Pressure induced nucleus DNA fragmentation

Cezary Grygoruk · Piotr Sieczynski ·
Jacek A. Modlinski · Barbara Gajda · Pawel Greda ·
Izabela Grad · Piotr Pietrewicz · Grzegorz Mrugacz

Received: 27 July 2010 / Accepted: 14 December 2010 / Published online: 14 January 2011
© Springer Science+Business Media, LLC 2011

Abstract

Purpose The present study was designed to investigate the impact of pressure on nuclear DNA integrity in viable cells of mouse blastocysts.

Methods The blastocysts of hybrid F1 females [(C57Bl/10J × CBA-H); $N=15$] aged 2–3 months were exposed into the pressure impulse lasting ~0.021 s and characterized by a positive pressure peak of ~76 mmHg. The nuclear DNA fragmentation index of mouse blastocysts was assessed by TUNEL assay within 60 s after exposure to pressure impulse. **Results** The mean nuclear DNA fragmentation index was significantly higher in the experimental group (83%) than in the control group (19.7%); $p<0.001$.

Capsule A low magnitude pressure impulse can induce nucleus DNA fragmentation. Compression and decompression forces can be responsible for the observed DNA shearing.

C. Grygoruk (✉) · P. Pietrewicz · G. Mrugacz
Center for Reproductive Medicine Bocian,
Akademicka 26,
15-267 Białystok, Poland
e-mail: cezary.grygoruk@gmail.com

P. Sieczynski
Center for Reproductive Medicine Kriobank,
Stoleczna 11,
15-879 Białystok, Poland

J. A. Modlinski · P. Greda
Department of Experimental Embryology, Institute of Genetics
and Animal Breeding of the Polish Academy of Sciences,
Jastrzebiec, Postępu 1,
05-552 Wolka Kosowska, Poland

B. Gajda · I. Grad
Department of Biotechnology of Animal Reproduction, National
Research Institute of Animal Production,
Krakowska 1,
32-083 Balice/Krakow, Poland

Conclusion(s) A low magnitude pressure impulse can induce nuclear DNA fragmentation in mouse blastocysts. The compression and decompression forces appearing during pressure fluctuations are responsible for the observed DNA shearing.

Keywords Embryo cell apoptosis · Pressure impulse · Pressure induced DNA fragmentation · Pressure DNA shearing · PDS

Introduction

In the process of the in vitro fertilization, the gametes and embryos are exposed into the extracorporeal environment, where many physical factors can affect the growth and development of cells. The retrieved gametes and then embryos are usually cultured in liquid-based media. The medium surrounding the gametes and embryos exerts pressure in a direction perpendicular to the surface of the cell. Any change in pressure applied at any given point of the culture medium is transmitted very rapidly, equally and undiminished throughout the fluid toward the surface of the cell [1]. Therefore, it is reasonable to suspect that the pressure changes may influence the vital function of the cell. According the recent studies, pressure fluctuations can induce cell membrane disruption, cytoskeleton disorganization and enzymes inactivation [2–6]. There are also some evidences about the pressure induced fragmentation of DNA isolated from cells [7–9]. However, little is known on the influence of the pressure changes on the nucleus DNA integrity of the living cells. Therefore, a study was designed to examine the impact of the pressure fluctuations on the nucleus DNA integrity in the viable cells of the mouse blastocysts.

Material and methods

Collection and culture of embryos

The experiments were performed according to the rules of the Polish Government Act for Animal Care and were approved by the Local Ethics Committee for Animal Care. All animals originated from the mouse-raising facility in the Department of Experimental Embryology IGAB PAS. Mice were kept in a temperature-controlled room with a 12 h light:12 h darkness starting at 7 am. Food (Labofeed H, Kcynia, Poland; metabolic energy of 13.0 MJ/kg) and water were available *ad libitum*. Hybrid F1 (C57Bl/10 J $\times$ CBA-H) females aged 2–3 months served as donors of embryos. Females were superovulated by injection of 7.5 IU of pregnant mare serum gonadotrophin (PMSG—Folligon, Intervet) followed by 7.5 IU of human chorionic gonadotrophin (hCG—Chorulon, Intervet, Boxmeer, Holland) given 48 h apart and mated with F1 (C57Bl/10 J $\times$ CBA-H) males. Zygotes at the early pronuclear stage were used for generation of blastocysts in similar developmental stage. Donor females were killed by cervical dislocation 21 h after hCG injectin. Oviducts were excised into M2 manipulation medium (HEPES-buffered M16) [10] and zygotes, surrounded by cumulus cells, were collected from oviducts by puncturing the wall of ampulla. For removal cumulus cells zygotes were treated with hyaluronidase (150 i.u./ml; Sigma Chemical Co., St. Louis, MO, USA) and gently pipetted. Cumulus-free zygotes were rinsed twice with M2 medium, placed in drops of KSOM medium (KCl-enriched simplex optimized medium; Specialty Media, Phillipsburg, NJ, USA) [11] under paraffin oil (Sigma) in Petri dishes (Corning) and cultured at 37°C, in an atmosphere of 5% CO₂ in air until they reached blastocyst stage.

