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Analysis of *in vivo*- and *in vitro*-derived pig expanded blastocysts based on DNA fragmentation*

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The aim of the study was to investigate the competence of *in vivo*- and *in vitro*-derived pig expanded blastocysts by analysing DNA fragmentation using TUNEL. A total of 533 porcine expanded blastocysts were examined, and results were evaluated using Fisher's test. Significant differences in the incidence of fragmented nuclei (detected by the TUNEL reaction) and all nuclei (detected by DAPI) were identified between *in vivo*- and *in vitro*-derived embryos at the expanded blastocyst stage. The total numbers of nuclei observed in *in vivo*-derived embryos were significantly different from those in *in vitro*-cultured embryos (89.1±13.4 and 47.7±25.1, respectively). TUNEL index in *in vitro*-cultured embryos (28.3%) was significantly higher (P<0.01) than in *in vivo*-derived blastocysts (4%). These findings indicate that *in vivo*- and *in vitro*-derived expanded blastocysts consisting of a small number of cells are characterized by a high incidence of DNA fragmentation. The total number of nuclei in *in vivo*- and *in vitro*-derived blastocysts correlated negatively with the number of TUNEL-positive nuclei (r = -0.51; P<0.0001) and the TUNEL index (r = -0.69; P<0.0001), whereas the number of TUNEL-positive nuclei was positively correlated with the TUNEL index (r = 0.95; P<0.0001). Moreover, significant differences were observed between embryos collected from individual experiments.

KEY WORDS: blastocysts / DNA fragmentation / *in vitro* / *in vivo* / porcine expanded blastocysts / TUNEL

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Advances in the biotechnology of porcine reproduction have led to methods that enable better use of female reproductive potential. These methods are superovulation, *in vitro* embryo collection and transfer, oocyte and embryo cryopreservation, embryonic and somatic cloning, and transgenesis. The application of these methods in female reproduction has increased the demand for embryos and female gametes, for both breeding and experimental purposes. 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 still is a problem in obtaining high-value embryos developing to the blastocyst stage as well as in improving their quality and developmental competence.

Quality of embryos based on morphological criteria. Additional criteria is timing of development and the total number of nuclei per blastocyst [Lonergan *et al*. 1999, Mateusen *et al*. 2005, Hardy *et al.* 2003]. Important indicator of mammalian embryo quality is also the blastomere fragmentation rate [Mateusen *et al*. 2005]. It has been suggested that DNA fragmentation indicating cell death is a common feature of the development of mammalian embryos [Hardy *et al.* 2003, Pomar *et al.* 2004]. The indicators of apoptosis in embryos include nuclear fragmentation (identifiable with dyes that specifically bind to DNA such as 4,6-dimanino 2-phenylindole - DAPI) and the presence of single- and double-strand DNA breaks detectable by terminal dUTP nick and labelling (TUNEL) – Pomar *et al.* [2005]. Deoxynucleotidyl transferase (TdT) catalyses the incorporation of biotinylated deoxyuridine at the sites of the DNA breaks. The signal is amplified by avidinperoxidase, enabling detection by fluorescent microscopy [Neuber *et al*. 2002]. Positive signals for these biochemical markers were detected in at least one nucleus of every *in vivo* and *in vitro* blastocyst from many mammalian species, including pigs [Long *et al*. 1998, Hao *et al*. 2003, Pomar *et al*. 2004, 2005, Mateusen *et al*. 2005]. This indicates that nuclear fragmentation occurred during normal embryos development. However, *in vitro*-derived blastocysts show a higher degree of fragmentation than their *in vivo* counterparts. During *in vitro* embryos culture this process is probably caused by suboptimal conditions and, therefore, may also be an indicator of embryo quality [Pomar *et al*. 2005].

Jurisicova *et al.* [1996] using TUNEL method presented evidence of apoptotic changes in human fragmented embryos. Apoptotic changes have been shown in about 75% of fertilized *in vitro* embryos with morphological fragmentation. DNA fragmentation was furthermore described in 56-71% of *in vivo*-derived [Fabian *et al.* 2005] and in 90% of *in vitro*-derived [Long *et al.* 1998] embryos.

A small number of cells undergoing DNA degeneration can be seen even in high-quality, normal human embryos [Hardy 1997, 1999]. 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 of embryos and their subsequent

survival are total cell number and fragmentation rate [Mateusen *et al*. 2005]. A high cell count at any given time and a concurrent low fragmentation rate indicate good embryo quality and a higher chance for successful development and implantation. Despite the importance of fragmentation as a morphological indicator of embryonic viability, the origin of fragmentation is still unclear [Mateusen *et al.* 2005].

