Lycopene-loaded nanoliposomes improve the performance of a modified Beltsville extender for the cryopreservation of semen from broiler breeder roosters

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ABSTACT

Antioxidants might ameliorate the effects of the freeze-thawing stress on cryopreserved spermatozoa. Lycopene is an effective antioxidant yet to be tested for rooster sperm cryopreservation. Moreover, nanoliposomes, a technology recently applied to sperm cryopreservation, might improve conservation and antioxidant delivery to spermatozoa. Thus, we evaluated the effect of 0.1, 0.2 and 0.3 mM lycopene and lycopene-loaded nanoliposomes (LnL) on the cryopreservation of rooster sperm in Beltsville extender. Post-thawing evaluation comprised: Sperm motility, membrane integrity, abnormal morphology, mitochondria activity, apoptotic status, malondialdehyde (MDA) and antioxidant activities: Gluthation peroxidase (GPx), superoxide dismutase (SOD) and total antioxidant capacity (TAC). Total and progressive motility, membrane integrity and mitochondria activity were higher with 0.2 mM lycopene and lycopene-loaded liposomes ($P < 0.05$), comparing to 0.1 and 0.3 mM (lycopene and LnL) and control group. A lower percentage of apoptotic sperm and increased GPx activity and TAC were observed in the 0.2 mM lycopene and LnL compared with the other treatments. The 0.2 mM concenctrations also showed reduced MDA levels in compared to 0.3 mM lycopene and LnL and control. Supplementation did not significantly affect activities of SOD nor the proportion of abnormal spermatozoa. A fertility trial showed that 0.2 mM lycopene increased the proportion of fertile and hatched eggs respect to the control, especially when using nanoliposomes. Therefore, the supplementation of Beltsville extender with 0.2 mM lycopene could improve the quality of rooster spermatozoa after freeze-thawing, whereas the combination with nanoliposomes seem promising to improve the performance of existing approaches.

Keywords: Nanoliposomes; lycopene; rooster sperm; apoptosis; cryopreservation

1. Introduction

Semen cryopreservation has a key role in spreading artificial insemination (AI), since, it allows long-term preservation of sperm fertility. However, the fertility of cryopreserved poultry semen is very variable and it is unreliable for commercial production or genetics preservation (Blesbois and Brillard, 2007). Sperm cryopreservation can result in the generation of reactive oxygen species (ROS) (Swami et al., 2017), which are an important factor in sperm damage during freezing and thawing (Zheng et al., 2017). Because of aerobic metabolism and lipid peroxidation (LPO), ROS are produced continuously during sperm cryopreservation, causing sperm death and sublethal damages, and thus affecting the usability of the thawed spermatozoa (Chatterjee et al., 2001). Although semen have antioxidant systems, such as catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx) and non-enzymatic antioxidants (Mehdipour et al., 2017), their activity can be affected by cryopreservation, resulting in a decreased response to ROS production. Thus, the supplementation of extenders with antioxidants has been proposed as a means of reducing the damage of the freezing-thawing process.

Lycopene is a lipophilic carotenoid with both antioxidant and pro-oxidant activities (Young and Lowe, 2001). It is the main carotenoid in tomatoes, also present in guava, watermelon, pink grapefruit and papaya (Stahl and Sies, 1996). Several studies have demonstrated that sperm motility, membrane integrity (Uysal and Bucak, 2007; Bucak et al., 2015) and DNA damage (Zini et al., 2010) can be improved by lycopene. Akain et al. (2016), reported that adding lycopene at 0.5 and 2 mM improved sperm quality while reducing oxidative stress parameters in cooled-stored ram semen. Lycopene has also showed promising results for the conservation of fowl spermatozoa (Mangiagalli et al., 2007; Rosato et al., 2012; Lotfi et al., 2017)

Additionally, liposomes have been used to improve the efficiency of some lipophilic and hydrophilic antioxidants (Minko et al., 2002). Encapsulation technologies are an effective approach for lengthening the life of bioactive ingredients, because the encapsulation provides a physical barrier against adverse environmental conditions during processing and storage (Tan et al., 2014). Moreover, liposomes could enhance water solubility, thermal and pH stability of antioxidants (Stone and Smith, 2004). Enzymatic (SOD and catalase) and non-enzymatic antioxidants (N-acetylcysteine, resveratrol, CoQ-10, curcumin, α-tocopherol, glutathione and γ-tocopherol) show enhanced therapeutic potential against oxidative-induced tissue damages once encapsulated in liposomes, because of expedited intracellular delivery and delayed release of the entrapped agents to the cell (Suntres, 2011).

