



Effect of resveratrol-loaded nanostructured lipid carriers supplementation in cryopreservation medium on post-thawed sperm quality and fertility of roosters

Abouzar Najafi^a, Hossein Daghigh Kia^{a,*}, Hamed Hamishehkar^b,
Gholamali Moghaddam^a, Sadeg Alijani^a

^a Department of Animal Science, College of Agriculture, University of Tabriz, Tabriz, Iran

^b Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran



ARTICLE INFO

Keywords:

NLC
Rooster
Sperm
Resveratrol
Cryopreservation

ABSTRACT

The objective of this study was to assess the effect of resveratrol and resveratrol-loaded nanostructured lipid carriers (NLC) supplementation of semen extender on values for fertility variables of cryopreserved rooster semen. Rooster semen was cryopreserved in modified Beltsville extender containing 0 (control group), resveratrol at 20, 40 and 60 μM and resveratrol-loaded NLC at 20, 40 and 60 μM . After thawing, motility properties, abnormal morphology, viability, membrane functionality, mitochondrial activity, apoptotic status, malondialdehyde (MDA) and antioxidant activities (glutathione peroxidase (GPx), superoxide dismutase (SOD), and total antioxidant capacity (TAC)) and fertility potential (fertility and hatchability rates) were assessed. Using 40 μM resveratrol and resveratrol-loaded NLC improved total motility. The results indicated that sperm fertility and hatching rate, viability and membrane functionality were greater in the 40 μM resveratrol and resveratrol-loaded NLC compared to control group. The percentage of apoptotic spermatozoa in 40 μM resveratrol and resveratrol-loaded NLC group was less compared with the 60 μM resveratrol, 60 resveratrol-loaded NLC and control groups. Mitochondria activity was greater in the 40 μM resveratrol and resveratrol-loaded NLC extender group compared to 20 and 60 μM resveratrol, 60 resveratrol-loaded, and control groups. Also, the 40 μM resveratrol and resveratrol-loaded NLC extender group had a greater TAC and reduced MDA. Morphology and SOD were not affected by dose of resveratrol. The results indicate supplementation of the modified Beltsville extender with 40 μM resveratrol and resveratrol-loaded NLC resulted in a greater quality of frozen-thawed rooster sperm.

1. Introduction

The importance of genetic conservation and biodiversity in food production system is obvious, although the efforts in this regard are mostly focused on endangered species, these have a very important purpose in domestic animals, especially in poultry breeding (Madeddu et al., 2016).

The optimization of sperm cryopreserving from a particular rooster is important in genetic conservation. In sperm cryopreservation, due to the relatively greater amount of polyunsaturated fatty acids and lesser amounts of antioxidants, the susceptibility to

* Corresponding author.

E-mail addresses: daghighkia@tabrizu.ac.ir, hdk6955@gmail.com (H. Daghigh Kia).

<https://doi.org/10.1016/j.anireprosci.2018.12.006>

Received 11 August 2018; Received in revised form 5 December 2018; Accepted 14 December 2018

Available online 15 December 2018

0378-4320/ © 2018 Elsevier B.V. All rights reserved.

oxidative damage is relatively greater (Aitken et al., 2012). Oxidative stress occurs when production of reactive oxygen species (ROS) increases or antioxidant defenses decrease. The concentration of ROS is managed by antioxidant defenses containing various enzymatic systems such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and non-enzymatic antioxidants such as glutathione (GSH) (Santiani et al., 2014). The ROS are reactive molecules and may detrimentally affect fertility and function of sperm (Lotfi et al., 2017). Antioxidants have various functions such as eliminating ROS or inhibiting ROS toxicity in the sperm of different poultry or mammalian species (Safa et al., 2016; Zadeh Hashem and Eslami, 2016; Longobardi et al., 2017).

Without an effective antioxidant defense system, the peroxidation of the plasma membrane phospholipids can cause serious cell damage (Finkel and Holbrook, 2000). Resveratrol is known to have an effective inhibition of cell membrane lipid peroxidation, besides protein oxidation owing to its potential capacity to eliminate a variety of ROS, containing hydroxyl and superoxide radicals (Leonard et al., 2003). Resveratrol is the most prevalent non-flavonoid polyphenol contained in almost all grapes, which has multiple effects such as antioxidant, anti-cancer, anti-aging, cardioprotective, anti-inflammatory, and neuroprotective effects (Longobardi et al., 2017). Resveratrol has been observed to protect the plasma membrane integrity from oxidative damage, but did not affect the magnitude of decrease of motility caused by cryopreservation of human semen (Garcez et al., 2010). In addition, resveratrol is efficient in reducing post-thawed DNA damage in human sperm (Branco et al., 2010) and improves post-thawed bull sperm quality regarding sperm DNA integrity, motility and mitochondrial activity (Bucak et al., 2015). The beneficial effects of resveratrol supplementation on sperm medium have been reported in several species, including mice (Mojica-Villegas et al., 2014), rams (Sarlos et al., 2002), humans (Collodel et al., 2011), and bulls (Bucak et al., 2015), but inconsistent results occur (Silva et al., 2012; Gadani et al., 2017).

Although resveratrol is an effective antioxidant, it is unstable and has less than desirable bioavailability and poor water solubility (Walle et al., 2004; Vitaglione et al., 2005; Teskač and Kristl, 2010). The effect of loading resveratrol to nanocarriers to investigate if this approach allows for overcoming the problems and results in enhancing the antioxidant capacity of this compound. It is extremely important to maintain pharmacological properties of a substance such as resveratrol while developing the site-specific drug delivery systems for improving its bioavailability (Lu et al., 2009; Shao et al., 2009). Nanostructured lipid carriers (NLC), as a new delivery system, are developmental stages. The NLC are composed of solid and liquid lipids, surface active agents and water, in which a liquid lipid core is surrounded by solid lipid matrix. It immobilizes the bioactive compound in the solid particle matrix and protects the incorporated material from degradation. The lipid system functions as a physical barrier that may protect an encapsulated sensitive bioactive component from detrimental factors in aqueous phase. These carriers have advantages in comparison to other carriers among which the most important are: colloidal stability against gravitational separation due to the greater density of solid lipids, greater chemical stability due to the greater stability of the solid lipid, possibility of sterilization, not using organic solvents in its production and great encapsulation efficiency. The incorporation of these bioactive materials into lipid carriers increases their stability and bioavailability and improves the activities of resveratrol as an antioxidant. The NLC is a useful option for handling and transmission of lipophilic biomaterials and other fat-like substances in water-based medium (Karimi et al., 2018). The inclusion of antioxidant loaded with NLC in extenders is one of the possibilities to increase the efficiency of the freezing process of sperm and this technique is suitable for most lipophilic antioxidants. Nanocarriers have scarcely been considered in sperm cryopreservation.

