

Full Length Research Paper

High efficacy of fibroblast and hepatocyte growth factors on the *in vitro* blastocyst production of post-thaw mouse two-cell embryo: The cryotop method

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There is great need to improve our understanding of what increases an embryo's development potential, after vitrification-thawing processes. For this subject, 358 two-cell stage embryos were collected from oviduct of pregnant two-day old mice and vitrified. After thawing, embryos were cultured in Tyrode's (T6) medium supplemented with different doses of fibroblast growth factor (FGF; 0, 10, 20, 50 and 100 ng/ml) and hepatocyte growth factor (HGF; 0, 10, 20, 50 and 100 ng/ml) until the blastocyst stage. To determine quality of blastocysts, blastocysts were stained with hoechst and propidium iodide. After culture for 24 h, 92.65% of treated embryos with 20 ng/ml of FGF had higher ($P < 0.05$) survival rate in comparison to the control group (84%). Blastocyst embryo formation rate were 79.43% ($P < 0.01$), and 67.46% ($P < 0.05$) in the treated groups with 20 ng/ml of FGF and 20 ng/ml of HGF, respectively, which were significantly different from the control (56%). In the treatment group with 20 ng/ml of FGF, blastocysts with >64 cells had a significantly higher inner cell mass (ICM) in comparison to the control group ($P < 0.01$). In conclusion, in this experiment, addition of growth factors in the culture had favorable effects on post-thawed cleavage of vitrified 2-cell embryos and blastocyst quality.

Key words: Blastocyst quality, growth factors, preimplantation development, vitrification.

INTRODUCTION

Following scientific efforts, more logical and economical procedures in order to maintain and promote the success

of assisted reproduction techniques are discovered. One of these procedures is cryopreservation of oocytes, zygotes and embryos (Al-Hasani et al., 2007). The first report about successful frozen-thawed human embryos was in the early 1980s (Al-Hasani et al., 2007). Vitrification with a rapid cooling rate and high concentrations of CPAs could be used. Complete deletion of ice crystal formation (Bahadori et al., 2009; Kasai and Mukaida, 2004; Vajta and Nagy, 2006), spending a shorter time, independency to a programmable temperature container (Karlsson, 2002), its feasibility (Zhang et al., 2009) and handleability by only one embryologist (Kuwayama et al., 2005b) are the other advantages of this method. Recently, changes in the culture systems and cryo-

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Abbreviations: FGF, Fibroblast growth factor; HGF, hepatocyte growth factor; hCG, human chorionic gonadotrophin; ART, assisted reproductive techniques; TE, trophoectoderm; ICM, inner cell mass; GUMS, Guilan university of medical science; PMSG, gonadotrophin; EG, ethylene glycol; DMSO, dimethyl sulphoxide; TCN, total cell number.

preservation methods appear to improve *in vitro* production of cryopreserved embryos (Khurana and Niemann, 2000; Mtango et al., 2003). There is great need to improve our understanding of what increases an embryo's development potential, after vitrification-thawing process. A few studies were performed with FGF and HGF. FGFs are potent physiological regulators of growth and proliferation of the cells (Detillieux et al., 2003) and it is known to be a powerful mitogen for granulosa cells of various species in culture (Quennell et al., 2004). However, Bieser et al. (1998) reported that the role of modulating growth factor like FGF was essential for extra-cellular proteolysis occurring during *in vitro* maturation of cumulus oocyte complexes. FGF is key-players in the processes of proliferation and differentiation of oocytes (Pandey et al., 2009). But, a few studied has been shown the effect of FGF as proliferation agent in embryo. Nandi et al. (2003) observed that FGF significantly increased cleavage and blastocysts rates of buffalo embryos as compared to the control.

Several lines of studies have investigated HGF in mitogenic, motogenic and morphogenic functions on various epithelial cells derived from rodents and human (Osuga et al., 1999). It has been reported that HGF is present in follicular fluid and its expression is stimulated by human chorionic gonadotrophin (hCG) (Osuga et al., 1999). It has been shown that FGF stimulate HGF expression in non-reproductive cells and tissues (Osuga et al., 1999). HGF-induced signaling motifs coupled to the regulation of cell cycle progression, morphogenesis and cell survival (Zachow and Uzumcu, 2007). So, that might impress proliferation, cell cycle progression and survival of embryos.