As a part of our interest in the synthesis of nanostructures and investigation of their application in diagnosis and treatment (Taheri et al., 2016a; b; Bagdeli et al., 2017; Fasihi-Ramandi et al., 2017; Khansary et al., 2017; Taheri et al., 2017), in this work we have focused on the effects of lycopene, free and loaded in nanoliposomes, on the post-thawing quality of rooster semen. We evaluated not only conventional sperm parameters such as motility and viability, but also the presence of apoptotic markers and mitochondrial activity (markers of sublethal damage) and direct markers of the oxidative status, such as malondialdehyde (MDA) levels and antioxidants GPx, SOD and TAC (total antioxidant capacity). Since lycopene shows pro-oxidant effects in some circumstances, we tested a range of concentrations in order to find out which one could be most appropriate, and if the delivery by nanoliposomes affected its response. Moreover, this is the first attempt to apply nanoliposomes, a relatively novel technology in the field of spermatology, to the cryopreservation of bird semen.

2. Materials and methods

2.1. Chemicals

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

2.2. Animals and semen collection

The experiment was carried out using 10 mature (2-year-old) broiler roosters (Ross 308) kept individually in cages (70 × 95 × 85 cm) at 18– 20 °C, under a 14 L:10 D photoperiod. Feeding was a commercial formula for breeding fowl, and water was provided ad libitum. All procedures were performed with the agreement of the Baqiyatallah University of Medical Sciences, Tehran, Iran.

2.3. Preparation of lycopene liposomes

Lycopene-loaded liposomes were prepared by thin-layer hydration. A weighed amount of soybean lecithin (50 mg) and lycopene (0.1, 0.2, and 0.3 mM final concentration) were mixed, then 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 PBS, shaking 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 minutes at 60 °C, and to sonication (Vibra Cell-Sonics & Material, 130 W, 20 kHz, USA) for 10 minutes (70% power). These treatments

yielded a one-phase dispersion or nanoliposomes containing lycopene. The lycopene-loaded nanoliposome (LnL) solution was stored at 4 °C.

2.4. Semen collection and processing

Semen was collected twice a week by the massage method (Fattah et al., 2017). Only samples with ≥300 × 10^6 spermatozoa/mL, ≥90% normal morphology, and ≥80% motility, were selected for this experiment.

The components of modified Beltsville extender were sodium glutamate (51.28 mM), fructose (27.75 mM), sodium acetate (3.9 mM), TES [n-tris (hydroxymethyl) methyl 1-2 amino ethane 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 pH of 7.1 and osmolarity of 310 mOsm/kg. Glycerol was added to this basic medium at 8% (v/v).

Each pooled ejaculate was distributed into seven equal aliquots, each one being extended (final concentration of 100×10^6 sperm/ml) in Beltsville extender containing lycopene or lycopene-loaded liposomes, at 0.1, 0.2 and 0.3 mM. The remaining aliquot was extended with non-supplemented Beltsville, as a control. The extended semen was cooled gradually down to 4 °C for 3 h. The samples were loaded into 0.25 mL straws (IMV, L'Aigle, France), and sealed with polyethylene glycol. Next, the straws were cryopreservation in liquid nitrogen (LN) vapors (4 cm above the LN for 7 min) and then plunged into LN for storage. After cryopreservation (one week), the frozen straws were thawed separately at 37 °C for 30 s in a water bath before assessment.

2.5. Sperm motility

Assessment of sperm motility was conducted using the Sperm Class Analyzer (SCA) software (Version 5.1; Microptic, Barcelona, Spain). At least 200 sperm were tested for each sample using standard settings (37 °C, 60 frames/s). The proportions of total motile spermatozoa (MOT, %), and progressive spermatozoa (PROG, %), were determined. The motility parameters measured for each sperm included the curvilinear velocity (VCL, μm/s), the straight-line velocity (VSL, μm/s), the average path velocity (VAP, μm/s), the linearity (LIN, %; VSL/VCL), the straightness (STR, %; VSL/VAP), the beat cross frequency (BCF, Hz) and the amplitude of lateral head displacement (ALH, μm).

