



Original article

Edible bird's nest supplementation in chilled and cryopreserved Arabian stallion semen

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ARTICLE INFO

Article history:

Received 1 September 2021

Revised 5 October 2021

Accepted 4 November 2021

Available online 12 November 2021

Keywords:

Arabian stallion

Antioxidant

Cryopreserved Semen

Edible Bird's Nest

EquiPlus[®]INRA[®]

ABSTRACT

Diluents and various biological products have been used in different animal species, with promising outcomes in post-thaw sperm quality. Nevertheless, only a few reports are available for the semen of Arabian horses. Edible bird's nest (EBN) – a product of the salivary secretions of swiftlet species is widely known to have both antioxidant and anti-inflammatory properties. Presently, there is no data available on the role of EBN supplemented in different extenders and its effect on semen quality in stallion semen. Two *in vitro* experiments were conducted to examine the effects of edible bird's nest (EBN) on the quality of chilled and post-thawed cryopreserved Arabian stallion spermatozoa. In experiment one, 10 ejaculates were collected, divided into two equal parts, diluted using EquiPlus[®] and INRA 96[®] and supplemented with 0 % (control), 0.12 %, 0.24 % EBN concentrations. The semen samples were stored at 5 °C and observed at 0, 24, and 48 h. Sperm kinetics variables (% total motility [TM] and progressive motility [PM], curvilinear velocity; VCL, straightness; VSL, average path velocity; VAP) were analyzed using computerized assisted sperm analysis. For chilled semen, there was no significant difference in any of the sperm quality parameters between control (0 %), 0.12 %, and 0.24 % EBN supplementation either in INRA96[®] or EquiPlus[®]. In experiment two, nine ejaculates were diluted and cryopreserved using EquiPlus Freeze[®] and INRA Freeze[®] containing 0 %, 2.4 %, and 4.8 % EBN, and evaluated after thawing. Sperm kinetics, DNA integrity and antioxidant capacity - Biological Anti-oxidant Potential (BAP) and Reactive Oxygen Metabolites (d-ROMs) test were evaluated. In chilled semen, there was no significant difference in any of the sperm quality parameters between control (0 %), 0.12 %, and 0.24 % EBN supplementation either in INRA96[®] or EquiPlus[®]. For frozen semen supplemented with 2.4 % and 4.8 % EBN had higher sperm motility parameters compared to control in INRA Freeze[®] and EquiPlus Freeze[®], but the values were not statistically significant ($P > 0.05$). Also, EBN supplementation had no significant effects on the DNA integrity, biological antioxidant potential, and reactive oxygen metabolites. EBN supplementation had no significant effects on sperm quality and antioxidant status in chilled and frozen Arabian Stallion semen. Future studies might consider different methods of EBN preparation and concentrations to elucidate the potential biological impact of EBN in Arabian stallion semen.

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Abbreviations: EBN, Edible Bird Nest; ROS, Reactive oxygen species; d-ROMs, Determination of Reactive Oxygen Metabolites; BAP, Biological Anti-Oxidant Protection; DNA, Deoxyribonucleic Acid; AS, Arabian Stallion.

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Peer review under responsibility of King Saud University.



1. Introduction

Advances in the cryopreservation of stallion semen have led to a significant improvement in equine breeding over the past years. With the worldwide demand for stallion spermatozoa, cryopreservation is beneficial in terms of long-term preservation, transportation, and availability of important genetic resources, as well as reducing the chances of disease transmission associated with natural breeding (Alvarenga et al. 2016). Nevertheless, there are concerns regarding the low fertility of frozen-thawed stallion semen.

<https://doi.org/10.1016/j.sjbs.2021.11.009>

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compared with other domestic species (Gibb et al. 2017; Gibb, Lambourne, and Aitken 2014). Fertility problems arise from the low survival of stallion spermatozoa during freezing (Alvarenga et al. 2016), which are attributed to alterations such as osmotic stress, the formation of intracellular ice crystals, and toxicity of cryoprotectants (Alvarenga et al. 2016). The underlining mechanisms related to the variability are not fully understood (Ortega Ferrusola et al. 2009). There are various degrees of damage experienced by cooled or frozen-thawed spermatozoa ranging from reduced sperm motility to defective membrane integrity or even sperm death (Gibb et al. 2013).

Oxidative stress is one of the events affecting the fertility and physiology of frozen spermatozoa (Aitken and Krausz 2001). Oxidative stress results from an overproduction of reactive oxygen species (ROS) beyond the antioxidant capacity of the body cells (Halliwell 2006), thereby leading to substantial damages of spermatozoa such as lipids peroxidation (Ball 2008) protein oxidation (Morielli and O'Flaherty 2015) deoxyribonucleic acid (DNA) fragmentation (Rui et al. 2018), and reduced activity of the mitochondrial membrane (Len, Koh, and Tan 2019; López-Fernández et al. 2008). Endoplasmic reticulum is a known source of ROS (Tu and Weissman 2004), and its hemostasis is also perturbed during cryopreservation (Zhao et al. 2015). Detection of oxidative damage in cryopreserved spermatozoa is an important parameter to quantify the extent of the damage. The degree of DNA damage in cells was significantly increased by cryopreservation as assessed by DNA fragmentation. However, it is proven that using slow-freezing rate of cells can reduce the damage of DNA due to the activation of p53 and RAD51 which considered to be the guardians of the genome (Xu et al. 2010).

Edible bird's nest (EBN) – a product of the salivary secretions of swiftlet species is recognized for its medicinal and nutritional benefits and demonstrated as a potential antioxidant (Ghassem et al. 2017; Marcone 2005). Several authors have reported that EBN possesses anti-aging, anti-cancer, antioxidative, and anti-inflammatory properties (Hamzah et al. 2013; Hou et al. 2015; Yida et al. 2015). EBN contains numerous components such as carbohydrates which make up 27–58% of the constituents (Yida et al. 2015). Specifically, the carbohydrates elements consist of 9.0% sialic acid, 7.2% galactosamine, 5.3% glucosamine, 16.9 % galactose, and 0.7 % fructose. Sialic acid (SA) is also considered an immune moderator which affects mucus' ability to remove harmful microbes (Yagi et al. 2008). Sialic acid is well-known to have both metabolic enhancing (Abdul Khalid et al. 2019; Yusop et al. 2016) and antioxidative ability (Ghassem et al. 2017; Hou et al. 2015). This antioxidative property contributed to the preservation of morphological characteristics of stallion semen during cryopreservation (Yusop et al. 2016). Also, monosaccharide (glucose) and disaccharide (trehalose) combinations increased semen performance after cryopreservation (Naing et al. 2010). Moreover, several studies have reported the beneficial effects of sugars on the post-thawed viability of animal sperm in semen-freezing extenders (Panyaboriban et al. 2015). A maximum of 18 amino acids has been identified in EBN (Abdul Khalid et al. 2019). Amino acids are important for energy production, facilitating cell function regulation, and building the immune system through the production of immunoglobulins and antibodies (Chua and Zukefli 2016). Reports suggested amino acid supplementation enhanced sperm motility, viability, and acrosome integrity (Khalili et al. 2010). Szczesniak-Fabianczyk et al. found that the addition of cysteine in semen extender improved the viability, structure of chromatin, and integrity of boar sperm cells during liquid preservation (Szczesniak-Fabianczyk et al. 2003).

In line with the benefits stated above, there is an evidence suggesting that a combination of different antioxidants could induce additive protective effects against cryopreservation compared with

those used solely (Uysal and Bucak 2007). For instance, a combination of superoxide dismutase (SOD) and catalase led to a significant improvement in oocyte survivability compared to when the two treatments were used individually (Dinara et al. 2001). Relating this to human sperm cryopreservation, a combination of SOD and catalase enhanced sperm motility, which was far better than the sole supplementation of each treatment (Trzcińska and Bryła 2015). In stallion semen, freezing extenders made up of INRA96® + INRA Freeze® or BotuSemen + BotuCrio led to improved semen quality than those containing EquiPlus® and Gent® (Neuhauser et al. 2019).

