Effect of cryopreservation on the properties of human endometrial stromal cells used in embryo co-culture systems

Ivan Bochev¹ · Kalina Belemezova² · Atanas Shterev¹ · Stanimir Kyurkchiev^{2,3}

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Abstract

Purpose Along with comparative investigation of the decidualization potential and IL-6 secretion by fresh and frozen ESCs, we also aimed to evaluate the effectiveness of co-culture systems based on fresh or frozen ESCs in terms of clinical pregnancy rates.

Methods Outcome analysis of a total of 215 IVF cycles with co-culture with fresh or frozen ESCs was performed. Endometrial tissue was obtained from 17 healthy donors. Concentrations of secreted prolactin, IGFBP-1, and IL-6 in conditioned media from cultured fresh and frozen ESCs (decidualized or not) were measured using ELISA or ECLIA. **Results** Embryo co-culture with frozen ESCs resulted in a much lower pregnancy rate compared to the alternative system using fresh ESCs. Furthermore, cultivated frozen ESCs showed considerably decreased release of prolactin, IGFBP-1, and IL-6 compared to fresh ESCs, indicating that cryopreservation negatively affects their decidualization potential and cytokine production.

Conclusions Altogether, this data illustrates the need for optimization and improvement of the existing autologous endometrial co-culture systems.

Keywords Embryo co-culture · Endometrial stromal cells · Cryopreservation · Decidualization · Cytokine production · Pregnancy rate

Introduction

Co-culture of human embryos in the presence of another autologous cell type (feeder cells) was introduced for the first time more than 25 years ago [1, 2] and has since been established as a routine assisted reproductive technology (ART) procedure that aims to improve the preimplantation development and overall quality of the embryos. The development of such kind of embryo-supportive techniques based on the surrounding feeder cells represents just one of the various strategies for improving the suboptimal conditions provided by the artificial media for in vitro embryo culture. The main idea behind co-culture as a concept is that the supportive cells exert their beneficial effect by enhancing the in vitro conditions through detoxification of the culture medium and removal of potentially noxious agents such as heavy metals and free radicals and by providing the necessary signals (via cell-to-cell interactions) or/and release of a broad spectrum of embryotrophic factors (e.g., growth factors; cytokines; nutrients) [3–6]. The final result should be mimicking the optimal physiological conditions of the maternal environment within the uterine cavity and by reproducing the intimate in vivo cross-communication links between the embryo and the cells of the receptive endometrium.

Capsule Altogether, this data illustrates the need for optimization and improvement of the existing autologous endometrial co-culture systems.

✉ Ivan Bochev
lakatush@yahoo.com

¹ IVF Department, Ob/Gyn Hospital Dr. Shterev, 25-31 Hristo Blagoev Str., 1330 Sofia, Bulgaria

² Tissue bank BULGEN, 25-31 Hristo Blagoev Str., 1330 Sofia, Bulgaria

³ Institute of Reproductive Health, 25-31 Hristo Blagoev Str., 1330 Sofia, Bulgaria

To date, a wide variety of co-culture systems have been reported to have favorable effects on preimplantation embryo development such as improved growth [3, 5, 7–9], morphological characteristics [10], and blastocyst development [11]; decreased incidence of fragmentation [3, 5, 12] and apoptosis [13]; and higher pregnancy [4, 10] and delivery rates [14]. Among the broad range of somatic cell lines with well-determined embryotrophic properties, Vero cells [15–18], cumulus–granulosa cells [19–23], and endometrial cells from the lining of the uterus [3–5, 11, 12, 24, 25] could be defined as the most popular and commonly used in clinical in vitro fertilization programs.

In this respect, of particular significance is the notion that the routine application of monolayers of human autologous endometrial (epithelial or stromal) cells in the context of the so-called autologous endometrial co-culture (AECC) system could provide a number of additional benefits including sufficient cell availability, relatively easy and atraumatic access to the cell source, high expandability of the isolated cells, and avoided risk of cross-contamination and immune rejection. However, the most valuable advantage of endometrial cells is their origin from the natural site in which the peri-implantation period of embryonic development takes place, and thus their usage allows for the re-creation of conditions most closely resembling the in vivo environment in the receptive endometrium [26, 27].