The need for efficient cryopreservation of oocytes and embryos is enormous both for theoretical and practical reasons. However, in spite of the vast efforts invested, advances are rather slow. The main problems are lack of consistency as well as differences in survival and developmental rates after warming between species, developmental stages and quality (Kuwayama, 2007). The suboptimal culture condition in which fertilization and early embryonic growth occur affected the success rates of assisted reproductive techniques (ART). There are the problems such as embryo fragmentation, cell irregularity, reduction in total cell numbers, retarded cleavage rate and blastulation in *in vitro* blastocysts growth, attributed to suboptimal *in vitro* conditions, which in turn contribute significantly to reduce embryo quality and implantation rate following embryo transfer (Cheung et al., 2003). The aim of this study was to determine whether the exposure of embryos to growth factors prior to implantation, would affect thawed embryo development rates. As a second objective, total cell number, TE (trophoectoderm) and ICM were assessed to determine whether the growth factors can promote the reduced cell number in blastocysts derived from vitrified mouse embryos.

MATERIALS AND METHODS

Animals and embryo recovery

All of the reagents were purchased from Sigma (Germany) except hCG that was purchased from Organon (Holand), HGF and bFGF-4 from R and D systems (USA), Cryotop from Kitazato Company (Japan) and NMRI mice from the Razi Institute of Karaj (Iran). 30 female mice aged six to eight weeks old were kept under the controlled light and temperature conditions with free access to water and food. All animals were treated in accordance with the guidelines of the Guilan University of Medical Science (GUMS) Ethics Community Standards on the Care and Use of Laboratory Animals. They were super-ovulated by intra-peritoneal injection of 5 IU of pregnant mare's serum gonadotrophin (PMSG), and followed by injection of 5 IU of hCG for 48 h. On the same day of hCG injection, designated as day 0, the female mice were paired with male mice of the same strain to mate. The mated females were then killed 48 h after hCG administration and two-cell stage embryos (Figure 1a) were harvested from the oviducts in pre-warmed T6 medium. After washing several times, the obtained 2-cell embryos with the normal morphology were prepared for vitrification.

Vitrification and thawing procedures

The vitrification/thawing protocol was performed according to the method described by kuwayama (2007). The two-cell stage embryo were incubated in equilibration solution comprising 7.5% ethylene glycol (EG) and 7.5% dimethyl sulphoxide (DMSO) in Ham's F-10 media for 5 to 15 min (depending on the time needed for re-expansion of the cell) at room temperature. After an initial shrinkage and recovery, they were then aspirated and placed into the vitrification solution (15% EG, 15% DMSO, 0.5 M sucrose) in Ham's F-10 medium for 50 to 60 s at room temperature. As soon as cellular shrinkage was absorbed (Figure 1b), embryos were aspirated and placed on the tip of the Cryotop (Figure 1c). Cooling of the embryos was done by direct contact with liquid nitrogen. The Cryotops were stored in liquid nitrogen for at least two weeks. Warming of embryos was performed by placing the Cryotop in thawing solution (1 M sucrose) for 50 to 60s at room temperature and then into dilution solution (0.5 M sucrose) for 3 min, followed by another dilution solution of 0.25 M sucrose for another 3 min, both at room temperature. The warmed embryos were placed four to five times into washing solution (Ham's F-10) before incubation. The embryo quality and survival rate were assessed morphologically.

Fibroblast growth factor and hepatocyte growth factor

To demonstrate any positive effects of growth factors supplementation in culture medium on survival and development after thawing, thawed two-cell stage embryos were incubated for 72 h in medium at 37°C in a humidified 5% CO₂ air as the following treatment groups: T6 + 5mg/ml of bovine serum albumin (BSA) (control), T6 + 5mg/ml of BSA + different doses of FGF (1 to 4 treated groups: 10, 20, 50 and 100 ng/ml, respectively) and T6 + 5 mg/ml of BSA + different doses of HGF (1 to 4 treated groups: 10, 20, 50 and 100 ng/ml, respectively). The embryos survival was defined as the percentage of vitrified embryos that re-expanded after 24 h. The thawed embryos were cultured until blastocyst stage.

