Ann. Anim. Sci., Vol. 11, No. 2 (2011) 261–271

APOPTOSIS IN PORCINE BLASTOCYSTS DERIVED FROM 2-4 CELL *IN VIVO* PRODUCED EMBRYOS SUBSEQUENTLY CULTURED *IN VITRO*

Magdalena Bryła, Monika Trzcińska

Department of Biotechnology of Animal Reproduction, National Research Institute of Animal Production, 32-083 Balice n. Kraków, Poland
Corresponding author: Magdalena Bryła, Department of Biotechnology of Animal Reproduction, National Research Institute of Animal Production, 32-083 Balice/Kraków, Poland
Phone: +48 666081266. Fax: +48 12258838. E-mail: mbryla@izoo.krakow.pl

Abstract

The aim of this study was to assess the quality of *in vitro* produced porcine expanded blastocysts. The quality of the preimplantation embryos was determined by counting the number of cells, observing a TUNEL-positive reaction and by caspase-3 labelling. A total of 394 porcine expanded blastocysts were examined, and results were evaluated using Student's t-test. In our study the development from the two- to four-cell embryos fertilized *in vivo* to the expanded blastocyst stage was different between the days post insemination (pi) (Day 7: 28.5%; Day 8: 41.0%; Day 9: 17.7%). The blastocysts from Day 7 pi had higher ability to expand, contained more cells and were less predisposed to DNA fragmentation than blastocysts from Days 8 and 9 pi. Therefore, the analysis of embryo quality based on total cell number, TUNEL-positive nuclei and caspase-3 activity revealed significant differences in quality of blastocysts from Day 7 of culture compared to Day 9 pi. Moreover, the percentage of caspase-3 activity in embryos increased between Days 7, 8 and 9 pi (56.9, 58.0, 80.0 respectively). We found that expanded blastocysts consisting of a small number of cells are characterized by a high incidence of TUNEL-positive nuclei and caspase-3 activity. These results revealed a positive correlation between the percentage of blastocysts with TUNEL-positive nuclei and the percentage of blastocysts with caspase-3 activity (r = 0.9918; P<0.0001). The number of cells, TUNEL-positive nuclei and caspase-3 activity revealed that the best quality was characteristic of blastocysts from Day 7 pi. Our results showed that expanding blastocysts obtained on Day 7 pi were of the highest quality based on the three-point scale. Such embryos can be used in further biotechnological procedures and have the greatest chance of post-implantation development.

Key words: porcine expanded blastocysts, *in vitro*, DNA fragmentation, TUNEL, caspase-3

Over the last twenty years, foundations were laid for better utilization of female reproductive potential and ability, resulting in considerable breeding progress. Despite these achievements, there is still a problem of obtaining high-value embryos

developing to the blastocyst stage and improving their quality and developmental competence.

The preimplantation embryo is extremely sensitive to environmental factors, so deficiencies in culture conditions often lead to aberrant embryo development that manifests in lower frequency of blastocyst formation and lower cell count and can affect fetal as well as postnatal life. Current research is oriented towards minimizing the negative effect of *in vitro* culture conditions on quality of the embryos obtained. Augustin et al. (2003) reports that bovine blastocysts derived from cultures supplemented with insulin showed a significant decrease in apoptosis determined by the TUNEL assay. Another factor that may be involved in the process of apoptosis in embryo is fetal calf serum (FCS). Fouladi-Nashta et al. (2005) reported no differences in the number of apoptotic nuclei between embryos cultured in medium supplemented with FCS and without serum. Meanwhile, Warzych et al. (2007) demonstrated lower blastocyst rate and elevated apoptotic index in embryos derived from oocytes matured with PVP40.

