Global Veterinaria 4 (3): 237-241, 2010
ISSN 1992-6197

The Effect of Protein Supplements on *In-vitro* Development of Preimplantation Mouse Embryo

[1]*Rahim Beheshti,* [1]*Jamshid Ghiasi Ghalehkandi and* [2]*Parviz Tajik*

[1]Department of Veterinary Science, Islamic Azad University- Shabestar Branch, 53815-159 Shabestar, Iran
[2]Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tehran, Iran

Abstract: Murine 2-cells embryos were collected 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 4 mg mL^{-1} bovine serum albumin (BSA) or different concentrations of bovine follicular fluid (bFF) and bovine fetal cord serum (BCoS). Significantly higher ($p<0.05$) ≥4-cell embryos were developed when embryos were cultured in 20%bFF (84.33%) as comparing to 10 and 15% bFF (48.33 and 69.33%, respectively) as well as 4 mg mL^{-1} BSA (65.66%). Morula rates were also lower in 10% bFF (22.33%) as comparing to the other groups and were similar in 15 and 20% bFF (62.66 and 72.33%, respectively) as well as BSA containing medium (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%) as comparing to 15% bFF (33.66%) or 4 mg mL^{-1} BSA. When embryos cultured in BCoS, no significant different was observed in different culture media (58.66, 68.33 and 77% ≥4-cell embryos in 10, 15 and 20% bFF and 4 mg mL^{-1} BSA, respectively). Morula rates were also similar in all groups (52.66, 36.33 and 68% morularates for 10, 15 and 20% BCoS and 4 mg mL^{-1} BSA, respectively). Blastocysts rates were higher ($p<0.05$) in 20% BCoS than other groups and were similar in 10 and, 15% BCoS and control group (28.33, 23.33 and 59.33% for 10, 15 and 20% BCoS and 4 mg mL^{-1} BSA, respectively). The hatching rate of blastocysts in 10% concentrations of FF and BCoS were significantly ($p<0.05$) lower than all groups. In conclusion, 20% bFF could be substituted for BSA when *in vitro* culture of murine embryos is carried out.

Key words: Murine embryos · IVC · BFF · BcoS · 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 mouse morula developed into blastocysts in complex medium or in simple medium consisting mainly of Kreb's Ringer bicarbonate [1]. Studies on culture of preimplantation embryos become considerably advanced after the development of biological medium containing egg white egg yolk and a chemically semi-defined medium with BSA for mouse embryos [2].

Mammalian embryos are generally cultured in medium supplemented with serum as protein supplement [3 - 5]. the use of serum involves the addition of a wide range protein, hormones and other elements which may vary widely from batch to batch [6]. Serum is an extremely fluid containing variety of energy substrates, amino acids, vitamins and growth factors that may support survival and growth of mammalian cells in culture [2]. Many reports indicated that exposure of 2- to 8 cell embryos to fetal calf serum is detrimental to their development to blastocyst *in vitro* [7, 8] indicating that sera may contain toxic factors. On the other hand, Pinyopummintr and Bavister [9] reported that serum had a biphasic effect on bovine embryo development, inhibiting the first cleavage of 1-cell embryos and have 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 have been reported in pigs [10] and mice [11]. In our previous study [12] murine fertilized egg could reach to blastocyst stage in medium with estrous cow serum.

It has been hypothesized that protein in embryo culture medium may function as a fixed nitrogen source

Corresponding Author: Dr. Rahim Beheshti, Department of Veterinary Science, Islamic Azad University, Shabestar Branch, 53815-159, Shabestar, Iran

[13]. The use of fetal cord serum (FCoS) was first described at the year 1980 by Hsu, who demonstrated its benefit for development of mouse post-implantation embryos. Fetal cord serum for supporting post-implantation development of mouse embryos can be replaced by high-molecular-eight constituents of fetal bovine serum [14].

Follicular fluid is a complex mixture of products of follicular secretion and to a lesser extent blood serum filtration. This fluid contains a complex mixture of serum proteins, proteins secreted by follicular cells, steroid hormones and other molecules, some still unknown.

In the past few years, the impact of diluted and pure FF on *in vitro* oocyte maturation, fertilization and embryonic development has been extensively studied in cattle and, buffalo, swine and horses. Serum albumin, on the other hand, is a relatively pure fraction, although its content can also be very variable [7]. The varying conditions present current embryo culture systems may contribute to the poor cultured embryos [15].

