

Assessment of Epidermal Growth Factor (EGF) Effects on Development of Vitrified Mouse Morulae to the Blastocyst Stage

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ABSTRACT

Many investigators are interested in finding the new cultural systems that can support the *in vitro* development of pre-implantation embryos better. Previous studies suggested that growth factors such as epidermal growth factor (EGF) are important in pre-implantation embryo development and implantation process. On the other hand, it is very important to support post thaw development of frozen embryos. The purpose of this study was to determine if the developmental potential of mouse morulae survived after vitrification could be increased using medium containing EGF. Mouse morulae were divided into vitrified and non-vitrified groups. Vitrification procedure was carried out using a combination of 40% ethylene glycol, 30% ficoll and 0.5 M sucrose (EFS40) as cryoprotectant. The embryos were warmed rapidly using 0.5 M sucrose. The survived embryos were cultured either on T6 or T6+EGF media. Accordingly, the embryos of the non-vitrified group were also cultured. The developmental rates in all groups were daily recorded and compared statistically using Chi-square test. The results showed that after 4 days of culture, the developmental potential of non-vitrified embryos cultured on T6+EGF was significantly increased. There was no significant difference between vitrified embryos cultured on T6 and T6+EGF media. In conclusion, the developmental potential of vitrified-warmed embryos does not increase in the medium containing EGF, even though there was significant increased developmental potential of non-vitrified embryos after culture on medium containing EGF. It is needed to do more study about the changes which will probably happen on the embryo EGF receptors following vitrification. *Iran. Biomed. J. 8 (2): 77-82, 2004*

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INTRODUCTION

In assisted reproductive technology (ART), cryopreservation of embryos has several advantages. Vitrification is an inexpensive and simple method for cryopreservation of mammalian embryos which was described for the first time by Rall and Fahy [1].

In vitrification, the selection of a cryoprotectant requires care because at high concentration, cryoprotectants are toxic [2] and should be exposed either at low temperature or for short period of time [3]. Kasai *et al.* [4] described a cryoprotectant solution containing ethylene glycol, ficoll and sucrose (EFS) that could retain high survival rate after warming.

Despite many reports on successful vitrification of mammalian embryos, the rate of development of

vitrified- warmed embryos is still lower than that of non-vitrified embryos [5, 6]. On the other hand, interest in the culture and transfer of human embryos at the blastocyst stage has been spurred by the availability of a newer culture medium formulation than better support the nutritional requirements of the developing embryo. Data supporting a vital role for growth factors during early embryonic development have been steadily accumulating [7, 8]. Among the growth factors, epidermal growth factor (EGF) stimulates cellular proliferation and differentiation [9], enhances maturation of cytoplasm in immature oocytes of mice [10] and human [11]. Trials with growth factor supplementation of human embryo culture medium have been very limited but appear quite promising [12].

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Rieger *et al.* [13] showed that addition of EGF to medium of immature cattle oocytes could help them to mature and after fertilization, they had greater developmental rate to form expanded blastocysts. Kim *et al.* [14] concluded that EGF might not influence blastulation of a 8-cell mouse embryo, but it could increase the percentage of hatched blastocysts. Desai *et al.* [12] compared the effects of some growth factors with co-culture of frozen-thawed mouse morula, as a model for human embryo study and concluded that amongst the factors tested, insulin like growth factor (IGF I and II) and EGF had the greatest impact.

As there was no report on the effect of addition of EGF to culture medium of vitrified embryos, we propose to determine if direct supplementation of EGF could improve the post-thaw development of vitrified mouse morula.

MATERIALS AND METHODS

Embryos. Six to eight-week-old female NMRI mice were induced to superovulate with an injection of 7.5 IU human menopausal gonadotropin (hMG; Serono), followed by 7.5 IU human chorionic gonadotropin (hCG; Serono), given 48 h apart. Females were mated with males from the same strain and inspected for the presence of vaginal plug following day. Mice with the presence of vaginal plug considered as a pregnant and sacrificed 78-82 h post hCG injection by cervical dislocation. Morulae were flushed from oviduct and uterus horns using T6 medium [15] supplemented with 5 mg/ml bovine serum albumin (BSA; Sigma, USA). Morphologically, normal embryos were washed and pooled in fresh T6 medium before use.