Blastocyst cell number determination

To determine blastocyst cell numbers (vitrified and non-vitrified)

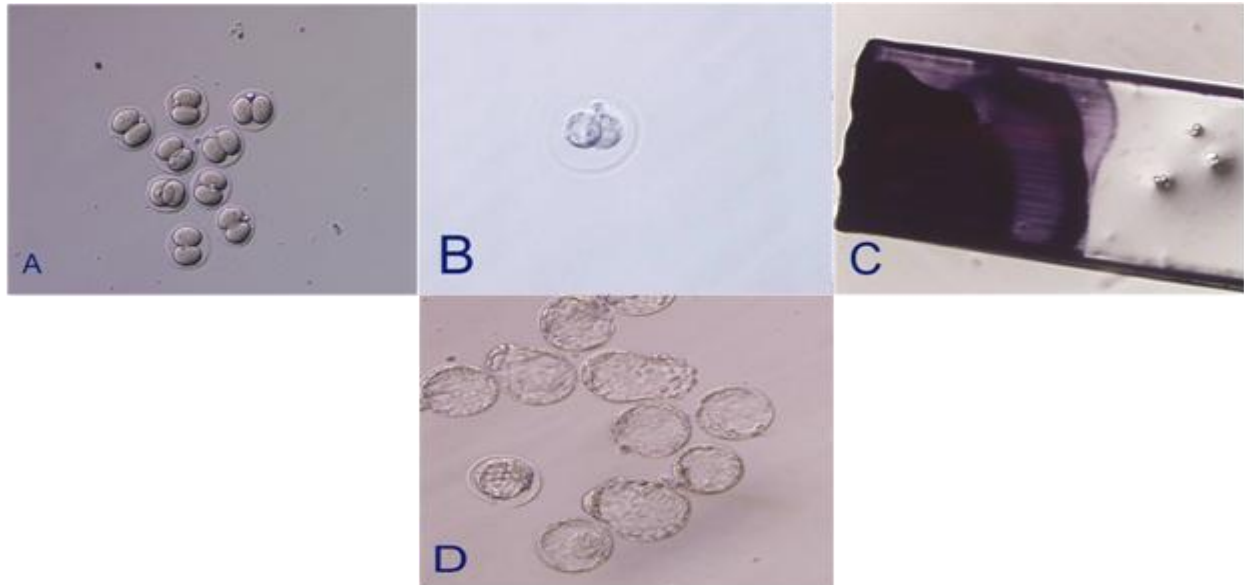


Figure 1. Different stages of culture and freeze of mouse embryo; A Mouse embryos at two-cell stage; A two-cells vitrifying and cellular shrink ageing; B placed on the tip of the cryotop; C and blastocyst stage (D) in control group. Magnification; $\times 100$ (A-B) and magnification $\times 200$ (C-D).

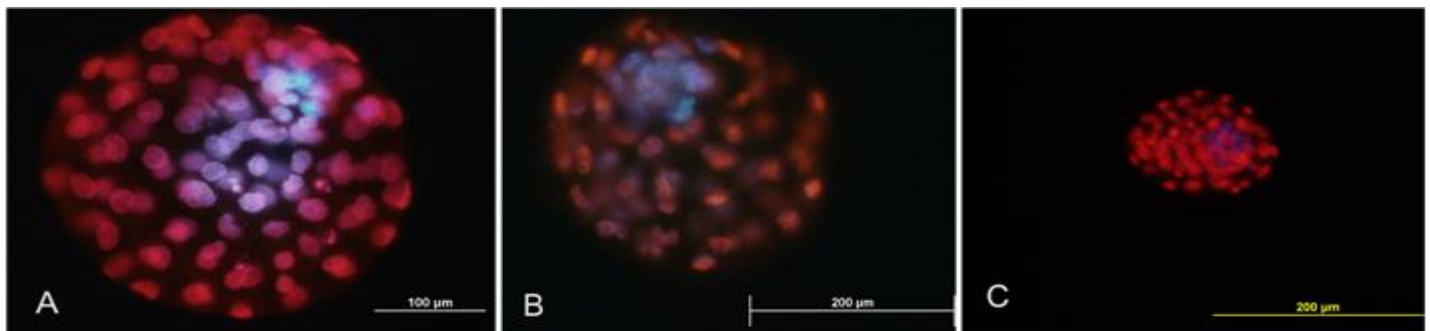


Figure 2. Mouse blastocysts. A, treated with 20 ng/ml of FGF; B treated with 20 ng/ml of HGF; C, control group at day 3 after thawing were stained with Hoechst 33342 and propidium iodide; observed under the fluorescence microscope. Blue, inner cell mass; red, trophoectoderm.

