Expression of HSP90 Gene in the Cryopreserved Bovine Spermatozoa

Filip Benko[1*], Alexandra Geschwandtnerová[1], Jana Žiarovská[2], Norbert Lukáč[1], Eva Tvrdá[1]

[1]Department of Animal Physiology, Slovak University of Agriculture, 949 76 Nitra, Tr. A. Hlinku, 2, Slovakia
[2]Department of Genetics and Plant Breeding, Slovak University of Agriculture, 949 76 Nitra, Tr. A. Hlinku, 2, Slovakia

Abstract
Cryopreservation is a technique, commonly used for a long-term storage of male gametes as an effective way to preserve their longevity and vitality. During cryopreservation and following the thawing process, sperm cells are exposed to thermal stress, which may lead into disruptions of their morphological structures and a subsequent functional dysfunction. The objective of our study was to monitor HSP90 (heat shock protein 90) gene expression in cryopreserved bovine spermatozoa. HSP90 is one of the main heat-related cellular proteins, which belongs into chaperone protein family. It is involved in the formation of protein complexes, which stabilize proteins against heat stress and aid in the protein degradation. For the experimental purposes were used semen samples from 12 sexually mature Holstein-Friesian bulls. The samples were divided into two identical fractions, one fraction was cryopreserved and kept in liquid nitrogen at -196°C, while the other fraction was processed as non-cryopreserved fresh semen. The sperm motility rate was evaluated using CASA (Computer Assisted Semen Analysis). Quantification of isolated RNA was performed by real-time PCR and the expression of the HSP90 gene was assessed with the Western blot technique. For a proper analysis, we compared cryopreserved sperm samples with fresh ones. The data from our study indicate a reduction in the sperm motility ($P<0.01$) and HSP90 gene expression by approximetly 50% in the cryopreserved samples ($P<0.01$ for real-time PCR; $P<0.05$ for Western blot). Based on the results, we may confirm that the gene expression is reduced in the cryopreserved samples, which leads into a decrease of the protective properties of HSP90 due to the impact of low temperatures.

Keywords: cryopreservation, HSP90, heat shock, spermatozoa, bulls

1. Introduction

At present, cryopreservation is considered to be an effective method used in animal reproduction for a genetic improvement of domestic animals and preservation of genetic material from rare breeds. Cryopreserved techniques are closely related with artificial insemination, which accelerates the distribution of genetically suitable animals around the world [1,2]. However, cryopreservation has been shown to be detrimental for the sperm integrity due to irreversible changes in the membrane structure and cell metabolism. Temperature changes during cooling, freezing or thawing, high concentrations of cryoprotectants and the presence of ice crystalization leads into heat and osmotic stress [3-5]. Cryodamage of bovine spermatozoa causes motility loss, elimination of the acrosomal cap and a release of enzymes into the extracellular space [6]. Heat shock proteins (HSP) provide natural protection for cells against temperature stress. These proteins can be found in the cytosol, mitochondria, endoplasmatic reticulum or cellular nucleus. HSP90 is a chaperone protein, which is a higly expressed cellular protein across all species and

* Corresponding author: MSc. Filip Benko, Tel: +421-37-641-4918, Email: filip.benko276@gmail.com

weighs roughly 90 kiloDaltons (kDa). It is involved in the formation of protein complexes, protein degradation and stabilization of proteins against heat stress. The expression of HSP90 naturally increases when cells are exposed to stress conditions [7-9]. The aim of this study was to monitor and compare of HSP90 gene expression in bovine spermatozoa before and after cryopreservation.

2. Materials and methods

Sample collection, distribution and freezing

Semen samples were obtained from 12 sexually mature Holstein-Friesian bulls at a local breeding station (Slovenské biologické služby, a.s., Lužianky) using an artificial vagina. After collection, all samples were checked for their quality. The ejaculates had to accomplish the required qualitative criteria, including motility, morphology, volume, concentration and density. Following the quality control, the samples were divided into two identical fractions, one was subsequently diluted with a diluent consisting of triladyl (Minitube, Tiefenbach, Germany), distilled water and egg yolk for later freezing, and the second one stayed fresh. The optimal ratio of sample to diluent was determined based on the sperm concentration. Then, diluted samples were filled into pellets, identified with the date of collection, cooled down to 4 °C, frozen using a digital freezing machine (Digitcool 5300 ZB 250; IMV, France) and stored in liquid nitrogen at -196°C at least for one month. Fresh semen fractions were immediately transpotred to the laboratory and diluted using phosphate buffer without calcium and magnesium (PBS; Sigma-Aldrich, St. Louis, USA), while the cryopreserved samples were removed from the liquid nitrogen and thawed at 37 °C.

