Slov Vet Res 2014; 51 (1): 35-42
UDC 636.3.082.453.5:612.616.2:57.086.13

Original Scientific Article

THE EFFECT OF Equex STM® IN FREEZING MEDIA ON POST THAW MOTILITY, VIABILITY AND DNA INTEGRITY OF FROZEN - THAWED RAM SPERMATOZOA

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Summary: In this study we investigated the effect of Equex STM® on quality and in-vitro survival of ram spermatozoa frozen in Tris egg yolk based extender. Ejaculates from 6 crossbreed rams were frozen according to the standard procedure after two step dilution with Tris-egg yolk extender (1). The second extender, added to the semen at 5° C, contained 14 % of glycerol and was supplemented with detergent 0.75 % Equex STM® (group OEP) or contained no detergent (control group). After thawing the samples were incubated in a water bath at 37° C and analysis were performed 10 minutes, 6, 12 and 24 hours later. Motility and the viability (Viadent®) of the semen were analysed with Hamilton Thorne Biosciences, Version 12.3 and membrane integrity with HOS (hypoosmotic swelling test). DNA fragmentation (DFI %) of F/T spermatozoa was analyzed 10 minutes and 3 hours after thawing using sperm chromatin structure assay (SCSA™). The sperm membrane integrity was analysed 15 minutes and 3 hours after thawing by Sybr-14/PI test. Percentage of motile spermatozoa was significantly higher in OEP group in comparison to control group at 0, 6, 12 and 24h ($P < 0.001$). Viability of spermatozoa was significantly higher ($P < 0.001$) in OEP compared to control group in all analysed times after thawing. Percentage of HOS positive spermatozoa was significantly higher in OEP compared to control group respectively for 0 ($P = 0.001$), 6 ($P =< 0.001$), 12 and 24h ($P = 0.002$) after thawing.

Key words: Semen; ram; Equex STM®; flow cytometry; Viadent®; SCSA™; Sybr-14/PI

Introduction

Freezing of the semen in liquid nitrogen enable long term storage of fertile spermatozoa from different animal species (2, 3, 4, 5). The process of cryopreservation involves different steps which are harmful to spermatozoa and consequently reduce their quality and fertility. Another reason for reduced fertility in sheep inseminated intra-cervical with frozen-thawed semen is the anatomical structure of the ewe's cervix and passage of viable spermatozoa through the cervix. Polyunsaturated fatty acids in the membranes of spermatozoa, which are exposed to lipid peroxidation during freezing and thawing process are believed to be one of the main reasons for reduced fertility (6). Deep freezing of spermatozoa increases the concentration of reactive oxygen species (ROS) in the semen from various species (7, 8, 9). ROS represent a wide variety of different free radicals. Among the most common forms of ROS, which affect the viability and functionality of spermatozoa, include hydroxyl radicals (OH •), superoxide radicals (O2 •), hydrogen

Received: 26 June 2013
Accepted for publication: 25 September 2013

peroxide (H2O2), peroxide radicals (ROO •), hypochlorite radicals, etc.(10, 11). ROS inactivate various proteins and promote the peroxidation of unsaturated fatty acids in cell membranes (12). Sperm membrane is very susceptible to lipid peroxidation because of its high content of unsaturated fatty acids (13). Peroxidation of unsaturated fatty acids leads to loss of integrity and consequently functions of the spermatozoa membrane**.** Freezing and oxidative stress cause decrease of spermatozoa motility, fertilizing ability (14) and also effects the stability of DNA (15).

However, minor concentrations of ROS have a positive effect on some of the vital function of spermatozoa. They help to regulate the function of spermatozoa, for example hydrogen peroxide in small quantities stimulates capacitation of spermatozoa, their hiperactivation, acrosomal reaction and fertilization (16, 17). Dead and damaged spermatozoa represent a source of ROS which have a detrimental effect on motility and viability of spermatozoa (18), adverse affect on the integrity of the spermatozoa membrane (19) and on the integrity of the DNA (20).

