Boar freeze-dried semen in medium with antioxidants evaluated based on DNA integrity after a long-time preservation

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Abstract
Sperm freeze-drying is considered an alternative method to preserve male gametes in refrigeration or at room temperature condition. In order to preserve sperm integrity special protection is required. The aim of our research was to examine the effect of vitamin C (0.5 mM) and rosmarinic acid (105 µM) on the DNA spermatozoa integrity after freeze-drying and 36 months of preservation at refrigerator temperature. Our results indicates that more than 90% of DNA boar spermatozoa integrity is not affected by long-time preservation with small differences between experimental groups: with +0.59% higher DNA integrity in AR group from Duroc boar, with +2.83% higher DNA integrity in AR group from Landrace boar and with no differences regarding DNA integrity in group supplemented with vitamin C. The main conclusion of these preliminary results is that DNA integrity of boar freeze-dried semen is not affected by long-time preservation and it can be used further for intracytoplasmatic sperm injection technique.
***Key words**: DNA-integrity, freeze -dry, antioxidants, boar semen*

Introduction

There are several methods by which semen can be preserved. Some of them are used in current practice, others used only in research. Among the cryopreservation methods there are: slow freezing, fast freezing, vitrification (ultra-fast freezing), freezing of a small number of sperm and lyophilization. Lyophilization (freeze-drying) is a technique that does not require liquid nitrogen, the samples can be stored at refrigerator temperature, they can be transported at room temperature. DNA damage is much lower than in conventional freezing techniques, but sperm are immobile, so they can only be used in fertilization by the intracytoplasmic sperm injection (ICSI) technique (Hazavehei et al.,2018).

Semen lyophilization was applied to several species, such as mouse, rat, cat, rabbit, boar, monkey with different results of the percentage of blastocysts obtained and gestation rates (Patrick et al, 2017). In 2019 Wakayama and Yanahimachi were the first researchers to obtain new-borns through ICSI with lyophilized mouse sperm (Wakayama şi Yanahimachi, 2019).

Negative effects on DNA during lyophilization can occur due to the activation of endogenous nucleases, but also oxidative stress (Shahabna et al., 2016). Different solutions such as EGTA, EDTA, but also different antioxidants are used to ensure sperm protection (Shahabna et al. 2016; Olaciregui et al., 2017).

Boar semen is more sensitive to cryopreservation than other species, due to the high content of unsaturated phospholipids and the low level of cholesterol in the plasma membrane. Thus, during cooling-thawing procedures, changes in the sperm membrane lead to a destabilization, affecting calcium homeostasis, acrosome integrity, but also a disorganization of the lipid membrane (Yeste et al., 2015).

The imbalance between the presence of ROS and the antioxidant activity of sperm is the main cause of the effects of cryopreservation on sperm. To support this balance, antioxidants are used, such as rosmarinic acid, which added to the diluent improves motility and prevents peroxidation of boar sperm (harvested from the epididymis), there is a significant correlation between it and the concentration of malonaldehyde (MDA - highlights lipid peroxidation)(Malo et al., 2011). Studies on bull semen have shown that rosmarinic acid in a concentration of 10g/L added

to the diluent increases the viability, motility and speed of sperm after thawing (Daghigh et al., 2014).

Vitamin C (ascorbic acid), another antioxidant, plays an important role in the integrity of sperm and their fertility by increasing testosterone levels and preventing agglutination. Physiologically, in the seminal plasma, it is 10 times higher than in the blood serum and contributes up to 65% of the total antioxidant capacity of the seminal plasma. In human medicine it is used to improve the quality of semen, in treatment for 3 months with 500 mg /day vitamin C along with zinc and vitamin E (Cyrus et al., 2015).

The aim of the research is to evaluate the antioxidant effects of vitamin C and rosmarinic acid on the integrity of sperm DNA in diluted boar semen preserved by lyophilization, after a period of 36 months, kept at $+3^0$C.

Materials and methods

The research was carried out on four samples of diluted and refrigerated sperm obtained from four different breeds (Pietrain, Large White, Duroc and Landrace).

Sperm samples originated from Semest-BVN Targu-Mures and were transported within 24 hours under appropriate conditions ($15\text{-}17^0$C) at the CLC Assisted Reproduction Laboratory, USAMVB Timisoara. Each semen sample was divided in 3 groups: control group (M group), vitamin C group (C group) and rosmarinic acid group (RA group). In control group was no antioxidant, in group C we added 0.5 mM vitamin C and in group RA we added 105 μM rosmarinic acid. Totally we analyzed 24 samples.