Vitrification solution and procedure. Embryos were vitrified using the vitrification method described by Kasai [16]. Briefly, the vitrification solution (EFS40) was prepared using 30% (w/v) ficoll 70 (average molecular weight 70,000), 0.5 M sucrose and 10% (V/V) ethylene glycol in PBI [modified Dulbecco's phosphate-buffered saline] [16]. The embryos were transferred to EFS40 at room temperature and held for 2 min. Using a tuberculin syringe, 0.25 ml French straws (A-201-Instruments de medicine Veterinaire, France) was profiled with approximately 100 μ l of 0.5 M sucrose solution in PBI, followed by a short column of air and approximately 20 μ l of EFS40. Then groups of 10 ± 2 embryos were loaded into each straw and a bubble was added followed by a short column of 0.5 M sucrose in PBI. The loaded straws

were sealed with a plug and plunged into liquid nitrogen.

Warming solution and procedure. After 1-2 weeks, straws were transferred from liquid nitrogen to room temperature in air for 10 sec, followed by shaking in a 20°C water-bath for 20 sec. The contents of each straw were then transferred into a Petri dish containing 1 ml 0.5 M sucrose in PBI and kept for 5 min. With emptying the contents of each straw into the Petri dish, the column of the sucrose solution was mixed with the column of the vitrification solution and diluted ethylene glycol out of the embryos. After that, the embryos were transferred to about 2 ml of fresh PBI, kept at 18°C to rehydrate for another 5 min. All embryos were scored for survival and then transferred to drops of T6 medium supplemented with 5 mg/ml BSA with or without EGF (Sigma, USA).

Treatment with EGF. EGF (Sigma, St. Louis, Mo, USA) was prepared as 1000 ng/ml stock solution diluted in T6 media containing 5 mg/ml BSA and stored in 1 ml aliquots at -20°C until use. After 1 hour of culture in T6 medium, the embryos were transferred to the media supplemented with the concentration of 10 ng/ml. It is necessary to mention that the experiment was applied for non-vitrified embryos using concentration of 1, 4 and 10 ng/ml EGF and cultured for four days. The results showed that the addition of 10 ng/ml EGF could improve the developmental rate of control embryos, so it was chosen for the vitrified embryos.

Experimental design. Controls and non-vitrified embryos were randomly allocated into the drops of T6 medium with 5 mg/ml BSA (control 1) and drops of the above medium supplemented with 10 ng/ml EGF (control 2). After warming, vitrified embryos were also randomly allocated into the drops of T6 medium with 5 mg/ml BSA (experimental 1) and drops of the above medium supplemented with 10 ng/ml EGF (experimental 2). The experiments were replicated seven and five times for non-vitrified and vitrified embryos, respectively. The embryos were observed and recorded every 24 h for four days.

Blastocyst staining. Ten blastocysts of each group were randomly selected on the second day of cultivation and stained using Fong and Bongso procedure. Briefly, the blastocysts were incubated in 0.5% sodium nitrate solution at 37°C for 15-30 min. Then the embryos were removed using a Pasteur pipette, placed on a clean slide and the

excess sodium nitrate removed from the slide. Before drying of the embryos, a few fixative (Acetic Acid 1: Methanol 3) were added to the blastocysts. After air drying, the slides were stained with 5% Giemsa (Sigma, USA) in PBS for 15-30 min and washed with distilled water. Nuclei of the blastomeres were count using a light microscope with magnification of 100 ×.

Statistical analysis. Statistical comparison of developmental rates was done using Chi-Square analysis between four groups. Mean ± standard deviations of numbers of blastomeres were analyzed by one-way analysis of variances (ANOVA).

RESULTS

Embryo collection and the effect of vitrification on embryos survival rate. Six hundred forty two morulae were obtained from superovulated female mice and checked for any morphological abnormality. Six hundred ten (95%) embryos appeared normal under an inverted microscope and were randomly allocated to non-vitrified and vitrified groups (Table 1). The embryos from vitrified group were cryopreserved as described under materials and methods. After warming, 275 out of 295 embryos (93%) were survived and considered as normal.

Development of non-vitrified and vitrified morulae in media containing EGF and EGF free following a 96-h culture. During the cultivation period, inclusion of EGF in medium improved the development of non-vitrified embryos (Table 1).

In the first day of culture, the second control group (T6+10 ng/ml EGF) was compared with medium alone (first control group). No embryos ceased at morula stage (0 versus 2.3%) and also fewer embryos became blastocysts stages (53.8 versus 77%; $p<0.001$) while, more embryos were at hatched and hatching stages (39.5 versus 19.5%; $p<0.001$). After 48 h in culture, the development rate of hatched and hatching blastocyst was significantly higher in second control group than that of the T6 medium alone (78.2 versus 58.5%; $p<0.01$). After 72 h in culture, the rate of hatching and hatched stages for the second control group was significantly higher than embryos of EGF free medium (83.2 versus 66%; $p<0.01$) and no blastocyst was remained in the second control group.

