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INFLUENCE OF ALPHA LINOLENIC ACID ON THE MOTILITY, VIABILITY, ANTIOXIDANT ACTIVITY AND FERTILITY OF FROZEN-THAWED NEW ZEALAND WHITE RABBIT BUCK SEMEN

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Abstract: Freezing and thawing processes result in production and accumulation of high concentrations of reactive oxygen species that are detrimental to spermatozoal motility and fertility. Therefore, supplementation of exogenous source of antioxidants to freezing diluent is crucial. The aim of the present study was to investigate for the first time whether supplementation of semen diluent with alpha linolenic acid (ALA) can improve motility, viability, membrane integrity, antioxidant status and fertility of post-thaw rabbit spermatozoa. Semen was collected and pooled from fifteen New Zealand white rabbit bucks. Semen samples were diluted with a tris-citrate-glucose (TCG) extender supplemented with ALA (0, 50, 75 and 100 µmol). Then, extended rabbit semen was cooled at 5°C and cryopreserved in liquid nitrogen. After thawing, spermatozoal quality parameters (individual motility %, viability %, osmotic resistance %, and acrosome integrity %), antioxidant activity (SOD, CAT, and GSH activities), lipid peroxidation (malondialdehyde) and fertility (conception and kindling rates) were evaluated. Results revealed that supplementation of rabbit semen extender with 50 µmol ALA significantly ($P<0.05$) increased spermatozoal characteristics including motility (56.54%), viability (60.01%), acrosome status (72.66%) and membrane integrity (59.13%). The activity of semen antioxidant enzymes (SOD, CAT, and GSH) showed a significant improvement with a marked decrease in lipid peroxidation. Moreover, the conception (73.30%) and kindling (70.00%) rates were significantly ($P<0.05$) higher in does inseminated with thawed semen treated with 50 µmol ALA in comparison with other concentrations (0, 75 and 100 µmol). In summary, supplementation of rabbit semen extender with 50 µmol ALA improved motility, viability, membrane integrity, acrosome integrity, antioxidant enzymes activity and fertility of post-thaw rabbit spermatozoa. Our findings suggested that higher concentrations of ALA are detrimental to post-thaw characteristics of New Zealand white rabbit buck spermatozoa. To achieve better results, the semen freezing extender should be supplemented with ALA at lower concentrations, especially 50 µmol.

Key Words: rabbit semen, cryopreservation, alpha linolenic acid, antioxidant, fertility.

INTRODUCTION

Sperm cryopreservation is a great way to conserve the genetic constitution of many species (Bailey *et al*., 2000). In rabbit, the results of artificial insemination using frozen - thawed buck semen are still not encouraging (Mocé and Vicente, 2009). In addition, the cryopreservation process results in accumulation of reactive oxygen species (ROS), which are detrimental to the motility, viability and fertility of spermatozoa (Watson, 2000). Moreover, excessive ROS production results in lipid peroxidation (LPO). Which leads to extensive structural alterations in plasma membrane fluidity (Bucak *et al*., 2007), permeability with a great loss of total phospholipids, ion exchange, and consequently impairs the membrane function (Watson, 2000). In addition, LPO is accompanied by DNA oxidation and fragmentation,

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which adversely affect the fertility of frozen-thawed spermatozoa (Lessard *et al.*, 2000). Therefore, addition of exogenous source of antioxidants to semen freezing diluent plays a vital role in protection rabbit spermatozoa against the harmful effect of ROS during the cryopreservation process. Alpha linolenic fatty acid (ALA) is one of the main polyunsaturated fatty acids (PUFA) present in the spermatozoal plasma membrane, which regulates membrane proteins, provides energy, preserves membrane function and maintains spermatozoal viability (Shevchenko and Simons, 2010). Previous studies were conducted to evaluate the effect of dietary supplementation of PUFA (*in vivo*) on the quality of frozen-thawed spermatozoal characteristics and revealed that PUFA dietary supplementation might lead to the improvement of post-thaw spermatozoal quality in rabbits (Castellini *et al.*, 2004, 2019), bulls (Gholami *et al.*, 2010), human (Conquer *et al.*, 1999), rams (Samadian *et al.*, 2010), boars (Rooke *et al.*, 2001) and bucks (Dolatpanah *et al.*, 2008). To the best of our knowledge, there are no works in the literature regarding the effect of supplementation of rabbit semen freezing extender with PUFA (*in vitro*), particularly ALA, on the semen characteristics, antioxidant status and fertility of post-thaw rabbit spermatozoa. Accordingly, this study was conducted to declare the influence of supplementation of rabbit semen extender with ALA on the motility, viability, membrane integrity, antioxidant activity and fertility of frozen-thawed rabbit buck spermatozoa.

