Year: 2009

Effect of antioxidants (sodium pyruvate and catalase) on post thaw motility

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Abstract: Freezing of the semen in liquid nitrogen is the only method for long term storage of fertile spermatozoa. The procedure involves different steps which are harmful to spermatozoa and consequently reduce their quality and fertility. In many animal species the addition of antioxidants to semen before freezing was found to have positive effect on the quality and fertility of frozen/thawed (F/T) spermatozoa. As in rams there are contradictory results in the literature concerning the influence of specific antioxidants on sperm quality of F/T spermatozoa, the present study was performed to evaluate the effects of two different antioxidants on post thaw motility, viability and DNA integrity of F/T ram spermatozoa. Ejaculates from six crossbreed rams were frozen according to the standard procedure after two step dilution with modified Tris-egg yolk extender. The second extender, which contained 14% of glycerol, was added to the semen at 5°C. Ejaculates were divided into three parts and extended with diluents that were either supplemented with 5mM sodium pyruvate (group 1), 20IU/ml catalase (group 2), both antioxidants together (group 3) or contained no antioxidants (control group). Diluted samples were loaded into 0.5ml straws and frozen in nitrogen vapour, 4cm above the liquid nitrogen. Frozen samples were kept in liquid nitrogen for at least two month before thawing and analysis. Frozen straws were thawed in water bath at 37°C for 17s. Motility and viability (Viadent®) of the F/T samples were analysed with Hamilton Thorne Biosciences, Version 12.3, after incubation in water bath at 37°C for 10min, 6,12 and 24h. The percentages of sperm showing a high DNA fragmentation (DFI%) of F/T spermatozoa was analysed 5min and 3h after thawing by using the flow cytometric sperm chromatin structure assay (SCSA™). Statistical analyses were performed with Sigma Stat (Version 3.10). Statistical difference between the groups was tested with Kruskal-Wallis One Way Analysis of Variance on Ranks. Values were considered to be statistically significant when $p<0.05$. There were no differences in percentages of motile (74.0±23.6% vs. 73.6±23.8 vs. 73.6±23.7% vs. 73.3±22.9% and 58.4±25.4% vs. 55.6±24.3% vs. 60.2±25.1 vs. 57.7±26.8% and 45.5±25.6% vs. 45.2±23.1% vs. 45.7±22.4 vs. 45.6±24.3% and 10.0±9.4% vs. 13.3±11.5% vs. 13.3±12.1 vs. 11.9±11.7% for group 1 vs. 2 vs. 3 vs. control respectively for 0, 6, 12 and 24h after F/T) and viable spermatozoa (81.0 ± 16.3% vs. 82.5 ± 13.5% vs. 82.7 ± 13.2 vs. 80.7±17.0% and 78.7±15.0% vs. 79.8±13.9% vs. 79.7±14.8 vs. 75.8±18.9% and 79.0 ± 13.0% vs. 77.9 ± 14.2% vs. 80.1 ± 12.2 vs. 77.6 ± 15.1% and 67.6 ± 26.2% vs. 71.4 ± 19.9% vs. 70.5 ± 23.4 vs. 66.4 ± 27.2% for group 1 vs. 2 vs. 3 vs. control respectively for 0, 6, 12 and 24h after F/T) in groups with antioxidants in comparison to control group at 0, 6, 12 and 24h ($p>0.44$). Analysis of SCSA™ also revealed no difference (1.7 ± 1.2% vs. 1.6 ± 0.9% vs. 1.7 ± 1.0 vs. 1.6 ± 1.1% and 1.8 ± 1.3% vs. 1.7 ± 1.0% vs. 1.9 ± 1.2 vs. 1.7 ± 1.3% for group 1 vs. 2 vs. 3 vs. control respectively for 0 and 3h after F/T) ($p>0.06$) the p-value is rather low and indicates that there is somewhere a difference by trend ($0.05<p<0.10$) in DFI%-values between groups with antioxidants compared to control group 0 and 3h after thawing. These results indicate no positive effect of pyruvate and catalase on motility, viability and chromosome integrity of F/T ram spermatozoa.

