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THE IMPACT OF HIGH HYDROSTATIC PRESSURE (40 MPa AND 60 MPa) ON THE APOPTOSIS RATES AND FUNCTIONAL ACTIVITY OF CRYOPRESERVED PORCINE MESENCHYMAL STEM CELLS*

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Abstract

The aim of the present study was to examine the influence of two varied high hydrostatic pressure (HHP) values on the apoptosis (assessing caspase-8, survivin, CAD, Bax, $Bclx_L$ and $Bclx_S$) and functional activity (using cocultures with bovine embryos) of porcine mesenchymal stem cells (pBMSCs). pBMSCs were isolated from porcine bone marrow and cultured *in vitro*. Before cryopreservation and storage in liquid nitrogen, pBMSCs were subjected to HHP values of 40 MPa and 60 MPa for 1 h at 24°C. After thawing, the cells were analysed for caspase-8 activity and protein expression of survivin, CAD, Bax, $Bclx_L$ and $Bclx_S$. To indirectly test the influence of HHP on the functional activity of pBMSCs, *in vitro* maturated bovine oocytes were fertilized *in vitro*, and the obtained embryos were cultured under 4 different conditions: 1. monoculture in SOF medium; 2. coculture with pBMSCs in SOF medium; 3. coculture with pBMSCs subjected to 40 MPa HHP in SOF medium and 4. coculture with pBMSCs subjected to 60 MPa HHP in SOF medium. The quality of the developed blastocysts was analysed by TUNEL assay. HHP did not induce apoptosis in pBMSCs, as no significant difference was noted in the expression of any of the analysed apoptosis-related proteins between pBMSCs subjected to HHP (40 MPa or 60 MPa) and control. The highest number of obtained blastocysts was observed when the embryos were cultured in SOF. A highly significant difference ($P<0.005$) was noted between embryos cultured in SOF and embryos cultured in the presence of pBMSCs subjected to 60 MPa HHP or untreated pBMSCs. A significant difference ($P<0.05$) was noted between embryos cultured in SOF and embryos cultured in the presence of pBMSCs subjected to 40 MPa HHP. In conclusion, HHP does not induce apoptosis in pBMSCs. The obtained results of the blastocysts cocultured *in vitro* with pBMSCs (HHP-treated and untreated cells) imply that coculture with pBMSCs has a negative impact on the developmental rates of blastocysts.

Key words: pBMSCs, HHP, cryopreservation, apoptosis, IVF

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The mechanism of improving the efficiency of cryopreservation by subjecting cells to high hydrostatic pressure (HHP) is becoming better understood. The responses of cells to different types of stresses can be similar, providing a possibility for 'cross-protection'. HHP, by stimulating the defensive reaction of cells to stress, increases their resistance to other adverse factors. HHP treatment causes small transcriptional changes in genes involved in cell death, survival, RNA processing, cell cycle and cell proliferation (Jiang et al., 2016). Molecular chaperones induced by HHP can provide cellular protection and maintain homeostasis, and they can even be applied in cryoprotective therapy (Pribenszky et al., 2011). The HHP method has been of interest to researchers over the past few years. This has led to numerous discoveries in both basic and applied research involving the production of food and medicine (Demazeau and Rivalain, 2011). The HHP method has also been used in assisted reproduction techniques to improve post-cryopreservation survival of *in vitro* cultured cells and to aid in somatic cell nuclear transfer or insemination (Pribenszky et al., 2010). HHP has been chosen in this study as a stressor because of its unique features such as instant and uniform action at every point of the cell and lack of gradient effect; HHP can be applied with high precision, consistency, reliability and safety, and it acts with an extremely high safety margin. Moreover, it has a wide therapeutic range and no or minimal cell to cell variation (Pribenszky et al., 2010).

We decided to examine the influence of two varied high hydrostatic pressure values on apoptosis and functional activity in an experimental model of porcine mesenchymal stem cells (pBMSCs). pBMSCs are non-haematopoietic stem cells that are able to regenerate tissues due to their self-renewal and differentiation mechanisms, and they can support cell survival and angiogenesis and even limit inflammation (Mao et al., 2017). These types of cells have been used in regenerative therapy, such as in bone and cartilage regeneration, as well as in the treatment of disorders such as multiple sclerosis and Crohn's disease (Rohban and Pieber, 2017). Furthermore, the ability of pBMSCs to not induce immune responses is conducive to their use for transplantation purposes, including both autotransplantation and allotransplantation. Similar anatomical and physiological parameters as humans have made pigs model animals for the study of obesity, food intake (Stachowiak et al., 2016) and cirrhosis for liver regeneration (Lee et al., 2017). Moreover, pigs are the most suitable species for transplantation using an animal-to-human model. Double-transgenic pigs expressing α1,2-fucosyltransferase and α-galactosidase have been used for xenotransplantation studies (Zeyland et al., 2014).

pBMSCs can be isolated from the bone marrow, which allows large amounts of material to be obtained from one animal. However, this creates a need for storage of unused cells. It has been shown that as a result of cryopreservation, the number of stem cells after thawing can decrease by as much as one-fifth of the initial population (Bourin et al., 2008). The low cryotolerance is caused by mechanic damages during the freezing and thawing procedure. In addition, cryopreservation also induces cell apoptosis (Gala et al., 2009). There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The extrinsic apoptotic pathway is activated by death receptors on the cell surface that bind ligands. Receptor-ligand interaction leads to the formation of the DISC (death-induc-