MATERIAL AND METHODS

This study was carried out at the Theriogenology department, Faculty of veterinary medicine, Cairo University. Experimental techniques were reviewed and approved by the Animal Ethics Committee at Cairo University (VET-IACUC: CU III 8 422).

Animals and semen collection

The study was carried out on fifteen healthy adult New Zealand white rabbit bucks (14 mo of age and weighing 4-6 kg) that were independently housed in wire cages, fed a standard commercial diet in accordance with NRC, 1977 recommendations and exposed to temperatures ranging from 19°C to 25°C. Freshwater was accessible *ad libitum*. Semen was collected from bucks using a pre-warmed (45°C) and lubricated artificial vagina on a regular basis (twice/buck week). Sixteen replicates (240 ejaculates/15 rabbit bucks) were conducted from September -November, 2020. Directly after collection, the gel plug was separated and the ejaculates were pooled to avoid individual variations among rabbit bucks.

Experiment 1

This experiment was carried out to study the effect of different concentrations of ALA on the post-thawed quality parameters of rabbit buck spermatozoa.

Semen processing

Pooled semen sample was divided into four aliquots and then diluted in tris-citrate glucose (TCG); composed of glucose 9.31 g/L, tris 30.28 g/L, citric acid 16.90 g/L, 20% egg yolk, 50 000 IU of penicillin plus 25 mg of gentamicin/100 mL (Roca *et al.*, 2000) and fortified with cryoprotectants N,N-dimethylformamide 4%+dimethyl sulfoxide 4% (Fadl *et al.*, 2019). Tris-citrate glucose was supplemented with ALA (Sigma Aldrich, Spain) at different concentrations (0, 50, 75 and 100 µmol). After that, the extended semen samples were gradually cooled to +5°C within 90 min and preserved in the refrigerator at +5°C for 15 min. Plastic straws (0.5 mL) were loaded with diluted cooled semen for the cryopreservation process, sealed with a sealing powder and horizontally placed above the liquid nitrogen vapour for 10 min. Then, straws were kept in the liquid nitrogen tank (Cristanelli *et al.*, 1985). After at least 2 wk storage, frozen straws were thawed at 50°C (water bath) for 7 s for evaluation (laffaldano *et al.*, 2014).

Evaluation of quality of Post thawing Rabbit buck semen parameters

Individual motility

A 10-15 µL drop of frozen-thawed semen sample was placed directly onto a pre-warmed microscope glass slide and covered by a cover slip. Using an optical microscope (Olympus BH-2, made in Japan) provided with a warm

stage (400×), progressive motility of the spermatozoa was assessed subjectively (via visual estimation). Then, the percentage of motile population was calculated after examination of several microscopic fields.

Sperm viability and sperm abnormalities evaluation

Using Eosin-nigrosin stain, the percentages of live and abnormal spermatozoa were evaluated according to Evans and Maxwell (1987). Briefly, a drop of semen was mixed with 2 drops of eosin-nigrosin and examined under a bright field microscope (1000× with oil immersion). A total of 200 spermatozoa were evaluated in five different microscopic fields. Live spermatozoa appeared white in coloration (intact membrane, so staining is rejected), whereas dead spermatozoa were pink (lost membrane integrity, so eosin stain is absorbed). The percentage of live spermatozoa was recorded. Using the same slide as for viability evaluation, abnormal spermatozoa with defects including head (giant, double), mid-piece (double, broken) and tail (coiled, bent) were assessed. The percentage of abnormal spermatozoa was calculated.

Hypo osmotic swelling test (HOST)

To evaluate the membrane functional integrity of spermatozoa, a hypo-osmotic swelling test (HOST) was performed according to Iaffaldano *et al.* (2014). The test was carried out mixing thawed semen (10 µL) with distilled water (80 µL) and incubating for 5 min at 37°C. Under a phase-contrast microscope, a total of 200 spermatozoa were examined in five different microscopic fields and calculated. Spermatozoa with normal intact membrane showed swelling and coiling of the tail.

Acrosome integrity

To assess the acrosome integrity, a specific Spermac (FertiPro NV Belgium) stain was used as described by Chan *et al.* (1999). In brief, dried smears of frozen-thawed semen sample were prepared, fixed in 10% formalin solution for 10 min and passed through staining solutions A, B, and C for 1 min at room temperature. Under oil immersion (×1000), 200 spermatozoa were examined and counted. The normal intact acrosome looked like a typical ovoid head with red-pink post acrosomal areas and dark green anterior acrosome regions.