2.6. Assessment of sperm abnormalities

For the evaluation of sperm morphology, 15 μl were added into tubes containing 1 ml of Hancock solution (426 mM sodium, 21.4 mM formalin, 304.29 mM Na_2HPO_4 and 99.42 mM K_2HPO_4) (Bucak et al., 2007). A minimum of 200 spermatozoa were examined for morphologic abnormalities under a phase contrast microscope (×1000 magnification, oil immersion).

2.7. Plasma membrane functionality

The plasma membrane functionality was assessed by the hypo-osmotic swelling test (HOS test) as described for chicken sperm (Shanmugam et al., 2014). Ten μl of thawed semen and 300 μl of HOS solution (100 mOsm/kg; sodium citrate 1.9 mM, fructose 5.0 mM) were mixed and incubated for 60 min at 37 °C. The samples were evaluated under a phase-contrast microscope at ×400, examining at least 200 spermatozoa. The percentage of sperm having coiled tails was considered as HOS+.

154 *2.8. Sperm viability*

155 Sperm viability was assessed by staining with nigrosin–eosin (Mehdipour et al., 2016).
156 Sperm smears were prepared by mixing 10 µl of semen with 20 µl of stain on a warm slide and
157 immediately smearing with a second slide. Viability was evaluated by examining 200 cells under
158 a phase-contrast microscope (×400). Spermatozoa displaying partial or whole purple staining were
159 considered non-viable and only sperm showing strict exclusion of the stain were considered to be
160 viable.

161

162 *2.9. MDA concentration*

163 MDA concentration, as an indicator of the lipid peroxidation in the semen samples, was
164 measured using the thiobarbituric acid (TBA) reaction (Esterbauer and Cheeseman, 1990). Semen
165 samples from each treatment (250 µL, containing thus 25×10^6 spermatozoa) were centrifuged at
166 1200×g for 5 min. The supernatant was discarded and 1 mL of trichloroacetic acid was added to
167 the pellet. The mixture was centrifuged at 12,000×g for 10 min and then the supernatant was
168 discarded and 1 mL thiobarbituric acid (0.375%) was added. The tubes were incubated in boiling
169 water for 10 min. After the samples cooled to room temperature, they were analyzed using a
170 UV/Visible spectrophotometer (T80 UV/VIS PJ Instruments Ltd, UK) at 532 nm.

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172 *2.10. TAC, GPx and SOD determination*

173 The antioxidant system was analyzed by determining the TAC, and GPx and SOD activities
174 (Mehdipour et al., 2017). They were determined spectrophotometrically by using Randox kits
175 (RANDOX Laboratories Ltd.) and an Olympus AU 400 automatic biochemistry analyzer
176 (Olympus, Tokyo, Japan), converting absorbance to specific units with a calibration curve. 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 is reduced by GPx with a concomitant oxidation of NADPH to NADP+, determining the decrease in absorbance at 340 nm. SOD determination is based in 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.

2.11. Sperm viability, apoptotic status and mitochondrial activity determination by flow cytometry

For determination of sperm apoptosis (Mehdipour et al., 2017), the thawed semen was washed in calcium buffer and readjusted to 100 µl at 1×10^6 sperm/mL, followed by the addition of 10 µL Annexin V–FITC (A; 0.01 mg/mL stock). Samples were incubated at room temperature for 20 min. After that, we added 10 µL of propidium iodide (PI; 1 mg/mL stock) and incubated 10 more minutes. In the flow cytometry analysis, spermatozoa were classified into four groups: viable non-apoptotic cells (A-/PI-); early apoptotic (A+/PI-); late apoptotic (A+/PI+); and necrotic (A-/PI+). Late apoptotic and necrotic cells were categorized together as dead cells.