Only morphologically normal blastocysts were included into the study. Assessments of embryos were carried out under Nikon SMZ 1500 (Japan) stereomicroscope. Photographs were made using Axiovert 200 M inverted microscope (Zeiss, Germany) equipped with Orca –ER camera (Hamamatsu Photon, Japan).

Experimental groups

The mouse blastocysts were randomly divided into the experimental group A, exposed to the pressure impulse, and the control group B, not exposed to the pressure fluctuations.

Experimental setup

The pressure impulse was generated with the use of the standard catheter for embryo transfer (Labotect, Bovender-Gottingen, Germany) connected to 1 ml insulin syringe (Polfa Lublin, Poland). The reference loading of the syringe-catheter complex was arranged in the following order from the catheter tip: 0.1 μ l air, 1.5 μ l liquid (embryo culture medium), 0.2 μ l air, 1.5 μ l liquid (mouse blastocyst in embryo culture medium), 0.2 μ l air, 1.5 μ l liquid (embryo culture medium) and 25 μ l air, Fig. 1.

The mouse blastocysts from the experimental group A were loaded individually into the embryo transfer catheter. Then, a tip of a loaded catheter was positioned in the center of a well (four-well multidishes, Nunc, Denmark) filled with the M2 manipulation medium (HEPES-buffered M16) incubated at 38°C for 1 h. Then each blastocyst was injected individually into the medium. After the exposition of the embryo into the pressure impulse, the embryo was within 60 s fixed in paraformaldehyde and investigated for signs of apoptosis.

The blastocysts from the control group B were not exposed to the pressure impulse and were only fixed in paraformaldehyde and studied for signs of apoptosis.

DNA fragmentation assessment

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 [13]. The expanded blastocysts were fixed in 4% paraformaldehyde in PBS (SIGMA) for 1 h at room temperature. Then the embryos were washed twice in PBS-PVP (1 μ g/ml polyvinylpyrrolidone in PBS, SIGMA), and permeabilized with 0.1% Triton X-100 (SIGMA) in PBS for 30 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 three times in PBS-PVP solution and transferred trough a

Fig. 1 The reference loading of the syringe-catheter complex used for generation of the pressure impulse

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) [14].

Pressure measurement

The pressure changes during the mock transfers, without embryos, were registered with a Mikro-Tip® Pressure Transducer Catheter (Millar, Houston, Texas, USA) located 1 mm from the tip of a loaded syringe catheter-complex immersed in M2 manipulation medium (HEPES-buffered M16) [10]. 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 (embryo culture medium), 0.2 µl air, 1.5 µl liquid (embryo culture medium) and 25 µl air, Fig. 1. The pressure sensor was connected to the pressure control unit (PCU-2000 Pressure Control Unit with Patient Isolation Millar, Houston, Texas, USA) and data acquisition system (PowerLab 4/30, Millar, Houston, Texas, USA). The injection time of a transferred volume was calculated with the use of LabChart and Scope software (Millar, Houston, Texas, USA). The mock transfer procedures were performed by a doctor blinded to the real-time pressure recordings. The term “transferred volume” represents the overall transferred volume of liquid with air.

Statistical analysis

Statistical evaluation was performed using Student’s *t*-test and chi-square test. Differences were considered significant at $p < 0.05$. Statistical analyses were performed with the SPSS package.

Table 1 The apoptotic changes in the experimental group A and the control group B

	Group A	Group B
Number of blastocysts in groups	40	40
Mean number of cells per blastocyst (SD)	30.7 (4.3)	27.3 (6.9)
Mean number of apoptotic nuclei (SD)	25.4 (3.8)*	5 (3.6)
Mean apoptotic index (SD)	83 (8.6)*	19.7 (14)

* $p < 0.001$

Table 2 Pressure changes, time and speed of injection of transferred volume recorded during 20 mock embryo transfers

	Mean	SD
Peak pressure during transfer [mmHg]	75.6	34.1
Pressure increase slope [mmHg/s]	25 979	16 650
Pressure decrease slope [mmHg/s]	59 938	32 801
Time of injection of transferred volume [s]	0.022	0.006
Speed of injection of transferred volume [m/s]	12.1	3.9

Result

There were 40 blastocysts in each group. A mean number of cells per blastocyst in the groups was comparable between the experimental group and control group, 30.7 and 27.3 respectively; $p > 0.05$. The mean number of

Fig. 2 A paradigm recording of pressure impulse generated with the use of the insulin syringe and the catheter for embryo transfer. The registered pressure values were as follows: peak pressure 76 mmHg, pressure increase slope 27 156 mmHg/s, pressure decrease slope 51 156 mmHg, pressure wave duration 0.021 s

apoptotic nuclei was 25.4 in the experimental group and was significantly higher than in the control group, 5; $p < 0.001$. The mean nucleus DNA fragmentation index was significantly higher in the experimental group (83%) than in the control group (19.7%); $p < 0.001$, Table 1, Fig. 3. The detailed data are presented in Table 1.

