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BOAR SEMEN CRYOPRESERVATION – SOME LINES OF RESEARCH*

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W hodowli trzody chlewnej wykorzystanie mrożonego nasienia knura do zabiegów inseminacyjnych ogranicza się do zwiększenia postępu genetycznego zwierząt. Brak praktycznego wykorzystania nasienia mrożonego w porównanie z nasieniem przechowywanym w stanie płynnym wynika z podatności plemników knura na uszkodzenia kriogeniczne i skomplikowaną procedurę mrożenia. Proces schładzania, zamrażania i rozmrażania indukuje fizyczne i chemiczne zmiany w błonie komórkowej plemników. Co więcej, wywołany procedurą kriokonserwacji szok chłodowy i stres oksydacyjny, wpływając na błony plazmatyczne plemników, obniżają ich żywotność i zmniejszają efektywność mrożenia. Ponadto, po użyciu do inseminacji nasienia zamrożonego-rozmrożonego uzyskuje się niskie wskaźniki rozrodcze i mniejszą liczebność miotu. Celem artykułu jest podsumowanie wybranych kierunków badań nad czynnikami, które mogą zwiększyć efektywność kriokonserwacji nasienia knura. Jednocześnie została przedstawiona metoda mrożenia nasienia knura opracowana w Instytucie Zootechniki PIB.

Słowa kluczowe: knur, nasienie, rozcieńczalniki mrożeniowe, kriokonserwacja

Despite numerous studies on the development of an effective cryopreservation method, the use of frozen boar semen is still limited practice-wise and remains below 1% of the total number of performed insemination procedures. Low reproductive rates obtained after insemination of sows with frozen semen are mainly due to the fact that the cellular membranes of boar's spermatozoa are characterized by high susceptibility to cryogenic damage (Groβfeld et al., 2008). Boar spermatozoa are much more sensitive to freezing factors than spermatozoa of other farm animal species. The involvement of polyunsaturated fatty acids in lipids and their specific composition is an important factor causing increased sensitivity of spermatozoa to freezing. Polyunsaturated fatty acids (C22:5 and C22:6) in the lipids of board spermatozoa are responsible for 65% of the total fatty acid content. The quantitative ratio of

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unsaturated to saturated fatty acids in boar spermatozoa is 3.5, while in case of bulls and rams it is 2.8. Osmotic stress, cold shock and formation of ice crystals within the cells cause damage to plasma membranes and other cellular organs of boar spermatozoa organelles (Holt, 2000). As a consequence, following the freezing procedure, the spermatozoa demonstrate changes associated with a decrease in the sphingomyelin content with simultaneous increase in the phosphatidylcholine level. On the other hand, changes in the interactions of plasmalemma lipids in the cryopreserved spermatozoa and shifting their susceptibility to Ca^{2+} ion results in the fertilization process disturbances (Berger et al., 1996).

The first attempts to freeze mammalian semen were made in the eighteenth and nineteenth centuries, but it was only in the twentieth century that effective methods of cryopreservation of the bull semen (Polge & Rowson, 1952) and the stallion semen (Polge, 1957) were developed. In 1957, Hess team first obtained piglets after insemination of sows with thawed boar sperm. Over the years, intensive research on the effective and reproducible method of cryopreservation of boar semen has led to the development of two methods: American (Pursel & Johnson, 1975) and German (Westendorf et al., 1975).

Since then, many research teams have attempted to increase cryopreservation of boar semen by modifying the composition of the freezing extender, as well as developing technological procedures for handling sperm.

This article presents selected issues concerning cryopreservation of boar semen, as well as the freezing method developed at the National Research Institute of Animal Production.