In addition, embryo quality and frequency of DNA fragmentation may depend on the other external factors such as weight and age of embryo donors and season of the year in which embryos are obtained. Following Smorag *et al*. [1999], gilts weighing 90-100 kg and aged 6 to 8 months are most suitable as embryo donors and the higher superovulation rates are obtained during the summer-autumn period.

The aim of this study was to compare the developmental competence of *in vivo* and *in vitro* porcine embryos based on total number of cells and DNA fragmentation.

Also, to recognize whether any correlation exists between total numbers of nuclei, TUNEL-positive nuclei and TUNEL index in *in vivo*- and *in vitro*-derived expanded pig blastocysts.

Material and methods

In vivo collection of blastocysts

Expanded blastocyts were collected from 24 donor gilts (six gilts in each experiment). All aged 6-8 months, weighed 90-100 kg, remained in similar body condition and were offered the same diet. Donors from four experimental groups were inseminated at regular two-week intervals in the autumn and were superovulated by injection of 1500 IU PMSG (Serogonadotropin, BIOWET, Poland) and 1000 IU of hCG (Choluron, BIOMED, Poland) administered 72 hours later with the standard dose of semen. At the onset of oestrus (24 h post hCG administration) the gilts were artificially inseminated, twice at 12-hour intervals, with the standard dose of semen.

Immediately after stunning and bleeding of gilts (approximately 168 h after hCG injection), their *uteri* were removed and flushed with Dulbecco's PBS (DPBS) obtained from INVITROGEN, USA for embryo collection. Embryos were transferred in a thermobox to the laboratory in PBS (SIGMA, St. Louis, USA) supplemented with 20% foetal calf serum (FCS, SIGMA, St. Louis, USA) at 38°C and examined with TUNEL method.

In vitro-deriving of blastocysts

Experiments were performed on two- to four-cell embryos obtained from 24 donor gilts (six gilts in each experiment). All gilts used were at the age of 6-8-months, weighed 90-100 kg, remained in similar body condition and were offered the same diet. Donors were inseminated at regular two-week intervals in the autumn and 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 oestrus (24 h after hCG administration) the gilts were artificially inseminated twice, at 12-hour intervals, with the standard dose of semen.

Embryos at the two- to four-cell stage were collected 48 h post-insemination by flushing the fallopian tubes with PBS supplemented with 20% FCS at 38°C. Embryos at the two- to four-cell stage were cultured *in vitro* in North Carolina State University (NCSU) -23 medium [Peters and Wells 1993] for six days at 38.5°C under 5% CO_2 in air, up to expanded blastocysts stage and examined with TUNEL method.

Detection of DNA fragmentation by TUNEL method

DNA fragmentation of *in vivo*- and *in vitro*-derived expanded blastocysts was examined with a combined technique based on simultaneous nuclear staining and TUNEL using a modification of the procedures by Brison and Schultz [1997].

For the preparation for TUNEL, embryos were fixed for 1h at room temperature in 4% paraformaldehyde in PBS (SIGMA Chemical Company, St. Louis, USA). The embryos were washed three times in 50 µl drops of PBS-PVP (1 µg/ml polyvinylpyrrolidone in PBS, SIGMA Chemical Company, St. Louis, USA), then permeabilized in a humidified box with 0.1% Triton X-100 in PBS (SIGMA Chemical Company, St. Louis, USA) for 30 min at the room temperature, and finally washed in drops of PBS-PVP solution. The embryos were incubated in fluorescein-conjugated dUTP and TdT (TUNEL reagent; In Situ Cell Detection kit, ROCHE Diagnostics, Germany) for 1h at 38.5°C and 5% CO_2 in air. As a positive control, one or two embryos per each group for TUNEL analysis were incubated in 50 U/ml DNase I ROCHE Diagnostics, Germany) for 20 min, at 38.5°C. As a negative control, one or two embryos per each group for 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 and transferred through 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 nuclei per expanded blastocyst (determined by nuclear staining with DAPI) and the 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).

Investigation was approved by the Local Ethics Committee for Animal Experimentation, Cracow, Poland, Cert. No. 376 of July 21, 2005.

