Improvement of post-thawed sperm quality in broiler breeder roosters by ellagic acid-loaded liposomes

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ABSTRACT Liposomes could improve the delivery of substances to sperm. This study was conducted to investigate the effect of the antioxidant ellagic acid and ellagic acid-loaded liposomes on post-thawed sperm quality in broiler breeder roosters. Semen was diluted in Beltsville extender containing ellagic acid or ellagic acid-loaded liposomes (ellagic acid at 0 (control), 0.5, 1, and 2 mM) and cryopreserved. Sperm quality was evaluated post-thawing: motility characteristics (Computer-Assisted Semen Analysis), membrane functionality (HOS test), abnormal morphology, mitochondrial activity (Rhodamine 123), apoptotic status (Annexin V/Propidium iodide), malondialdehyde, and antioxidant activities (glutathione peroxidase (**GPx**), superoxide dismutase (**SOD**), and total antioxidant capacity (**TAC**)). The results showed that 1 mM ellagic acid-loaded liposomes improved total motility, membrane functionality, and viability comparing to 0.5 and 2 mM ellagic acid, 2 mM ellagic acid-loaded liposomes, and control group. Mitochondrial activity was significantly higher for 1 mM ellagic acid-loaded liposomes compared to the rest of the treatments, except 1 mM ellagic acid. Ellagic acid at 1 mM in both forms significantly increased GPx and TAC after freeze-thawing (no significant variation for SOD), and also yielded the lower proportion of apoptotic and dead cells. In conclusion, ellagic acid improved post-thawed sperm quality in broiler breeder roosters. The use of liposomes could further enhance the effects of ellagic acid.

Key words: ellagic acid, liposome, rooster, sperm quality, post-thawed

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INTRODUCTION

Cryopreservation of rooster sperm ensures the availability of male gametes at the time of artificial propagation and facilitates genetic and breeding studies (Gliozzi et al., 2011). However, cryopreservation decreases the fertility of sperm samples, due to the physiological and chemical stress on the sperm, in part related to increased oxidative stress (Chatterjee et al., 2001).

The commonest approach is the incorporation of protective compounds into cryopreservation extenders (Ball, 2008; Pérez-Cerezales et al., 2009; Mata-Campuzano et al., 2015), including antioxidants to counteract the effects of free radicals (Najafi et al., 2014b; Sharafi et al., 2015). Our group previously showed that rooster sperm are highly susceptible to cryodamage, especially regarding sperm motility (Fattah et al., 2017).

Ellagic acid is a potent antioxidant found in many, nuts, and many other food sources. Ellagic acid exhibits antioxidant, radical scavenging, antiproliferative, and antiapoptotic properties (Turk et al., 2010; Rehman et al., 2012). This molecule contains four hydroxyl groups and two lactone groups. These hydroxyl groups enable the antioxidant activity, preventing lipid peroxidation, and protecting cells from oxidative damage (Pari and Sivasankari, 2008). It has protective effects against the anti-neoplastic drug cisplatin, by scavenging of free radicals and suppression of oxidative damage to DNA (Turk et al., 2008).

Poor absorption of ellagic acid influences in vivo antitumourigenic activity, preventing appropriate concentrations in plasma or target cells (Madrigal-Carballo et al., 2010). The low concentrations of free ellagic acid in

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plasma have been attributed to its low solubility in water (Lei et al., 2003), and may also be due to metabolic transformation and degradation prior to absorption. To overcome this restriction, a novel formulation of ellagic acid loaded into liposomes has been proposed, investigating the effect of this nanoparticle system on ellagic acid delivery at different physiological environments. Liposomes are spherical bilayer vesicles made by dispersion of certain polar lipids in aqueous solvents, which have attracted extensive attention in the biomedical, food, and agricultural industries in the last years because of their capability to act as targeted and/or sustained carrier systems. Liposomes can be used to deliver both water and oil-soluble functional compounds such as antimicrobials, flavors, antioxidants, and bioactive compounds to cells (Laye et al., 2008). Antioxidant liposomes have carried out an important role in the treatment of several diseases related to oxidative stress (Stone and Smith, 2004).

To our knowledge, there are no reports on the effects of ellagic acid and ellagic acid-loaded liposomes on the cryopreservation of rooster sperm. As a part of our interest in the synthesis of nanostructures and investigation of their application in diagnosis and treatment (Bagdeli et al., 2017; Taheri et al., 2017; Fasihi-Ramandi et al., 2018; Hajipour et al., 2018), the purpose of this study was to test the effects of different concentrations (0.5, 1, and 2 mM) of ellagic acid and ellagic acid-loaded liposomes on the post-thawed quality of rooster sperm. Our hypothesis is that there is an optimal concentration of ellagic acid that might improve rooster sperm post-thawing quality and that the liposome formulation could enhance its effects.

