Comparing the effect of rooster semen extender supplemented with gamma-oryzanol and its nano form on post-thaw sperm quality and fertility

Abouzar Najafi ,*,[1] Hossein Daghigh-Kia,[†] Mahdieh Mehdipour,[†] Hossein Mohammadi,[‡] and Hamed Hamishehkar[§]

[*]*Department of Animal and Poultry Science, College of Aburaihan, University of Tehran, Tehran, Iran;* [†]*Department of Animal Science, College of Agriculture, University of Tabriz, Tabriz, Iran;* [‡]*Department of Animal Science, Faculty of Agriculture and Natural Resources, Arak, University, Arak, Iran; and* [§]*Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*

ABSTRACT Antioxidant nanoparticles include the potential for improving sperm cryopreservation. The aim of performing this study was to evaluate the effects of gamma-oryzanol (**GO**) at 0 (C) (control group), 20 (GO20), 40 (GO40), 60 (GO60), 80 (GO80), and 100 (GO100) μM and gamma-oryzanol nanoparticles (GON) at 0 (CN), 20 (GON20), 40 (GON40), 60 (GO60), 80 (GON80), and 100 (GON100) μM on post-thawed sperm quality and fertility of rooster sperm. Sperm motility, plasma membrane integrity, total abnormality, mitochondrial activity (Rhodamine 123), apoptotic features (Annexin V/Propidium iodide), reactive oxygen species (**ROS**) production, ATP content and the fertility and hatchability were evaluated after thawing. Total motility in GON60 and GON80 were significantly higher compared to control groups (C and CN). GON80 showed the greatest percentages of progressive motilities. When GO80, GON60, and GON80 were added to the cryopreservation medium, the plasma membrane functionality of the semen samples improved. The minimum abnormality of spermatozoa is observed in the group treated with GON80. The groups treated with GON60 and GON80 had greater ($P < 0.05$) mitochondrial activity. The level of sperm ROS after cryopreservation was significantly lower in GON60 and GON80 groups. Live sperm was significantly higher ($P < 0.05$) in GON60 and GON80 group compared to other groups. GON60 and GON80 groups also led to the lowest significant percentage of apoptosis-like change sperm. Greater fertility percentages were observed ($P < 0.05$) when sperm were stored in extenders treated with GON60 and GON80. GON80 resulted in significantly improved hatched eggs compared to C, GO60, GO180 and CN. In conclusion, supplementation of Lake extender with 60 and 80 μM gamma-oryzanol nanoparticles could be a proper process to improve freeze-thawing rooster sperm quality leading to better freeze/thaw characteristics.

Key words: nanoparticles, sperm, rooster, cryopreservation, gamma-oryzanol

2022 Poultry Science 101:101637
https://doi.org/10.1016/j.psj.2021.101637

INTRODUCTION

Cryobiology is the study of the physical and biological activity of live cells and tissues at extremely low temperatures. Sperm cryopreservation is critical for the production of high-quality breeder animals, as well as the transmission of genetic material to coming generations. Cold shock and oxidative stress during cryopreservation, on the other hand, induce cryodamage to spermatozoa. The use of this technology in cattle has increased fast, while sperm freezing in poultry is limited due to the high costs of semen processing and decreased fertility as a result (Zhandi et al., 2020).

Cold shock in spermatozoa is demonstrated to lead in oxidative stress and dead spermatozoa which are proposed to be responsible for the production of reactive oxygen species (**ROS**) (Bucak et al., 2012). In the presence of thermal changes, and exposure to free radicals, oxidative stress is the most detrimental and unavoidable factor of sperm damage. Antioxidants are naturally present in semen, although they diminish after dilution and cryopreservation. Therefore, exogenous antioxidants in extension may be a potential method to reduce ROS-induced damage (Awan et al., 2018).

Gamma-oryzanol is a phytochemical compound found in rice bran oil and rice germ. Gamma-oryzanol is an

Received July 29, 2021.
Accepted November 25, 2021.

[1]Corresponding author: abozar.najafi@ut.ac.ir

inexpensive derivative of β-oryzanol with potential biological applications in the field of antioxidants and anti-inflammation (Silva et al., 2021). Kaeoket et al. (2012) improved the quality of frozen−thawed boar sperm by adding gamma-oryzanol-rich rice bran oil to lactose −egg yolk freezing extender (progressive motility, viability, and acrosome integrity). For boar semen cryopreservation in lactose−egg yolk freezing extender, the optimum concentration of gamma-oryzanol depends not only on the individual boar but also the breed of boar (Chanapiwat and Kaeoket, 2015).

