Influence of pressure changes during embryo transfer on blastocyst viability

Mrugacz G.[1], Grygoruk C.[1*], Gajda B.[2], Grad I.[2], Grusza M.[1], Sieczynski P.[3]

[1] Center for Reproductive Medicine BOCIAN, Akademicka 26, 15-267 Bialystok, Poland
[2] National Research Institute of Animal Production, Department of Biotechnology of Animal Reproduction, Krakowska 1, 32-083 Balice/Krakow, Poland
[3] Center for Reproductive Medicine KRIOBANK, Stoleczna 11, 15-879 Bialystok, Poland

ABSTRACT

Purpose: The embryo transfer into the uterus by a transcervical catheter is the final stage of in-vitro fertilization procedure. So far, a little attention has been placed on the impact of embryo transfer procedure on embryo viability. The study was design to measure the morphological changes and apoptosis rate in embryos exposed to embryo transfer in in-vitro conditions.

Methods: Morphologically normal rabbit blastocysts were divided randomly into control (48) and experimental (48) groups. The experimental group blastocysts were exposed to embryo transfer in in-vitro conditions. Morphological changes in response to embryo transfer were assessed 5 and 60 minutes after ET. The apoptosis rate was measured one hour after embryo transfer.

Results: Morphological changes in response to embryo transfer were more prevalent in the experimental group; 14 shrunken and one collapsed blastocysts in experimental group and only two shrunken blastocysts in the control group. The mean DNA fragmented nucleus index in the experimental group was 37.7 % and was significantly higher than in the control group, 8.1 %.

Conclusions: Embryo transfer can trigger both morphological and apoptotic changes in rabbit blastocysts. The local pressure fluctuations during embryo transfer could be a major factor responsible for the above-mentioned changes.

Key words: apoptosis, blastocyst, embryo transfer, pressure.

***Corresponding author:**
Cezary Grygoruk,
Center for Reproductive Medicine Bocian
26 Akademicka str, 15-267 Bialystok, Poland
Tel/Fax: +48857447700
e-mail: cezary.grygoruk@gmail.com

Received: 04.03.2013
Accepted: 24.06.2013
Progress in Health Sciences
Vol. 3(1) 2013 pp 89-93

INTRODUCTION

The procedure of embryo transfer (ET) is the final manual intervention in in-vitro fertilization procedure (IVF). During ET a newly formed embryo is placed within the uterus by a transcervical catheter. The catheter loaded with embryos is inserted into the uterine cavity through the cervical canal of the uterus. Then, the load is injected and the catheter is removed from the cavity. The fact that high rates of fertilization in the laboratory result in a relatively low rate of take home babies have led investigators to focus the blame on various features of the ET procedure [1-4]. Several factors, having an impact on the ET success, have been described as for example the number, quality and stage of development of the embryos, the receptivity of the uterus, the risk of expulsion of the embryos from the uterine cavity, the instrumentations used as well as the ability and specific experience of the operators. It was also suggested that mechanical factors, such as catheter type, method of loading the catheter, placement of the catheter tip may be the cause for the relatively low pregnancy rates [1-4]. So far, a little attention has been placed on the physical forces acting on embryo in the process of embryo transfer. The impact of the ET itself on viability of the embryo is not well understood nor it has been investigated to date. Therefore, in the current work, a laboratory setting for in vitro simulation of ET was developed in order to investigate the morphological changes and the apoptosis rate in embryos in response to physical conditions generated during ET. A rabbit model was chosen because of the morphological similarities between human and rabbit blastocysts.