MATERIALS AND METHODS

Chemicals

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of Ellagic Acid-loaded Liposomes

Ellagic acid-loaded liposomes were prepared by the thin-layer hydration method with a slight modification (Mohammadi et al., 2014). A weighed amount of soybean lecithin (50 mg) and ellagic acid (0.5, 1, and 2 mM final concentration) were mixed, dissolved in 8 mL of ethanol, and transferred to a round-bottom flask. Ethanol was evaporated off at 40°C under vacuum (rotary evaporator, Heidolph, Germany). The dried lipid film was hydrated with 10 mL phosphate-buffered saline, shaken for producing an emulsion. This emulsion was dispersed for liposome formation. The liposomal size was decreased by submitting the emulsion to a homogenizer (20,000 rpm, Heidolph, Germany) for 20 min at 60°C, and to sonication (Vibra Cell-Sonics and Material, 130 W, 20 kHz, USA) for 10 min (70% power). These treatments yielded one-phase dispersion or nanoliposomes containing ellagic acid. The ellagic acid nanoliposome solution was stored at 4°C.

Animals, Semen Collection and Sperm Handling

This experiment was performed on 10 mature males (30 wk of age) of the Ross 308 breed, which were individually maintained in cages (70 × 95 × 85 cm) at 18°C to 20°C, under a 14 L:10 D artificially induced photoperiod. The roosters fed on commercial feed and water was provided ad libitum. Ejaculates were collected twice a week from each rooster using the dorso-abdominal massage method (Fattah et al., 2017), with a total of five ejaculates per rooster (10 ejaculates per session). The collection was always performed by the same technician and under the same conditions. Only samples with $\geq 300 \times 10^6$ sperm/mL, ≥90% normal morphology, and ≥80% motility were selected for this experiment. To eliminate individual differences and obtain sufficient sperm for analysis, we pooled the 10 ejaculates obtained in each session, which were split according to the number of treatments. Therefore, the experiment was replicated five times with pooled semen from the 10 roosters. Fresh pooled semen was diluted in Beltsville extender containing ellagic acid or ellagic acid-loaded liposomes, at 0.5, 1, and 2 mM. The remaining aliquot was extended with non-supplemented modified Beltsville extender, as a 0 mM control (final sperm concentration of 100×10^6 cells/mL). The composition of the modified Beltsville extender (Fattah et al., 2017) is: sodium glutamate (51.28 mM), fructose (27.75 mM), sodium acetate (3.9 mM), TES [n-tris (hydroxymethyl) methyl 1-2 aminoethane sulfonic acid] (13.95 mM), dipotassium phosphate (43.57 mM), monopotassium phosphate (5.14 mM), magnesium chloride (0.35 mM), potassium citrate (2.08 mM), with a pH of 7.1 and an osmolarity of 310 mOsm/kg.

Freezing/Thawing Sperm

Initially, semen was extended with half of the volume of Beltsville freezing extender (with one-fourth of the final glycerol concentration) and cooled for 2 h at 4°C. In the next step, the extended semen was further extended by adding the same volume of Beltsville, containing three-fourth of the final concentration of glycerol, and the completely extended semen was equilibrated for 1 h at 4°C. The treatment groups were loaded into 0.25-mL straws after equilibration, which were layered horizontally in racks and placed 4 cm above the surface of liquid nitrogen vapor for 7 min, after which they were directly plunged in liquid nitrogen. After storing for 1 wk, the frozen straws were thawed individually at 37°C for 30 s in a water bath and their contents analyzed.

Sperm Viability

Sperm viability was evaluated via eosin–nigrosin staining on smears (Campbell et al., 1956). A minimum of 200 sperm was assessed in each sample. Esosin-stained sperm were considered "dead," whereas unstained sperm (excluding eosin) were considered "alive."

Sperm Membrane Functionality

Sperm membrane functionality was assessed with the hypoosmotic (**HOS**) test, as described by Lotfi et al. (2017). Ten microliters of semen were mixed with 200 mL of a HOS solution (100 mOsm/kg; sodium citrate 1.9 mM, fructose 5.0 mM) and incubated at 37°C for 60 min. A drop of diluted semen was placed on a clean slide and covered with a coverslip. A total of 200 sperm were calculated in different fields at 40× under phase contrast microscope, and the percentage of sperm positive to HOS test (having coiled tails) was assessed.

