Effect of Vero Cell Coculture on the Development of Frozen–Thawed Two-Cell Mouse Embryos

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Purpose: *Our purpose was to evaluate the beneficial effects of long-term coculture of Vero cells on the development of frozen–thawed two-cell mouse embryos.*
Methods: *Two-cell mouse embryos were frozen slowly with 1,2-propandiol and sucrose as cryoprotectants and thawed rapidly, followed by stepwise dilution. Vero cells were cultured in drops of RPMI 1640 to establish monolayers. Frozen–thawed embryos were cultured alone (control) or cocultured with Vero cells. The rate of development in both groups was compared.*
Results: *After 4 days of culture, significantly more embryos in coculture were developed to expanded blastocysts (61 vs 37% for controls; $P \leq 0.0001$). In addition, on the fifth day of cultivation, more embryos in coculture showed the potential of hatching from the zona pellucida (26 vs 7% in controls; $P \leq 0.0001$). The rate of degeneration in coculture was also much lower than in controls (6 and 15%, respectively).*
Conclusions: *Coculture of cryopreserved preimplantation-stage embryos with Vero cells seems to be a useful tool to eliminate the postthaw deleterious effect of freezing and also to obtain better-quality embryos appropriate for transfer.*

KEY WORDS: Vero cells; coculture; cryopreservation; mouse embryos.

INTRODUCTION

To date, cryopreservation of embryos from many species is a well-established procedure that has allowed embryo transfer practitioners to transfer them at the desired time. Despite many reports on successful freezing of embryos, the rate of progression of frozen–thawed embryos is lower than that in nonfrozen embryos in various species, e.g., mouse (1), cow (2), and human (3).

Recently, the employment of coculture systems using various somatic helper cells from established (4,5) and nonestablished (6,7) cell lines has been suggested to improve the development of embryos to the blastocyst stage. Some helper cells from different origins have been shown to have the potential to overcome the embryonic block that occurs following culture of embryos at the time of genomic activation in different species: mouse (8), equine (9), ovine (10), and human (11) embryos.

The concept that somatic helper cells may facilitate embryonic development, especially under suboptimal conditions, has resulted in various investigations. However, even under precisely controlled conditions, the in vitro environment seems to be rather suboptimal. Zygotes produced after subzonal injection of human spermatozoa showed a greater potential for development to cleaved embryos and pregnancy when cocultured with Vero cells (12). Utilization of coculture after partial zona dissection of mouse embryos has resulted in greater numbers of hatched blastocysts (13). In a similar study embryos from couples with three or more failed attempts at IVF were treated with coculture on bovine oviduct epithelial cells (BOEC) followed by assisted hatching. This treatment resulted in a higher pregnancy rate (14).

Short-term coculture of microinjected (ICSI) oocytes on Vero cells has also improved the pregnancy rate in humans (15). Coculture of human embryos with Vero cells in patients with repeated IVF failures has yielded high numbers of blastocysts appropriate for freezing. Frozen–thawed blastocysts replaced in the

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uterus on days 5–6 of the luteinizing hormone (LH) peak resulted in an 18.2% clinical pregnancy rate (4).

Despite current studies on benefits of coculture on embryonic development, there have been few reports on coculture of frozen–thawed embryos with somatic helper cells. Takagi *et al.* (16) reported a beneficial effect of bovine cumulus cells on postthaw development of 7 to 8-day-old bovine embryos. Another study (17) has shown that coculture of frozen–thawed bovine embryos with BOEC resulted in a greater number of expanded blastocysts. Coculture of frozen–thawed human zygotes on monolayers of Vero cells (18) has also increased the pregnancy rate to 27%, which has been reported to be higher than in controls (12%). During the cryopreservation procedure some embryos may degenerate at the time of thawing, and dilution or freezing may alter further development of embryos, resulting in underdevelopment of embryos following succeeding days in culture.

The present study was designed to assess the feasibility of Vero cell coculture to eliminate deleterious postthaw effects of cryopreservation using mouse embryos as a model.

MATERIALS AND METHODS

Embryos. Five- to eight-week-old female Swiss white mice were induced to superovulate with an injection of 7.5 IU pregnant mare serum gonadotropin (PMSG; Sigma) followed by 7.5 IU human chorionic gonadotropin (hCG; Serono), given 48 hr apart. Females were mated with males from the same strain. Mice were killed 44–48 hr post hCG by cervical dislocation and two-cell embryos were flushed from oviduct with Earl's balanced salt solution (EBSS) supplemented with 10% human albuminar 5 (Ha-5) containing 5% human serum albumin (HSA; Blood Research and Fractionation Center, Tehran, Iran). Morphologically normal embryos were washed and pooled in fresh EBSS before use.

