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THE INTEREST OF HYPEROSMOLAR EXTENDERS IN ROOSTER SPERM CRYOPRESERVATION

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Abstract. The aim of the present study was to investigate thepotential benefit of hyperosmolar extender during the freezing-thawing process in rooster sperm. The goal was to minimize the amount of intracellular water and reduce the impact of intracellular icecrystal during the freezing process. A total of five 45-week old Hubbard commercial broilers were subjected to bi-weekly semen collections. Collected sperm was pooled and divided in three aliquots. The control aliquot was diluted with Tris-extender without further supplementation at 300 mOsm (Control). The two (2) other aliquots were diluted with Tris-extender at 300 mOsm containing vitamin E (Vit E) or with Tris-extender without Vitamin E but at osmolarity of 450 mOsm (Hyper). After incubation at 22 °C for 15 min, all aliquots were cooled and then frozen in liquid nitrogen. A Computer Aided Semen Analysis (CASA) was used to investigate the impact on different motility parameters. After thawing, Hyperosmolar (Hyper) and vitamin E extenders showed the highest values in terms of sperm motility preservation. Hyperosmolar extender (Hyper) showed particularly the highest values in terms of VSL velocities and progressive motile spermatozoa, known as indicators of sperm quality. In conclusion, the present results revealed that a significant impact was observed when using extenders at osmolarity of 450 mOsmin rooster sperm, in the same manner and even better then when using vitamin E. The positive impact is probably related to the reduction of intracellular ice formation.

Key words: Poultry, sperm, cryopreservation, hyperosmolar extender, oxidative stress, cold chock, spermatozoa, intracellular water

INTRODUCTION

It is well-known in rooster that artificial insemination (AI) is routinely carried out after short term storage at 4°C and it evidenced that fertilizing capacity of freshly collected avian semen is dramatically lost after half an hour [1] compromising consequently fertility outputs. In addition, fertility success of frozen poultry semen is far lower than any of the domesticated mammalian species. It has been estimated that post-thawing chicken semen retains only 1.6% of the fertilizing capacity of fresh semen [2].

Therefore, development of semen extenders, including investigation of different active compounds, was considered worldwide by research groups to enhance hatchability results and to protect post-thawed sperm motility.The strategies are essentially focused to protect cell membrane against cold shock and oxidative stress [3, 4].

Oxidative stress, targeting cell membranes, has been identified as the main factor damaging phospholipids in different animal species including chicken [5, 6] and turkey [7]. Particularly, semen preservation for extended period causes several time-dependent structural and biochemical damages in avian [8, 9] and other mammalian species [10]. In avian, related to the high proportion of PUFA [11—13], cells membranes are more subjected to oxidative stress damages [14, 15].

There is also evidence that during cryopreservation, membrane damage caused by intracellular ice formation in spermatozoa varies to a great extent [16, 17] with the membrane damage related to cold shock and oxidative stress being [18, 19]. The main damage caused by cold shock is observed during the cooling process, when membrane lipids undergo a phase transition from a liquid to a gel state with subsequent disturbance of the membrane structure [20, 21].

During the cryopreservation phase when there is ice crystal formation, there is simultaneously excessive generation of reactive oxygen species (ROS) with a minimal sperm antioxidant capacity existing in the gametes [22]. The first targets of ROS are membrane lipids, particularly polyunsaturated fatty acids (PUFA), inducing the peroxidation and alteration in membrane fluidity and permeability [23—25].

The current study aimed to reduce intracellular ice formation by subjecting rooster spermatozoa to hyperosmolarity (450 mOsm) to increase a driving force for water efflux from the cell. We assume that reducing intracellular ice formation will reduce sperm cells injuries and consequently will protect sperm motility, known as the major factor conditioning fertility outputs.

MATERIALS AND METHODS

All experiments were conducted in accordance with the legislation governing the ethical treatment of animals. A total of five 45-weeks old Hubbard commercial broiler reproductive cocks were used during the experiment. The animals were housed in conventional individual cages under 14 hours of daily illumination, and fed with a standard commercial food at the rate of 155 g/day/animal.

The roosters were subjected to bi-weekly semen collections by dorso-abdominal massage as described by Burros and Quinn [26]. The collected ejaculates were then pooled and analysed. In order to minimize animal stress, the collection was carried out by the same operator and under the same conditions. Necessary precautions were taken during collection to avoid contamination by cloaca fluids. Sperm motility was calculated using a computer-assisted sperm analyser (Sperm class analyser, SCA Microptic, S.L., Version 3.2.0, Barcelona, Spain).