Sperm Motility Analysis

Sperm motion variables were assessed using a Computer-Assisted Semen Analysis (**CASA**) system fitted with the Sperm Class Analyzer (**SCA**) software (Version 5.1; Microptic, Barcelona, Spain). CASA configuration consisted on: frame rate 53 Hz and an acquisition time of 1 s. Each sample was adjusted to 20 × 10^6 sperm/mL and loaded in a Leja chamber (depth: 10 μm), with a phase-contrast microscope (Nikon Eclipse, Japan) on a 37°C thermostatized stage, and images were captured using a video camera (Basler Vision Tecnologies A312fc, Ahrensburg, Germany), with a ×10 objective (negative phase contrast). Motility variables were total motility (**MOT**, %), progressive motility (**PROG**, %), curvilinear velocity (**VCL**, μm/s), straight-line velocity (**VSL**, μm/s), average path velocity (**VAP**, μm/s), amplitude of lateral head displacement (**ALH**, μm), linearity (**LIN**, %), straightness (**STR**, %), wobble (**WOB**, %), and beat cross frequency (**BCF**, Hz). For each evaluation, five microscopic fields were analyzed to include at least 200 cells.

Assessment of Sperm Abnormalities

For evaluation of total abnormalities, 5 μL of semen were pipetted into 1 mL Hancock's solution (Schäfer and Holzmann, 2000). Hancock's solution consisted of 426 mM sodium, 21.4 mM formalin, 304.29 mM Na_2HPO_4, and 99.42 mM K_2HPO_4. To record sperm abnormalities, 10 μL of processed sperm were placed on a slide, and the percentage of total sperm abnormalities (head abnormalities, detached heads, abnormal mid-pieces, and tail defects) was recorded by counting a total of 200 sperm under a phase-contrast microscope (×1000 magnification; oil immersion).

MDA Concentration

Amounts of MDA were measured using the thiobarbituric acid reaction, using the method described by (Najafi et al., 2014b). Briefly, the sperm was added to the thiobarbituric solution, mixed, and then double distilled water was added, and the mixture was shaken. Tubes were heated in boiling water, then cooled and centrifuged for 10 min at 1200 g. The absorbance of the supernatant was read at 532 nm wavelength. After the samples cooled to room temperature, they were analyzed using a UV/Visible spectrophotometer (T80 UV/VIS PJ Instruments Ltd, UK) at 532 nm.

TAC, GPx and SOD Determination

The antioxidant system was analyzed by determining the total antioxidant capacity (**TAC**) and the glutathione peroxidase (**GPx**) and superoxide dismutase (**SOD**) activities (Mehdipour et al., 2017). They were determined spectrophotometrically by using Randox kits (RANDOX Laboratories Ltd.) and an Olympus AU 400 automatic biochemistry analyzer (Olympus, Tokyo, Japan), converting absorbance to specific units with calibration curves. TAC was assessed by adding the reactive provided in the kit and measuring the absorbance at 600 nm, converting absorbance to mmol/L. 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. SOD determination is based on the degree 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 reduction of INT by 50% under the conditions of the assay. The absorbance was recorded at 505 nm.

Sperm Viability, Apoptotic Status and Mitochondrial Activity Determination by Flow Cytometry

Phosphatidylserine presence on sperm was detected by an Annexin-V/propidium iodide kit (Mehdipour et al., 2017). Briefly, the samples were re-suspended in calcium buffer to a concentration of 10 × 10^6 sperm/mL. Ten microliters of semen samples were mixed with Annexin V–FITC (A; 0.01 mg/mL stock) incubated for 15 min at room temperature (25°C) in the dark. After that we added 10 μL of propidium iodide (PI; 1 mg/mL stock) and incubated 10 more minutes. Sperm were classified into four groups: live cells (A−/PI−); live, early apoptotic (A+/PI−); and two categories of PI+ dead cells, the late apoptotic or

Table 1. Effect of ellagic acid and ellagic acid-loaded liposomes on motility parameters of rooster thawed semen, analyzed by CASA (n = 5).

Antioxidant	mM	MOT[1] (%)	PROG[2] (%)	VAP[3] (μm/s)	VSL[4] (μm/s)	VCL[5] (μm/s)	LIN[6] (%)	STR[7] (%)	ALH[8] (μm)	BCF[9] (Hz)
Control	0	54.1^{c,d}	18.5^{b}	28.8	17.9	51.9	31.0	55.9	5.1	16.1
Ellagic acid	0.5	61.5^{b,c}	23.3^{a,b}	31.1	19.1	56.3	31.5	57.0	5.2	15.6
	1	69.2^{a,b}	28.7^{a,b}	34.0	16.2	57.5	32.4	58.1	4.6	17.8
	2	50.4^{d}	19.0^{b}	29.3	17.5	56.5	28.7	56.6	5.6	15.9
Ellagic acid-loaded liposomes	0.5	63.6^{a-c}	25.0^{a,b}	31.4	20.5	55.0	32.2	55.4	5.0	18.3
	1	71.3^{a}	31.6^{a}	35.8	16.5	60.4	33.7	59.6	4.5	15.8
	2	56.4^{c,d}	20.1^{b}	32.5	16.0	59.1	29.1	51.1	4.9	15.8
SEM		2.2	2.4	1.6	1.4	2.0	2.5	4.4	0.4	2.0