Experiment 2

The experiment was carried out to study the influence of different concentrations of ALA on antioxidant enzyme activity (superoxide dismutase and catalase activity) and malondialdehyde (MDA) concentrations as an indication of lipid peroxidation of the rabbit buck semen.

Evaluation of antioxidant activity of the semen

A frozen-thawed semen sample was centrifuged (3000 rpm) and the seminal plasma was stored at –20°C before assaying of superoxide dismutase (SOD), catalase activity and reduced glutathione activity.

Seminal plasma superoxide dismutase (SOD, U/mL)

Superoxide dismutase activity was assessed colorimetrically by spectrophotometer according to Nishikimi *et al.* (1972) using a SOD Assay Kit (Thermo Fisher Scientific Company LLC, CA, USA). The assay basically depends on the capability of the SOD to inhibit the reduction of nitro blue tetrazolium.

Seminal plasma catalase activity (CAT, U/L)

CAT activity was measured colorimetrically by spectrophotometer as described previously by Aebi (1984) using a Catalase Assay Kit (Thermo Fisher Scientific Company LLC, CA, USA). In brief, CAT reacted with a recognised amount of H_2O_2. Catalase inhibitor was used to stop the reaction after 1 min. After that, residual H_2O_2 reacted with 4-aminophenazone (AAP) and 3, 5 dichloro-2-hydroxy benzene sulfonic (DHBS) in the presence of peroxidase (HRP) to produce a chromophore (its colour intensity is proportional to the amount of catalase).

Seminal plasma reduced glutathione activity (GR, mg/dL)

GR activity was determined using a GH reductase assay kits (Thermo Fisher Scientific Company LLC, CA, USA). In short, GR catalysing the reduction of oxidised glutathione (GSSG) in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) which is oxidised, the absorbance is measured at 340 nm. It was assayed colorimetrically by spectrophotometer according to Goldberg and Spooner (1974).

Lipid peroxidation assay (LPO, nmol/mL)

MDA was measured colorimetrically as described previously by Ohkawa *et al.* (1979). The assay mainly depended on the reaction of the thiobarbituric acid (TBA) with MDA to produce thiobarbituric acid reactive product at a temperature of 95°C for 1 h and in acidic medium. In short, 1 mL of seminal plasma was mixed with 1.5 mL of 0.8% of TBA with addition of 0.4 mL of 8.1% of SDS and 1.5 mL of acetic acid. The mixture was placed in a hot water bath at 95°C for 1 h and then mixed with n-butanol and pyridine (15:1, v/v) after cooling and centrifuged at 4000 rpm for 10 min. The absorbance of the upper layer was read against blank by the spectrophotometer at 532 nm.

Experiment 3 (Evaluation of the fertility of the semen)

To assess the fertility of the frozen-thawed rabbit spermatozoa, 120 New Zealand white multiparous lactating rabbit does (12-14 mo) were allocated to four groups (n=30 for each group) and artificially inseminated with frozen-thawed rabbit buck semen supplemented with ALA at different concentrations (G0 as a control concentration, G1- 50 µmol, G2- 75 µmol, G3- 100 µmol). After one-minute post-thawed, does were deeply inseminated with 0.5 mL extended semen containing about 50×10^6 spermatozoa using an inseminating pipette (Imporvet, S.A.). For induction of ovulation, every doe was injected with buserelin acetate 1.0 µg/doe (Di Iorio *et al.*, 2018). In addition, abdominal palpation was carried out 15 d after insemination to assess pregnancy and calculate conception rate. Kindling rate was also recorded at parturition.

Statistical analysis

All data were achieved as mean±standard error of mean. Data were normalised using arcsine transformation. Analyses were attained using the SPSS program (SPSS, V16.0, SPSS Inc., USA). Simple one-way ANOVA was used to compare between the sperm quality parameters, antioxidant activity parameters and fertility results followed by Duncan's comparison test. Significance differences were measured at $P<0.05$.

RESULTS

Post-thawed semen quality parameters, antioxidant activity and lipid peroxidation

As depicted in Table 1, the individual motility, viability, normal morphology, membrane integrity and acrosome integrity of frozen-thawed spermatozoa showed a significant improvement ($P<0.05$) when rabbit spermatozoa were extended with TCG supplemented with ALA at a concentration of 50 µmol in comparison with the other concentrations of ALA (0, 75, 100 µmol). Moreover, addition of ALA at concentration of 50 µmol to freezing extender resulted in a significant

Table 1: Quality parameters of frozen/thawed extended rabbit semen supplemented with different concentrations of ALA (mean±standard error).