Freeze–Thaw Procedure. Embryos were frozen using the slow freezing method reported elsewhere (19). Briefly, embryos were exposed to 1.5 *M* 1,2-propandiol (PROH) in EBSS + 20% Ha-5 for 10 min, then transferred to 1.5 *M* PROH and 0.1 *M* sucrose in EBSS + 20% Ha-5 for 10 min. Embryos were then loaded into 0.2-ml plastic straws (Erlangen, Stromberg, Germany; CTE 880-0.2-EC) and heat-sealed. Using a biological freezer (Erlangen; CTE 880, Cryo-Technic), straws were cooled at −1 to −7°C, held for 5 min at −7°C for seeding, then at −0.3 to −30°C, followed by a plunge into liquid nitrogen. For thawing, straws were transferred from liquid nitrogen to room temperature for 10 sec, followed by shaking in a 30°C water bath for 20 sec. Cryoprotectant was removed by passing the embryos through decreasing concentrations of PROH (1, 0.5, 0.25, and 0 *M*) in 0.2 *M* sucrose in EBSS + 20% Ha-5. After being washed twice in EBSS + 10% Ha-5, all embryos were scored for survival and then transferred randomly to drops of RPMI (GIBCO) + 10% Ha-5 with and without (control) a monolayer of Vero cells.

Cell Culture. Vero cells are derived from green monkey kidney and were handled at passage 183. We used the method described by Menezo (19) with some modifications. Briefly, cells were cultured in Roswell Park Memorial Institute (RPMI) (GIBCO) supplemented with 10% fetal calf serum (FCS; GIBCO) in 50-ml flasks for 3–4 days. Cells then were trypsinized with 0.5% trypsin and 0.2 mg/L EDTA (GIBCO) in phosphate buffer solution. After two successive washes, cells were cultured in drops of 100 μl RPMI + 10% Ha-5 at a density of 10^6 cells ml in culture dishes. A drop of the same medium was placed adjacent to the former and both were overlaid with light paraffin oil (Sigma). Frozen–thawed embryos were transferred into the coculture and control drops simultaneously and were incubated in a humidified 37°C incubator with an atmosphere of 5% CO_2 in air.

Assessment of Development and Data Analysis. Experiments were repeated eight times and embryos were observed every 24 hr for 5 days. The rate of development of embryos to a higher stage was recorded daily. Data were analyzed by χ^2 test.

RESULTS

Embryo Collection and the Effect of Cryopreservation on Embryo Survival (Table I)

Four hundred ninety two-cell-stage embryos were flushed from the oviducts of superovulated female mice. Embryos were observed carefully for detection of any morphological abnormality. Four hundred forty-six (91%) appeared normal under an inverted microscope and were cryopreserved as described under Materials and Methods (mean number of embryos/straw = 18.32 ± 2.39). Three hundred seventy-two

Table I. Embryo Collection and Effect of the Freeze–Thaw Procedure on the Number of Surviving Embryos

Number of embryos flushed	490
Morphologically normal embryos	446 (91%)
Number of cryopreserved embryos	446
Number of embryos recovered after thawing	372 (83%)
Number surviving	335 (90%)
Number degenerated	37 (10%)
Number in coculture group	182
Number in control group	145

embryos were recovered after thawing (83%). Of these, 335 (90%) embryos were considered normal after thawing and 37 (10%) were degenerated. Survived embryos were randomly allocated in control ($n = 145$) and coculture ($n = 182$) groups. In one experiment embryos ($n = 19$) in the control group degenerated due to contamination and were excluded from the results.

Embryo Development in Coculture and Controls Following 120-hr Culture

The results are summarized in Table II. At the initial stage of cultivation (day 1), Vero cells did not significantly stimulate the rate of development to four-cell- and eight-cell-stage embryos. In coculture 80 (44%) and 55 (30%) embryos (total, 135; 74%) reached the four-cell and eight-cell stage, respectively. This was nearly identical to the control group, in which 52 (41%) and 37 (29%) embryos reached the four-cell and eight-cell stage, respectively (total, 89; 71%). The rate of development to morula and early blastocyst at day 2 was significantly higher ($P < 0.05$) in coculture than controls. One hundred thirty-seven (75%) embryos in coculture versus 73 (58%) in controls developed beyond the morula stage. However, the percentage of embryos developed to early blastocysts in coculture was lower than in controls: 25 (14%) in coculture versus 25 (19%) in controls.

On day 3, more embryos in coculture developed to expanded blastocysts compared to controls: 69 (38%) and 33 (26%), respectively ($P < 0.05$). Ninety-six hours after culture 111 (61%) embryos in coculture were developed to expanded blastocysts, which was significantly higher than the number in controls: 46 (37%) ($P < 0.0001$).

The rate of hatched blastocysts also increased daily from day 3 till 120 hr, both in coculture and in controls. Sixteen (9%), 34 (19%), and 48 (26%) cocultured embryos developed to hatching blastocysts at days 3, 4, and 5, respectively, which was significantly higher ($P < 0.0001$) than controls, in which 3 (2%), 8 (6%), and 9 (7%) embryos hatched after 72, 96, and 120 hr, respectively.

The coculture system decreased the rate of embryo degeneration. In the coculture group 11 (6%) embryos degenerated during 5 days of culture, while in the control group 25 (15%) embryos underwent degeneration ($P < 0.05$).