Motility analysis

Evaluation of the sperm motility rate was evaluated by CASA (Computer Assisted Semen Analysis) HTM system (Hamilton-Thorne Biosciences; IVOS, Animal Version 12.3D Bild 002; INC, Beverly USA) [10].

Genetical analysis

Isolation of RNA was performed by a modified DDT (dithiotreitol; Sigma-Aldrich, St. Louis, USA) and β-ME (β-mercaptoethanol; Sigma-Aldrich, St. Louis, USA) method due to a strong sulfide bonds in the sperm nucleus. The final protocol followed a modification of the basic isolation kit Qiagene RNeasy Mini Kit (Qiagene, Hilden, Germany). After the isolation, the yield and purify of the isolated RNA were determined on the P-Class nanophometer (Implen, Munich, Germany). All samples were normalized to 60 ng/µl and transcribed into cDNA using the Tetro cDNA Synthesis Kit and oligo dT primer. The final transcription product was cDNA, which was quantified with the real-time qPCR (Quantitative Polymerase Chain Reaction) method. cDNA samples were mixed with the components of a commercial kit, which contained SYBR Green (Express SYBR Green kit, Invitrogen, Carlsbad, USA) for the visualisation of double-stranded DNA (dsDNA). PCR reactions were performed in plates with a thermocycler under specific conditions (2 min./50 °C; 2 min./95 °C; 40 replicates). The specificity of the amplicon in the optimalization phase was monitored by agarose electrophoresis and subsequent dissociation analysis of the resulting amplicons.

Western blot

For the detection of proteins isolated from intact sperm cells the Western blot technique was applied. The extraction of proteins was performed with RIPA lysation buffer (Sigma-Aldrich, St. Louis, USA) enriched with protease inhibitor (Sigma-Aldrich, St. Louis, USA) for the prevention of protein degradation. Each sample had to be normalized to the same protein concentration, for which the commercial Total protein kit (DIA SYS, Holzheim, Germany) was used and the concentration of proteins was analyzed by the RX Monza automatic spectrophotometer (Randox, Crumlin, UK). Then, the samples were treated with β-ME and heat (100 °C), loaded into a polyacrylamide gel (Mini-PROTEAN TGX Stain-free, Biorad, Hercules, USA) for polyacrylamide vertical electrophoresis (PAGE) run for 2 hours at 90 V. Following electrophoresis, the gel was visualized and checked in the ChemiDoc Imaging System (Biorad, Hercules, USA). The proteins were transfered from the gel into a PVDF membrane, using the Trans Blot Turbo Transfer System (Biorad, Hercules, USA), set to 7 min./25 V/2.5 A. An electric current passing through the gel caused that the proteins attached in the gel were

transfered to the membrane. The membrane was blocked with a blocking buffer and incubated with a specific primary antibody (D1A7 mAb rabbit antibody, Cell Signaling Technology, Beverly, USA) over night at 4°C. The next day, the membrane was washed in the washing buffer at room temperature using a shaker. After that, incubation with a secondary antibody (anti-rabbit, GE Healthcare, Chicago, USA) was performed and the membrane was washed again from excess and unbound secondary antibody. For the final protein visualization, the membrane was incubated with the ECL substrate (GE Healthcare, Chicago, USA) for 5 min. in the dark and finally visualized with the ChemiDoc Imaging System. Protein expression was evaluated with the BioRad Image Software 5. 2. 1. based on the light signal transmitted by the membrane.

Statistical analysis

The obtained data were statistically processed with the GraphPad Prism (version 5.02 for Windows; GraphPad Software, La Jolla, California, USA, www.graphpad.com). For advanced statistical evaluation the paired t-test was used, and the level of significance was set at ***P<0.001; **P<0.01; *P<0.05.