	0 µmol ALA control	50 µmol ALA	75 µmol ALA	100 µmol ALA
Individual motility %	$35.24^{b}\pm1.23$	$56.54^{d}\pm1.35$	$44.25^{c}\pm1.46$	$30.22^{a}\pm1.15$
viability %	$38.63^{b}\pm1.31$	$60.01^{d}\pm1.52$	$48.42^{c}\pm1.27$	$34.12^{a}\pm1.64$
Abnormal sperm %	$31.32^{c}\pm1.43$	$17.21^{a}\pm1.73$	$22.84^{b}\pm1.35$	$36.81^{d}\pm1.26$
HOST %	$37.41^{b}\pm1.71$	$59.13^{d}\pm1.45$	$45.32^{c}\pm1.08$	$33.02^{a}\pm1.11$
Acrosome integrity %	$54.06^{b}\pm1.22$	$72.66^{d}\pm1.23$	$64.43^{c}\pm1.87$	$49.35^{a}\pm1.37$

^{abcd}Mean values in the same row not sharing superscripts are significant ($P<0.05$).
HOST: hypo-osmotic swelling test. ALA: alpha linolenic acid.

Table 2: Antioxidant activity parameters of frozen/thawed buck semen diluted with TCG supplemented with ALA.

	0 µmol ALA control	50 µ mol ALA	75 µ mol ALA	100 µ mol ALA
SOD (U/mL)	$72.35^{c}\pm1.52$	$104.23^{a}\pm2.33$	$88.43^{b}\pm2.12$	$60.23^{d}\pm2.07$
GSH (mg/dL)	$2.18^{c}\pm0.22$	$4.97^{a}\pm0.62$	$2.92^{b}\pm0.28$	$1.74^{c}\pm0.86$
Catalase (U/L)	$131.93^{c}\pm1.54$	$217.52^{a}\pm2.03$	$162.34^{b}\pm2.12$	$109.00^{d}\pm1.91$
MDA (nmol/mL)	$5.18^{c}\pm1.67$	$2.04^{a}\pm1.54$	$3.14^{b}\pm1.78$	$5.92^{c}\pm1.89$

[abcd]Mean values in the same row not sharing superscripts are significant ($P<0.05$).
TCG: tris-citrate-glucose; ALA: alpha linolenic acid; SOD: super oxide dismutase; GSH: glutathione reductase; MDA: malondialdehyde.

increase ($P<0.05$) of the antioxidant enzyme activity of frozen-thawed spermatozoa, which was expressed by the higher values of catalase, GSH and SOD enzymes (Table 2).

Concerning lipid peroxidation, a higher MDA value ($P<0.05$) was observed when ALA was added to rabbit spermatozoa at 100 µmol concentration before freezing compared to the other ALA concentrations (0, 75, 50 µmol), as shown in Table 2.

In vivo reproductive performance

As illustrated in Table 3, the conception and kindling rates of G2 (rabbit does inseminated with frozen-thawed spermatozoa diluted with TCG extender supplemented with 50 µmol of ALA) revealed a significant ($P<0.05$) increase compared to the other groups (G1, G3, G4).

DISCUSSION

The plasma membrane of spermatozoa is composed mainly of n-3 long-chain PUFA, which cannot be synthesised inside the body, as it lacks the suitable fatty acid elongase and desaturase enzymes (Conquer *et al.*, 1999). However, PUFAs maintain the function, structure and fluidity of spermatozoal membrane; the plasma membrane is predisposed to peroxidation particularly after the dramatic stressful results from freezing and thawing processes (Alvarez *et al.*, 1987; James *et al.*, 1999; Lenzi *et al.*, 2002; Aksoy *et al.*, 2006). In the current study, supplementation of freezing extender (TCG) with alpha linolenic acid (ALA) at 50 µmol concentration resulted in a significant improvement in post-thaw individual motility, viability, normal morphology, functional membrane integrity and acrosome integrity compared to other concentrations of ALA (0, 75, 100 µmol, respectively). Our findings are in accordance with previous studies in rabbits (Castellini *et al.*,2003; Conquer *et al.*, 2000), bulls (Gholami *et al.*, 2010; Nasiri *et al.*, 2011; Towhidi and Park, 2013; Kiernan *et al.*, 2013; Kaka *et al.*, 2015a,b), rams (Abdi-Benemar *et al.*, 2015; Masoudi *et al.*,2016) and goat bucks (Ansari *et al.*, 2012; Yimer *et al.*, 2014). This improvement is attributed to the incorporation of ALA in the spermatozoal plasma membrane, which offers greater energy and fluidity to the membrane and protects it against the harmful effect arising from cryopreservation, consequently maintaining motility and viability of the spermatozoa (Erickson, 1998; Watson, 2000; Medeiros *et al.*, 2002). Contrary to our findings, previous studies in boars (Maldjian *et al.*, 2005; Chanapiwat *et al.*, 2009) and in bulls (Abavisani *et al.*, 2013; Kandelousi *et al.*, 2013), in which soft gels containing n-3 and n-3 fatty acids were added to citrate extenders, resulted in decreasing viability and motility of post-thawed spermatozoa. The disparity between the results may be due to species differences and the types of extenders used. Moreover, the addition of ALA to a freezing extender at a concentration of 50 µmol resulted in a significant