With regard to the functional role of the endometrial stromal cells (ESCs) their transformation into decidual stromal cells represents one of the foremost events associated with decidualization, and it is of crucial importance for successful embryo implantation and maintenance of pregnancy [28, 29]. Decidualization of the human uterine endometrium is a tissue remodeling process that begins in the early secretory phase of the menstrual cycle under the influence of ovarian sex steroid hormones independently of the presence of a conceptus. The process of decidualization is characterized by dramatic morphological and biochemical transformation of ESCs into secretory decidual cells that is associated with the production of cytokines, growth factors, and inflammatory mediators essential for blastocyst development and implantation [28, 30, 31]. For instance, ESCs have been demonstrated to release a number of factors like leukemia inhibitory factor (LIF) [32], granulocyte-macrophage colony-stimulating factor (GM-CSF) [33], insulin-like growth factor (IGF) [34], epidermal growth factor (EGF) [35], and IL-6 [36, 37] that have been found to exert a direct beneficial effect on embryonic development, growth rates, and implantation potential [3, 4, 26, 38, 39].

IL-6 is a pleiotropic cytokine with a pivotal role in a multitude of processes including regulation of the immune response, inflammation, hematopoiesis, cell survival, apoptosis, cell proliferation, and oncogenesis [40, 41]. IL-6 is produced by a wide range of immune and non-immune cell types [42,

43]. In a normal human endometrium, it is expressed at the highest levels during the mid- to late secretory phases of the cycle and can be produced by both epithelial and stromal cells [36, 37, 44, 45]. Furthermore, receptors for IL-6 have been found on the endometrium and human embryo from the blastocyst stage onwards [45, 46]. All these findings suggest that this cytokine may play a role in processes that prepare endometrium and embryo for implantation. Moreover, secreted IL-6 has been demonstrated to be present in conditioned culture media from cell lines with embryotrophic properties that are routinely used for human embryo co-culture [47, 48].

In some cases, autologous endometrial cell co-culture is performed with fresh primary ESCs isolated from biopsied tissue taken in the menstrual cycle which immediately precedes the controlled ovarian hyperstimulation and then maintained in culture for up to 3–4 weeks prior to beginning the co-cultivation procedure. In those patients who have one or more cycles passed between biopsy and ART procedure, the co-culture is performed using cryopreserved autologous ESCs. Although fresh and frozen cells might exhibit similar morphological patterns and even comparable proliferation rates [49, 50], it is quite possible that the cryopreservation process, as previously demonstrated with other cell types such as hepatocytes [51, 52], chondrocytes [53], granulocytes [54], and endothelial cells [50], might affect their functional state and, in the case of ESCs, might influence their ability to support and improve embryo development in vitro.

In the present study, we investigated the effect of cryopreservation on some functional properties of human ESCs that are essential for embryonic development and successful implantation, such as decidualization potential and production of IL-6. Additionally, the effectiveness of co-culture systems using post-thawed ESCs was evaluated. This was accomplished by a comparative analysis of the clinical pregnancy rates achieved after embryo co-culture with fresh and frozen ESCs.

Materials and methods

Endometrial tissue samples were collected from 17 healthy, regularly cycling donors (aged 28–42 years) after having obtained their signed consent according to the requirements of the Ethics Committee of Ob/Gyn Hospital “Dr. Shterev”, Sofia. The samples were delivered to the laboratory and processed within 15 min after collection.

In addition, the outcome of a total of 215 in vitro fertilization (IVF) cycles having undergone co-culture with fresh ($n = 86$ cycles) or frozen ($n = 129$ cycles) autologous ESCs during the period 1 January 2010 through 31 March 2015 was analyzed. Endometrial tissue was obtained from each patient in a non-medicated cycle prior to initiating the IVF procedure

by a luteal phase endometrial biopsy (Ob/Gyn Hospital “Dr. Shterev”, Sofia) and processed as described below.

Isolation and culture of human endometrial stromal cells

Endometrial tissue specimens were obtained in a spontaneous cycle by a luteal phase endometrial aspiration biopsy (between days 20–23 of the menstrual cycle) using an endometrial suction curette (Probet, Gynetics, Belgium). After collection, tissue samples were washed in Dulbecco’s modified Eagle’s medium/Ham’s Nutrient Mixture F-12 (DMEM/F-12; PAN-Biotech, Germany), minced into small pieces of less than 1 mm^3 , and subjected to collagenase digestion (0.25 % Collagenase IA; Sigma-Aldrich) for 45 min at 37°C with agitation. The digested tissue was passed through a $100\text{-}\mu\text{m}$ nylon cell strainer (Corning, USA) to remove non-dispersed tissue fragments. The strained cell suspension was then centrifuged at 300 g for 10 min, and the cell pellet was re-suspended in culture medium DMEM/F-12 supplemented with 10 % fetal bovine serum (FBS; PAN-Biotech, Germany) and 1 % Antibiotic/Antimycotic Solution (Sigma-Aldrich, Germany). The isolated cells were plated into 10-cm^2 6-well plates (Orange Scientific, Belgium) and cultivated at 37°C in a humidified atmosphere of 5 % CO_2 . Medium exchange was performed every 4 days of culture. When the culture reached 80–90 % confluence, the cells were harvested after trypsinization (0.05 % trypsin/0.5 mM EDTA; Irvine Scientific, USA) at 37°C for 3 min and subsequently replated in DMEM/F-12 + 10 % FBS or cryopreserved in a 10 % dimethyl sulfoxide solution in FBS and stored in liquid nitrogen for later use. For comparative experimental analyses, fresh and cryopreserved ESCs from each individual cell line at second, third, and fourth passages were used. For cytokine production experiments, culture supernatants from ESC monolayers were collected after 48 h of culture, centrifuged, and stored at -20°C for future analyses. The morphological characteristics of cultured ESCs were repeatedly examined under an inverted phase contrast microscope (Axiovert 40, Zeiss, Germany).