Statistical

Significance of differences in the total number of nuclei, TUNEL-positive nuclei and the TUNEL index per blastocyst between in *in vivo*- and *in vitro*-derived expanded blastocysts. was assessed using Fisher's tests. Significant differences were considered to be highly significant at $P<0.01$ and significant at $P<0.05$. Correlation between the total number of nuclei, TUNEL-positive nuclei and the TUNEL index was estimated

based on Pearson's rank test. Computations were performed using Statistical Analysis System [2001].

Results and discussion

In this experiment 260 *in vivo* and 273 *in vitro* expanded blastocysts were examined (Tab. 1), derived from 414 two- to four-cell stage embryos. Expanded blastocysts subjected to preincubation in DNaseI (positive controls) displayed the TUNEL reaction in all nuclei, whereas when terminal transferase was omitted (negative controls), no labelling of any nucleus was observed. Determined was the number of nuclei per blastocyst (by nuclear staining with DAPI) and the number of TUNEL-positive nuclei (fragmented and condensed).

Table 1. The incidence of DNA fragmentation in *in vivo* and *in vitro*-derived porcine expanded blastocysts

Embryos source	No. of blastocysts expanded		No. of cells related to one blastocyst	TUNEL index (%)[1]	No. of TUNEL-positive nuclei related to one blastocyst[2]	TUNEL index related to one blastocyst[3]	No. of TUNEL-positive nuclei related to all blastocysts (%)
Derived *in vivo*	260	mean	89.1^{A}	4.0	3.6^{A}	4.3^{A}	51.9
		SD	13.4^{A}		5.5	7.1	
Derived *in vitro*	273	mean	47.7^{B}	28.3	13.5^{B}	38.6^{B}	94.1
		SD	25.1		7.7	35.0	

[1]No. of TUNEL-positive nuclei, fragmented / (total no. of nuclei) × 100 per all analysed blastocysts.
[2]Nuclei were recorded as positive for TUNEL labelling when they had visible green fluorescence (cells with DNA strand breaks and condensed nuclei).
[3]Mean TUNEL index = (no. of TUNEL-positive nuclei, fragmented) / (total no. of nuclei) × 100 per individual blastocysts
[AB]Within columns means bearing different superscripts differ significantly at $P \leq 0.01$.

The incidence of DNA fragmentation in *in vivo* and *in vitro* expanded blastocysts is presented in Table 1 showing that 3.6 and 13.5 nuclei were found to be TUNEL-positive *in vivo* and *in vitro*, respectively. *In vivo* derived blastocysts contained significantly more nuclei (89.1±13.4) than their *in vitro* counterparts (47.7±25.1; $P<0.01$). Significant differences in the percentage of TUNEL-stained nuclei were observed between the *in vivo*-derived and *in vitro*-cultured blastocysts (4.0 and 28.3%, respectively). The following percentages of expanded blastocysts containing at least one TUNEL-positive nucleus were found: 51.9 and 94.1% in *in vivo* and *in vitro*-derived embryos, respectively (Tab. 1).

From all four groups of *in vivo*-derived expanded blastocysts, the highest total number of cells was found in embryos from III experimental group (93.6±12.6) – Table 2. Significant differences in mean number of TUNEL-positive nuclei per expanded blastocyst were identified between experimental groups. Differences were also observed in percentage of TUNEL index between expanded blastocyts derived from four experimental groups of inseminated gilts (Tab. 2).

Table 2. *In vivo* derived expanded blastocysts characterized in relation to groups of donor gilts

Experimental group of inseminated donor gilts (6 per group)	No. of blastocysts expanded		No. of cells related to one blastocyst	TUNEL index (%)[1]	No. of TUNEL-positive nuclei related to one blastocyst[2]	TUNEL index related to one blastocyst[3]
I	59	mean	83.2^{B}	6.9	5.7^{Aa}	7.8^{A}
		SD	13.3		7.4	11.0
II	70	mean	86.6^{B}	4.0	3.5^{ABa}	4.3^{B}
		SD	13.6		5.0	6.5
III	62	mean	93.6^{A}	3.1	2.9^{B}	2.9^{B}
		SD	12.6		4.5	4.2
IV	69	mean	92.6^{A}	2.8	2.6^{B}	2.6^{B}
		SD	11.9		4.3	4.0

[1]No. of TUNEL-positive nuclei, fragmented / (total no. of nuclei) × 100 per all analysed blastocysts.
[2]Nuclei were recorded as positive for TUNEL labelling when they had visible green fluorescence (cells with DNA strand breaks and condensed nuclei).
[3]No. of TUNEL-positive nuclei, fragmented) / (total no. of nuclei) × 100 per individual blastocysts
[AB]Within columns means bearing different superscripts differ significantly at: small letters – P≤0.05; capitals – P≤0.01.