ing signalling complex) and activation of the initiator caspase-8 (Hay-Koren et al., 2017). The intrinsic pathway of apoptosis is caused by an imbalance between levels of pro- and anti-apoptotic proteins representing the BCL2 family (e.g., Bax, $Bclx_L$ and $Bclx_S$) The level of expression of pro- and anti-apoptotic proteins and caspases has already been used in the qualitative assessment of pBMSCs (Son et al., 2013). Other important proteins involved in the regulation of apoptosis are the inhibitors of apoptosis (IAPs). These proteins are thought to inhibit apoptosis in two ways: they bind to procaspases to prevent their activation, and they bind to caspases to inhibit their activity. Survivin is a member of the IAP family, which blocks the activation of caspase-3 and caspase-9 and inhibits the mitochondrial- and AIF (apoptosis inducing factor)-dependent apoptotic pathways. Because survivin is more strongly expressed in good quality rather than in poor quality COCs (cumulus-oocyte complexes), it is suggested that survivin may be a good marker for embryo quality (Jeon et al., 2008). The intrinsic pathway is initiated by intracellular stimuli. This leads to dimerization of the pro-apoptotic proteins Bax and Bak and permeabilization of the outer mitochondrial membrane. The intrinsic and extrinsic pathways of apoptosis have the same execution pathway. The execution pathway is initiated by the cleavage of caspase-3 and results in fragmentation of DNA, degradation of cytoskeletal and nuclear proteins and formation of apoptotic bodies, leading to the uptake by phagocytic cells (Elmore, 2007). One of the major nucleases responsible for internucleosomal DNA fragmentation is called caspase-activated DNase (CAD) (Liu et al., 1997). Considering the complex nature of the apoptotic process, we selected proteins that are activated in different points of apoptosis and engaged in different pathways to determine the impact of HHP on cell quality. Moreover, the real impact of a procedure can be only estimated when functional studies are performed. As the proportion of bovine blastocysts obtained *in vitro* does not exceed 30–40% (Kątska et al., 1995), scientists are seeking new embryo culture methods and new kinds of *in vitro* culture systems to increase the efficiency of embryo development. Embryos cocultured with various cells such as granulosa cells (Maeda et al., 1996), oviductal epithelial cells (Rief et al., 2002), BRL cells (buffalo rat liver cells) (Duszewska et al., 2000; Zhang et al., 1994) and VERO cells (kidney cells extracted from the African green monkey) (Duszewska et al., 2000; Kątska-Książkiewicz et al., 2007) have been successfully used so far and significantly increased the proportion of developed blastocysts. In our study, we proposed the use of pBMSCs to coculture bovine embryos due to their multipotential character and ability to produce haematopoietic and nonhaematopoietic growth factors, as well as to secrete interleukins and chemokines (reviewed in Opiela and Samiec, 2013). All the mentioned factors are beneficial for the development of totipotent embryos.

Material and methods

Animal welfare

Two-month-old male and female Polish Large White (PLW) pigs weighing 15-20 kg each were maintained under conventional conditions in a pigsty at the Depart-

ment of Biotechnology of Reproduction and Cryopreservation, National Research Institute of Animal Production in Balice, Poland. The protocol was approved by the Local Animal Care Ethics Committee No. II in Kraków. The reagents used in the present study were purchased from Sigma-Aldrich (Poznań, Poland), unless otherwise indicated.

Isolation and *in vitro* culture of pig mesenchymal stem cells (pBMSCs)

The isolation of MSCs from porcine bone marrow was carried out as described by Samiec et al. (2015). Cells were resuspended in DMEM containing 10% foetal bovine serum (FBS) and 1% antibiotics. The cells were seeded in T-75 cm^2 flasks (T75 flask). After 24 h of incubation, nonadherent cells were discarded, and fresh medium was added to the cultures. The cells were trypsinized at confluency using trypsin/EDTA and passaged. Afterwards, the cells were seeded at a concentration of 0.25 to 0.5 × 10^6 in T75 flasks.

Treatment of cells with HHP

pBMSCs were trypsinized and resuspended in HEPES-buffered TCM-199 supplemented with 2% FBS. Then, pBMSCs were transferred into 0.5 ml plastic straws with 2 ml syringes. The straws were placed inside the pressure chamber (Cryo-Innovation Inc., Budapest, Hungary) filled with distilled water. The cells were subjected to 40 MPa and 60 MPa pressure for 60 min at room temperature. After treatment, the pBMSCs were released from straws and incubated for 30 min at 39°C in DMEM.

Cryopreservation and thawing of pBMSCs

The cells were trypsinized and washed twice with HEPES supplemented with 10% FBS by centrifugation at 500×g for 5 min. Then, the pBMSCs were mixed with freezing medium (90% FBS/10% DMSO) and transferred to cryovials (Thermo Scientific, Roskilde, Denmark). The cryovials were stored at -20°C for 2 h. Then, the cryovials were plunged into liquid nitrogen (LN_2) and stored there.

Detection of caspase-8 activity

Caspase-8 activity was detected by Image–iT LIVE Green Caspase-8 Detection Kit, for microscopy (Life Technologies, Warszawa, Poland) according to the manufacturer's instructions. This methodology is based on the use of a non-cytotoxic and cell permeable fluorochrome inhibitor of caspases (FLICA) that binds covalently to active caspases and emits green fluorescence. Cells with activated caspase-8 were observed using a fluorescence microscope (FAM-FLICA emits at 520 nm). Nuclear counterstaining was performed by incubation with 1 µM Hoechst 33342 (emits at 461 nm). For induction of apoptosis, cells were incubated in the presence of staurosporine at a concentration of 1M for 2 h (Wang et al., 2013). Data is the mean from three independent experiments.