The concentrations was chosen based on literature data regarding their use as antioxidants for animal semen (Olaciregui et al., 2017; Varo et al., 2014; Fanaei et al., 2014)

Lyophilization was performed with Ilshin FD 5512, GF3100, at -52°C and 5 mTorr. After 36 months from lyophilization, each sample of sperm was reconstituted in 10 ml DPBS (D-8662, Sigma Aldrich),

Evaluation of sperm DNA integrity was performed using the Halomax kit. This assessment is based on the differential response of sperm chromatin with or without fragmented DNA to a protein depletion treatment. In the absence of massive DNA breakage, the removal of nuclear proteins produces intensely stained nucleoids with very small haloes of DNA loops emerging from a central and compact core. However, nucleoids from sperm containing fragmented DNA show a big and faintly stained halo of diffusion of DNA fragments emerging from a residual central core.

The DNA fragmentation analysis in all groups was performed following the manufacturer's instructions. In brief, the lysis solution was placed at room temperature (22°C). Then, an Eppendorf tube containing agarose was placed in a water bath at 95°C–100°C for five minutes, and then transferred in a water bath at 37°C for five minutes. Meanwhile, 25 µl of each diluted sperm sample was added to an empty eppendorf tube, and 50 ml of liquefied agarose was then transferred into the tube and gently mixed. The temperature of the tubes was maintained at 37°C. Then, a drop of 2 µl of the cell suspension was placed onto marked wells and each drop was covered with a 24 × 24 mm glass coverslip. The slides were held in a horizontal position throughout the entire process. The slides were placed on a cold surface precooled at 4°C in a fridge to solidify the agarose. After 5 min the slides were taken out of the fridge and the coverslips were gently removed. Then, the slides were fully immersed horizontally in 10 ml of lysis solution for five minutes. Subsequently, the preparation was introduced into a bath of distilled water for 5 min and then dehydrated by immersion in 2 successive baths of ethanol at 70% and 100% for 2 min each. Finally, the slides were allowed to air-dry before staining. All the slides were stained using a commercial kit for red fluorescence staining (Fluored, HT-FR100, Halotech DNA SL, Spain). 2 µl

of red fluorochrome and mountain medium (1:1; vol/vol) was placed into the well of slide for fluorescent staining of sperm chromatin. The samples were evaluated using fluorescent microscopy (Leica DMI 4000) at magnification 20X and a minimum of 200 spermatozoa were counted per semen sample. Five replicated trials were carried out for each group. Sperm showing a small and compacted halo around a compacted nuclear core contained intact DNA and sperm that displayed a large and spotty halo around the nuclear core corresponded to those sperm with fragmented DNA.

Results and discussions

The results presented in figure 1 shows that over 90% of the sperm analyzed have intact DNA after lyophilization even after a period of 36 months, kept at +3^{0}C. There are individual variations, depending on the breed, but these were not significant. The most important observation found after analyzing these samples is that the semen can be stored for a long time at a temperature of 3^{0}C without affecting the sperm DNA, which can ensure a fertilizing capacity during use in the technique of intracytoplasmic sperm injection (ICSI). The rate of sperm DNA damage was between 10.58% in the Large White sample and 2.29% in the Landrace sample.