Motility was assessed using a Computer Assisted Sperm Analyzer (CASA; Sperm class analyzer, SCA Microptic, S.L., Version3.2.0, Barcelona, Spain). To facilitate the image capture, the samples were diluted (10—20 · 10^6 Spz/ml) using Tris-extender. Subsequently, 5 μl of each sample was placed onto a warmed (37 °C) 20 μm Leja® 3 chamber slide (Leja Products B.V., Nieuw-Vennep, The Netherlands). Leja® slide was placed under a phase-contrast microscope (Nikon E200®-LED microscope) on a warmed stage (37 °C) and images were captured using a video camera (Caméra Digital Basler A312 fc Germany) at magnification ×10. Four sequences were scanned and

at least 200 spermatozoa were analyzed. The standard settings were set at 25 frames/s, 20—90 μm^2 for head area and VCL > 10 μm/s to classify a spermatozoa as motile. Kinetic variables that were assessed were: total motility (TM%), progressive motility (PM%), movement linearity (LIN%); straightness (STR%); wobble (WOB%); curvilinear velocity (VCL μm/s); straight linear velocity (VSL μm/s); average path velocity (VAP μm/s); amplitude of lateral movement of the head (ALH μm); beat cross frequency (BCF Hertz), % rapid gametes (fraction of cells moving with VCL > 75 μm/s), % moderately motile gametes (fraction of cells moving with 45 < VCL < 75 μm/s) and % slow gametes (fraction of cells moving with 10 < VCL < 45 μm/s). Total motility (TM) was defined as the percentage of spermatozoa with VCL > 10 μm/s, and progressive motility (PM) was defined as the percentage of spermatozoa with VCL > 25 μm/s and STR > 80%.

All samples had a minimum of 70% motile sperm. The sperm samples were then pooled and divided into three aliquots. The control aliquot was diluted with Tris-egg yolk extender without further supplementation at 300 mOsm(Control). The two (2) other aliquots were diluted with Tris-egg yolk extenders at 300 mOsm, containing vitamin E (Vit E) or with Tris-egg yolk extenders without Vitamin E but at osmolarity of 450 mOsm (Hyper). Tris-extender was composed of 300 mM tris[hydroxymethyl]aminomethane, 95 mM citric acid monohydrate, 28 mM D-(+)-Glucose.

All samples were equilibrated for 2 h at 4 °C and then packaged into 0.25 ml straws and frozen in liquid nitrogen vapor using a programmable freezer (CryoMed, Thermo Fisher Scientific, Waltham, MA, USA). Frozen straws were then thawed in a water bath at 5 °C for 3 minutes and sperm motility analyzed using a Computer Aided Sperm Analyzer (CASA).

Calculation of means, standard error of the mean (SEM), and statistical analysis were performed using Statview 4.02 software (Abacus Concepts Inc., Berkeley, CA, USA). Values for each variable were expressed as the mean ± SEM. Variables used for comparison purposes were the three sperm extenders (control, Hyper, Vit E). Differences between treatments were assessed using one-way ANOVA, followed by *posthoc* Fisher's test. Values were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Percentages of total motility and progressive motility

The data regarding the effect of different sperm extenders are presented in figure 1. Compared to the control, the results indicated that Hyperosmolar and vit E extenders protected all CASA motility variables.

Concerning TM, a significant difference ($P < 0.05$) was observed between the control and the two others tested extender (Hyper and Vit E), with means of 11.11, 48.89 and 44.87%, respectively. It's obvious that the control group frozen by a standard extender with 300 mOsmshowed the lowest total motility values. No significant difference was observed when comparing Hyperosmolar (Hyper) and vitamin E (Vit E) extenders. Concerning the progressive motility (PM), these two treatments showed sensibly the same values (12.82 and 11.11%), values significantly higher than those of the control group (2.85%).