Immunofluorescence staining

Fluorescence staining of treated and untreated cells was carried out on glass coverslips after fixing (1% paraformaldehyde in 4°C) and permeabilizing (0.2% Triton

X-100 in PBS + 0.1% Tween for 15 min) the cells. The pBMSCs were blocked in 3% bovine serum for 30 min and incubated overnight at 4°C with primary antibodies against the following: survivin (sc-10811; rabbit polyclonal, Santa Cruz Biotechnology, Dallas, USA) and CAD (sc-8342; rabbit polyclonal, Santa Cruz Biotechnology). The secondary goat anti-rabbit antibody was conjugated to fluorescein (sc-2012, Santa Cruz Biotechnology). DAPI was subsequently added for nuclear staining. Microscopic analysis was performed using a fluorescence microscope. For induction of apoptosis, cells were incubated in the presence of staurosporine at a concentration of 1M for 2 h (Wang et al., 2013). All counts were performed in triplicate.

Western blotting analysis

Cells were collected in minimal volumes of PBS (without Mg^{2+} and Ca^{2+}), snap frozen in LN_2 and stored at –80°C until use. Total proteins of the cells were prepared using tissue protein extraction reagent T-PER and protease inhibitor cocktail (Calbiochem, German). After 5 min of incubation, the samples were sonicated for 10 sec using an ultrasonic cell disrupter (TORBEO, 36810; Cole-Parmer, Vernon Hills, USA) and denatured. The protein lysates were separated in discontinuous SDS gel system consisting of a 4% polyacrylamide stacking and a 12% separating component. After electrophoresis, the proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride membranes (Bio-Rad, Warszawa, Poland) in 10 mM 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) containing 10% methanol using a semidry electroblotting apparatus (Bio-Rad, Warszawa, Poland) at 50 V for 50 min.

The membranes were blocked with 0.2% (w/v) skimmed milk in TBS-T (25 mM Tris, 0.5 M NaCl, pH 7.5 + 0.1% Tween 20). Blocking, washing and incubating were done at room temperature in the Snap i.d system (Millipore, Warszawa, Poland). The membranes were incubated with primary antibodies against Bax (sc-493, rabbit polyclonal, Santa Cruz Biotechnology, Inc, USA) or $Bclx_{L/S}$ (sc-634, rabbit polyclonal, Santa Cruz Biotechnology, Inc, USA) at a concentration of 14 µg/ml for 10 min. Then, the membranes were re-incubated for 10 min with secondary goat anti-rabbit antibody (sc-2004, Santa Cruz Biotechnology, Inc, USA) conjugated to horseradish peroxidase (HRP) at a concentration of 0.15 µg/ml. The proteins were visualized using a chemiluminescence kit (Pierce Biotechnology, Inc. Rockford, IL, USA) on X-ray films according to the manufacturer's instructions. For stripping off the bound antibody, the membranes were washed in stripping buffer (100 mM 2-mercaptoethanol, 20% SDS, 62.5 mM Tris–HCl, pH 6.7) at 56°C for 30 min.

After stripping, the membranes were washed six times in TBS-T and blocked. Then, they were incubated with primary antibodies against actin (sc-1615 goat polyclonal, Santa Cruz Biotechnology, Inc, USA) at a concentration of 0.4 µg/ml for 10 min. Secondary donkey anti-goat antibody (sc-2020, Santa Cruz Biotechnology, Inc, USA) at a concentration of 0.1 µg/ml was added for 10 min. Actin was visualized by chemiluminescence.

Optical density (OD) of the bands was measured using Fusion FX (Vilber Lourmat, Marne La Vallée, France). The ratio of OD of Bax, $Bclx_L$ or $Bclx_S$ to that of actin is presented in the form of bar charts. Data is the mean from three independent experiments.

Collection of oocyte-granulosa cell complexes

Bovine ovaries were obtained from a local abattoir and transported to the laboratory. After 3 washes in physiological saline (PBS) containing 0.5 ml of kanamycin solution, the bovine oocytes were collected by aspiration of follicular fluid of the ovarian follicles. In holding medium (TCM 199 Earle's salt with 25 mM of HEPES containing 10% foetal calf serum), oocytes with intact cumulus cells and evenly granulated cytoplasm (COCs) were selected.

Isolation and culture of granulosa cells (GCs)

After washing, ovaries were transferred into PBS supplemented with 0.5 ml of kanamycin solution. Using a pair of scissors and forceps, follicles of 4- to 6-mm diameter were dissected from the surrounding connective tissues. Individual follicles were completely trimmed from the remaining connective tissues. Nonatretic follicles were selected on the basis of morphological criteria including translucency, lack of free particles, and presence of blood vessels. Then, the follicles were transferred into holding medium, and GCs were released by manually puncturing the ovaries with precision tweezers. The obtained granulosa cells were suspended in 2 ml of *in vitro* maturation (IVM) medium at concentrations of 3 to 5 × 10^6 granulosa cells/ml.

***In vitro* maturation**

For the culture of COCs, IVM medium was prepared. The commercial medium M-199 buffered with sodium bicarbonate and supplemented with L-glutamine, 20% FBS, 0.15 IU/1 ml of FSH and 10 μg/1 ml of β-estradiol was used. The selected COCs were cultured for 22 h in 2 ml of medium containing the prepared granulosa cells.

***In vitro* fertilization**

For *in vitro* fertilization, straws with frozen semen were thawed in a water bath at 37°C. Motile sperm were selected by centrifugation (300 g, 30 min) using a two-step gradient with 1 ml of 90% and 1 ml of 45% Percoll (Pharmacia, Sweden). After washing in 1 ml fertilization medium containing 10 μg/ml of heparin and a mixture of penicillamine (20 μM), hypotaurine (10 μM), and epinephrine (1 μM), the spermatozoa were suspended at a concentration of 1 to 2 × 10^6 spermatozoa/ml of fertilization medium. After maturation culture, oocytes were cocultured with spermatozoa in 40 μl droplets (10 oocytes/droplet) of fertilization medium covered with mineral oil in a humidified atmosphere at 39°C with 5% CO_2 for 20–24 h.

