Zygote **24** (April), pp. 259–265. © Cambridge University Press 2015
doi:10.1017/S0967199415000155 First Published Online 23 June 2015

Effect of alpha-lipoic acid on boar spermatozoa quality during freezing–thawing

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Date submitted: 19.11.2014. Date revised: 26.03.2015. Date accepted: 06.04.2015

Summary

Alpha-lipoic acid (ALA) is known to be a natural antioxidant. The aim of the present study was to evaluate the cryoprotective effect of ALA on the motility of boar spermatozoa and its antioxidant effect on boar spermatozoa during freezing–thawing. Different concentrations (2.0, 4.0, 6.0, 8.0 or 10.0 mg/ml) of ALA were added to the extender used to freeze boar semen, and the effects on the quality and endogenous antioxidant enzyme activities of frozen–thawed spermatozoa were assessed. The results indicated that the addition of ALA to the extender resulted in a higher percentage of motile spermatozoa post-thaw ($P < 0.05$). The activities of superoxide dismutase, lactate dehydrogenase, glutamic-oxaloacetic transaminase and catalase improved after adding ALA to the extender ($P < 0.05$). Artificial insemination results showed that pregnancy rate and litter size were significantly higher at 6.0 mg/ml in the ALA group than in the control group ($P < 0.05$). In conclusion, ALA conferred a cryoprotective capacity to the extender used for boar semen during the process of freezing–thawing, and the optimal concentration of ALA for the frozen extender was 6.0 mg/ml.

Keywords: Alpha-lipoic acid, Antioxidants, Artificial insemination, Boar spermatozoa, Cryopreservation

Introduction

Artificial insemination is widely used in animal reproduction because of its greater efficiency compared with natural service. Artificial insemination also allows the flexible selection of males with superior traits and performance for fertilization of a plentiful number of females as compared with natural service. Liquid-extended semen has been a great resource for artificial insemination; however, its use in commercial swine production has been limited because of low pregnancy rates and litter sizes. Boar spermatozoa are especially susceptible to cold shock (White, 1993). During freezing and thawing, the physical and chemical properties of spermatozoa are changed and the cells become susceptible to serious cold damage. The production of reactive oxygen species (ROS) is a normal physiological event during spermatozoa cryopreservation, but overproduction of ROS can cause injury via peroxidation damage. Therefore, antioxidant compounds need to be added to the frozen diluents to overcome the detrimental effects of ROS and protect spermatozoa during freezing–thawing.

Alpha-lipoic acid (ALA) is an organosulfur compound derived from octanoic acid, it is naturally produced in animals and acts as an essential cofactor for many enzymes. ALA has a role in Krebs cycle where it helps in the oxidative decarboxylation of pyruvate to acetyl CoA (Perera *et al.*, 2011); it can also help restore other important antioxidants, such as glutathione, vitamin C and vitamin E (Packer *et al.*, 1995). Furthermore, ALA plays an important role in mitochondrial dehydrogenase and ROS reactions

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(Arivazhagan *et al.*, 2001). Recent studies have revealed that ALA performs antioxidant activities, such as enhancing antioxidant defence in erythrocytes (Lexis *et al.*, 2006), endothelial cells (Jones *et al.*, 2002) and testicular cells (Ashour *et al.*, 2011). Therefore, this characteristic of ALA could protect spermatozoa from oxidation damage. Ma *et al.* (2011) found that ALA could improve motility and protect goat spermatozoa from oxidative damage during sperm-mediated gene transfer. Ibrahim *et al.* (2008) incubated buck spermatozoa with 0.02 mmol/ml and noted that ALA could improve motility and reduce DNA damage during spermatozoa cryopreservation.

However, the use of ALA as a cryoprotective agent for boar spermatozoa has not yet been reported. This study evaluated the effectiveness of ALA, when added to boar semen extender, for increasing the motility of boar frozen–thawed spermatozoa and decreasing the damage induced by cryopreservation.