[1]MOT: Total motility (MOT, %)
[2]PROG: Progressive motility
[3]VAP: Average path velocity
[4]VSL: Straight-line velocity
[5]VCL: Curvilinear velocity
[6]LIN: Linearity
[7]STR: Straightness
[8]ALH: Mean amplitude of the lateral head displacement
[9]BCF: Mean of the beat cross frequency. Different superscripts within the same column indicate significant differences among groups ($P < 0.05$).

early necrotic cells (A+/P+); and late necrotic cells (A−/PI+). Late apoptotic and necrotic cells were categorized together as dead cells.

Mitochondrial activity was assessed with rhodamine 123 and PI (Mehdipour et al., 2017). Five microliters of R123 solution (0.01 mg/mL stock) and PI solution (1 mg/mL stock) were added to 250 μL of diluted semen (50×10^6 sperm/mL) and incubated at 37°C for 15 min in the dark before flow cytometry. We recorded the proportion of sperm with high R123 fluorescence and no PI fluorescence (alive with active mitochondria).

Flow cytometry analyses were performed using a FACSCalibur (Becton Dickinson, San Jose, CA, USA), with an Argon ion 488 nm laser. Sperm cells were separated from debris by using a forward/side-scatter gate. Green fluorescence (Rhodamine-123 and Annexin-V) was detected with a band-pass filter (530/30 nm), and red fluorescence (propidium iodide) was detected using a long-pass filter (610 nm). Acquisitions were done using the CellQuest 3.3 software (Becton Dickinson). At least 10,000 events were acquired for each sample.

Statistical Analysis

Statistical analyses were performed using SAS software (version 9.1). PROC UNIVARIATE and the Shapiro–Wilk test assessed all data for normal distribution. Then, linear mixed-effect models (PROC MIXED) were used to test the effects of the treatments. Different pools were used as the random effect of the statistical model. Since interactions were non-significant, we also tested if the overall effect of using liposomes was significant. Tukey's test was used to compare treatments when the models were significant. The significance level was $P < 0.05$. Results are shown in mean ± standard error of the mean (**SEM**).

RESULTS

In general, 1 mM ellagic acid-loaded liposomes exerted a positive effect on post-thawing quality (overall, significant differences when compared to the control and the 0.5 and 2 mM treatments, ellagic acid alone or liposome-loaded). The 1 mM ellagic acid treatment showed slightly lower average values, but this effect was non-significant. The 0.5 mM concentration exerted an overall significantly positive effect comparing to the control, whereas 2 mM showed no significant differences with the control (except for the MDA levels of ellagic acid-loaded liposomes).

Table 1 shows CASA results. Total and progressive motility followed the above described pattern, with 1 mM having a significantly positive effect, whereas the effects on the kinematic parameters were non-significant. The effects of ellagic acid-loaded liposomes on sperm viability, abnormal morphology, membrane functionality, and mitochondrial activity are shown in Table 2. The distribution was very similar to that of total motility for viability, functional membranes, and active mitochondria sperm, with an inverse pattern for abnormal forms. Ellagic acid also improved the occurrence of apoptosis (Table 3), with 1 mM not only increasing viable sperm but also significantly decreasing the proportion of early apoptotic sperm.

The antioxidant status of the thawed samples was improved by ellagic acid, and more pronouncedly if delivered in liposomes (Table 4). All treatments reduced the mean MDA concentration respect to the control, although 0.5 and 2 mM results were only significantly lower in the liposome treatments. Ellagic acid at 1 mM showed the most pronounced effect for all parameters, except for SOD activity, which was not modified by any treatment.

Finally, sinceinteractions between ellagic acid concentrations and liposome/no liposome treatments were not significant, we carried out an overall effect

Table 2. Effect of ellagic acid and ellagic acid-loaded liposomes on viability (eosine/nigrosine), plasma membrane functionality, abnormal forms, and mitochondrial activity of rooster thawed semen (n = 5).