Доклади на Българската академия на науките
Comptes rendus de l'Académie bulgare des Sciences

Tome 62, No 5, 2009

BIOLOGIE

Biotechnologie

EFFECT OF ANTIOXIDANTS (SODIUM PYRUVATE AND CATALASE) ON POST THAW MOTILITY, VIABILITY AND DNA INTEGRITY OF RAM SPERMATOZOA

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(*Submitted by Academician Z. Chinkowski on February 24, 2009*)

Abstract

Freezing of the semen in liquid nitrogen is the only method for long term storage of fertile spermatozoa. The procedure involves different steps which are harmful to spermatozoa and consequently reduce their quality and fertility. In many animal species the addition of antioxidants to semen before freezing was found to have positive effect on the quality and fertility of frozen/thawed (F/T) spermatozoa. As in rams there are contradictory results in the literature concerning the influence of specific antioxidants on sperm quality of F/T spermatozoa, the present study was performed to evaluate the effects of two different antioxidants on post thaw motility, viability and DNA integrity of F/T ram spermatozoa. Ejaculates from six crossbreed rams were frozen according to the standard procedure after two step dilution with modified Tris-egg yolk extender. The second extender, which contained 14% of glycerol, was added to the semen at 5 °C. Ejaculates were divided into three parts and extended with diluents that were either supplemented with 5 mM sodium pyruvate (group 1), 20 IU/ml catalase (group 2), both antioxidants together (group 3) or contained no antioxidants (control group). Diluted samples were loaded into 0.5 ml straws and frozen in nitrogen vapour, 4 cm above the liquid nitrogen. Frozen samples were kept in liquid nitrogen for at least two month before thawing and analysis. Frozen straws were thawed in water bath at 37 °C for 17 s. Motility and

This scientific paper is presented on the 1st Conference of the Balkan Network for Animal Reproduction Biotechnology within project 4-01-2008 under the European programme "Research Cooperation and Networking between Austria and South Eastern Europe" supported by the Austrian Science and Research Liaison Offices.

viability (Viadent®) of the F/T samples were analysed with Hamilton Thorne Biosciences, Version 12.3, after incubation in water bath at 37 °C for 10 min, 6, 12 and 24 h. The percentages of sperm showing a high DNA fragmentation (DFI%) of F/T spermatozoa was analysed 5 min and 3 h after thawing by using the flow cytometric sperm chromatin structure assay (SCSA™). Statistical analyses were performed with Sigma Stat (Version 3.10). Statistical difference between the groups was tested with Kruskal-Wallis One Way Analysis of Variance on Ranks. Values were considered to be statistically significant when $p < 0.05$. There were no differences in percentages of motile ($74.0 \pm 23.6\%$ vs. 73.6 ± 23.8 vs. $73.6 \pm 23.7\%$ vs. $73.3 \pm 22.9\%$ and $58.4 \pm 25.4\%$ vs. $55.6 \pm 24.3\%$ vs. 60.2 ± 25.1 vs. $57.7 \pm 26.8\%$ and $45.5 \pm 25.6\%$ vs. $45.2 \pm 23.1\%$ vs. 45.7 ± 22.4 vs. $45.6 \pm 24.3\%$ and $10.0 \pm 9.4\%$ vs. $13.3 \pm 11.5\%$ vs. 13.3 ± 12.1 vs. $11.9 \pm 11.7\%$ for group 1 vs. 2 vs. 3 vs. control respectively for 0, 6, 12 and 24 h after F/T) and viable spermatozoa ($81.0 \pm 16.3\%$ vs. $82.5 \pm 13.5\%$ vs. 82.7 ± 13.2 vs. $80.7 \pm 17.0\%$ and $78.7 \pm 15.0\%$ vs. $79.8 \pm 13.9\%$ vs. 79.7 ± 14.8 vs. $75.8 \pm 18.9\%$ and $79.0 \pm 13.0\%$ vs. $77.9 \pm 14.2\%$ vs. 80.1 ± 12.2 vs. $77.6 \pm 15.1\%$ and $67.6 \pm 26.2\%$ vs. $71.4 \pm 19.9\%$ vs. 70.5 ± 23.4 vs. $66.4 \pm 27.2\%$ for group 1 vs. 2 vs. 3 vs. control respectively for 0, 6, 12 and 24 h after F/T) in groups with antioxidants in comparison to control group at 0, 6, 12 and 24 h ($p > 0.44$). Analysis of SCSA™ also revealed no difference ($1.7 \pm 1.2\%$ vs. $1.6 \pm 0.9\%$ vs. 1.7 ± 1.0 vs. $1.6 \pm 1.1\%$ and $1.8 \pm 1.3\%$ vs. $1.7 \pm 1.0\%$ vs. 1.9 ± 1.2 vs. $1.7 \pm 1.3\%$ for group 1 vs. 2 vs. 3 vs. control respectively for 0 and 3 h after F/T) ($p > 0.06$) the p-value is rather low and indicates that there is somewhere a difference by trend ($0.05 < p < 0.10$) in DFI%-values between groups with antioxidants compared to control group 0 and 3 h after thawing. These results indicate no positive effect of pyruvate and catalase on motility, viability and chromosome integrity of F/T ram spermatozoa.