Materials and methods

Reagents and chemicals

Alpha-lipoic acid and all chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and stored in 4°C, unless otherwise stated.

Diluent preparation

The freezing extender for the treatments used in this study was Tris-citric acid-glucose solution (TCG), which consists of 1.48 g citric acid, 1.1 g glucose, 2.42 g Tris, 25 mg gentamicin, 50,000 IU penicillin, and 20 ml egg yolk in 100 ml deionized water. It was supplemented with different concentrations of ALA (2.0, 4.0, 6.0, 8.0 or 10.0 mg/ml). The freezing extender for the control contained the same ingredients as those for the treatment, minus the ALA. All extenders contained 3% (v/v) glycerol.

Semen collection and cryopreservation

Five sexually mature Swagger boars (aged 1.5–2 years) were used in this study. All the boars had proven fertility following artificial insemination with extended liquid semen. Only samples with more than 70% motile spermatozoa and more than 80% morphologically normal spermatozoa were used for this experiment.

After collection, the semen samples were randomly divided and transferred into 50 ml pre-warmed tubes, and equilibrated for 30 min at room temperature. Subsequently the samples were added to equal volumes of Beltsville thawing solution (BTS), the tubes were cooled and maintained at 15°C for 3 h. After standing, the semen was centrifuged at 1600 *g* for 5 min. The supernatant was then removed and the concentrated semen was diluted to a volume of 1 ml with the cooling diluent at 15°C. The diluted semen was gently mixed and cooled slowly to 5°C for 2 h. The suspension was subsequently transferred to 0.25 ml polyvinyl chloride straws (0.25 ml; IMV Technologies France, L'Aigle, France) and cooled from 4 to –5°C with a cool controlled rate freezer (Mini Digit cool 1400, IMV, France). The freezing rate was 1°C/min. All straws were then placed in contact with nitrogen vapour for 20 min, about 3 cm (–120°C) above the liquid nitrogen level followed by immersion in liquid nitrogen (–196°C) for storage.

After storage, samples were moved from liquid nitrogen and thawed in a water bath at 37°C for 45 s.

Analysis of sperm motility

The thawed semen was diluted with BTS at 37°C to a concentration of 1×10^6 to 5×10^6/ml. A 6 μl sample was placed on a slide, covered with a glass coverslip and spermatozoa movement and progression was estimated using a light microscope (COIC XSZ-G, Chongqing Guangdian Co. Ltd., China) equipped with a warm stage (37°C) at ×400 magnification. Spermatozoa motility was assessed by determining the percentage showing any movement of the flagellum [total percentage of motile (TM%)] and the percentage showing linear movement (LM%).

Analysis of plasma membrane integrity

Plasma membrane integrity was evaluated using the hypo-osmotic swelling test (HOST; (Osinowo *et al.*, 1982). Fifty microlitres of thawed semen was mixed with 1 ml hypo-osmotic solution (7.35 g sodium citrate $2H_2O$ and 13.51 g fructose in 1 litre of distilled water) and incubated at 37°C for 30 min (Revell & Mrode, 1994; Buckett *et al.*, 1997). After incubation, 15 μl of well-mixed sample was observed under a light microscope (Shanghai Optical Instrument Factory, Shanghai, China) at ×400 magnification at 37°C (on a warm slide). Membrane-intact spermatozoa had coiled tails after HOST. At least 200 spermatozoa were counted per slide.

Assessment of mitochondrial activity

The mitochondrial activity of the spermatozoa was evaluated by Rhodamine 123 (Rh123) staining (Garner *et al.*, 1997). Fifty microlitres of thawed semen were added into 100 μl pre-warmed Rh123 solution (1 μg/ml). After 10 min of incubation at 37°C in the dark, the sample was observed under a fluorescence microscope (COIC TBE 2000, Chongqing, China) at ×400 magnification. Spermatozoa showing green fluorescence in the midsection were considered to

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be positive for mitochondrial activity. At least 200 spermatozoa were counted per slide.