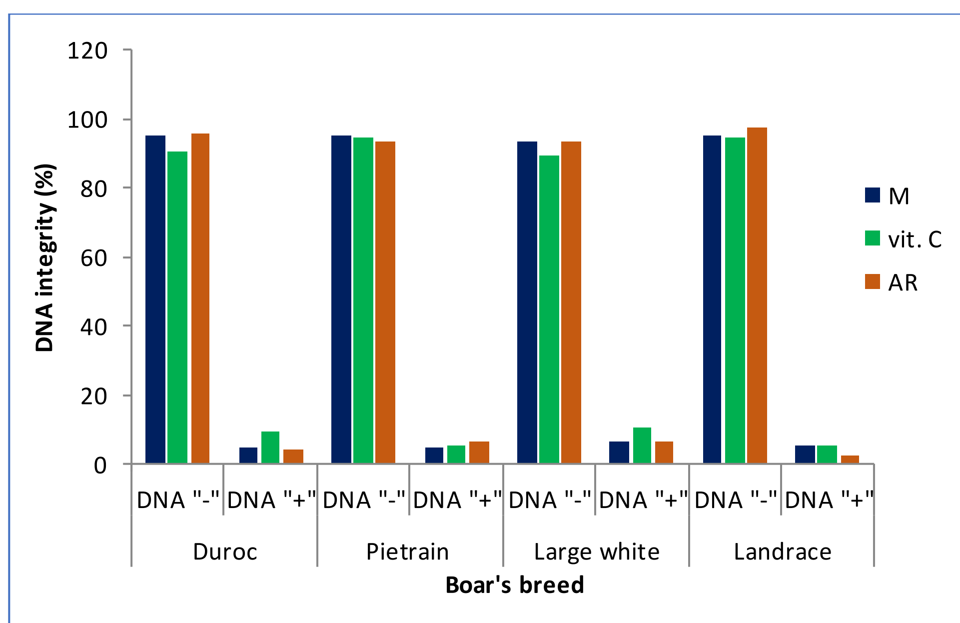

Fig.1. Boar sperm DNA integrity evaluation after 36 months from liophilization (DNA"-" = unafected DNA integrity, DNA "+" = affected DNA integrity)

The protective effect of rosmarinic acid is probably due to the blockade of hydrogen peroxide, which reduces the motility of sperm through the xanthine-xanthine oxidase system. (Malo et al., 2011). Good results were obtained with the help of rosmarinic acid (2.5g/100 ml, 5g/100 ml or 10g/100ml) and on boar spermatozoa harvested from the epididymis. Boar spermatozoa harvested from the epididymis have a higher resistance to cryopreservation probably due to the lack of seminal plasma which is rich in polyunsaturated acids. By determining the concentration of malonaldehyde (MDA) it was observed that there is a significant correlation, so a decrease in MDA was observed when rosmarinic acid was added in higher concentration (Malo et

al., 2011). Similar studies in the literature support the antioxidant effect of rosmarinic acid (105 µM) on the rate of DNA oxidation in boar samples after thawing (Luno et al., 2014).

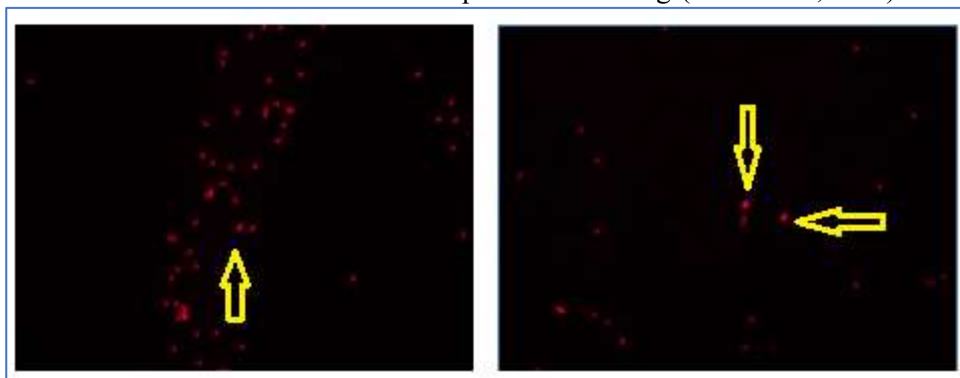

Fig.2. Lyophylized boar spermatozoa stained with Fluored after 36 months of preservation at 3^0C (arrow indicates spermatozoa with DNA integrity affected)(20X)

In 2019, among the few existing studies worldwide on the influence of rosmarinic acid on sow oocytes, Zhang et al. (2019) showed beneficial effects, but in much lower doses (5 µM), effects quantified by tracking developmental competence, bastocyst formation rate, blastocyst hatching rate, blastocyst diameter, total number of blastomeres in the blastocyst, rate of embryos obtained by nuclear somatic cell transfer. Beneficial effects of rosmarinic acid (105 µM) on the quality of sow oocytes after *in vitro* maturation for 44 h, we also observed by quantifying the gene expression of PTX3, p53, BAX, BCL-2, results being published. Similar results were obtained by Luno et al. (2014) with the help of rosmarinic acid in different concentrations (0 µM, 26.25 µM, 52.5 µM and 105 µM) added to boar semen diluted with egg lactose-egg yolk and observed after thawing that the rate of DNA oxidation at 120 and 240 minutes after thawing, respectively, was the lowest in the group supplemented with 105 µM, even if immediately after thawing there were no differences between groups.