Mitochondrial activity was assessed with rhodamine 123 and PI. Five µL 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 spermatozoa/ml) and incubated at 37 °C for 15 min in the dark before flow cytometry. Spermatozoa were classified into three groups: viable low-mitochondrial-potential (R123-/PI-); viable high-mitochondrial-potential (R123+/PI-); dead spermatozoa (PI+). We used the proportion of R123+/PI- in this study (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 10000 events were acquired for each sample.

2.12. Artificial insemination

Artificial insemination was accomplished as described by Lotfi et al. (2017) with minor modifications. To assess fertility, 70 Ross breeder hens were divided into 7 groups of 10 hens. Hens were housed in separate cages (70 × 70 × 85 cm) and inseminated with thawed semen from the 7 treatments. AI was performed immediately after thawing on certain days (twice a week for approximately 2 weeks) with insemination of 100×10^6 sperm/straw obtained from each treatment. The eggs were collected up to 5 days after the last artificial insemination. For each group, 45 eggs in 2-weeks were randomly selected for incubation. After egg collection, they were set on turning trays and disinfected for 15 minutes. Afterwards, eggs were set in a common incubator for 18 days at 37.7 °C. After incubation, the eggs were transferred to the hatcher for the remaining 3 days of incubation. Eggs were assessed for fertilization on day 7 of incubation by candling. Hatched eggs were counted after 21 days of incubation, and the hatching ratio was calculated based on the number of fertilized eggs.

2.13. Statistical analysis

The data were analyzed with SAS software (version 9.1). The Shapiro-Wilk test was used to check the normality of the data. Then, linear mixed-effect models (PROC MIXED; SAS, 2002) were

223 used to test the effects of the treatments. Tukey's test was used to compare treatments when the
224 models were significant. Fertility data were analyzed
225 GENMOD, chi-square test). The significance level was $P < 0.05$. Results are shown as mean±SEM.

226

227 **3. Results**

228 In general, the 0.2 mM concentration, both in lycopene and in LnL, improved sperm quality
229 after freezing-thawing. The 0.1 mM concentration yielded mixed results, and the 0.3 mM
230 concentration was similar to the control, with a lower quality overall.

231 Table 1 shows the results of the CASA analysis. The freezing extender supplemented with
232 0.2 mM LnL led to higher total and progressive motility, followed by the same concentration of
233 lycopene ($P>0.05$), being both significantly higher than the control and 0.3 mM. Kinematic
234 parameters were not significantly affected by lycopene or LnL supplementation, although 0.2 mM
235 yielded higher means except for ALH.

236 The sperm viability, membrane functionality and mitochondrial status were also higher
237 with 0.2 mM lycopene and LnL treatments, whereas no differences were noted for abnormal forms
238 (Table 2). Viability assessed by eosin/nigrosin was significantly higher for 0.2 mM vs. the rest of
239 the treatments, irrespective of the type of supplementation. For membrane functionality and active
240 mitochondria, LnL 0.2 mM was significantly higher than any other concentration or control,
241 whereas lycopene 0.2 mM yielded a slightly lower mean value, not significantly different than
242 LnL 0.1 mM. For mitochondrial activity, lycopene 0.2 mM was also not significantly different
243 than lycopene 0.1 mM.

244 The influence of the antioxidants on MDA levels and GSH-Px and SOD activities and TAC
245 in thawed rooster sperm are showed in Table 3. Lycopene and LnL at 0.2 mM lowered MDA levels

and increased GPx activity and TAC ($P < 0.05$ comparing to Control and 0.3 mM concentrations). SOD activity was not affected by any supplementation.

Results were similar in the sperm populations obtained in the apoptosis assay by flow cytometry (Table 4). A higher percentage of the live subpopulation (and a corresponding lower proportion of the dead one), was observed in 0.2 mM ($P<0.05$ comparing to the Control and 0.3 mM). Apoptotic spermatozoa were significantly reduced in the 0.2 mM concentrations compared with the other treatments.

The results of the fertility trial (Table 5) showed a positive effect of lycopene 0.2 mM, especially when delivered in nanoliposomes. In the control, 38% of eggs were fertilized and 16% hatched (hatchability of 41%), and these numbers were higher with any lycopene supplementation and even higher in the nanoliposome group. Indeed, the 0.2 mM lycopene in nanoliposomes group was significantly higher than any other group (62% fertilized and 47% hatched eggs, with 75% hatchability).