Key words: sodium pyruvate, catalase, ram spermatozoa, freezing procedure, motility, viability, defragmentation index

Introduction. Cryopreservation increases the concentration of reactive oxygen species (ROS) in the semen of different animal species [1,2]. ROS inactivates different proteins and induce peroxidation of unsaturated lipids in membranes [3]. Membrane of spermatozoa contains high percentage of polyunsaturated fatty acids and is therefore very vulnerable for lipid peroxidation [4]. Cryopreservation also has effect on the stability of DNA and promotes DNA damage [5]. Natural mechanisms for spermatozoa protection against lipid peroxidation, i.e. superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase, is therefore highly important for sperm protection [6]. The addition of different antioxidants to extenders used for semen conservation has also shown their positive effect on the viability of spermatozoa in different animal species [7,8]. Despite the positive effect in other animal species, on ram spermatozoa the addition of antioxidants to the extenders produced some contradictory results [9,10].

Therefore, the main goal of this study was to analyse the effect of two different antioxidants on post thaw motility, viability and DNA integrity of ram spermatozoa.

Materials and methods. ***Reagents and media.*** Chemicals and enzymes used in this study were obtain from Sigma – Aldrich Chemical Co. (St. Louis, USA), Merck (Darmstadt, Germany), Calbiochem®, Nova Chemical Sales Inc. (Scituate, USA) and Kemika (Zagreb, Croatia).

Semen collection, processing and sperm cryopreservation. The animals were housed in the Clinic for Reproduction and Horses, Vet Faculty, University of Ljubljana, Slovenia. Semen (7 ejaculates per ram) was collected with electroejaculation from six cross-breed rams. Immediately after collection, the ejaculates were transferred into a tube and kept in a water bath at 27 °C until analysis and further processing. Sperm concentration was measured with spectrophotometer (photometer SDM 5, Mini Tüb) and the motility was analysed with phase contrast microscope (Olympus BX 40). After the analysis, the ejaculates were diluted and frozen according to two-step procedure with modified Tris egg yolk extender (Tris 263 mM, citric acid 85 mM, fructose 73 mM, egg yolk 20%: 340 mOsm, pH 7.0). Each ejaculate was divided into four parts. Aliquots (200 µL) of fresh semen were diluted with extender I at 27 °C. Extender I either contained no antioxidants (control group) or was supplemented with antioxidants (group 1: 5 mM of sodium pyruvate; group 2: 20 IU/ml catalase; group 3: both antioxidants together). Diluted samples were placed in 90 ml water bath, which enabled slow cooling (2 h) to 5 °C. The second extender was added to the semen after cooling to 5 °C. It contained 14% glycerol, 0.75% of Equex STM® and the same concentration of antioxidants per group as in extender I. Diluted samples were loaded into 0.5 ml straws and frozen in nitrogen vapour, 4 cm above the liquid nitrogen. Frozen samples were kept in liquid nitrogen for at least two months before thawing and analysis. Frozen straws were thawed in water bath at 37 °C for 17 s.

Analysis of motility and viability (Viadent®). Spermatozoa motility and viability was analysed after incubation of the samples in water bath at 37 °C for 10 min, 6, 12 and 24 h. Analysis was performed with a computer assisted analyser (Hamilton Thorne Biosciences, Version 12.3, Beverly, MA) in a counting chamber (Makler counting chamber®). Five automatically selected fields were analysed per sample. Spermatozoa viability was analysed with Viadent® (Hamilton Thorne Biosciences, Version 12.3, Beverly, MA) assay according to the directions of the manufacturer.

Chromatin structure assay (SCSA™). The SCSA™ procedure was performed 5 min and 3 h after thawing by using the flow cytomety (EPICS XL – MCL, Beckman Coulter), based on protocol described previously [11, 12].

Results. See Figure 1.

Discussion. The main objective of this study was to analyse the effect of different antioxidants on motility, viability and chromosome integrity of F/T ram spermatozoa. Previous studies in different animal species showed the positive effect of sodium pyruvate [13, 14] and catalase [7, 8] on motility and viability of