The Department of Biotechnology of Animal Reproduction of the National Research Institute of Animal Production has for many years investigated pig embryo cultures in different culture media. A study by Gajda (1998) and Gajda and Smorąg (2004) showed that the highest percentage of embryos developing to the blastocyst stage was observed for NCSU-23 medium. These embryos had the highest total number of cells on average compared to blastocysts cultured in other media. Embryo quality is usually evaluated based on morphological evaluation, but increasingly often one parameter of particular interest in the pig is apoptosis, as *in vivo*-produced blastocysts present few or no apoptotic cells, whereas *in vitro*-produced embryos show a much higher incidence (Kidson et al., 2004; Bryła et al., 2009; 2010). *In vitro* culture conditions are thought to increase apoptotic incidence, decrease embryonic cell number, decrease implantation rates and increase fetal resorption (Gjørret et al., 2003).

It has been speculated that apoptosis also plays a function in eliminating defective embryos (Jurisicova and Acton, 2004). During *in vitro* embryo culture apoptosis is probably caused by suboptimal conditions and may therefore also be an indicator of embryo quality (Pomar et al., 2005). DNA fragmentation is the typical sign of degeneration phase of apoptotic cell death. This process has been demonstrated to occur during pre-implantation development both *in vivo* and *in vitro* and it is believed to contribute to early embryonic loss. However, *in vitro*-derived blastocysts show a higher degree of fragmentation than their *in vivo* counterparts. During *in vitro* embryo culture this process is probably caused by suboptimal conditions and may therefore also be an indicator of embryo quality (Pomar et al., 2005). DNA fragmentation was furthermore described in 56–71% of porcine *in vivo*-derived embryos (Fabian et al., 2005) and 90% of porcine *in vitro*-derived embryos (Long et al., 1998). Basal levels of TUNEL-positive nuclei in the embryo may be important in the developmental processes by selectively removing cells from the inner cell mass that have retained trophoectodermal competence (Gjørret et al., 2003). DNA fragmentation is also the means by which inherently incompetent cells and/or embryos are eliminated. The two major criteria that influence the quality of the preimplantation

embryos and their subsequent survival are total cell number and degree of fragmentation (Mateusen et al., 2005). A high cell count at any given time and a concurrent low degree of fragmentation indicate good embryo quality and a higher chance for successful development and implantation.

Several studies with preimplantation embryos have suggested an association between apoptosis and fragmentation of DNA and/or caspase-3 activation. Caspase-3 holds a central position in the apoptotic cascade (Hangartner, 2000; Mehmet, 2000). One of the critical substrates cleaved by effector caspases is the inhibitor of caspase-activated DNase (ICAD). Cleavage of ICAD by caspase-3 releases the enzyme caspase-activated DNase (CAD), which is responsible for nucleosomal DNA fragmentation during apoptotic changes (Enari, 1998; Sakahira et al., 1998). Assessment of cells with fragmented DNA was made by incorporating fluorescein-dUTP at 3’OH DNA ends using the Terminal Deoxynucleotidyl Transferase (TdT) enzyme. Therefore, use of these two markers is important to investigate the competence of preimplantation embryos. Developmental potential of a postimplantation embryo is affected by apoptotic incidence in preimplantation stages (Loureiro et al., 2007). The degree and pattern of programmed cell death in early embryos significantly impact implantation and pregnancy. Therefore, use of several concurrent markers is needed for adequate identification of the apoptotic changes in preimplantation embryos.

In our experiment, a three-point scale of embryo quality evaluation was used for the first time in porcine embryos: total cell count, number of apoptotic nuclei based on TUNEL reaction and activity of the caspase-3. The aim of this study was to assess the quality of expanded blastocyst stage embryos obtained from *in vitro* culture of 2-4 cell embryos fertilized *in vivo*.

Material and methods

In vitro-produced porcine blastocysts

Experiments were performed on two- to four-cell embryos fertilizing *in vivo* obtained from 30 synchronized gilts of the same age (6 to 8 months old). The crossbreds of Polish Landrace and Polish Large White, weighing 90–100 kg, were in similar condition and were offered the same diet. Donor gilts were kept at the Experimental Station in Żerniki Wielkie.