Assessment of acrosome integrity

Acrosome integrity was evaluated by the fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA, 100 μg/ml; Aboagla & Terada, 2003). The thawed semen samples were added into 2 ml of 3% polyvinylpyrrolidone fluid and centrifuged twice at 800 *g* for 3 min. After centrifugation, the sediments were diluted in phosphate-buffered saline (PBS) and 30 μl was used to make smears. After air drying for 15 min, spermatozoa smears were fixed with pure methanol for 10 min. Thirty microlitres of FITC-PNA solution was spread over a microscope slide. The slides were then incubated in a dark, moist chamber for 30 min at 37°C. After incubation, the slides were washed with PBS and air dried for 15 min. The slides were then mounted with 10 μl of anti-fade solution to preserve fluorescence. Next, the sample was observed using a fluorescence microscope (COIC TBE 2000, Chongqing, China) at ×400 magnification. Entire acrosomes were visualized with strong green fluorescence under the fluorescence microscope. At least 200 spermatozoa were counted per slide.

Biochemical assay

The thawed semen (120 μl) was centrifuged at 1600 *g* for 5 min at 25°C, and the sediment was diluted in 360 μl 1% TritonX-100. After standing for 20 min, the sample was centrifuged at 4000 *g* for 30 min. The supernatants were collected as the crude extracts of enzymes in spermatozoa.

The activities of superoxide dismutase (SOD), lactate dehydrogenase (LDH), glutamic-oxalacetic transaminase (GOT) and catalase (CAT) were detected using specific kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and measured on a spectrophotometer (Shanghai Spectrophotometer Co. Ltd., Shanghai, China).

The activities of SOD were measured using the method of Flohe & Otting (1984). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of nitro blue tetrazolium chloride reduction. The LDH activity was measured using the method of Gibson & Fuller (2000). One unit of enzyme was considered to be the amount producing an optical density change of 2.07 /min at 340 nm, which corresponded to oxidation of 1 μmol nicotinamide adenine dinucleotide (NADH) under the conditions of the assay. The LDH activity was measured using the method of Borque & Ayllón (1996). One unit of GOT activity was equivalent to a change in optical density of 0.001/min at 340 nm. All enzyme activities were expressed as U/ml.

Artificial insemination

Sixty standing estrus gilts were prepared for artificial insemination. The semen in each thawed straw (5 ml) were diluted with 95 ml BTS in a 100 ml plastic artificial insemination bottle at 37°C. Gilts were inseminated using a DeepBlue deep intrauterine artificial insemination catheter (Minitube of America, Verona, WI, USA) with 100 ml of the resulting suspension of spermatozoa. After 24 h, a second insemination was performed. After farrowing, the number of piglets born alive from each pregnant gilt was calculated as the litter size.

Statistical analysis

All statistical analyses were performed using Statistical Product and Service Solutions (SPSS Inc., Chicago, IL, USA). All results were expressed as the mean values ± standard error of the mean (SEM). The mean values of the percentages of motile sperm, acrosome-intact, plasma membrane-intact and mitochondrial activity sperm were compared using Duncan's multiple range tests by analysis of variance (ANOVA), where the F-value was significant ($P < 0.05$).

Results

Effects of ALA on spermatozoa motility

The effects of ALA on the percentages of total motile spermatozoa (TM%), percentages of linear movement spermatozoa (LM%), plasma membrane integrity (MI), mitochondrial activity (MA) and acrosome integrity (AI) of frozen–thawed boar spermatozoa were evaluated and the results are shown in (Table 1.

All motility values in the fresh semen groups were higher than in the cryopreserved semen groups. In the cryopreserved semen groups, the extender containing 6.0 mg/ml ALA conferred the highest spermatozoa motility. The TM and LM of frozen–thawed spermatozoa in the extender supplemented with 6.0 mg/ml ALA were significantly higher than those of other groups ($P < 0.05$). The extender containing 6.0 mg/ml of ALA also led to the highest MA and acrosome integrity, in comparison with those of other treatment groups and the control group ($P < 0.05$). The extenders containing 4.0 and 6.0 mg/ml of ALA had the highest spermatozoa MI percentage ($P < 0.05$).