3. Results and discussion

One of the most important parameters, which indicate the quality of ejaculate, is the sperm motility. As Figure 1 shows, the motility rate in the cryopreserved samples was only 50% when compared to the control, where the motility was around 80%. The motility of the cryopreserved bovine specimens was significantly lower (P<0.01) against the control group, due to temperature changes during the freezing and thawing process. Studies from the field of cryobiology and animal andrology have already confirmed that temperature changes during the cryopreservation process lead to a reduction of the bull sperm motility at ± 40% against fresh ejaculates [11,12]. HSP90 belongs into ATP-dependent proteins. ATP is produced as a result result of oxidative phosphorylation, which is important for the sperm motility. The levels of ATP decreased after freezing and thawing, which could cause an abrupt decrease of the sperm motion [13].

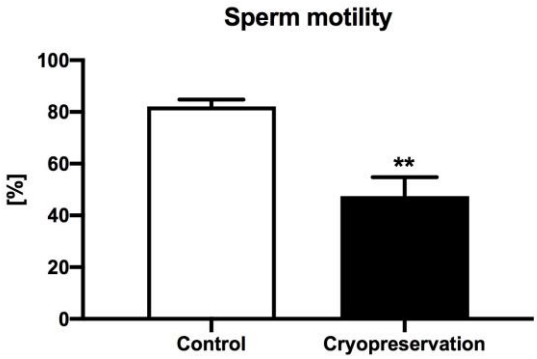

Figure 1. Percentage of sperm motility, average ± S.D. P<0.01

According to the data obtained from qPCR (Figure 2), the expression of HSP90 was significantly decreased (P<0.01) in cryopreserved bovine spermatozoa when compared to the control. The cryopreservation caused that the HSP90 gene expression was reduced after thawing and the volume of isolated mRNA was lower when compared to the fresh bull ejaculate. A significant reduction of ± 50% in HSP90 expression could be related with the reduction of sperm motility after thawing [13,14].

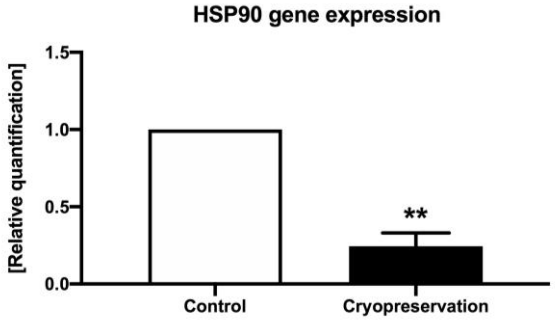

Figure 2. Expression of HSP90 gene, average ± S.D. P<0.01

For the monitoring of the HSP90 gene expression, an intern control of the Wester blot was applied using the housekeeping β-actin protein (beta-actin rabbit Polyclonal Antibody; Bioss, Woburn, USA), which was analyzed simultaneously with the HSP90 protein. Based on the light signal from chemiluminescence (Figure 3), the concentration of β-actin was the same in both types of samples but in the case of HSP90, signal was weaker in the cryopreserved samples. However, in the case of HSP90 (Figure 4), the expression of cryopreserved samples was significantly reduced (P<0.05) against the control. These findings indicate that

the HSP90 gene expression in the cryopreserved samples was altered, which leads into the loss of sperm protective properties against heat stress, and the proteins are more vulnerable to degradation. The expression of HSP90 could be therefore used as a marker for the determination of the bull sperm freezing resistance [15].

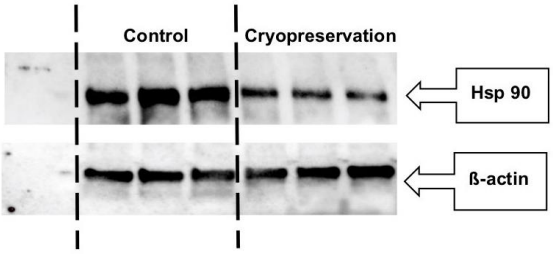

Figure 3. HSP90 and β-actin chemiluminescence visualization in the membrane

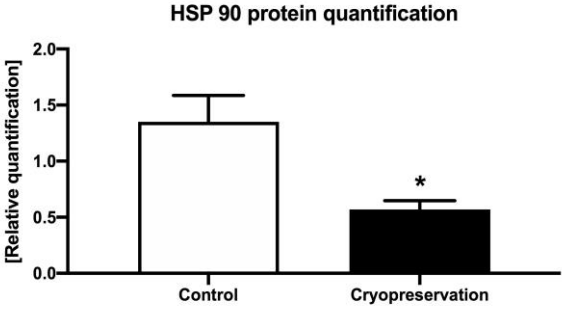

Figure 4. HSP90 protein quantification, average ± S.D. P<0.05

4. Conclusions

The process of cryopreservation negatively affected the sperm motility and reduced the expression of HSP90 gene in the cryopreserved bull spermatozoa against fresh samples. Summarizing our data, HSP90 gene was probably damaged due to temperature changes and lost its protective properties important for the bull sperm resistance against thermal shock.