MATERIALS AND METHODS

Collection and culture of embryos

The experiments were carried out, after receiving consent from the local ethics committee, in 96 rabbit blastocysts. Rabbit females (*New Zealand*, N = 4) 4.5– 6.5 months of age and weighing 3–4 kgs were used as donors. Females were superovulated by IM injection of 100 IU of PMSG (Serogonadotropin; Biowet, Poland), followed by 100 IU of hCG (Biogonadyl, Biomed, Poland), injected 72 hours later. Immediately, after hCG injection, donors were inseminated with semen collected from two New Zealand bucks.. The embryos at the one-cell stage were collected at 18 to 20 hours after insemination. Only morphologically normal zygotes (with single cell, normally formed cytoplasm and visible polar bodies) were selected for culture. The embryos were recovered at room temperature by flushing the Fallopian tubes with 10 mL of Dulbecco's solution (PBS, Sigma) supplemented with 20% fetal calf serum (Sigma). The recovered embryos were examined morphologically under a stereomicroscope. All the embryos were washed in a B2 medium (INRA, Ménézo, bioMerieux, France) [9] and cultured in 1.0mL of the medium in four-well multidishes (Nunc, Denmark) in a CO_2 incubator (5% CO_2 in an air at 38°C) for 3 to 4 days until they reached a blastocyst stage Morphologically normal blastocysts, obtained 90–106 h post insemination were randomly divided into the control and the experimental group. Assessments of embryos were carried out under a stereomicroscope (Nikon) [5].

Embryo transfer

The insulin syringe (1mL) was connected to an embryo transfer catheter (Labotect, Bovender-Gottingen, Germany) and then loaded with a combination of air and liquid based on a routine clinical protocol. The reference loading of the syringe-catheter complex was arranged in the following order from the catheter tip: 0.1 µl air, 1.5 µl liquid (embryo culture medium), 0.2 µl air, 1.5 µl liquid (rabbit blastocyst in the embryo culture medium), 0.2 µl air, 1.5 µl liquid (embryo culture medium) and 25 µl air. A tip of a loaded syringe catheter-complex was positioned in the center of a well (four-well multidishe, Nunc, Denmark) filled with 1.0mL of the B2 medium (INRA, Ménézo, bioMerieux, France) and then the transfer load was injected into the medium. The procedure was performed by a medical doctor specialized in reproductive medicine according to his routine clinical practice. After ET, the embryos were left in the wells, one embryo per one well, and cultured in a CO_2 incubator (5% CO_2 in the air at 38°C) for 1 hr. After that time, the embryos were investigated for signs of apoptosis.

ET procedure was performed only in the experimental group. The control group blastocysts were moved individually using automatic pipette (HTL, Poland) into a well (four-well multidishes, Nunc, Denmark) filled with 1.0mL of the B2 medium (INRA, Ménézo, bioMerieux, France) . and then investigated for signs of apoptosis after one hour culture in the CO_2 incubator (5% CO_2 in the air at 38°C) .

Embryo assessment

a. Morphology

A natural response of the blastocyst to a stressful environmental factor is a shrinkage (author's observation). Therefore, we decided to use the blastocysts as a study model in order to follow the shrinkage response of embryos in reaction to ET. The blastocysts shape and the blastocele size were assessed before, 5 and 60

minutes after transfer. The blastocele size, its shortest diameter, was measured with the use of an inverted microscope (Nikon Diaphot 200) equipped with a digital camera (DCM-500) connected to a computer with installed measuring software (ScopePhoto). The shrinkage of blastocyst was noted when the blastocele size diminished over 20% after the embryo transfer. The blastocyst shrinkage response (BSR) to transfer was classified accordingly:

- no visible changes in shape of the blastocyst, blastocele size unchanged (0 on Fig.1)
- shrinkage of the blastocyst, blastocele size reduced (I on Fig.1a),
- collapse of the blastocyst, no blastocele observed (II on Fig.1b).