We expect that the chemical and physical properties of resveratrol and also its antioxidant effects will increase through use of nanoparticle systems. Indeed, many antioxidants have been tested for use in fowl semen preservation. There, however, have been two issues that support the importance and worthiness of the topic described in the present research. Firstly, this is the first report of the use of resveratrol for rooster sperm cryopreservation. The effect often varies of different treatments of sperm for different species, and, therefore, there is a necessity for specific species validation for use in semen preservation. Secondly, the main focus of the research is not only using an antioxidant to improve the cryopreservation of rooster semen, but also to apply this relatively new technology to rooster semen. This is the first study where NLC has been used for the cryopreservation of bird semen. The primary purpose of this study, therefore, was to use resveratrol and a novel resveratrol nanodelivery system mostly based on lipid nanoparticles to enable resveratrol to be used in sperm medium and for studying the effectiveness of resveratrol and resveratrol-loaded NLC on sperm properties, fertility and hatchability rates of eggs fertilized using sperm the were stored using these treatments.

2. Materials and methods

2.1. Chemicals

All materials used in this study were purchased from Sigma (St.Louis, Mo, USA) and Merck (Darmstadt, Germany) unless indicated.

2.2. Animal ethics

This trial was conducted following approval distributed by Animal Care Committee and Animal Research Ethics University of Tabriz, Iran.

2.3. Preparation of resveratrol-loaded nanostructured lipid carriers) NLC (and resveratrol encapsulation efficiency (EE) assessment

For NLC, a hot homogenization procedure was used to produce the NLC. In brief, a weighed amount of 1 mg of resveratrol (4.38×10^{-3} mmol) was dissolved in 20 mg liquid oil (Miglyol) and the mixture was added into 80 mg melted solid lipid (Precirol)

(ratio of resveratrol to carrier 1/100). The hot aqueous surfactant solution was subsequently prepared by dissolving 75 mg of poloxamer 407 in 10 ml distilled water and it was added gradually into the lipid phase (at the same temperature as melted lipids) while homogenization occurred (Silent crusher M, Heidolph, Nuremberg, Germany) at 20,000 rpm for 15 min. The hot nanoemulsion was cooled to 24 °C in lipid phase recrystallization to obtain the eventual formation of NLC. Afterward, resveratrol-loaded nanostructured lipid carriers were stored at 4 °C for further use.

Resveratrol encapsulation efficiency (EE) was determined using a centrifugal filter tube (Amicon® Ultra-15 with molecular weight cut-off of 100 kDa, Millipore, USA) (Hajipour et al., 2018). To determine the amount of non-encapsulated resveratrol (free resveratrol), 1 ml of the formulation was passed through Amicon® filters by centrifugation at 5000 rpm for 10 min to separate the free from the encapsulated resveratrol. The solution in the bottom of Amicon® tube was used for resveratrol determination by ultraviolet-visible spectrophotometer (Ultrascope 2000®, Pharmacia Biotech, UK) at 276 nm. The average value of EE was 94%.

2.4. Farm management, semen collection, extender preparation and sperm cryopreservation

The experiment was conducted using ten roosters (28 weeks old) of the Ross 308 breed kept individually in cages (70 × 70 × 85 cm) with a controlled photoperiod program (14 L: 10 D), and the birds being provided a standard diet and water ad libitum.

The experiment was performed in five replicates there being 5 days for semen collection with a frequency of twice per week. Ejaculates were collected using the dorso-abdominal massage method and collection was always performed by the same person and with the same conditions being imposed. After collection, the ejaculates were placed in a thermal flask containing water at a temperature 37 °C and then transferred to the laboratory within 5 min after collection for primary evaluation. To eliminate individual differences and obtain sufficient sperm for analysis, in each replicate, the ejaculates of the ten roosters were briefly inspected and ejaculates with $\geq 300 \times 10^6$ spermatozoa/mL, $\geq 90\%$ normal morphology and $\geq 80\%$ motility were then pooled. For all replicates, three ejaculates (of all 50 ejaculates) were rejected. There was subsequent aliquoting of the pooled semen sample according to the number of treatments (seven equal aliquots). The semen was extended in two steps with Beltsville freezing extender (Table 1). In the first step, the volume of pooled semen was extended at 37 °C with (one part of semen and four of extender) Beltsville extender containing 0 (control), 20, 40 or 60 μM resveratrol (plain or in NLC), and 2% (v/v) glycerol. The pre-diluted semen was then cooled for 2 h at 4 °C. In the next step, the pre-diluted semen was further extended 1:1 with Beltsville extender, but containing 6% (v/v) glycerol and no resveratrol, giving a final glycerol concentration of 3.8%. The completely extended semen was equilibrated for 1 h at 4 °C and then the diluted semen was aspirated into 0.25 mL plastic straws at final concentration of 100×10^6 sperm/mL and then were placed 4 cm above the surface of the liquid nitrogen for 7 min and were instantly immersed in liquid nitrogen. For assessment, the cryopreserved straws were thawed in a water bath at 37 °C for 30 s.

2.5. Sperm motility

Sperm motility was evaluated using the computer assisted sperm analysis (CASA V 12.2; Hamilton Thorne Biosciences, Beverly, MA, USA). In brief, 5 μL sperm ($10\text{--}20 \times 10^6$ sperm/mL) was placed in a counting chamber (10 μm depth) and examined at $\times 10$ (negative phase contrast) using a microscope with a warmed stage (37 °C). Sperm motility variables included motility (TM%), progressive motility (PM%), curve-linear velocity ($\mu\text{m/s}$), average path velocity ($\mu\text{m/s}$), straight-line velocity ($\mu\text{m/s}$), amplitude of lateral head displacement (μm), straightness (%), linearity (%), and beat cross frequency (Hz) were evaluated.