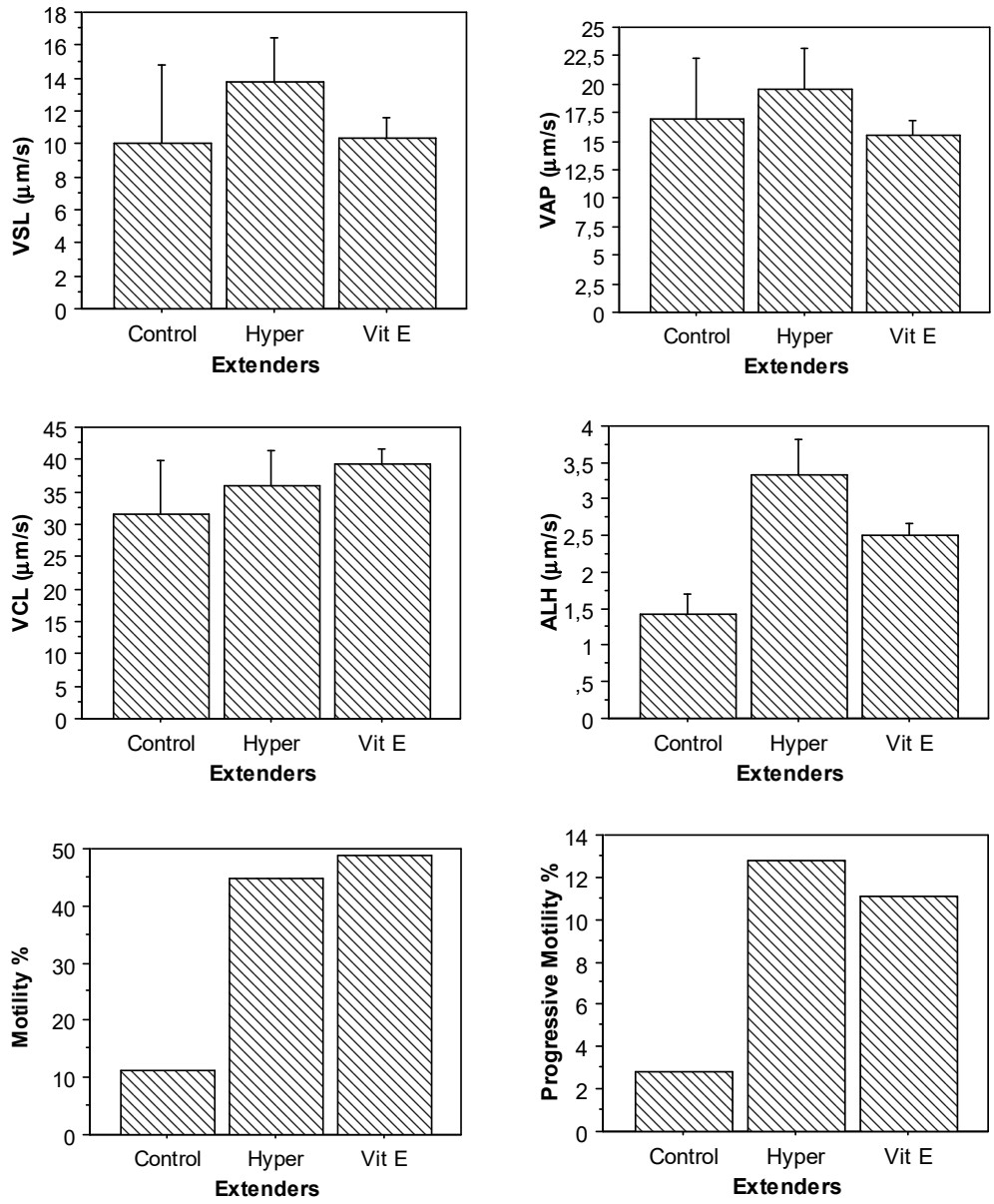

Fig. 1. Percentages (Mean ± S.E.M.) of total motility (TM), progressively motility (PM), curvilinear velocity (VCL), straight linear velocity (VSL), average path velocity (VAP), linearity lateral movement of the head (ALH) after cryopreservation of rooster sperm in 300 mOsm extender (control) hyper-osmolar extender with 450 mOsm (hyper) and extender containing vitamin E (Vit E). Values are presented as Mean ± S.E.M

Kinematic parameters (VCL, VAP, VSL, ALH)

The impact of investigated treatments on VCL, VSL, VAP and ALH are presented in Fig. 1. The lowest values were observed in the control group with 31.58 ± 8.35 µm/s, 10.01 ± 4.78 µm/s, 17.01 ± 5.31 µm/s and 1.43 ± 0.28 µm. Among all treatments, hyperosmolar extender showed the highest values,except for VCL, followed by Vitamin E

extender with 35.93 ± 5.35 μm/s versus 39.18 ± 2.34 μm/s, 13.75 ± 2.69 μm/s versus 10.35 ± 1.19 μm/s, (19.59 ± 3.53 μm/s versus 15.59 ± 1.27 μm/s and (3.33 ± 0.5 versus 2.5 ± 0.16 μm, for VCL, VSL, VAP and ALH, respectively.

The aim of the present experiment was to improve rooster sperm cryopreservation by lowering the amount of intracellular water formation through the use of hyperosmolar extender at 450 mOsm. Two controls were concomitantly used, an isotonic extender with 300 mOsm and an extender containing vitamin E.

Vitamin E is one of the most potent molecules known to inhibit production of ROS and LPO. Vitamin E, a lipophilic molecule, is used both as an antioxidant [27], to combat LPO, and as a stabilizer of plasma membranes [28]. The beneficial effect of vitamin E on sperm was previously observed [29, 30]. Vitamin E (Vit E) increased significantly VCL, VAP, ALH and BCF when compared to the control. This is in accordance with the previous reports where vitamin E was found to increase sperm motility and membrane integrity [31, 32].