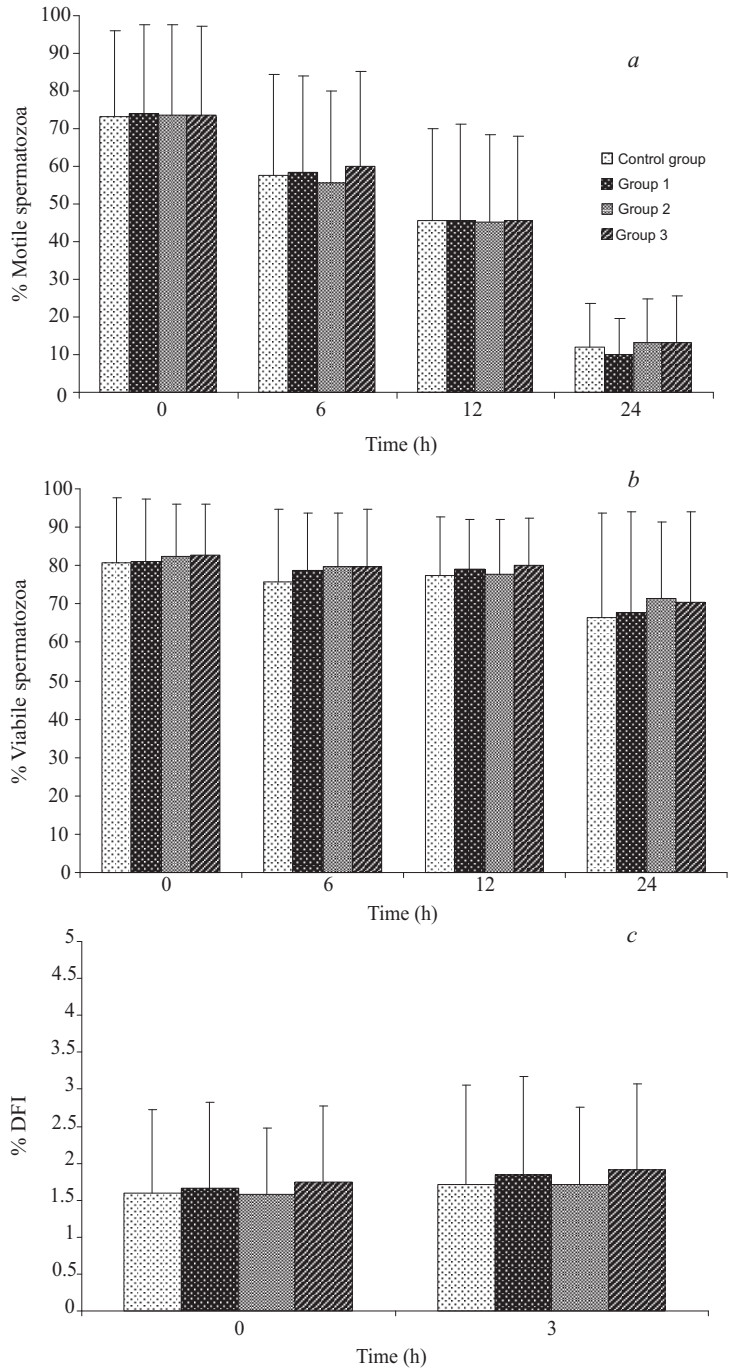

Fig. 1. Post thaw motility (a), viability (b) and DNA integrity (c) of frozen/thawed ram spermatozoa. Differences between the control group, group 1 (sodium pyruvate), group 2 (catalase), group 3 (both antioxidants together) ($n = 42$)

spermatozoa. To the contrary, addition of sodium pyruvate and catalase to the extenders used for ram spermatozoa did not produce repeatable positive effect. In some studies on ram spermatozoa sodium pyruvate produced positive effect [10,15], but addition of catalase to ram semen did not [9,16]. In our research we could not find any statistical significant effect of these two antioxidants on the motility and viability of spermatozoa. Although the fertilizing capacity was not analysed, our results indicate that antioxidants used in this study probably do not have any effect on fertilizing capacity of F/T ram spermatozoa. To prove this hypothesis further analysis of fertility should be performed.

Similar results were obtained after analysis of chromatin stability. SCSA™ is a technique used to study sperm chromatin structure based on the metachromatic properties of acridine orange to distinguish denatured (fluoresces red) from intact (fluoresces green) DNA in spermatozoa [17]. Some previous studies showed the negative correlation between the percentage of spermatozoa with denatured DNA and fertilizing capacity of spermatozoa [18,19]. Although there was no significant difference between groups which contained antioxidants and the control group, the p value was rather low ($p = 0.06$) in some instances. The difference was highest between the control group and group 1 (sodium pyruvate) and 2 (sodium pyruvate and catalase). This indicates that there is a tendency of positive effect of sodium pyruvate on the DNA stability, but this cannot be proved without a doubt.

Despite this conclusion motility, viability and % DFI in all groups including the control group of frozen/thawed spermatozoa was relatively high. From this findings and the data from literature it can be assumed that addition of Equex STM® is responsible for relatively high quality of F/T spermatozoa in this research [20].

Acknowledgements. The authors are thankful to Drago Kompan, Dušan Birtič and Domen Drašler (Department of Animal Science, Biotechnical Faculty, University of Ljubljana) for the animals used in this study.

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