2.6. Sperm viability

The sperm viability was assessed using the eosin-nigrosin stain method (Khiabani et al., 2017). Briefly, Sperm suspension smears were prepared by mixing 10 μL of sperm sample with 20 μL of eosin-nigrosin and then, spread on a warm slide. Subsequently, sperm viability was evaluated for 200 sperm (viable and non-viable) using phase-contrast microscopy at a magnification of $400 \times$.

Table 1
Composition of the Beltsville extender.

Ingredient	
Potassium citrate tribasic monohydrate (mM)	2.08
Sodium-L-glutamate (mM)	51.28
Magnesium chloride anhydrous (mM)	0.35
D-(–)-Fructose (mM)	27.75
Potassium phosphate dibasic trihydrate (mM)	43.57
Potassium phosphate monobasic (mM)	5.14
N-[Tris (hydroxymethyl) methyl]-2 (mM)	13.95
Sodium acetate trihydrate (mM)	3.9
Soybean lecithin (g/100 mL)	1
pH	7.1
Osmolality (mOsm/kg)	310

2.7. Plasma membrane functionality

Plasma membrane functionality was evaluated using the hypo-osmotic swelling (HOS) Test as previously described by Feyzi et al. (2018). In brief, this test was conducted by incubating 10 μ L of sperm with 100 μ L of a hypo-osmotic solution (fructose (5 mM) and sodium citrate (1.9 mM); 100 mOsm) at 37 °C for 30 min. Afterward, 10 μ L of the suspension was spread with a cover slip on a warm slide (37 °C). Sperm ($n = 200$) were assessed using a phase-contrast microscopy at 400 \times magnification to determine the fraction of sperm with osmotically active membranes (swollen or coiled tails).

2.8. Total abnormalities

Cold shock during semen cryopreservation may result in some abnormalities in sperm. For the assessment of sperm total abnormalities in the semen samples, at least 10 μ L of the semen was transferred into Eppendorf tubes, including 1000 μ L Hancock's solution (Najafi et al., 2018). Ten μ L of this suspension was placed on a slide and a cover slip was applied. The percentage of sperm total abnormalities (head abnormalities, detached heads, abnormal midpieces and tail defects) ($n = 200$ /slide) was determined using a phase-contrast microscope at 400 \times magnification.

2.9. TAC, GPx and SOD assessments

The TAC, SOD and GPx activities were evaluated spectrophotometrically using Randox kits (RANDOX Laboratories Ltd.) (Mehdipour et al., 2017), and an Olympus AU 400 automated biochemistry analyzer (Olympus, Tokyo, Japan). The TAC was assessed by adding the reactive provided in the kit and measuring the absorbance at 600 nm, converting absorbance to mmol/l. The GPx was measured in the presence of oxidized glutathione (cumene hydroperoxide) and NADPH. The oxidized glutathione was then reduced by GPx with a concomitant oxidation of NADPH to NADP⁺, determining the decrease in absorbance at 340 nm. The SOD determination is based on the extent of inhibition of the oxidation of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazoliumchloride (INT) to the red formazan dye by superoxide radicals (produced by a xanthine/xanthine oxidase system). One unit of SOD prevents the reduction of INT by 50% under the conditions of the assay. The absorbance was recorded at 505 nm.

2.10. Malondialdehyde (MDA)

The MDA concentrations in diluted semen were measured as an index of lipid peroxidation using the thiobarbituric-acid reaction (Seifi-Jamadi et al., 2016). Lipid peroxidation was assessed by TBARS assay. In brief, 1000 μ L of diluted sperm (200 $\times 10^6$ sperm/ml) was mixed with 1000 μ L of cold 20% (w/v) trichloroacetic acid (TCA) to precipitate protein. The precipitation was pelleted by centrifuging with 1200 rcf for 10 min, and 1000 μ L of the supernatant was incubated with 1 ml of 0.67% (w/v) TBA in a water bath at 100 °C for 10 min. The absorbance was subsequently measured using a T80 UV/VIS PG spectrophotometer at 532 nm. The results are described as nmol/ml.

2.11. Sperm apoptosis

The detection of phosphatidylserine translocation as an index of apoptosis changes in the sperm cells was conducted using an Annexin V-FITC detection kit (IQP, Groningen, The Netherlands) with the methodologies being used as previously described Mehdipour et al. (2018) with a slight modification. The semen samples were re-suspended in buffer to a concentration of 1 $\times 10^6$ sperm/mL and 10 μ L of Annexin V (AV) (0.01 mg/mL) was subsequently added to 100 μ L of the sperm samples, which was then incubated for 15 min. Then, 10 μ L of propidium iodide (PI) (1 mg/mL) was added and incubated for a minimum of 10 min. Subsequently, the mixture was calculated by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). After the flow cytometric process was completed, the sperm were categorized into four subpopulations: (1) AV and PI negative staining was classified as live non-apoptotic sperm (AV-/PI-); (2) AV positive and PI negative staining classified as early apoptotic sperm (AV+/PI-); (3) AV positive and PI positive staining classified as late apoptotic sperm (AV+/PI+); and (4) AV negative and PI positive sperm classified as necrotic sperm (AV-/PI+). Late apoptotic sperm and necrotic sperm were classified as being dead sperm.

2.12. Active mitochondria

Rhodamine 123 (R123) and PI staining was used to measure sperm mitochondrial activity. In brief, 5 μ L of R123 solution (0.01 mg/ml) and 5 μ L of PI were mixed with 250 μ L of diluted sample (50 $\times 10^6$ sperm/ml) and incubated in dark place at 37 °C for 15 min. The percentage of sperm mitochondria activity was assessed using a flow cytometer (positive for R123 and negative for PI) (Najafi et al., 2017).

2.13. Fertility, and hatchability

Artificial insemination (AI) was performed using the technique of Long and Kulkarni (2004) with a slight modification. Breeder hens (Ross) were categorized into three treatments (ten hens in each treatment), which were kept in individual cages (70 \times 70 \times 85 cm). The subsequent three treatments were selected using results of in vitro sperm determination. There was then

Table 2

Effect of resveratrol and resveratrol-loaded nanostructured lipid carriers (NLC) on values for motility variables of rooster sperm assessed by CASA after freeze-thawing.