In recent years, in the case of nutrients with low water solubility, nanoparticle delivery systems are already more used. Nanoparticle delivery systems offer benefits such as improved surface and reactivity, improving solubility and bioavailability of nutraceutical components, in particular poorly soluble molecules like as functional lipids and natural antioxidants, as well as reduced harmful effects (Acosta, 2009; Ting et al., 2014). Gamma-oryzanol may be restricted by low water solubility, poor bioavailability, and quick metabolism in the use of medicinal and functional food systems (Kim et al., 2010). In order to overcome this restriction, nanoemulsion compounds can be utilized to enhance and regulate the distribution of water-insoluble medicines, nutraceuticals, and other therapeutic substances (Mason et al., 2006).

To the best of our knowledge, there is no study investigating the effects of gamma-oryzanol and also gamma-oryzanol nanoparticles on post-thawed sperm quality and fertility of rooster sperm. Therefore, the present study was considered to investigate the effect of adding gamma-oryzanol gamma-oryzanol nanoparticles to extender on rooster sperm quality parameters after the freeze-thawing process.

MATERIAL AND METHODS

Chemicals

All the chemicals were bought from Merck (Darmstadt, Germany). The research was authorized by the Committee on Animal Care and carried out at the Animal Science College, University of Tehran, Iran.

Gamma-Oryzanol Nanoemulsion Preparation

Gamma-oryzanol emulsion was produced mixing ultrapure water and palm olein as the continuous phase and the disperse phase, respectively. Gamma-oryzanol was initially combined with pure palm olein (15% w/w). The obtained solution was added to purified water (82.5% w/w) which included Tween 80 and lecithin as the emulsifier (2.5% w/w). A high shear homogenizer (Silverson Homogenizer, Chesham, UK) at 3,000 rpm for 5 min was applied to homogenize the premix and then, high pressure homogenizer (model APV 1000, APV Systems, Albertslund, Denmark) was utilized. Then, it was applied to sonication (Vibra Cell-Sonics & Material, Newtown, CT, 130 W, 20 kHz) for 10 min (70% power). A laser light scattering particle size analyzer (SALD 2101, Shimadzu, Tokyo, Japan) was used to determine the particle size of the nanoparticles.

Animal Handling

In this research, 10 Ross broiler breeder roosters (30 wk of age) were applied. All of the roosters were kept in separate cages (70 × 60 × 75 cm; photoperiod = 15 L: 9 D; temperature = 18−22°C) and the diet used for feeding them included 0.7% calcium, 12% crude protein, 2750 kcal energy/kg maintenance, and 0.35% phosphorus available. For obtaining sperm, the abdominal massage was utilized twice a week (Najafi et al., 2020). Following sample preparation, they were transferred to the laboratory for primary assessment at 37°C for 5 min. Samples with the following criteria were chosen for further use in the study: sperm volume (>0.2 mL); normal morphology (>90%) total motility (>80%), and sperm concentration (>3 × 10^9 spermatozoa/mL).

The buffer selected for this experiment was Lake buffer which contained D-fructose (44.40 mM), sodium glutamate (113.53 mM), magnesium acetate (4.91 mM), polyvinylpyrrolidone (0.3 mM), potassium citrate (16.31), and glycine (0.187 g/l). Osmolarity and pH were set at 7.1 and 310 mOsm/kg, respectively. Fresh semen was diluted in the prepared Lake extender including gamma-oryzanol (GO) at 0 (C) (control group), 20 (GO20), 40 (GO40), 60 (GO60), 80 (GO80), and 100 (GO100) μM and gamma-oryzanol nanoparticles (GON) at 0 (CN), 20 (GON20), 40 (GON40), 60 (GO60), 80 (GON80), and 100 (GON100) μM. Soybean lecithin (P3644 Sigma L-α-phosphatidylcholine from soybean) and glycerol were added to the basic extender at 1% (wt/vol) and 3.8% (v/v), respectively.

Extender Preparation and Cryopreservation

After sample dilution, they were aspirated into 0.25 milliliter French straws. Eventually, the straws were equilibrated at 4°C for 3 h, after being sealed with polyvinyl alcohol. Following equilibration, the straws were positioned 4 cm above the sample surface of the liquid nitrogen for 7 min, and then they were dipped into the liquid nitrogen (Najafi et al., 2020). For carrying out the assessments, the straws were separately thawed (37°C) for 30 s in a water bath, and then they were analyzed independently.

Motility Parameters

The sperm motility has been determined by a computer-assisted sperm analyzer (CASA; 12.3 CEROS, Hamilton Thorne Biosciences, Beverly, MA). For preparing the samples for motion characteristics, they were diluted with PBS (1:10) buffer. Afterward, 3 μL of the diluted sample (37°C, Leja 4; 20 mm height; Leja, Luzernestraat B.V., Holland) was injected into a chamber slide. At least 6 fields comprising almost 300 sperm were

analyzed for parameters such as sperm total motility (**TM**), progressive motility (**PM**), straight linear velocity (**VSL**, μm/s), average path velocity (**VAP**, μm/s), amplitude lateral head displacement (**ALH** μm), and curvilinear velocity (**VCL**, μm/s) (Mehdipour et al., 2020b).