from each group, embryos were placed in drops supplemented with $1\mu\text{g/ml}$ of propidium iodide (sigma) at 37°C for 20 to 50 s. This was followed by incubation in $5\mu\text{g/ml}$ bisbenzimidazole (Hoechst 33342, sigma) in absolute ethanol overnight at 4°C . The propidium iodide stained only the nucleus of non-viable cells without an intact plasma membrane, whereas bisbenzimidazole stained the nucleus of both viable and non-viable cells. Hence, the TE will be stained by both propidium iodide and bisbenzimidazole, whilst the intact ICM will be stained only by bisbenzimidazole. Embryos were mounted on microscope slides with glycerol, a cover-slip was placed on the top of the embryos, and initially examined with the whole mount. Under fluorescence microscopy (excitation filter at 420 nm, barrier filter at 365 nm), the outer TE cells were identified by the pink fluorescence of propidium iodide, whereas the ICM cells were recognized by the blue fluorescence of the bisbenzimidazole (Figure 2). The numbers of ICM and TE nuclei were then counted under the inverted fluorescence microscope (IX71, Olympus, Japan) (Zhang et al., 2009).

Statistical analysis

The differences between growth factors treated and control groups in total cell numbers, cleavage, development to blastocyst and survival rates after vitrification were analyzed by the Chi-square (χ^2) analysis and a probability level of $P < 0.05$ was considered statistically significant.

RESULTS

A total of 358, two-cell embryos were vitrified and 100% was recovered after warming. After culture for 24 h, the survival rate of treated embryos with 20 ng/ml of FGF (92.65%) was higher than that of the control group (84%, $P < 0.05$), while HGF (84.92%) did not show significant

Table 1. Effect of fibroblast growth factor and hepatocyte growth factor on the blastocysts quality of *in vitro* thawed 2-cell embryos in comparison to control group.

Group (dose)	Embryos (n)	TE (Mean ± SD)	ICM (Mean ± SD)	TCN (Mean ± SD)	Ratio ICM/TE (Mean ± SD)
FGF (20 ng/ml)	29	68.1 ± 10.12 ^a	41.3 ± 8.21 ^a	109.4 ± 12.3 ^a	0.60 ± 0.15
HGF (20 ng/ml)	25	55.64 ± 8.7	33.76 ± 5.9	89.4 ± 10.32	0.60 ± 0.13
Control (0)	33	56 ± 9.4	30.4 ± 7.03	88.4 ± 12	0.52 ± 0.16

TCN, total cell numbers; ICM, inner cell mass; TE, trophoectoderm. Values are represented as mean ± standard deviation; a, compare to control group; a, $P < 0.05$.

Table 2. Cell number categories in blastocysts derived from thawed two-cell mouse embryos among different groups.

Group (doses)	Number of blastocyst	% of blastocyst		
		≤ 32 cells	33-64 cells	>64 cells
FGF (20 ng/ml)	29	8	25	67 ^a
HGF (20 ng/ml)	25	19	33	48
Control (0)	33	23	37	40

FGF, fibroblast growth factor; HGF, hepatocyte growth factor. a: $P < 0.01$.

effects compared to the control ($P > 0.05$). When cleavage rates were analyzed, FGF and HGF groups differed significantly ($P < 0.05$) from the control group. The morulae formation rates for treated groups with 20 ng/ml of FGF and 20 ng/ml of HGF and control group were 83.17%, 72.22% and 60% respectively ($P < 0.01$, Fig. 3). For further evaluation of viability and cleavage rates of morulae embryos after addition of growth factors in culture media, they were cultured until blastocyst stage (Figure 1d). Addition of 20 ng/ml of FGF and 20 ng/ml of HGF increased the development rate of morulae embryos to blastocyst stage when compared to the control group ($P < 0.01$). The percentage of blastocyst formation in treated groups with 20 ng/ml of FGF and 20 ng/ml of HGF and the control group were 79.43, 67.46 and 56%, respectively.