***In vitro* embryo culture and pBMSCs preparation**

Briefly, after co-incubation with spermatozoa, presumptive zygotes were freed from cumulus cells. The cumulus-free zygotes were cultured in 50 μl droplets of synthetic oviductal fluid media (SOF) supplemented with 0.8 mg/ml BSA covered with mineral oil in a humidified atmosphere at 39°C with 5% CO_2, 5% O_2, and 90% N_2 for 24 h. The number of cleaved embryos was recorded on day 2 following fertilization, and embryos were cultured under 4 different conditions: 1. monoculture

in SOF medium (control); 2. coculture with pBMSCs (SOF/MSC) in SOF medium; 3. coculture with pBMSCs subjected to 40 MPa HHP in SOF medium (SOF/MSC/HHP40) and 4. coculture with pBMSCs subjected to 60 MPa HHP in SOF medium (SOF/MSC/HHP60). SOF medium was supplemented with 10% FBS and 0.8 mg/ml BSA. The cultures were covered with mineral oil in a humidified atmosphere at 39°C with 5% CO_2, 5% O_2, and 90% N_2 for 7-9 days. The medium in the culture drops was partially changed at intervals of 48 h. At the end of the culture period, total blastocyst formation rate and hatching rate were recorded. Data is the mean from three (for SOF, SOF/MSC and SOF/MSC/HHP60) and four (for SOF/MSC/HHP40) independent experiments.

pBMSCs were seeded at a concentration of 1×10^2 cells in 100 µl of medium per drop. The cultures were incubated in SOF medium supplemented with 10% FBS and 0.8 mg/ml BSA at 39°C in a humidified incubator with 5% CO_2, 5% O_2, and 90% N_2. On day 2, monolayers were rinsed with fresh medium and allowed to equilibrate for at least one hour in the incubator before initiating coculture. Cells were not older than passage 3.

Terminal deoxynucleotidyl transferase dUTP nick-end labelling

Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) was performed according to the protocol described in Warzych et al. (2007) using a Dead End Fluorometric TUNEL System (Promega, Poland). Embryos were fixed in 4% paraformaldehyde (PFA) for 1 h at room temperature; then, they were transferred in 1% PFA at 4°C. On the day of experiment, fixed embryos were washed three times with PVP/PBS before being permeabilized with 0.2% Triton X-100 solution for 5 min at room temperature. After three washes, the embryos were incubated in a reaction mixture consisting of equilibration buffer for 10 min, followed by incubation in the dark for 1 h at 39°C in a buffer consisting of equilibration buffer and a nucleotide mix containing fluorescein-dUTP and terminal deoxynucleotidyl transferase. The final incubation was in a solution of 2× SSC for 15 mins. TUNEL-stained embryos were then washed three times in PBS/PVP for at least 5 min. Finally, the blastocysts were placed in a drop of Vectashield + DAPI solution and mounted onto glass slides, and their nuclear configuration was analysed. Embryos were analysed on a Nikon Eclipse E600 fluorescence microscope. The TUNEL index was determined by calculating the percentage of TUNEL-positive to DAPI-labelled cells.

Statistical analysis

Significant differences in proteins levels and TUNEL results regarding blastocyst nuclei, apoptotic nuclei, and DCI were calculated. The results were assessed by Tukey's post-hoc one-way ANOVA. Differences in blastocyst rates were assessed using chi-square test (x^2). In all tests, differences with a probability value of 0.05 or less were considered significant.

Results

Effect of HHP treatment on apoptosis in pBMSCs

Apoptosis in cells was analysed by the expression of pro-apoptosis and anti-apoptosis proteins.

Effect of HHP treatment on caspase-8 activity

Induction of apoptosis by the intrinsic or extrinsic pathway is precisely mediated by caspase cascade events. The expression of caspase-8 was similar among the groups (Figure 1).

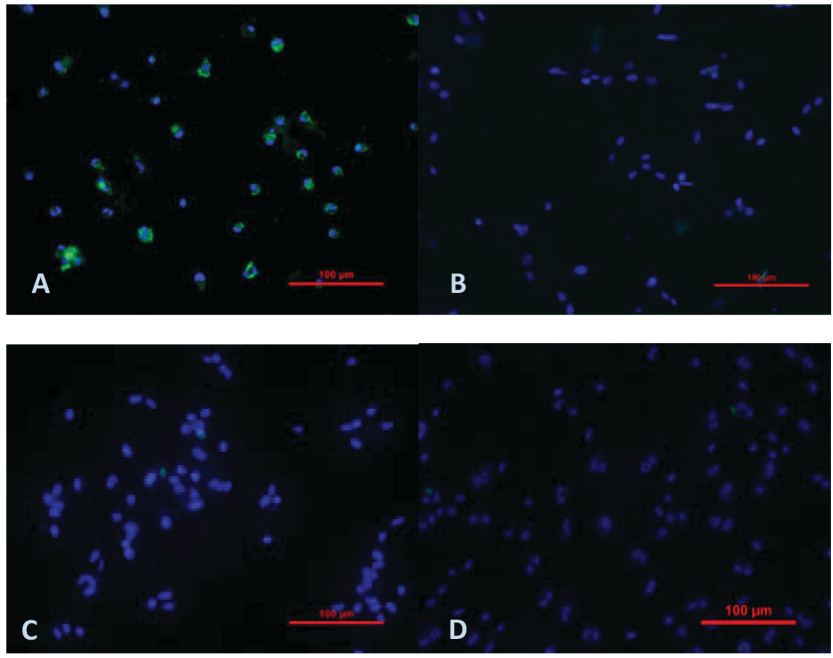

Figure 1. Caspase-8 activity measured in pBMSCs after HHP and cryopreservation. A – positive control (×200); B – pBMSCs subjected to 40MPa HHP (×200); C – pBMSCs subjected to 60MPa HHP (×200); D – control pBMSCs (×200)

Effect of HHP treatment on survivin and CAD proteins

The analysed genes of pBMSCs including survivin and CAD were detected by immunofluorescence. The immunofluorescence staining results showed no significant difference in survivin and CAD protein expression between pBMSCs subjected to HHP (40 MPa or 60 MPa) and control (Figure 2).

