very expensive and hard to prepare. However, we (Tajik and Niwa, 1998) have shown that bovine oocytes can be fertilized and embryos will develop to blastocyst in the complete absence of any exogenous fixed nitrogen source, although a high molecular weight such as polyvinylpyrrolidone was added to the culture media as a replacement for BSA. The aim of the present study was to examine development of mouse 2-cell embryos in the presence different concentrations of follicular fluid and estrus cow serum (of bovine source) as substitutions for BSA.

MATERIALS AND METHODS

Animals and embryo collection: This study was conducted during 1 year almost from spring to winter 2006. Randomly bred female mice (BALB/c), 8-10 weeks old, were superovulated by an intraperitoneal injection of 5 IU of human menopausal gonadotrophin (hMG, Humegun, Zaran, Iran), followed 48 h later by 5 IU of human chorionic gonadotrophin (hCG; Oregone, Holland). After the injection human chorionic gonadotropin (hCG) (purchased from Darou Pakhsh, Iran). Superovulated females were caged overnight with males. Insemination was verified the following morning finding a copulation plug in the vagina. Embryos were recovered 40 h after hCG injection by flushing uterine. Any embryos appearing degenerate or abnormal were discarded. Normal 2-cell embryos different litters were pooled, washed 3 times and transferred to Ham's F10 medium. Groups of 15 embryos were placed in drops with different culture media (Ham's F10) in 35 mm Petri dishes of each protein supplemented media and three replicates were done.

Follicular fluid collection and preparation: Mixed bovine follicular fluid was retrieved from slaughterhouse ovaries. Follicles with diameters between 3 and 15 mm were aspirated by an 18 gauge needle attached to a 10 mL syringe the pooled follicular fluid was then centrifuged at 4000 rpm, the supernatant stored at -20°C, until use. All experiments were performed with the same batch.

Estrus cow serum collection: Serum used in this experiment was obtained from six estrous cows, heat-inactivated (56°C, 30 min), pooled, filtered with a 0.22 μm membrane (Millipore, Brussels, Belgium) and frozen in 1.5 mL vials until used.

Culture treatments: For embryo culture, Ham's F-10 medium was supplemented with either estrous cow serum (ECS) at three concentrations of 10, 15 and 20% (v/v) or bovine follicular fluid (bFF) at three concentrations of 10, 15 and 20% (v/v). Each 10-15 2-cell harvested embryos were randomly allocated into 50 μL media droplets of the mentioned culture media in a polystyrene culture dish (35×10 mm). The dishes were kept in a CO_2 incubator (5% CO_2 in air at 37°C) for about 2 h before embryos were added. After 24, 48 and 72 h culture of embryos in CO_2 incubator embryos were observed under a stereomicroscope (Nikon co. ltd, Japan). Embryonic development was scored every 24 h and the proportion the 4 to 8-cell, morula and blastocyst stages were recorded.

Statistical analysis: The proportions of total embryos in each stages of development were subjected to an arc-sin transformation and the transformed values were analyzed using a mathematical model that included fixed effect due to treatment (serum concentrations) and residual error. When the analysis revealed a significant effect, the values were compared by Duncan's multiple range test.

RESULTS

When murine 2-cell embryos were cultured in Ham's F-10 medium supplemented with different concentrations of sera, 22.33, 23.33 and 15.33% were blocked and did not

Table 1: The 2-cell block in different concentrations of bovine follicular fluid (bFF) and estrous cow serum (ECS) in Ham's F-10 medium 48 h post-culture

	Proportions of 2-cell block in bFF and ECS	
Concentrations (%)	bFF	ECS
10	22.33	14.33
15	23.33	20.00
20	15.33	15.50
Control	25.66	

Table 2: The proportion of ≥4-cell in different concentrations of bovine follicular fluid (bFF) and estrous cow serum (ECS) in Ham's F-10 medium 24 h post-culture

	Proportions of ≥4-cell block in bFF and ECS	
Concentrations (%)	bFF	ECS
10	48.33^{a}	76.66^{b}
15	69.33^{ab}	72.33^{b}
20	84.33^{b}	82.50^{b}
Control	65.66^{ab}	

$^{a-b}$Values in rows, columns and in control group with different superscript are significantly different ($p<0.05$)

developed to higher stages in !0, 15 and 20% bFF. These values were 14.33, 20 and 15.5% for 10, 15 and 20% ECS respectively. More embryos were blocked in medium containing BSA. However, the different was not significant (Table 1).

Significantly ($p<0.05$) lower development rat (48.33%) was observed in 10% bFF comparing to 20% bFF (84.33%) or different concentrations of ECS studied (76.66, 72.33 and 82.5% development rates for 10, 15 and 20% ECS respectively). This value was not either significantly lower than the medium supplemented with BSA (65.66%) (Table 2).