Donors were superovulated by injection of 1500 IU PMSG (Serogonadotropin, Biowet, Poland) and 1000 IU of hCG (Choluron, Biomed, Poland) administered 72 hours later. At the onset of estrus (24 h after hCG administration) the gilts were artificially inseminated at regular two-week intervals twice at 12-hour intervals with the standard dose of semen.

Embryos at the two- to four-cell stage were collected 48 h after insemination (Day 2 post-insemination (pi)). *In vitro* culture of 2-4 cell embryos flushed from the oviduct was cultured *in vitro* in NCSU-23 medium (North Carolina State University) (Peters and Wells, 1993) with bovine serum albumin-fraction V (BSA-V, Sigma Chemical Company, St. Louis, MO, USA) for 7 days (Day 9 pi) at 38.5°C under 5% CO_2 in air till the expanded blastocyst stage.

The cultured embryos were evaluated at 24-hour intervals until the expanding blastocyst stage. At each of three inspection points (total cell number per blastocyst, TUNEL-positive nuclei, caspase-3 activity) embryos at the stage of expanded blastocyst were removed from the culture and their quality assessed.

Morphological classification of expanded blastocysts

Blastocysts were classified on the basis of their morphological appearance using stereo microscope. Blastocysts that were symmetrical and spherical with a clear inner cell mass (ICM), intact trophectoderm and containing no visible extruded blastomeres were categorized as having a normal morphology.

Detection of DNA fragmentation by TUNEL

DNA fragmentation in blastomeres nuclei was analysed by using a combined technique for simultaneous nuclear staining and TUNEL by a modification of the procedures used by Brison and Schultz (1997).

For the preparation for TUNEL, embryos were fixed in 4% paraformaldehyde in PBS, pH 7.4 for 1 h at room temperature. The embryos were washed three times in 50 µl drops of PBS-PVP (1 µg/ml polyvinylpyrrolidone in PBS), then permeabilized in a humidified box with 0.1% Triton X-100 in PBS for 30 min at room temperature, and finally washed again in drops of PBS-PVP solution. Embryos were then incubated in fluorescein-conjugated dUTP and TdT (TUNEL reagent; In Situ Cell Detection kit, Roche Diagnostics, Germany) for 1 h in an incubator at 38.5°C and 5% CO_2 in air. As a positive control, one or two embryos per TUNEL analysis were incubated in 50 U/ml DNase I (Promega) for 20 min at 38.5°C. As a negative control, one or two embryos per TUNEL analysis were incubated in fluorescein-dUTP in the absence of TdT. After the reaction, the embryos were washed three times in drops of PBS-PVP solution, transferred through a gradient of Vecta-Shield with DAPI (Vector Laboratories, Burlingame, CA) 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 nuclei per blastocyst (determined by nuclear staining with DAPI) and the number of nuclei positive for TUNEL labelling (TUNEL+) were counted (nuclei were recorded as positive for TUNEL when they had visible green fluorescence cells with DNA strand breaks and condensed nuclei). The DNA-fragmented nucleus index (TUNEL index) of the embryos was calculated as follows: TUNEL index = (no. TUNEL-positive nuclei, fragmented and condensed)/(total no. of nuclei) × 100.

The embryos after labelling were identified to two categories: i) lack of TUNEL-positive nuclei; ii) TUNEL-positive nuclei.

Caspase-3 activity

Activity of caspase-3 in expanded blastocysts was detected using PhiPhiLux G_2D_2 kit (Calbiochem, Germany). This kit contains a peptide substrate for caspase-3. The substrate GDEVDGI (the caspase cleavage site is underlined) has two rhodamine fluorophores conjugated on each side of the caspase-3 cleavage site. The fluorescence is quenched in intact substrate due to the folded peptide structure. Upon

cleavage by the activity of caspase-3, high intensity red rhodamine fluorescence can be obtained. The blastocysts were washed in 50 µl drops of PBS-PVP (1 µg/ml polyvinylpyrrolidone in PBS) and incubated in RPMI 1640 medium with 10 µM peptide substrate + 10% FCS at 38.5°C, 5% CO_2 for 60 minutes. After incubation period embryos were washed in PBS-PVP three times to reduce the background fluorescence and were then mounted on a glass slide with coverslip. The slides were examined under fluorescence microscope (Nikon Eclipse E600) using rhodamine filter combination (EX 510-560, BA 590). After labelling, embryos were classified to one of two categories: lack of caspase-3 activity and presence of the enzyme activity.