Natural mechanisms which protect spermatozoa against lipid peroxidation include various antioxidants and enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase, etc. These antioxidants are very important for the protection of spermatozoa in different animal species (21, 22, 23, 24). Addition of detergent to the extender for freezing represents another mechanism for membrane protection during freezing. Cryopreservation process induces serious detrimental changes in sperm function. The cell and acrosomal membranes of spermatozoa are considered to be the primary site of these modifications due to thermal, mechanical, chemical and osmotic stresses and are critical for semen survival. Equex STM® improves the post-thaw survival of spermatozoa by acting as a surfactant to stabilize cell membranes, particularly acrosomal membranes, and to protect spermatozoa against the toxic effects during the freeze-thaw process (25, 26). It is well known that small amount of detergent added to the extender that contains egg yolk, have a positive effect on membrane stability during freezing /thawing process. Equex STM® added to extender for freezing the dog semen protect spermatozoa from damage incurred during the deep freezing process (27).

Materials and methods

Semen Collection, Processing and Sperm Cryopreservation

The animals were housed on the Clinic for Reproduction and Horses, Vet Faculty, University of Ljubljana, Slovenia. Semen (6 ejaculates per ram) was collected with electro–ejaculation from 6 cross-breed rams. Immediately after collection, the ejaculates were transferred into tube and kept in a water bath at 27° C until the analysis and further processing.

Analysis of the semen concentration and motility was performed before further processing.

Semen concentration was measured with spectrophotometer (photometer SDM 5, Mini Tüb) and motility was analysed with phase contrast microscope (Olympus BX 40). After 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 (Merck, Darmstadt, Germany). Each ejaculate was divided into two parts. Aliquots (200µL) of fresh semen were diluted with 1800µL of extender I warmed to 27° C. Diluted samples were placed in 90 ml water bath, which enable slowly cooling (2h) to +5° C. After cooling to +5° C, 2000 µL of the second extender was added to the semen. It either contained no Equex STM® (control group) or was supplemented with Equex STM® (0.38 % final Equex STM® concentration; Nova Chemical Sales Inc., Scituate, USA). Both extenders also contained 14 % of glycerol (Kemika, Zagreb, Croatia). Diluted samples were aspirated into 0.5ml 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 second.

Analysis of Motility and Viability (Viadent®)

Semen motility and viability was analysed after incubation of the samples in water bath at 37° C for 10 minutes, 6, 12 and 24 hours. Analysis was performed with a computer assisted analyzer (Hamilton Thorne Biosciences, Version 12.3, Beverly, MA) in a counting chamber (Makler counting chamber®). Five automatic selected

fields were analysed per sample. Semen 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 is an acridine orange (AO) staining technique which uses a metachromatic dye, AO (chromatographically purified No 04539, Polysciences Inc., *Eppelheim, Warrington, PA, USA*) to evaluate the ratio of single- (abnormal) and double-stranded (native) DNA present in individual spermatozoa. Abnormal chromatin structure was defined as the susceptibility of spermatozoa DNA to undergo acid-induced denaturation in situ. Following the exposure of the prepared DNA to AO, the degree of chromatin integrity (percentage of DNA fragmentation index (% DFI)) was analysed by flow cytometric measurement of the metachromatic shift from green (stable, double-stranded DNA) to red (denaturated, single-stranded DNA) AO fluorescence emitted by each individual spermatozoa. The SCSA™ procedure was performed 10 minutes and 3 hours after thawing using the flow cytometry (EPICS XL – MCL, Beckman Coulter). Samples were extended immediately after thawing (0 h) to a final concentration of $2x10^6$ spermatozoa/mL using TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1mM EDTA, pH 7.4). Two aliquots of each sample were evaluated for their DNA fragmentation, using the SCSA™ as previously described by Evenson and Jost (28, 29). Acid-induced denaturation of DNA in situ was achieved by adding 0.4 mL of acid-detergent solution (0.1 % (v/v) Triton X-100, 0.15 M NaCl, 0.08 N HCl, pH 1.2) to 200 µL of extended semen sample. After 30 s, semen was stained by adding 1.2 mL of AO staining solution containing 6µg purified AO per mL of buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, pH 6.0). Stained semen was incubated on ice for 3 min before flow cytrometric analysis.

