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The use of butylated hydroxytoluene in cryopreservation of boar semen*

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The objective of the study was to determine the effect of butylated hydroxytoluene (BHT) on the quality and fertilizing capacity of frozen-thawed (FT) boar semen. Semen from five boars (36 ejaculates) was resuspended in lactose-egg yolk-glycerol extender supplemented with 0 (control), 1.0 (R1), 1.5 (R2) or 2.0 mM BHT (R3). Sperm quality was assessed based on motility (CASA; TM: total motility; PM: progressive motility), phosphatidylserine (PS) translocation across the plasma membrane (Annexin-V-FLuos Staining Kit) and DNA fragmentation (TUNEL Assay). The FT semen was also used for intrauterine artificial insemination (AI) of synchronized gilts. The fertilizing capacity of the FT semen was assessed on the basis of the gilt insemination rate and the number of morphologically normal embryos. The quality of the preimplantation embryos was determined by observing a TUNEL-positive reaction. The highest percentage of progressive motile and viable spermatozoa was noted in extender R3 (74.8 ±4.4% and 63.7 ±5.8%), as compared with the control (38.3 ±2.8% and 36.1 ±2.6%). The addition of BHT to the extender did not increase early apoptotic changes in the frozen-thawed spermatozoa with respect to the control. Irrespective of the variant of the extender, cryopreservation and thawing did not induce fragmentation in the boar spermatozoa. The highest number of morphologically normal embryos from inseminated gilts was observed in the case of semen cryopreserved in extender supplemented with 1.5 mM BHT. No significant differences were observed in DNA fragmentation in the expanded blastocysts from gilts inseminated with FT semen cryopreserved in the extenders analysed.

KEY WORDS: boar / cryopreservation / butylated hydroxytoluene / embryos / TUNEL

Cryopreservation of biological material is one of the technologies used for conservation of genetically determined diversity. Freezing of semen, which until now has

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been the only means of preserving it, enables rational control of reproduction and significantly contributes to breeding progress. Cryopreserved semen can be stored and used use for artificial insemination for a virtually unlimited time. This makes it possible to use genetic material from particularly valuable males in any place and at any time.

Boar sperm are much more sensitive to factors associated with freezing and thawing than the sperm of other farm animals. One reason for this is their high proportion of unsaturated acids, which pose a threat to plasma membrane structure in sperm due to susceptibility to peroxidation. An excess amount of reactive oxygen species (ROS) or exhaustion of the compensatory potential of the antioxidant system in the semen leads to lipid oxidation [5]. This causes a loss of cell membrane integrity, lipid asymmetry, DNA damage, cell death by apoptosis, and reduced biological value of the semen. Adding antioxidant compounds to extenders used in freezing boar semen may minimize the negative effects of ROS and increase the efficiency of cryopreservation [11].

Butylated hydroxytoluene (BHT) is an organic lipophilic compound used for its antioxidant properties in the food, fodder and chemical industries. In animal reproduction it is used to minimize cold shock during cryopreservation of the semen of various animals, including goats [7], bulls [12], dogs [9], water buffalo [6] and boars [11, 13].

The aim of the study was to modify the composition of a freezing extender by adding butylated hydroxytoluene as an antioxidant substance. Semen quality following the freezing and thawing procedure was evaluated by standard methods and with selected apoptotic markers. At the same time, in order to test the fertilization capacity of the boar sperm following cryopreservation, we evaluated the quality of the embryos obtained from gilts inseminated with semen frozen in the modified extenders.

Material and methods

The semen of 5 boars of interbreed crosses (Polish Landrace x Polish Large White) with body weight of 252.5 ±34.6 kg and at the age of 17.8 ±0.8 months was used in the experiment. The boars were kept at the Boar Exploitation Station in Klecza Dolna. Semen was collected twice a week from October to November. In total 40 ejaculates (8 per boar) were studied. For the remainder of the experiment ejaculates were used in which the percentage of sperm with progressive motility was at least 70% and the percentage of sperm with normal morphological structure was at least 80%. Thirty-six ejaculates qualified for further analysis. The boar semen freezing procedure was carried out according to our own method [16].

The semen (sperm-rich fraction) was collected by the manual method on a phantom and diluted 1:1 in Biosolwens Plus extender (Biochefa, Poland). The diluted semen was chilled to 15°C and equilibrated for 60 min. To separate the sperm from the plasma and extender, the semen was centrifuged at 15°C at 800 g for 25 min. The sperm suspension was diluted to a concentration of 1.5×10^9 cells/ml using lactose-egg yolk (LEY) extender. The diluted semen was chilled to 5°C and equilibrated for 120 min. The sperm suspension was diluted

with an extender with glycerol (LEYG: 89.5% LEY containing 9% glycerol and 1.5% Equex-STM®; Nova Chemical Sales, USA) to obtain a final sperm concentration of $1x10^9$/ml and 3% glycerol.