The study showed that the total number of nuclei in *in vivo*- and *in vitro*-derived blastocysts was negatively correlated with the number of TUNEL-positive nuclei (r= -0.51; P<0.0001) and the TUNEL index (r= -0.69; P<0.0001), whereas the number of TUNEL-positive nuclei was positively correlated with the TUNEL index (r=0.95; P<0.0001) – data not tabulated.

Significant differences were observed between *in vivo*-produced blastocysts from different experimental groups of inseminated gilts (Tab. 2). The data showed a negative correlation between the total number of nuclei per blastocyst and the TUNEL index (r= -0.25; P<0.0001) and a positive correlation between the TUNEL index and number of TUNEL-positive nuclei (r= 0.96; P<0.0001) – data not tabulated.

The development from the two- to four-cell embryos to the expanded blastocyst stage was different in each of four experimental groups of inseminated gilts (80%, 75.5%, 55.4% and 51.1% in group I, II, III and IV, respectively) – Table 3. The number of nuclei in each blastocyst was different within individual experimental groups. Moreover, blastocysts consisting of a high number of nuclei, presented a low incidence of DNA fragmentation (TUNEL index for I experimental group – 58.8 and 17.2%, and for II experimental group – 55.3, 24.8%, respectively) – Table 3. The data showed a negative correlation between the total number of nuclei per blastocyst and the TUNEL index (r = -0.56; P<0.0001) and a positive correlation between the TUNEL index and number of TUNEL-positive nuclei (r = 0.72; P<0.0001) – data not tabulated.

Table 3. Effect of *in vitro* culture on porcine blastocyst development and the incidence of DNA fragmentation

Experimental group of inseminated donor gilts (6 per group)	Total no. of 2-4 cell stage embryos	No. of blastocysts expanded (%) of total		No. of cells related to one blastocyst	TUNEL index (%)[1]	No. of TUNEL-positive nuclei related to one blastocyst[2]	TUNEL index related to one blastocyst[3]
I	120	96 (80%)	mean SD	58.8^{A} 22.7	17.2	10.1^{A} 6.3	19.9^{A} 15.4
II	90	68 (75.5%)	mean SD	55.3^{A} 31.4	24.8	13.7^{Ba} 8.1	34.4^{B} 26.9
III	112	62 (55.4%)	mean SD	33.6^{B} 12.9	47.9	16.1^{B} 7.6	54.7^{C} 37.0
IV	92	47 (51.1%)	mean SD	32.8_{B} 12.7	50.6	16.6^{Bb} 7.3	61.4^{C} 47.0

[1]No. of TUNEL-positive nuclei, fragmented / (total no. of nuclei) × 100 per all analysed blastocysts.
[2]Nuclei were recorded as positive for TUNEL labelling when they had visible green fluorescence (cells with DNA strand breaks and condensed nuclei).
[3]No. of TUNEL-positive nuclei, fragmented) / (total no. of nuclei) × 100 per individual blastocysts.
[AB]Within columns means bearing different superscripts differ significantly at: small letters – P≤0.05; capitals – P≤0.01.

The TUNEL method has been used by several laboratories to assess DNA fragmentation in mammalian embryos [Hardy *et al*. 2003, Kamjoo *et al* 2002, Paula-Lopes and Hansen 2002, Pomar *et al*.2005]. The incidence of DNA-fragmented nuclei may be a good indicator of health status of *in vivo*- and *in vitro*-derived blastocysts, especially in relation to the culture conditions [Fabian *et al.* 2005]. This will yield information about the competence of the embryos, their implantation ability and future growth [Pomar *et al*. 2005].

The number of cells and number of TUNEL-positive nuclei in the embryos are important indicators of embryo development and health [Pomar *et al*. 2005]. It is thought that embryos with a large number of cells are more likely to implant and give rise to live offspring [Long *et al.* 1998].

In this study the TUNEL-positive nuclei were observed in 51.9% of all *in vivo*-produced blastocysts (Tab. 1). These results are lower than those reported by Fabian *et al*. [2005] in which nuclear changes were observed in approximately 71% of all *in vitro*-derived porcine blastocysts. In the present report the total number of nuclei per each *in vivo* expanded blastocyst was 89.1, and the TUNEL index (4%) was similar to the level naturally occurring in blastocysts from different species [Mullen and Critser 2004].