Membrane Functionally (Hypo-Osmotic Swelling Test)

The hypo-osmotic swelling test was performed on the basis of the percentage of observed curled and swollen tails in spermatozoa (Mehdipour et al., 2018). This test was carried out by incubating 10 μL of semen at 37°C for 30 min at room temperature with 100 μL of 100 mOsm/L hypoosmotic solution comprising 5 mM fructose and 1.9 mM sodium citrate (Nadri et al., 2019). Using a phase contrast light microscope (Labomed LX400; Labomed Inc., Culver City, CA), at least 7 separate microscopic areas, 400 spermatozoa were counted in total.

Morphology

After being thawed, 15 μL of the semen were placed into Eppendorf tubes that included one milliliter of Hancock solution involving 150 mL of sodium saline solution, 500 mL of double-distilled water, 62.5 mL of formalin (37.5%), and 150 mL of the buffer solution. An overall abnormality of the sperm was diagnosed by loading a slide with 10 mL of processed sperm and counting a total number of 300 individual spermatozoa under a phase contrast microscope (Labomed LX400; Labomed Inc., Culver City, CA) (magnification 400x) to determine the percentage of sperm with an abnormal Acrosome (such as defects in the mid-pieces and tails, detached heads, and other acrosomal abnormalities)

Determination of ATP in Sperm

The amount of ATP in the sperm was determined using the technique developed by Mehdipour et al. (2020a). First, 5 microliters of each sample were diluted in 750 microliters of Lake buffer. Then, 5 mL of the diluted sample was pipetted into 190 mL of perchloric acid to perform the reaction. The tubes were then centrifuged at 12,000 rcf for 2 min at room temperature. To neutralize the top phase (180 mL), it was mixed with 10.7 mL of 2 M KCl, 58.7 mL of 1 M KOH, 10.7 mL of saturated Tris, and 1 mg/mL of red phenol. Then 100 μL luciferin−luciferase reconstituted reagent (100 mM glycine, 20 mM $MgSO_4$, pH 7.4) was added for evaluation (measured bioluminescence using a luminometer). The amount of ATP present in the sperm was represented as pmol ATP/10^6 sperm.

Determination of ROS

ROS was assessed by the method of Mehdipour et al. (2020a). Briefly, the incubation of semen samples was done for 20 min in 250 μL of PBS at 37°C and then they were centrifuged at 300 × g for 7 min, and supernatant was discarded. Following the addition of 3 mL of PBS to the pellet, centrifugation at 300 g for 7 min was performed on the mixture. By diluting the sperm with PBS, the concentration was adjusted to 20 × 10^6 mL^{-1}. Then, 10 μL of luminol were added to 400 μL of sample and finally the tubes were placed in an Orion II Microplate Luminometer (Berthold Detection Systems Gmbh, Pforzheim. Germany) for evaluation. The obtained results were presented as 10^3 cpm/10^6 spermatozoa.

Apoptotic Like Changes

At first, washing the semen samples was done in calcium buffer to assess the externalization of phosphatidyl serine as the earliest predictor of apoptotic-like modifications (Najafi et al., 2021). Then 10 μL of Annexin V-FITC (0.01 mg/mL) was added to 100 μL of sperm suspension. This was then incubated for 20 min. In addition, 10 μL of propidium iodide (PI; 1 mg/mL) was applied to the suspension of the sperm and incubated for at least 10 min. Then, a flow cytometer (Becton Dickinson System, San Jose, CA) equipped with an argon-ion laser at 488 nm was used to evaluate the suspension. The results obtained from flow cytometer were expressed as 3 groups (viable, apoptotic, and dead sperm).

Mitochondrial Activity

Rhodamine 123 (R123; Invitrogen TM, Eugene, OR) and PI were utilized to test the function of mitochondria (Mehdipour et al., 2021). In a dark room, 10 microliters of R123 were applied to 200 μL of diluted sperm and incubated for 20 min. Following resuspension, 10 μL of PI was applied to the mixture, and active mitochondrial sperm was recorded. Analysis of the flow cytometer was carried out with a flow cytometer from FacsCalibur (Becton Dickinson, San Jose, CA). The function of mitochondria in sperm was recognized by a positive Rh123 signal and a negative PI signal.

Analysis of Reproductive Performance

In 180 broiler breeder hens with the same strain, artificial insemination was done after 1 mo of no contact with the roosters. For AI, 30 hens were inseminated (100 × 10^6 sperm/hen) for each treatment. Artificial insemination (**AI**) was conducted twice a week (for a total of 4 d in 2 wk) at the same time and on the same day of the week. Up to 5 d after the last AI, eggs were collected daily. The levels of fertility and hatchability were defined using eggs candled on d 7 and the number of hatched chicks on d 21.