SYBR-14/PI test

A combination of stains, one specific for live spermatozoa - SYBR-14 and the other specific for spermatozoa that lost membrane integrity -propidium iodide (Invitrogen™, Molecular Probes Inc., Eugene, OR, USA) were used to determine the proportion of live spermatozoa. SYBR 14 is a membrane permeant stain, fluorescenting bright green, which binds to DNA of all spermatozoa. While PI is a red fluorescence stain and binds to DNA in spermatozoa with damaged membranes (30). A third population of spermatozoa is moribund and stains with both red and green (doubly-stained). The SYBR 14/PI procedure was performed 15 minutes and 3 hours after thawing using flow cytometer (EPICS XL – MCL, Beckman Coulter). For analysis, five microliters of 10 µM SYBR 14 in DMSO and 3µl PI were added to 500µl of semen samples diluted to the concentration of 5×10^6 spermatozoa mL with Tyrode's salt solution (Sigma – Aldrich Chemical Co., St. Louis, USA). Samples were analysed with the flow cytometer following 15 minutes incubation on 37°.

Hypoosmotic swelling test (HOS)

The HOS test was used as an assay to evaluate the functional activity of the spermatozoa membrane. The procedure used was that described by Jeyendran et al. (31), adapted for ram semen by Garcia Artiga (32). An aliquot of 50µL of frozen-thawed semen was diluted in 500 µL of hypoosmotic solution and incubated at 37° C for 30 min. A total of 200 spermatozoa were counted. Percentage of spermatozoa population with swollen and/or coiled tail was scored under a phase contrast microscope (400x magnifications).

Statistical analyses

All statistical analyses were performed using Sigma Stat Version 3.1 (Systat Software Inc., Chicago, IL, USA). In results average values are expressed as mean ± SD. Differences between the two extenders at 0, 6, 12 and 24 hours were analysed using one way analysis of variance (HOS, progressive motility at T0) or Kruskal-Wallis test (motility, progressive motility at T6, T12 and T24, viability, PMI and DFI), depending on the distribution of the data. All values of $P < 0.05$ were considered significant.

Results

Percentage of motile spermatozoa was significantly higher in OEP group in comparison to control group (78.4 ± 15.7 vs 43.3 ± 23.9; 66.5 ± 16.9 vs. 15.7 ± 20.6; 46.8 ± 26.3 vs. 7.0 ± 16.6