Antioxidant	mM	Viability (%)	Membrane functionality (%)	Abnormal forms (%)	Mitochondrial activity (%)
Control	0	60.9^{c}	49.9^{c}	16.5^{a}	47.2^{c}
Ellagic acid	0.5	64.3^{c}	57.7^{b,c}	14.0^{a,b}	55.6^{b,c}
	1	75.9^{a,b}	64.2^{a,b}	9.5^{b,c}	65.9^{a,b}
	2	60.7^{c}	49.2^{c}	15.0^{a}	49.7^{c}
Ellagic acid-loaded liposomes	0.5	65.9^{b,c}	61.0^{a,b}	13.4^{a,b}	54.5^{c}
	1	77.5^{a}	70.5^{a}	8.6^{c}	70.7^{a}
	2	62.8^{c}	55.3^{b,c}	14.4^{a}	53.0^{c}
SEM		2.5	2.3	1.0	2.3

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

Table 3. Effect of ellagic acid and ellagic acid-loaded liposomes on viable, apoptotic, and dead spermatozoa in rooster thawed semen, as assessed by flow cytometry (n = 5).

Antioxidant	mM	Live (%)	Early apoptosis (%)	Dead (%)
Control	0	39.3^{c}	26.2^{a}	34.5^{a}
Ellagic acid	0.5	49.8^{b,c}	25.1^{a}	25.1^{a,b}
	1	65.4^{a}	13.4^{b}	21.2^{a,b}
	2	41.2^{c}	24.5^{a}	34.3^{a}
Ellagic acid -loaded liposomes	0.5	53.9^{b}	18.6^{a,b}	27.5^{a,b}
	1	68.4^{a}	12.9^{b}	18.7^{b}
	2	43.2^{b,c}	22.3^{a}	34.5^{a}
SEM		2.4	1.9^{a,b}	3.1

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

Table 4. Effect of ellagic acid and ellagic acid-loaded liposomes on malondialdehyde concentration (MDA), glutathione peroxidase (GPx), and superoxide dismutase (SOD) activities and total antioxidant capacity (TAC) of rooster thawed semen (n = 5).

Antioxidant	mM	MDA (nmol/mL)	GPx (U/mg protein)	SOD (U/mg)	TAC (mmol/l)
Control	0	4.1^{a}	50.4^{b}	111.8	1.1^{c}
Ellagic acid	0.5	2.8^{a-c}	57.1^{a,b}	116.7	1.5^{b,c}
	1	1.4^{d,e}	64.83^{a}	133.0	2.2^{a}
	2	3.6^{a,b}	53.2^{b}	114.3	1.5^{b,c}
Ellagic acid-loaded liposomes	0.5	2.4^{c-e}	58.7^{a,b}	123.8	1.4^{a,b}
	1	1.3^{e}	66.7^{a}	137.5	2.4^{a}
	2	2.5^{b-d}	53.6^{b}	116.3	1.5^{b,c}
SEM		0.3	2.4	8.1	0.1

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

analysis on the effect of the use of liposomes. This analysis showed a significant effect of using liposomes (irrespective of ellagic acid concentration), with higher membrane integrity ($P = 0.005$) and lower occurrence of early apoptosis ($P = 0.048$) and MDA concentration ($P = 0.026$).

DISCUSSION

Our results show that supplementing the extender with ellagic acid at 1 mM, especially loaded in liposomes, could improve the post-thawing quality of rooster sperm. Several studies have shown that extenders supplemented with antioxidants are beneficial for inhibition of reactive oxygen species (ROS) generation in avian sperm (Partyka et al., 2013; Fattah et al., 2017; Lotfi et al., 2017). Nevertheless, there are no studies describing the effect of antioxidant encapsulation in bird semen extenders and their effect on the cryopreservation of rooster sperm. Our results show that liposomes can be used to enhance the effects of ellagic acid. Even though the two formulations of 1 mM ellagic acid did not differ significantly, the ellagic acid-loaded liposomes consistently yielded higher quality, and the overall comparison of both formulations showed an advantage for the liposomes formulation for membrane integrity, early apoptosis and MDA concentration.

To our knowledge, there is no report in the literature indicating the effects of ellagic acid in cryopreservation of rooster sperm or other poultry species, and there is only a previous study on the effects of ellagic acid on sperm cryopreservation in sheep. Omur and Coyan (2016) reported that freezing ram semen with an extender supplemented with 1 or 2 mM ellagic acid allowed to higher post-thawing sperm motility, membrane, and acrosomal integrity when compared to an untreated control. However, there are some differences between that study and ours, possibly due to the different animal groups considered and highlighting the need for confirming findings from different species, especially when using a cell type such as the sperm, subject to strong evolutionary forces. Thus, the optimal ellagic acid concentration for freezing rooster sperm seems to be around 1 mM, with 2 mM no offering advantage comparing to the control. It is not unusual that high antioxidant doses show a decreasing benefit (Anel-Lopez et al., 2012; Najafi et al., 2014a), and it is possible that 2 mM ellagic acid exerts negative effects that counteract the positive ones showed at 1 mM. Indeed, Omur and Coyan (2016) obtained worse results when using 4 mM ellagic acid. Toxic effects of ellagic acid at higher concentrations cannot be discarded, as showed in previous studies (Mata-Campuzano et al., 2012, 2015).