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 ad hard to prepare. However, Tajik, *et al.* [16] have shown that bovine oocytes can be fertilized and embryos will develop to blastocysts in the complete absence of any exogenous fixed nitrogen source, although a high molecular weight colloid (polyvinylpyrolidone) was added to the culture media as a replacement for BSA.

In the present study, we have examined the development of mouse embryos in the presence of different follicular fluid and fetal cord serum (of bovine source) as substitutions for BSA.

MATERIALS AND METHODS

Animal and Embryo Collection: Female mice were induced to superovulate by an intraperitoneal injection of 5 IU of Human menopausal gonadotrophin (hMG, Humegun, Tehran. Iran), followed by 48 h later by 5 IU of human chorionic gonadotrophin (hCG; Oregone, Holland). After the hCG injection, these females were caged with fertile males and examined for the presence of a vaginal plug, which was taken as evidence of mating. Fourteen hours later, mice were euthanized by cervical dislocation and embryos were collected by retrograde flushing of their oviducts. Any embryos appearing degenerate or abnormal were discarded. Normal two cell embryos were washed 3 times in culture medium and then were transferred into culture treatments. The proteins to be examined were prepared in Ham's F-10 medium. In each protein solution, three replicates of 15 two-cell mouse embryos were cultured *in vitro* to test the capacity of the solution to enable embryo development to the blastocyst stage.

Bovine Fetal Cord Serum: BCoS was obtained by drawing venous blood from the cord into sterile tubes immediately after delivery of the Calves. After centrifuging the blood sample at 700 *g* for 5 min, serum was removed with a Pasteur pipette and heat-inactivated at 56°C for 30 min. The serum was sterilized through a 0.22 μm filter.

Culture Treatments: For embryo culture, Ham's F-10 medium was supplemented with either BCoS at three concentration of 10, 15 and 20% (v/v) and BSA at a concentration of 0.4% (w/v). Harvested embryos were randomly allocated into these culture treatments and cultures for 120 h at 37 °C in 5% CO2 in air. A control medium with BSA was also included in each experiment. Embryonic development was scored every 24 h and the proportion the 4-to 8-cell, morula and blastocyst stages were recorded.

Statistical Analyses: The proportions of the ≥4cell, Morula, Blastocyst stages and rate of hatching Blastocyst were expressed as mean±SEM. Comparison of means was carried out using ONE WAY ANOVA and the Dunkan test. A significance level of $P<0.05$ was used throughout this study.

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 developed to next stages in 10, 15 and 20% bFF. These values were 26.66, 20.66 and 16.33% for 10, 15 and 20% BCoS, respectively. High percent of embryos were blocked in medium containing BSA. However, the different was not significant (Table 1).

Significantly ($p<0.05$) lower development rate (48.33%) was observed in 10% bFF as comparing to 20% bFF (84.33%). This Value was not either significantly lower than the medium supplemented with BSA (65.66%) or different concentration of BCoS studied (58.66, 68.33, 77% development rates for 10, 15 and 20% BCoS, respectively) (Table 2).

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

	Proportions of 2-cell block in bFF and BCoS	
Concentrations (%)	bFF	BCoS
10%	22.33	26.66
15%	23.33	20.66
20%	15.33	16.33
Control	25.66	

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

	Proportions of ≥4-cell in bFF and BcoS	
Concentrations (%)	bFF	BCoS
10%	48.33^{a}	58.66^{ab}
15%	69.33^{ab}	68.33^{ab}
20%	84.33^{b}	77.00^{ab}
Control	65.66^{ab}	

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

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

	Proportions of morula in bFF and BCoS	
Concentrations (%)	bFF	BCoS
10%	22.33^{a}	52.66^{abc}
15%	62.66^{bc}	36.33^{ab}
20%	72.33^{c}	68.00^{bc}
Control	55.33^{abc}	

$^{a, b-c}$ 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 bovine cord serum (BCoS) in Ham's F-10 medium 72h post-culture

	Proportions of blastocyst in bFF and BCoS	
Concentrations (%)	bFF	BCoS
10%	15.00^{a}	28.33^{a}
15%	33.66^{a}	23.33^{a}
20%	64.33^{b}	59.33^{b}
Control	29.66^{a}	

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

Table 5: The rate of Blastocyst hatching in different concentrations of bovine follicular fluid (bFF) and bovine cord serum (BCoS) in Ham's F-10 medium 96h post-culture

	The rate of Blastocyst hatching in bFF and BCoS	
Concentrations (%)	bFF	BCoS
10%	0.00^{a}	4.33^{ab}
15%	0.00^{a}	12.00^{bc}
20%	62.63^{d}	20.66^{c}
Control	14.66^{bc}	

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

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, 20% bFF and 20% BCoS. Morula developed in 20% BCoS was significantly higher from 15% BCoS and 10% bFF ($p<0.05$).