Statistical analysis

The results are expressed as the means ± SD. Statistical analyses were performed using the Statistica 6.0 program (StatSoft, Tulsa, OK, USA). The significance of the differences between the means was tested using Student's t-test. The statistical differences were considered to be significant at P≤0.05. Statistical relationships between the fluorescence parameters were assessed using Pearson's correlation coefficient.

Results

A total of 453 two- to four-cell stage embryos (Day 2 pi) collected from 30 donor gilts (15.1 embryos per donor) were cultured *in vitro* until the expanded blastocyst stage. The rate of expanded blastocysts developed from 2-4 cell embryos was different at Days 7, 8 and 9 pi (Day 7: 28.5%; Day 8: 41.0%; Day 9: 17.7%) (Table 1).

The percentage of developing embryos from the two- to four-cell embryos to the expanded blastocyst was different between the days of culture.

Altogether 394 expanded blastocysts were subjected to investigation: 196 of them were analysed by TUNEL for incidence of apoptotic nuclei and the total cell count whereas in the remaining 198 the caspase-3 activity was measured. In this study the total number of nuclei per blastocyst (determined by nuclear staining with DAPI) and the number of TUNEL-positive nuclei (fragmented and condensed nuclei) was determined. Significant differences were observed in the number of nuclei in *in vitro*-produced blastocysts on Day 7 pi compared to Day 9 (P≤0.05) (Table 2), (Figures 1 and 2). Moreover, blastocysts derived after Day 7 pi consist of a high number of nuclei and have a low incidence of TUNEL-positive nuclei compared to blastocysts after Day 9 pi (P≤0.05). The remaining *in vitro*-cultured expanded blastocysts (198) were analysed by caspase-3 activity. The rate of expanded blastocysts showing TUNEL-positive nuclei and activity of the caspase-3 (Table 2) increased with the time of embryo culture. The following percentages of expanded blastocysts showing caspase activity were found: 56.9; 58.0 and 80.0% on Day 7, 8 and 9 pi, respectively (Table 2). As culture time increased the highest percentage of expanded blastocysts revealed TUNEL-positive nuclei and activity of caspase-3 (Table 2).

Table 1. Developmental competence of 2- to 4-cell porcine embryos (Day 2 pi) till the expanding blastosyst stage

Developmental competence/ Day post insemination (pi)							
Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
Total no. of 2- to 4-cell stage embryos (%)	Total no. of 5-to 8-cell stage embryos (%)	Total no. of 9- to 15- cell stage embryos (%)	Total no. of 16- to 32-cell stage embryos (%)	No. of morulae (%)	No. of expanded blastocysts (%)		
453 (100)	409 (90.3)	401 (88.5)	398 (87.7)	397 (87.6)	129 (28.5)	185 (41.0)	80 (17.7)

Table 2. The incidence of apoptosis of expanded blastocysts derived after Day 7, 8 and 9 post insemination assessed by cell number, TUNEL-positive nuclei and caspase-3 activity

Days post insemination	TUNEL method						Caspase-3		
	No. of analysed expanded blastocysts	No. of cells related to one blastocyst[1]	No. of positive nuclei	TUNEL index (%)2	Lack of TUNEL-positive nuclei	TUNEL-positive nuclei	No. of analysed expanded blastocysts	Lack of activ-ity (%)	Presence of activity (%)
Day 7	64	66,5±24.0 a	8.8±12.5 a	13.2	37 (57.9)	27 (42.2)	65	28/43.1	37/56.9
Day 8	92	65.9±21.8	11.1±15.0	16.8	43 (47.1)	18 (53.3)	93	39/42.0	54/58.0
Day 9	40	54.8±15.9 b	16.2±14.9 b	29.8	12 (32.0)	28 (70.0)	40	8/20.0	32/80.0