An antioxidant was added to the sperm suspension diluted in the LEY and LEYG extenders to obtain the following final BHT concentrations: 0 mM (control); 1.0 mM (extender R1); 1.5 mM (extender R2); and 2.0 mM (extender R3). Straws 0.5 ml in volume were filled with the diluted semen. The straws were frozen in liquid nitrogen vapour at about –120°C for 15 min in a hard polystyrene foam container. The frozen straws were stored in a container with liquid nitrogen (–196°C) for two weeks. They were thawed in a water bath at 37°C for 40 seconds. Then the contents of the straws were transferred to Biosolwens Plus extender at 37°C.

The cryopreserved semen was evaluated after 15 minutes of incubation at 37°C. Sperm quality was evaluated on the basis of motility, using the CASA system (SM-CMA, MTM, Switzerland) to determine total motility (TM%) and progressive motility (PM%). Sperm quality was also analysed by fluorescence using selected apoptotic markers. Apoptotic changes were evaluated on the basis of identification of phosphatidylserine residues on the surface of the sperm cell membrane using an Annexin V-Fluos Staining Kit (Roche, Germany), distinguishing the following subpopulations of cells: live (AnV^-/PI^-), early apoptotic (AnV^+/PI^-), late apoptotic/early necrotic (AnV^+/PI^+), and dead sperm cells (AnV^-/PI^+) [13]. The degree of DNA fragmentation was evaluated by the TUNEL method using an In Situ Cell Death Detection Kit (Roche, Germany), by determining the percentage of sperm exhibiting DNA fragmentation ($TUNEL^+$) [13]. Individual subpopulations of sperm were observed under a Nikon Eclipse E600 epi-fluorescence microscope (Japan) with appropriate filters: with a wavelength of 520 ±20 nm to detect green fluorescence and with a wavelength of >620 nm to detect red fluorescence. The analysis was performed on 200 sperm from each sample.

Twenty gilts of different breeds (Pietrain x Duroc, Polish Landrace and Polish Large White), at the age of 6 months and body weight from 85 to 90 kg, were selected for insemination with the frozen and thawed semen. The gilts were kept in standard conditions at the National Research Institute of Animal Production Experimental Station in Żerniki Wielkie. The gilts underwent standard synchronization by intramuscular injection with 750 IU of PMSG (Folligon, Intervet B.V., Netherlands). After 72 hours 500 IU of hCG (Chorulon, Intervet B.V., Netherlands) was administered. On the day of onset of oestrus (24 hours after administration of hCG) the gilts were prepared for intrauterine insemination according to our own method [14]. A blunt-tip needle with a syringe containing thawed semen ($1x10^9$ sperm in 3 ml Biosolwens Plus extender) was inserted into each uterine horn (near the tubouterine junction). The gilts were isolated from the rest of the animals and 5.5 days after insemination embryos in the expanding blastocyst stage were obtained. Embryos were obtained by washing the uterus twice with supplemented PBS (Invitrogen, USA) at 38ºC, using about 500 ml PBS for each uterine horn. Embryos were detected and their morphology was evaluated under a stereoscopic microscope (100 x magnifi-

cation). Then the embryos were transferred to PBS supplemented with 20% foetal bovine serum (Sigma, Germany). The embryos were transferred to a laboratory in a special chamber maintaining a constant temperature of 38ºC and the degree of DNA fragmentation was determined.

Consent for the experiment was obtained from the Local Ethics Committee in Krakow based at the Institute of Pharmacology of the Polish Academy of Sciences.

The results of the evaluation of semen quality were analysed statistically by one-way ANOVA (according to the formula $y_{ij} = \mu + a_i + e_{ij}$; where: y – variable evaluated, μ – mean, a_i – type of extender, e_{ij} – random error) using Statistica 10 software (StatSoft, Tulsa, USA). Significance of differences between means in groups was estimated by the Duncan Multiple Range Test. Differences between parameters at a level of $P<0.01$ were considered highly statistically significant. The results of the evaluation of the fertilization rate and embryo quality were analysed by the χ^2 test, and differences at $P<0.05$ were considered statistically significant.

Results and discussion

The results of the evaluation of semen cryopreserved in modified extenders are presented in Table 1. The study showed that the addition of butylated hydroxytoluene to the freezing extender significantly improves sperm motility parameters (TM% and PM%) after the freezing and thawing procedure in comparison with the control. This increase was observed in the case of the 1.0, 1.5, and 2.0 mM addition of butylated hydroxytoluene. However, among these concentrations the highest percentage of sperm with total and progressive motility was obtained following the use of 2.0 mM BHT, i.e. 80.9 ±5.2% and 74.8 ±4.4%, respectively. Both our own research [13] and studies by Bamba and Cran [1] and Roca et al. [11] have shown that adding BHT to freezing extender significantly improves motility of boar sperm following freezing and thawing.

