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Effects of Vitamin C and Vitamin E on Cryopreservation of Guanzhong Donkey Semen

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ABSTRACT

Sperm cryopreservation offers an effective method to protect Guanzhong donkey, a precious species. The objective of this study was to reveal the effect of different antioxidants on the semen cryopreservation in Guangzhong donkey by testing indices of sperm motility, mitochondrial activity, membrane integrity and arosome integrity. The results illustrated that compared with controls, above-mentioned indices of sperm were significantly improved under concentrations of Vitamin C (400 mg/L) and Vitamin E (600 mg/L) ($P < 0.05$). Moreover, physiological indices of donkey semen were significantly higher than the control groups ($P < 0.05$) when Vitamin C (200 mg/L) and Vitamin E (200 mg/L) were present simultaneitily in the medium. Overall, the results suggested both antioxidants (Vitamin C and Vitamin E) could increase frozen semen quality and the ability of antioxidant. Nevertheless, the effect on compatibility of the two antioxidants was better than single addition.

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Authors' Contribution
XY and XS conceived and designed the research. MK conducted the experiments. LW provided experimental materials. XY wrote the manuscript and YZ and SW edited it. SH performed the statistical analysis.

Key words
Guanzhong donkey, Semen, Cryopreservation, Vitamin C and E.

INTRODUCTION

Guanzhong donkey lives in Guanzhong Weihe River basin of China at the earliest. During late 1980, traditional function of donkey was replaced by agricultural mechanization, resulting in donkey's population decreasing year by year. The potential value of Guanzhong donkey in meat puts importance to protecting, developing and utilizing Guanzhong donkey. Studies of donkey artificial insemination began with 1960s-1970s in China, but some causes such as low reproduction rate, long pregnancy, sensitivity of sperm to low temperature blow, easy damage of sperm during the process of freezing-thawing, and hydroperoxidation restricted the development of frozen semen in donkey, which provides a new method finding appropriate cryoprotectants to improve semen quality. Under chemical, osmotic, thermal and mechanical stress, sublethal sperm injury has been generated, which may result in destruction of acrosomal plasma membrane (Johnston *et al*., 2012). Adding Vitamin C to semen extender improved sperm motility and viability after cryopreservation of bull semen (Beconi *et al*., 1993; Mittal *et al*., 2014). The addition of Vitamin C to an extender can reduce sperm cell damage through its continuous radical-scavenging action (Reza *et al*., 2011). Vitamin E can be used to reduce the damage caused by high concentrations of free radicals, providing protection to the semen DNA and cellular membranes against peroxidation (Nordberg and Arner, 2001). However, reports about the use of Vitamin C and Vitamin E in donkey's pcryoprotectant solution are scarce. This study aims at selecting an optimum semen extender and demonstrate the best type and concentration of antioxidants for donkey by semen.

MATERIALS AND METHODS

Animals and semen collection

Semen was collected by artificial vagina from four mature Guanzhong donkeys that had gross sexual appetites and strong build in Shaanxi Agriculture And Animal Husbandry Seed Multiplication Farm. Donkeys were conducted according to international ethical standard and we took our efforts to minimize donkeys' discomfort. Semen quality was assessed by observing the morphology of sperm under the microscope at 400X and evaluating its motility subjectively. The density of sperm was determined by counting number of sperms in cell counting chamber after diluting the semen. Ultimately, only the semen free from extraneous odour, milk-white in colour, with abnormal morphology of less than 15% and sperm vitality more than 0.75 was used in the study.

For semen dilution, five formulae which are usually used in *Equus* were adopted in this study (Cui *et al*., 2010; Zhou *et al*., 2006; Zhao *et al*., 2010). Refrigerating fluid which contained 75% of basic liquid (Table I), 25% of egg yolk, 5% of glycerine was used in all experiments.