Another difference with that study on ram sperm is that we have reported an increase on the proportion of sperm with high mitochondrial activity in 1 mM ellagic acid. Mitochondria are not only the powerhouse of the cell, but it is also a key on apoptosis regulation (Castedo et al., 2002). In fact, not only viability, membrane functionality and motility accompanied this improvement on mitochondrial status, but apoptosis was also prevented when using 1 mM ellagic acid for freezing rooster sperm.

These positive effects could be due to the antioxidant properties of ellagic acid. Çeribaşı et al. (2012) indicates that this polyphenol not only exhibits antioxidative and antiapoptotic properties, but it also can chelate metal ions and therefore prevent iron- and copper-catalyzed ROS generation. These authors and others reported a protective effect when treating rats with chemotoxic compounds (Çeribaşı et al., 2012; Türk et al., 2008), and Atessahin et al. (2007) observed that ellagic acid protected cisplatin-induced testicular and sperm toxicity by decreasing lipid peroxidation and increasing scavenging enzymes.

Cryopreservation generates ROS and reduces antioxidants level. ROS eventually lead to loss of membrane integrity, reduced sperm motility, leakage of intracellular enzymes, and damage of the sperm DNA, through the oxidative stress and the production of cytotoxic aldehydes (Baspinar et al., 2011), ultimately resulting in lower fertility. We have obtained a higher TAC in the 1 mM treatments, which was accompanied by a higher GPx activity. The first defense lines against the detrimental effects of ROS produced in rooster semen are antioxidant enzymes such as SOD and GPx (Partyka et al., 2013; Rad et al., 2016). SOD scavenges superoxide radicals and generates hydrogen peroxide, which is in turn processed by GPx (Cerolini et al., 2001). SOD was not affected, similarly to previous studies (Mehdipour et al., 2016), but the improving effect in GPx could be responsible of the prevention of membrane peroxidation (lower MDA, especially in 1 mM). Indeed, GPx are key enzymes in sperm membrane protection, allowing an improved redox balance, evidenced by the higher TAC (Godeas et al., 1997). Moreover, adding 1 mM ellagic acid to the extender reduced sperm total abnormalities, consistent with findings in previous studies in ram (Daghigh Kia et al., 2017). This is an interesting fact to highlight, since abnormal sperm could be a source of ROS (Seifi-Jamadi et al., 2016), and therefore reducing their occurrence could help ameliorating the oxidative stress associated to freezing–thawing.

The overall positive effect of liposomes as a delivery vehicle for ellagic acid (not explored before) could be due to an enhancement on the intracellular levels of this antioxidant, as described in other cell models (Stone and Smith, 2004). Ellagic acid could then better maintain mitochondrial activity, preventing apoptosis and ROS production, and therefore cell viability. However, we cannot discard a protective effect of the liposomes by themselves. Thus, our group previously established a positive effect of nanoliposomes on TAC and sperm motility and viability in ram sperm (Mehdipour et al., 2017).

In conclusion, ellagic acid at 1 mM seems to be an effective supplement for the cryopreservation of rooster semen, resulting on a higher motility and general viability than the extender alone. The use of liposomes for delivering ellagic acid resulted in enhanced results, which should be confirmed in future studies. The practical use of these new formulations should be tested in fertility trials, to evaluate their cost-efficiency balance compared to standard protocols.

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REFERENCES

Anel-Lopez, L., M. Alvarez-Rodriguez, O. Garcia-Alvarez, M. Alvarez, A. Maroto-Morales, L. Anel, P. de Paz, J. J. Garde, and F Martinez-Pastor. 2012. Reduced glutathione and Trolox (vitamin E) as extender supplements in cryopreservation of red deer epididymal spermatozoa. Anim. Reprod. Sci. 135:37–46.

Atessahin, A., A. O. Ceribasi, A. Yuce, O. Bulmus, and G. Cikim. 2007. Role of ellagic acid against cisplatin-induced nephrotoxicity and oxidative stress in rats. Basic Clin. Pharmacol. Toxicol. 100:121–126.