In research on improving cryopreservation techniques it is important to develop methods enabling identification of subpopulations of frozen and thawed sperm with high fertilization capacity. A study by Pena et al. [10] showed that the use of Annexin V and propidium iodine enables precise evaluation of the quality of thawed boar semen. In the present study, statistically significant differences were observed in the percentage of AnV^-/PI^-, AnV^+/PI^-, AnV^+/PI^+ and AnV^-/PI^+ sperm cells between the control and modified extenders. The lowest percentage of early apoptotic sperm (6.8 ±2.3%) and late apoptotic sperm (23.7 ±4.1%) was noted for extender R3. The study indicates that the use of BHT at concentrations from 1.0 mM to 2.0 mM most likely significantly reduces the loss of lipid asymmetry in the sperm cell induced by accumulation of reactive oxygen species during the freezing process in comparison with the control extender containing no antioxidant substances. A study by Roca et al. [11] showed that the use of butylated hydroxytoluene in concentrations ranging from 0.2 to 0.8 mM ensures the highest quality of semen after thawing.

Table 1
The results of quality assessment of boar semen cryopreserved in modified extenders

Sperm quality parameters		Extenders			
		control	R1 extender 1	R2 extender 2	R3 extender 3
Motility	Total motility (%)	42.3 ±5.3	78.3 ±5.2**	77.5 ±4.8**	80.9 ±5.2**
	Progressive motility (%)	38.3 ±2.8	72.4 ±3.5**	71.7 ±4.2**	74.8 ±4.4**
Annexin V Fluos Staining/PI	Viable spermatozoa (AnV^-/PI^-) (%)	36.1 ±2.6	62.8 ±4.2**	63.5 ±5.1**	63.7 ±5.8**
	Early apoptotic spermatozoa (AnV^+/PI^-) (%)	19.8 ±3.8	7.1 ±1.9**	6.9 ±2.1**	6.8 ±2.3**
	Late apoptotic spermatozoa (AnV^+/PI^+) (%)	33.2 ±4.6	24.3 ±6.7**	23.9 ±3.7**	23.7 ±4.1**
	Nonviable spermatozoa (AnV^-/PI^+) (%)	13.4 ±4.4	5.8 ±1.9**	5.7 ±2.1**	5.8 ±1.2**
TUNEL	$TUNEL^+$ (%)	2.1 ±0.9	1.9 ±0.5	2.0 ±0.4	1.8 ±0.2

**Values expressed as means ±SD in rows differ significantly (P<0.01) from control

It was also demonstrated that the cryopreservation procedure does not induce DNA fragmentation in boar sperm in either the control or modified extenders. The level of sperm DNA fragmentation ranged from 1.8 ±0.2% to 2.1 ±0.9%. A similar result was obtained by Chanapiwat et al. [3], who conducted a study on cryopreserved boar semen, and by Martin et al. [8], who evaluated frozen bull semen. On the other hand, Fraser and Strzeżek [4] found that the freezing and thawing process destabilized sperm chromatin structure, which increases susceptibility to DNA fragmentation.

To determine the fertilization capacity of semen cryopreserved in the extenders, the quality of embryos obtained from gilts inseminated with the frozen semen was evaluated. The results are presented in Table 2.

Table 2
Conception rate and quality of embryos obtained from gilts inseminated with cryopreserved semen

	Extenders			
	control	R1 extender 1	R2 extender 2	R3 extender 3
Number of inseminated gilts (head/%)	5	5	5	5
Number of successfully inseminated gilts (head/%)	3 / 60	4 / 80*	5 / 100*	5 / 100*
Number of total blastocysts/blastocysts per gilt	35 / 11.7	54 / 13.5*	70 / 14.0*	69 / 13.8*
Number of morphologically normal blastocysts (no./%)	27 / 77.1	49 / 90.7*	65 / 92.9*	64 / 92.7*
TUNEL$^+$ (%)	2.8	2.1	2.1	2.3

*Values in rows differ significantly (P<0.05) from control

The lowest fertilization rate, 60%, was noted in the gilts inseminated with semen cryopreserved in the control extender. A high fertilization rate was obtained with the semen frozen in extenders R1, R2 and R3: 80%, 100% and 100%, respectively. The most embryos in the expanding blastocyst stage were obtained following insemination of gilts with semen frozen in the extender with 1.5 mM BHT, and 92.9% of these were defined as morphologically normal. Moreover, statistically significant differences in the total number of blastocysts and morphologically normal embryos were noted between the control extender and the other extenders used to inseminate gilts. A study by Roca et al. [11] showed that the use of semen frozen in an extender with BHT for in vitro fertilization increases the developmental competence of the embryo up to the blastocyst stage. At the same time, our own study [15] found a high farrowing rate following insemination with semen cryopreserved in an extender with BHT. Analysis of embryo quality by the TUNEL method showed no statistically significant differences in the degree of DNA fragmentation between blastocysts obtained from gilts inseminated with semen frozen in the control extender and in the extenders containing various concentrations of BHT. The percentage of DNA fragmentation in the embryos was low, ranging from 2.1% to 2.8%.

In conclusion, the results of the study indicate that the addition of butylated hydroxytoluene to freezing extender protects boar sperm against cryogenic damage. The cryopreservation procedure was not found to induce DNA fragmentation in sperm. At the same time, the use of semen cryopreserved in an extender with BHT for insemination ensures a higher fertilization rate and more morphologically normal embryos in comparison with semen frozen in a standard lactose-egg yolk-glycerol extender.

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