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Semen processing and subpackaging

Semen samples were centrifuged at 900 RPM for 10 min at 20 to 25 °C. A part of the super-natant was removed. The remaining super-natant alongwith the pellet was mixed with diluent at a ratio of 1:1 and then placed at 4 °C for 60 min. Next 5% glycerinum was added to diluent and kept at 4 °C for 60 min. Later, semen was suctioned into 0.25 mL precooled thin tube for the final semen subpackage. In the process of packing, 1 cm interval of the thin tube was reserved to accommodate the expansion of the frozen semen. Finally the tubes were sealed by heated tweezers and properly marked and labelled.

Semen freezing

For freezing semen, liquid nitrogen was used. For this purpose, a certain volume of liquid nitrogen was placed in the incubator and then self-made floating plate with cryogenic thermometer probe was placed on the surface of liquid nitrogen. Then thin tubes were placed on the hot plate at 4 °C. When the temperature was about 130 °C, the hob was put on the floating plate immediately. Foam insulation box was capped tightly and then fumigated for 10-15 min with the thermometer changing between -100 °C to -130 °C during freezing. The change of freezing temperature and freezing time should be recorded and controlled. After the fumigation, thin tube was quickly plunged into liquid nitrogen for storage.

For thawing semen, frozen tubes were immersed in water bath at 37 °C for about 20 sec.

Effect of vitamins C and E on cryopreservation of semen

Different concentrations of vitamin C (200, 400, 600, 400 mg/L) and Vitamin E (200, 400, 600, 400 mg/L) were added in the selected diluent and their protective effect was determinded on sperms.

For sperm quality evaluation sperm motility, sperm acrosome integrity (FITC-PNA staining method), mitochondrial membrane integrity (Rh123-PI combination dyeing) and plasma membrane integrity (HOST) to determine thoroughly and comprehensively the sperm quality after freezing-thawing.

Statistical analysis

All analysis was performed using SPSS18.0 version for Windows Software. Data of semen analysis were expressed as means ± SEM. Comparison of means values among treatments was performed using One-way ANOVA. The level of significance was set at P value < 0.05.

RESULTS

Effect of different diluents on cryopreservation of spermatozoa

Table II shows that among five frozen diluents, the sperm living rate, the acrosome intact rate, the rate of plasma membrane integrity and mitochondrial activity were significantly higher in group II than in other groups ($P < 0.05$). Group II worked best in all groups, therefore the formula of group II was used as based fluid in the subsequent experiments.

Effect of vitamin C on cryopreservation of spermatozoa

Table III showed that after adding different concentrations of Vitamin C in the diluent, the sperm viability, the acrosome integrity, plasma membrane integrity and mitochondrial activity were all significantly improved after thawing compared with the control group ($P < 0.05$). Vitamin C concentration of 400 mg/L and 600 mg/L gave the best results.

Effect of vitamin E on cryopreservation of spermatozoa

Table III shows the effect of different concentrations of vitamin E in diluent on sperm activity indicators. Vitamin E concentration of 600 mg/L gave best results.

Table I.- Components of base liquids.

Components	I	II	III	IV	V
Distilled-water (mL)	100	100	100	100	100
Sucrose (g)	11	7.3	7.3	-	-
Glucose (g)	-	2.0	2.0	3.0	6.0
Fructose (g)	-	-	-	-	2.0
Sodium citrate (g)	-	0.12	-	1.4	-
$NaHCO_3$ (g)	-	0.04	0.04	-	-
EDTA (g)	-	0.13	0.13	-	1.0
Penicillin sodium (IU)	25000	25000	25000	10000	10000
Streptomycin sulfate (g)	-	-	0.04	0.04	0.04

Table II.- Effect of different diluents on cryopreservation of Guanzhong donkey spermatozoa.