Bagdeli, S., A. H. Rezayan, R. A. Taheri, M. Kamali, and M. Hosseini. 2017. FRET- based immunoassay using CdTe and AuNPs

for the detection of OmpW antigen of *Vibrio cholerae*. J. Lumin. 192:932–939.
Ball, B. A. 2008. Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. Anim. Reprod. Sci. 107:257–267.
Baspinar, N., K. Coyan, M. N. Bucak, and P. B. Tuncer. 2011. Effects of dithioerythritol on ram semen after the freeze–thawing process. Cryobiology 63:152–156.
Campbell, R. C., H. M. Dott, and T. D. Glover. 1956. Nigrosin eosin as a stain for differentiating live and dead spermatozoa. J. Agric. Sci. 48:1–8.
Castedo, M., K. Ferri, T. Roumier, D. Métivier, N. Zamzami, and G. Kroemer. 2002. Quantitation of mitochondrial alterations associated with apoptosis. J. Immunol. Methods 265:39–47.
Çeribaşı, A. O., F. Sakin, G. Türk, M. Sönmez, and A. Ateşşahin. 2012. Impact of ellagic acid on adriamycin-induced testicular histopathological lesions, apoptosis, lipid peroxidation and sperm damages. Exp. Toxicol. Pathol. 64:717–724.
Cerolini, S., A. Maldjian, F. Pizzi, and T. M. Gliozzi. 2001. Changes in sperm quality and lipid composition during cryopreservation of boar semen. Reproduction 121:395–401.
Chatterjee, S., E. de Lamirande, and C. Gagnon. 2001. Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: protection by oxidized glutathione. Mol. Reprod. Dev. 60:498–506.
Daghigh Kia, H, Z. Blooki, H. Vaseghi Dodran, and M. Mahdipour. 2017. Effect of adding coenzyme Q10 and Ellagic acid during cryopreservation on post-thaw quality of ram semen. Iran J. Appl. Anim. Sci. 7:445–451.
Fasihi-Ramandi, M., H. Ghobadi-Ghadikolaee, S. Ahmadi-Renani, R. A. Taheri, and K. Ahmadi. 2018. *Vibrio cholerae* lipopolysaccharide loaded chitosan nanoparticle could save life by induction of specific immunoglobulin isotype. Artif. Cells Nanomed. Biotechnol. 46:56–61.
Fattah, A., M. Sharafi, R. Masoudi, A. Shahverdi, V. Esmaeili, and A. Najafi. 2017. l-Carnitine in rooster semen cryopreservation: flow cytometric, biochemical and motion findings for frozen-thawed sperm. Cryobiology 74:148–153.
Gliozzi, T. M., L. Zaniboni, and S. Cerolini. 2011. DNA fragmentation in chicken spermatozoa during cryopreservation. Theriogenology 75:1613–1622.
Godeas, C., F. Tramer, F. Micali, M. Soranzo, G. Sandri, and E. Panfili. 1997. Distribution and possible novel role of phospholipid hydroperoxide glutathione peroxidase in rat epididymal spermatozoa1. Biol. Reprod. 57:1502–1508.
Hajipour, H., H. Hamishehkar, S. Nazari Soltan Ahmad, S. Barghi, N. F. Maroufi, and R. A. Taheri. 2018. Improved anticancer effects of epigallocatechin gallate using RGD-containing nanostructured lipid carriers. Artif. Cells Nanomed. Biotechnol.1–10, doi: 10.1080/21691401.2017.1423493.
Laye, C., D. J. McClements, and J. Weiss. 2008. Formation of biopolymer-coated liposomes by electrostatic deposition of chitosan. J. Food Sci. 73:N7–N15.
Lei, F., D. M. Xing, L. Xiang, Y. N. Zhao, W. Wang, L. J. Zhang, and L. J. Du. 2003. Pharmacokinetic study of ellagic acid in rat after oral administration of pomegranate leaf extract. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 796:189–194.
Lotfi, S., M. Mehri, M. Sharafi, and R. Masoudi. 2017. Hyaluronic acid improves frozen-thawed sperm quality and fertility potential in rooster. Anim. Reprod. Sci. 184:204–210.
Madrigal-Carballo, S., S. Lim, G. Rodriguez, A. O. Vila, C. G. Krueger, S. Gunasekaran, and J. D. Reed. 2010. Biopolymer coating of soybean lecithin liposomes via layer-by-layer self-assembly as novel delivery system for ellagic acid. J. Funct. Foods 2:99–106.
Mata-Campuzano, M., M. Alvarez-Rodriguez, M. Alvarez, L. Anel, P. de Paz, J. J. Garde, and F Martinez-Pastor. 2012. Effect of several antioxidants on thawed ram spermatozoa submitted to 37°C up to four hours. Reprod. Domest. Anim. 47:907–914.
Mata-Campuzano, M., M. Alvarez-Rodriguez, M. Alvarez, J. Tamayo-Canul, L. Anel, P. de Paz, and F Martinez-Pastor. 2015. Post-thawing quality and incubation resilience of cryopreserved ram spermatozoa are affected by antioxidant supplementation and choice of extender. Theriogenology 83:520–528