Forty hour post-culture only 22.33% of embryos in 10% bFF developed to morula stage. However, these values were significantly ($p<0.05$) higher in 15 and 20% bFF (with 62.66 and 72.33% development rates). The development rates in different concentrations of ECS are also increasing in a dose dependency (Table 3).

Observation for detection of blastocyst on 72 h post-culture showed that 64.33% of embryos reached to blastocyst stage in 20% bFF. This value was significantly higher than blastocyst rates in all other groups except in 20% ECS in which 44.50% embryos reached to blastocyst (Table 4).

Table 3: The proportion of morula in different concentrations of bovine follicular fluid (bFF) and estrous cow serum (ECS) in Ham's F-10 medium 48 h post-culture

	Proportions of morula in bFF and ECS	
Concentrations (%)	bFF	ECS
10	22.33^{a}	32.33^{a}
15	62.66^{b}	41.66^{ab}
20	72.33^{b}	66.25^{b}
Control	55.33^{b}	

$^{a-b}$Values in rows, columns and in control group with different superscript are significantly different ($p<0.05$)

Table 4: The proportion of blastocyst in different concentrations of bovine follicular fluid (bFF) and estrous cow serum (ECS) in Ham's F-10 medium 72 h post-culture

	Proportions of blastocyst in bFF and ECS	
Concentrations (%)	bFF	ECS
10	15.00^{a}	15.33^{a}
15	33.66^{a}	27.00^{a}
20	64.33^{b}	44.50^{ab}
Control	29.66^{a}	

$^{a-b}$Values in rows, columns and in control group with different superscript are significantly different ($p<0.05$)

DISCUSSION

Follicular fluid is instrumental in the nutritional and developmental support of the oocyte. Follicular maturation and the maturation of its oocyte are parallel events and also functionally related. Malekshah *et al.* (1996) have reported that developments of 2-cell mouse embryos are possible in human heat inactivated follicular fluid (hFF). In their study, the development of 2-cell embryos to 4 cells or beyond is likely supported in 15% FF comparing to the control group. In the present study regarding development of embryos to ≥4-cell stage, no significant difference was observed between 10 and 15% bFF with control. However, the medium with 20% bFF, significantly ($p<0.05$) supported embryonic development to ≥4-cell.

It has been reported that there is a significant improved development of ICR mouse 2-cell embryos to 8-cell and morula by the addition of amino acids and in the presence of 10% hFF comparing to the medium supplemented with BSA (Cho *et al.*, 2002).

Recent studies have shown that the follicular fluid derived from small, medium, large and pre-ovulatory follicles supplemented to the maturation medium at the range of 10% (Carolan *et al.*, 1996; Elmileik *et al.*, 1995; Sirard *et al.*, 1995), 20% (Romero-Arredondo and Seidel, 1996) and 100% (Choi *et al.*, 1998) improved the developmental capacity of bovine oocytes. In swine, also a medium of 100% follicular fluid supplemented with FSH 0.12 IU mL^{-1} was used for oocyte maturation, markedly improved male pronucleus formation (Naito *et al.*, 1988, 1989).

However, 60% of bovine follicular fluid derived from small or large follicles had a detrimental effect on embryonic development (Elmileik *et al.*, 1995; Kim *et al.*, 1996). In present, development of mice 2-cell embryos to and beyond the 4-cell stage was not inhibited when they were cultured in HF-10+20% ECS or HF-10+20% FF. It was also observed that, protein supplementation has a beneficial effect on embryo development from 2-cell embryos to blastocyst stages.

The BSA used was 98% pure and we consider that the 2% of uncharacterized impurities are the probable source of the variability between batches of BSA and may also be important essential factors for the hatching process.

Albumin, on the other hand, has the advantage of being a single protein that is commercially available. The source of albumin can be human or bovine. Ashwood-Smith *et al.* (1989) reported on the outcome of embryo development comparing embryo culture in Earle's medium with either Albuminar-5 or patient's serum as protein source. No significant differences were found regarding fertilization rate and implantation rates. In the present study the proportions of ≥4-cell in BSA 65.66% was not significantly different with dose in different concentrations of bFF and ECS. The proportion of morula (55.33%) and blastocyst (29.66%) in control medium containing BSA were also similar to those of cultured in 15 and 20% of bFF and ECS, which is in agreement with Ashwood-Smith *et al.* (1989). However, Staessen *et al.* (1990) found that the morphological appearance and the pregnancy rate were significantly higher in the Albuminar group.

In conclusion, media supplemented with concentration of 20% bFF and ECS is likely to support murine 2-cell embryos development to blastocyst stage. These observations could have important implications for human *in vitro* fertilization and development

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