DISCUSSION

The precise mechanism by which feeder cells promote development of mammalian embryos in vitro is not fully determined. Two mechanisms are suggested for the coculture system's effect on improvement of embryo quality and development. Feeder cells secrete embryotrophic substances such as insulin-like growth factor (IGF) (20), insulin-like growth factor binding-proteins (21), and tubal epithelial proteins 1 and 2 (22) into the culture medium, which is called positive conditioning. Or they may reduce or change the concentration of potentially harmful substances to levels more conducive for embryo development, which is called negative conditioning. Taurine, an antioxidant agent that is secreted by many feeder cells (8), may decrease the harmful effect of hydroxyl radicals (23)

Table II. Development of Frozen–Thawed Two-Cell Embryos in Coculture with Vero Cells and Controls Following 120-hr Cultivation[a]

Group	Number	24 hr (4 to 8 cell)	48hr (M & Eb)	72 hr		96 hr		120 hr	
				Exp. b	Hb	Exp. b	Hb	Hb	D
Control	126	89 (71)	73 (58)	33 (26)	3 (2)	46 (37)	8 (6)	9 (7)	25 (15)*
Coculture	182	135 (74)	137 (75)*	69 (38)*	16 (9)**	111 (61)**	34 (19)**	48 (26)**	11 (6)

[a] Values in parentheses are percentages. M, morula; Eb, early blastocyst; Exp. b, expanded blastocyst; Hb, hatched blastocyst; D, degenerated embryos.
* $P < 0.05$ (χ^2 test).
** $P < 0.0001$ (χ^2 test).

in the culture system; a high concentration of glucose in the medium is also inhibitory to embryo development (24). Most helper cells metabolize glucose readily to pyruvate and reduce the toxicity of glucose (8); coculture of mouse embryos previously contaminated with human papillomavirus DNA increased blastocyst transformation in comparison with noncocultured contaminated embryos (25).

Although methods of cryopreservation of mammalian embryos have been extremely refined, the rate of survival and development for cryopreserved embryos is lower than that for fresh embryos. Embryos as a whole or cytoplasmic elements of intact blastomeres may be affected during freezing. This could be due to high osmotic pressure, toxicity of cryoprotectants, and swelling during thawing (16). In addition, the embryonic stage (26) and the arrangement of the cytoskeleton (27) in each blastomere have been shown to affect the success of cryopreservation. Such suboptimal conditions may cause degeneration of embryos immediately after thawing or influence further embryonic development. Our preliminary results (28) on coculture of noncryopreserved embryos indicated that the development of two-cell mouse embryos in the presence of Vero cell monolayer was not significantly higher than in controls (80 versus 75% blastocyst transformation, respectively), while our data in the present study show that 61% of frozen–thawed embryos in coculture developed to expanded blastocysts, which was significantly higher than in the control group (37%). In addition, Vero cells decreased degeneration of embryos toward the end of the culture period. The rate of hatching was much higher in coculture, due possibly to the higher number of blastomeres in each embryo, thinness of the zona pellucida (unmeasured observations) in coculture, and change in the ultrastructure of the zona pellucida, which may facilitate hatching. These findings in combination with our earlier study on nonfrozen embryos cocultured with Vero cells may support a negative conditioning role of somatic cells in coculture systems.

The type of medium used is an important factor in coculture systems; the medium selected should contain the necessary metabolic requirements for both embryo development and feeder cells. In our experiment we first studied the ability of three commonly used media (EBSS, Ham's F10, and RPMI 1640, all with 10% Ha-5) to determine which medium was most appropriate for mouse embryo culture. The rate of development to expanded blastocysts and hatching was higher in EBSS, followed by RPMI and Ham's F10 (data not shown). In a parallel experiment Vero cells were also cultured in the same media and the rate of confluency and degenerated/surviving cells was evaluated. Vero cells grew much better and faster in RPMI than Ham's F10 and EBSS (data not shown), so we selected RPMI for coculture of embryos with Vero cells.

Application of coculture and freezing is by no means a new concept but it has been used most often for embryo development through the blastocyst stage prior to freezing (10,29). However, due to ethical problems associated with freezing cleaving human embryos, many IVF clinics prefer frozen human zygotes instead of cleaved embryos. More importantly, the pronuclear oocyte may well be the choice stage of biological development in terms of maintaining chromosomal integrity (30), and its survival is easy to diagnose because transition through syngamy to the first cleavage verifies viability. In addition, although freezing blastocyst-stage embryos with the aim of coculture systems or sequential media (31,32) is applicable, the quality of frozen–thawed blastocyst-stage embryos is not well known because there is not enough time to evaluate the viability of blastocysts before transfer is performed. When zygotes or embryos at an earlier stage are frozen, the embryo quality can be determined following culture of zygotes/embryos during several days in the coculture system. Another problem with freezing human embryos at the blastocyst stage is inadequacy of common culture conditions for maintenance of embryo development from the pronuclear to the blastocyst stage. Even in the most improved culture systems, only low numbers of human embryos have shown the competence to develop to expanded blastocysts appropriate for freezing at the blastocyst stage.

CONCLUSIONS

Coculture of cryopreserved embryos after thawing is a promising system and may decrease injurious post-thaw effects of cryopreservation and concomitantly provide more normal embryos at different stages appropriate for transfer.

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