Composition of boar semen freezing extender

The basic composition of the extender used for freezing boar semen contains sugars, cryoprotective substance, egg yolk and sodium dodecylosulphate (SDS) synthetic detergent (Equex STM or Orvus ES Paste). The freezing extender is most often composed of simple sugars (glucose, galactose, fructose, sorbitol) or disaccharides (lactose, lactulose, trehalose, melibiosis). They are not only a source of energy, but also have a positive effect on spermatozoa by protecting them from dehydration and ice crystals formation (Yeste, 2015). Research performed by Gómez-Fernández et al. (2012) shows that the use of disaccharides, especially trehalose, ensures high efficiency of cryopreservation. On the other hand studies by Malo et al. (2010 a) have shown that the use of spermatozoa cryopreserved in freezing extender containing trehalose for *in vitro* fertilization ensures higher fertilization rate (44.8%) compared to extender with lactose (28.6%) or glucose (34.4%).

Protective compounds are used in order to protect plasma membranes of spermatozoa from cryogenic damage, including: dimethylformamide, dimethylacetamide, dimethylsulfoxide, and glycerol. As can be seen from the studies conducted by Buhr et al. (2001), glycerol with a concentration of more than 4% affects negatively the changes in the lipid structure of spermatozoa

plasmalemma, reducing the effectiveness of cryopreservation. Glycerol added directly before freezing stage with a concentration of 3% (Holt, 2000) or between 2% and 3% (Zeng et al., 2014) ensures optimal protection of spermatozoa cell membranes against cryogenic lesions.

In order to maintain the appropriate osmotic pressure and reduce sperm cell membrane damage caused by free oxygen radicals (ROS), low-density lipoproteins (LDL) of bird egg yolk origin are added to the freezing extender, In order to protect the sperm from cold shock, the addition of egg yolks to the freezing extender is most often used. However, experiments carried out by Fraser & Strzeżek (2005) have shown that LDL extracted from African ostrich egg yolk (LPFo) reduce the negative effects of cold shock on boar spermatozoa to a greater extent than the LDL extracted from hen egg yolk. The addition of LPFo to freezing extender increases the percentage of spermatozoa with integral DNA (24.4%), compared to standard extender containing hen egg yolk (17.8%). Currently, studies conducted by Wang et al. (2014) comparing the effect of LDL fraction extracted from egg yolk of various species of birds (hen, ostrich, duck, quail, pigeon) demonstrated that the addition of lipoprotein of pigeon yolk to the freezing extender allows to obtain 43.2% of mobile spermatozoa after thawing, 52.57% of live spermatozoa with integral acrosome, and 48.13% of spermatozoa with integral cell membrane.

Regardless of the lipoprotein source used during the cryopreservation process, lipoprotein aggregates are formed after mixing bird egg yolk with semen plasma proteins (Holt, 2000). Because of that, the addition of sodium dodecylosulphate synthetic detergent (SDS) (Equex STM or Orvus ES Paste) is used in the composition of the freezing extender in order to limit this phenomenon (Shimazaki et al., 2015). At the same time, studies are conducted to replace this component of the freezing extender with plant proteins (Masoudi et al., 2016).

Over the last years a lot of work has been devoted to modification of the composition of the freezing extender based on the addition of substances with an antioxidant effect and shielding the spermatozoa plasma membranes. The addition of antioxidant compounds reduces the negative effects caused by reactive oxygen species (ROS) and contributes to the increase in cryopreservation efficiency. The most common antioxidants used to improve the quality of boar's sperm after thawing include glutathione (Zhang et al, 2012); α-tocopherol (Satorre et al., 2012); superoxide dismutase and/or catalase (Roca et al., 2005); ascorbic acid in combination with glutathione (Giaretta et al., 2015). The addition of butylated hydroxytoluene (Roca et al., 2004) and L-cysteine (Malo et al., 2010 b) to freezing extender allows to obtain higher percentage of spermatozoa with integral acrosome compared to control group (60.7% vs. 44.07% and 61.2% vs. 46.0%. Hyaluronic acid (Peña et al., 2004) and gamma-oryzanol (Kaeoket et al., 2012) are compounds presenting properties of plasma membrane protection of spermatozoa, which found use in cryopreservation of boar semen. At the same time, enrichment of the freezing extender with 1,000

µg/ml of hyaluronic acid or 0.1 mg/ml of gammae-orynazol increases the percentage of spermatozoa with a progressive movement after thawing compared to the extender with no addition (54.8% vs. 31.9% or 48.5% vs. 34.0%.