Most Arabian mares in the Kingdom of Saudi Arabia are bred naturally and a considerable number of stallions were reported to have poor semen quality and fertility after thawing (Ali, Alamaary, and Al-Sobayil 2014; Waheed et al. 2016). Diluents and several biological products have been used in different animal species, with promising outcomes in post-thaw sperm quality, but only a few reports are available for the semen of Arabian horses. Presently, there is no data available on the role of EBN supplemented in different extenders and its effect on semen quality in stallion semen. We hypothesized that the addition of EBN to extended semen would limit oxidative damage and osmotic shock, which are the main events responsible for sperm damage during the freezing and cooling process. The present study aimed to evaluate the effect of different concentrations of EBN added into EquiPlus®, EquiPlus Freeze®, INRA 96® and INRA Freeze® extenders on the quality of chilled and cryopreserved Arabian stallion spermatozoa.

2. Materials and methods

2.1. Animals

A convenient sample size of nine Arabian stallions aged between 6 and 13 years (median 9 years) with weight ranging from 400 to 540 kg were enrolled in this study. Prior to the experiment, the stallions were examined for semen quality which included total motility (TM), progressive motility (PM), morphology, and concentration. Each stallion was housed in 20 m² stables at the Mounted Police Division in the Royal Oman Police area, Muscat, Oman. They were all provided with food and water. The daily diet was made up of dry grass Katambora, oats, and concentrated ration cubes of HAVENS® basis-sport which contain carbohydrates (51 %), crude protein (12.6 %), crude fat (3.4 %), calcium (10.4 g) and phosphorus (4.7 g). The experiment was conducted during the breeding season between January to May 2019. All the procedures involving animal use were approved by the Animal Welfare Committee at Animal Health Research Center (AHRC) No 54.

2.2. Preparation of EBN and extenders

EquiPlus® - Equiplus Freeze® (Minitube, Germany) and INRA 96® - INRA Freeze® (IMV Technologies Group, France) were the four commercial extenders used in this experiment. The supplement was a hydrolyzed form of EBN (a product from house nest swiftlet [*Aerodramus fuciphagus*]) obtained from the University Kebangsaan Malaysia. To prepare stock A, B, and C of hydrolyzed EBN solutions, 0.48 g (480 mg), 0.24 g (240 mg), 0.12 g (120 mg) of hydrolyzed EBN were added, respectively into corresponding 10 ml of physiological buffer saline (PBS) leading to the formation of 4.8 %, 2.4 %, and 1.2 % concentrations of hydrolyzed EBN solution aliquots.

Ten ml of stock A, B and C were refrigerated at – 20 °C in smaller fractions of 40 µl and 400 µl Eppendorf tubes before use. The aliquots were introduced into a water bath at 37 °C for 3 min before

Table 1a

Semen extenders used for chilled and frozen stallion semen in the present study.

| Components | Manufacturer |
|---|---|
| Extender for chilled semen INRA 96® EquiPlus® | Purified fractions of the milk micellar protein, penicillin, gentamicin, and amphotericin B. Caseinates derived from various fractions of milk casein, lincomycin and spectinomycin. IMV technologies, France Minitub, Tiefenbach, Germany |
| Extender for frozen semen INRA Freeze® EquiPlus Freeze® | Purified fractions of the milk micellar protein, sterilized egg yolk plasma (2%), Sugars, Salts, Buffer, penicillin, gentamicin, and amphotericin B, Glycerol (2.5%). Skim milk, Sugars, Buffer, Gentamicin sulfate Glycerol (3%) IMV technologies, France Minitub, Tiefenbach, Germany |

Table 1b

Composition of bioactive edible bird's nests (EBN) hydro-peptides analyzed in UKM UNIQEQ.

| Parameter, Unit | Result | Test Method |
|-----------------------------|---------------|--|
| Sialic Acid, % | 16.4 | HPLC |
| Protein, g/100 g | 64 | Method No. STP/Chem/A03 based on AOAC 16th Edi. 981.10 |
| Total Fat, g/100 g | 0.6 | Method No. STP/Chem/A02(ii) based on the Chemical Analysis of Food, David Pearson, 6th Edition: Page13 |
| Total Carbohydrate, g/100 g | 26.6 | Method No. STP/Chem/A06 based on Promerance Food Analysis: Theory and Practice, 2nd Ed. (pg 637) |
| Total Ash, g/100 g | 2.2 | Method No. STP/Chem/A05 based on AOAC 16th Edi. 923.03 |
| Moisture, g/100 g | 6.6 | Method No. STP/Chem/A04 based on AOAC 16th Edi. 950.46 |
| Energy, kcal/100 g | 368 (1546 kJ) | Method No. STP/Chem/A03 based on Person's The Chemical Analysis of Foods (6th Edition, page 578) |

adding them into the extenders. For chilled semen experiment, 40 µl of the hydrolyzed EBN (24 µg/µl) from stock B solution was added into 3,960 µl of each extender (EquiPlus® and INRA 96®) to make the 0.24 % hydrolyzed EBN treatment. The same quantity of EBN from stock C solution (12 µg/µl) was added to 3,960 µl of each extender to make the 0.12 % of hydrolyzed EBN treatment. For the frozen semen experiment, 400 µl of the hydrolyzed EBN (48 µg/µl) from stock A solution was added into 3600 µl of each extender (EquiPlus Freeze® and INRA Freeze®) to make the 4.8 % hydrolyzed EBN treatment. About 400 µl of the hydrolyzed EBN (24 µg/µl) from stock B solution was added into 3600 µl of each extender to make the 2.4 % hydrolyzed EBN treatment. The components and Physico-chemical properties of the extenders and EBN used in this study are presented in Table 1a and 1b.

2.3. Semen collection and processing

A total of 19 ejaculates were collected from 9 stallions using a Hannover model artificial vagina (AV; Mini-tube of America, Verona, VT), lubricated using a non-spermicidal lubricant (Minitube, Germany), and pre-warmed at 48 °C. An inline filter fitted into the artificial vagina (Mini-tube, Germany) was employed in separating the ejaculate from the gel. A phantom was used to stimulate the stallion in the presence of an estrous mare. Thereafter, the samples were transported to the laboratory immediately before processing. The semen volume was read using a graduated cylinder and inspected for contaminants, residue, odor, or abnormal appearance.

The ejaculates were checked for sperm kinetic parameters using the Computer-Assisted Sperm Analyzer (CASA; CEROS, Version12, Hamilton Thorne Biosciences, USA). Sperm concentration was determined using SDM1-Photometer (REF. 12300/0101, Minitube, Germany). Only ejaculates with sperm motility higher than 60 % and concentrations of 200 × 10⁶ sperm cells/ml were used for the experiment.

2.4. Experimental design

To evaluate the effect of EBN in the extenders used for liquid storage and cryopreserved semen, the samples were divided and

allocated to two main groups for both extenders (EquiPlus® and INRA 96®). Each sample was diluted with semen extenders and divided into two parts for two corresponding tests. For the first experiment, 10 selected ejaculates from five stallions were divided into two equal parts and diluted using extenders EquiPlus® and INRA96® to obtain semen concentration of 50 × 10⁶. Both extenders contained 0 % (control), 0.12 %, and 0.24 % of EBN and were stored at 5 °C with a cooling rate of 0.3 °C/min for 48 h (0 h after equilibration period of 90 min at 5 °C).

