

## Effect of plant protein supplementation on *in vitro* development of porcine embryos

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The aim of the study was to investigate the possibility of using plant protein (PP) substitute instead of bovine serum albumin (BSA) in the culture of porcine embryos *in vitro*. The experiment was done on pig zygotes collected from superovulated gilts at 24-26 h after insemination. Zygotes were cultured *in vitro* in NCSU-23 medium supplemented with 0.004 g PP/ml (experimental group) or 0.004 g BSA/ml (control group). Embryo quality criteria were: cleavage, morula and blastocyst rates, timing of development, total cell number per blastocyst and degree of apoptosis assessed by TUNEL method. Results were analysed by chi-square and ANOVA tests. There were no differences in cleavage rate between embryos cultured in NCSU-23 medium supplemented with PP (88.1%) and BSA (87.7%). The percentage of embryos developed to the morula and blastocyst stage was 83.4 and 67.7 for experimental group (PP) and 76.6 and 61.7% for control group (BSA), respectively (intergroup differences not significant). Timing of development of embryos for group PP and BSA was on the same level. There was no differences in total number of cells per blastocyst between experimental and control groups. Differences were noticed ( $P < 0.05$ ) in the apoptotic index between experimental (19.7%) and control group (11.2%). It is concluded that the possibility exists of using plant protein in *in vitro* culture of pig embryos. Further studies to optimize the concentration of PP in culture medium and to examine the *in vivo* developmental potential of porcine embryos cultured in medium with PP are required.

**KEY WORDS:** apoptosis / embryo / *in vitro* culture / pig / plant protein /

*In vitro* culture of embryos is a method which has found very wide application in biotechnology of reproduction. Outside mother's body, embryos must develop in environmental conditions that enable subsequent mitotic divisions until they reach the

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most advanced preimplantation stage known as the blastocyst. At present, embryos can be produced using three different culture systems: culture in reproductive organs of intermediate recipients, co-culture with somatic cells or tissues and *in vitro* culture in defined media.

In the first experiments on *in vitro* culture of pig embryos it was demonstrated that their development is limited and only a small proportion of zygotes reach the 4-cell stage [Davis and Day 1978]. The progress in *in vitro* culturing of porcine embryos was noticed when culture medium based on a simple Krebs-Ringer fluid was used. [Davis 1985]. In subsequent studies, the culture medium was supplemented with bovine serum albumin (BSA) resulting in the increase of developmental potential of early-stage porcine embryos [Youngs and McGinnis 1990]. Addition of oviductal fluid also had a positive effect on the development and quality of porcine blastocysts produced *in vitro* [Lloyd *et al.* 2009]. In the nineties, porcine embryos were cultured *in vitro* using chemically defined media such as Whitten's medium, Eagle's medium (MEM) or Brinster's medium (BMOC-3) – [Petters and Wells 1993], Tyrode's medium (TL) – Hagen *et al.* [1991], Beltsville's medium (BECM-3) – [Dobrinisky *et al.* 1996], Porcine Zygote Medium (PZM) – [Yoshioka *et al.* 2002] and North Carolina State University media – 23 and NCSU-37) – [Petters and Wells 1993]. Chemically defined medium enables to analyse inorganic components, energy sources, hormones and vitamins active during preimplantation development of embryos while eliminating the effect of non-specific factors present in serum. Very important components of culture media are animal proteins such as serum or bovine serum albumin. Serum proteins are responsible to acid-alkali stability and osmotic pressure while being a source of amino acids. As many as 60% of serum proteins are albumins, which are responsible for hormone and fatty acid transport, and for stabilization of osmotic pressure and pH.

Experiments on porcine embryos [Gajda *et al.* 2008] showed that protein in the form of bovine serum albumin (BSA) or foetal calf serum (FCS), supplemented to culture medium, increases the percentage of *in vitro* blastocysts obtained compared to medium without protein supplementation. Efforts to replace bovine serum in *in vitro* culture medium with macromolecules such as polyvinyl alcohol (PVA) or polyvinylpyrrolidone (PVP) failed to produce positive results because the percentage of blastocysts obtained *in vitro* was lower than in medium with animal protein [Orsi and Leese 2004, Lim *et al.* 2007].