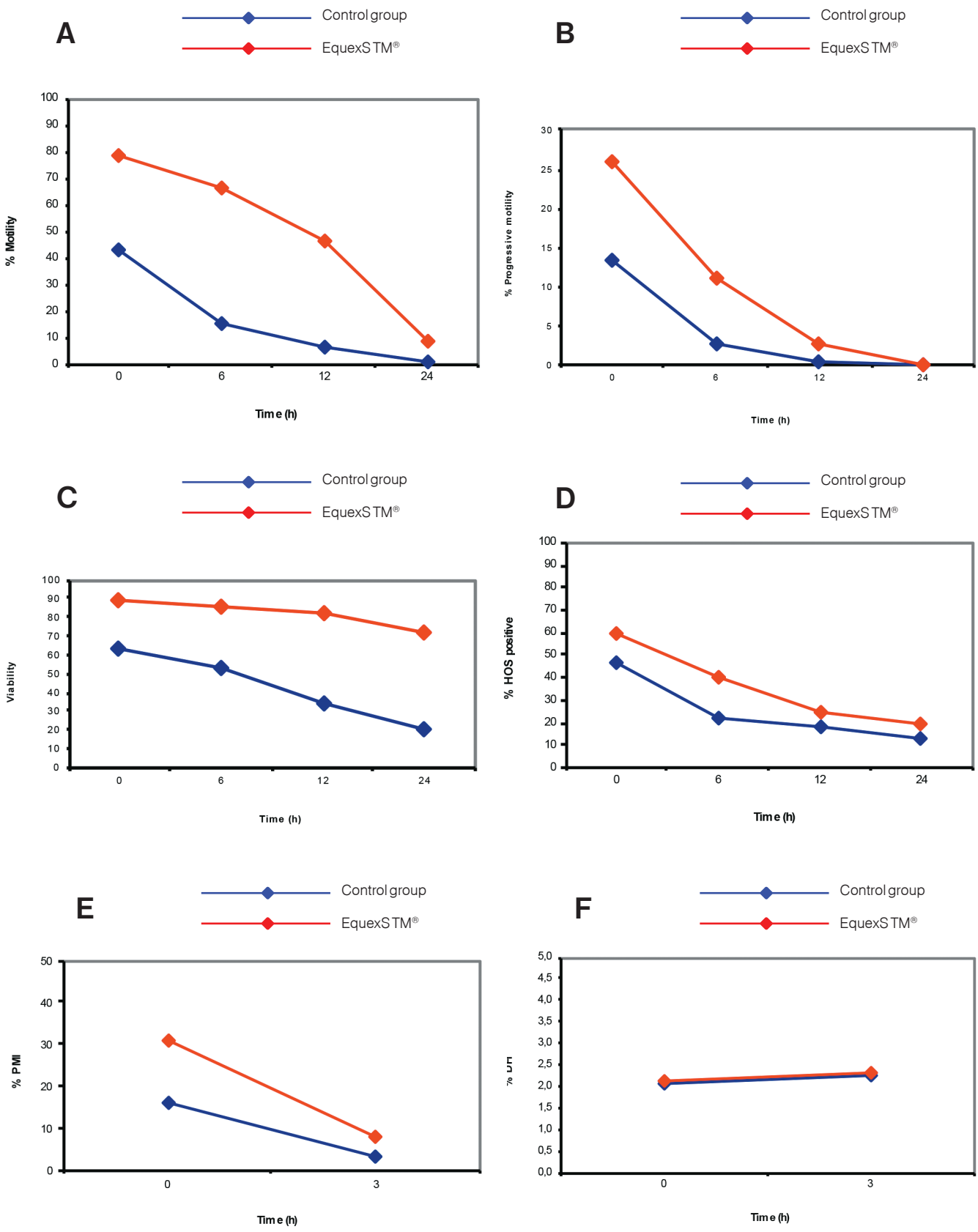

Figure1: Changes in the sperm data after thawing: (A) motility (a:b = p =< 0.001), (B) progressive motility (a:b = p < 0.05), (C) viability (a:b = p =< 0.001), (D) HOS positive (a:b = p < 0.05), (E) membrane integrity (a:b = p =< 0.001) and (F) DNA integrity (a:b = p>0.05)

and 9.3 ± 11.4 vs. 0.8 ± 3.5) respectively for 0, 6, 12 and 24h after F/T (P =< 0.001). Analysis of progressive motility also revealed significantly higher percentage in OEP compared to control group (26.1 ± 9.2 vs. 13.6 ± 9.2; 11.3 ± 6.8 vs. 2.8 ± 6.3; 2.8 ± 2.9 vs. 0.3 ± 0.8; 0.2 ± 0.4 vs. 0.0 ± 0.2) respectively for 0 (P = 0.025), 6, 12 and 24h after F/T (P =< 0.001). Analysis of viability also revealed significantly higher (P =< 0.001) percentage of viable spermatozoa in OEP compared to control group (88.4 ± 8.3 vs. 63.5 ± 17.7; 85.1 ± 11.8 vs. 52.9 ± 21.2; 83.1 ± 14.0 vs. 33.6 ± 30.3; 72.5 ± 23.1 vs. 21.1 ± 26.1) respectively for all times. Percentage of HOS positive spermatozoa was also significantly higher in OEP compared to control group respectively (60.0 ± 16.2 vs. 46.8 ± 16.9; 40.8 ± 12.5 vs. 22.5 ± 11.5; 25.1 ± 9.0 vs. 17.9 ± 9.4; 19.4 ± 7.8 vs. 13.5 ± 7.6) for 0 (P = 0.001), 6 (P =< 0.001), 12 and 24h after F/T (P = 0.002). Percentage of plasma membrane integrity (PMI) was significantly higher in OEP compared to control group respectively (30.6±14.2 % vs. 16.1±13.3 %; 7.8±3.3 vs. 3.2±4.1 %) for 0 and 3h after F/T (P =< 0.001). Analysis of SCSA™ revealed no difference (2.1±1.3 % vs. 2.0±1.3 %; 2.3±1.1 vs. 2.3±1.2 %) in DFI %-values between OEP group compared to control group respectively for 0 (P = 0.599) and 3h (P = 0.760) after thawing.