[1]Nuclei were recorded as positive for TUNEL labeling (TUNEL+) when they had visible green fluorescence (cells with DNA strand breaks and condensed nuclei).
[2]TUNEL index = (no. of TUNEL-positive nuclei, fragmented and condensed)/(total no. of nuclei) x 100 per all analysed blastocysts.
a, b – Within columns means bearing different superscripts differ significantly at $P \leq 0.05$.

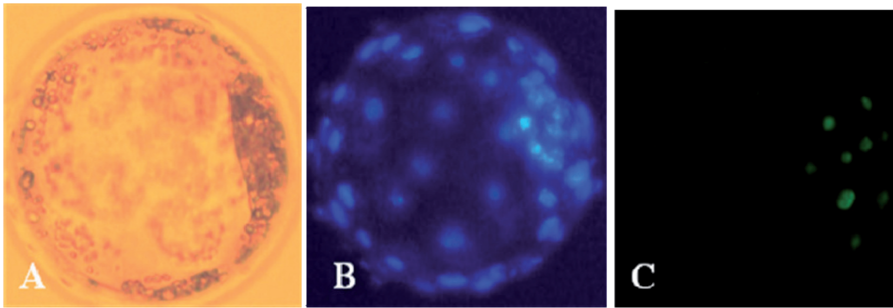

Figure 1. The same porcine expanded blastocyst produced *in vitro* on Day 5 of culture (A) in phase-contrast microscope, (B) counterstaining with DAPI (blue fluorescence) and (C) TUNEL-positive nuclei (green fluorescence; original magnification: X 200)

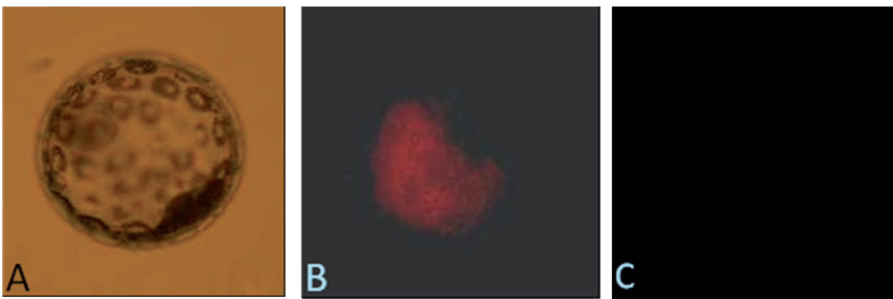

Figure 2. Porcine expanded blastocyst produced *in vitro*. The expanded blastocyst on Day 5 of culture (A) in phase-contrast microscope, (B) Expanded blastocyst with activity of caspase-3 (C) Expanded blastocyst with absence of caspase-3 activity (original magnification: X 200)

Correlation coefficient

The data showed a negative correlation between the total cell count per blastocyst and the TUNEL index (r = –0.8236; P<0.0001). The TUNEL index and TUNEL-positive nuclei (r = 0.9622; P<0.0001) showed a positive correlation - data not tabulated.

The study showed that the total number of nuclei in *in vitro*-produced expanded blastocysts was negatively correlated with the number of TUNEL-positive nuclei (r = –0.7685; P<0.0001) and the TUNEL index (r = –0.7752; P<0.0001), whereas the number of TUNEL-positive nuclei was positively correlated with the TUNEL index (r = 0.9664; P<0.0001) – data not tabulated.

Our results revealed a positive correlation between percentage of blastocysts with TUNEL-positive reaction and percentage of blastocysts with activity of caspase-3 (r = 0.9918; P<0.01) – data not tabulated.

