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Influence of Adding Green Synthesized Gold Nanoparticles to Tris-Extender on Sperm Characteristics of Cryopreserved Goat Semen

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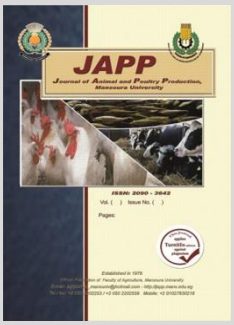

ABSTRACT

There are various challenges during semen cryopreservation used for artificial insemination, oxidative stress is considered as the major factor. Usage of nano-particle materials is an efficient tool to overcome these challenges. In the present article, effects of adding green synthesized gold nanoparticles (GSGNPs, 5; 10ppm/mL) to Tris-extender on sperm characteristics in post-equilibrated and post-thawed semen. Apoptosis and sperm ultrastructure post-thawed were also examined as well as enzyme activity and antioxidant status in post-thawed extender of goat semen were evaluated. Supplementation of GSGNPs (10 ppm) increased ($P<0.05$) motility, livability, and membrane integrity post-equilibration, and also in addition to acrosome integrity post-thawing. Also, viable sperm increased ($P<0.05$), early apoptotic, apoptotic, and necrotic sperm decreased ($P<0.05$) by 10 ppm GSGPNs. In the extender of thawed semen, AST and ALT activities were not affected, total antioxidant capacity and catalase activity were higher ($P<0.05$), while malondialdehyde decreased ($P<0.05$) by 10 ppm GSGNPs. Frequency distribution of sperm cells with intact plasma or acrosomal membranes increased ($P<0.05$), while of those with swollen plasma membranes or typical acrosome reaction decreased ($P<0.05$). However, frequency distribution of sperm cells with slightly swollen or lost plasma membranes as well as atypical acrosome reaction and lost acrosomal membrane was not affected by GSGNPs. Transmission electron microscopy examination revealed no entry of GSGNPs with sperm cells. The present article may suggest considerable addition of GSGNPs at a level of 10 ppm/mL to Tris-extender of cryopreserved goat semen during cryopreservation.

Keywords: Goat, semen, freezing, nano-gold, motility, sperm ultrastructure.

INTRODUCTION

The mammalian sperm cells contain a high ratio of polyunsaturated fatty acids, and they are highly susceptible to cryopreservation process. During semen cryopreservation, a loss of membrane integrity and DNA fragmentation may lead to a reduction in movement, function and fertilizing ability of sperm cells (Bucak *et al*., 2008; El-Badry *et al*., 2016; Sardoy *et al*., 2008) as a result of peroxidative damage and oxidative stress (OS). This is mainly related to the reduced temperature (Stradaioli *et al*., 2007).

The OS resulted from inadequate balance of the antioxidant defense system with free radicals production (reactive oxygen species, ROS) in sperm cells. To improve the motility, livability, and membrane integrity of cryopreserved sperm cells, several studies have also been conducted by adding antioxidants such as glutathione, zinc, selenium, and vitamins C and E to semen extenders of different species (Barkhordari *et al*., 2013; El-Harairy *et al*., 2016; Kotdawala *et al*., 2012).

Nanoparticles (NPs) having 1–100 nm in diameter, have become increasingly common in a variety of medical areas (Barkhordari *et al*., 2013), and the benefits on freezing ability and fertility of nano-elements like Se and Zn in extenders of post-thaw semen of different animal species of cryopreserved semen were recently reported (Khalil *et al*., 2019; Shahin *et al*., 2020). Nano-elements have low toxicity and high bioavailability, because they exhibit novel characteristics, such as a specific more surface area, numerous active surface centers, high surface activity, high catalytic efficiency, and strong adsorption ability (Peters *et al*., 2004; Shi *et al*., 2010).

Gold is an outstanding metal of option in the organic system field, existing organism and medicinal field (Prasannaraj and Venkatachalam, 2017). Gold in the powder form used immunologically as an agent for treating several cases of deficiency, like male impotency and many diseases in South Asian (Astruc *et al*., 2004).