A total of 20 mock embryo transfers were performed in order to evaluate the pressure changes during embryo transfer. A mean value of the recorded peak pressure was 75.6 mmHg (SD 34). The mean pressure increase slope was 25 979 mmHg/s (SD 16650) and the mean pressure decrease slope was 59938 mmHg/s (SD 32801). The mean time of injection of the transferred volume was 0.022 s (SD 0.006) and the mean speed of injection of the transferred volume was 12.1 m/s (SD 3.9). The detailed data are presented in Table 2. A paradigm recording of pressure impulse generated with the use of the insulin syringe and the catheter for embryo transfer during one of the mock embryo transfers is presented in Fig. 2.

Discussion

In the present study, a nucleus DNA fragmentation was assessed in the mouse blastocysts exposed to the pressure

impulse in *in-vitro* conditions. The obtained results indicated that the pressure fluctuations undergoing in a very short period of 0.02 s characterized by a positive pressure peak of 76 mmHg caused the nucleus DNA shearing. The mean nucleus DNA fragmentation index in the blastocysts exposed to the pressure impulse was fourfold higher than in the control group, Fig. 3.

An abrupt pressure fluctuation in the cell environment may influence many vital aspects of the cell anatomy and physiology. For example, the cell membrane of the pulmonary epithelial cells could be disrupted by the steep pressure gradient appearing during airway reopening [3, 6]. Furthermore, cytoskeletal damage *in vitro* was demonstrated after the impact of 16 MPa (120000 mmHg) on a human renal carcinoma cell line [4]. Interestingly, the spindle microtubules depolymerization and assembly depends on the hydrostatic pressure as well [15]. It was also demonstrated that the high pressure could inactivate intracellular enzymes [2, 5]. There is also some information about the impact of pressure fluctuations on DNA integrity [7, 9, 16]. Kochanski et al. demonstrated that shock waves, generated with an experimental lithotripter could cause a severe double strand DNA fragmentation in the high weight DNA isolated from the white blood cells [9]. The experimental lithotripter used in their experiment produced

Fig. 3 DAPI nuclear staining (blue) and TUNEL assay (green) of mouse blastocysts from the control group (A, A1) with nucleus DNA fragmentation index of 18.5% and the experimental group (B, B1) group with nucleus DNA fragmentation index of 74.5%

the shock waves characterized by the positive pressure peak of 30 MPa (225 018 mmHg) and negative one of 9 MPa (67 505 mmHg). According to Kochanski et al. the possible mechanisms for the DNA damage during shock waves exposure depended mainly on the presence of cavitation effect and generation of sonochemicals such as oxygen superoxide. Whether this mechanism could play any role in the nucleus DNA fragmentation observed in the present study is rather doubtful. In our experiment, the pressure fluctuations were not sufficient to create neither shock waves or cavitation effect as well as sonochemical. The other mechanism which might be responsible for the DNA damage is an activation of the apoptotic cascade enzymes. However, taking under consideration that the blastocysts were fixed in less than 60 s after the exposure to the pressure impulse, it is reasonable to assume that the time was insufficient for a complete activation of the apoptotic cascade. The most possible mechanism which could be responsible for the nucleus DNA fragmentation is a mechanical disruption of the double strand DNA by the compression and decompression forces evoked by the pressure impulse, Fig. 2. It is certain that positive pressure does not actually cause damage to the cells but the steep increase in pressure (compression), followed by negative pressure (decompression), does cause damage since biological structures can only be damaged by shear or extension and not by positive pressure [17]. The pressure impulse applied in our study was characterized by a steep increase in pressure (compression) ~26 000 mmHg/s followed by a rapid decrease in pressure (decompression) ~60 000 mmHg/s, Fig. 2. Such abrupt pressure fluctuation undergoing in very short period, 0.02 s, might not leave enough time for the DNA molecular structure to adapt and could cause a disruption of DNA strands. The mechanical DNA shearing induced by pressure was described by Schrieffer et al. as a useful alternative to sonic and enzymatic DNA fragmentation methods [8]. In Schrieffer et al. study, the isolated DNA samples were passed through a small French pressure chamber at a variety of low to intermediate pressures (from 5 000 to 50 000 mmHg). In the present study, much lower pressure changes, ~76 mmHg, were able to cause pressure DNA shearing (PDS) inside the nucleus of a living cells. According to our recent studies, the pressure fluctuations of comparable magnitude could also appear during embryo transfer procedure (ET) and cause both morphological and apoptotic changes in the embryos exposed to ET [12, 18]. Taking under consideration the entire process of extracorporeal fertilization, the significant pressure fluctuations could also emerge during oocyte pickup, oocyte decoronation and embryo pipetting. Any of these procedures, if performed too vigorously, could evoke pressure fluctuations able to reach PDS threshold. The appropriate embryo growth is strictly