Conclusions

1. examining the integrity of sperm DNA is a necessary and more accurate parameter for assessing the quality of semen
2. lyophilized semen can be stored at refrigerator temperature (+3^0C) without significantly affecting DNA integrity
3. there are individual variations between boar breeds regarding sperm DNA integrity
4. lyophilization can be considered a suitable method for preserving boar semen used in intracytoplasmic sperm injection (ICSI) techniques

Acknowledgments

The research was carried in the IVF (In Vitro Fertilization) laboratory and Antioxidant Research Laboratory (A1c) from Horia Cernescu Research Unit – USAMVBT, equipped through POSCCE 2669 program.

References

1. Cyrus A, Kabir A, Goodarzi D, Moghimi M. (2015) - *The effect of adjuvant vitamin C after varicocele surgery on sperm quality and quantity in infertile men: A double blind placebo controlled clinical tria*l. International Brazilian Journal of Urology, 41,230-238
2. Daghigh-Kia H, Olfati-Karaji R, Hoseinkhani A, Ashrafi I. (2014) - *Effect of rosemary (Rosmarinus officinalis) extracts and glutathione antioxidants on bull semen quality after cryopreservation*, Spanish Journal of Agricultural Research, 12, 98-105
3. Fanaei H, Khayat S, Halvaei I, Ramezani V, Azizi Y, Kasaeian A, Mardaneh J, Parvizi Mr, Akrami M. (2014) - *Effects of ascorbic acid on sperm motility, viability, acrosome reaction and DNA integrity in teratozoospermic samples*, Iranian J Reprod Med., 12: 103-110.
4. Hazavehei, M., Sharafi ,M., Kouchesfahani, H.M., Henkel, R., Agarwal, A., Esmaeili, V., Shahverdi, A. (2018) - *Sperm cryopreservationȘ Areview on current molecular cryobiology and advanced approaches*, RBMO, 37, 3, 327-340
5. Luño, V.; Gil, L.; Olaciregui, M.; González, N.; Jerez, R. A.; De Blas, I., (2014) - *Rosmarinic acid improves function and in vitro fertilising ability of boar sperm after cryopreservation*, Cryobiology, *69*, 157–162.
6. Malo C, Gil L, Cano R, Martinez F, Gale I. (2011) - *Antioxidant effect of rosemary (Rosmarinus officinalis) on boar epididymal spermatozoa during cryopreservation*. Theriogenology, 75, 1735-1741
7. Olaciregui,M., Luno, Victoria, Domingo, Paula., Gonzalez, Noelia, Gil, Lydia (2017) – *In vitro developmental ability of ovine oocytes following intracytoplasmatic injection with freeze-dried spermatozoa*, Scientific reports, 7, 1096, DOI:10.1038/s41598-017-00583-0,
8. Patrick ,J., Comizzoli, P., Elliott, G. (2017) - *Dry preservation of spermatozoa: considerations for different species*, Biopreservation and biobanking, 15(20).
9. Saragusty, J., Loi, P. (2019) – *Exploring dry storage as an alternative biobanking strategy inspired by Nature*, Theriogenology, 126, 17-27.
10. Shahba, M.I., El-Sheshtawy, R., El-Azab, A.S.I., Abdel-Ghaffar, A.E., Ziada, M.S., Zaky, A.A. (2016) – *The effect of freeze-drying media and storage temperature on ultrastructure and DNA of freeze-dried buffalo bull spermatozoa*, Asian Pacific Journal of Reproduction, 5(6), 524-535.
11. Varo-Ghiuru,F., Miclea Ileana, Hettig,A., Ladosi,I., Miclea, V., Egerszegi,I., Zahan, M. (2015) - *Lutein, trolox, ascorbic acid and combination of trolox with ascorbic acid can improve boar semen quality during cryopreservation*, CryoLetters, 36, 1, 1-7.
12. Wakayama T, Yanagimachi R., (1998) - *Development of normal mice from oocytes injected with freeze-dried spermatozoa*. Nat Biotechnol, 16(7), 639-641.
13. Yeste, M. (2015) – *Recent advances in boar sperm cryopreservationȘ state of the art and current perspective*s, Reprod Dom Anim, 50, 2, 71-79.
14. Zhang, Y.; Guo, J.; Nie, X. W.; Li, Z. Y.; Wang, Y. M.; Liang, S.; Li, S. (2019) - *Rosmarinic acid treatment during porcine oocyte maturation attenuates oxidative stress and improves subsequent embryo development in vitro*, *PeerJ* , 1–15.