The fact that it is not possible to standardize the conditions of *in vitro* culture with animal protein supplementation and concern about virus or prions transmission [Lane *et al.* 2003] have increased interest in developing chemically defined medium containing substitute of animal origin components. It is now believed that an alternative are plant proteins which basic function in tissues is to regulate osmotic pressure and to bind nutritive and regulatory elements. The most common animal proteins substitutes are plant proteins such as plant peptones, which are formed during partial hydrolysis of plant proteins. They contain numerous free amino-acids, short oligopeptides, some vitamins and sometimes carbohydrates. Depending on origin

and degree of hydrolysis, plant peptones can provide nutrients and similar growth factors [Heidenmann *et al.* 2000]. Research showed that hydrolyzed plant proteins have antiapoptotic properties [Schlaeger 1996], and some peptides have a protective function over lipids and metal ions [Siemensma *et al.* 2002]. In earlier research on the possibility of using plant protein in medium to culture somatic mammalian cells *in vitro* used were rice and soybean peptones [Heidemann *et al.* 2000, Chun *et al.* 2007], or wheat and cotton proteins to culture bovine embryos *in vitro* [George *et al.* 2009], and wheat peptones to cryoconserve bovine embryos [George *et al.* 2006]. The use of medium with plant protein additive will make it possible to create reproducible culture conditions, eliminate the possible presence of non-specific or pathogenic factors and reduce the cost of *in vitro* embryo culture.

This study attempted at replacing bovine serum albumin with plant protein in NCSU-23 medium to culture porcine embryos *in vitro* and to evaluate the quality of blastocysts obtained *in vitro* based on the timing of development, total cell number per blastocyst and apoptosis rate.

### **Material and methods**

All inorganic and organic compounds used in this study were purchased from SIGMA Chemical Company, St Louis, MO, USA), unless otherwise indicated.

#### **Embryo donors**

Six-month-old gilts at 90-110 kg body weight were used as donors, superovulated by i.m. injection of 1500 IU of PMSG (Serogonadotropin, BIOWET, Poland) followed by 1000 IU of hCG (Biogonadyl, BIOMED, Poland) administered 72 h later. At the onset of oestrus (24 h after hCG administration) the gilts were artificially inseminated twice at 12 h intervals with the standard dose of semen. The gilts were operated 24-26 h after insemination.

#### **Obtaining embryos**

Embryos were obtained surgically by flushing oviducts with PBS medium supplemented with 20% foetal calf serum (FCS) at about 30°C. The obtained embryos were examined morphologically under a stereomicroscope, in a laminar chamber at 30°C.

#### **Embryo culture *in vitro***

The culture medium NCSU (North Carolina State University)-23 [Petters and Wells 1993] was prepared in Institute's laboratory and its components were dissolved in H<sub>2</sub>O. Prior to the use the media were filtered through the Millipore 0,22 µm filter (MILLEX-GS, France) to plastic 4-well dishes (NUNC, Denmark), to contain about 0.7 ml of the medium. Ten to twenty zygotes were placed in each well. Then the dishes with embryos were placed in CO<sub>2</sub> incubator (5% CO<sub>2</sub> in air at 39°C) for 96 to 168 h.

Morphologically normal zygotes or 2-cell embryos were cultured *in vitro* in NCSU-23 medium supplemented with 0.004 g Plant Protein /ml (Plant Protein – Animal Farma BV, The Netherland) in experimental group, or 0.004 g/ml bovine serum albumin (BSA) in control group. The PP is a mixture of several plant proteins and soya lecithin prepared using a high pressure homogenization process.

#### Embryo evaluation

During culture the embryos were assessed for morphological stage of development 1-7 days after being transferred to culture media. The assessment was made every day under the stereomicroscope. The assessment criteria were successive stages of embryos development: 2-4 blastomeres, 8-16 blastomeres, morula and blastocyst.