FGF increase the quality of *in vitro* produced blastocysts

Blastocysts quality was promoted among 20 ng/ml of fibroblast growth factor-treated group in comparison to HGF treated and untreated embryos (Table 1). Interestingly, total cell number (TCN), TE and ICM of treated blastocysts with 20 ng/ml of FGF were higher in this group compared to the control group ($P < 0.05$). Additionally, our results show that there was no significant difference in TCN between treatment groups with HGF and untreated group. The mean cell number ± SD in *in vitro* cultured blastocysts derived from thawed two-cell embryos of 20 ng/ml of HGF treated group was 89.4 ± 10.32 with 55.64 ± 8.7 cells in the TE and 33.76 ±

5.9 in the ICM (Table 1). The ICM: TE ratio was not significantly different for blastocysts of different groups.

Blastocysts categories on basis of cell number

Categories of blastocysts with increasing cell numbers were defined according to the expected cell number, before the fifth cleavage, between fifth and sixth, and following the sixth cleavage, been, respectively ≤ 32, 33 to 46 and >64 cells according to the categories described by Van der Elst (1998). In the control group, 23% of blastocysts had ≤ 32 cells, 37% had 33 to 46 cells and 40% had >64 cells (Table 2). In the treated group with 20 ng/ml of FGF, 8, 25 and 67% of blastocysts had ≤ 32, 33 to 46 and >64 cells, respectively; the percentage of blastocysts with >64 cells was significantly different in comparison to the control group ($p < 0.01$). The blastocysts rate with ≤ 32, 33 to 46 and >64 cells was 19, 33 and 48%, respectively in 20 ng/ml of HGF treated group; there was no significantly different in comparison to the control group. In the FGF treated group, blastocysts with >64 cells had a significantly higher ICM in comparison to the control group (Table 3, Figure 3).

DISCUSSION

The results of this study clearly demonstrate that the post thaw development of *in vitro* vitrified embryos can be greatly improved by the addition of growth factors. The results of this study indicate that, survival rate and the blastocyst formation of vitrified embryos were 84 and

Table 3. Percentage of inner cell mass (ICM) in different cell number categories of blastocysts derived from thawed two-cell mouse embryos among the different groups.

Groups (doses)	% ICM in blastocyst		
	≤ 32 cells	33-64 cells	>64 cells
FGF (20 ng/ml)	31	39	44 ^a
HGF (20 ng/ml)	30	44	36
Control (0)	28	40	30

ICM, Inner cell mass; FGF, fibroblast growth factor; HGF, hepatocyte growth factor. a: $P < 0.01$.

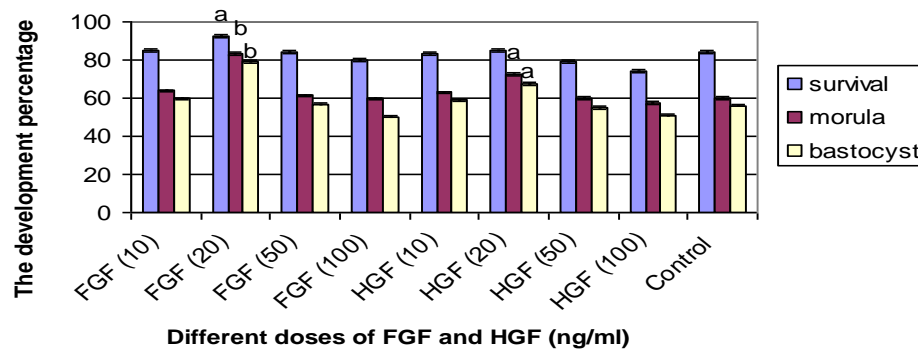


Figure 3. The rate of post-thawing mouse embryos survival, morula and development to blastocyst cultured in media supplemented with different doses of FGF and HGF and control group. Treated embryos with 20 ng/ml of FGF had higher ($P < 0.05$) survival rate in comparison to control group. Addition of 20 ng/ml of FGF ($P < 0.01$) and 20 ng/ml of HGF ($P < 0.05$) increased the cleavage of 2-cell embryo rate and development rate of Morula embryos to blastocyst stage when compared to control group.