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 groups expect in 20% BCoS in which 59.33% embryos reached to blastocyst (Table 4).

The hatching rate of blastocysts in 10 or 15% concentrations of FF and 10% BCoS were significantly ($P<0.05$) lower than all groups. Significantly ($p<0.05$) highest hatching rate was in 20% bFF treatment ($P<0.05$). This value in 20% BCoS was higher than 10, 15% bFF and 10% BCoS ($P<0.05$) (Table 5).

DISCUSSION

In the present study regarding development of embryos to ≥4-cell stage, no significant difference was observed between different concentrations of bFF, BCoS with control. The 20% bFF group showed significantly higher rate of ≥4-cell development than the 10% bFF groups ($P<0.05$).

The medium with 20% bFF and BCoS better supported embryonic development to ≥4-cell. Follicular fluid is critical in the nutritional and developmental support of the oocyte. Follicular maturation and the maturation of its oocyte are parallel events and also functionally related. In swine, 100% follicular fluid from medium follicles supplemented with FSH 0.12 IU ml was used as oocyte maturation medium and markedly improved male pronuclear formation was observed [17].

Recent studies have shown that the follicular fluid derived from small, medium, large and pre-ovulatory follicles supplemented to the maturation medium at 10% [18 - 20] and 20% [21] improved the developmental capacity of bovine oocytes. However, 60% of bovine follicular fluid derived from small or large follicles had a detrimental effect on embryonic development [19, 22]. In the present study, development of mice 2-cell embryos to and beyond the 4-cell stage was not inhibited when they were cultured in HF-10+BCoS or HF-10+FF.

However, protein supplementation has a beneficial effect on embryo development from 2-cell embryos to blastocyst stages. The development rate of blastocyst ($P<0.05$) in 20% concentration of supplements was higher

than of lower supplement concentrations in medium and control. Blastocyst formation rates among 10 and 15% concentrations of treatments were not significantly ($P<0.05$) different. Blastocysts hatching rate in low concentrations (10 and 15%) of bFF is lower than that of control ($P<0.05$). There was significant difference in mean scores per drop between 20% bFF and other groups ($P>0.05$). Tornesi and Archer [11] demonstrated that serum improves the hatching rate of embryos *in vitro* compared to BSA, but embryos can develop from the morula stage to the expanded blastocyst stage without serum or BSA.

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 [2]. However, many reports indicated that exposure of 2- to 8-cell embryos to FBS is detrimental to their development to blastocysts *in vitro* [7, 8], indicating that serum also contains toxic factors. Pinyopumminar and Bavister [9] 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 blastocysts. The beneficial effect of serum on advanced-stage embryos also been reported in pigs [10] and mice [11].

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.* [23] and Staessen, *et al.* [24] 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 although, Staessen, *et al.* [26] found that the morphological appearance and the pregnancy rate are significantly higher in the Albuminar group.

It is concluded that protein supplementation of culture medium may beneficial affect embryo development and hatching. These observations could have important implications for investigations on *in vitro* fertilization.