4. Discussion

The main factors reducing the motility and viability of cryopreserved sperm are the alterations in membrane integrity owing to damages induced by cold shock, ice formation, osmotic stress, cryoprotective agents (Maxwell and Watson, 1996) and reactive oxygen species, that end in lipoperoxidation (Anel-Lopez et al., 2012). The results of this study showed that supplementing the freezing extender with lycopene, and especially with lycopene-loaded liposomes, significantly improved sperm quality, possibly due to the improvement of the redox balance and membrane protection. Other studies in mammals, such as ram (Akain et al., 2016), and bull (Bucak et al., 2015), also found that lycopene improved semen cryopreservation, possibly through similar

processes. Lycopene displays an antioxidative function in cells by quenching and neutralizing free radicals before they can damage cells (Di Mascio et al., 1989).

Lycopene did also show positive effects on fowl spermatozoa. Mangiagalli et al. (2007) found that the supplementation with lycopene in rooster semen during chilled storage improved sperm viability, but it did not improve motility or progressive motility. However, in that study lycopene was added as part of a mixture in a vitamin complex, and these authors did not cryopreserved the semen, making it difficult to compare with our results. In another study, Mangiagalli et al. (2010) reported that feed supplemented with lycopene also improved the viability of poultry spermatozoa. Rosato et al. (2012) showed some positive effects of lycopene on chilled turkey spermatozoa, but they did not obtain significant effects after cryopreservation. These results highlight the influence of different experimental conditions and the requirement for testing these treatments even in related species.

A question remains, how lycopene, which is insoluble in water, could be stable and benefit spermatozoa. We hypothesise that lycopene could be stabilized in solution by the lecithin, likely the same mechanism acting in other studies testing lipophilic antioxidants for sperm cryopreservation (de Vasconcelos Franco et al., 2016). This could also explain, at least in part, the enhanced effects of lycopene when combined with the nanoliposomes. In fact, we have observed a direct effect of lycopene on the antioxidant status of the semen samples, by enhancing of GPx and TAC, and the decrease of MDA levels, a proxy of peroxidative damage. These parameters are related to improved sperm quality. For instance, Am-In et al. (2011) found a higher concentration of TAC in the seminal plasma of wild boars with a normal sperm motility, in contrast to those with a lower percentage of motile spermatozoa. In that study, TAC positively correlated with sperm motility, viability and normal morphology. Rosato et al. (2012) reported that a concentration of

lycopene similar to the 0.2 mM assayed in our study (0.1 mg/ml) preserved the sperm membrane integrity and functionality of chilled turkey samples. However, it failed to enhance the sperm quality in cryopreserved spermatozoa, although this 0.1 mg/ml dose seemed to keep the DNA integrity after thawing.

Our results show that lycopene protects sperm mitochondria, reduces MDA levels and prevents apoptosis. The cryopreservation process could reduce flippase activity in the plasma membrane, resulting in the translocation of phosphatidylserine (PS) to the outer leaflet, which is an early apoptotic event (Anzar et al., 2002). Furthermore, lipid peroxidation of the plasma membrane, which was showed in our results and in other studies on rooster semen cryopreservation (Fattah et al., 2017), may cause apoptosis in these cells. Sperm apoptosis is also inversely correlated with mitochondrial activity (Mehdipour et al., 2017) and plasma membrane integrity (Khan et al., 2009). Indeed, these mitochondrial membrane alterations could initiate apoptosis in the spermatozoa (Najafi et al., 2017). Our hypothesis is that lycopene could reduce apoptotic markers such as PS translocation due achieve these effects to an individual or combined effect by stabilizing the sperm plasma membrane by reducing lipoperoxidation, stabilizing the plasmalemma and/or modulating apoptosis signalling pathways (Tuncer et al., 2014), a process in which mitochondrial protection could play a crucial role.