In vitro decidualization of endometrial stromal cells

At near confluency, fresh and frozen ESCs from every individual cell line were decidualized in phenol red-free DMEM-high glucose (PAN-Biotech, Germany) supplemented with 2 % charcoal-stripped FBS (cs-FBS; PAN-Biotech, Germany), $1\text{ }\mu\text{M}$ Medroxyprogesterone 17-acetate (MPA; Sigma-Aldrich, Germany), 10 nM β -Estradiol (Sigma-Aldrich, Germany), and 1 % Antibiotic/Antimycotic Solution (Sigma-Aldrich, Germany) for 17 days at 37°C and 5 % CO_2 . During the differentiation period, the non-induced-toward-decidualization control cells were maintained only in phenol red-free DMEM-high glucose containing 2 % cs-FCS, 1 % Antibiotic/Antimycotic Solution, and 0.1 % ethanol vehicle (control). Each experiment was conducted in triplicate, and the media were changed every third day. At the end of the induction period, culture supernatants were collected and stored at -20°C until assayed. Endpoint analyses were measurements of protein concentrations of secreted prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1) in conditioned media.

Enzyme-linked immunosorbent assay

Concentrations of IGFBP-1 and IL-6 in the culture supernatants were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (DuoSet ELISA Development kit, R&D Systems, USA, and Ready-SET-Go! ELISA Set, eBioscience, USA, respectively) according to the manufacturer’s instructions. All samples were assayed in duplicate. The minimum detection limits for IGFBP-1 and IL-6 assays were 30 and 2 pg/ml, respectively.

Electro-chemiluminescence immunoassay

PRL levels in the conditioned media from decidualized ESCs were measured by an electro-chemiluminescence immunoassay (ECLIA; Elecsys, Roche Diagnostics, Switzerland).

Fig. 1 Morphology and proliferation rates of fresh and frozen ESCs. Fresh (a) and thawed (b) ESCs (passage 3) exhibited similar fibroblast-like spindle-shaped morphology; magnification, $\times 160$. c The average

doubling time of fresh ESCs cultures was significantly shorter than that of the thawed cultures (46.3 ± 0.8 versus 76.8 ± 4.3 h, respectively; $*P < 0.05$)

Fig. 2 PRL production by fresh and frozen ESCs after in vitro decidualization. Decreased PRL concentrations were detected in all 17 thawed ESC cultures as compared to the corresponding fresh cultures (a).

The mean levels of secreted PRL in fresh ESC cultures were significantly higher in comparison to the mean values obtained for the thawed ESCs (b; 46.2 ± 35.5 versus 6.3 ± 7.7 ng/ml, respectively; $*P < 0.05$)

land) on the Roche Elecsys 2010 Immunoassay Analyzer (Roche Diagnostics, Switzerland). The minimum detection limit was 0.47 ng/ml.

Proliferation Assay

Fresh and frozen ESCs (passage 2) were seeded at a density of 2000 cells/cm² in 10-cm² 6-well plates (Orange Scientific, Belgium). Cultivation was performed in DMEM/F-12 + 10 % FBS and 1 % Antibiotic/Antimycotic Solution at 37 °C/5 % CO₂. At each time point (24, 48, 72, 96, 120, 144, and 168 h), cells were trypsinized and counted using an automated cell counter (LUNA-II; Logos Biosystems, Anyang, South Korea). The exponential growth curve was generated, and doubling time was calculated using GraphPad Prism software, version 5.00 (GraphPad Software Inc., San Diego, USA).