Cryopreservation technology for boar semen

In Poland, the first attempts to develop a method of cryopreservation of boar semen were made in Poznań and Olsztyn centers. In 1985, the team was lead by prof. Jerzy Strzeżek presented a comprehensive method of boar semen cryopreservation called the "Kortowo method of boar semen freezing" (Strzeżek et al., 1985). As a result of gilt insemination with frozen semen using the Kortowo method, a farrowing index of 77.7% and an effective reproductive rate of sows with 11.35 live piglets in litter were obtained (Strzeżek, 2011).

Despite technological differences, all cryopreservation methods consist of similar stages of semen handling: collection, cooling, thickening, equilibration, glycerolisation, freezing and thawing.

For cryopreservation, the dense fraction of ejaculate is used, and the use of the first 10 ml of this fraction for freezing (Saravia et al., 2010) and the uptake of the ejaculate in winter and spring period (Barranco et al., 2013) increases the effectiveness of this process. The dense fraction must be stored until the procedure start at 15–17°C (Rodriguez-Gil, 2006; Casas & Althouse, 2013). The minimum recommended storage time for boar semen in commercially available extenders is 1 to 3 hours, but most freezing protocols suggest that this time should be extended even up to 24 hours. As reported by Casas and Althouse (2013), this treatment may additionally stabilize lipids in cell membranes of boar spermatozoa, improving the effectiveness of cryopreservation.

Ejaculates, in which a minimum of 70% of mobile spermatozoa and 80% of morphologically normal spermatozoa are found are qualified for cryopreservation. At the same time, due to individual variability in semen freezing between boars, an additional selection of individuals is introduced based on the mobility of sperm evaluated after thawing. Roca et al. (2006) divide boars into three groups with high ("good freezers"), medium ("moderate freezers") and low ("poor freezers") freezing utility, in which the mobility of sperm after thawing is >60%, 40–60% and <40%, respectively. The use of the first group of boar sperm for insemination allows higher fertility rate (53.8%) compared to the results obtained after insemination with lower quality semen (26.3%) (Casas et al., 2009). At the same time, studies carried out by Hernández et al. (2007) have shown that the addition of 5% high quality boar sperm plasma to the freezing extender reduces spermatozoa susceptibility to cold shock. Latest researches carried out by Fernández-Gago et al. (2016) demonstrated that a 4-hour incubation of spermatozoa after thawing in the extender with 50% semen plasma addition reduces the susceptibility of sperm chromatin to damage during the cryopreservation process.

Another technological solution for the cryopreservation of semen is to use

semen dialysis as a step in the process. Dialysis can be used to reduce peptide substances and free inorganic ions which are harmful to the spermatozoa. According to studies conducted by Fraser et al. (2007), the use of dialysis results in a higher percentage of spermatozoa with integral cell membranes (52.8%) after thawing compared to semen not subjected to dialysis (43.7%).

Correct selection of the semen packaging method is an important step in the cryopreservation process. This may include large rounded-macro straws and micro-tubes, flat straws, spheres or plastic bags. Straws with a smaller capacity (0.5 ml) are used at the moment, reducing differences in the flow rate of the freezing and thawing temperatures within the straw by increasing the surface to volume ratio (Didion et al., 2013). The recommended freezing temperature for semen in such straws is 30°C/min at 3% glycerol (Fiser & Fairfull, 1990). The use of 0.5 ml straws increases the viability of spermatozoa after thawing in comparison to frozen and thawed spermatozoa in large straws of 5–6 ml capacity. The freezing temperature in these straws should be 16°C/min at 3.3% glycerol concentration (Park & Pursel, 1985).