dependent on the DNA, which constitute the main source of genetic instruction for synthesis of RNA and proteins. The damage of DNA structure can result in either delay or arrest of the embryo development depending on the damage extent and the embryo's ability to repair the damaged DNA. Therefore, it could be advised to handle the gametes and embryos with minimal exposition to pressure fluctuation throughout the whole process of in-vitro fertilization. For example, in the case of ET the pressure build up in the transferred liquid is proportional to the speed of ejection of the transferred load. For that reason, it is reasonable to suggest transferring the embryos at the lowest possible ejection speed [12, 18].

In conclusion, the nucleus DNA fragmentation in a viable cell can be triggered by a relatively low pressure fluctuation. The compression and decompression forces appearing during pressure fluctuations are most probably responsible for the observed DNA shearing. According to our best knowledge, it is a first report concerning influence of the pressure changes on the nucleus DNA integrity of the living cells.

References

1. Bloomfield L. How things work: the physics of everyday life. Wiley, 2006, pp. 153.
2. Dreyfus G, Guimaraes-Motta H, Silva JL. Effect of hydrostatic pressure on the mitochondrial ATP synthase. *Biochemistry*. 1988;27:6704–10.
3. Kay SS, Bilek AM, Dee KC, Gaver 3rd DP. Pressure gradient, not exposure duration, determines the extent of epithelial cell damage in a model of pulmonary airway reopening. *J Appl Physiol*. 2004;97:269–76.
4. Moosavi-Nejad SF, Hosseini SH, Satoh M, Takayama K. Shock wave induced cytoskeletal and morphological deformations in a human renal carcinoma cell line. *Cancer Sci*. 2006;97:296–304.
5. Paladini Jr AA, Weber G. Pressure-induced reversible dissociation of enolase. *Biochemistry*. 1981;20:2587–93.
6. Bilek AM, Dee KC, Gaver 3rd DP. Mechanisms of surface-tension-induced epithelial cell damage in a model of pulmonary airway reopening. *J Appl Physiol*. 2003;94:770–83.
7. Miller DL, Thomas RM, Buschbom RL. Comet assay reveals DNA strand breaks induced by ultrasonic cavitation in vitro. *Ultrasound Med Biol*. 1995;21:841–8.
8. Schrieffer LA, Gebauer BK, Qui LQ, Waterston RH, Wilson RK. Low pressure DNA shearing: a method for random DNA sequence analysis. *Nucleic Acids Res*. 1990;18:7455–6.
9. Kochanski AM, Mejnartowicz JP, Latos-Bielenska A, Etienne J, Filipczynski L. DNA damage induced by lithotripter generated shock waves: short report. *Int Urol Nephrol*. 2001;32:419–22.
10. Fulton BP, Whittingham DG. Activation of mammalian oocytes by intracellular injection of calcium. *Nature*. 1978;273:149–51.
11. Lawitts JA, Biggers JD. Culture of preimplantation embryos. *Meth Enzymol*. 1993;225:153–64.
12. Grygoruk C, Sieczynski P, Pietrewicz P, Mrugacz M, Gagan J, Mrugacz G. Pressure changes during embryo transfer. *Fertil Steril* 2010; in press.
13. Brison DR, Schultz RM. Apoptosis during mouse blastocyst formation: evidence for a role for survival factors including

- transforming growth factor alpha. *Biol Reprod.* 1997;56:1088–96.
14. 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 Krakow.* 2008;56:57–63.
  15. Salmon ED. Pressure-induced depolymerization of spindle microtubules. I. Changes in birefringence and spindle length. *J Cell Biol.* 1975;65:603–14.
  16. Endl E, Steinbach P, Hofstadter F. Flow cytometric analysis of cell suspensions exposed to shock waves in the presence of the radical sensitive dye hydroethidine. *Ultrasound Med Biol.* 1995;21:569–77.
  17. Sellier K, Kneubuehl BP. *Wound Ballistics and the Scientific Background*: Elsevier Health Sciences, 1994.
  18. Grygoruk C, Sieczynski P, Modlinski JA, Gajda B, Greda P, Grad I, Pietrewicz P, Mrugacz G. Influence of embryo transfer on blastocyst viability. *Fertil Steril* 2010; in press.