Parameters	Treatments							SEM
	Control	Resveratrol (μM)			Resveratrol-loaded NLC (μM)			
		20	40	60	20	40	60	
TM (%)	51.9 ^c	58.7 ^{bc}	64.3 ^{ab}	54.1 ^{bc}	61.0 ^{bc}	73.0 ^a	55.5 ^{bc}	2.5
PM (%)	17.4 ^b	24.2 ^{ab}	27.4 ^{ab}	17.7 ^b	23.3 ^{ab}	31.3 ^a	18.9 ^b	2.3
VAP ($\mu\text{m/s}$)	30.7	33.1	35.9	31.3	33.7	37.1	32.3	1.6
VSL ($\mu\text{m/s}$)	17.2	18	20.9	17.4	19	21.8	17.7	1.5
VCL ($\mu\text{m/s}$)	56.8	57.9	59.5	57.5	58.5	60.7	56.9	2.5
LIN (%)	30.8	31.1	36.1	30.2	32.7	36	31.3	2.9
STR (%)	56.4	56.8	58.3	56.3	56.4	59.1	55.3	5.3
ALH (μm)	6	4.7	4.8	5.1	5.3	5.2	5	0.4
BCF (Hz)	15.3	16.4	18.2	15.6	16.9	18.8	16.2	1.6

TM: Total motility; PM: Progressive motility; VSL: straight–line velocity; VAP: Average path velocity; VCL: curvilinear velocity; LIN: Linearity; STR: Straightness; ALH: Mean amplitude of the lateral head displacement; BCF: Mean of the beat cross frequency. Different superscripts within the same row indicate differences among groups ($P < 0.05$).

assessment of fertility using 40 μM of semen from the resveratrol, resveratrol-loaded NLC, and control group to compare the fertility capacity of rooster sperm on which the treatments had been imposed to that of the control group.

Glycerol was removed using a discontinuous Accudenz gradient, which contained a 30% (0.5 mL) layer under a 12% (5.0 mL) layer. After centrifugation of semen (1200 rcf; 20 min), the glycerol and extender were placed above the 12% layer, whereas the sperm cells were deposited between the 12% and 30% layers. The upper layer was subsequently removed and the 30% layer was aspirated. After use of the Accudenz gradient, as much as 500 μL of extender was added to the semen layer.

Artificial insemination was performed after thawing at the 1500 h on certain days (twice a week for approximately 1 month) with insemination of 100×10^6 sperm per hen obtained from each treatment. The eggs were collected up to 5 days after the last AI. The eggs were incubated in the setter for 18 days and moved into hatchery for the remaining 3 days. After 7 days of incubation, the fertility rate (by candling of eggs) [(embryonated eggs/total eggs) $\times 100$], and after 21 days hatchability of fertile eggs [(hatched chick numbers/fertilized) $\times 100$] were evaluated.

2.14. Statistical analysis

All data were analyzed for normal distribution by PROC UNIVARIATE and the Shapiro–Wilk test using SAS software (version 9.1). The data were analyzed using the mixed procedure. Differences among groups were assessed by Tukey's test and $P < 0.05$ was recorded to be significant. Results are shown as a mean \pm SEM. Also, fertility and hatching rate were analyzed by GENMOD procedure using the chi-square test.

3. Results

The higher percentages of TM was observed when sperm were cryopreserved with 40 μM resveratrol and resveratrol-loaded NLC (Table 2). The use of the 40 μM of resveratrol-loaded NLC resulted in improvement of PM compared to the control group, whereas cryopreservation with resveratrol had no effect on values for kinematic variables. The data for sperm abnormal structures, membrane integrity, viability and mitochondrial activity after cryopreservation are presented in Table 3. After thawing, the spermatozoa viability and membrane integrity were greater in the group with 40 μM resveratrol and resveratrol-loaded NLC extender group

Table 3

Effect of resveratrol and resveratrol-loaded NLC on viability (eosine/nigrosine), plasma membrane functionality, abnormal forms and mitochondrial activity of rooster thawed semen.

Parameters	Treatments							SEM
	Control	Resveratrol (μM)			Resveratrol-loaded NLC (μM)			
		20	40	60	20	40	60	
Viability (%)	59.6 ^c	62.2 ^{bc}	70.8 ^{ab}	60.1 ^c	67.2 ^{abc}	75.0 ^a	60.2 ^c	2.2
Plasma membrane integrity (%)	50.6 ^c	55.9 ^{bc}	61.2 ^{ab}	53.2 ^{bc}	59.4 ^{bc}	70.0 ^a	57.6 ^{bc}	2.0
Abnormal forms (%)	20.2	18.0	16.7	18.6	17.7	16.5	18.0	1.7
Mitochondria activity (%)	41.3 ^d	53.8 ^{bc}	64.5 ^a	48.5 ^{cd}	59.4 ^{ab}	67.3 ^a	49.2 ^{cd}	2.3

Different superscripts within the same row indicate differences among groups ($P < 0.05$).

Table 4

Effect of resveratrol and resveratrol-loaded NLC on live, apoptotic and dead spermatozoa in thawed rooster thawed as assessed by flow cytometry.

Parameters	Treatments								SEM
	Control	Resveratrol (μM)			Resveratrol -loaded NLC (μM)				
		20	40	60	20	40	60		
Live (%)	44.6 ^d	51.51 ^{cd}	61.0 ^{ab}	44.4 ^d	57.4 ^{ab}	68.0 ^a	46.9 ^{cd}	2.3	
Early apoptosis (%)	28.8 ^a	21.2 ^{abc}	17.3 ^{bc}	25.6 ^a	21.7 ^{bc}	13.1 ^c	23.3 ^{ab}	1.8	
Dead (%)	26.5	27.2	21.5	29.8	20.7	18.8	29.6	2.7	

Viable (%), AnnexinV – /PI –), apoptotic (%), AnnexinV + /PI –) and dead (%), PI +) variables were analyzed. Different superscripts within the same row indicate differences among groups ($P < 0.05$).

compared with that of the control group. For total abnormalities, there was no difference ($P > 0.05$) in the percentage of total abnormalities with use of all experimental extenders.