Effects of ALA on antioxidant activities

The effect of various concentrations of ALA supplementation in the extender on antioxidant activity are shown in Table 2.

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Table 1 Effects of different concentrations of Alpha-lipoic acid on the boar sperm total motile sperm (TM), linear movement sperm (LM), plasma membrane integrity (MI), mitochondrial activity (MA) and acrosome integrity (AI) after freezing–thawing

Groups (ALA concentration)	TM (%)	LM (%)	MI (%)	MA (%)	AI (%)
Control	43.79 ± 0.56^{a}	41.58 ± 0.70^{a}	45.35 ± 0.80^{a}	44.36 ± 0.71^{a}	46.19 ± 0.72^{a}
2.0 mg/ml	47.06 ± 0.75^{b}	45.28 ± 0.90^{c}	49.05 ± 0.81^{c}	47.06 ± 0.25^{b}	49.45 ± 0.99^{b}
4.0 mg/ml	51.27 ± 0.59^{e}	49.40 ± 0.71^{d}	52.31 ± 0.61^{d}	50.08 ± 0.75^{c}	53.02 ± 0.99^{d}
6.0 mg/ml	52.85 ± 0.48^{f}	50.52 ± 0.62^{e}	52.54 ± 0.63^{d}	50.89 ± 0.51^{d}	53.87 ± 0.48^{e}
8.0 mg/ml	49.12 ± 0.76^{d}	46.17 ± 0.50^{c}	48.95 ± 0.51^{c}	47.55 ± 0.79^{b}	50.63 ± 1.40^{c}
10.0 mg/ml	47.94 ± 0.78^{c}	45.17 ± 0.91^{b}	47.95 ± 0.83^{b}	47.01 ± 0.74^{b}	49.62 ± 0.65^{b}
Fresh semen	77.60 ± 0.65^{g}	75.31 ± 0.99^{f}	76.07 ± 0.55^{e}	76.06 ± 0.88^{e}	78.30 ± 0.77^{f}

Note: Values in the same column with different letters indicates differences ($P < 0.05$). Values are mean ± standard error of the mean (SEM) of thawed boar spermatozoa in all the concentrations of ALA. All treatments were replicated 10 times.

Table 2 Effects of different concentrations of Alpha-lipoic acid on the boar sperm superoxide dismutase (SOD), lactate dehydrogenase (LDH), glutamic-oxalacetic transaminase (GOT) and catalase (CAT) activity after freezing–thawing

Groups (ALA concentration)	SOD (nmol/ml)	LDH (nmol/ml)	GOT (nmol/ml)	CAT (nmol/ml)
Control	74.03 ± 2.00^{a}	2.51 ± 0.08^{a}	34.87 ± 0.68^{a}	2.65 ± 0.09^{a}
2.0 mg/ml	77.75 ± 2.05^{b}	2.80 ± 0.07^{c}	$37.65 \pm 1.09^{b,c}$	3.47 ± 0.09^{c}
4.0 mg/ml	80.57 ± 1.92^{c}	3.16 ± 0.09^{e}	39.92 ± 0.90^{d}	3.80 ± 0.09^{d}
6.0 mg/ml	81.64 ± 1.99^{c}	3.32 ± 0.06^{f}	40.01 ± 0.55^{d}	3.91 ± 0.08^{e}
8.0 mg/ml	78.15 ± 1.16^{b}	2.87 ± 0.0d	38.34 ± 0.90^{c}	3.49 ± 0.10^{c}
10.0 mg/ml	77.87 ± 1.09^{b}	2.71 ± 0.07^{b}	37.11 ± 0.72^{b}	3.33 ± 0.08^{b}
Fresh semen	127.58 ± 1.02^{d}	5.03 ± 0.07^{g}	55.85 ± 0.93^{e}	6.52 ± 0.08^{f}

Note: Values in the same column with different letters indicates differences ($P < 0.05$). Values are mean ± standard error of the mean (SEM) of thawed boar spermatozoa in all the concentrations of ALA. All treatments were replicated 10 times.