Dilutents	Sperm viability (%)	Acrosome integrity (%)	Membrane integrity (%)	Mitochondrial activity (%)
I	31.00±1.57^{d}	47.22±0.88^{d}	48.20±1.50^{e}	38.13±1.14^{d}
II	44.32±0.76^{a}	62.75±0.88^{a}	63.49±1.26^{a}	50.17±1.78^{a}
III	37.91±1.05^{b}	56.19±1.07^{b}	54.75±0.97^{b}	46.88±1.15^{b}
IV	25.64±1.46^{e}	41.14±1.17^{e}	39.00±1.21^{d}	36.88±0.92^{d}
V	35.58±0.65^{c}	51.38±1.41^{c}	50.87±0.77^{c}	42.13±0.98^{c}

The different letters of each column in the table are on behalf significant difference (P>0.05), the same letter indicate the difference was not significant (P>0.05).

Table III.- Effect of vitamins C and E, alone and in concentration on cryopreservation of Guanzhong donkey spermatozoa.

Concentration (mg/L)	Sperm viability (%)	Acrosome integrity (%)	Membrane integrity (%)	Mitochondrial activity (%)
Vitamin C				
Control	44.32±0.76 ^{b}	62.75±0.88 ^{c}	63.49±1.26 ^{b}	50.17±1.78 ^{b}
200	44.33±0.84 ^{b}	62.90±0.46 ^{c}	63.45±0.70 ^{b}	50.22±0.79 ^{b}
400	46.13±1.18 ^{a}	65.75±0.49 ^{a}	66.17±0.71 ^{a}	54.25±0.97 ^{a}
600	45.05±0.89 ^{ab}	64.53±0.72 ^{b}	65.91±0.77 ^{a}	53.73±0.45 ^{a}
800	44.33±1.05 ^{b}	62.92±0.68 ^{c}	62.92±0.85 ^{b}	50.28±1.32 ^{b}
Vitamin E				
Control	44.32±0.76 ^{b}	62.75±0.88 ^{c}	63.49±1.26 ^{b}	50.17±1.78 ^{b}
200	44.37±0.84 ^{b}	62.78±0.61 ^{c}	63.51±0.77 ^{b}	50.22±0.65 ^{b}
400	45.01±1.15 ^{ab}	65.53±0.72 ^{b}	64.90±1.24 ^{a}	53.36±0.90 ^{a}
600	46.21±1.11 ^{a}	65.72±0.41 ^{a}	66.10±0.60 ^{a}	54.17±0.86 ^{a}
800	44.31±1.18 ^{b}	62.92±0.68 ^{c}	62.92±0.85 ^{b}	50.28±1.32 ^{b}
Vit. C+ Vit. E				
0+400	45.01±1.15 ^{b}	64.51±0.68 ^{c}	64.90±1.24 ^{cd}	53.36±0.90 ^{ab}
600+0	45.05±0.89 ^{b}	64.53±0.72 ^{c}	65.91±0.77 ^{abc}	53.73±0.45 ^{ab}
200+200	46.97±1.02 ^{a}	66.22±1.06 ^{a}	66.59±0.42 ^{a}	54.95±1.69 ^{a}
200+400	45.87±0.94 ^{ab}	65.66±0.63 ^{ab}	66.14±0.71 ^{ab}	53.74±0.87 ^{ab}
600+200	45.39±0.94 ^{b}	64.71±0.90 ^{ba}	65.37±0.85 ^{bcd}	53.80±1.58 ^{ab}
600+400	44.51±1.04 ^{b}	64.65±0.65 ^{ba}	64.73±0.71 ^{d}	52.49±1.05 ^{b}

The different letters of each column in the table are on behalf significant difference (P>0.05), the same letter indicate the difference was not significant (P>0.05).

Combined effect of vitamins C and E on cryopreservation of spermatozoa

Table III shows that the combined effect of vitamins C and E improved the indicators of frozen sperm to a certain extent. In addition, the groups Vit. C + Vit. E (200+200) and Vit. C + Vit. E (200+400) proved to be best preservatives with living sperm rate, acrosome intact rate, mitochondria intact rate higher than other groups. However, the former is more superior to the latter in spite of some indicators having no significant difference (P> 0.05).