There were greater ($P < 0.05$) mitochondrial activities (Table 3) and number of live sperm (Table 4) with use of the 40 μM resveratrol and resveratrol-loaded NLC extender compared with use of the semen of the 20 and 60 μM resveratrol and 60 μM resveratrol-loaded and control groups.

Data in Table 4 indicate the effect of resveratrol on Annexin V/PI analysis following sperm cryopreservation. The lesser percentage of sperm in the early apoptotic stages was greater in the 40 μM resveratrol and resveratrol-loaded NLC group compared with the 60 μM resveratrol and 60 μM resveratrol-loaded NLC and control group. For dead sperm, there was no difference ($P > 0.05$) in the percentage of dead sperm with use of all experimental extenders.

Data on the effect of resveratrol and resveratrol-loaded NLC extender on SOD, GPx, TAC and MDA are shown in Table 5. The results indicate that MDA concentrations were less ($P < 0.05$) in semen with 40 μM resveratrol and resveratrol-loaded NLC extender compared to that in the control group. Also, the concentrations of GPx was greater with use of the 40 μM resveratrol-loaded NLC extender compared to the control group. Furthermore, there were no significant differences for GPx concentrations for the semen of the various semen treated with resveratrol and resveratrol-loaded NLC. The TAC was greater ($P < 0.05$) in sperm stored in the extender containing 40 μM resveratrol and resveratrol-loaded NLC compared with the 60 μM resveratrol or control group. For SOD, there were no differences ($P > 0.05$) among the groups.

The data on fertility and hatchability of eggs fertilized with rooster spermatozoa after cryopreservation are presented in Table 6. The fertility and hatchability of eggs were greater when the semen was treated with 40 μM resveratrol or resveratrol-loaded NLC compared with that of the control group. The difference in values for these variables was not significant when there was comparison of all groups treated with resveratrol and resveratrol-loaded NLC.

4. Discussion

The results of the current study indicated that with the use of resveratrol there was a reduction in post-thawing oxidative damages and, therefore, this compound may be important for inclusion when freezing of poultry semen. In the present study, protective effects of 40 μM resveratrol and resveratrol-loaded NLC were observed on viability and plasma membrane integrity. It was hypothesized that an effectiveness of resveratrol would be enhanced by its inclusion in nanoparticles due to an increased compound stability. In addition, the function of NLC is based on its potential capacity to enhance antioxidant delivery into cells, while minimizing systemic exposure and, therefore, decreasing non-specific toxicity (Sabzichi et al., 2016). Lipid nanoparticles are thought to be submicron colloidal carriers composed of biodegradable and biocompatible lipids that are generally recognized as safe and suitable for the incorporation of lipophilic and relatively water insoluble active ingredients such as resveratrol (Neves et al., 2013; Yameen et al., 2014; Barbosa et al., 2016). Lipid nanoparticles have a superior capacity to penetrate cell membranes, allowing for increased cellular uptake of compounds loaded in the nanoparticles (Acosta, 2009). The cell membrane functions as a barrier separating the external

Table 5

Effect of resveratrol and resveratrol-loaded NLC on malondialdehyde concentration (MDA), glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities and total antioxidant capacity (TAC) of thawed rooster semen.

Parameters	Treatments								SEM
	Control	Resveratrol (μM)			Resveratrol-loaded NLC (μM)				
		20	40	60	20	40	60		
MDA (nmol/mL)	3.7 ^a	2.7 ^{ab}	1.6 ^b	2.9 ^{ab}	2.2 ^{ab}	1.3 ^b	2.7 ^{ab}	0.3	
GPx (U/mg protein)	54.0 ^b	59.5 ^{ab}	64.2 ^{ab}	57.6 ^{ab}	62.7 ^{ab}	67.4 ^a	60.0 ^{ab}	2.3	
SOD (U/mg)	107.4	113.1	125.3	114.0	119.9	132.1	118.1	10.1	
TAC (mmol/l)	1.1 ^c	1.7 ^{abc}	2.0 ^{ab}	1.4 ^{bc}	1.85 ^{ab}	2.1 ^a	1.5 ^{abc}	0.1	

Different superscripts within the same row indicate differences among groups ($P < 0.05$).

Table 6
Effect of resveratrol and resveratrol-loaded NLC on fertility and hatchability rates of rooster semen after freeze-thawing.

Parameters	Treatments		
	Control	Resveratrol-loaded NLC (40 μ M)	Resveratrol (40 μ M)
Fertility (%)	40 ^b	67.2 ^a	58.1 ^a
Hatchability of fertile egg (%)	45.4 ^b	75.6 ^a	68.7 ^a

Different superscripts letters within row are different ($P < 0.05$).

environment from the inside of the cell, allowing substances to be concentrated in cells or excluded from cells (Kettler et al., 2014). One mechanism to overcome this barrier is endocytosis. When nanoparticles reach the exterior membrane of a cell there can be an interaction with components of the plasma membrane or extracellular matrix that facilitates entry into the cell, mainly through endocytosis. Endocytosis leads to the engulfment of nanoparticles in membrane invaginations, followed by the budding and pinching off to form endocytic vesicles, which are then transported to specialized intracellular compartments (Moretti et al., 2013; Zhang et al., 2015; Behzadi et al., 2017). Endocytosis of NLCs is described as the main mechanism for improved cellular uptake in cells (Jyoti et al., 2015; Silva et al., 2017). In the present study, only small differences were detected between the use of resveratrol alone and resveratrol-loaded NLC, which may be due to the fact that resveratrol is somewhat lipophilic and can partition into the lipid membrane bilayer which may lead to sufficient cell uptake without use of the nanocarrier.

We hypothesize that the protective effect of resveratrol on the motility of sperm can be due to its scavenging properties. Data from the present study confirm that addition of 40 μ M resveratrol and resveratrol-loaded NLC to semen samples results in a reduction of free radical production, which is associated with the reduction of lipid peroxidation and as a consequence preserves sperm motility. The addition of 40 μ M resveratrol and resveratrol-loaded NLC prevented the detrimental action of oxidative stress in the present study.