All antioxidant activity in the fresh semen groups was higher than in the cryopreserved semen groups. In the cryopreserved semen groups, the extenders with 4.0 and 6.0 mg/ml of ALA showed significantly improved SOD activities as compared with other treatment groups and the control group ($P < 0.05$). Those containing 6.0 mg/ml of ALA in the extender conferred the highest LDH and CAT activity ($P < 0.05$). Additionally, the extenders containing 4.0 and 6.0 mg/ml ALA had a higher GOT activity than other groups. However, there were no significant differences in SOD activity between the 2.0, 8.0 and 10.0 mg/ml concentrations of ALA and no significant differences for GOT activity between 2.0, 8.0 and 10.0 mg/ml concentrations of ALA.

Effects of ALA on artificial insemination

Artificial insemination results using boar semen are shown in Table 3. The pregnancy rate and litter size was higher ($P < 0.05$) in the fresh semen groups compared with other groups. However, the pregnancy rate and litter size of cryopreserved boar sperm was higher ($P < 0.05$) in the extenders containing 6.0 mg/ml of ALA as compared with the control group.

Discussion

ALA is an organosulfur compound derived from octanoic acid. It is soluble in water and lipids and distributed in the intracellular membrane (Wada *et al.*, 1997). It has been suggested that lipid-soluble compounds can be incorporated into cell membranes and prevent the damaging activity of lipid peroxyl radicals (Aitken & Clarkson, 1988). Some researchers have demonstrated the antioxidant function of ALA, and predicted it to be a potential ROS scavenger (Cao *et al.*, 2003; Jana *et al.*, 2014). However, there have been some reports on the protective effect of ALA on mammalian spermatozoa. This is the first report on the effect of ALA on boar spermatozoa during the freezing–thawing process. In the present research, our results showed that motility was well protected by ALA treatments (Table 1). The TM%, LM%, plasma MI, MA and acrosome integrity were significantly improved by adding ALA to the extender. However, we did find that motility decreased at high concentrations of ALA after cryopreservation, possibly due to overly high concentrations. In the present study, the optimum concentration of ALA was 6.0 mg/ml.

https://doi.org/10.1017/S0967199415000155 Published online by Cambridge University Press

Table 3 Pregnancy rate and litter size of different treatment on boar sperm after artificial insemination

Groups	Pregnancy rate	Total piglets born /litter
Control	60% (12/20)	8.6 ± 1.4^a
6.0 mg/ml ALA	75% (15/20)	10.1 ± 1.1^b
Fresh semen	80% (16/20)	11.3 ± 1.1^c

Note: Values in the same column with different letters indicates differences ($P < 0.05$). Values are mean ± standard error of the mean (SEM) of total fetuses per litter. Control group $n = 12$, 6.0 mg/ml ALA $n = 15$, fresh semen $n = 16$.