Comprehensive technology of boar semen cryopreservation developed in National Research Institute of Animal Production

Researches involving the composition of the freezing extender, as well as the proceedings with boar semen during freezing have been conducted in the Department of Animal Reproduction Biotechnology at the National Research Institute of Animal Production for several years. Conducting the works was possible within the framework of the research project No. N311 524840: “Antioxidants and new shielding compounds in cryopreservation of boar semen evaluated with the use of apoptotic markers”, as well as statutory activities of the Institute. The improvement in cryoconcentration efficiency for boar semen was planned to be achieved through the addition of antioxidant substances and substances with shielding properties protecting spermatozoa plasma membranes sensitive to cryogenic damage. The studies included supplementation of yolk-lactose-glycerol extender with selected antioxidants (glutathione, butylated hydroxytoluene, superoxide dismutase and/or catalase) and the sodium salt of hyaluronic acid as a protecting substance. The quality of semen after freezing-thawing was assessed on the basis of the percentage of spermatozoa showing total and progressive movement, the percentage of live spermatozoa evaluated with apoptotic markers (YOPRO-1/PI, Annexin V-Fluos/PI), the percentage of live spermatozoa with integral acrosome (PNA-FITC/PI), spermatozoa with a high mitochondrial potential (JC-1), and the percentage of spermatozoa not demonstrating DNA fragmentation (TUNEL). Based on the conducted studies (Trzcińska & Bryła, 2015; Trzcińska et al., 2015; Bryła & Trzcińska, 2014), a new composition of freezing extender and boar semen cryopreservation technology has been developed. The new developed extender for cryoconservation of boar semen contains the following components:

– ethanol-extracted hydroxytoluene (BHT; 2,6-bis(1,1-dimethylethyl)-4-

methylphenol) added in the amount of 1 mmol to thinner containing glycerol;

– yolk-lactose extender with composition of 80% lactose and 20% egg yolks;

– extender containing glycerol as a shielding compound obtained by adding 9% glycerol and 1.5% Equex-STM® to 89.5% yolk-lactose extender.

The technology of boar sement cryopreservation according to own method is presented in the implementation manual (Trzcińska et al., 2013). Frozen semen was stored in liquid nitrogen (-196°C) for two weeks (Trzcińska et al., 2015). In order to determine the effectiveness of the used freezing extender, a method of surgical insemination of the gilts was developed for the new technology of boar semen cryopreservation (Trzcińska et al., 2014). Thanks to this insemination method, it was possible to introduce a small dose of thawed semen (1×10^9) into the female reproductive tract. At the same time, in order to optimize fertility results, the procedure of gilt insemination with cryopreserved semen was performed approximately 4–6 hours before ovulation. Performed inseminations with cryopreserved semen in a yolk-lactose-glycerol extender with addition of 1 mM butylated hydroxytoluene allowed to obtain a fertility index of 86.7% and an average of 10.8 ± 1.6 piglets in the litter. Lower fertility rates and mean number of piglets in the litter after insemination with cryopreserved semen were obtained by both Roca et al. (2003) (70% and 9.25±0.23) and Bolarin et al. (2008) (71.3% and 10.3±0.3). On the other hand, after insemination with frozen semen in the extender with the addition of 2 mM glutathione, Estrada's team (2014) achieved a higher fertility rate (92.7%) and a higher number of piglets born in the litter (13.0 ± 1.0).

The developed own method of boar semen freezing allows to obtain satisfactory fertility rates comparable to those obtained after liquid semen insemination (Trzcińska et al., 2011; Bryła & Trzcińska, 2015) and can find use in animal biological material banks.

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Boar semen cryopreservation – some lines of research

SUMMARY

In commercial pig production, frozen-thawed boar semen for artificial insemination is mostly used to improve the genetic progress. This semen has not been used under production conditions as efficiently as liquid-preserved semen, due to the high susceptibility of boar spermatozoa to damage during cryopreservation and a complicated process of deep freezing. The processes of cooling, freezing, and thawing produce physical and chemical stress on the sperm membrane. Moreover, the cryopreservation protocol produced cold shock and oxidative stress on the sperm membrane, which decreased sperm survival and freezing effectiveness. Therefore, the artificial insemination with frozen-thawed boar semen still results in low conception rate and small litter sizes. The purpose of this paper is to summarize selected knowledge about factors that may contribute positively to increasing the effectiveness of boar semen cryopreservation. The review also represents the methodology of boar semen cryopreservation developed by the National Research Institute of Animal Production.

Key words: boar, semen, freezing extenders, cryopreservation