

Antioxidant Effect of Elamipretide on Bull's Sperm Cells During Freezing/Thawing Process

Alicja Kowalczyk (✉ med.inseminacja@gmail.com)

Uniwersytet Przyrodniczy we Wrocławiu <https://orcid.org/0000-0003-1908-2942>

Czerniawska Piątkowska Ewa

Zachodniopomorski Uniwersytet Technologiczny w Szczecinie

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Abstract

Background: Spermatozoa are subjected to drastic changes in temperature, ice crystal formation, and diverse types of stresses (chemical, physical, osmotic, and oxidative) during the cryopreservation process, which severely compromise sperm quality and fertility. In this study, we aimed to investigate the protective role of Elamipretide in the cryopreservation of bull's sperm.

Materials and methods: The study included 36 healthy Simmental bulls with an average age of 2 ± 0.5 years housed individually in pens. Two ejaculates were collected from each bull using an artificial vagina at 7 a.m. Subsequently, the semen was extended with animal protein-free commercial BIOXcell® extender (IMV Technologies, L'aigle, France) to a final concentration of 160×10^6 spermatozoa/mL, and rated in terms of motile sperm percentage, progressive motility, viability and abnormality of spermatozoa. Semen samples that showed more than 60% motility and 60% viability, were selected for the experiment. The fresh semen was then divided into five equal fractions. The first fraction was left for the control group (without Elamipretide), to the next were added in succession 0.1; 1; 5; and 10 μM of Elamipretide TFA (Trifluoroacetic) (MedChemExpress, USA). After that semen was subjected to freezing and thawing. Next semen was assessed for motility, viability, and antioxidant activity (SOD, CAT, MDA).

Results: It has been shown that a concentration of 5 and 10 μM proved to be the most effective in terms of tested parameters of the quality of sperm cells subjected to cryopreservation.

Conclusion: In conclusion, addition of the Elamipretide to the cryopreservation extender significantly improved frozen-thawed sperm cells quality and their function. The results of this study indicate that Elamipretide can be used as a cryoprotective agent to protect cells against the devastating effects of oxidative stress and increasing sperm survival after cryopreservation.

1. Introduction

Spermatozoa are subjected to drastic changes in temperature, ice crystal formation, and diverse types of stresses (chemical, physical, osmotic, and oxidative) during the cryopreservation process, which severely compromise sperm quality and fertility [1,2,3]. Although the continuous optimization of cryopreservation methods has improved the quality of sperm after freezing, changes in sperm structure, epigenetic modification and the long-term effects caused by freezing injury including enzyme inactivation, ion changes, and oxidative stress cannot be ignored, and the optimization of the freezing system is still an ongoing task that needs further research [4,5]. Despite the development of many agents such as different proteins, antioxidants and cryoprotective agents (incorporated into the freezing medium) for increasing sperm cryosurvival have not yet reached the desired level because many sperm still lose their viability after cryopreservation [6].

Elamipretide (formerly referred to as Bendavia, MTP-131, and SS-31) is an aromatic-cationic tetrapeptide that readily penetrating cell membranes and transiently localizing to the inner mitochondrial membrane where it is associating with cardiolipin. Through this, elamipretide is able to restore energy production, to

reduce the production of reactive oxygen species, and ultimately to increase the energy (adenosine triphosphate [ATP]) supplied to affected cells [7,8]. As recent studies show can increase the synthesis of ATP and reduced reactive oxygen species (ROS) production independently of the specific mitochondrial abnormality causing the impaired mitochondrial respiration [9-10].

However, there are no reports on the application of Elamipretide in bull's spermatozoa preservation and it is unclear whether it can effectively protect sperm from cryodamage during freezing. In this study, we aimed to investigate the protective role of Elamipretide in the cryopreservation of bull's sperm.

2. Materials And Methods

2.1 Semen processing

The experiment has been performed as part of routine activities during the current semen production in the reproductive station and did not require the approval of the ethics committee.

These experiments were performed on the Breeding and Insemination Centre 'MCB' (Krasne, Poland). The study included 36 healthy Simmental bulls with an average age of 2 ± 0.5 years housed individually in pens. Two ejaculates were collected from each bull using an artificial vagina at 7 a.m. The semen was held in a water bath at 37°C, where the sperm concentration and initial percentage of motile spermatozoa were estimated. Sperm concentration was assessed using a digital photometer (Dr Lange, LP 300 SDM; Minitube, Tiefenbach b. Landshut, Germany) at 560 nm.

Semen samples were immediately after collection transferred into graduated test tubes, placed in a water bath at 37°C. The fresh undiluted semen was then evaluated microscopically (Nikon E 200, China) for mass motility. Subsequently, the semen was extended with animal protein-free commercial BIOXcell® extender (IMV Technologies, L'aigle, France) to a final concentration of 160×10^6 spermatozoa/mL, and rated in terms of motile sperm percentage, progressive motility, viability and abnormality of spermatozoa. Semen samples that showed more than 60% motility and 60% viability, were selected for the experiment. After a positive evaluation, semen samples were pooled to eliminate individual differences.

