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Effect of Cumulus cell co-culture and Protein Supplement on Success of *in vitro* Fertilization and Development of Pre-implanted Embryos in mice

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:
Cumulus) .
In vitro fertilization;) (cells
. (IVF
(Hyaluronidase)
(modified Earl's medium; MEM) .
(Inactivated bovine amniotic fluid; 10%)
. (MEM) .
(% 10) (MEM)
(MEM) .
(Paraffin oil) (6-5) .
. (20-18) (CO_2) 37
.
.
. (P<0.05)
(P<0.05)
.

.

ABSTRACT: Successful oocyte fertilization and normal embryonic development of mice were considered the most important diagnostic criteria for the safety of materials and tools used for human *in vitro* fertilization and embryo transfer (IVF-ET). Therefore, we studied the influence of cumulus cells co-culture and protein supplement within culture medium on percentages of *in vitro* fertilization (IVF) and normal development of early stages of mouse embryo later. Oocytes were collected and treated with hyaluronidase to remove cumulus cells. Oocytes were divided into four groups namely: Group-1: Oocytes incubated within modified Earl's medium (MEM) supplied with 10% inactivated bovine amniotic fluid as a protein source and cumulus cells; Group-2: Oocytes incubated with MEM supplied with cumulus cells only; Group-3: Oocytes incubated with MEM supplied with 10% inactivated bovine amniotic fluid only; and Group-4: Oocytes incubated with MEM free of both protein source and cumulus cells. For IVF, 5-6 oocytes were incubated with active spermatozoa under paraffin oil for 18-20 hours at 37° ºC in 5% CO_2. Percentages of IVF and embryonic development were then recorded. Best results for IVF and normal embryonic development were achieved from oocytes of Group-1 when compared to the other groups. As compared to Group-1, the percentage of IVF for Group-2 and Group-3 were decreased insignificantly and significantly ($P<0.002$), respectively. Significant ($P<0.01$) reduction in the percentages of IVF and normal embryonic development were reported in Group-4 as compared to Group-1. Therefore, it was concluded that the presence of cumulus cells co-culture and bovine amniotic fluid as a protein source within culture medium may have an important role on the fertilizing capacity of spermatozoa and oocytes and normal development of pre-implanted mouse embryo later.

KEYWORDS: Mouse, fertilization, embryo, culture medium, IVF.

1. Introduction

In vitro fertilization (IVF) is a procedure that involves retrieving oocytes and spermatozoa from the female and male respectively, and placing them together in a laboratory dish to facilitate fertilization. Fertilized eggs are then allowed to develop *in vitro* and after several days are transferred into a female's uterus where implantation and embryo development can occur. A number of advances have been made concerning the laboratory aspects of IVF, and one of these advances was the development of better culture media for both fertilization and embryonic growth (Yeung and Ng, 2000).

Bavister (1995) reported that the programs of IVF use different types of culture media based on animal and human studies. However, culture media are classified into two types based on composition. The first one is a simple salt solution formulated with the addition of pyruvate, lactate, glucose and albumin. Others are complex media such as Ham's F-10 which are suitable for long term serum-free cultures and contain nutrients such as vitamins, amino acids and other metabolites (Mahadevan *et al.* 1997; Duran, 2000).

Certainly, it is important to provide optimum constituents when using *in vitro* culture techniques to create more natural conditions, which has led to the development of several types of culture media and various additives to optimize fertilization and embryo development (Bae and Foote, 1980; Motlik and Fulka, 1981). Efforts have been made to supplement the culture media with natural additives like serum (Kane and Headon, 1980; Totey *et al.* 1993), follicular fluid (Calvo *et al.* 1989), oviductal fluid

(Zhu *et al.* 1994), human tubal fluid (Maeda *et al.* 1996; Osheroff *et al.* 1999), cumulus cells (Hartshorne, 1989; Xu *et al.* 1992; Madan *et al.* 1994; Watson *et al.* 1994), granulosa cells (Motlik and Fulka, 1981; Vongpralub and Koyanagi, 1994), and oviductal cells (Minami *et al.* 1994; Li *et al.* 2001). However, the use of bovine amniotic fluid as a constituent of culture medium for *in vitro* maturation, IVF and embryonic development has not been reported, although, amniotic fluid contains different electrolytes, non-protein and protein components (Johnson and Everitt, 1988). But, it was reported that the exact requirements for growing embryos through its different developmental stages are not fully known (Yadav *et al.* 1998).