Embryo co-culture with autologous ESCs

Each patient included in the study had at least two previously failed attempts at IVF or intracytoplasmic sperm injection (ICSI) with poor preimplantation embryo development. Embryos were placed on a confluent monolayer of autologous

ESCs (fresh or frozen/thawed) the day after oocyte retrieval and IVF procedure (IVF or ICSI) when fertilization was determined. The co-culture continued until the day of embryo transfer. From the pronucleate stage to day 3, embryos were co-cultured in G-1 medium (Vitrolife, Sweden) at 37 °C and 6 % CO₂. From day 3 to the blastocyst stage, co-culture was performed in G-2 medium (Vitrolife, Sweden) at 37 °C and 6 % CO₂ and 5 % O₂. Embryo quality was evaluated according to the Istanbul consensus criteria [55]. A clinical pregnancy was confirmed by transvaginal ultrasonography at least 5 weeks after embryo transfer.

Statistical analysis

Quantitative data is expressed as means ± standard deviations (SDs). Statistical significance of difference between diverse data sets was assessed by Student's *t* test, Mann–Whitney rank sum test, or Wilcoxon signed-rank test. Categorical data were compared using χ^2 analysis and Fisher's exact test. The data was analyzed using STATGRAPHICS Centurion XV (Statpoint Technologies, Inc.) and SigmaPlot (Systat Software, Inc.) statistical software. A level of $P \leq 0.05$ was considered statistically significant.

Fig. 3 IGFBP-1 production by fresh and frozen ESCs after in vitro decidualization. Comparison of IGFBP-1 protein levels measured in conditioned media from fresh and matched frozen ESC cultures at

individual level (a). The mean IGFBP-1 levels in frozen ESC cultures were 4.4-fold lower than those in fresh cell cultures (b; 14.8 ± 28.4 vs 65.0 ± 76.2 ng/ml, respectively; $P = 0.054$)

Results

Morphological characteristics and growing rates of fresh and frozen ESCs

Comparative microscopic examination of confluent monolayers of ESCs at passages 1 to 4 revealed a close morphological resemblance between pre- and post-cryopreserved ESCs. In both cases, ESCs exhibited a characteristic fibroblast-like spindle-shaped morphology (Fig. 1a, b). In contrast to their morphological features which were not affected by the cryopreservation process, frozen ESCs demonstrated significantly decreased proliferation capacity compared to their fresh counterparts. For pre-cryopreserved ESCs at second passage, an average doubling time of 46.3 ± 0.8 h was assessed, whereas for post-thawed ESCs this value was 76.8 ± 4.3 h ($P < 0.05$) (Fig. 1c).

Effects of cryopreservation on ESC decidualization

Decidualization capacity of pre- and post-cryopreserved ESCs from 17 different donors was compared. Conditioned media from ESCs decidualized with MPA and β -Estradiol were assayed for secretion of PRL and IGFBP-1, which are well-established biochemical markers and quantitative indicators for decidualization [56]. Decreased PRL protein levels were measured in all 17 post-cryopreserved ESC cultures as compared to corresponding fresh ESCs at individual level (Fig. 2a). The mean PRL levels in fresh ESC cultures (46.2 ± 35.5 ng/ml) were significantly higher compared to the frozen ESCs (6.3 ± 7.7 ng/ml, $P < 0.05$; Fig. 2b).

In 7 of 10 in vitro decidualized ESC cultures, the cryopreservation process was found to result in a decrease of the IGFBP-1 concentrations (Fig. 3a). Increased IGFBP-1 protein levels in conditioned medium from frozen ESCs (versus corresponding fresh ESC culture) were detected in only one of the cases, and in 2 of 10 cases, there was no difference observed in the amounts of secreted IGFBP-1 between pre- and post-cryopreserved ESCs (Fig. 3a). Nevertheless, the average IGFBP-1 concentration in frozen ESC cultures was found to

Fig. 5 Clinical pregnancy rates obtained after embryo co-culture with fresh or frozen ESCs. The difference of about 10 % was in favor of the group of cycles in which the AECC was performed with fresh ESCs

be 4.4-fold lower than that in fresh cultures (14.8 ± 28.4 vs 65.0 ± 76.2 ng/ml, respectively; $P = 0.054$) (Fig. 3b).

IL-6 production by fresh and frozen ESCs

Protein concentrations of IL-6 were determined in conditioned media from 11 ESC lines (fresh and thawed; passage 2). When IL-6 production was compared between fresh and frozen ESCs from each individual cell line, it was found that in 7 of 11 cases, non-cryopreserved ESCs secreted higher amounts of IL-6. In one case, IL-6 levels were comparable between the two ESC cultures (Fig. 4a). Non-cryopreserved ESC cultures had mean IL-6 levels of 193.3 ± 163.8 pg/ml. In contrast, after thawing, the mean IL-6 concentration in ESC cultures was measured to be considerably (although not statistically significantly) lower (114.9 ± 80.3 pg/ml) in comparison to the fresh cell cultures (Fig. 4b).