After 96 h in culture, less embryos of medium

alone (first control group) were at hatching and hatched blastocyst compared with embryos in T6+EGF group and the difference was significant (70.6 versus 83.2%; $p<0.05$). At the end of cultivation, the rate of the embryo degeneration was nearly identical (16.8 versus 16%) (Table1).

During the first 24 h of embryo cultivation in EGF free medium, for the vitrified group (exp.1), the development rate to blastocyst stage (early and late) was nearly identical to control 1 (74 versus 77%). Whereas the rate of hatching development and hatched blastocyst in the vitrified group was significantly lower than non-vitrified group (2.4 versus 19.5%; $p<0.01$). After 48 h and 72 h in culture, there were no significant differences of blastocyst formation rates between vitrified and non-vitrified groups (exp. 1 versus control 1). The rate of hatching and hatched blastocyst at the end of culture was identical (Table 1).

During the first day of culture, nearly identical embryos of the first experimental group were at hatching and hatched stage versus vitrified embryos cultured in T6+EGF (exp.2) (2.4 versus 3%).

After a 48-h culture, the rate of development to blastocyst stage in the vitrified embryos cultured in T6+EGF was significantly higher than that of T6 alone (51 versus 37.3%; $p<0.05$) and less embryos of exp.2 hatched in comparison with exp.1 and there was significant difference between two groups for hatching rate (30 versus 50.7%; $p<0.001$).

During the next 48 h of culture, there were no significant differences comparing developmental rates of two vitrified groups. The rate of embryo degeneration, from the first day of cultivation until the end of culture was higher in exp. 2 group and in the last day, there was significant difference (23% for exp.1 versus 27% for exp. 2; $p<0.01$).

Number of cells of the blastocysts produced in media containing EGF and EGF free. After 48 h of culture, the mean cell number of non-vitrified embryos cultured in T6+EGF was significantly higher than non-vitrified embryos cultured in T6 alone (97 ± 21.66 versus 72.5 ± 20.2 ; $p<0.01$). There was no significant difference between two experimental groups (vitrified embryos cultured in T6: 79 ± 22.5 versus vitrified embryos cultured in T6+EGF: 72 ± 20.12), but there was significant was no significant difference between two experimental groups (vitrified embryos cultured in T6: 79 ± 22.5 versus vitrified embryos cultured in T6+EGF: 72 ± 20.12), but there was significant difference between the second control group with the both experimental groups (Table 2).

Table 1. Development of vitrified and non-vitrified morulae in media containing EGF and EGF free following a 96-h cultivation.

Treatment	Groups	No.	24 h				48 h				72 h			96 h		
			M (%)	Eb+Lb (%)	Hgb+Hgd (%)	D (%)	Eb+Lb (%)	Hgb+Hgd (%)	D (%)	Eb+Lb (%)	Hgb+Hgd (%)	D (%)	Eb+Lb (%)	Hgb+Hgd (%)	D (%)	
Non-vitrified	Control 1	174	4 (2.3)	134 (77)	34 (19.5)	2 (1.2)	57 (33)	102 (58.5)	15 (8.5)	32 (18.5)	115 (66)	27 (15.5)	23 (13.4)	123 (70.6)	28 (16)	
	Control 2	119	0 (0)	b*** 64 (53.8)	b*** 47 (39.5)	b* 8 (6.7)	b*** 12 (10)	b** 93 (78.2)	14 (11.8)	b*** 0 (0)	b** 99 (83.2)	20 (16.8)	b*** 0 (0)	b** 99 (83.2)	20 (16.8)	
Vitrified	Exp. 1	126	a* 23 (18)	93 (74)	a** 3 (2.4)	7 (5.6)	47 (37.3)	102 (50.7)	15 (11)	21 (16.6)	83 (65.9)	22 (17.5)	a*** 8 (6.5)	89 (70.5)	29 (23)	
	Exp. 2	129	c*** 14 (11)	101 (78)	4 (3)	10 (8)	c* 66 (51)	c*** 50 (39)	13 (10)	31 (24)	72 (56)	26 (20)	11 (9)	83 (64)	c** 35 (27)	

Control and Exp. 1, embryos culture in EGF free medium; Control 2 and Exp. 2, embryos culture in medium containing EGF, Values in parentheses are percentages; M, morula; Eb, early blastocyst; Lb, late blastocyst; Hgb, hatching blastocyst; Hgd, hatched blastocyst; D, degenerated embryo. a, Exp. 1 versus Control 1; b, Control 2 versus Control 1; c, Exp. 2 versus Exp. 1; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 2. Number of cells of the blastocysts produced in media containing EGF and EGF free.