The use of pre-implantation mouse embryo culture as a quality control and educational model for technicians to acquire necessary embryo handling skills for human IVF programs has been advocated (Pike and Alikani, 1990). More is known about the embryology of the laboratory mouse than that of any other mammal, and almost all mammalian embryological techniques were originally devised for, and practiced on, the mouse. Even now, only few methods are possible on the mouse, and some other techniques may be less successful in other species (Buehr, 1995). Therefore, this study was designed to investigate the influence of cumulus cells co-culture and bovine amniotic fluid supplemented to the culture medium on the success of IVF and pre-implantation embryonic development in mice.

2. Materials and Methods

2.1 Animals and Superovulation Regimen

Twenty four healthy, mothered mice (age: 15-16 weeks) of Swiss albino strain were kept in an air-conditioned room at a temperature of (26$\pm$2) ^{o} C and exposed to 12-14 hour day light program (Petter and Pearson, 1971). Females were intraperitoneally (IP) injected with 10 IU of Urofollitrophin FSH-HP (Metrodin, Serono, Italy). 72 hours after the first injection, the same females were injected IP with 10 IU of Chorionic gonadotrophin (Chorulon; Intervet, Holland). Oocytes were flushed by using modified Earl's medium (MEM) (Earl's Balanced Salts, ICN Flow, ICN Biomedicals Inc., Costa Mesa, CA, USA) from both oviducts 16-18 hours after the last injection, and then treated with hyaluronidase (Medi-Cult, Denmark) to remove cumulus cells. Recovered oocytes were pippeted and washed two times with MEM and classified into immature, mature and atretic depending on the presence of first polar body and morphological features.

2.2 Cumulus Cells Preparation and Culture

Cumulus cells were removed from collected oocytes by using hyaluronidase. Cumulus cells were pooled and washed with MEM twice. Then, the cumulus cells were diluted and adjusted to a concentration of 1 X 10^5 cells/ml. Cumulus cells were cultured according to the procedure reported in details by Weiss and Eckert (1989) with slight modifications.

2.3 Collection and Preparation of Bovine Amniotic Fluid

Reproductive systems were obtained from pregnant bovine (10-12 weeks gestation) after slaughter directly, and then transferred to the laboratory within a 1-1.5 hour period. Bovine amniotic fluid (BAF) was collected and centrifuged (1200 g for 15 min. at 4 ^{o}C) to remove cells and debris. Then, BAF was inactivated at 56 ^{o}C for 30 min. and filtered by using Millipore filter (0.22 µm; Acrodisc-DLL, Gelman Sciences). Prepared BAF was divided into aliquots (10 ml) and stored at – 4 ^{o}C for later use.

2.4 Experimental Design

The present study was designed to examine the effects of cumulus cells co-culture and presence of 10% inactivated bovine amniotic fluid (BAF) as a protein source within MEM on percentages of IVF and normal development of pre-implanted mouse embryos later. Accordingly, oocytes were divided into the following four groups including :
Group-1 (G-1): Oocytes (No. = 108) were incubated with MEM supplied with protein source and cumulus cells;
Group-2 (G-2): Oocytes (No. = 112) were incubated with MEM supplied with cumulus cells only;
Group-3 (G-3): Oocytes (No. = 103) were incubated with MEM supplied with protein source only; and Group-4 (G-4): Oocytes (No. = 113) were incubated with MEM free of both protein source and cumulus cells.

2.5 Sperm Collection and Function Tests (SFTs)

Spermatozoa were flushed from both vas deferens of male mouse with 1ml of sperm preparation medium (Medi-Cult, Denmark). According to the procedure mentioned in-details by Fakhrildin (2000), sperm function tests including sperm concentration, percentage of sperm motility, sperm grade activity and percentage of sperm viability were examined after one hour (Table 1). Then, vassal spermatozoa was adjusted to 5 $X10^6$ sperm / ml within sperm preparation medium.