In Experiment two, nine selected ejaculates from four stallions were divided into two equal parts and diluted at 1:1 with INRA 96® and EquiPlus® extenders. The samples were then centrifuged at 1,000 g for 10 min to remove 90 to 95 % of seminal plasma. The supernatant was removed and the sperm pellets containing 5 to 10% of seminal plasma were resuspended with specific freezing extenders (INRA freeze® + 0 % [control], INRA freeze® + 2.4 %, INRA freeze® + 4.8 %, EquiPlus freeze® + 0 % [control], EquiPlus freeze® + 2.4 %, and EquiPlus freeze® + 4.8 %) to obtain 200 × 10⁶ sperm/ml. Each diluted aliquots were cooled in a cold handling cabinet rate 0.3 °C/min (IMV, France) for equilibration to 5 °C over 2 h. Equilibrated semen samples were filled in 0.5 ml French straws (IMV, France) and frozen using programmable freezer – equine fast rate (5 °C/minute from + 4 °C to –10 °C, 60 °C/minute from –10 °C to –140 °C) (Minitube, Germany) and the straws were plunged into LN2 (–196 °C) for storage. Thereafter, the samples were thawed after a week at 37 °C for 30 sec. Sperm motion kinetics (CASA), Reactive Oxygen Metabolites (d-ROMs), Biological Antioxidant Potential (BAP) (Diacron, Grosseto, Italy) and sperm DNA integrity (Halomax DNA integrity test kit, Minitube, Tiefenbach, Germany) were assessed at 37 °C immediately after thawing.

2.5. Analysis of sperm motility and sperm kinetics

Diluted, liquefied semen samples were further diluted with extender to 50 × 10⁶ spermatozoa/ml. Three microliters of semen was placed on a pre-warmed 20 µm standard count analysis chamber (Leja, Nieuw-Vennep, The Netherlands). Six randomly selected microscopic fields were scanned and approximately 600 spermatozoa counted. The total motility (TM), progressive motility (PM), average path velocity (VAP), progressive velocity (VSL) and track

speed (VCL), lateral head amplitude (ALH), beat cross frequency (BCF), straightness (STR) and linearity (LIN) of spermatozoa were analysed. The CASA settings are summarized in Table 2.

2.6. Sperm viability and DNA integrity (DNA fragmentation)

Hoechst33342/PI fluorescent staining kit (Minitube, Tiefenbach, Germany) was applied to assess sperm viability. The staining was used to distinguish semen cells from the intact and damaged membrane and analyzed with the module “Membrane Integrity” (Viability) of AndroVision®. Initially, the semen sample (50 µl; 200 million spermatozoa/ml concentration) was warmed at 38 °C. Then 1.5 µl of stain was added to the 50 µl sample and mixed well before incubation for 15 min. A drop of the mixed semen sample was added onto a microscope slide. Six randomly selected microscopic fields were scanned and approximately 600 spermatozoa counted using AndroVision® Florecent microscope – Neofluar objective (20x) (Minitube, Tiefenbach, Germany).

DNA sperm integrity was analyzed using the Halomax DNA integrity kit (Minitube, Tiefenbach, Germany) by following the manufacturer’s description. The sperm DNA fragmentation was performed immediately after warming and thawing of the samples prepared in experiments 1 and 2, respectively. The lysis solution was prepared by setting the base lysis solution (BLS), reconstituted to lysis solution (LS), followed by the addition of 3 µl of reducing agent (RA), and mixed to ensure homogenization. An agarose microgel was used to measure the final LS and prepared samples. The semen samples were further diluted to a concentration of 15–20 million sperm/ml. An Eppendorf tube (ACS) containing agarose (500 µl) was placed in the float and incubated in a microwave oven. About 25 µl of each semen sample was transferred to an empty Eppendorf tube, 50 µl of liquefied agarose was added and mixed. Next, a drop of 2 µl of cell suspension was dropped onto the marked wells and covered with glass coverslips, placed on a glass plate before removing the coverslips. The slide was placed horizontally on the float into a petri dish, followed by applying LS over the wells. The sample was incubated for 5 min, drained, and staged horizontally again directly on the float. The slide was washed for 5 min with distilled water using a disposable pipette, rinsed, and placed horizontally on top of the float. Dehydration was conducted for 2 min using 10 % ethanol, drained, and allowed to dry. The slides were stained using 2 µl of sybergreen prepared at a final 40x concentration in distilled water, and mixed with antifading mounting media (i.e. vectashield). The stained slides were views under the AndroVision® Florecent microscope – Neo-

fluor objective (20x) (Minitube, Tiefenbach, Germany). The analysis of sperm DNA fragmentation entailed a minimum number of 300 sperm/sample. Interpretation of results was based on sperm with fragmented DNA; sperm indicating nucleoids having a large and spotty halo of chromatin, and sperm without fragmented DNA; those showing nucleoids with a small and compact halo of dispersed chromatin.

2.7. Reactive oxygen Metabolites (dROMs) and biological antioxidant potential (BAP) test

Frozen stallion semen samples were thawed at 37 °C for 30 sec before conducting d-ROMs and BAP tests. A 500 µl of thawed semen sample (200×10^6 sperm/ml) was centrifuged at 14,000 g for 10 min, and the supernatant was collected and stored at –20 °C until analysis. The concentration of extracellular ROMs was determined using the d-ROMs commercial kit and FREE System (Diacron, Grosseto, Italy). The results were expressed in arbitrary units called ‘Carratelli unit’ (U CARR), where 1 Carratelli unit corresponds to 0.08 mg/100 ml of hydrogen peroxide (Ferramosca et al. 2013).

Total antioxidant capacity or biological antioxidant potential (BAP) was measured by a spectrophotometric assay method using the Free Radical Elective Evaluator (FREE) photometer (Diacron International, Grosseto, Italy). The reagent consisted of 10 µl of the diluted sample, 1 ml of antioxidant solution, and 10 µl of chromogenic substance (N, N-diethyl paraphenylenediamine). Also included was a standard and a blank reagent obtained after replacing the sample with distilled water. Absorbance was measured at 505 nm by using the same FREE System. The absorbance of the blank reagent was subtracted from those of the samples and standard. Results were expressed as moles of HClO consumed per ml of the sample.

2.8. Data analysis

All the data were analyzed using the statistical package for social science (SPSS, Version 24, IBM, USA). The data were checked for normality based on the level of kurtosis and skewness. Log transformation was conducted when necessary. All the CASA parameters were analyzed using repeated-measures analysis of variance (ANOVA) under the General linear model. For variables that failed to conform to normal distribution after log transformation, Kruskal-Wallis test to determine the difference in the means between various EBN concentrations. Time (0, 24, and 48 h), extenders (EquiPlus® and INRA 96®), EBN concentration (0 %, 0.12 %, and 0.24 %), and temperature for chilled and frozen semen were treated as fixed effects in the model. Replicates were considered as random effects. $P < 0.05$ was considered for statistical significance in all the analyses. Possible interactions between the fixed factors were checked.

3. Results

3.1. Effects of supplementation of semen extender with EBN on the quality of chilled Arabian stallion spermatozoa.

Table 3 shows the average sperm kinetics following 0, 24, and 48 h storage. The TM and PM values were not significantly different ($P > 0.05$) between the control and EBN supplementation irrespective of storage time. Likewise, EBN supplementation of semen extended with INRA 96® had no significant effect on any of the other sperm quality parameters. For EquiPlus®, similar findings were observed as sperm quality parameters were not significantly different ($P > 0.05$) between the control and higher EBN supplementations.