Table 1. Effect of different levels of gamma-oryzanol and gamma-oryzanol nanoparticles on motility parameters of rooster sperm assessed by CASA after freeze-thawing.

	Treatments												
Parameters	C	GO20	GO40	GO60	GO80	GO100	CN	GO20N	GON40	GON60	GON80	GON100	SEM
TM (%)	37.3^{e}	40.9^{de}	43.5^{de}	52.4^{c}	60.2^{b}	39.5^{e}	38.6^{e}	43.2^{de}	47.8^{cd}	66.3^{ab}	71.7^{a}	41.8^{de}	1.59
PM (%)	16.9^{d}	17.3^{cd}	18.2^{cd}	18.5^{cd}	22.4^{bc}	17.2^{cd}	17.0^{d}	18.1^{cd}	19.1^{cd}	26.8^{b}	34.9^{a}	17.3^{cd}	1.14
VAP (μm/s)	30.5^{c}	30.7^{c}	31.1^{c}	31.8^{c}	33.1^{bc}	30.3^{c}	30.1^{c}	30.9^{c}	32.6^{bc}	36.3^{ab}	38.2^{a}	30.1^{c}	0.88
VSL (μm/s)	16.8^{c}	17^{bc}	17.4^{bc}	18.3^{bc}	19.1^{abc}	16.9^{c}	16.9^{c}	17.3^{bc}	18.6^{bc}	20.9^{ab}	22.8^{a}	16.9^{c}	0.83
VCL (μm/s)	54.2^{b}	54.4^{b}	55.2^{ab}	56.1^{ab}	57.6^{ab}	54.3^{b}	54.3^{b}	54.8^{b}	56.9^{ab}	57.5^{ab}	60.3^{a}	54.8^{b}	1.14
LIN (%)	30.9	30.9	30.9	30.9	30.9	30.9	30.9	30.9	30.9	30.9	30.9	30.9	1.47
STR (%)	55.4	56.1	56.3	57.7	58	56	56.5	56.4	57.1	58.2	59.6	56.1	3.1
BCF (Hz)	16.2	16.4	16.7	16.9	17.1	16.4	16.3	16.8	17	17.7	18.4	16.5	0.94
ALH (μm)	5.5	5.3	5.1	5	4.6	5.2	5.4	5.1	4.8	4.5	4.3	5.1	0.31

Abbreviations: LIN, linearity; PM, progressive motility; TM, total motility; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity.

Lake extender including gamma-oryzanol (GO) at 0 (C) (control group), 20 (GO20), 40 (GO40), 60 (GO60), 80 (GO80), and 100 (GO100) μM and gamma-oryzanol nanoparticles (GON) at 0 (CN), 20 (GON20), 40 (GON40), 60 (GO60), 80 (GON80), and 100 (GON100) μM.

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

Statistical Analysis

All data were tested for normality by UNIVARIATE procedure and ShapiroeWilk test, and all percentage data were standardized by $\sqrt{x}$ Arcsin transformation. All data were analyzed using Proc Mixed of SAS 9.1 (SAS Institute, version 9.1, 2002, Cary, NC). These findings were compared with the Tukey-test at $P < 0.05$ and evaluated based on mean ± standard error of the mean. The fertility and hatchability rates were evaluated by the GENMOD protocol and the findings were compared by the chi-squared test at $P < 0.05$.

RESULTS

The effect of gamma-oryzanol and gamma-oryzanol nanoparticles on sperm motility and kinematic parameters after cryopreservation are presented in Table 1. Total motility in GON60 and GON80 were significantly higher compared to control groups (C and CN). Sperm cryopreserved in a medium containing GON80 had the greatest percentages of progressive motilities compared with the other treatment.

Significantly higher percentages of VSL were noticed in samples treated with GON60 and GON80 compared to C, GO100, CN and GON100. Moreover, significantly improved percentages of VCL and VAP were observed in GON60 and GON80 compared to C, GO100, CN, GON20 and GON100. LIN, BCF, STR, and ALH were not significantly ($P > 0.05$) affected by different treatment.

In Table 2, there is depiction of the effect of gamma-oryzanol and gamma-oryzanol nanoparticles on plasma membrane functionality, morphology, Mitochondria activity, ATP and ROS of rooster spermatozoa. Cryopreservation media supplements with GO80, GON60, and GON80 improved membrane functioning in semen samples after thawing when compared to cryopreservation media supplemented with C, GO20, GO40, GO60, GO100, CN, GON20, and GON100. According to the findings given in Table 2, the least amount of abnormalities in spermatozoa was observed in the group treated with GON80, whereas the greatest amount of abnormality was observed in the sperm in the control groups (C and CN). The sperm stored in the extender treated with GON60 and GON80 had a greater ($P < 0.05$) mitochondrial membrane potential compared to C, GO20, GO40, GO60, GO100, CN, GON20, and GON100. The highest significant ($P < 0.05$) ATP was observed in GON80 compared to C, GO20, GO40, GO60, GO100, CN, GON20, and GON100. The levels of sperm ROS after frozen thawing were significantly lower in GON60 and GON80 group compared to C, GO20, GO40, GO60, GO100, CN, GON20, and GON100.