DISCUSSION

Vitamin C that plays a regulatory role in the oxidation reduction and metabolic reactions is a very common water-soluble vitamin. It has already been widely used in many laboratories for animal sperm cryopreservation. The experiments proved that vitamin C improved the sperm activity of cow after thawing (Foote *et al*., 2002; Singh and Sharma, 2018). Cheede's (2011) study showed that vitamin C also had good effect on the preservation of semen in buffalo, and it could improve the conception rate of

buffalo after artificial insemination. Moreover, the vitamin C also had the ability of reducing the cohesion of thawed sperm, which was very helpful to sperm liquefaction after thawing (Mustafa and Esref, 2004). The effect of vitamin C on semen cryopreservation in Guanzhong donkey has not been reported so far. This experiment aims to detect all indices by adding different concentrations of vitamin C into donkey semen to suggest its antioxidant effect in Guanzhong donkey semen cryopreservation and reveal the optimal concentration. The reactive oxygen species composition would be stimulated during semen cryopreservation. Jones and Mann (1997) reported that vitamin C could not only neutralize the reactive oxygen species composition that did not reach the dynamic balance but also could effectively remove free radicals. Besides, it can prevent the peroxidatic reaction of sperm phospholipid, and can effectively maintain the integrity of sperm DNA (Mangoli *et al*., 2014). Certain concentration of vitamin C could significantly improve sperm living rate, acrosome integrity rate and mitochondrial activity in this experiment, suggesting that vitamin C in sperm cryopreservation plays a protective role mainly by reducing the oxidative damage. Vitamin C is also easy to be catalyzed to generate hydrogen peroxide and hydroxyl radicals in the presence of metal ions and increasing peroxide stress of sperm, which is likely to explain why the effect of group with high concentration was inferior to the one of 400 mg/L.

Vitamin E known as alpha tocopherol is a kind of fat-soluble vitamins, and it is also one of the most common type of antioxidants. Its antioxidant capacity is derived from its own structure that there is a lively hydroxyl on the benzene ring. In addition, the saturated carbon chain on one side of five carbon ring brings the fat-soluble feature to vitamin E. When oxygen free radicals (ROS) entered into fat, Vitamin E could neutralize ROS immediately to conduct the antioxidant effect. Vitamin E as an antioxidant was applied widely in frozen semen (Ollero, 1998), and its role adjusting sexual function and extending the life of sperm in terms of reproductive function has been recognized by academic circles. It can be concluded from this experiment that the addition of 600 mg/L of vitamin E could have very good protection effect on sperm, consistant with many results from researchers before (Cui, 2010; Zhou, 2006; Zhao, 2010). But when the concentration of Vitamin E in semen diluent is in excess, the sperm may die.

In previous experiments, vitamin C and vitamin E were applied to bull semen diluent to study *in vitro* fertilization rate. The results showed that the combination of the two increased the fertilization rate to 74%. The use of vitamin C or vitamin E separately resulted in 50% and 47% fertilization rates, respectively which showed that the two vitamins had a synergistic effect (Dalvit, 1998). The mechanism of synergy is not clear, hence further investigations are required in this directions. In future study, L-tryptophan , which can be used in activation medium for trouts, could be considered potentially positive effect on cryopreservation of Guanzhong donkey semen (Filiz, 2018).

CONCLUSION

Addition of antioxidants vitamin C and vitamin E can improve the quality of frozen thawed semen, and the optimum amount is 400 mg/L and 600 mg/L, respectively. The effect of combination of two antibiotics is apparently better, which can significantly improve the indicators of sperm, superior to adding alone. The best compatibility concentration of vitamin C and vitamin E are 200 mg/L for vitamin C and 200 mg/L for vitamin E.

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Statement of conflict of interest

The authors declare no conflict of interest.

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