FGF: fibroblast growth factor, HGF: hepatocyte growth factor. ^a, $P < 0.05$ ^b, $P < 0.01$

56%, respectively (in control group). In general, Zhang et al. (2009) reported that survival rate and blastocyst formation of vitrification of early stages (two-cell) of mouse embryos were 96 and 69.4%, respectively; our control group results is almost similar to their results. But it was reported that the blastocyst formation rate of vitrified two-cell stage embryos was significantly lower than that of four-cell or eight-cell stage embryos. Our results suggest that addition of growth factors can eliminate these decreasing effects significantly. So, our findings demonstrate that the presence of FGF (83.17%) and HGF (67.46%) in developmental medium influenced the ability of post-thaw embryo to undergo cleavage and this resulted to blastocysts development. Our results are similar to the reported results in 2003 that shows that the addition of growth factor to the culture medium resulted in higher developmental capacities of thawed blastocysts than that of the control group (Mtango et al., 2003). Also, it was reported that the total cell numbers in blastocysts of vitrified two-cell embryos was 85.4 (Zhang et al., 2009), while our results show that the total cell numbers in embryos increased when treated with 20 ng/ml of FGF

(109.4 ± 12.3 , $P < 0.05$) compared to the control group (88.4 ± 12). Also, it has been shown that addition of EGF and growth hormone increased TCN in thawed blastocysts when compared to the controls (Mtango et al., 2003).

Freezing and thawing procedures usually results in cell loss or damage and it is likely that such damage may also result in alterations in autocrine secretion of growth factors (Mtango et al., 2003). This is clearly shown in our results where cell number after vitrification was decreased immensely but addition of growth factor on culture media can promote this problem. FGF increase the percentage of blastocysts with more than 64 cells and their ICM. It has been acknowledged without doubt that the routine use of a good, reliable, and safe cryopreservation programmer leads to improved cumulative clinical outcomes of IVF and ICSI cycles (Vajta and Nagy, 2006). The cost of a live birth could also be reduced by both preventing the repetitive use of expensive induction agent and avoiding the expensive clinical follow-up of ovulation induction cycles. However, the possibility of cell damage caused by cryopreservation is a major issue. Thus,

several strategies used to prevent cell damage have led to the introduction of different cryopreservation techniques over the past decades (Kuwayama et al., 2005a). Vitrification is a non-equilibrium method and may be regarded as a radical approach in which ice crystal formation is totally eliminated. Nevertheless, it requires an extremely high cooling rate alongside much higher concentrations of cryoprotectant when compared with slow-rate freezing (Vajta and Nagy, 2006). Therefore, there is an increased probability of cell injury due to factors other than the formation of ice crystals.

In 1985, vitrification was reported for the first time as an effective method in mouse embryo cryopreservation (Rall and Fahy, 1985). Afterwards, Mukaida et al. (1998) reported successful vitrification of human 4 to 8 cell embryos using the method developed for mouse embryos and vitrification was then suggested as a viable and real alternative to slow-rate freezing. By far, the largest reported survey is that of cryotop method (Kuwayama et al., 2005b). The cryotop method probably needs the least minimum volume among vitrification approaches (Kuwayama, 2007). The role of growth factors in embryo production became an active area of research in the recent years. The hormones and growth factors present in the culture media or those secreted from oocytes seem to play a pivotal role in acquisition of developmental competence (Combelles et al., 2002). Fewer reports were available on the effects of FGF and HGF on the development of embryos. Further studies should focus on the *in vivo* development competence upon transfer of the vitrified-warmed embryos at supplemented medium with different growth factors, hormones and antioxidant, as well as elimination of the any damage from vitrification. Although it is unclear how our findings can be extrapolated to human embryos, it is hoped that these findings provide a reference point and the media most suitable for the culture of human vitrified-warmed embryos should be investigated.

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