Discussion

In present study we analysed the effect of Equex STM® on freezability of ram semen. With great certainty we found that addition of 0.38 % detergent into egg yolk based extender has a beneficial effect on the quality of F/T ram semen. This improvement was seen as higher motility, plasma membrane integrity and survival rate of spermatozoa in F/T samples after addition of specific detergent. The results of this study demonstrate that the addition of Equex STM® in the freezing extender protect spermatozoa during freezing and thawing process. These results are in agreement with the study of Akourki et al. (33). Although in our study the initial motility after thawing was higher in OEP group in comparison to the results of Akourki et al. (33). Further analysis after thawing and incubation on 37° C for 24 hours also revealed beneficial effect of Equex STM® on motility, progressive motility, viability, HOS and PMI. In assessing semen quality, animal and human fertility was developed the SCSA test which measure semen DNA integrity. SCSA data on thousands of semen samples from bulls, stallions, boars and exotic cats show the clinical value of this assay for animal fertility assessment. SCSA can utilize a fresh or frozen-thawed semen sample and using the features of flow cytometry, collect and analyse data on 5000 or more cells within a few min of time to evaluate semen quality and further define the relationship of semen quality to fertility (34).

However, we could not find any positive effect on DNA integrity. Some previous studies have shown the negative correlation between the percentage of spermatozoa with denaturated DNA and fertilizing capacity of spermatozoa (35, 36). In our previous research we compared extenders with two different antioxidants and there was no significant difference of DFI between groups which contained antioxidants and control group (37). In present research integrity of DNA was in comparison to Bucak et al. (38), relatively high in all samples and we could not find any effect of detergent on this parameter. Results of DNA integrity and results for other parameters (motility, progressive motility, viability, PMI and HOS) suggest that even a standard protocol used in our study enabled high quality freezing of ram spermatozoa and that the detergent additionally improved the freezing capacity of ram spermatozoa.

These results suggest that spermatozoa frozen in the presence of Equex STM® also have a better fertility compared to the samples that were frozen without the addition of detergent. Pursel et al. (39) found a positive correlation between percentages of motile spermatozoa after thawing and their fertilizing capacity. Intravaginal insemination of bitch with frozen canine semen supplemented with detergent resulted in an overall pregnancy rate, similar to that obtained after natural mating (40). The addition of Equex STM® to the freezing extender had a positive effect on the motility, PMI immediately after freezing-thawing in different species (25, 26, 39, 41). Active compound in Equex STM® is sodium dodecyl sulphate (SDS), a water-soluble anionic detergent that solubilizes active molecules but have a toxic effect on spermatozoa membranes (26, 39, 42). The detergent functions are through the modification of egg-yolk components, this increase sperm membrane permeability and reduce osmotic stress during the freezing–thawing process (26, 39). The cryoprotective effect of Equex

STM® is only seen in the presence of egg yolk and that indicate to be exerted by modification of egg yolk lipoproteins (26, 43). Increased stability of spermatozoa plasma membrane integrity was also seen in our study based on Tris egg yolk extender supplemented with Equex STM®. This positive effect of Equex STM® on post-thaw quality of spermatozoa was found in studies from different animal species, i.e. dog (27, 40, 43), bull (26) , boar (39, 44, 45, 46), stallion (25) and cat (47).