Controlling cell metabolism in spermatozoa is associated with cellular organelles, such as mitochondria, which are essential structures for a successful aerobic metabolism (Rodríguez-Gil, 2006). Cryodamage can negatively affect the generation of mitochondrial ATP and subsequently the mitochondrial membrane potential (Tchir and Acker, 2010). In the current study, supplementation of the semen extender with 40 μ M resveratrol and resveratrol-loaded NLC before cryopreservation improved mitochondrial activity. Results of the present study suggest that sperm viability and motility may be somewhat related to the activity of mitochondria. In the current study, results are inconsistent with the previous results of Silva et al. (2012) where addition of 5–20 μ g/mL of resveratrol to the cryopreservation extender resulted in a decrease in ram spermatozoa mitochondrial activity. The reasons for this inconsistency are not clear but it seems that application of different extenders along with protocols and different species can affect the mitochondrial activity. Furthermore, resveratrol partitions in the membranes and enhances membrane fluidity, which may increase its capacity to protect more efficiently against free radical formation in a disrupted lipid bilayer (Brittes et al., 2010).

It has been often reported that the apoptosis-like phenomena is accelerated during cryopreservation (Martin et al., 2004). In the present study, adding 40 μ M resveratrol and resveratrol-loaded NLC before cryopreservation resulted in a decrease in apoptotic sperm. The result of the current study is consistent with those of Attia (2012), where there were indications that translocation of phosphatidylserine was reduced, due to resveratrol scavenger activity. Resveratrol has been reported to reduce doxorubicin-induced testicular damage by its anti-apoptotic effect in rats (Türedi et al., 2015). Furthermore, resveratrol functions to protect sperm against apoptosis that results from physiological disruptions (Jiang et al., 2008). Uguralp et al. (2005) reported that treated with resveratrol resulted in a decrease in the incidence of apoptosis in the testis following testicular ischemia/reperfusion damage.

The results of the present study indicate that 40 μ M resveratrol and resveratrol-loaded NLC extender prevents the MDA production in sperm, indicating the antioxidant properties of resveratrol as a ROS scavenger. This antioxidant property of resveratrol may result from its phenolic groups (Burkitt and Duncan, 2000). There were differing results in the present study when resveratrol was in relatively greater concentrations. The greater concentrations of resveratrol did not have a differential effect on sperm when compared with treatment with 40 μ M resveratrol and resveratrol-loaded NLC which when used reduced lipid peroxidation compared to the control group. In a previous study (Najafi et al., 2018), however, treatments with greater concentrations of lycopene resulted in an increase in lipid peroxidation compared to the group with lesser lycopene (group which had less lipid peroxidation compared to control group). This difference in results of these two studies is thought to be due to the different antioxidants and nanocarriers used in the two studies.

Garcez et al. (2010) reported that with use of resveratrol there is a capacity to avoid post-thawing oxidative damages indicating there could be use of this compound in human sperm cryopreservation processes. Earlier studies have indicated that with use of resveratrol there is less lipid peroxidation stimulated by tert-butyl hydroperoxide in human sperm (Collodel et al., 2011), and that resveratrol has a lipophilic structure that inhibits lipid peroxidation induced by Fenton reaction products (Berrougui et al., 2009). This protective function results from the enzymatic activity of GPx in sperm. The GPx activity was greater in the group supplemented with 40 μ M resveratrol-loaded NLC. There, however, were not any differences in SOD activity as a result of resveratrol treatments in the present study.

Fertility preservation is an important factor during the cryopreservation. The current study assessed the effect of resveratrol and resveratrol-loaded NLC on fertility and hatchability rate. In the present study, the greatest fertility rate was detected with use of 40 μ M resveratrol and resveratrol-loaded NLC. These data support the sperm quality results. The freezing and thawing process markedly

decreases rooster semen fertilizing capacity. Semen properties associated with fertility such as sperm total and progressive motility, viability, mitochondrial activity, and apoptosis may affect the cervical mucus penetration and are thus important for sperm preservation and fertilizing capacity (Ansari et al., 2017). It, therefore, appears that the greater sperm quality with the 40 μ M resveratrol and resveratrol-loaded NLC treatments leads to greater fertility and hatchability in treated groups as a result of anti-apoptotic and anti-peroxidative effects during cryopreservation.

5. Conclusion

In the present study, there was a quality of post-thawed rooster sperm when the cryopreservation extender was supplemented with 40 μ M resveratrol and resveratrol-loaded NLC. The supplementation with this concentration increased motility, viability, membrane functionality, mitochondrial activity, and total antioxidant capacity (TAC), while there was a decreased apoptotic status and malondialdehyde (MDA). Furthermore, when there was supplementation of extender with 40 μ M resveratrol and resveratrol-loaded NLC, there was a positive effect on fertility rate as a result of anti-apoptotic and anti-peroxidative effects.

Conflicts of interest

None of the authors have any conflict of interest to declare.