Gold nanoparticles (GNPs) are used widely in industry and science. The GNPs, with diameter of <10 nm, became surprisingly active. This is particularly true at low temperatures, for many reactions, such as CO oxidation and propylene epoxidation (Haruta, 2003). GNPs are inert, non-toxic in nature, possess many related medical applications, exert biological effects (Tripathi *et al*., 2015), can penetrate plasma membrane, and were detected inside the nucleus of human spermatozoa (Moretti *et al*., 2013). No systematic studies of GNPs spermatoxicity have been reported, but penetration of GNPs to spermatozoa could result in fragmentation. However, the possible spermatotoxicity of GNPs in industrial use has been reported elsewhere as a cause of male sterility and, possibly of epididymitis (Manin *et al*., 2007). The cytotoxic effect of GNPs on human sperm motility and viability occurred in a significant dose-dependent manner, 30, 60, 125, 250 and 500 µM (Moretti *et al*., 2013). In the same way, Wiwanitkit *et al*. (2009) observed a decrease in

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sperm motility after the exposure of human semen to 9-nm-sized GNPs (44 ppm). The results of most available reports on the impaired effects of GNPs were for total semen incubated with high levels of GNPs, not on diluted frozen semen.

We thought that reducing level of GNPs in Tris-extender may exhibit the beneficial effects of GNPs, as an antioxidant on improving semen quality of cryopreserved semen. Therefore, the objective of this study was to assess the effect of adding GSGNPs at levels of 5 or 10 ppm/mL to semen extender on the quality of frozen goat semen.

MATERIALS AND METHODS

The experimental work was carried out at the Laboratory of Animal Physiology and Biotechnology, Animal Production Department, Faculty of Agriculture, Mansoura University, from August 2019 to February 2020.

Preparation of *Chenopodium murale* and green synthesis of gold nanoparticles:

According to Abdelghany *et al*. (2019) fifteen gram of completely cleaned leaf in a 250 ml pitcher heated in 60 ml double distilled water for 2 h was used to prepare fresh leaf extract which utilized for the green synthesis of GNPs. Amputation leaf was purified with Whatman No. 40 filter paper. One hundred ml of 4mM water solution of $AuCl_4$ was prepared in a Stoppered flask. Four ml of extract was added to gold chloride at room temperature. The pinkish red color showed that the creation of gold nanoparticles was detected next 18 hours in the dark.

Ultra-morphology of nanoparticles of green synthesized gold:

The morphological evaluation of the NPs extract was performed by transmission electron microscopy (TEM) (JEOL-JEM-2100, JEOL Ltd, Tokyo, Japan) at 160 kV (EM-Unit at Mansoura University). For this procedure, 1 mL of the NPs dispersion sample was properly diluted with double deionized water and sonicated for 2 min using an ultrasonic bath. After dilution, one drop of the NPs was added to a carbon-coated copper grid and the excess material was removed, leaving a thin film stretched over the holes. This was allowed to dry at room temperature before the image was captured and analyzed in the Gatan software (Version 2.11. 1404.0).

The average diameter of the nanoparticles of green synthesized gold was 19.28 nm, (Figure 1).

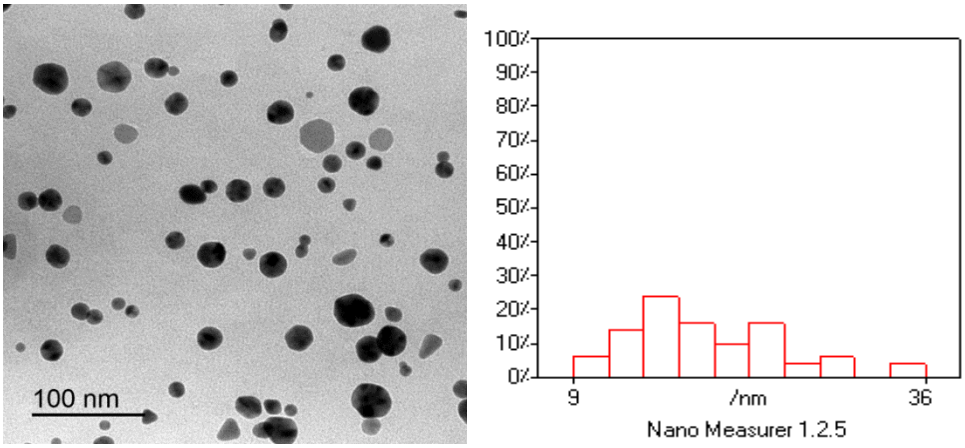

Figure 1. Ultramorphology and histogram of the particle size of green synthesized gold nanoparticles by transmission electron microscopy.