In previous studies it was found that optimal concentration of detergent in freezing extender varied between 0,5 and 1 % for extenders containing 20 % egg yolk (39). Higher concentration of the detergent had a detrimental effect on membrane stability and quality of spermatozoa (26). Axner et al. (48) concluded that addition of Equex STM® to the freezing extender reduces acrosome damage but decreases spermatozoa longevity during post-thaw in vitro incubation of cat epidydimal spermatozoa. However, this result is contradictory with experiments in other species such as bull (26), dog (40) and ram (33). The benefficial effects of Equex STM® on motility, progressive motility, viability and acrosome integrity on buck spermatozoa were found especially during the first two hours after thawing. The long incubation of buck spermatozoa in detergent had an adverse effect during the three hours incubation on 37° C (49). Detergent has also a beneficial effect on post thaw motility of alpaca spermatozoa, while acrosome integrity was unaffected (50). Similar results for motility were also found in our study.

In conclusion, this data clearly indicate that the addition of Equex STM® to the modified Tris egg yolk extender used for freezing of ram spermatozoa significantly improves post-thaw semen quality.

Further studies are necessary to verify, if addition of detergent to the egg yolk based extenders used for freezing of ram semen, would have the same positive effect on fertility of frozen-thawed ram semen.

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, as well as to Christel Hettel, technical assistant in the Sperm Laboratory, Clinic for Cattle, University of Veterinary Medicine Hannover, for her friendly support.

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VPLIV DETERGENTA EQUEX STM® NA GIBLJIVOST, VITALNOST IN INTEGRITETO DNK ZAMRZNJENIH/ODMRZNJENIH OVNOVIH SEMENČIC

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Povzetek: V raziskavi smo preučevali vpliv Equex STM® na kakovost in preživetje ovnovih semenčic, zamrznjenih v razredčevalcu z dodanim jajčnim rumenjakom. Ejakulati 6 ovnov so bili zamrznjeni po standardnem dvostopenjskem postopku z razredčevalcem Tris z dodatkom jajčnega rumenjaka (1). Drugi razredčevalec je bil dodan semenu pri 5 °C in je dodatno vseboval le glicerol (kontrolna skupina) ali glicerol in Equex STM® (skupina OEP). Po odmrzovanju smo vzorce inkubirali v vodni kopeli pri 37 °C. Analize smo opravili po 10 minutah ter 6, 12 in 24 urah. Analiza vzorcev na gibljivost in test na preživitveno sposobnost semenčic (Viadent®) sta bila opravljena z računalniško analizo semena (Hamilton Thorne Biosciences, Version 12.3, Beverly, MA), integriteta membrane semenčic pa s hipoozmotskim testom (HOS). Test integritete DNK semenčic (SCSA™) je bil opravljen z uporabo pretočne citometrije v času 10 minut in 3 ur z določanjem DNK fragmentacijskega indeksa (DFI%). Test integritete membrane semenčic (Sybr-14/PI) je bil opravljen z uporabo pretočne citometrije v času 15 minut in 3 ur po tajanju. Odstotek gibljivih semenčic je bil pri OEP v primerjavi s kontrolno skupino 0, 6, 12 in 24 ur po tajanju statistično značilno višji ($P<0.001$). Analiza preživitvene sposobnosti semenčic je prav tako pokazala statistično značilno višji odstotek ($P<0.001$) vitalnih semenčic pri OEP v primerjavi s kontrolno skupino. Odstotek pozitivnih semenčic pri testu HOS je bil tudi statistično značilno višji pri OEP v primerjavi s kontrolno skupino, in sicer 0 ($P=0.001$), 6 ($P=<0.001$), 12 in 24 ur ($P=0.002$) po tajanju.

Ključne besede: seme; oven; Equex STM®; pretočna citometrija; Viadent®; SCSA™; Sybr-14/PI