Discussion

The present study analysed the quality of *in vitro*-produced embryos based on total cell number, TUNEL-positive nuclei and caspase-3 activity Our results demonstrated that blastocysts produced *in vitro* from 2–4 cell embryos fertilized *in vivo* were of different quality. These differences in quality of embryos were due to time of reaching the expanding blastocyst stage. In our study, cultured 2–4 cell embryos reached the expanding blastocyst stage *in vitro* on Days 7, 8 or 9 pi.

In our experiment high developmental rates were observed (86.9%). These results are higher than those reported by Mateusen et al. (2005) in which developmental rates to the blastocyst stage after *in vitro* culture were observed in approximately 70%. These results are in contrast to those reported by Abeydeera (2002) in which after culture of IVF pig embryos, the blastocyst rates were about 25%. These differences are not surprising because procedure of IVF including maturation and fertilization of oocyte causes a decrease in developmental rate.

Embryonic fragmentation is the most important morphological parameter of embryo quality. The porcine embryo fragmentation is a dynamic feature in which the location and size of fragmentation can vary in time (Mateusen et al., 2005). In our experiment, the blastocyst from Day 7 and Day 8 pi had a similar number of cells (66.5 and 65.9) and these results are higher than in blastocysts produced on Day 9 pi. Our result does not agree with the findings of Papaioannou and Ebert (1988), who showed no differences in the number of blastocyst nuclei obtained on different days of culture. Moreover, the blastocysts from Day 7 pi had higher ability to expand, contained more cells and were less predisposed to DNA fragmentation and had the lowest caspase-3 activity. Therefore, the longer period of *in vitro* culture increased the percentage of embryos that show DNA fragmentation and caspase-3 activity. This is probably the result of embryo reaction to suboptimal embryo culture conditions. It is well known that culture conditions cause cellular trauma in the embryo that can be manifested in different ways, and the overall result is the developmental retardation of the cultured embryos (Machaty et al., 1998).

Our results showed that the percentage of the embryos that had TUNEL-positive reactions was similar to the percentage of embryos exhibiting caspase-3 activity. Moreover, a strong correlation was observed between the blastocysts that had TUNEL-positive nuclei and the blastocysts that exhibited caspase-3 activity. Our observations suggest that DNA fragmentation takes place shortly after caspase-3 activation. Because the analysis was not performed on the same embryos, it is possible that the degradation of the DNA and the changes in nuclear morphology may occur either before, concurrent with or after activation of caspase-3 (Darzynkiewicz et al., 2001). In this study caspase-3 activity was only detected in a small percentage of blastocysts, indicating that most of caspase-3 protein was still in zymogen form. Most of the literature data suggest that morphologically normal embryos show low or lack of caspase-3 activity. Jurisicova et al. (2003) found caspase-3 activity to increase in embryos showing DNA fragmentation, whereas lack of caspase-3 activity occurred for embryos showing no fragmentation. Our results demonstrated that

TUNEL-positive nuclei were associated with caspase-3 activity in porcine expanded blastocysts.

In our experiment, a three-point scale of qualitative evaluation of pig embryos obtained at the expanding blastocyst stage after *in vitro* culture of 2–4-cell embryos (fertilized *in vivo*) was used for the first time. Our study showed that expanding blastocysts obtained on Day 7 pi were of the highest quality based on the three-point scale. Such embryos can be used in further biotechnological procedures and have the greatest chance of post-implantation development.

Acknowledgments

This study was financed by NRIAP statutory project no. 3340.1 and statutory project no. 3428.1 founded by the Ministry of Science and Higher Education. All animal procedures were approved by the Local Ethics Board for Animal Experiments in Kraków (Poland). The authors would also like to thank Jarosław Wieczorek PhD and Waldemar Kowalski PhD for their help in obtaining embryos from gilts.