Table 2

Settings of the Hamilton Thorne CEROS animal software (Version 12) used to assess stallion sperm kinematics.

| Variable | Setting |
|-------------------------------------|----------|
| Frame rate | 60 Hz |
| Number of frames required | 45 |
| Minimum contrast | 55 |
| Minimum cell size (pixels) | 6 |
| Progressive path velocity cut-off | 30 µm/s |
| Progressive straightness | 60% |
| Slow average path velocity cutoff | 10 µm/s |
| Slow straight-line velocity cutoff | 5 µm/s |
| Static average path velocity cutoff | 4 µm/s |
| Static average line velocity cutoff | 1 µm/s |
| Non-motile head size (pixels) | 0.5–4.8 |
| Non-motile head intensity | 0.25–1.8 |
| Non-motile head intensity | 1.87 x |
| Video frequency | 60 |
| Illumination intensity | 2300 |
| Temperature | 37 °C |

3.2. Effects of supplementation of semen extenders (INRA Freeze® and EquiPlus Freeze®) with EBN on frozen-thawed sperm kinetics and antioxidant properties

Table 4 shows the comparisons of sperm kinetics of frozen-thawed semen extended with INRA Freeze® and EquiPlus Freeze® and supplemented with 0 % (control), 2.4 %, and 4.8 % EBN. There was no significant difference (P > 0.05) in any of the sperm motility parameters between the control, 2.4 %, and 4.8 % EBN supplementation in the semen extended with INRA Freeze® or EquiPlus Freeze®. Table 5 shows the comparisons of sperm kinetic parameters between the different concentrations of EBN after equilibration period and before freezing. For INRA Freeze®, both TM and PM were not different (P > 0.05) between the control and various EBN supplementation. Numerically, VAP, VSL, and VCL were higher in 4.8 % EBN compared to the control, but the differences were not statistically significant (P > 0.05). Likewise, for the frozen semen diluted with EquiPlus Freeze®, no significant difference (P > 0.05) was observed in any of the sperm motility parameters between the control, 2.4 %, and 4.8 % EBN supplementation. Comparisons of DNA integrity, BAP, and d-ROMs between the control and various EBN treatments are shown in Fig. 1. There was no significant difference in any of the antioxidant indicators between the various EBN treatment and control for frozen semen either diluted with INRA Freeze® or EquiPlus Freeze® (P > 0.05).

4. Discussion

To the best of our knowledge, there is no available data on the impact of EBN on the quality of either chilled or frozen equine semen treated with commercial extenders (i.e., INRA 96®, INRA Freeze® and EquiPlus Freeze®). In chilled semen, the majority of

sperm kinetics were maintained at various storage times following dilution with both extenders and EBN supplementation. These results highlighted the beneficial effect of extenders on sperm kinetics, which is consistent with reports from other studies (Al-Bulushi et al. 2019; Alamaary et al. 2019; Contri et al. 2010). The findings may be associated with the composition of INRA 96® and EquiPlus®, which could be protective to sperm membranes during preservation (Pillet et al. 2008).

The protective effects of INRA96® on sperm membranes during preservation have been reported in previous studies (Pillet et al. 2008). Semen extenders have intrinsic features that result in varying effects on the percentage of viable spermatozoa in horses (Alamaary et al. 2019; Waheed et al. 2016). For instance, the integrity of the acrosome membrane may be compromised by the local production of ROS (Len et al. 2019; Whittington and Ford 1999), which leads to oxidative stress and marked reductions in sperm viability (Aitken and Krausz 2001). The capacity to reduce or limit these underlying mechanisms varies between extenders (Alamaary et al. 2019; Neuhauser et al. 2019). In previous studies, there was no significant difference in post-thawed TM, PM, and membrane integrity between stallions' semen extended with INRA 96® and E-Z mix® (Alamaary et al. 2019). A recent study conducted in Arabian stallion found that INRA Freeze® and EquiPlus® performed similarly in improving PM compared to HF-20 extender (Glucose 5 g, Lactose 0.3 g, Raffinose 0.3 g, Sodium citrate 0.15 g, Sodium phosphate 0.05 g, Potassium sodium tartrate 0.005 g and egg yolk 10%) (Alamaary et al. 2019).

However, EBN had no significant effect on the sperm quality of chilled Arabian stallion semen in this study. Furthermore, when sperm kinetics were compared between the various EBN concentrations and control in both extenders, although some of the sperm motility parameters had higher values at higher EBN concentration

Table 3 Effects of supplementation of semen extender with EBN on the sperm kinetics and viability of chilled Arabian stallion spermatozoa.

| Variable | Time | INRA 96® | | | EquiPlus® | | |
|----------------------------------|------|--------------|--------------|--------------|--------------|--------------|--------------|
| | | Control | 0.12 % | 0.24 % | Control | 0.12 % | 0.24 % |
| Total motility (%) | 0 | 96.2 ± 1.7 | 96.8 ± 1.8 | 92.8 ± 2.7 | 95.7 ± 1.5 | 96.4 ± 0.8 | 94.7 ± 1.1 |
| | 24 | 83.0 ± 6.4 | 79.2 ± 8.6 | 84.2 ± 6.2 | 67.5 ± 7.8 | 67.0 ± 7.5 | 70.1 ± 8.1 |
| | 48 | 63.4 ± 16.5 | 62.8 ± 15.6 | 68.2 ± 13.3 | 34.5 ± 7.9 | 29.5 ± 9.3 | 38.0 ± 12.4 |
| Progressive motility (%) | 0 | 54.0 ± 7.0 | 55.0 ± 6.4 | 49.1 ± 6.1 | 55.1 ± 2.1 | 58.2 ± 1.8 | 54.8 ± 2.7 |
| | 24 | 38.0 ± 5.1 | 36.7 ± 5.7 | 38.8 ± 4.9 | 20.7 ± 4.2 | 23.2 ± 4.6 | 28.0 ± 2.0 |
| | 48 | 21.0 ± 6.8 | 21.4 ± 6.5 | 24.6 ± 5.83 | 6.8 ± 2.2 | 5.5 ± 2.1 | 8.7 ± 3.3 |
| Path Velocity (VAP, μm/s) | 0 | 159.2 ± 23.5 | 165.1 ± 26.1 | 158.9 ± 19.3 | 161.5 ± 5.8 | 172.9 ± 4.6 | 161.0 ± 6.4 |
| | 24 | 130.2 ± 9.4 | 132.9 ± 11.9 | 132.5 ± 9.6 | 81.5 ± 4.8 | 88.6 ± 8.4 | 93.8 ± 10.0 |
| | 48 | 87.1 ± 11.7 | 98.0 ± 7.2 | 99.4 ± 4.0 | 64.7 ± 1.6 | 58.6 ± 1.7 | 60.7 ± 3.7 |
| Progressive velocity (VSL, μm/s) | 0 | 127.1 ± 20.3 | 131.4 ± 22.5 | 126.2 ± 16.7 | 126.7 ± 5.8 | 136.5 ± 3.7 | 126.9 ± 5.9 |
| | 24 | 101.4 ± 8.0 | 105.2 ± 10.6 | 104.1 ± 9.4 | 59.2 ± 4.4 | 64.8 ± 7.8 | 70.3 ± 9.0 |
| | 48 | 64.2 ± 9.7 | 73.4 ± 5.6 | 73.8 ± 4.1 | 40.4 ± 2.3 | 38.3 ± 1.0 | 38.7 ± 3.7 |
| Curvilinear velocity (VCL, μm/s) | 0 | 257.7 ± 41.3 | 263.2 ± 45.5 | 255.0 ± 32.8 | 259.6 ± 13.9 | 288.5 ± 8.5 | 260.5 ± 16.7 |
| | 24 | 224.7 ± 14.0 | 227.8 ± 19.3 | 226.1 ± 14.9 | 142.1 ± 7.6 | 153.8 ± 13.5 | 161.5 ± 16.5 |
| | 48 | 161.0 ± 21.4 | 174.4 ± 12.2 | 177.5 ± 16.8 | 110.4 ± 6.5 | 107.4 ± 3.1 | 108.7 ± 6.1 |
| Lateral head amplitude (ALH, μm) | 0 | 8.6 ± 1.3 | 8.3 ± 1.3 | 8.9 ± 1.0 | 9.4 ± 0.4 | 9.8 ± 0.5 | 9.6 ± 0.5 |
| | 24 | 8.8 ± 0.4 | 8.9 ± 0.3 | 8.5 ± 0.3 | 6.4 ± 0.2 | 6.6 ± 0.4 | 6.7 ± 0.4 |
| | 48 | 7.3 ± 0.8 | 8.0 ± 0.4 | 7.8 ± 0.4 | 6.0 ± 0.4 | 5.7 ± 0.2 | 5.3 ± 0.1 |
| Beat cross frequency (BCF, Hz) | 0 | 13.1 ± 2.5 | 13.9 ± 2.2 | 13.4 ± 1.9 | 21.7 ± 3.0 | 20.5 ± 2.9 | 22.0 ± 2.9 |
| | 24 | 16.1 ± 1.3 | 17.0 ± 1.5 | 16.1 ± 1.3 | 14.2 ± 0.8 | 13.9 ± 1.1 | 14.2 ± 1.1 |
| | 48 | 11.2 ± 2.3 | 15.4 ± 1.2 | 14.8 ± 1.6 | 14.7 ± 0.9 | 15.0 ± 2.2 | 14.4 ± 2.4 |
| Straightness (STR, %) | 0 | 75.6 ± 1.6 | 76.6 ± 2.1 | 75.6 ± 1.3 | 74.5 ± 2.3 | 75.2 ± 2.1 | 74.3 ± 2.3 |
| | 24 | 72.4 ± 1.3 | 73.1 ± 1.5 | 73.5 ± 1.7 | 57.7 ± 1.6 | 67.0 ± 1.9 | 69.0 ± 1.9 |
| | 48 | 65.6 ± 1.8 | 70.0 ± 0.7 | 74.0 ± 3.9 | 62.7 ± 3.0 | 63.7 ± 2.5 | 63.7 ± 1.6 |
| Linearity (LIN, %) | 0 | 48.6 ± 1.6 | 50.2 ± 2.4 | 47.8 ± 1.6 | 44.7 ± 1.8 | 45.7 ± 1.5 | 44.7 ± 1.6 |
| | 24 | 42.7 ± 1.2 | 43.3 ± 0.9 | 44.1 ± 1.2 | 40.5 ± 1.1 | 40.1 ± 1.1 | 41.7 ± 1.2 |
| | 48 | 38.8 ± 0.5 | 40.8 ± 0.7 | 41.0 ± 1.4 | 38.0 ± 1.5 | 39.0 ± 2.6 | 38.5 ± 1.3 |
| Viable spermatozoa (%) | 0 | 75.8 ± 7.6 | 68.2 ± 8.7 | 67.4 ± 8.3 | 53.3 ± 8.3 | 59.3 ± 9.7 | 60.3 ± 8.3 |
| | 24 | 50.2 ± 8.2 | 42.1 ± 6.0 | 49.1 ± 8.4 | 44.5 ± 9.0 | 35.7 ± 8.4 | 38.5 ± 5.3 |
| | 48 | 36.6 ± 8.2 | 42.4 ± 10.9 | 36.4 ± 9.4 | 24.4 ± 12.4 | 25.2 ± 11.7 | 32.2 ± 14.3 |