Mehdipour, M., H. Daghigh Kia, A. Najafi, H. V Dodaran, and O. Garcia-Alvarez. 2016. Effect of green tea (*Camellia sinensis*) extract and pre-freezing equilibration time on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender. Cryobiology 73:297–303..
Mehdipour, M., H. Daghigh Kia, M Nazari, and A. Najafi. 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.
Mohammadi, M., B. Ghanbarzadeh, and H. Hamishehkar. 2014. Formulation of nanoliposomal vitamin D3 for potential application in beverage fortification. Adv. Pharm. Bull. 4:569–75.
Najafi, A., H. Daghigh Kia, H Mohammadi, M. H. Najafi, Z. Zanganeh, M. Sharafi, F. Martinez-Pastor, and H. Adeldust. 2014. Different concentrations of cysteamine and ergothioneine improve microscopic and oxidative parameters in ram semen frozen with a soybean lecithin extender. Cryobiology 69:68–73.
Najafi, A., M. H. Najafi, Z. Zanganeh, M. Sharafi, F. Martinez-Pastor, and H. Adeldust. 2014. Cryopreservation of ram semen in extenders containing soybean lecithin as cryoprotectant and hyaluronic acid as antioxidant. Reprod. Dom. Anim. 49:934–940.
Omur, A. D., and K. Coyan. 2016. Protective effects of the antioxidants curcumin, ellagic acid and methionine on motility, mitochondrial transmembrane potential, plasma membrane and acrosome integrity in freeze-thawed Merino ram sperm. Vet. Med. (Praha). 61:10–16.
Pari, L., and R. Sivasankari. 2008. Effect of ellagic acid on cyclosporine A-induced oxidative damage in the liver of rats. Fundam. Clin. Pharmacol. 22:395–401.
Partyka, A., W. Nizanski, J. Bajzert, E. Lukaszewicz, and M. Ochota. 2013. The effect of cysteine and superoxide dismutase on the quality of post-thawed chicken sperm. Cryobiology 67:132–136.
Pérez-Cerezales, S., S. Martínez-Páramo, E. Cabrita, F. Martínez-Pastor, P. De Paz, and M. P. Herráez. 2009. Evaluation of oxidative DNA damage promoted by storage in sperm from sex-reversed rainbow trout. Theriogenology 71:605–613.
Rad, H. M., M. Eslami, and A. Ghanie. 2016. Palmitoleate enhances quality of rooster semen during chilled storage. Anim. Reprod. Sci. 165:38–45.
Rehman, M. U., M. Tahir, F. Ali, W. Qamar, A. Lateef, R. Khan, A. Quaiyoom, and S. Sultana. 2012. Cyclophosphamide-induced nephrotoxicity, genotoxicity, and damage in kidney genomic DNA of Swiss albino mice: the protective effect of ellagic acid. Mol. Cell Biochem. 365:119–127.
Schäfer, S., and A. Holzmann. 2000. The use of transmigration and Spermac^{TM} stain to evaluate epididymal cat spermatozoa. Anim. Reprod. Sci. 59:201–211.
Seifi-Jamadi, A., H. Kohram, A. Zareh-Shahne, P. Dehghanizadeh, and E. Ahmad. 2016. Effect of various concentrations of butylated hydroxyanisole and butylated hydroxytoluene on freezing capacity of Turkman stallion sperm. Anim. Reprod. Sci. 170:108–113.
Sharafi, M., M. Zhandi, and A. Akbari Sharif. 2015. Supplementation of soybean lecithin-based semen extender by antioxidants: complementary flowcytometric study on post-thawed ram spermatozoa. Cell Tissue Bank 16:261–269.
Stone, W. L., and M. Smith. 2004. Therapeutic uses of antioxidant liposomes. Mol. Biotechnol. 27:217–230.
Taheri, R. A., A. H. Rezayan, F. Rahimi, J. Mohammadnejad, and M. Kamali. 2017. Evaluating the potential of an antibody against recombinant OmpW antigen in detection of Vibrio cholerae by surface plasmon resonance (SPR) biosensor. Plasmonics 12:1493–1504.
Turk, G., A. Atessahin, M. Sonmez, A. O. Ceribasi, and A. Yuce. 2008. Improvement of cisplatin-induced injuries to sperm quality, the oxidant-antioxidant system, and the histologic structure of the rat testis by ellagic acid. Fertil. Steril. 89:1474–1481.
Turk, G., M. Sonmez, A. O. Ceribasi, A. Yuce, and A. Atessahin. 2010. Attenuation of cyclosporine A-induced testicular and spermatozoal damages associated with oxidative stress by ellagic acid. Int. Immunopharmacol. 10:177–182.