#### Evaluation of blastocyst quality

- a. On the basis of timing of development.** Timing of development of embryos to the blastocyst stage in experimental and control groups was determined by total number of expanded blastocysts developed on day 6, 7 and 8.
- b. On the basis of DNA-fragmented nuclei.** DNA fragmentation of embryos was analysed by 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, expanded blastocysts were fixed for 1 h at room temperature in 4% paraformaldehyde in PBS. The embryos were washed three times in 50 µl drops of PBS-PVP (1 µg/ml polyvinylpyrrolidone PBS) then permeabilized with 0.5% Triton X-100 in PBS for 30 min at the room temperature in a humidified box and washed again in drops of PBS-PVP solution. Then, the embryos were incubated in fluorescein-conjugated dUTP and TdT (TUNEL reagent; In Situ Cell Detection Kit, Roche Diagnostics, Germany) for 1 h at 38,5°C and 5% CO<sub>2</sub> in air. The number of cells with DNA-fragmented nuclei was counted under a microscope with epifluorescent illumination (NIKON Eclipse 600) at a wavelength of 520 nm (green fluorescence of apoptotic cells).
- c. On the basis of total cell number.** After TUNEL, the embryos were washed three times in drops of PBS-PVP solution and transferred through a gradient (50-70-100% in PBS) of Vecta-Shield with DAPI (VECTOR Laboratories, Burlingame, USA) in order to visualize blue fluorescence of all cells in expanded blastocysts. The total number of cells was counted under a microscope with epifluorescent illumination (NIKON Eclipse 600) at a wavelength of 358-461 nm (blue fluorescence of all nuclei in embryos).
- d. On the basis of DNA-fragmented nucleus index.** The total number of cells and number of cells with DNA-fragmented nucleus were counted, and DNA- fragmented nucleus index was calculated by dividing the number of cells with fragmented DNA by the total number of cells, which included DNA-fragmented nuclei.

### Statistical

Results were analysed with chi-square and ANOVA tests.

### Results and discussion

The first experiments concerning *in vitro* culture of mouse embryos [Whittingham 1969] showed a beneficial effect of bovine serum albumin (BSA) supplementation on the inhibition the first cleavage division. Successive studies confirmed this effect on *in vitro* developmental competence of bovine [Kircher *et al.* 1999] and porcine embryos [Youngs and McGinnis 1990, Bavister 1995, Gajda and Smorağ 2004, Gajda *et al.* 2008]. It was found that medium supplemented with albumin has a beneficial effect on pig embryos, as resulted in total cell number per blastocyst and apoptosis digree [Kircher *et al.* 1999, Gajda *et al.* 2008]. Due to interest in the development of chemically defined media, attempts were also made to replace animal protein with high-molecular weight synthetic substances such as polyvinylpyrrolidone (PVP), polyvinylalcohol (PVA) – Orsi and Leese [2004], Lim *et al.* [2007] – and aspartic acid [Spate *et al.* 2009]. The literature contains little information concerning the supplementation of *in vitro* culture medium with plant protein. The first experiments in which bovine serum albumin was replaced with plant protein in medium for bovine embryo cryoconservation were performed by George *et al.* [2006]. Recently, a replacement of BSA with plant proteins in medium for *in vitro* culture of bovine embryos has been reported[George *et al.* 2009].

In the present study the possibility was demonstrated of using plant proteins as substitute of animal proteins in development of porcine embryos from zygotes to blastocyst stage *in vitro*. Obtained was similar percentage of cleaved embryos in medium supplemented with plant proteins (PP group, 88.1%) and animal proteins (BSA, control group, 87.7%) – Table 1, whereas percentage of morulae and blastosysts cultured *in vitro* in medium with PP was slightly higher (83.4 and 67.7%) compared to control group (76.6 and 61.7%, respectively), difference not-significant. George *et al.* [2009] showed a beneficial effect of plant protein supplementation on first divisions of bovine embryos. On the other hand, they reported slightly lower percentage of

**Table 1.** *In vitro* development of porcine embryos cultured in NCSU-23 medium supplemented with plant protein (PP, experimental group) and bovine serum albumin (BSA; control group)

Group	No. of embryos cultured per replic.	No. of embryos cleaved (%)	No. of morulae (%)*	No. of expanded blastocysts (%)*
Experimental (NCSU-23+PP)	151/8	133 (88.1)	111 (83.4)	90 (67.7)
Control (NCSU-23+BSA)	146/8	128 (87.7)	98 (76.6)	79 (61.7)

\*number of embryos cleaved = 100.

blastocysts developed in medium with plant protein (49 vs. 61%), regardless of concentration, compared to medium with animal protein (66 vs. 70%).

Loneragan *et al.* [2003] showed that the timing of embryo development *in vitro* may be one of the factors determining the possibility of full *in vivo* development. Results of the present study demonstrate that regardless the type of protein used for supplementation (PP or BSA), most porcine embryos cultured *in vitro* reached the blastocyst stage on day 6 (51.5%) or 7 (37.3%). There was a difference ( $P < 0.01$ ) in proportion of expanded blastocysts developed between 6 and 8 or 7 and 8 days of culture (51.1 and 11.2 % or 37.3 and 11.2%, respectively). The timing of embryo development observed in the present study in experimental group occurred similar to that found in control groups. The study of George *et al.* [2009] indicated that most of bovine blastocysts developed on day 7 and 8 of *in vitro* culture. In addition, the mentioned authors found no differences in the timing of development between embryos cultured with PP or BSA supplement..