The fresh semen was then divided into five equal fractions. The first fraction was left for the control group (without Elamipretide), to the next were added in succession 0.1; 1; 5; and 10 μ M of Elamipretide TFA (Trifluoroacetic) (MedChemExpress, USA). Semen was automatically packed (Bloc Machine FIN, IS 4, France) into polyvinyl chloride (PVC) straws (0.25 mL) (IMV, France) which were filled and equilibrated for 1.5 h at 4°C. After equilibration, the straws were frozen in liquid nitrogen vapour using a computer controlled automatic freezer from 4°C to -15°C at the rate of -3°C/min and from -15°C to -80°C at the rate of -10°C/min (IMV Technologies, France). After reaching -80°C, semen straws were plunged into liquid nitrogen and packaged in plastic goblets for 24 hours of storage in the liquid nitrogen container. After one day, the straws were thawed in a water bath at 38°C for 20 sec and then were examined.

2.2 Computerized assessment of sperm motility

Sperm motility was examined using a Sperm Class Analyzer (SCA, version 5.1, Microptic, Barcelona, Spain), a light microscope (Nikon Eclipse E200). Just prior to analysis, semen was diluted 1:10 in a warm (25°C) physiological solution (sodium chlorate 0.9%). Then, 2 IL of the prepared sample was placed in a Leja 4 analysis chamber (Leja Products B.V., Holland) of a thickness of 20.0 μ m. The slide was placed on a stage warmer (38°C). The following motility parameters were included in this study: percentage of motile sperm, curvilinear velocity (VCL), straight-line velocity (VSL), path velocity (VAP), linearity (LIN) and amplitude of lateral head displacement (ALH). Minimum 500 cells were evaluated, and depending on sperm concentration, five analyses were performed per sample.

2.3 Viability

The double stain SYBR-14 with propidium iodide (L-7011 LIVE/DEAD Sperm Viability Kit; Invitrogen, Molecular Probes, Barcelona, Spain) using flow cytometer was applied (CytoFlex Beckman Coulter, B3-R1-V0, China). For this purpose, 50 mL of thawed semen was measured (37°C for 20 sec) and 940 μ l NaCl (0.9%) and 5 mL SYBR14 were added. The whole was thoroughly mixed and then incubated (36°C for 10 min) without light access. Subsequently, 5 μ l of PI was remixed and incubated 3min without light, followed by a test.

2.4 Biochemical assays

Semen samples were centrifuged at 750 rpm for 10 min to remove the supernatants, obtained cell pellets were washed three times with PBS and then incubated with 0.2% Triton X-100 on ice for 20 min. The supernatants were pipetted for subsequent biochemical analyses. Superoxide dismutase (SOD) and Catalase (CAT) activities were examined using the spectrophotometric method by commercially available enzyme activity test kits (Jiancheng Bioengineering Institute, China), Malondialdehyde (MDA) content was determined using the thiobarbituric acid (TBA) method by chemical reaction kits (Jiancheng Bioengineering Institute, China), which performed following manufacturer's instructions as described previously [11].

2.5 Statistical analysis

Data are presented as mean \pm standard error of the mean (SEM). Analysis of variance (ANOVA) was used to assess differences between concentrations of Elamipretide supplementation on all semen characteristics. When the F ratio was significant ($p < 0.05$), Duncan's multiple range test was used to compare treatment means. The statistical analysis of the results was performed with Statistica 12.0 (StatSoft, Poland).

3. Results

Tables 1 and 2 present the results of the bull's semen quality analysis before and after the cryopreservation process.

Table 1 shows the effects of different concentrations of Elamipretide on sperm cell motility and the stability of their plasmic membrane. The highest percentage of motile sperm was observed in the group with the addition of 10 μM of Elamipretide (increase by 8.98%). Significantly higher ($P < 0.05$) cell motility was observed in samples containing 1; 5 and 10 μM of Elamipretide relative to the additive-free group. Plasmic membrane stability increased in all groups studied, the best sperm viability result was obtained in the group with the addition of 10 μM of Elamipretide (50.50%), which was higher by 10.84% than the result obtained in the control group (39.66%). In both parameters tested, the lowest concentration of Elamipretide (0.01 μM) did not significantly affect the results obtained.

Table 2 shows the influence of Elamipretide addition on SOD, CAT activity and intracellular MDA in frozen/thawed sperm cells. After cryopreservation, the antioxidant enzyme SOD and CAT activities were also reduced, in all study groups relative to the control group, while the concentration of MDA was significantly lowered ($P < 0.05$). Significant differences between the study groups with Elamipretide supplement and the control group within SOD activity were observed in samples containing 1; 5 and 10 μM of Elamipretide. In terms of CAT enzymatic activity, significant differences were observed in samples containing 0.1; 1; 5 and 10 μM of Elamipretide relative to the group not containing the additive. MDA concentration in samples containing 1; 5 and 10 μM of Elamipretide significantly ($P < 0.05$) lowered relative to the control group by respectively 0.63, 0.64 and 0.72 nmol/ml.