REFERENCES

1. Loutradis, D.K., D. John and A.A. Kiessling, 1987. Hypoxanthine causes 2-cell block in random-bred mouse embryo. Biology of Reproduction, 37: 311-316.
2. Han, M.S. and K. Niwa, 2003. Effect of BSA and fetal bovine serum in culture medium on development of rat embryos. J. Reproduction and Development, 49: 235-242.
3. Babaei, H., S.N. Nematallahi and A. Kheradmand, 2006. The effect of vitamin A administration on the development of vitrified-warmed mouse blastocyst. Animal Reproduction Sci., 95: 125-133.
4. De Santis, L., G. Coticchio, S. Paynter, K. Albertini, L. Hutt, M. Iaccarino, C. Gambardella, C. Flamigni and A. Borini, 2007. Permibility of human oocytes to ethylene glycol and their survival and spindle configuration after slow cooling cryopreservation. Human Reproduction, 10: 1776-1783.
5. Banwell, K.M., M. Lane, K.L. Russell and J.G. Thompson, 2007. Oxygen concentration during mouse oocyte *in-vitro* maturation affects embryo and fetal development. Human Reproduction, 10: 2768-2775.
6. Barnes, D. and G. Sato, 1980. Serum-free cell culture: a unifying approach. Cell, 22: 649-655.
7. Bavister, B.D., 1995. Culture of preimplantation embryos: facts and artifacts. Human Reproduction, 1: 91-148.
8. Maurer, H.R., 1992. Towards serum-free chemically defined media for mammalian cell culture. Animal Cell Culture, Oxford University Press.
9. Pinyopummintr, T. and B.D. Bavister, 1991. *In-vitro* matured / *in-vitro* fertilized bovine oocytes can develop into morula/blastocystes in chemically define, protein-free culture media. Biology of Reproduction, 45: 736-742.
10. Polard, J.W., C. Plante and S.P. Leibo, 1995. Comparison of development of pig zygotes and embryos in simple and complex culture media. J. Reproduction and Fertility, 103: 331-337.
11. Torensi, M.B. and J. Archer, 1996. The early development of mouse embryos *in vitro* in medium supplemented with different batches of serum and bovine serum. Veterinary Research Communication, 20: 15-19.
12. Tajik, P., R. Beheshti-Govij, J. Soleimani-Rad, H. Ghasemzade-Nava and A. Mohammadi-Roshandeh, 2008. Effect of different concentrations

of bovine follicular fluid and estrus cow serum on development of murine2-cell embryos. Pakistan J. Biological Sci., 11: 628-32.

13. Fissore, R.A., K.V. Jackson and A.A. Kiessling, 1989. Mouse zygote development in culture medium without protein in the presence of ethylenediaminetetraacetic acid. Biology of Reproduction, 41: 835-841.
14. Lavarge, H., P. De Sutter, R. Desmet, J. Vander Elst and M. Dhont, 1997. Prospective randomized study comparing human serum albumin with fetal cord serum as protein supplement in culture medium for *in vitro* fertilization. Human Reproduction, 12: 2263-2266.
15. Racowsky, C., 2002. High rates of embryonic loss, yet high incidence of multiple births in human art: is this paradoxical? Theriogenol., 57: 87-96.
16. Tajik, P. and K. Niwa, 1998. Effects of caffeine and/or heparin in a chemically defined medium with or without glocuse on *in vitro* penetration of bovine oocytes and their subsequent development. Theriogenol., 49: 771-777.
17. Naito, K., Y. Fukuda and I. Ishibashi, 1989. Developmental ability of porcine ova matured in porcine follicular fluid *in vitro* and fertilized *in vitro*. Theriogenol., 31: 1049-1057.
18. Carolan, C., P. Lonergan, P. Monget, D. Monniaux and P. Mermillod, 1996. Effect of follicle size and quality on the ability of follicular fluid to support cytoplasmic maturation of bovine oocytes. Molecular Reproduction and Development, 43: 477-483.
19. Elmileik, A.M.A., T. Maeda and T. Terada, 1995. Higher rates of development into blastocyst following the *in vitro* fertilization of bovine oocytes matured in a medium supplemented with the fluid from large bovine follicles. Animal Reproduction Sci., 38: 85-96.
20. Sirard, M.A., F. Roy, B. Patrick, P. Mermillod and L.A. Guilbault, 1995. Origin of the follicular fluid added to the media during bovine IVM influences embryonic development. Theriogenol., 44: 85-94.
21. Romero-Arredondo, A. and G.E. Seidel, 1996. Effects of follicular fluid during *in vitro* maturation of bovine oocytes on *in vitro* fertilization and early embryonic development. Biology of Reproduction, 55: 1012-1016.
22. Kim, K.S., N. Mitsumizo, K. Fujita and K. Utsumi, 1996. The effects of follicular fluid on *in vitro* maturation, oocyte fertilization and the development of bovine embryos. Theriogenol., 45: 787-799.
23. Ashwood-Smith, M.J., P. Hollands and R.G. Edwards, 1989. The use of Albuminar-5 (TM) as a medium supplement in clinical IVF. Human Reproduction, 4: 702-705.
24. Staessen, C., E. Van den Abbeel and M. Carle, 1990. Comparison between human serum and albuminar-20TM supplement for *in-vitro* fertilization. Human Reproduction, 5: 336-341.