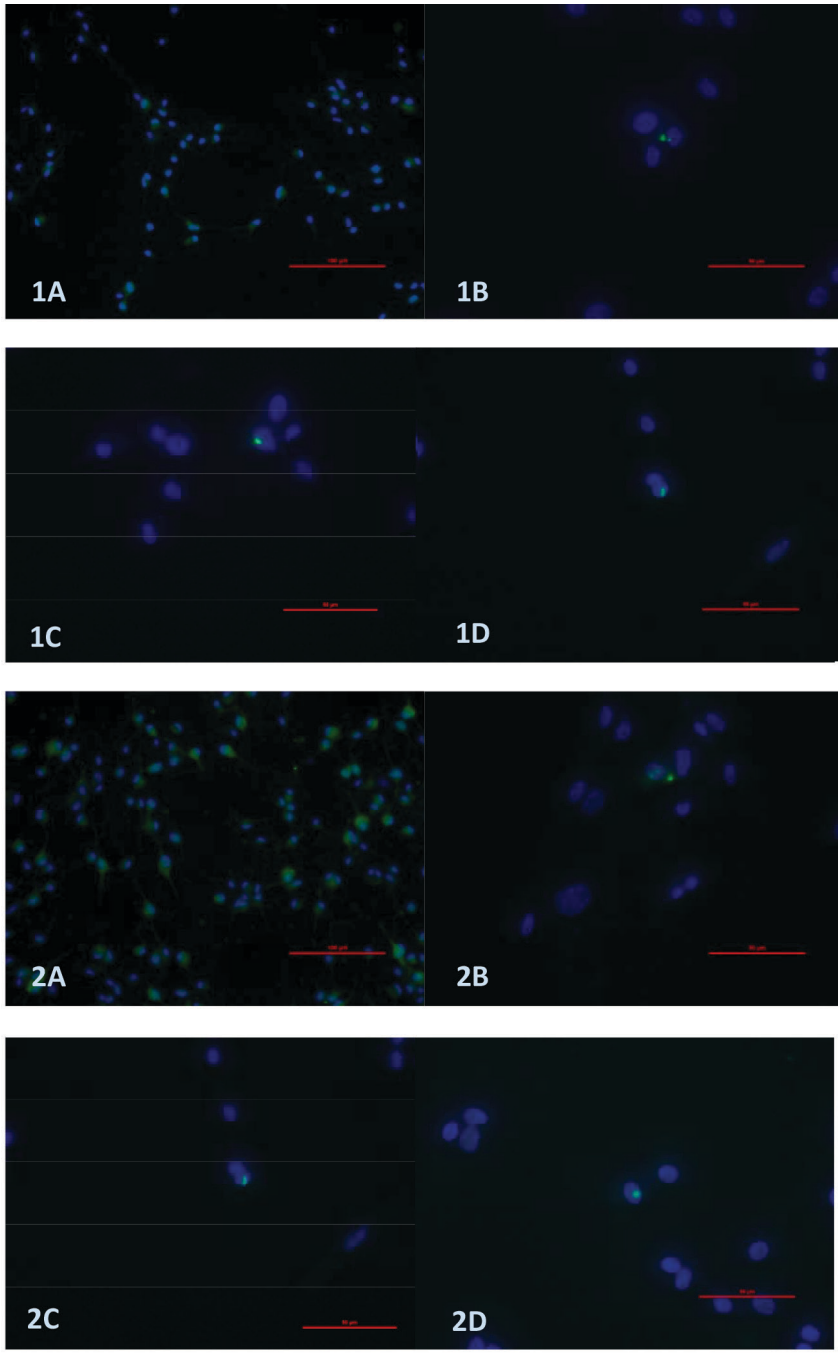

Figure 2. Survivin (1) and CAD (2) proteins expression measured in pBMSCs after HHP and cryopreservation. A – positive control (×100); B – pBMSCs subjected to 40MPa HHP (×400); C – pBMSCs subjected to 60MPa HHP (×400); D – control pBMSCs (×400)

Effect of HHP treatment on Bax, $Bclx_L$ and $Bclx_S$ proteins

Bax, $Bclx_L$ and $Bclx_S$ proteins were expressed in all the analysed groups. Western blotting analysis showed no significant difference in the Bax, $Bclx_L$ and $Bclx_S$ protein expression between pBMSCs subjected to HHP and control. The product of Bax gene is a band of size 23 kDa, that of $Bclx_L$ gene is a band of size 30 kDa, while that of $Bclx_S$ gene is a band of size 18 kDa. The product of actin gene is a band of size 46 kDa. Specific protein bands for Bax, $Bclx_L$ or $Bclx_S$ and actin were determined by applying a marker visible on the X-ray film using appropriate secondary antibodies (Figure 3).