The *in vitro* system profoundly affects the competence to form blastocysts, the number of cells of the whole embryo and the incidence of fragmentation. In the present experiment, the mean number of cells per blastocyst was significantly lower in the *in vitro*-derived blastocysts (47.7±25.1) than in those derived *in vivo* (89.1±13.4). The lower cell number of embryos developed *in vitro* is not surprising. Papaioannou *et al.* [1988] reported that cultured embryos were retarded in terms of development of total cell number compared to *in vivo* embryos of the same chronological age.

The results presented here show that TUNEL-positive nuclei occurred in 94.1% of all *in vitro*-derived blastocysts. This result is similar to that reported by Long *et al.* [1998], in which similar nuclear changes were observed in approximately 90% of all *in vitro*-derived porcine blastocysts. Approximate values of the TUNEL index in this experiment are higher than those given by Pomar *et al*. [2005] who reported 7.3% for all *in vitro*-derived blastocysts. Significant differences in the TUNEL index were observed between the groups of *in vitro*-cultured embryos. Moreover, in this study, the number of nuclei was higher (58.8) – Table 3 – in some of the *in vitro*-cultured porcine blastocysts, and these embryos had fewer TUNEL-stained nuclei (10.1) compared to another *in vitro*-cultured blastocysts with fewer total (32.8) and TUNEL-stained nuclei (16.6). Probably this observation resulted from additional influences of the culture conditions and the developmental abilities of these embryos. It was shown that culture conditions have a negative effect on cell number and the incidence of nuclear fragmentation in *in vitro*-derived expanded *vs*. in vivo-derived expanded blastocysts.

Significant differences in cell number per blastocyst, TUNEL-positive nuclei and the TUNEL index were also observed in *in vivo*- and *in vitro*-derived expanded blastocysts among different experimental groups of inseminated gilts (Tab. 2 and 3). Probably, these differences were associated with the individual quality of superovulated gilts per experimental group although they were at the same age, of similar body condition and were fed the same diet.

The study showed that the total number of nuclei was negatively correlated with the number of TUNEL-positive nuclei and the TUNEL index, whereas the number of TUNEL-positive nuclei was positively correlated with the TUNEL index. DNA fragmentation and the values of the TUNEL index occurred higher in *in vitro*-cultured embryos then in those derived *in vivo*. A relationship was identified between the progression of these changes and the quality of the embryos obtained.

These results allow a conclusion that the TUNEL procedure can be used as an additional criterion for evaluating the quality and developmental capacity of preimplantation pig embryos.

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Ocena fragmentacji jądrowego DNA w zarodkach świni w stadium ekspandującej blastocysty uzyskanych *in vivo* i *in vitro*

S t r e s z c z e n i e

Celem pracy była ocena jakości zarodków świni uzyskanych *in vivo* i hodowanych *in vitro*, ocenianych na podstawie fragmentacji DNA z zastosowaniem metody TUNEL i całkowitej liczby jąder (wyznakowanych barwnikiem DAPI). Badania przeprowadzono na 533 zarodkach w stadium ekspandującej blastocysty, a uzyskane wyniki szacowano testem Fishera. Istotnie wyższą całkowitą liczbę jąder i wielkość indeksu apoptotycznego zaobserwowano w zarodkach poddanych hodowli *in vitro* (odpowiednio 47,7±25,11 i 28,3%) w porównaniu z zarodkami uzyskanymi *in vivo* (odpowiednio 89,1±13,45 i 4%). Uzyskane wyniki wskazują też, że niskiej całkowitej liczbie jąder towarzyszy nasilenie fragmentacji DNA zarówno w grupie zarodków uzyskanych *in vivo* jak i *in vitro*. Analiza zarodków wykazała ponadto ujemną korelację między całkowitą liczbą jąder a liczbą jąder apoptotycznych (r = -0,51, P<0,0001) i indeksem apoptotycznym (r = -0,69, P<0,0001). Stwierdzono dodatnią korelację między liczbą jąder apoptotycznych a indeksem apoptotycznym (r = 0,95, P<0,0001). Dodatkowo wykazano istotne różnice między jakością zarodków (*in vivo* i *in vitro*) uzyskanych w poszczególnych doświadczeniach.