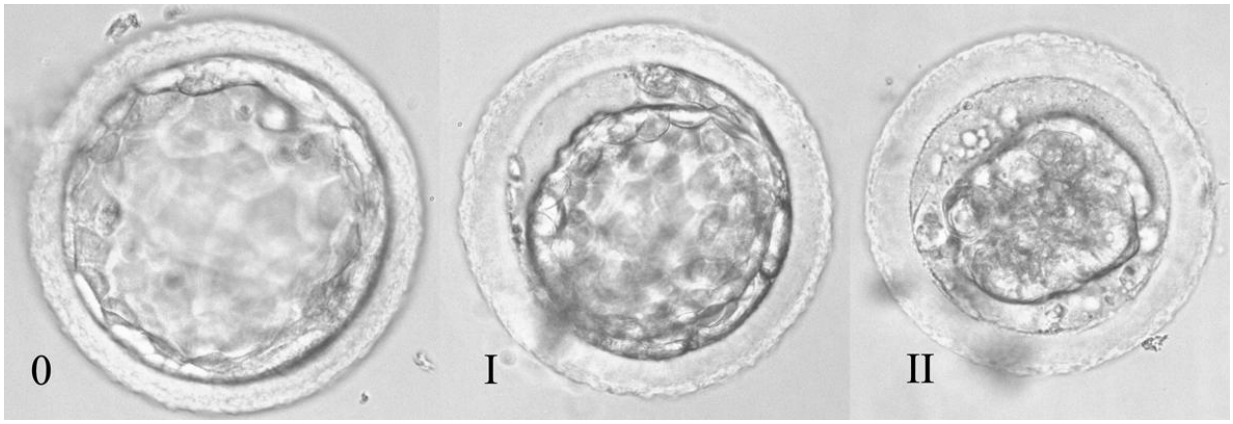

Figure 1a. Blastocyst shrinkage response to a stressful environmental factor. 0 - no visible changes in shape of the blastocyst, blastocele size unchanged, I - shrinkage of the blastocyst, blastocele size reduced, II - collapse of the blastocyst, no blastocele observed.

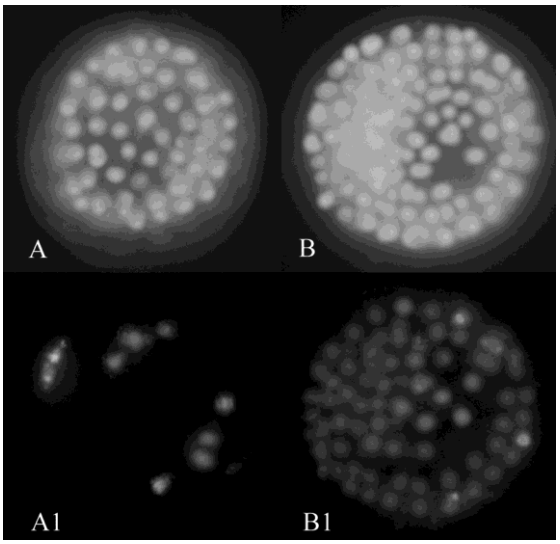

Figure 1b. DAPI nuclear staining (A, B) and TUNEL assay (A1, B1) of rabbit blastocysts from the control (A, A1) and the experimental (B, B1) group.

b. Apoptosis

DNA fragmentation of embryos was analyzed by using a combined technique for simultaneous nuclear staining and TUNEL by a modification of the procedure used by Brison and Schultz [6]. The expanded blastocysts (Day 4) with intact zonae pellucidae were fixed in 4% paraformaldehyde in PBS (Sigma) for 1 h at room temperature. Then the embryos were washed twice in PBS-PVP (1µg/ml polivinylpyrrolidone in PBS, Sigma), and permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 45 min at room temperature in a humidified box and washed again in PVP solution. Next the embryos were incubated in fluorescein-conjugated dUTP and TdT (TUNEL reagent; In situ Cell Detection kit, Roche Diagnostic, Germany) for 1 h at 38°C and 5% CO2 in the air. Positive controls were incubated in 50 U/ml DNAse (Roche Diagnostic, Germany) for 20 min at 38°C. Negative controls were incubated in fluorescein-dUTP in the absence of TdT. After the reaction, the embryos were washed tree times in PBS-PVP solution and transferred trough a gradient of Vecta-Shield with DAPI (Vector Laboratories, Burlingame, USA) at 75 and 100% (v/v) in PBS/PVP and mounted on a glass slide. Labelled nuclei were examined under a NIKON Eclipse E600 microscope fitted with epifluorescent illumination. The total number of cells per blastocyst (determined by nuclear staining with DAPI) and number of cells with DNA-fragmented nuclei were counted. The DNA fragmented nucleus index was calculated by dividing the number of cells with DNA fragmentation by the total number of cells (including DNA-fragmented nuclei) [7].

Statistical analysis

Statistical evaluation was performed using Student's t-test. Differences were considered significant at $p < 0.05$. Statistical analyses were

performed with the SPSS package (IBM, Chicago, USA).