Table 1. The sperm function tests of vassal spermatozoa after one hour incubation

Sperm function tests	Range
Sperm concentration ($X10^6$ sperm/ ml)	26 - 38
Percentage of sperm motility	68 - 76
Sperm grade activity	2.5 - 3.5
Percentage of sperm viability	80 - 88

2.6 *In vitro* fertilization technique

For IVF, each 5-6 oocytes/0.5 ml of any group were incubated with active vassal spermatozoa (0.5 $X10^6$ motile sperm/0.1 ml) under paraffin oil (Medi-Cult, Denmark) for 18-20 hour at 37 ºC in 5% CO_2. The culture medium for each of four types was changed for *in vitro* developed embryos two times daily. Percentages of IVF and embryonic development were recorded.

2.7 Statistics

Data are presented as mean and standard error of mean (SEM). The data were statistically analyzed by using Chi-square test to compare the level of significance among different means of each group (SPSS Data Editor).

3. Results

3.1 Percentages of *in vitro F*ertilization (IVF)

The results of the present study show that the highest percentage of IVF for oocytes was obtained with MEM supplied with cumulus cells co-culture and inactivated bovine amniotic fluid as a protein source (group-1). The percentage of IVF for oocytes of group-1 have either insignificant differences as compared to group-2, or significant ($P<0.01$) differences as compared to group-3 and

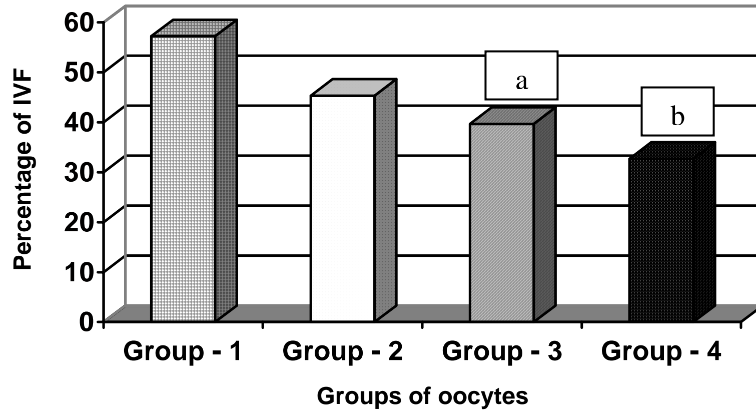

Figure 1. Percentages of IVF for oocytes divided according to the presence of cumulus cells and/or protein source in culture medium. a: Means significantly (P<0.01) decreased when compared to group-1. b: Means significantly (P<0.03) decreased when compared to groups-2 and 3.

group-4 (Figure 1). However, there were insignificant differences in the percentages of IVF between oocytes of group-2 and group-3.

The lowest percentage of IVF was recorded for the oocytes which were cultured with MEM without the presence of cumulus cells co-culture and protein source (group-4) when compared to other groups (1, 2 and 3) of oocytes (Figure 1).

3.2 Percentages of Progressive Embryonic Development

The progressive development of pre-implanted embryos is presented in table 2. Best embryonic development was recorded in the group-1 oocytes where more than 24 %, 31 % and 21 % of produced embryos were developed into 8 cells, 16 cells and 32 cells embryonic stages; respectively. Meanwhile in the same embryonic stages, it was noticed that the lowest embryonic development occurred in oocytes of group-4.

Table 2. Percentages of embryonic development produced from groups of oocytes, divided according to the presence of cumulus cells and/or protein source in culture medium

Groups of ooytes	IVF (%)	No. of developed embryos	Percentages of embryonic development					
			1-cell embryo	2 – cells embryo	4 – cells embryo	8 – cells embryo	16 – cells embryo	32-cells embryo
Group-1	57.24	62	1.61	9.68	12.90	24.19	30.64	20.98
Group-2	45.33	51	7.84	29.41	23.53	21.57	9.80	7.85
Group-3	39.70	41	4.88	9.76	21.95	21.95	26.83	14.63
Group-4	32.69	36	15.79	28.95	26.32	21.05	7.89	0