Effects of gamma-oryzanol and gamma-oryzanol nanoparticles on viability and apoptosis-like changes of

Table 2. Effect of different levels of gamma-oryzanol and gamma-oryzanol nanoparticles on plasma membrane functionality and abnormal forms of rooster thawed semen.

	Treatments												
Parameters	C	GO20	GO40	GO60	GO80	GO100	CN	GO20N	GON40	GON60	GON80	GON100	SEM
Plasma membrane functionality (%)	35.8^{d}	37.4^{d}	39.1^{d}	47.5^{c}	58.4^{ab}	36^{d}	36.5^{d}	41.2^{cd}	46.1^{c}	63.2^{ab}	69.7^{a}	39.1^{d}	1.32
Abnormal forms (%)	17.4^{a}	17^{ab}	16.7^{ab}	16.3^{ab}	15.6^{ab}	16.9^{ab}	17.3^{ab}	16.5^{ab}	15.9^{ab}	12.8^{ab}	11.5^{b}	16.5^{ab}	1.21
Mitochondria activity (%)	34.9^{e}	38.8^{de}	41.1^{cde}	43.7^{dc}	56^{b}	35.2^{e}	35.1e	40.9^{cde}	45.9^{c}	62.1^{ab}	65.6^{a}	35.8^{e}	1.44
ROS (10^3 cpm/10^6 sperm)	4.1^{a}	4.1^{a}	4^{a}	3.6^{ab}	2.9^{bc}	4^{a}	4.1^{a}	3.8^{a}	3.7^{a}	2.8^{c}	2.6^{c}	3.8^{a}	0.14
ATP (pmol/10^6 sperm)	88.7^{e}	91^{de}	94.8^{cde}	102.7^{bcd}	105^{abc}	89.5^{e}	89.3^{e}	92.9^{cde}	95.8^{cde}	110.6^{ab}	117.2^{a}	90.5^{de}	2.69

Lake extender including gamma-oryzanol (GO) at 0 (C) (control group), 20 (GO20), 40 (GO40), 60 (GO60), 80 (GO80), and 100 (GO100) μM and gamma-oryzanol nanoparticles (GON) at 0 (CN), 20 (GON20), 40 (GON40), 60 (GO60), 80 (GON80), and 100 (GON100) μM.

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

Table 3. Effect of different levels of gamma-oryzanol and gamma-oryzanol nanoparticles on live, apoptotic and dead spermatozoa in rooster thawed semen, as assessed by flow cytometry.

	Treatments												
Parameters	C	GO20	GO40	GO60	GO80	GO100	CN	GO20N	GON40	GON60	GON80	GON100	SEM
Live (%)	37.3^{e}	38.5^{e}	40.7^{de}	48.1^{c}	56.5^{b}	37.9^{e}	37.4^{e}	41.9^{cde}	45.8^{cd}	62.3^{ab}	65.7^{a}	38.1^{e}	1.31
Apoptotic (%)	24.7^{a}	23.9^{a}	22.8^{a}	21.3^{ab}	16.6^{bc}	23.1^{a}	24.1^{a}	23.2^{a}	21.7^{ab}	13^{c}	12^{c}	23.3^{a}	1.22
Dead (%)	37.9^{a}	37.5^{a}	36.3^{a}	30.5^{abcd}	26.8^{bcd}	38.9^{a}	38.4^{a}	34.8^{ab}	32.3^{abc}	24.6^{cd}	22.2^{d}	38.5^{a}	1.88

Viable (%, AnnexinV−/PI−)), apoptotic (%, AnnexinV+/PI−)) and dead (%, PI+) parameters were analyzed. Lake extender including gamma-oryzanol (GO) at 0 (C) (control group), 20 (GO20), 40 (GO40), 60 (GO60), 80 (GO80), and 100 (GO100) μM and gamma-oryzanol nanoparticles (GON) at 0 (CN), 20 (GON20), 40 (GON40), 60 (GO60), 80 (GON80), and 100 (GON100) μM.

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

rooster sperm following cryopreservation are depicted in Table 3. The percentage of live sperm was significantly higher ($P < 0.05$) in GON60 and GON80 groups compared to other groups. GON60 and GON80 groups also led to the lowest significant percentage of apoptosis-like change sperm compared to C, GO20, GO40, GO60, GO100, CN, GON20, and GON100. Moreover, the lowest percentage of dead sperm was obtained in GON80 compared to C, GO20, GO40, GO100, CN, GON20, and GON100.