References

- Acosta, E., 2009. Bioavailability of nanoparticles in nutrient and nutraceutical delivery. *Curr. Opin. Colloid Interface Sci.* 14, 3–15.
- Aitken, R.J., Jones, K.T., Robertson, S.A., 2012. Reactive oxygen species and sperm function—in sickness and in health. *J. Androl.* 33, 1096–1106.
- Ansari, M., Zhandi, M., Kohram, H., Zaghari, M., Sadeghi, M., Sharafi, M., 2017. Improvement of post-thawed sperm quality and fertility of Arian rooster by oral administration of d-aspartic acid. *Theriogenology* 92, 69–74.
- Attia, S.M., 2012. Influence of resveratrol on oxidative damage in genomic DNA and apoptosis induced by cisplatin. *Mutat. Res. Toxicol. Environ. Mutagen.* 741, 22–31.
- Barbosa, J.P., Neves, A.R., Silva, A.M., Barbosa, M.A., Reis, M.S., Santos, S.G., 2016. Nanostructured lipid carriers loaded with resveratrol modulate human dendritic cells. *Int. J. Nanomed.* 11, 3501.
- Behzadi, S., Serpooshan, V., Tao, W., Hamaly, M.A., Alkawareek, M.Y., Dreaden, E.C., Brown, D., Alkilany, A.M., Farokhzad, O.C., Mahmoudi, M., 2017. Cellular uptake of nanoparticles: journey inside the cell. *Chem. Soc. Rev.* 46, 4218–4244.
- Berrougui, H., Grenier, G., Loued, S., Drouin, G., Khalil, A., 2009. A new insight into resveratrol as an atheroprotective compound: inhibition of lipid peroxidation and enhancement of cholesterol efflux. *Atherosclerosis* 207, 420–427.
- Branco, C.S., Garcez, M.E., Pasqualotto, F.F., Erdtman, B., Salvador, M., 2010. Resveratrol and ascorbic acid prevent DNA damage induced by cryopreservation in human semen. *Cryobiology* 60, 235–237.
- Brittes, J., Lúcio, M., Nunes, C., Lima, J., Reis, S., 2010. Effects of resveratrol on membrane biophysical properties: relevance for its pharmacological effects. *Chem. Phys. Lipids* 163, 747–754.
- Bucak, M.N., Ataman, M.B., Baspınar, N., Uysal, O., Taspınar, M., Bilgili, A., Ozturk, C., Gungor, S., Inanc, M.E., Akal, E., 2015. Lycopene and resveratrol improve post-thaw bull sperm parameters: sperm motility, mitochondrial activity and DNA integrity. *Andrologia* 47, 545–552.
- Burkitt, M.J., Duncan, J., 2000. Effects of trans-resveratrol on copper-dependent hydroxyl-radical formation and DNA damage: evidence for hydroxyl-radical scavenging and a novel, glutathione-sparing mechanism of action. *Arch. Biochem. Biophys.* 381, 253–263.
- Collodel, G., Federico, M.G., Geminiani, M., Martini, S., Bonechi, C., Rossi, C., Figura, N., Moretti, E., 2011. Effect of trans-resveratrol on induced oxidative stress in human sperm and in rat germinal cells. *Reprod. Toxicol.* 31, 239–246.
- Feyzi, S., Sharafi, M., Rahimi, S., 2018. Stress preconditioning of rooster semen before cryopreservation improves fertility potential of thawed sperm. *Poult. Sci.* 97, 2582–2590.
- Finkel, T., Holbrook, N.J., 2000. Oxidants, oxidative stress and the biology of ageing. *Nature* 408, 239.
- Gadani, B., Buccì, D., Spinaci, M., Tamamini, C., Galeati, G., 2017. Resveratrol and Epigallocatechin-3-gallate addition to thawed boar sperm improves in vitro fertilization. *Theriogenology* 90, 88–93.
- Garcez, M.E., dos Santos Branco, C., Lara, L.V., Pasqualotto, F.F., Salvador, M., 2010. Effects of resveratrol supplementation on cryopreservation medium of human semen. *Fertil. Steril.* 94, 2118–2121.
- Hajipour, H., Hamishehkar, H., Nazari, S., Ahmad, S., Maroufi, N.F., Taheri, R.A., 2018. Improved anticancer effects of epigallocatechin gallate using RGD-containing nanostructured lipid carriers. *Artif. Cells Nanomed. Biotechnol.* 1–10. <https://doi.org/10.1080/21691401.2017.1423493>.
- Jiang, Y., Peng, T., Luo, Y., Li, M., Lin, Y., 2008. Resveratrol reestablishes spermatogenesis after testicular injury in rats caused by 2, 5-hexanedione. *Chin. Med. J. (Engl.)* 121, 1204–1209.
- Jyoti, K., Kaur, K., Pandey, R.S., Jain, U.K., Chandra, R., Madan, J., 2015. Inhalable nanostructured lipid particles of 9-bromo-noscapine, a tubulin-binding cytotoxic agent: in vitro and in vivo studies. *J. Colloid Interface Sci.* 445, 219–230.
- Karimi, N., Ghanbarzadeh, B., Hamishehkar, H., Mehramuz, B., Kafil, H.S., 2018. Antioxidant, antimicrobial and physicochemical properties of turmeric extract-loaded nanostructured lipid carrier (NLC). *Colloid Interface Sci. Commun.* 22, 18–24.
- Kettler, K., Veltman, K., van de Meent, D., van Wezel, A., Hendriks, A.J., 2014. Cellular uptake of nanoparticles as determined by particle properties, experimental conditions, and cell type. *Environ. Toxicol. Chem.* 33, 481–492.
- Khiabani, A.B., Moghaddam, G., Kia, H.D., 2017. Effects of adding different levels of Glutamine to modified Beltsville extender on the survival of frozen rooster semen. *Anim. Reprod. Sci.* 184, 172–177.
- Leonard, S.S., Xia, C., Jiang, B.-H., Stinefelt, B., Klandorf, H., Harris, G.K., Shi, X., 2003. Resveratrol scavenges reactive oxygen species and effects radical-induced cellular responses. *Biochem. Biophys. Res. Commun.* 309, 1017–1026.
- Long, J.A., Kulkarni, G., 2004. An effective method for improving the fertility of glycerol-exposed poultry semen. *Poult. Sci.* 83, 1594–1601.
- Longobardi, V., Zullo, G., Salzano, A., De Canditiis, C., Cammarano, A., De Luise, L., Puzio, M.V., Neglia, G., Gasparini, B., 2017. Resveratrol prevents capacitation-like changes and improves in vitro fertilizing capability of buffalo frozen-thawed sperm. *Theriogenology* 88, 1–8.
- Lotfi, S., Mehri, M., Sharafi, M., Masoudi, R., 2017. Hyaluronic acid improves frozen-thawed sperm quality and fertility potential in rooster. *Anim. Reprod. Sci.* 184, 204–210.
- Lu, X., Ji, C., Xu, H., Li, X., Ding, H., Ye, M., Zhu, Z., Ding, D., Jiang, X., Ding, X., 2009. Resveratrol-loaded polymeric micelles protect cells from A β -induced oxidative stress. *Int. J. Pharm.* 375, 89–96.
- Madeddu, M., Mosca, F., Abdel Sayed, A., Zaniboni, L., Mangiagalli, M.G., Colombo, E., Cerolini, S., 2016. Effect of cooling rate on the survival of cryopreserved rooster sperm: comparison of different distances in the vapor above the surface of the liquid nitrogen. *Anim. Reprod. Sci.* 171, 58–64.
- Martin, G., Sabido, O., Durand, P., Levy, R., 2004. Cryopreservation induces an apoptosis-like mechanism in bull sperm. *Biol. Reprod.* 71, 28–37.