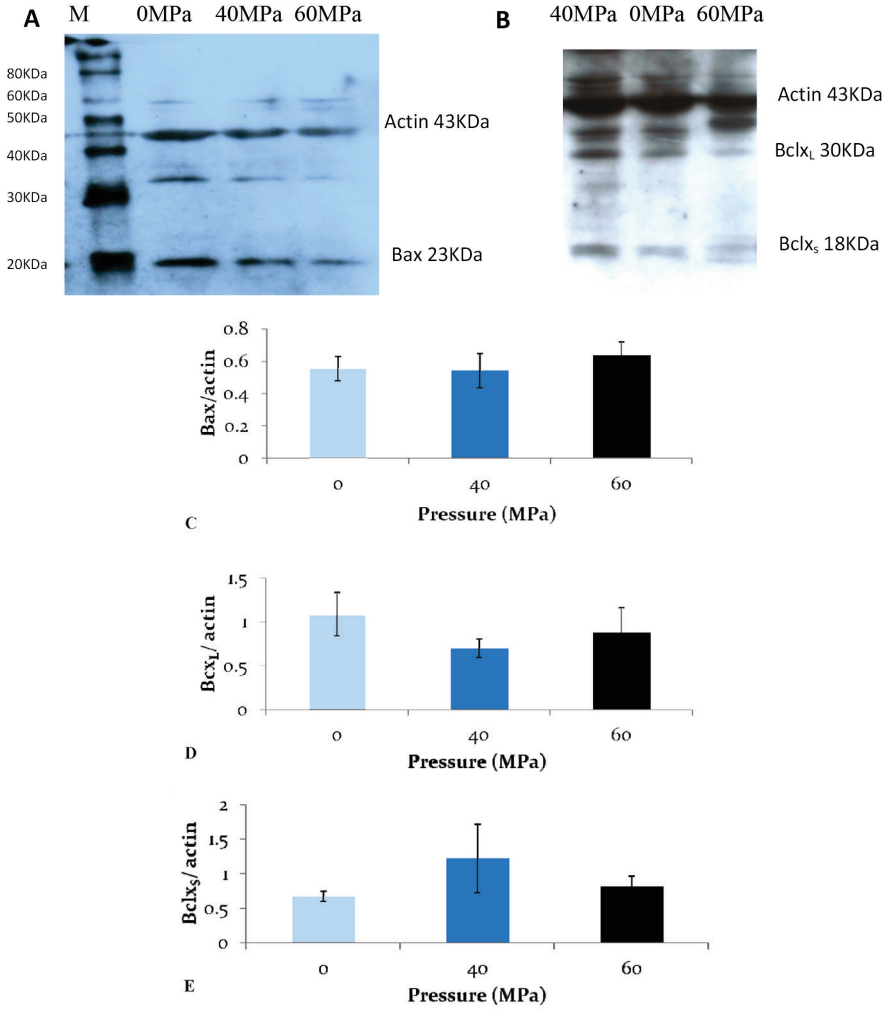

Figure 3. The picture of Bax (A), $Bclx_L$ and $Bclx_S$(B) proteins bands on X-ray film after western blotting; and analysis of Bax (C), $Bclx_L$ (D) and $Bclx_S$ (E) proteins expression in pBMSCs subjected to 0 MPa (control), 40 MPa, 60MPa HHP before cryopreservation. M – marker

The effect of HHP treatment on developmental competence of bovine oocytes

A total of 479 immature oocytes with a compact cumulus were collected. The maturation rate was 91%, and the embryo cleavage rate was nearly 80% (Table 1). For the coculture systems, 89 (control), 85 (SOF/MSC), 90 (SOF/MSC/HHP40) and 82 (SOF/MSC/HHP60) cleaved oocytes were used. The highest number of obtained blastocysts was observed when the embryos were cultured under control conditions in relation to embryos cocultured with pBMSCs subjected to 60 MPa HHP and embryos cocultured with untreated pBMSCs ($P<0.005$). Additionally, a significant difference ($P<0.05$) was noted between embryos in the control group and embryos cultured in the presence of pBMSCs subjected to 40 MPa HHP (Table 2).

Table 1. *In vitro* development of bovine oocytes

Total n of oocytes	N (%) of oocytes for IVF	N (%) of cleaved oocytes
479	435 (90.81%)	346 (79.5%)

n: number.

Table 2. *In vitro* development of bovine embryos cultured in 4 different systems

Culture system	N (%) of cleaved oocytes	N (%) of blastocysts (Mean±S.D.)
SOF	89	22 (24.7) (9±2.3) Aa
SOF/MSC	85	12 (14.1) (4±2.6) B
SOF/MSC/HHP40	90	8 (8.7) (2±1.4) b
SOF/MSC/HHP60	82	4 (4.9) (1.33±0.57) B

A, B – $P<0.005$; a, b – $P<0.05$ (test x^2).

n: number.

Data is the mean from three (for SOF, SOF/MSC and SOF/MSC/HHP60) and four (for SOF/MSC/HHP40) independent experiments.

The effect of HHP treatment on blastocyst quality measured by TUNEL assay

No significant difference was noted in the mean number of apoptotic nuclei per blastocyst and in the mean number of nuclei per blastocyst in any of the analysed group of blastocysts (Figure 4). Additionally, the SOF/MSC/HHP40 and SOF/MSC/HHP60 blastocysts had lower DCI compared to the control and SOF/MSC blastocysts ($P<0.05$) (Table 3).

Table 3. The effect of different culture systems on blastocyst quality measured by DNA fragmentation of blastomeres

Culture system	n blastocysts	Mean n of nuclei per blastocyst ± S.D.	Mean n of apoptotic nuclei per blastocyst ± S.D.	Mean DCI per blastocyst ± S.D.
SOF	15	78.8±51.15	3.27±3.17	3.74±2.77 b
SOF/MSC	12	79.75±58.24	2.58±2.87	3.24±3.9 b
SOF/MSC/HHP40	8	61.8±33.12	1.8±2.49	2.24±2.74 a
SOF/MSC/HHP60	4	51±40.59	2±2.31	2.34±2.7 a

a, b – $P<0.05$ ANOVA followed by Tukey's post hoc test.

n: number; DCI: death cell index.