Table 3: Reproductive performance of does inseminated with diluted thawed semen supplemented with different concentrations of ALA.

N=30	0 µmol ALA control	50 µ mol ALA	75 µ mol ALA	100 µ mol ALA
Conception rate n (%)	14 (46.6)[b]	22 (73.3)[c]	16 (53.3)[b]	7 (23.3)[a]
Kindling rate n (%)	12 (40.0)[b]	21 (70.0)[c]	13 (43.3)[b]	5 (16.6)[a]

[abc]Values in the same row not sharing superscripts are significant ($P<0.05$).
N=Number of does in each treatment=30.
ALA: alpha linolenic acid.

increase in the activity of an anti-oxidative enzyme (GSH, SOD, and catalase) and decreased spermatozoal lipid peroxidation compared to other concentrations (0, 75, 100 µmol, respectively). These results are in agreement with studies in bulls (Towhidi and Park, 2013; Kaka *et al.*, 2015b), which revealed that addition of n3 PUFA to the semen extender produced high amounts of MDA in a dose dependent manner. In contrast, the results for anti-oxidative activity and lipid peroxidation do not chime with those obtained by Rodrigues *et al.* (2016) in stallions and Gürler *et al.* (2015) in bulls. They reported that the antioxidants (SOD and glutathione peroxidase) in bovine and equine seminal plasma were not affected by the additional feeding of n-3 polyunsaturated fatty acids. The discrepancy in results may be credited to the species variation difference and the way in which fatty acid is supplemented (Castellano, *et al.*, 2010; Kandelousi *et al.*, 2013). Concerning *in vivo* reproductive performance results, the inclusion of ALA at a concentration of 50 µmol in rabbit semen freezing extender resulted in a significant increase in conception and kindling rates. The possible mechanisms through which ALA (50 µmol) results in improvement of the fertility results are explained as follows: first, incorporation of ALA in the plasma membrane provides greater fluidity and excellent membrane fusion during fertilisation (Conquer *et al.*, 2000). Second, Addition of ALA (50 µmol) results in enhancement of the spermatozoal quality parameters (motility, normal morphology and intact acrosome) that are crucial for successful fertilisation. Third, the activity of antioxidant enzymes is increased by supplementation of ALA (50 µmol) to freezing extender, and consequently protects the spermatozoal membrane against lipid peroxidation by ROS. So, ALA maintains normal function of the plasma membrane. Moreover, our results suggested that the beneficial effect of supplementation of rabbit semen extender with ALA was achieved by the addition of a lower concentration of ALA (50 µmol) for greater fluidity of the plasma membrane and, consequently, greater capacity to withstand cryopreservation stress. In addition, a higher concentration of ALA (100 µmol) resulted in a detrimental effect on the rabbit spermatozoal quality, antioxidant activity and fertility parameters. The harmful effect of higher concentrations of ALA might be attributed to making rabbit spermatozoa more susceptible to lipid peroxidation (imbalance between saturated and unsaturated fatty acids). So, the influence of supplementation of semen extender with ALA on the rabbit spermatozoal characteristics differed according to the concentrations of ALA used.

CONCLUSIONS

To our knowledge, this is the first study on the effect of supplementation of semen extender with ALA on the motility, viability, membrane integrity, normal morphology, acrosome integrity, antioxidant enzyme activities and fertility of frozen/thawed rabbit spermatozoa. The findings provide the optimum concentration of ALA (50 µmol) as a potent antioxidant that protects spermatozoa against oxidative stress, lipid peroxidation and maintains motility, viability and fertility of rabbit spermatozoa through freezing and thawing processes.

Conflict of interest: The authors declare that they do not have any conflict of interest

Authorship statement: Aya M. Fadl: Semen collection, evaluation, writing, review & editing. Khaled H. Shahat: Writing - review & editing. Elshymaa A. Abdelnaby: Statistical analysis, visualisation, data curation & editing.

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