It is known that blastocyst cultured *in vitro*, characterized by a normal number of cells result in full development *in vivo* [Rubio Pomar *et al.* 2005]. Several authors reported that blastocysts derived from *in vitro* culture showed a lower total cell number compared to non-cultured blastocysts. The lower quality of embryos obtained *in vitro* may result from suboptimum culture conditions [Papaioannou and Ebert 1988]. Moreover, reduced is the ability of embryos to implant. On the other hand, it was demonstrated that a similar proportion of pregnancies could be obtained after transfer of porcine embryos cultured in NCSU-23 medium for 4 days as after transfer of non-cultured embryos [Prather and Day 1998]. This is the evidence of the possibility of full *in vivo* development of pig embryos cultured in NCSU-23. In the present experiment, a similar mean number of cells was found in blastocysts cultured in NCSU-23 medium supplemented with PP or BSA (28.2 and 31.5, respectively) – Table 2. Similar observations for the total cell number in pig blastocysts cultured in NCSU-23 medium with BSA were made by Gajda *et al.* [2008], while Bryła *et al.* [2009] observed higher total cell number (47) in cultured porcine embryos. However, in the present study as well as in that of George *et al.* [2009], there was no difference in total cell number in blastocysts cultured in medium supplemented with plant or animal protein.

The evaluation of embryos quality with TUNEL method was based on analysis of expanded blastocysts (Photo 1). This stage is characterized by full activation of the embryo genome, which enables apoptotic changes to be estimated. In this study the TUNEL-positive nuclei were observed in 95% of all *in vitro* cultured blastocysts. These results are similar to those reported by Long *et al.* [1998]. In the present study a difference was observed between experimental and control group in the number of apoptotic nuclei (5.5 and 3.3,  $P < 0.01$ , respectively) and in apoptotic index (19.7 and 11.2%,  $P < 0.05$ , respectively) – Table 2. Meanwhile, for bovine embryos George *et al.* [2006] reported no difference in apoptotic index between blastocysts cultured in medium with plant or animal protein.

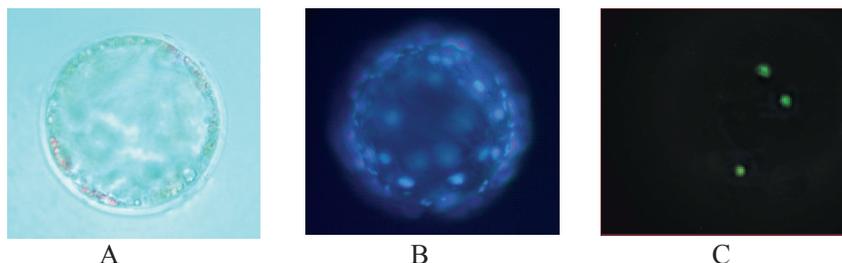


Photo 1. Pig expanded blastocyst from the control group. A – Normal light microphotograph (x100). B – DAPI staining for total number of cells counted. C – TUNEL staining for number of cells with DNA-fragmented nucleus counted.

**Table 2.** The quality of porcine expanded blastocysts cultured in NCSU-23 medium supplemented with plant protein (PP, experimental group) and bovine serum albumin (BSA, control group)

Group	No. of blastocysts assessed per replic.	Mean number of cells per blastocyst	Mean number of apoptotic nuclei per blastocyst	Apoptotic index (%)
Experimental (NCSU-23+PP)	20/4	28.2±7.53	5.5 <sup>A</sup>	19.7 <sup>c</sup>
Control (NCSU-23+BSA)	12/3	31.5±7.86	3.3 <sup>B</sup>	11.2 <sup>d</sup>

<sup>aA</sup>–Means in columns bearing different superscripts are significantly different: capitals – P≤0.01, small letters – P≤0.05.

In conclusion, porcine embryos can be cultured *in vitro* in NCSU-23 medium supplemented with plant protein in place of bovine serum albumin. Further studies to determine the optimum concentration of plant protein as a substitute for animal protein in medium for *in vitro* culture of porcine embryos that can survive after embryo transfer are needed.

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