Note: Comparisons are between groups of treatment across the rows for each extender and storage time. For all the variable assessed, no significant difference were found between supplemented and control groups.

Table 4
Comparisons of frozen-thawed sperm kinetics processed with INRA Freeze® and EquiPlus Freeze® extenders supplemented with EBN.

| Variables | INRA Freeze® | | | EquiPlus Freeze® | | |
|----------------------------------|--------------|-------------|-------------|------------------|------------|-------------|
| | Control | 2.4 % EBN | 4.8 % EBN | Control | 2.4 % EBN | 4.8 % EBN |
| Total motility (%) | 63.6 ± 8.5 | 56.1 ± 7.1 | 53.4 ± 8.6 | 44.2 ± 9.3 | 53.9 ± 6.6 | 50.5 ± 9.2 |
| Progressive motility (%) | 25.3 ± 3.5 | 19.6 ± 3.0 | 21.2 ± 3.4 | 13.6 ± 3.7 | 16.5 ± 3.7 | 16.3 ± 3.4 |
| Path Velocity (VAP, μm/s) | 99.1 ± 7.8 | 89.3 ± 4.8 | 97.1 ± 5.1 | 73.2 ± 4.5 | 74.4 ± 2.6 | 78.7 ± 4.2 |
| Progressive velocity (VSL, μm/s) | 77.5 ± 7.2 | 69.6 ± 4.3 | 76.8 ± 4.0 | 54.4 ± 3.7 | 56.2 ± 2.2 | 60.9 ± 3.4 |
| Curvilinear velocity (VCL, μm/s) | 170.1 ± 11.3 | 153.4 ± 7.1 | 165.0 ± 7.7 | 126.7 ± 8.2 | 129.9 ± 3 | 136.2 ± 6.8 |
| Lateral head amplitude (ALH, μm) | 7.2 ± 0.3 | 7.0 ± 0.2 | 7.3 ± 0.2 | 6.5 ± 0.3 | 6.3 ± 0.1 | 6.8 ± 0.2 |
| Beat cross frequency (BCF, Hz) | 14.9 ± 0.9 | 15.9 ± 1.2 | 16.0 ± 1.2 | 13.9 ± 1.4 | 14.7 ± 0.9 | 16.1 ± 1.2 |
| Straightness (STR, %) | 73.1 ± 1.4 | 72.6 ± 0.9 | 74.4 ± 1.2 | 69.7 ± 1.0 | 70.5 ± 1.1 | 72.0 ± 1.2 |
| Linearity (LIN, %) | 44.0 ± 1.6 | 43.7 ± 0.7 | 45.1 ± 0.8 | 42.7 ± 0.9 | 42.0 ± 1.1 | 42.7 ± 0.6 |

Note: Comparisons are across the rows between the various EBN concentrations for each extender. Means were not statistically different between control and EBN concentrations in both extenders.

Table 5
Comparisons of sperm kinetics between INRA Freeze® and EquiPlus Freeze® following supplementation of frozen-thawed Arabian stallion semen with 0 %, 2.4 % and 4.8 % EBN.

| Variables | INRA Freeze® | | | EquiPlus Freeze® | | |
|----------------------------------|--------------|--------------|--------------|------------------|--------------|--------------|
| | Control | 2.4 % EBN | 4.8 % EBN | Control | 2.4 % EBN | 4.8 % EBN |
| Total motility (%) | 92.7 ± 2.9 | 96.0 ± 1.5 | 94.8 ± 5.6 | 85.7 ± 6.7 | 92.0 ± 2.1 | 89.3 ± 3.4 |
| Progressive motility (%) | 59.4 ± 6.6 | 71.9 ± 4.3 | 67.8 ± 6.7 | 41.7 ± 4.4 | 40.7 ± 2.3 | 41.3 ± 3.4 |
| Path Velocity (VAP, μm/s) | 165.1 ± 10.1 | 173.6 ± 7.5 | 178.5 ± 8.3 | 131.3 ± 8.0 | 125.9 ± 4.5 | 128.7 ± 7.7 |
| Progressive velocity (VSL, μm/s) | 122.0 ± 8.2 | 129.6 ± 5.6 | 130.5 ± 5.5 | 99.2 ± 4.6 | 94.3 ± 1.9 | 96.3 ± 3.9 |
| Curvilinear velocity (VCL, μm/s) | 283.4 ± 14.8 | 292.7 ± 15.2 | 303.0 ± 16.9 | 225.0 ± 16.7 | 219.4 ± 11.3 | 221.5 ± 17.1 |
| Lateral head amplitude (ALH, μm) | 11.3 ± 0.6 | 11.2 ± 0.7 | 11.4 ± 0.7 | 9.0 ± 0.7 | 9.0 ± 0.7 | 8.9 ± 0.8 |
| Beat cross frequency (BCF, Hz) | 15.2 ± 3.7 | 14.3 ± 2.7 | 12.6 ± 2.8 | 18.2 ± 2.7 | 18.2 ± 3.3 | 18.2 ± 3.0 |
| Straightness (STR, %) | 69.0 ± 2.0 | 70.6 ± 2.1 | 69.7 ± 2.1 | 71.6 ± 2.2 | 69.5 ± 2.3 | 70.3 ± 2.1 |
| Linearity (LIN, %) | 40.6 ± 2.0 | 42.6 ± 1.9 | 41.6 ± 1.8 | 43.0 ± 1.9 | 40.8 ± 2.0 | 42.2 ± 1.7 |

Note: Comparisons are across the rows between the various EBN concentrations for each extender. Means were not statistically different between control and EBN concentrations in both extenders. Values within a column with different superscripts differ (P < 0.05).

compared to the control, the results were not statistically significant. These results indicate that EBN supplementation did not yield better sperm motility in any of the extenders. These outcomes were not expected based on the positive effects of EBN reported in several animal models, which include potent antioxidative and anti-inflammatory properties (Hamzah et al. 2013; Yida et al. 2015). The constituents of EBN such as sugar fractions, proteins, sialic acid are expected to interact positively to improve the quality of chilled semen during storage (Hou et al. 2015; Yagi et al. 2008). For instance, sialic acid has been reported to enhance metabolism and antioxidant capacity (Abdul Khalid et al. 2019; Yagi et al. 2008), as seen in the preservation of sperm morphology and better performance in other animal species such as bovine semen during freezing (Naing et al. 2010; Yusop et al. 2016) Likewise, an example

of the sugars present in EBN, treehouse, was demonstrated to improve semen performance post-freezing (Panyaboriban et al. 2015). Moreover, the commercial extenders used in this study have been shown to be effective not only for the preservation of stallion semen but also improving semen quality during chilled and frozen storage.