Medium	Non-vitrified embryos (No.)	Vitrified embryos (No.)
T6	72.5 ± 20.2 ^a (10)	79.1 ± 22.5 ^a (10)
T6 + EGF	97.0 ± 21.66 (10)	72.0 ± 20.12 ^a (10)

Values are mean ± S.D. Number of examined embryos is in parentheses. a, significant difference with non-vitrified embryos cultured on T6 + EGF.

DISCUSSION

Our results indicate that EGF can enhance development of mouse embryo to blastocyst stage in a dose-dependent manner. These results are in agreement with the other investigators [12-14]. Kim *et al.* [14] reported the lower dosage (0.1 ng/ml) compared to the other doses (1 ng/ml and 100 ng/ml) was more effective for eight cell mouse embryo to enhance hatching. Desai *et al.* [12] observed that EGF with 4 ng/ml can increase developmental rate of mouse morulae. We compared the addition of 1, 4 and 10 ng/ml EGF to the medium of non-vitrified mouse morulae and concluded that the higher dose of EGF (10 ng/ml) can improve the development rate of mouse morulae. This dose also increased the total cell number of the produced blastocysts (data were not shown). The optimal dose of EGF depends on the mouse strain as Gardner and Lane [17] indicated that different mouse strains show different sensitivity to the changes occurred in the culture conditions. Successful development of embryo is dependent on genomic expression, regulatory hormone, energy source and growth factors and cytokines secreted from fallopian tube, uterus and embryo itself [14]. EGF with stimulation of cellular proliferation and differentiation acts as a mitogen that promotes mitosis and seems to have some roles on compaction and blastulation of early embryos [14].

In the present study, we selected mouse morula because after an 8-cell stage, there are little effects of spermatozoa or oocyte genomes on embryos compared to earlier stage embryos. After 8-cell stage, it is known that genomic expression of gamete is finished. Theoretically, the selection of morula is more accurate for EGF effect on embryonic development [14].

The results also showed that there was significant difference on development of hatching and hatched stage of vitrified embryos treated with EGF in comparison with vitrified morulae cultured on EGF free medium only on the second day of culture and less embryos of exp.2 get to this developmental

stage (Table 1). This result disagreed with authors who claimed the beneficial effects of growth factors inclusion on post-warming culture system [12]. These contradictory results may be related to the mode of freezing procedure. Desai *et al.* [12] did slow freezing but we did vitrification. Maybe, the vitrification procedure can produce some damages to the embryonic EGF receptors, so they are not able to respond to exogenous EGF properly. As others indicated structural changes [18], poor activity of both Na/H antiporter and HCO₃⁻/Cl⁻ exchanger [19], changes in the integrity of cellular membranes and intracellular organelles injuries can happen following vitrification. However, we did not study the effects of other doses of EGF on post-thaw development of vitrified embryos. Maybe there is need to add more than 10 ng/ml EGF in the culture medium to get optimum developmental rate.

More studies needed regarding embryonic EGF receptors following vitrification. Vitrification procedures often result in cell loss or damage. It is likely that such damages may also result in alterations in autocrine secretion of growth factors and therefore heighten the impact of the post-warming culture environment [12].

In this study, cell proliferation in non-vitrified embryos treated with EGF was significantly stimulated by just 48 h of exposure to EGF, but not for vitrified embryos. It is possible that vitrification procedure can produce some changes in the embryonic cells that growth factor (EGF) cannot imply its mitogenic effect. However, differential staining of treated blastocysts and analysis of cell distribution in both trophectoderm and inner cell mass (ICM) compartments upon growth factor treatment will be needed to interpret correctly.

The results also showed that embryonic degeneration rate was higher in EGF treated groups especially during the first day of cultivation. For non-vitrified group, the rate became lower from the second day until the last day. It was nearly identical to non-EGF treated, but for vitrified embryos the increase rate continued until the last day of cultivation. It seems the addition of EGF to culture medium induces some biochemical changes especially for vitrified embryos. In the embryos, the cryoprotectant comes out gradually from the embryonic cells and so makes the environment unappreciated for embryo development. Maybe, the daily medium change or shortening EGF treatment could improve the results.

In conclusion, EGF with specific concentration might have a positive effect on embryonic development at later stages. The effect on post-thaw vitrified embryos is not the same as EGF effects on

fresh embryos. Maybe it is due to the damages resulted from vitrification procedure. Therefore, further investigation is needed to study the mechanism and the functional role of EGF and EGF receptors in pre-implantation cooled-warmed embryos and implantation process.

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