The phospholipidic fraction of spermatozoa cell membranes characterized by a high proportion of polyunsaturated fatty acids (PUFA) [33] is essential to membrane fluidity particularly in fusion events including acrosome reaction, sperm egg-interaction and motility [34, 35]. Accordingly, high membrane concentration in PUFA makes sperm cells more susceptible to lipid peroxidation, especially, during the different stages of *in vitro* preservation [36]. In this respect, vitamin E showed in turkey significant positive effect on sperm mobility and viability during storage for 48 h at 5 °C [37] and enhanced fertility outputs [38]. As well, similar positive impacts have been observed in avian diet supplementation with alpha-tocopherol [39]. Similarly, dietary supplementation with other antioxidants, including vitamin E, selenium, vitamin C and enzymatic antioxidant systems improved significantly animal health and sperm quality [40, 41].

In the current experiment hyperosmolar extender showed sensibly the same protective effects as vitamin E treatment. It seems that the rate of water efflux from intracellular compartment was significantly enhanced in these hyperosmolar conditions by increasing permeability to the water in spermatozoa cell membranes. Contrary, in the control group, during cryopreservation the cell may not be able to loseenough intracellular water; the cytoplasm becomes super cooled and can freeze, which usually results in a lethal injury as previously demonstrated [42].

The susceptibility of sperm membrane to cold shock and lipid peroxidation (LPO) depends on cholesterol, a membrane stabilizer at low temperature and PUFA [43]. It is well established that cryopreservation induces cholesterol depletion from the membrane which could exacerbate sperm cold shock damage [44, 45], probably by reducing the amount of intracellular ice formation, such membrane cells alteration could be significantly avoided.

The current results showed that rooster sperm cells could be preserved in the same extent by the use of a potent antioxidant molecule (vitamin E) and by reducing intracellular ice formation. Consequently, these results open interesting alternatives in developing new rooster sperm diluents by a simultaneous use of hyperosmolar solutions at 450 mOsm and antioxidant molecules, mainly vitamin E. However, further research is warranted particularly in terms of fertility outputs after artificial insemination.

CONCLUSIONS

The current results showed that rooster sperm cells could be preserved in the same extent by the use of a potent antioxidant molecule (vitamin E) and by reducing intracellular ice formation. Consequently, these results open interesting alternatives in developing new rooster sperm diluents by a simultaneous use of hyperosmolar solutions at 450 mOsm and antioxidant molecules, mainly vitamin E. However, further research is warranted particularly in terms of fertility outputs after artificial insemination.

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ГИПЕРПОСМОЛЯРНЫЕ НАПОЛНИТЕЛИ СПЕРМЫ ПЕТУХОВ ПРИ ЕЕ КРИОКОНСЕРВАЦИИ

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Целью настоящей работы было исследование потенциального преимущества гиперосмолярного наполнителя в процессе замораживания—оттаивания спермы петухов. Цель заключалась в том, чтобы свести к минимуму количество внутриклеточной воды и уменьшить влияние внутриклеточного льда в процессе замораживания. В общей сложности у пяти 45-недельных коммерческих бройлеров кросса «Hubbard» были взяты образцы спермы в течение двухнедельного периода. Собранную сперму объединяли и делили на три равные части. Контрольный образец разбавляли трис-наполнителем без дополнительных добавок при 300 mOsm (контроль). Два других образца разбавляли трис-наполнителем при 300 мОм, содержащим витамин Е (Vit E), или с трис-наполнителем без витамина Е, но при осмолярности 450 mOsm (Hyper). После инкубации при 22 °С в течение 15 мин все образцы охлаждали и затем замораживали в жидком азоте. Для исследования влияния на различные параметры моторики был использован анализ спермы с использованием метода (CASA). После оттаивания наполнители с Hyperosmolar (Hyper) и витамином Е показали наилучшие значения с точки зрения сохранения подвижности сперматозоидов. Гиперосмолярный наполнитель (Hyper) показал особенно высокие значения в отношении скорости движения VSL и прогрессирующих подвижных сперматозоидов, известных как показатели качества спермы. Представленные результаты показали, что при использовании наполнителей спермы петухов при осмолярности, равной 450 mOsm, наблюдали существенное улучшение показателей сперматозоидов, которое было даже лучше, чем при использовании витамина Е. Положительное влияние, вероятно, связано с уменьшением внутриклеточного образования льда.

Ключевые слова: птица, сперма, криоконсервация, гиперосмолярный наполнитель, окислительный стресс, холодный поднос, сперматозоиды, внутриклеточная вода