4. Discussion

In this study, dose-related improvement in bulls' semen quality parameters was observed. Analyzes in the field of motility and viability of sperm cells and antioxidant activity of frozen/thawed semen showed that the addition of Elamipretide positively affects the percentage of live spermatozoa obtained after cryopreservation by an average of about 10%, which was also associated with an increased percentage of motile cells by an average of 9% in relative to the control group. Due to the fact that sperm cells has less cytoplasm and the plasma membrane is rich in unsaturated fatty acids, it is susceptible to oxidative stress damage during cryopreservation [12,13]. Moreover, reduced antioxidant activity of SOD observed in bull and ram sperm after cryopreservation, could explain in part the increased susceptibility of frozen-thawed sperm to oxidative damage [14,15].

So far it has been shown Elamipretide effectively resists stress and fights diseases in the myocardium, nervous and endocrine systems [16,17,18]. In research Bai et al., [19] shown that Elamipretide supplement significantly improve motility and viability of human sperm post-thawing, accompanied by reduced mitochondrial dysfunction and reactive oxygen species (ROS) production. In own research was observed the decrease in SOD and CAT activity and the accumulation of MDA which is also confirmed by other researchers [20,21]. Production of ROS triggers the oxidative attack of polyunsaturated phospholipids, resulting in the formation of malondialdehyde (MDA) [22]. Own research has shown that concentration of MDA decreased as the concentration of the Elamipretide additive increased.

The present study showed that samples containing Elamipretide at a concentration of 5 i 10 μM proved to be the most effective in terms of tested parameters of the quality of sperm cells subjected to cryopreservation. No toxic or limited effect on the spermatozoa of the above additive at concentration 10 μM has been demonstrated.

Conclusion

In conclusion, addition of the Elamipretide to the cryopreservation extender significantly improved frozen-thawed sperm cells quality and their function. The results of this study indicate that Elamipretide can be used as a cryoprotective agent to protect cells against the devastating effects of oxidative stress and increasing sperm survival after cryopreservation.

Declarations

Availability of data and material: The datasets used during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate: The experiment has been performed as part of routine activities during the current semen production in the reproductive station and did not require the approval of the ethics committee.

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Author contributions statement: We declare that all authors made substantial contributions to this manuscript. A.K. in conducting the experiment, in the conception and design of the study, and in establishing the methodology. E.C-P, in the collection, assembly of data and in the analysis and interpretation of results. A.K., E.C-P, in the preparation of the manuscript.

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Tables

Table 1

Effect of Elamipretide addition on the quality of cryopreserved bull's spermatozoa

| Groups (concentration; μM) | Motility (%) | Viability (%) |
|--|-------------------------------|--------------------------------|
| Control (0) | 50.03 ^A \pm 1.1 | 39.66 ^C \pm 0.099 |
| 0.1 | 52.18 ^{AC} \pm 2.2 | 41.12 ^C \pm 0.076 |
| 1.0 | 55.21 ^B \pm 2.4 | 45.90 ^B \pm 0.087 |
| 5.0 | 57.64 ^B \pm 3.1 | 49.32 ^A \pm 0.064 |
| 10.0 | 59.01 ^D \pm 3.3 | 50.50 ^A \pm 0.082 |
| Explanations: a, b, c, d – means with different superscript letters in the same row differ significantly at $P < 0.05$. The values are expressed as mean \pm SD. | | |

Table 2

Antioxidant potential of Elamipretide in bull's sperm cells

| Groups (concentration; μM) | SOD (U/ml) | CAT (mU/ml) | MDA (nmol/ml) |
|--|-------------------------------|---------------------------------|------------------------------|
| Control (0) | 3.50 ^C \pm 0.31 | 89.11 ^C \pm 8.80 | 2.12 ^A \pm 0.19 |
| 0.1 | 3.73 ^{BC} \pm 0.44 | 98.83 ^B \pm 9.63 | 1.70 ^A \pm 0.22 |
| 1.0 | 3.84 ^{AB} \pm 0.37 | 103.56 ^A \pm 10.10 | 1.49 ^B \pm 0.24 |
| 5.0 | 3.89 ^A \pm 0.32 | 103.79 ^A \pm 9.98 | 1.48 ^B \pm 0.21 |
| 10.0 | 3.93 ^A \pm 0.41 | 104.99 ^A \pm 13.14 | 1.40 ^B \pm 0.23 |
| Explanations: a, b, c, d – means with different superscript letters in the same row differ significantly at $P < 0.05$. The values are expressed as mean \pm SD. | | | |