However, antioxidants can have toxic or pro-oxidant effects when used at high doses or in the presence of other substances, and lycopene is known for presenting this dual effect (Young and Lowe, 2001). In our study, lycopene at 0.3 mM (the highest concentration), either free or in nanoliposomes, abolished the beneficial effects observed in 0.1 and 0.2 mM. Our findings are in agreement with studies on ruminants (Anel-Lopez et al., 2012; Mata-Campuzano et al., 2012; Najafi et al., 2014), in which higher doses of antioxidants were harmful to sperm viability. Uysal

315 and Bucak (2007) reported that lycopene at 3200 μg/ml (almost 6 mM) led to deleterious effects
316 on frozen-thawed ram semen, especially on viability. Nevertheless, 800 and 1600 μg/ml, still much
317 higher than our 0.3 mM, showed positive effects. Thus, spermatozoa from different species might
318 have different tolerance to this antioxidant, or maybe the experimental conditions (extender, etc.)
319 might modulate lycopene activity.

320 As relevant as the positive effects found for lycopene are the enhancement of its effect
321 when combined with nanoliposomes. The encapsulation of lycopene in nanoliposomes showed
322 some non-significant (when comparing with the same dose of lycopene) but promising effects in
323 sperm quality, especially regarding motility and mitochondrial activity. This suggests that an
324 enhanced delivery of lycopene could affect the redox balance and the energetic metabolism of the
325 cells. The nanoliposomes could enhance or modulate the delivery of lycopene to the spermatozoa,
326 but these effects must be tested in studies focused on specific cellular aspects (e.g., intramembrane
327 or intracellular content of lycopene, ATP production). An effect of the nanoliposomes in further
328 membrane stabilization can be discarded, since the flow cytometry subpopulations (viable,
329 apoptotic, dead) seemed to be affected by lycopene concentration alone, and not by the means of
330 delivery.

331 Finally, the fertility trial confirms that lycopene at 0.2 mM could improve AI results in
332 farms. Whereas previous studies with lycopene in fowl reported benefits for sperm quality
333 (Mangiagalli et al., 2010; Rosato et al., 2012), this is the first report confirming these results in a
334 fertility trial. Even though the effects of lycopene were most clearly observed in that concentration,
335 the addition of any of the lycopene concentrations had a positive effect in the numbers of fertilized
336 and hatched eggs, and in hatchability, that is, the survival of the embryo to hatch. The hatchability
337 improvement indicates that lycopene is possibly protecting sperm chromatin, as observed in other

species (Mangiagalli et al., 2010; Rosato et al., 2012), and therefore increasing embryo viability. In addition, the effect of the nanoliposome combination was positive in all lycopene groups, supporting the potential of this technology.

Although the detailed mechanism of sperm transport in the reproductive tract of hens is not fully understood, higher sperm motility and membrane integrity are essential for sperm uptake into sperm storage tubules (Sasanami et al., 2013). The greater sperm progressive motility and membrane integrity resulting from 0.2 mM lycopene-loaded nanoliposomes treatment likely increased the population of functional sperm in the sperm storage tubules and thus improved fertility. Additionally, improvement in cellular parameters of sperm through reinforcement of the antioxidant system and mitochondria activity potentially will increase the function of sperm during passage through the reproductive tract (Zhandi et al., 2017). This fertility enhancement was likely not only due to increasing the number of live spermatozoa after freeze-thawing but also by improving their functionality.

5. Conclusion

Supplementation of that 0.2 mM lycopene in the semen extender improved post-thawing quality and fertility in roosters. Nanoliposomes might convey advantages by delivering lycopene to the cells and enhancing the antioxidant effects. Future research should focus on some basic aspects of this kind of treatments, namely the degree of encapsulation achieved, how nanoliposomes interact with spermatozoa and how they can increase lycopene effects and how to improve this system to achieve optimal effects. Larger field trials are now required to confirm this fertility improvement and to assess the economic aspects of incorporating lycopene and nanoliposomes to AI practices to broiler breeding.