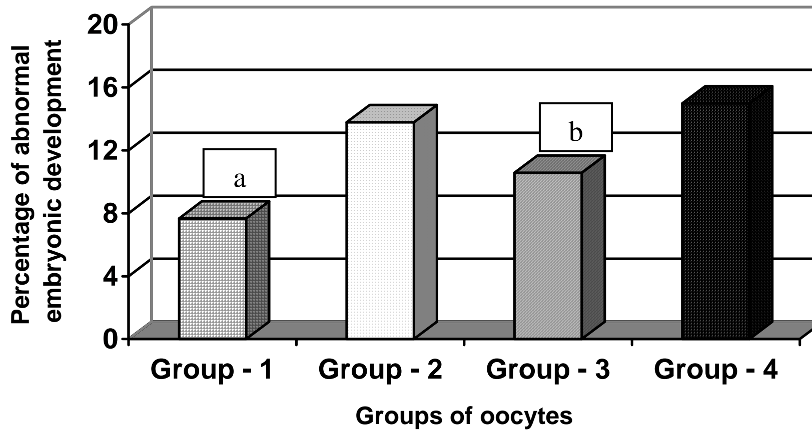

Figure 2. Percentages of abnormal produced from groups of oocytes were divided according to presence of cumulus cells and/or protein source in culture medium a: Means significantly ($P<0.01$) decreased when compared to groups-2 and 4. b: Means significantly ($P<0.05$) decreased when compared to groups-2 and 4.

In general, the oocytes of group-3 embryos developed into 8 cells, 16 cells and 32 cells embryonic stages better than the embryos which developed from oocytes of group-2 and group-4 (Table 2).

3.3 Percentages of Abnormal Embryonic Development

Figure 2 shows the percentages of abnormal embryonic development produced from four different groups of oocytes. Significant ($P<0.01$) reduction in the percentages of abnormal embryonic development was observed in the oocytes of group-1 and group-3 as compared to oocytes of group-2 and group-4. Non significant differences were observed in the percentages of abnormal embryonic development produced from oocytes of group-1 and group-3. Also, insignificant differences were found in the percentages of abnormal embryonic development between group-2 and group-4 oocytes.

4. Discussion

Since the earlier works of Steptoe and Edwards in i*n vitro* fertilization (IVF) (Steptoe and Edwards, 1978), numerous modifications have been introduced to the different stages of the technique. IVF and embryo transfer has become a widely accepted clinical method for the treatment of infertility, both for economic and academic purposes (Diedrich *et al.* 1988). Successful IVF requires appropriate preparation of sperm and oocyte, as well as culture conditions that are favorable to the metabolic activity of the male and female gametes (Duran, 2000). Because of the importance of the culture media for *in vitro* oocyte maturation, sperm preparation, *in vitro* fertilization and embryonic development (Pike and Alikani, 1990), considerable changes in composition and preparation of culture media, involving addition of the biological fluids and co-culture with granulose cells or oviductal cells or cumulus cells (Maeda *et al.* 1996; Conaghan *et al.* 1998), are necessary. The present study was designed to analyze the effects of supplementation of cumulus cells co-culture and inactivated bovine amniotic fluid (BAF) as a protein source with culture medium on percentages of IVF and embryonic development.

There are many reasons to select the BAF as a protein source in the present study. The most important reason is that amniotic fluid contains several electrolytes, non-protein and protein components. The concentration of protein content within amniotic fluid is near 3 % (Johnson and Everitt, 1988). Amniotic fluid is produced by the amniotic cells and is derived from maternal blood (Langman, 1981; Moore, 1988). Therefore, some contents of amniotic fluid are similar to maternal serum including albumin, globulin, glucose, prolactin, prostaglandins, estrogens and progesterone (Findlay, 1984; Arici *et al*. 1999; Yen and Jaffe, 1999).