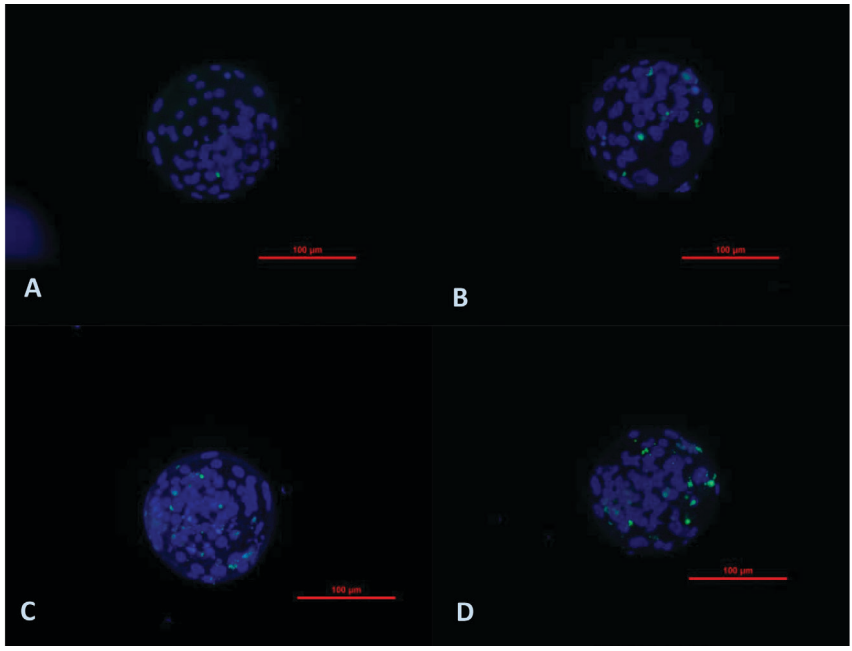

Figure 4. Bovine blastocyst after TUNEL staining (x400). A and B – high quality blastocyst cultured in coculture with mesenchymal stem cells (pBMSCs), C and D – poor quality blastocyst cultured in coculture with mesenchymal stem cells (pBMSCs)

Discussion

Because, to the best of our knowledge, no one has analysed the impact of HHP on the quality and functional activity of pBMSCs, it is difficult to discuss and compare our results with those of others. Wherever possible, we have referred to studies on gametes and embryos in which HHP was also used before cryopreservation as well as to studies in which other types of pressure were used. Our experiment assessed the apoptotic changes occurring in pBMSCs treated with HHP at 40 MPa and 60 MPa before cryopreservation. Apoptosis was estimated at the protein level by three different methods in its three phases: induction, decision and execution. Moreover, to check the functional abilities of pBMSCs in terms of secretion of beneficial factors into the medium, *in vitro* cultured bovine embryos were cocultured with pBMSCs subjected to HHP. Two HHP values were selected (40 and 60 MPa) based on previous results showing improved tolerance for pBMSCs cryopreservation (Romanek et al., 2017). Additionally, the quality of the obtained blastocysts was evaluated.

Caspase-8 is one of the initiator caspases, and it initiates caspase cascades leading to cell death. The results showed that HHP before cryopreservation did not affect the activity of caspase-8, indicating that subjecting cells to stresses such as HHP does not induce apoptosis.

In the case of survivin, a protein that inhibits *initiator and* executioner caspases, analysed by immunofluorescence, no significant differences were observed in the level of its expression in the HHP-treated (both 40 MPa and 60 MPa) and control cells. Positive fluorescent signal for survivin was observed in all groups. These results confirm the earlier statement that there is no induction of apoptosis as a result of HHP.

As a representative of the 3rd phase of apoptosis, the activity of CAD, which is responsible for oligonucleosomal DNA fragmentation, was determined. In this case, there were no significant differences in the level of CAD protein expression between HHP-treated and control cells, once again demonstrating the lack of pro-apoptotic effect of HHP. Interestingly, in HHP-treated and untreated cells, a positive fluorescent signal for CAD was observed in approximately 9% of pBMSCs. The observed CAD expression is not necessarily a sign of apoptosis. Larsen et al. (2010) demonstrated that myoblasts with reduced activity of caspase-3 and CAD showed decreased ability to differentiate. CAD affects the promotor of p21 gene, decreasing its expression. p21 is a strong inhibitor of cyclin-dependent kinases, and it binds with cyclin-CDK2 or CDK4 complexes inhibiting their activity and thus acts as a regulator of the G1 phase of cell cycle. These results indicate that caspase-3 and CAD affect differentiation of cells by modifying the expression of important regulatory genes (Larsen et al., 2010). From the literature, it is known that subjecting cells to hydrostatic pressure has a beneficial effect on their ability to differentiate (Dinnyes et al., 2010; Safshekan et al., 2012), but in the case of our study, additional validation including changes in caspase-3 expression are needed to assess the possible impact of HHP on the differentiation ability of pBMSCs.

There was also no difference in the expression of Bax protein between HHP-treated and control cells. This means that HHP does not affect the expression of Bax estimated after the action of two stressors: HHP and cryopreservation. We only found one study on the effect of HHP on Bax expression, which was performed in goat embryos, but they were not subjected to cryopreservation (Bogliolo et al., 2011). Embryos treated with 40 MPa HHP for 70 min showed reduced transcript levels of Bax ($P < 0.01$) compared to controls (Bogliolo et al., 2011). Such a decrease was not observed in pBMSCs, likely due to a higher sensitivity of blastocysts to changes in the *in vitro* culture conditions (Wolff et al., 2013). Additionally, applying HHP before pBMSCs cryopreservation did not affect the expression of anti-apoptotic protein $Bclx_L$ estimated after thawing. The short isoform of Bclx is a pro-apoptotic protein, known as $Bclx_S$. Little is known about the mechanism of action of this protein (Lindenboim et al., 2000). However, it is used as a marker of apoptosis (Malina and Hess, 2004). In our study, we also estimated this protein and found no significant differences among the assessed groups.