We designed this study to elucidate if EBN supplementation in extenders will improve semen quality and antioxidant properties during chilled and frozen storage based on earlier reports suggesting additive protective effects of such combination (Szczęśniak-Fabiańczyk et al. 2003). For instance, the combination of SOD and catalase significantly improved the survival rate of oocytes compared to individual treatments (Dinara et al. 2001). Also, stallion semen quality was enhanced following the combined effects of

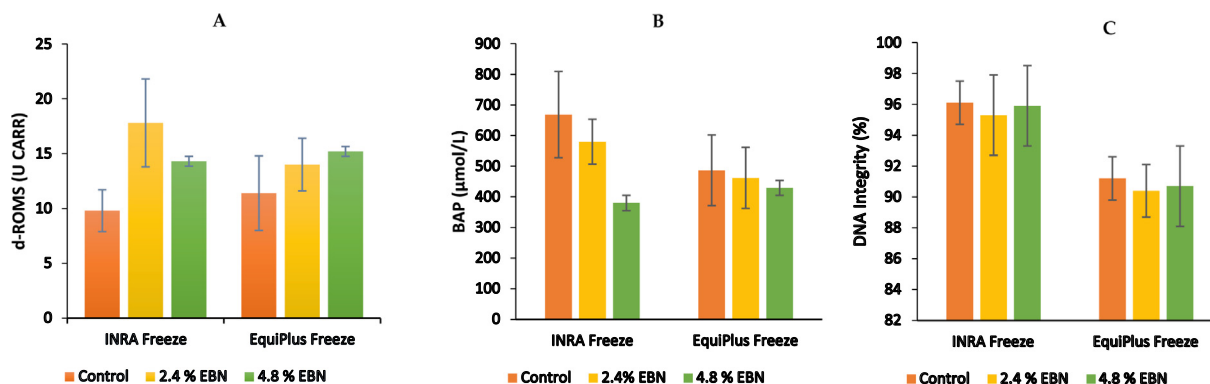


Fig. 1. Comparisons of the antioxidant properties and DNA integrity of frozen thawed Arabian stallion sperm processed with INRA Freeze® and EquiPlus Freeze® extenders supplemented with EBN. A = d-ROMS (Reactive oxygen metabolites), B = BAP (Biological antioxidative potential), C = DNA integrity. U CARR = Caratelli Units (1U CARR = 0.08 mg/100 ml H₂O₂). Note: Comparisons are between results (mean ± S.E) of the control and various EBN dose supplemented groups of the same extender. No significant difference (p > 0.05) were found for all the three variables (A, B, C) assessed.

freezing extenders, INRA96® + INRA Freeze® (Neuhauser et al. 2019). However, despite the numerous and promising findings in our experiment associated with EBN, its various constituents, and combined effects, semen quality and antioxidant properties were not significantly affected in the present study.

The reasons for the current findings which are against our hypothesis are not clearly understood. Nevertheless, certain factors such as the preparation of EBN, concentration of EBN, its compatibility with the commercial extenders may contribute to the results. EBN was prepared as described in our previous study (Al-Khalidi et al. 2021). The EBN was in a hydrolyzed form obtained from a reliable source and corresponding concentrations were prepared by adding 10 ml of PBS to produce the aliquots used in this study. Different concentrations ranging from 0.12 % to 0.24 % and 2.4 % to 4.8 % were used for the experiment one and two, respectively. These concentrations represent both low and high levels to elucidate whether the effects of EBN on Arabian stallion semen were dose-dependent. Such a dose-dependent effect was reported after investigating EBN supplementation in diabetic-induced rats (Yida et al. 2015). In our previous study, that supplementation with higher EBN concentration (0.24 %) had no significant effect on the total sperm motility. Moreover, extreme doses of antioxidants may not be effective in antagonizing the oxidative stress associated with the storage of chilled and cryopreserved semen.

The choice of extenders for stallion semen and their compatibility with EBN supplementation requires further investigation. Previous studies have demonstrated that the EquiPlus® extender was ineffective in preserving sperm quality after 24 h of storage (Neuhauser et al. 2019; Ghallab et al. 2017). Thus, the limitations of certain extenders in improving the semen quality may contribute to low synergic effects when combined with potential antioxidant agents such as EBN. In addition, the supplementation of extenders with antioxidants was shown to induce significant and positive effects on sperm quality at specific concentrations (Ghallab et al. 2017; de Oliveira et al. 2013). This reinstates the need for further research regarding EBN concentrations as a supplement for stallion semen either during cooling or freezing.

Sperm cryopreservation is associated with increased production of ROS, lower antioxidant levels and increased susceptibility of semen to lipid peroxidation (LPO) (Aitken and Krausz 2001; Len et al. 2019). These events precipitate premature aging and fragmentation of DNA in frozen equine spermatozoa (Len et al. 2019; Ortega Ferrusola et al. 2009). The d-ROMs test is vital for the identification of oxidative stress markers including malondialdehyde (an end product of LPO) and organic hydroperoxides (Costantini 2016). The BAP test uses photometrical reactions to quantify biological antioxidant potential in plasma based on the capacity to reduce iron from ferric (Fe³⁺) to ferrous form (Fe²⁺) (Celi, Sullivan, and Evans 2010; Tafuri et al. 2019). These events justified why we attempted to quantify the proportion of DNA fragmentation, BAP, and d-ROMs in this study.

The second experiment assessed sperm kinetics and antioxidant properties in frozen Arabian stallion semen extended with INRA Freeze® and EquiPlus Freeze® and supplemented with higher EBN concentrations (2.4 % and 4.8 %). In both extenders, no significant difference was observed in any of the sperm kinetic variables between the various EBN supplementation. In terms of sperm kinetics, although the variables were not significantly affected by EBN supplementation, the sperm quality was preserved during freezing. This finding highlights that EBN had no negative impact on sperm quality in Arabian stallion semen. However, the potential beneficial role of EBN supplementation requires further investigation.

The present study also revealed that EBN supplementation in extended semen had no significant impact on the proportion of fragmented DNA. DNA fragmentation results from high production

of ROS, reduced antioxidant levels and increased susceptibility of frozen semen to LPO (Aitken and Krausz 2001). EBN has been demonstrated in experimental studies to reduce inflammation and oxidative stress (Abdul Khalid et al. 2019; Yida et al. 2015). These mechanisms enhance the preservation of sperm membrane integrity and regulation of influx of intracellular ions during cryopreservation (Alvarenga et al. 2016). Furthermore, such an effect reduces the formation of intracellular ion crystals, cell death, and subsequently lower rates of DNA fragmentation (López-Fernández et al. 2008). It could be inferred from this study that antioxidant indicators were not significantly affected by EBN supplementation, however, no detrimental effects were observed. Other indications of antioxidant status such as BAP and d-ROMs were not significantly different between the various EBN supplementation. This result could have been influenced by the fact that the experimental animals were not randomly selected, test protocol, and the suitability of the test to assess antioxidant status in frozen semen. Various measurements such as BAP and d-ROMs tests provide a complete picture of antioxidant status in stallion semen (Tafuri et al. 2019). Hence, other parameters need to be considered in future research.