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Table 1

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

Antioxidant	mM	MOT (%)	PROG (%)	VSL (μm/s)	VAP (μm/s)	VCL (μm/s)	LIN (%)	STR (%)	ALH (μm)	BCF (Hz)
control	0	51.09^{c}	25.65^{c}	16.25	28.71	56.33	28.96	55.97	4.64	17.30
lycopene	0.1	54.24^{bc}	27.49^{c}	17.62	31.94	58.56	30.11	55.96	4.82	17.78
	0.2	64.08^{ab}	36.23^{ab}	19.54	35.18	61.90	31.69	56.64	3.80	18.61
	0.3	46.17^{c}	23.58^{c}	16.61	30.88	55.11	30.22	53.97	4.91	16.80
lycopene-loaded liposomes	0.1	55.34^{bc}	28.87^{bc}	17.49	32.24	59.59	29.70	55.18	4.51	17.64
	0.2	68.07^{a}	38.95^{a}	19.86	35.59	61.90	32.11	56.37	3.86	18.56
	0.3	48.80^{c}	24.05^{c}	15.83	30.08	54.89	28.96	53.74	4.75	17.19
SEM		2.82	1.86	0.95	2.19	2.22	1.78	4.48	0.36	1.07

MOT: Total motility (MOT, %); PROG: 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 column indicate significant differences among groups ($p < 0.05$).

Table 2

Effect of lycopene and lycopene-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 (%)	Active mitochondria (%)
control	0	55.60^{b}	44.80^{c}	16.03	44.51^{d}
lycopene	0.1	59.24^{b}	50.95^{c}	14.98	53.32^{bc}
	0.2	69.70^{a}	60.79^{ab}	13.14	59.22^{ab}
	0.3	55.07^{b}	42.46^{c}	16.11	48.12^{cd}
lycopene-loaded liposomes	0.1	60.70^{b}	51.82^{bc}	14.43	54.82^{bc}
	0.2	72.91^{a}	62.71^{a}	12.27	64.66^{a}
	0.3	53.70^{b}	45.57^{c}	15.78	49.02^{cd}
SEM		1.60	2.13	1.49	1.81

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

Table 3

Effect of lycopene and lycopene-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	3.60^{a}	54.80^{c}	104.76	1.28^{b}
lycopene	0.1	2.07^{cd}	62.05^{abc}	115.89	1.68^{ab}
	0.2	1.32^{d}	64.23^{ab}	124.31	2.01^{a}
	0.3	3.06^{abc}	53.38^{bc}	103.02	1.17^{b}
lycopene-loaded liposomes	0.1	2.27^{bcd}	64.92^{ab}	105.38	1.71^{ab}
	0.2	1.21^{d}	67.75^{a}	128.58	2.16^{a}
	0.3	3.42^{ab}	55.24^{bc}	107.93	1.33^{b}
SEM		0.29	2.39	7.58	0.12

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

Table 4

Effect of lycopene and lycopene-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	40.94^{c}	22.53^{a}	36.52^{a}
lycopene	0.1	50.00^{abc}	15.85^{ab}	34.13^{ab}
	0.2	60.29^{a}	14.59^{c}	25.12^{b}
	0.3	42.39^{bc}	21.16^{ab}	36.44^{a}
lycopene-loaded liposomes	0.1	52.40^{ab}	16.47^{ab}	31.12^{ab}
	0.2	61.26^{a}	13.72^{c}	25.01^{b}
	0.3	44.04^{bc}	20.75^{ab}	35.20^{a}
SEM		2.36	1.57	1.90

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

Table 5

Effect of lycopene-loaded liposomes on fertility and hatchability rates of rooster semen after freeze-thawing. Each experimental group contained 45 eggs initially. Numbers are absolute counts of eggs, with percentages (ratio respect to the initial egg count) between parentheses, except for the hatched eggs ratio.

Antioxidant	mM	Fertilized eggs	Hatched eggs	Hatched eggs ratio (hatched/fertilized, %)
control	0	17 (37.8)^{b}	7 (15.6)^{c}	41.2^{b}
lycopene	0.1	22 (48.9)^{ab}	10 (22.2)^{bc}	45.5^{ab}
	0.2	26 (57.8)^{ab}	16 (35.6)^{ab}	61.5^{ab}
	0.3	20 (44.4)^{ab}	9 (20)^{bc}	45.0^{b}
lycopene-loaded liposomes	0.1	24 (53.3)^{ab}	14 (31.1)^{abc}	58.3^{ab}
	0.2	28 (62.2)^{a}	21 (46.7)^{a}	75.0^{a}
	0.3	23 (51.1)^{ab}	12 (26.7)^{abc}	52.2^{ab}

Different superscripts letters within columns indicate significant differences between treatments ($P < 0.05$).