RESULTS

All together, 96 blastocysts, at the similar development stage, were included into the study, Each of two groups contained 48 blastocysts. The blastocyst shrinkage response to ET was more frequently observed in the experimental group than in the control group. The percentage of the morphologically altered blastocysts, collapsed and shrunken, in the experimental group was 23% in 5th min and 31% in 60th min after ET. On the contrary, in the control group, there were no shrunken or collapsed blastocysts in 5th min and only 4% of the shrunken blastocysts in 60th min after transfer. All together, one hour after transfer, there were 14 shrunken and one collapsed blastocysts in the experimental group and only two shrunken blastocysts in the control group, Table 1.

Table 1. The morphological changes in the rabbit blastocyst exposed to embryo transfer.

	Experimental Group		**Control Group**	
Time after blastocyst transfer (min)	5 min	60 min	5 min	60 min
Unchanged blastocysts (No.)	37	33	48	46
Shrunken blastocysts (No.)	8	14	-	2
Collapsed blastocysts (No.)	3	1	-	-
Shrunken & collapsed blastocysts (%)	23%	31%	-	4%

A mean number of cells per blastocyst in the groups was comparable, 68.1 in the study group and 74.5 in the control group; $p>0.05$. The DNA fragmented nucleus index was 25.1 in the experimental group and was significantly higher than in the control group 5.7; $p<0,001$. The DNA fragmented nucleus index was significantly higher in the experimental group 37.7% than in the control group 8.1%; $p<0.001$. The detailed data are presented in Table 2.

Table 2. The apoptotic changes in the rabbit blastocysts exposed to embryo transfer.

	Experimental Group	**Control Group**
No. of blastocysts	48	48
Mean number of cells per blastocyst (SD)	68.1 (16.4)	74.5 (16.7)
Mean number of apoptotic nuclei (SD)	25.1 (14.5)*	5.7 (2.7)
Mean apoptotic index [%] (SD)	37.7 (21.3)*	8.1 (4.5)
Minimal apoptotic index [%]	8.3	2.1
Maximal apoptotic index [%]	88.5	16.9

*$p<0,001$

DISCUSSION

The results of the present study indicate that ET can cause both morphological and apoptotic changes in the rabbit embryos. The most plausible factor responsible for the injury of the rabbit embryo in the process of ET is the pressure fluctuation appearing during injection of transferred load. The study conducted previously by our team [8] demonstrated that the pressure in the transferring load during ET could rise up to 155 mmHg in less than 0.1 s and the recorded pressure increase slope could reach up to 72 000 mmHg/s and the pressure decrease slope up to 144 000 mmHg/s. Moreover, the pressure buildup in the transferred liquid is proportional to the speed of ejection of the transferred load. Such rapid pressure changes in the environment of the embryo can result in its injury. So far, a little attention has been placed on the impact of the local pressure fluctuations occurring during final stages of ET on embryo viability. In the literature, there is some evidence for the cell damage caused by local pressure fluctuations. Bilek et al. investigated the surface-tension-induced human lung epithelial cell damage in a model of airway reopening, consisting of a semi-infinite bubble propagating in a narrow fluid-filled channel lined with pulmonary epithelial cells. They concluded that the steep pressure gradient near the bubble front was the most likely cause of the observed cellular damage. They demonstrated that there was a higher risk of the human pulmonary epithelial cell damage in the front meniscus than in the rear meniscus. According to them, the magnitude of the pressure gradient near the air bubble tip was directly correlated to the

human epithelial cell layer damage [9]. Similarly, Wong et al., in a study of cell damage caused by bubble motion in a circular-lumen endothelialized model of a microvessel demonstrated that bubble motion could cause significant damage to the human endothelial cells. According to Wong et al. the steep pressure gradient at the bubble front would be the most likely cause of cell damage [10]. Furthermore, it is certain that positive pressure does not actually cause damage but the steep increase in pressure, followed by negative pressure, does cause damage since biological structures can only be damaged by shear or extension and not by positive pressure (compression). A generalized consideration is that the degree of cell damage will depend upon the amplitude. Thus, low amplitude pressure fluctuations will only disturb cell functions (reversibly), while higher amplitudes pressure fluctuations can destroy cells and their functions. There is probably a threshold below which no cell reaction will take place. The morphological changes and an increased number of damaged cells in the rabbit embryos exposed to ET suggest that pressure fluctuations during ET are able to reach the threshold of cell injury.