In conclusion, EBN supplementation had no significant effect on sperm motility parameters in both chilled and cryopreserved semen during the various storage times. Similarly, antioxidant properties were not significantly enhanced following EBN supplementation in cryopreserved semen. Future studies might consider different methods of EBN preparation and concentrations to elucidate the potential biological impact of EBN in Arabian stallion semen.

5. Ethical animal research

All the procedures involving animal use were approved by the Animal Welfare Committee at Animal Health Research Center (AHRC) No 54.

Funding

This research was funded by Royal Oman Police – Mounted Police Division and Universiti Putra Malaysia – Veterinary Faculty

Informed consent

Informed consent was not required in this study. The approval to conduct the study was obtained from the Mounted Police Division and Directorate General of Veterinary Services, Royal Court Affairs, Muscat, the Sultanate of Oman.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank and acknowledge the supports provided by the team of the Veterinary Department, Mounted Police Division in the Royal Oman Police area, Muscat, Oman. We also thank the technical supports provided by Dr Naryan Paratab, the staff of the Laboratories and Animal Research Centre, Directorate General of Veterinary Services, Royal Court Affairs, Muscat, the Sultanate of Oman. The EBN sample used for the study was generously provided by the Mobile Harvest SDN BHD, Malaysia.

Author Contributions/roles

N. Yimer and K. Al-Khaldi conceptualized the study, N. Yimer, K. Al-Khaldi, and A. Babji designed the methodology; N. Yimer and S. Al-Bulushi and K. Al-Khaldi conducted the investigation; M.B. Sadiq and K. Al-Khaldi wrote the original draft. All authors contributed in reviewing and editing the manuscript.