- Mehdipour, M., Daghigh Kia, H., Nazari, M., Najafi, A., 2017. Effect of lecithin nanoliposome or soybean lecithin supplemented by pomegranate extract on post-thaw flow cytometric, microscopic and oxidative parameters in ram semen. *Cryobiology* 78, 34–40.
- Mehdipour, M., Daghigh Kia, H., Moghaddam, G., Hamishehkar, H., 2018. Effect of egg yolk plasma and soybean lecithin on rooster frozen-thawed sperm quality and fertility. *Theriogenology* 116, 89–94.
- Mojica-Villegas, M.A., Izquierdo-Vega, J.A., Chamorro-Cevallos, G., Sánchez-Gutiérrez, M., 2014. Protective effect of resveratrol on biomarkers of oxidative stress induced by iron/ascorbate in mouse spermatozoa. *Nutrients* 6, 489–503.
- Moretti, E., Terzuoli, G., Renieri, T., Iacoponi, F., Castellini, C., Giordano, C., Collodel, G., 2013. In vitro effect of gold and silver nanoparticles on human spermatozoa. *Andrologia* 45, 392–396.
- Najafi, A., Daghigh-Kia, H., Dodaran, H.V., Mehdipour, M., Alvarez-Rodriguez, M., 2017. Ethylene glycol, but not DMSO, could replace glycerol inclusion in soybean lecithin-based extenders in ram sperm cryopreservation. *Anim. Reprod. Sci.* 177, 35–41.
- Najafi, A., Taheri, R.A., Mehdipour, M., Farnoosh, G., Martinez-Pastor, F., 2018. Lycopene-loaded nanoliposomes improve the performance of a modified Beltsville extender broiler breeder roosters. *Anim. Reprod. Sci.* 195, 168–175.
- Neves, A.R., Lúcio, M., Martins, S., Lima, J.L.C., Reis, S., 2013. Novel resveratrol nanodelivery systems based on lipid nanoparticles to enhance its oral bioavailability. *Int. J. Nanomed.* 8, 177.
- Rodríguez-Gil, J.E., 2006. Mammalian sperm energy resources management and survival during conservation in refrigeration. *Reprod. Domest. Anim.* 41, 11–20.
- Sabzichi, M., Samadi, N., Mohammadian, J., Hamishehkar, H., Akbarzadeh, M., Molavi, O., 2016. Sustained release of melatonin: A novel approach in elevating efficacy of tamoxifen in breast cancer treatment. *Colloids Surf. B Biointerfaces* 145, 64–71.
- Safa, S., Moghaddam, G., Jozani, R.J., Daghigh Kia, H., Janmohammadi, H., 2016. Effect of vitamin E and selenium nanoparticles on post-thaw variables and oxidative status of rooster semen. *Anim. Reprod. Sci.* 174, 100–106.
- Santiani, A., Evangelista, S., Sepulveda, N., Risopatron, J., Villegas, J., Sanchez, R., 2014. Addition of superoxide dismutase mimics during cooling process prevents oxidative stress and improves semen quality parameters in frozen/thawed ram spermatozoa. *Theriogenology* 82, 884–889.
- Sarlos, P., Molnar, A., Kokai, M., et al., 2002. Comparative evaluation of the effect of antioxidants in the conservation of ram semen. *Acta Vet. Hung.* 50, 235–245.
- Seifi-Jamadi, A., Kohram, H., Zareh-Shahne, A., Dehghanizadeh, P., Ahmad, E., 2016. Effect of various concentrations of butylated hydroxyanisole and butylated hydroxytoluene on freezing capacity of Turkman stallion sperm. *Anim. Reprod. Sci.* 170, 108–113.
- Shao, J., Li, X., Lu, X., Jiang, C., Hu, Y., Li, Q., You, Y., Fu, Z., 2009. Enhanced growth inhibition effect of resveratrol incorporated into biodegradable nanoparticles against glioma cells is mediated by the induction of intracellular reactive oxygen species levels. *Colloids Surf. B Biointerfaces* 72, 40–47.
- Silva, E.C., Cajueiro, J.F., Silva, S.V., Soares, P.C., Guerra, M.M., 2012. Effect of antioxidants resveratrol and quercetin on in vitro evaluation of frozen ram sperm. *Theriogenology* 77, 1722–1726.
- Silva, E., Barreiros, L., Segundo, M.A., Lima, S.A.C., Reis, S., 2017. Cellular interactions of a lipid-based nanocarrier model with human keratinocytes: unravelling transport mechanisms. *Acta Biomater.* 53, 439–449.
- Tchir, J., Acker, J.P., 2010. Mitochondria and membrane cryoinjury in micropatterned cells: effects of cell–cell interactions. *Cryobiology* 61, 100–107.
- Teskač, K., Kristl, J., 2010. The evidence for solid lipid nanoparticles mediated cell uptake of resveratrol. *Int. J. Pharm.* 390, 61–69.
- Türedi, S., Yuluğ, E., Alver, A., Kutlu, Ö., Kahraman, C., 2015. Effects of resveratrol on doxorubicin induced testicular damage in rats. *Exp. Toxicol. Pathol.* 67, 229–235.
- Uguralp, S., Mizrak, B., Karabulut, A.B., 2005. Resveratrol reduces ischemia reperfusion injury after experimental testicular torsion. *Eur. J. Pediatr. Surg.* 15, 114–119.
- Vitaglione, P., Sforza, S., Galaverna, G., Ghidini, C., Caporaso, N., Vescovi, P.P., Fogliano, V., Marchelli, R., 2005. Bioavailability of trans-resveratrol from red wine in humans. *Mol. Nutr. Food Res.* 49, 495–504.
- Walle, T., Hsieh, F., DeLegge, M.H., Oatis, J.E., Walle, U.K., 2004. High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metab. Dispos.* 32, 1377–1382.
- Yameen, B., Choi II, W., Vilos, C., Swami, A., Shi, J., Farokhzad, O.C., 2014. Insight into nanoparticle cellular uptake and intracellular targeting. *J. Control. Release* 190, 485–499.
- Zadeh Hashem, E., Eslami, M., 2016. Kinetin improves motility, viability and antioxidative parameters of ram semen during storage at refrigerator temperature. *Cell Tissue Bank.* 19, 97–111.
- Zhang, S., Gao, H., Bao, G., 2015. Physical principles of nanoparticle cellular endocytosis. *ACS Nano* 9, 8655–8671.