References

Abeydeera L.R. (2002). *In vitro* production of embryos in swine. Theriogenology, 57: 257–273.

Augustin R., Pocar P., Wrenzycki C., Niemann H., Fischer B. (2003). Mitogenic and antiapoptotic activity of insulin on bovine embryos produced *in vitro*. Reproduction, 126 : 91–99.

Brison D.R., Schultz R.M. (1997). Apoptosis during mouse blastocyst formation: evidence for a role for survival factors including transforming growth factor. Biol. Reprod., 56: 1088–1096.

Bryła M., Trzcińska M., Wieczorek J. (2009). Analysis of *in vivo*- and *in vitro*-derived pig expanded blastocysts based on DNA fragmentation. Anim. Sci. Pap. Rep., 27: 59–68.

Bryła M., Trzcińska M., Wieczorek J., Słomski R., Smorąg Z. (2010). Quality of preimplantation embryos derived from gilts inseminated with transgenic and non-transgenic boars' semen. Anim. Reprod. Sci., 118: 77–82.

Darzynkiewicz Z., Bedner E., Traganos F. (2001). Difficulties and pitfalls in analysis of apoptosis. Methods Cell Biol., 63: 527–546.

Enari M., Sakahira H., Yokoyama H., Okawa K., Iwamatsu A., Nagata S. (1998). A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature, 391: 43–50.

Fabian D., Koppel J., Maddox-Hyttel P. (2005). Apoptotic processes during mammalian preimplantation development. Theriogenology, 64: 221–231.

Fouladi-Nashta A.A., Alberio R., Kafi M., Nicholas B., Campbell K.Y., Webb R. (2005). Differential staining combined with TUNEL labelling to detect apoptosis in the preimplantation bovine embryos. Reprod. Biomed. Online, 10: 497–502.

Gajda B., Smorąg Z. (2004). Cells number in pig blastocyst cultured in different media. Ann. Anim. Sci., 4: 315–320.

Gajda B. (1998). *In vitro* culture of pig embryos. Ann. Anim. Sci., 25: 31–38.

Gjørret J.O., Knijn H.M., Dieleman S.J., Avery B., Larsson L-I., Maddox-Hyttel P. (2003). Chronology of apoptosis in bovine embryos produced *in vivo* and *in vitro*. Biol. Reprod., 69: 1193–1200.

Hangartner M.O. (2000). The biochemistry of apoptosis. Nature, 407: 770–776.

Jurisicova A., Acton B.M. (2004). Deadly decisions: the role of genes regulating programmed cell death in human preimplantation embryo development. Reproduction, 128: 281–291.

Jurisicova A., Atenos M., Varmuza S., Tilly J.L., Casper R.F. (2003). Expression of apoptosis-related genes during human preimplantation embryo development: potential roles for the Harakiri gene product and Caspase-3 in blastomere fragmentation. Mol. Hum. Reprod., 9: 133–141.

Kidson A., Rubio-Pomar F.J., Van Knegsel A., Van Tol H.T., Hazeleger W., Ducro-Steverink D.W., Colenbrander B., Dieleman S.J., Bevers M.M. (2004). Quality of porcine blastocysts produced *in vitro* in the presence or absence of GH. Reproduction, 127: 165-177.

Long C.R, Dobrinsky J.R, Garrett W.M., Johnson L.A. (1998). Dual labeling of the cytoskeleton and DNA strand breaks in porcine embryos produced *in vivo* and *in vitro*. Mol. Reprod. Dev., 51: 59–65.

Loureiro B., Brad A.M., Hansen P.J. (2007). Heat shock and tumor necrosis factor-α induce apoptosis in bovine preimplantation embryos through a caspase-9 dependent mechanism. Reproduction, 133: 1129–1137.

Machaty Z., Day B.N., Prather R.S. (1998). Development of early porcine embryos *in vitro* and in vivo. Biol. Reprod., 59: 451–455.