References

- Khalid, A., Siti, A.R., Aziz, S., Ahmad, H., 2019. Effects of Sialic Acid from Edible Bird Nest on Cell Viability Associated with Brain Cognitive Performance in Mice. *World J. Traditional Chinese Med.* 5 (4), 214–219.
- Aitken, R.J., Krausz, C., 2001. Oxidative Stress, DNA Damage and the Y Chromosome. *Reproduction* (Cambridge, England) 122 (4), 497–506.
- Al-Bulushi, S., Manjunatha, B.M., Bathgate, R., Rickard, J.P., de Graaf, S.P., 2019. Liquid Storage of Dromedary Camel Semen in Different Extenders. *Anim. Reprod. Sci.* 207, 95–106.
- Al-Khaldi, K., Yimer, N., Al-Bulushi, S., Haron, A.W., Hiew, M., Babji, A.S., 2021. A Preliminary Study on the Effects of E-Z Mixin® and EquiPlus® Extenders Supplemented with Edible Bird's Nest on the Quality of Chilled Arabian Stallion Semen. *Anim. Reprod.* 18 (2). <https://doi.org/10.1590/1984-3143-ar2020-0027>.
- Alamaary, M.S., Wahid, H., Ali, M., Hiew, M.W.H., Adamu, L., Peter, I.D., 2019. Effects of Four Extenders on the Quality of Frozen Semen in Arabian Stallions. *Veterinary World* 12 (1), 34–40.
- Ali, A., Alamaary, M., Al-Sobayil, F., 2014. Reproductive Performance of Arab Mares in the Kingdom of Saudi Arabia. *Tierärztliche Praxis. Ausgabe G, Grosstiere/Nutztiere* 42 (3), 145–149.
- Alvarenga, M.A., Papa, F.O., Neto, C.R., 2016. Advances in Stallion Semen Cryopreservation. *The Veterinary Clinics of North America. Equine Practice* 32 (3), 521–530. <https://doi.org/10.1016/j.cveq.2016.08.003>.
- Ball, B.A., 2008. Oxidative Stress, Osmotic Stress and Apoptosis: Impacts on Sperm Function and Preservation in the Horse. *Anim. Reprod. Sci.* 107 (3–4), 257–267.
- Celi, P., Sullivan, M., Evans, D., 2010. The Stability of the Reactive Oxygen Metabolites (d-ROMs) and Biological Antioxidant Potential (BAP) Tests on Stored Horse Blood. *Vet. J.* 183 (2), 217–218.
- Chua, L.S., Zukefii, S.N., 2016. A Comprehensive Review of Edible Bird Nests and Swiftlet Farming. *J. Integrative Med.* 14 (6), 415–428.
- Contri, A., De Amicis, I., Veronesi, M.C., Faustini, M., Robbe, D., Carluccio, A., 2010. Efficiency of Different Extenders on Cooled Semen Collected during Long and Short Day Length Seasons in Martina Franca Donkey. *Anim. Reprod. Sci.* 120 (1–4), 136–141.
- Costantini, D., 2016. Oxidative Stress Ecology and the D-ROMs Test: Facts, Misfacts and an Appraisal of a Decade's Work. *Behav. Ecol. Sociobiol.* 70 (5), 809–820.
- Dinara, S., Sengoku, K., Tamate, K., Horikawa, M., Ishikawa, M., 2001. Effects of Supplementation with Free Radical Scavengers on the Survival and Fertilization Rates of Mouse Cryopreserved Oocytes. *Hum. Reprod.* 16 (9), 1976–1981.
- Ferramosca, A., Pinto Provenzano, S., Montagna, D.D., Coppola, L., Zara, V., 2013. Oxidative Stress Negatively Affects Human Sperm Mitochondrial Respiration. *Urology* 82 (1), 78–83.
- Ghallab, A.M., Shahat, A.M., Fadl, A.M., Ayoub, M.M., Moawad, A.R., 2017. Impact of Supplementation of Semen Extender with Antioxidants on the Quality of Chilled or Cryopreserved Arabian Stallion Spermatozoa. *Cryobiology* 79, 14–20.
- Ghassem, M., Arihara, K., Mohammadi, S., Sani, N.A., Babji, A.S., 2017. Identification of Two Novel Antioxidant Peptides from Edible Bird's Nest (Aerodramus fuciphagus) Protein Hydrolysates. *Food Funct.* 8 (5), 2046–2052.
- Gibb, Z., Butler, T.J., Morris, L.H.A., Maxwell, W.M.C., Grupen, C.G., 2013. Quercetin Improves the Postthaw Characteristics of Cryopreserved Sex-Sorted and Nonsorted Stallion Sperm. *Theriogenology* 79 (6), 1001–1009.
- Gibb, Z., Grupen, C.G., Maxwell, W.M.C., Morris, L.H.A., 2017. Field Fertility of Liquid Stored and Cryopreserved Flow Cytometrically Sex-Sorted Stallion Sperm. *Equine Vet. J.* 49 (2), 160–166.
- Gibb, Z., Lambourne, S.R., Aitken, R.J., 2014. The Paradoxical Relationship Between Stallion Fertility and Oxidative Stress. *Biol. Reprod.* 91 (3).
- Halliwel, B., 2006. Reactive Species and Antioxidants. *Redox Biology Is a Fundamental Theme of Aerobic Life. Plant Physiol.* 141 (2), 312–322.
- Hamzah, Z., Ibrahim, N.H., Sarojini, J., Hussin, K., Hashim, O., Lee, B.-B., 2013. Nutritional Properties of Edible Bird Nest. *Journal of Asian Scientific Research* 3 (6), 600–607.
- Hou, Z., Imam, M.U., Ismail, M., Azmi, N.H., Ismail, N., Ideris, A., Mahmud, R., 2015. Lactoferrin and Ovotransferrin Contribute toward Antioxidative Effects of Edible Bird's Nest against Hydrogen Peroxide-Induced Oxidative Stress in Human SH-SY5Y Cells. *Biosci. Biotechnol. Biochem.* 79 (10), 1570–1578.
- Khalili, B., Jafaroghli, M., Farshad, A., Pareskh-Khiavi, M., 2010. The Effects of Different Concentrations of Glycine and Cysteine on the Freezability of Moghani Ram Spermatozoa. *Asian-Australas J. Anim. Sci.* 23 (3), 318–325.
- Len, J.S., Koh, W.S.D., Tan, S.-X., 2019. The Roles of Reactive Oxygen Species and Antioxidants in Cryopreservation. *Biosci. Rep.* 39 (8).
- López-Fernández, C., Fernández, J.L., Gosálbez, A., Arroyo, F., Vázquez, J.M., Holt, W. V., Gosálbez, J., 2008. Dynamics of Sperm DNA Fragmentation in Domestic Animals: III. Ram. *Theriogenology* 70 (6), 898–908.
- Marcone, M.F., 2005. Characterization of the Edible Bird's Nest the "Caviar of the East". *Food Res. Int.* 38 (10), 1125–1134.
- Morielli, T., O'Flaherty, C., 2015. Oxidative Stress Impairs Function and Increases Redox Protein Modifications in Human Spermatozoa. *Reproduction* 149 (1), 113–123.
- Naing, S.W., Wahid, H., Mohd Azam, K., Rosnina, Y., Zuki, A.B., Kazhal, S., Bakar, M. M., Thein, M., Kyaw, T., San, M.M., 2010. Effect of Sugars on Characteristics of Boer Goat Semen after Cryopreservation. *Anim. Reprod. Sci.* 122 (1–2), 23–28.
- Neuhauser, S., Bollwein, H., Siuda, M., Handler, J., 2019. Comparison of the Effects of Five Semen Extenders on the Quality of Frozen-Thawed Equine Epididymal Sperm. *J. Equine Veterinary Sci.* 79, 1–8.
- de Oliveira, Rodrigo Arruda, Caroline Antoniazzi Wolf, Marco Antônio de Oliveira Viu, and Maria Lúcia Gambarini. 2013. 'Addition of Glutathione to an Extender for Frozen Equine Semen'. *J. Equine Veterinary Sci.* 33(12):1148–52.
- Ortega Ferrusola, C., González Fernández, L., Morrell, J.M., Salazar Sandoval, C., Macías García, B., Rodríguez-Martínez, H., Tapia, J.A., Peña, F.J., 2009. Lipid Peroxidation, Assessed with BODIPY-C11, Increases after Cryopreservation of Stallion Spermatozoa, Is Stallion-Dependent and Is Related to Apoptotic-like Changes. *Reproduction* (Cambridge, England) 138 (1), 55–63.
- Panyaboriban, Saritvich, Suwimonteerabutr, Junpen, Phutikanit, Nawapen, Swangchan-Uthai, Theerawat, Tharasanit, Theerawat, Techakumphu, Mongkol, 2015. Effect of Various Combinations of Sugar Supplementation in the Extender on Frozen-Thawed Ram Semen Quality and Fertility. *Thai J. Veterinary Med.* 45 (2), 229–237.
- Pillet, E., Battelier, F., Duchamp, G., Furstoss, V., Le Vern, Y., Kerboeuf, D., Desherces, S., Schmitt, E., Magistrini, M., 2008. High Fertility Rates with Stallion Sperm Cryopreserved in INRA96® Based Extender Are Not Predicted by in Vitro Parameters. *Animal Reprod. Sci.* 107 (3–4), 339–340.
- Rui, B.R., Angrimani, D.S.R., Bicudo, L.C., Losano, J.D.A., Nichi, M., Pereira, R.J.G., 2018. A Fast, Low-Cost and Efficient Method for the Diagnosis of Sperm DNA Fragmentation in Several Species. *Reprod. Domest. Anim.* 53 (1), 171–175.
- Szcześniak-Fabiańczyk, Barbara, Bochenek, Michał, Smorag, Zdzisław, Ryszka, Florian, 2003. Effect of Antioxidants Added to Boar Semen Extender on the Semen Survival Time and Sperm Chromatin Structure. *Reprod. Biol.* 3 (1), 81–87.
- Tafari, Simona, Natascia Cocchia, Domenico Carotenuto, Anastasia Vassetti, Alessia Staropoli, Vincenzo Mastellone, Vincenzo Peretti, Francesca Ciotola, Sara Albarella, Chiara Del Prete, Veronica Palumbo, Luigi Esposito, Francesco Vinale, and Francesca Ciani. 2019. 'Chemical Analysis of Lepidium Meyenii (Maca) and Its Effects on Redox Status and on Reproductive Biology in Stallions'. *Molecules* 24(10).
- Trzczińska, M., Bryła, M., 2015. Apoptotic-like Changes of Boar Spermatozoa in Freezing Media Supplemented with Different Antioxidants. *Polish J. Veterinary Sci.* 18 (3), 473–480.
- Tu, Benjamin P., Weissman, Jonathan S., 2004. Oxidative Protein Folding in Eukaryotes: Mechanisms and Consequences. *J. Cell Biol.* 164 (3), 341–346.
- Uysal, Ongun and Mustafa Bucak. 2007. 'Effects of Oxidized Glutathione, Bovine Serum Albumin, Cysteine and Lycopene on the Quality of Frozen-Thawed Ram Semen'. *Acta Veterinaria Brno - ACTA VET BRNO* 76:383–90.
- Waheed, M., Al-Khaldi, K., & Prapat, N. (2016). Effect of Extenders and Insemination Protocol on the Fertilizing Capacity of Cryopreserved Arabian Horse Semen. *SOJ Veterinary Sciences*: 1–8. <http://dx.doi.org/10.15226/2381-2907/2/2/00117>.
- Whittington, Kate and Ford. 1999. 'Relative Contribution of Leukocytes and of Spermatozoa to Reactive Oxygen Species Production in Human Sperm Suspensions'. *International Journal of Andrology* 22(4):229–35.
- Xu, Xia, Cowley, Sally, Flaim, Christopher J., James, William, Seymour, Leonard, Cui, Zhanfeng, 2010. The Roles of Apoptotic Pathways in the Low Recovery Rate after Cryopreservation of Dissociated Human Embryonic Stem Cells. *Biotechnol. Prog.* 26 (3), 827–837.
- Yagi, Hirokazu, Yasukawa, Naoko, Yu, Shin-Yi, Guo, Chao-Tan, Takahashi, Noriko, Takahashi, Tadanobu, Bukawa, Wakoto, Suzuki, Takashi, Khoo, Kay-Hooi, Suzuki, Yasuo, Kato, Koichi, 2008. The Expression of Sialylated High-Antennary N-Glycans in Edible Bird's Nest. *Carbohydr. Res.* 343 (8), 1373–1377.
- Yida, Zhang, Mustapha Umar Imam, Maznah Ismail, Der-Jiun Ooi, Nadarajan Sarega, Nur Hanisah Azmi, Norsharina Ismail, Kim Wei Chan, Zhiping Hou, and Norhayati Binti Yusuf. 2015. 'Edible Bird's Nest Prevents High Fat Diet-Induced Insulin Resistance in Rats' edited by J. F. Ndisang. *Journal of Diabetes Research* 2015:760535.
- Yusop, Dayang Rakhmioktaeawatty, Yimer, Nurhusien, Haron, Abd Wahid, Sharmila, Etty, Khushairay, Ibrahim, Babji, Abdul Salam, 2016. Edible Birds' Nest (EBN) Hydrolysate for Bovine Sperm Cryopreservation. *JSM Invitro Fertilization* 1 (1), 12–14.
- Zhao, Nan, Liu, Xue-Jun, Li, Jun-Tao, Zhang, Ling, Fu, Yang, Zhang, Ya-Jie, Chen, Ru-Xin, Wei, Xiao-Qing, Wang, Rui, Wang, Yu, Zhang, Jian-Min, 2015. Endoplasmic Reticulum Stress Inhibition Is a Valid Therapeutic Strategy in Vitriifying Oocytes. *Cryobiology* 70 (1), 48–52.