The data for fertility capacity containing fertility and hatching rates are shown in Table 4. When sperm from extenders treated with GON60 and GON80 were utilized, the fertility percentages were higher ($P < 0.05$) compared to the sperm from other treatments. Among treated groups, GON80 resulted in significantly improved hatched eggs compared to C, GO60, GO180, and CN. For Hatched eggs ratio (hatched/fertilized, %), higher significant rates of hatched eggs ratio were obtained in GON60 and GON80 compared to C and CN.

DISCUSSION

Because of the high amounts of polyunsaturated fatty acids (**PUFAs**) in Avian spermatozoa, they are more susceptible than other species to lipid peroxidation, which is thought to be a critical component in male fertility. Apoptosis was reduced in the current study due to the antioxidant and antiapoptotic properties of gamma-oryzanol nanoparticles and the ability of GON80 to reduce ROS production and maintain the integrity of the sperm plasma membrane. Numerous studies have shown that gamma-oryzanol can reduce oxidative damage by scavenging free radicals and reducing lipid peroxidation (Huang et al., 2020).

Table 4. Effect of gamma-oryzanol and gamma-oryzanol nanoparticles on fertility and hatchability rates of rooster semen after freeze-thawing.

	Parameters		
Treatments	Fertilized eggs	Hatched eggs	Hatched eggs ratio (hatched/fertilized, %)
C	79 (39.5)^{c}	46 (23)^{d}	58.2^{b}
GO60	110 (55)^{b}	74 (37)^{c}	67.2^{ab}
GO80	117 (58.5)^{b}	80 (40)^{bc}	68.3^{ab}
CN	80 (40)^{c}	47 (23.5)^{d}	58.7^{b}
GON60	137 (68.5)^{a}	98 (49)^{ab}	71.5^{a}
GON80	142 (71)^{a}	105 (52.5)^{a}	73.9^{a}

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

Each experimental group contained 200 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.

By loading the antioxidant gamma-oryzanol into nanocarrier in the present study, the beneficial effects of gamma-oryzanol increased post-thaw sperm quality and improving the sperm protection which can be used in poultry sperm cryopreservation parameters.

The current findings revealed that gamma-oryzanol nanoparticles had certain favorable impacts on motility (TM and PM) and velocity (VSL, VAP, and VCL). These beneficial profits can be attributed to the critical function that gamma-oryzanol nanoparticles play in promoting antioxidant capacity, which is crucial in preserving the sperm plasma membrane against reactive oxygen species (ROS). This was proven by the increases in acrosome integrity and viability observed with the addition of gamma-oryzanol to the extender, which in turn resulted in improvements in post-thaw motion variables (Chanapiwat and Kaeoket, 2015).

To attach to oocyte and begin an acrosome reaction, the sperm has to have its intact plasma membrane. Cryopreservation increases the risk of oxidation and phosphorylation in mitochondria, which leads to an increase in the leak of electrons and the production of superoxide radicals which ultimately impairs the quality of the sperm. The addition of GON80 considerably enhanced the membrane integrity compared to the control group. Gamma-oryzanol nanoparticles supplementation might prevent ROS-mediated peroxidative stress, helping to decrease the apoptotic cascade and enhance post-thaw semen quality. ATP synthase and oxidative phosphorylation activities in the mitochondria convert substances into energy sources that may be utilized by spermatozoa; however, thermal stress and excessive ROS generation have detrimental effects on mitochondrial functioning, resulting in impaired ATP transport efficiency (Fang et al., 2014). When there is an imbalance between the creation and elimination of free radicals, oxidative stress occurs, which leads to DNA damage and apoptotic-like alterations in spermatozoa. It has been shown that when there is significant oxidative stress, mitochondrial fission occurs, which results in a significant reaction in the mitochondria, including

mitochondrial aggregation and dysfunctions (Wang et al., 2014). An increase in mitochondrial fission is presumably associated with a reduction in ATP generation, which results in increased mitochondrial-derived apoptosis (Chen et al., 2005). According to Hagl et al. (2016), gamma-oryzanol (340 mg/kg/d for 3 wk) retrieved age-related mitochondrial dysfunction in the brains of aged NMRI mice by modulating the function of mitochondrial respiratory, peroxisome proliferator-activated receptor gamma coactivator1-alpha protein (PPARC1-alpha) expression, which is a sign of age-related mitochondrial dysfunction, and the mitochondrial marker enzyme citrate synthase activity.