Co-culture with fresh and frozen ESCs

The clinical outcome of 215 IVF cycles with autologous ESCs was evaluated. In the first group of 129 IVF cycles, co-culture was performed with frozen ESCs. Fresh ESCs were used in the other group of 86 cycles. The results showed that the clinical pregnancy rate achieved after embryo co-culture with fresh ESCs was much higher than that for the cryopreserved ESC group (38.4 vs 28.7 %, respectively) (Fig. 5). Although

Fig. 4 IL-6 secretion by fresh and frozen ESCs. **a** Amounts of IL-6 produced by fresh and frozen ESCs from each individual cell line. **b** Comparison between the mean values of IL-6 secreted by fresh and frozen ESCs cultures

quite marked, this difference in the pregnancy rate is not statistically significant.

Discussion

A main feature of the autologous endometrial co-culture system is that it implies the use of ESCs obtained in advance from the patients' own uterine lining. As a rule, primary ESCs are isolated during the cycle immediately preceding the ART procedure and initiation of COH. In such cases, freshly isolated ESCs need to be grown and maintained in culture for a period not longer than few weeks before their use in co-culture, which makes their cryopreservation unnecessary. Often, however, generally due to medical reasons, the period between isolation of ESCs and their co-cultivation with embryo/s could last up to several months. Since such a continuous maintenance of primary ESCs in culture is unfeasible, cryopreservation is required. Thus, depending on the circumstances, two essentially different systems (based on whether fresh or frozen cells are used) have to be routinely applied in the IVF programs, which logically raises a substantial need for a comparative assessment of their effectiveness in practice.

The analysis of pregnancy outcomes in 215 IVF cycles with AECC, routinely performed in our clinic during a period of about 4 years, showed a marked decrease in clinical pregnancy rate in those cases where frozen ESCs have been used for the co-cultivation process. Although not statistically significant, the difference in pregnancy outcomes is too substantial to be ignored. So it can be assumed that cryopreservation of ESCs might affect their feeder cell capacities to support in vitro development and to improve implantation competence of the embryo. Moreover, there is a substantial body of data indicating that freezing can have different adverse effects on the functions and activities of a wide variety of cell types. Thus, strongly reduced activity of xenobiotic metabolizing enzymes [51] and lowered responsiveness to enzyme-inducing agents [57] have been demonstrated in cryopreserved human hepatocytes compared with freshly isolated cells. Disturbances in cellular functions due to freezing have been also detected in human granulocytes and endothelial cells. Cryopreservation has been shown to result in decreased thrombomodulin activity and increased release of soluble thrombomodulin in endothelial cells, indicating impairment of their anticoagulant function [50]. Boonlayangoor et al. (1980) have reported a marked decrease in phagocytosis, killing activity, myeloperoxidase activity, nitroblue tetrazolium reduction, and chemiluminescence of cryopreserved granulocytes c.

All these and similar data, in addition to our outcome results from the practical use of the two different co-culture systems described above, prompted us to analyze the putative effects the freezing could exert on some specific properties of

human ESCs which are indicative of their functional status like decidualization and cytokine production. Firstly, however, we tried to determine their post-thaw condition based on common cell characteristics such as morphology and proliferation rates. We found that while cryopreserved ESCs retained their morphological characteristics, the in vitro growing capacity of the thawed cells was strongly reduced compared to fresh ESCs. Such retardation in the cell development in culture after freezing could be associated with damages to the cellular membranes and/or enzyme systems occurring during the freeze/thaw process.

Regarding the functional status of thawed ESCs, our results clearly demonstrate that cryopreservation negatively affects both of their properties analyzed—decidualization potential as assessed by release of PRL and IGFBP-1 and IL-6 production. This data is in good agreement with the previous findings of decreased pregnancy rates after co-culture with frozen ESCs and indirectly supports our assumption that cryopreservation exerts a negative influence on some functional properties of ESCs that are essential for their capacity to support development of the embryo in an AECC system. Obviously, on the basis only of the results presented here, this assumption remains quite speculative and further data and studies are needed in order to be confirmed. Nevertheless, our findings demonstrate the need for optimization of existing AECC systems and conditions (e.g., through optimization of ESC cryopreservation protocols or limited use in AECC of fresh ESCs only) in order to enhance the effectiveness of their use in ART.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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