By summarizing the above results on pro- and anti-apoptotic protein levels, it can be concluded that both 40 and 60 MPa HHP have no harmful effect on the quality of the pBMSCs measured by the level of induction and advancement of apoptosis.

Coculturing embryos with pMSCs pBMSCs seems to be an innovative approach for the continued problem of low efficiency of *in vitro* production (IVP). It is known that the coculture of buffalo rat liver (BRL) cells and Vero cells showed positive

results (Duszewska et al., 2000; Kątska-Książkiewicz et al., 2007). Bearing in mind the fact that the pBMSCs cultured *in vitro* produce haematopoietic and non-haematopoietic growth factors, interleukins, and chemokines, which may be more suitable for the development of totipotent embryos than factors secreted by differentiated cell lines, it can be assumed that the coculture of embryos with pMSCs pBMSCs may be more effective than coculture with BRL cells or VERO cells (Opiela and Samiec, 2013). Moreover, Park et al. (2013) reported positive results of several growth factors and cytokines, on porcine embryo development. Mouse oocytes maturated in medium conditioned by MSC produced a higher maturation rate and improved embryo development to blastocyst stage (Ling et al., 2008). Potapova et al. (2007) showed that the cocktail of factors produced by hMSC spheroids stimulates mitotic propagation, migration, and invasion of endothelial cells (Potapova et al., 2007). Also the granulosa cells apoptosis was decreased by coculture with pBMSCs, which also resulted in increased Bcl-2 expression *in vivo* and the function of rat ovaries treated with cyclophosphamide was improved after pBMSCs implantation (Fu et al., 2008). Accumulating evidence suggests that human umbilical cord Wharton's jelly-derived mesenchymal stem cells have antifibrotic properties. Culture of fibroblast keloids carried out in the presence of pBMSCs showed increased expression of plasminogen activator inhibitor-I and transforming growth factor-β2 (TGF-β2), interleukin (IL)-6, IL-8, TGF-β1, and TGF-β2 proteins, which increase the capacity of cells to undergo normal differentiation and reduce the chance of malignancies (Arno et al., 2014). In the case of pBMSCs cocultured with HeLa cells, a decrease in the expression of caspase-3 was noted ($P<0.05$) (Cui et al., 2013). In this study, an assessment of the quality of bovine embryos cocultured with pBMSCs without HHP (SOF/MSC) and pBMSCs subjected to HHP of 40 MPa (SOF/MSC/HHP40) and 60 MPa (SOF/MSC/HHP60) in SOF medium was performed. Control embryos were cultured in SOF medium (SOF). In the present study, we observed embryonic divisions at almost 80%. The obtained results of embryo development to the blastocyst stage in coculture systems with pBMSCs are not in line with our expectations. Unfortunately, the embryos cocultured with pBMSCs, regardless of the HHP treatment, exhibited significantly reduced numbers of developed blastocysts. The reduction was even higher when HHP-treated pBMSCs were used. The results are similar to those observed in the study by Opiela et al. (2018) in which highly statistically significant differences were observed in the number of blastocysts obtained between the groups cultured in SOF and cocultured with pBMSCs in SOF. In the studies where embryos were cocultured with Vero cells and Vero-BRL cells, a higher percentage of blastocysts was obtained in comparison to coculture with pBMSCs (Duszewska et al., 2007). We speculate that HHP may have changed the profile of factors secreted by pBMSCs into the medium, which may be less suitable for the embryo development. Therefore, it could be said that the use of the pBMSCs as a coculture system for the development of bovine embryos does not result in positive results. Due to the small number of developed blastocysts in SOF/MSC/HHP40 and SOF/MSC/HHP60 groups, TUNEL assay was performed on only 8 and 4 blastocysts, respectively. Interestingly, the blastocysts obtained in the above two culture systems showed lower apoptotic index ($P<0.05$) compared to the embryos cultured in SOF/MSC and SOF. Moreover, no significant

differences were noted between the analysed culture systems in the average number of total cells per embryo and in the average number of apoptotic cells per embryo. In addition, between blastocysts cultured in SOF and SOF/MSC, no differences were observed in the apoptotic index. Additionally, no significant differences between the analysed blastocysts cultured in SOF and cocultured with pBMSCs in SOF were observed in terms of the total number of nuclei or apoptotic features (Opiela et al., 2018). In a study by Hajian et al. (2011), where the apoptotic index was analysed in embryos cultured with VERO cells (6.1%) or in SOF alone (5.5%), the obtained results were higher than the results of our study. In summary, the results of TUNEL assay indicate that coculturing of embryos with pBMSCs does not adversely affect the quality of the embryos.

In summary, the coculture of embryos with pBMSCs adversely affects the development of blastocysts without a negative impact on their quality. The low percentage of developed blastocysts can also be explained by the fact that pBMSCs under certain conditions may inhibit proliferation as demonstrated for pBMSCs cultured with T cells *in vitro*, where inhibition of proliferation depended on the concentration of cells and culture conditions (Najar et al., 2010). On the other hand, in the case of pBMSCs cocultured with multiple myeloma cells, in addition to a decrease in proliferation rate, increased apoptosis was observed in tumour cells (Atsuta et al., 2013). Therefore, in light of the above findings, it can be speculated that the SOF medium is sub-optimal for the *in vitro* culture of pBMSCs and could contribute to the adverse effects of pBMSCs on dividing embryos.

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