The spontaneous contractions of the rabbit blastocysts are observed in *in-vitro* conditions [11]. It is believed, that weak contractions are physiological and promote hatching, while strong contractions have the influence of inhibiting hatching [12]. According to the results of the present study, there were three collapsed blastocysts after ET. Blastocysts physiologically rarely contract so severely that their blastocoels disappear [13]. Furthermore, the increased nucleus DNA fragmentation in the blastocysts exposed to ET may suggest that observed contractions are accompanied with cell structure injury. The wide range of apoptotic indexes, from 8 to 88%, in the experimental group resulted most probably from the position of the embryo in the dispersed liquid volume and the exposition to the pressure fluctuations at the time of ET. A further developmental potential of the blastocyst after ET would depend on the number and localization of the damaged cells as well as a potential of the remaining cells to replace the injured ones.

CONCLUSIONS

In conclusion, ET can induce morphological changes and trigger apoptotic processes in the embryos. Most likely, the pressure fluctuations during ejection of the transferred load are responsible for the embryo injury. Since, the pressure build-up in the transferred liquid is proportional to the speed of ejection of the transferred load, and the degree of cell damage depends upon the pressure amplitude, it is reasonable to advice to transfer embryos with minimal possible speed to avoid the embryo pressure injury.

Conflicts of interest

The authors have declared no conflicts of interest.

REFERENCES

1. Franco JG, Martins AM, Baruffi RL, Mauri AL, Petersen CG, Felipe V, Contart P, Pontes A, Oliveira JB. Best site for embryo transfer: the upper or lower half of endometrial cavity? Hum Reprod. 2004 Aug; 19(8):1785-90.
2. Kovacs GT. Which factors are important for successful embryo transfer after in-vitro fertilization? Hum Reprod. 1999 Oct; 14(10):2679.
3. Sallam HN. Embryo transfer: factors involved in optimizing the success. Curr Opin Obstet Gynecol. 2005 Jun; 17(3):289-98.
4. Schoolcraft WB, Surrey ES, Gardner DK. Embryo transfer: techniques and variables affecting success. Fertil Steril. 2001 Nov; 76(5):863-70.
5. Gajda B. SZ. Vitrification of non-cultured and cultured rabbit embryos. Anim Reprod Sci. 1991 Jun; 26(2):151-8.
6. Brison DR, Schultz RM. Apoptosis during mouse blastocyst formation: evidence for a role for survival factors including transforming growth factor alpha. Biol Reprod. 1997 May; 56(5):1088-96.
7. Gajda B, Bryla M, Smorag Z. Effects of protein source, vitamin E and phenazine ethosulfate on developmental competence and quality of porcine embryos cultured in vitro. Folia Biol. 2008 Jan; 56(1):57-63.
8. Grygoruk C, Sieczynski P, Pietrewicz P, Mrugacz M, Gagan J, Mrugacz G. Pressure changes during embryo transfer. Fertil Steril. 2011 Feb; 95(2):538-41.
9. Bilek AM, Dee KC, Gaver DP, 3rd. Mechanisms of surface-tension-induced epithelial cell damage in a model of pulmonary airway reopening. J Appl Physiol. 2003 Feb; 94(2):770-83.
10. Wong ZZ, Fowlkes JB, Bull JL. A study of cell damage caused by bubble motion in a circular-lumen endothelialized model of a microvessel. FASEB J. 2008 Mar, 2008;22(1):1220-5.
11. Lewis WH, Gregory PW. Cinematographs of Living Developing Rabbit-Eggs. Science. 1929 Feb; 69(1782):226-9.
12. Suzuki R, Niimura S. Hatching and distribution of actin filaments in mouse blastocysts whose activities of protein kinase A were suppressed by H-89. J Reprod Dev. 2010 Feb; 56(1):103-9.
13. Niimura S. Time-lapse videomicrographic analyses of contractions in mouse blastocysts. J Reprod Dev. 2003 Dec; 49(6):413-23.