Our findings demonstrated that pretreatment with gamma-oryzanol nanoparticles promoted ROS scavenging activity while simultaneously increasing mitochondrial activity. It was hypothesized that gamma-oryzanol nanoparticles prevented cell death by reducing ROS production, therefore controlling the opening of mitochondrial permeability transition pores and maintaining normal cellular activities. We also discovered that gamma-oryzanol nanoparticles were effective in preventing oxidative stress and apoptosis by inhibiting the accumulation of intracellular ROS and preserving mitochondrial activity, therefore restoring mitochondrial function which particularly revealing the reason of the observed improvement in motility parameters in the present research. According to Ismail et al. (2014), gamma-oryzanol exerts its neuroprotective benefits by maintaining mitochondrial metabolic enzyme function and decreasing apoptosis in SH-SY5Y cells when exposed to oxidative stress-induced neurotoxicity. Gamma-Oryzanol exhibits these properties due to activation of CAT, SOD1 and SOD2 genes, upregulation of antiapoptotic genes (extracellular signal regulated kinases and downregulation of proapoptotic genes [bax activator], bcl-2 homologous killer [bak1], bax, p21, and caspase-9), (c-Jun N-terminal kinases [JNK], TNF, an inhibitor of growth family member 3 [ING3]. The results of the current experiment revealed that the inclusion of gamma-oryzanol nanoparticles to the semen extender reduced the quantity of apoptotic sperm produced after thawing. Huang et al. (2020) discovered that the ratio of Bcl-2 to Bax increased significantly in cells pretreated with gamma-oryzanol, indicating that gamma-oryzanol regulated the expression of Bcl-2 and Bax, preventing activation of the mitochondrial apoptotic pathway in human hepatic L02 cells exposed to H_2O_2, consequently decreasing apoptotic cells which support our observations. The evaluation of post-thaw viability and apoptosis status indicated that the addition of gamma-oryzanol nanoparticles to the semen extender increased the number of living sperm while simultaneously decreasing the amount of apoptotic and dead sperm. According to the present research, the increased viability was most likely the result of antiapoptotic effect, which in turn resulted in increased motility and velocity of the cells. In several studies, it has been demonstrated that the amount of damage induced to the plasma membrane during the freeze-thaw process is adversely associated to post-thaw viability, motility, and fertilization capability (Bellagamba et al., 2007).

Evaluation of fertility via artificial insemination is a critical step in approving the in vitro results that have been acquired (Najafi et al., 2019). In other studies, high correlation was detected between different sperm parameters and reproductive performance in avian (Najafi et al., 2020). In order for spermatozoa to successfully fertilize an egg, they must have the optimum concentration, motility, plasma membrane integrity, and functioning. A positive correlation between the antioxidant and fertility rate has also been documented (Mehdipour et al., 2021). The fertility rate achieved in our study showed that gamma-oryzanol sperm nanoparticles contributed to the greater fertility rate after artificial insemination. Gamma-oryzanol nanoparticles treatment improved sperm motility and viability, and sperm plasma membrane functionality which subsequently enhanced the population of intact spermatozoa in the SSTs.

CONCLUSION

Taken together, these results suggest that gamma-oryzanol nanoparticles at 60 and 80 μM could be a suitable supplementation for cryopreservation of rooster sperm, because they improve post-thawed rooster sperm quality containing motility parameters, mitochondrial activity, apoptotic status, ATP and ROS which totally result in higher fertility success known as an important factor in poultry industry.

DISCLOSURES

The authors declare no conflicts of interest.

REFERENCES

Acosta, E. 2009. Bioavailability of nanoparticles in nutrient and nutraceutical delivery. Curr. Opin. Colloid Interface Sci. 14:3–15.

Awan, M. A., S. Akhter, A. U. Husna, M. S. Ansari, B. A. Rakha, A. Azam, and S. Qadeer. 2018. Antioxidant activity of nigella sativa seeds aqueous extract and its use for cryopreservation of buffalo spermatozoa. Andrologia:e13020.

Bellagamba, F., S. Cerolini, and L. G. Cavalchini. 2007. Cryopreservation of poultry semen: a review. Worlds. Poult. Sci. J. 49:157–166.

Bucak, M. N., N. Baspinar, P. B. Tuncer, K. Coyan, S. Sariozkan, P. P. Akalin, S. Buyukleblebici, and S. Kucukgunay. 2012. Effects of curcumin and dithioerythritol on frozen-thawed bovine semen. Andrologia 44(Suppl 1):102–109.

Chanapiwat, P., and K. Kaeoket. 2015. Breed of boar influences the optimal concentration of gamma-oryzanol needed for semen cryopreservation. Reprod. Domest. Anim. 50:221–226.

Chen, H., A. Chomyn, and D. C. Chan. 2005. Disruption of fusion results in mitochondrial heterogeneity and dysfunction. J. Biol. Chem. 280:26185–26192.

Fang, L., C. Bai, Y. Chen, J. Dai, Y. Xiang, X. Ji, C. Huang, and Q. Dong. 2014. Inhibition of ROS production through mitochondria-targeted antioxidant and mitochondrial uncoupling increases post-thaw sperm viability in yellow catfish. Cryobiology 69:386–393.

Hagl, S., D. Berressem, R. Grewal, N. Sus, J. Frank, and G. P. Eckert. 2016. Rice bran extract improves mitochondrial dysfunction in brains of aged NMRI mice. Nutr. Neurosci. 19:1–10.