The results of the present study showed that the highest percentage of IVF for oocytes were cultured with MEM supplied with cumulus cells co-culture and 10 % inactivated BAF as a protein source (group-1). The percentage of IVF for oocytes of group-1 have either insignificant differences as compared to group-2, or significant (P<0.01) differences as compared to group-3 and group-4 (Figure 1). These results emphasize the major role of cumulus cells co-culture with the culture medium in success of IVF. Also, our results are in agreement with different reports in the literature (Bornslaeger and Schultz, 1985; Hartshorne, 1989; Mori *et al*. 2000). It is suggested that the cumulus cells synthesize and secrete various substances like progesterone, polypeptides, cytokines, cAMP and MAP kinase which cause final maturation of oocyte, enhance fertilizing capacity of oocytes and spermatozoa and support *in vitro* development of early stages of embryos (Kol and Adashi, 1995; Shimada *et al*. 2001; Shimada and Terada, 2002; Su *et al*. 2002). In the present study, the use of inactivated BAF didn't have any species specific effect and improved the fertilizing capacity of both spermatozoa and oocytes *in vitro* (Figure 1). Possibly, the presence of several molecules in the 10 % inactivated bovine amniotic fluid may improve the physiology of sperm such as capacitation which is considered the first step for successful fertilization (Osheroff *et al*. 1999). However, there are no reports or data to show the use of inactivated bovine amniotic fluid with constituents of culture media for IVF and embryonic development.

The lowest percentage of IVF was recorded for oocytes that were cultured with MEM without the presence of cumulus cells co-culture and protein source (group-4) when compared to other groups (1, 2 and 3) of oocytes (Figure 1). It therefore appears that MEM has no growth factors or amino acids or hormones or cytokines or any protein source, which is considered very important for IVF and embryonic development. Indeed, the sub-optimal culture conditions are thought to contribute to these poor rates of development and consequently failure, following IVF (Lane and Gardner, 1997; Conaghan *et al*. 1998).

Best embryonic development was recorded in the group-1 oocytes where more than 24 %, 31 % and 21 % of produced embryos were developed into 8 cells, 16 cells and 32 cells embryonic stages; respectively. Meanwhile in the same embryonic stages, it was noticed that the lowest embryonic development was accrued from oocytes of group-4 (Table 2). Supplementation of the culture medium with both inactivated BAF and cumulus cells co-culture may have a direct or synchronized effect to enhance *in vitro* development of the embryonic stages. The presence of somatic cell monolayer in the culture medium during *in vitro* culture of the developing embryo was found to be very important in enhancing its development potential, which provides the developing zygotes with a suitable environment, and secretes some growth factors, or autocrine factors or has paracrine effect and balanced electrolytes support further *in vitro* development (Funahashi *et al*. 1997; Canipari, 2000; Duran, 2000; Emery *et al*. 2001).

The embryos that were produced from oocytes of group-3 developed into 8 cells, 16 cells and 32 cells embryonic stages were better than the embryos that were developed from oocytes of group-2 and group-4 (Table 2). These results demonstrated the significant role of protein source to support progressive growth and embryonic development. In contrast, it was reported that there was no significant difference in the proportion of embryos that continued to develop between the protein-free and protein-supplemented culture media, but no blastocysts in the protein-free medium hatched and only those embryos in protein-containing medium hatched that were from the zona pellucida (Pike

and Alikani, 1990). The presence of cumulus cells with culture medium only did not improve embryonic development, possibly as a result of the release of toxic metabolites or reduction of O_2 which led to rapid O_2 deprivation of the embryo. However, it remains possible that the local concentration of O_2 and lactic acid as well as a reduction in the partial pressure of O_2 may cause irreparable damage to the embryos during incubation (Barbehenn *et al*. 1974).

Significant ($P<0.01$) reduction in the percentages of abnormal embryonic development was observed from the oocytes of group-1 and group-3 as compared to oocytes of group-2 and group-4. Also, non significant differences were observed in the percentages of abnormal embryonic development produced from oocytes of group-1 and group-3 (Figure 2). These results indicate the importance and role of inactivated bovine amniotic fluid throughout progressive *in vitro* development of normal embryos, because BAF have important nutrients such as hormones, electrolytes, albumin and glucose, as well as the ability to chelate toxic metal ions and pyrogens. Graaff (2002) reported several beneficial functions of amniotic fluid for embryonic development that would ensure symmetrical growth, protection and consistent pressure and temperature.

5. Conclusion

It was concluded that the presence of cumulus cells co-culture and inactivated bovine amniotic fluid as a protein source with culture medium has an important role on the fertilizing capacity of spermatozoa and oocytes and normal development of pre-implanted mouse embryos. Inactivated bovine amniotic fluid has no-species-specific effect. Further work is required to assess the effect of cumulus cells co-culture and the presence of protein source with culture medium on implantation rate.

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