The freezing and thawing of boar spermatozoa causes considerable cell damage leading to severe reductions in farrowing rates and litter size after artificial insemination with frozen semen. The success of preservation by freezing depends on the susceptibility of spermatozoa cells to low temperature. Boar spermatozoa are especially susceptible to cold shock (White, 1993) because their membranes have a relatively high proportion of polyunsaturated fatty acids, which decreases significantly during the cryopreservation process, indicating lipid peroxidation (LPO; (Cerolini *et al.*, 2001). One of the deleterious effects that takes place during semen cryopreservation is oxidative stress elicited by ROS. LPO has been correlated to the exposure of spermatozoa to ROS (Chatterjee & Gagnon, 2001; Baumber *et al.*, 2003). ROS in the forms of superoxide anion ($\bullet O_2-$), hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2) are continuously generated by spermatozoa aerobic metabolism (Hu *et al.*, 2009). During the process of cryopreservation, spermatozoa are exposed to highly concentrated oxygen and ROS (Medeiros *et al.*, 2002). In spermatozoa, there are two channels for the production of ROS: via the mitochondria, through the electron transport chain, particularly when they suffer from freezing–thawing damage (Brouwers & Gadella, 2003); or via the spermatozoa plasma membrane, through the NADPH-dependent oxidase system (Agarwal *et al.*, 2005). In previous studies, mammalian spermatozoa membranes were particularly susceptible to LPO in the presence of ROS, with subsequent MI loss, impaired cell function and decreased motility (Lenzi *et al.*, 2002; Bucak *et al.*, 2007). Antioxidants can reduce the oxidative damage by scavenging ROS or inhibiting the generation of ROS. Recent studies have shown that ALA could protect endogenous antioxidant enzymes and scavenge free radicals. For example, ALA could increase SOD, GSH (reduced glutathione), and CAT (total antioxidant capacity) in rats with acute deltamethrin intoxication (Abdou & Abdel-Daim, 2014). Notably, Ashour *et al.* (2011) found that ALA increased testicular reduced glutathione (GSH) level and lactate dehydrogenase isoenzyme-x (LDH-x) activity in septic rats. ALA also reduce superoxide anion generation and oxidative stress in diabetic and non-diabetic kidneys (Bhatti *et al.*, 2005).

Our results showed that SOD, LDH, GOT and CAT activity was greater in the groups supplemented with ALA as compared with the control group ($P < 0.05$). This is in agreement with previous research (Ibrahim *et al.*, 2008; Ma *et al.*, 2011; Yeni *et al.*, 2012), in which it was inferred that ALA effectively protected spermatozoa from the adverse effects of ROS. However, the antioxidant activities of ALA decreased at high concentrations. This could possibly be because the greater amounts of antioxidant additives destroyed the mitochondria of the spermatozoa (Hu *et al.*, 2013).

It is important to note that total spermatozoa number was used for artificial insemination. Previous reports have recommended using frozen boar spermatozoa numbers equivalent to 1 to 3.0 × 10^9 motile cells or 5 to 6 × 10^9 total spermatozoa (Rodriguez *et al.*, 2010; Ringwelski *et al.*, 2013); in our study, we used the latter. Our artificial insemination results showed that the pregnancy rate and litter size all decreased following cryopreservation as compared with fresh semen. The possible reason was that the spermatozoa were damaged during the freezing–thawing process. However, the group with 6.0 mg/ml of ALA added to the extender was significantly better than the control group ($P < 0.05$). The reason might be that ALA has an antioxidant capacity; it can protect spermatozoa from oxidative damage during cryopreservation and subsequent artificial insemination. ALA's role in the Krebs cycle may aid in the production of ATP, and thus it may increase spermatozoa motility and help in fusion with the ovum. The results indicated that the addition of ALA to the extender could increase pregnancy rate and litter size of frozen–thawed boar spermatozoa. Therefore, 6.0 mg/ml of ALA could be a cryoprotectant in boar semen frozen extenders.

In conclusion, our results suggest that supplementation with ALA could provide greater cryoprotective capacity for frozen–thawed boar spermatozoa. It greatly improved spermatozoa motility, MI, MA and acrosomal integrity. ALA also improved spermatozoa antioxidant capacity, such as the activity of SOD, LDH, GOT and CAT. The addition of ALA to the extender significantly increased the pregnancy rate

https://doi.org/10.1017/S0967199415000155 Published online by Cambridge University Press

and litter size of cryopreserved spermatozoa in boar artificial insemination. The optimal concentration of ALA added into the extenders was 6.0 mg/ml. Further research is need to collate information concerning LPO and antioxidant capacities in cryopreserved boar semen, and to determine if ALA could provide greater effective cryoprotectant properties.

Acknowledgements

This research was supported by the 12th Five-year Plan Rural Areas State Science and Technology Support Projects (2011BAD19B04–3) and Science and Technology Innovation Project of Shaanxi Province (2011KTCL02–11).

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