Huang, L., W. Jiang, L. Zhu, C. Ma, Z. Ou, C. Luo, J. Wu, L. Wen, Z. Tan, and J. Yi. 2020. gamma-Oryzanol suppresses cell apoptosis by inhibiting reactive oxygen species-mediated mitochondrial signaling pathway in H2O2-stimulated L02 cells. Biomed. Pharmacother. 121:109554.

Ismail, N., M. Ismail, M. U. Imam, N. H. Azmi, S. F. Fathy, J. B. Foo, and M. F. A. Bakar. 2014. Mechanistic basis for protection of differentiated SH-SY5Y cells by oryzanol-rich fraction against hydrogen peroxide-induced neurotoxicity. BMC Complement. Altern. Med. 14:1–11.

Kaeoket, K., S. Donto, P. Nualnoy, J. Noiphinit, and P. Chanapiwat. 2012. Effect of gamma-oryzanol enriched rice bran oil on quality of cryopreserved boar semen. J. Vet. Med. 74 1149-1153.

Kim, J. S., J.-S. Lee, P.-S. Chang, and H. G. Lee. 2010. Optimization, in vitro release and bioavailability of γ-oryzanol-loaded calcium pectinate microparticles reinforced with chitosan. N. Biotechnol. 27:368–373.

Mason, T. G., J. N. Wilking, K. Meleson, C. B. Chang, and S. M. Graves. 2006. Nanoemulsions: formation, structure, and physical properties. J. Phys. Condens. Matter 18:R635.

Mehdipour, M., H. Daghigh Kia, G. Moghaddam, and H. Hamishehkar. 2018. Effect of egg yolk plasma and soybean lecithin on rooster frozen-thawed sperm quality and fertility. Theriogenology 116:89–94.

Mehdipour, M., H. Daghigh Kia, A. Najafi, H. Mohammadi, and M. Alvarez-Rodriguez. 2020b. Effect of crocin and naringenin supplementation in cryopreservation medium on post-thaw rooster sperm quality and expression of apoptosis associated genes. PLoS One 15:e0241105.

Mehdipour, M., H. D. Kia, A. Najafi, and F. Martínez-Pastor. 2021. Type III antifreeze protein (AFP) improves the post-thaw quality and in vivo fertility of rooster spermatozoa. Poult. Sci. 100:101291.

Nadri, T., A. Towhidi, S. Zeinoaldini, F. Martinez-Pastor, M. Mousavi, R. Noei, M. Tar, and A. Mohammadi Sangcheshmeh. 2019. Lecithin nanoparticles enhance the cryosurvival of caprine sperm. Theriogenology 133:38–44.

Najafi, A., H. Daghigh Kia, and H. Hamishehkar. 2021. Does alpha-lipoic acid-loaded nanostructured lipid carriers improve post-thawed sperm quality and ameliorate apoptosis-related genes of rooster sperm? Poult Sci. 100:357–365.

Najafi, A., H. Daghigh Kia, H. Hamishehkar, G. Moghaddam, and S. Alijani. 2019. Effect of resveratrol-loaded nanostructured lipid carriers supplementation in cryopreservation medium on post-thawed sperm quality and fertility of roosters. Anim. Reprod. Sci. 201:32–40.

Najafi, A., H. Daghigh Kia, M. Mehdipour, H. Hamishehkar, and M. Álvarez-Rodríguez. 2020. Effect of quercetin loaded liposomes or nanostructured lipid carrier (NLC) on post-thawed sperm quality and fertility of rooster sperm. Theriogenology 152:122–128.

Silva, N. C., M. R. Poetini, M. C. Bianchini, F. P. Almeida, M. M. M. Dahle, S. M. Araujo, V. C. Bortolotto, E. A. S. Musachio, B. P. Ramborger, D. R. Novo, R. Roehrs, M. F. Mesko, M. Prigol, and R. L. Puntel. 2021. Protective effect of gamma-oryzanol against manganese-induced toxicity in Drosophila melanogaster. Env. Sci Pollut Res. 28:17519–17531.

Ting, Y., Y. Jiang, C.-T. Ho, and Q. Huang. 2014. Common delivery systems for enhancing in vivo bioavailability and biological efficacy of nutraceuticals. J. Funct. Foods 7:112–128.

Wang, A., Á. V. Keita, V. Phan, C. M. McKay, I. Schoultz, J. Lee, M. P. Murphy, M. Fernando, N. Ronaghan, and D. Balce. 2014. Targeting mitochondria-derived reactive oxygen species to reduce epithelial barrier dysfunction and colitis. Am. J. Pathol. 184:2516–2527.

Zhandi, M., A. Talebnia-Chalanbar, A. Towhidi, M. Sharafi, A. R. Yousefi, and S. M. H. Hussaini. 2020. The effect of zinc oxide on rooster semen cryopreservation. Br. Poult. Sci. 61:188–194.