Acknowledgements

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References

1. Sharma, V., Sperm storage for cancer patients in the UK: a review of current practice, Human Reproduction, 2011, 26, 2935-2943.
2. Isachenko, E., Isachenko V., Katkov, I. and Nawroth, F., Vitrification of mammalian spermatozoa in the absence of cryoprotectants: From past practical difficulties to present success, Reproductive Biomedicine, 2003, 6, 191-200.
3. Baust, J. G., Gao, D. and Baust, J. M., Cryopreservation: An emerging paradigm change, Journal Organogenesis, 2009, 5, 90-96.
4. Peris-Frau, P., Soler, A. J., Iniesta-Guerda, M., Martín-Maestro, A., Sánchez-Ajofrín, I., Medina-Chávez, D. A., Fernández-Santos, M. R., García-Álvarez, O., Maroto-Morales, A., Montoro, V. and Garde, J. J., Sperm Cryodamage in Ruminants: Understanding the Molecular Changes Induced by the Cryopreservation Process to Optimize Sperm Quality, International Journal of Molecular Sciences, 2020, 21, 2781.
5. Khalil, W. A., El-Harairy, M. A., Zeidan A. E. B., Hassan, M. A. E. and Mohey-Elsaeed, O., Evaluation of bull spermatozoa during and after cryopreservation: Structural and ultrastructural insights, International Journal of Veterinary Science and Medicine, 2018, 6, 49-56.
6. Rasul, Z., Ahmad, N. and Anzar, M., Changes in motion characteristics, plasma membrane integrity, and acrosome morphology during cryopreservation of buffalo spermatozoa, Journal of andrology, 2013, 22, 278-283.
7. Chitti, F. and Dobson, C. M., Protein misfolding, amyloid formation, and human disease: A summary of progress over the last decade, Annual reviem of biochemistry, 2017, 86, 27-68.
8. Hoter, A., El-Sabban, M. E. and Naim, H. Y., The HSP90 Family: Structure, Regulation, Function, and Implications in Health and Disease, International Journal of Molecular Sciences, 2018, 19, 2560.
9. Condelli, V., Crispo, F., Pietrafesa, M., Lettini, G., Swann Matassa, D., Esposito, F., Landriscina, M. and Maddalena, F., HSP90 Molecular Chaperons, Metabolic Rewiring, and Epigenetics: Impact on Tumor Progression and Perspective for Anticancer Therapy, Cells, 2019, 8, 2-22.
10. Ďuračka, M., Debacker, M., Bučko, O., Lukáč, N. and Tvrdá, E., The effect of kaempferol and naringenin may improve the in vitro quality of stored boar semen, Journal of Central European Ariculture, 2019, 20, 1069.
11. Kumaresan, A., Johannisson, A., Humblot, P. and Berquis, A., Effect of bovine oviductal fluid on motility, tyrosine phosphorylation, and acrosome reaction in cryopreserved bull spermatozoa, Theriogenology, 2019, 124, 8-56.

12. Wang, P., Wang, Y. F., Wang, H., Wang, C. W., Zan, L. S., Hu, J. H., Wang-Li, Q., Jia, Y. H. and Ma, G. J., Hsp90 expression correlation with freezing resistance of bull sperm, Zygote, 2014, 22, 239-245.
13. Zhang, X. G., Hu, S., Han, C., Zhu, Q. C., Yan, G. J. and Hu, J. H., Association of heat shock protein 90 with motility of post-thawed sperm in bulls, Cryobiology, 2015, 70, 164-169.
14. Rajoriya, J. S., Prasad, J. K., Ghosh, S. K., Perumal, P., Kumar, A., Kaushal, S. and Ramteke, S. S., Studies on effect of different seasons on expression of Hsp70 and Hsp90 gene in sperm of Tharparkar bull, Asian pacific journal of reproduction, 2014, 3, 192-199.
15. Hong, W., Correlation study on HSP90 expression and bull sperm freezing resistance, Journal of Northwest Agriculture and Forestry University, 2013, 40, 31-37.