Mateusen B., Soom A.V., Maes D.G.D., Donnay I., Duchateau L., Lequarre A.S. (2005). Porcine embryo development and fragmentation and their relation to apoptotic markers: a cinematographic and confocal laser scanning microscopic study. Reproduction, 129: 443–453.

Mehmet H. (2000). Caspases find a new place to hide. Nature, 403: 29–30.

Papaioannou E.V., Ebert M.K. (1988). The preimplantation pig embryo: cell number and allocation to trophectoderm and inner call mass of the blastocyst *in vivo* and *in vitro*. Development, 102: 793–803.

Peters R.M., Wells K.D. (1993). Culture of pig embryos. J. Reprod. Fertil., 48: 61–73.

Pomar R.F.J., Teerds K.J., Kidson A., Colenbrander B., Tharasant T., Aguilar B., Roelen B.A.J. (2005). Differences in the incidence of apoptosis between *in vivo* and *in vitro* produced blastocysts of farm animal species: a comparative study. Theriogenology, 63: 2254–2268.

Sakahira H., Enari M., Nagata S. (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature, 391: 96–99.

Warzych E., Peippo J., Szydlowski M., Lechniak D. (2007). Supplements to in vitro maturation media affect the production of bovine blastocysts and their apoptotic index but not the proportions of maturated and apoptotic oocytes. Anim. Reprod. Sci., 97: 334–343.

Accepted for printing 4 IV 2011

MAGDALENA BRYŁA, MONIKA TRZCIŃSKA

Proces apoptozy w blastocystach świńskich uzyskanych *in vivo* z 2–4-komórkowych zarodków hodowanych *in vitro*

STRESZCZENIE

Celem badań była ocena jakości hodowanych *in vitro* zarodków świni w stadium ekspandującej blastocysty. Ocenę przeprowadzono na podstawie całkowitej liczby jąder, TUNEL-pozytywnych jąder i aktywności kaspazy-3. Ogółem analizie poddano 394 zarodki w stadium blastocysty ekspandującej, a uzyskane wyniki szacowano testem Studenta *t*. Zarodki 2–4-blastomerowe uzyskane po zapłodnieniu *in vivo*, hodowane następnie *in vitro*, osiągały stadium blastocysty ekspandującej w różnych dniach po inseminacji (pi) (dzień 7.: 28,5%; dzień 8.: 41,0%; dzień 9.: 17,7%). Zarodki uzyskane w 7. dniu pi posiadały wyższą całkowitą liczbę jąder oraz mniejszą predyspozycję do fragmentacji DNA w porównaniu z blastocystami uzyskanymi w 8. i 9. dniu pi. Jednakże kompleksowa analiza zarodków przeprowadzona na podstawie całkowitej liczby jąder, TUNEL-pozytywnych jąder i aktywności kaspazy-3 wykazała statystycznie istotne różnice tylko, w jakości blastocyst uzyskanych w 7. dniu w porównaniu z blastocystami uzyskanymi w 9. dniu pi. Co więcej, odsetek zarodków wykazujących aktywność kaspazy-3 wzrasta wraz z wydłużeniem czasu hodowli (dzień 7.: 56,9; dzień 8.: 58,0; dzień 9.: 80,0). Dodatkowo stwierdzono wyższy odsetek TUNEL-pozytywnych jąder oraz wyższą aktywność kaspa-

zy-3 w blastocystach z niższą całkowitą liczbę jąder. Wykazano dodatnią korelację pomiędzy odsetkiem blastocyst zawierających TUNEL-pozytywne jądra a odsetkiem blastocyst wykazujący aktywność kaspazy-3 (r = 0,9918; P<0,0001). Uzyskane wyniki wykazały, że blastocysty ekspandujące uzyskane w 7. dniu pi oceniane przy zastosowaniu trójstopniowej skali posiadały najwyższą jakość. Takie zarodki mają największe szanse na rozwój poimplantacyjny, dlatego można je wykorzystywać w dalszych procedurach biotechnologicznych.