Animals:

A total of 3 mature Baladi bucks (50-60 kg live body weight and 2-4 years of age), a local goat breed in Egypt were selected as semen donors; an artificial vagina was used to collect semen. All bucks were raised under the same environmental conditions, they were daily fed a concentrate feed mixture (CFM) at a level of 1.00 kg (14% CP) and 0.750 kg of Berseem hay, and they had free access to trace mineralized salt lick blocks and drinking water at all times. The study was approved by the Scientific Research Ethics Committee of Mansoura University in accordance with Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines.

Semen collection:

Semen was routinely collected from the 3 donors once weekly for 7 weeks before feeding at 7-8 a.m. by using the conventional artificial vagina method. Twenty-one semen ejaculates were transferred to the laboratory immediately after collection and placed in a water bath at 37 °C. Only semen with a mass motility of ≥ 70% was pooled, divided into the three extender types, and packaged in 0.25 mL straws.

Semen extender preparation:

The prepared control extender contained 3.025 g Tris (Sigma Chemical Co., St. Louis, MO, USA), 1.66 g citric acid monohydrate (Sigma), 1.25 g glucose (Sigma, Aldrich), 1% soybean lecithin (L-a-phosphatidyl choline, LAB, product number MC041), 5% glycerol, 100 IU/mL penicillin, and 100 µg/mL streptomycin. The same extender was supplemented with either 5 or 10 ppm/mL of green synthesized gold NPs (GSGNPs).

The extenders were gently shaken and warmed up to 37 °C in a water bath before use. The osmolarity and pH were measured before the addition of cryoprotectants and were adjusted to the osmolarity of 280-300 mOsmol (Micro-Osmometer, Loser Type 6, Löser Messtechnik, Berlin, Germany) and a pH value of 7.2-7.3 (PH/mV Temperature Meter, Jenway 3510, Jenway, Staffordshire, UK).

Semen freezing:

The tubes containing the extended semen were shaken gently immediately and placed in a water bath

containing warm water (37 °C). The pooled semen was diluted at a ratio of 1:20 (semen/extender) with different extender types. The diluted semen was cooled to 5 °C for 2 h (for equilibration) before being loaded into 0.25 mL straws. The straws were placed 4 cm over liquid nitrogen vapor for 10 min and then immersed in liquid nitrogen. The straws remained in liquid nitrogen until thawing at 37 °C in a water bath for 30 s.

Semen evaluation:

Sperm progressive motility:

A 10 µL aliquot of diluted semen was placed on a warm slide and covered with a coverslip; then, the number of spermatozoa exhibiting forward movement in a long semiarc pattern was determined in five fields, each containing 200 sperm cells, to calculate the percentage of progressive motility. A phase-contrast microscope supplied with a hot stage at 37 °C (DM 500; Leica, Switzerland) was used at 100× magnification. The same professional investigator performed the blind analysis that was conducted in triplicates.

Sperm livability and abnormality:

A smear of the diluted semen sample on a glass slide was stained with a dual staining procedure (5% eosin and 10% nigrosin) (Moskovtsev and Librach, 2013). Two hundred spermatozoa from each sample were examined under 400× magnification using a light microscope (Leica DM 500; Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany). The number of dead spermatozoa (red-stained) or live spermatozoa (unstained) was counted. Additionally, the morphological abnormalities of the spermatozoa (i.e., spermatozoa bearing head, tail, and cytoplasmic droplet abnormalities) were determined (Menon *et al*., 2011).

Plasma membrane integrity:

The hypo-osmotic swelling test (HOS-t) was used to evaluate the functional plasma membrane of spermatozoa (Neild *et al*., 1999). About 100 µL of a hypo-osmotic solution (6.75 g/L fructose and 3.67 g/L sodium citrate, for an osmolality level of 75 mOsmol/L) was added to 10 µL of diluted semen and incubated for 30 min at 37 °C. Then, 10 µL of the mixture was placed on a microscope slide and mounted with a coverslip. In a field of 300 spermatozoa (from each sample), swollen and coiled tails were evaluated under phase-contrast microscopy (Leica DM 500; Leica Mikrosysteme Vertrieb GmbH) under 400× magnification.

Acrosome integrity:

A drop of equilibrated or frozen-thawed semen was kept on a clean, grease-free, pre-warmed glass slide and was air-dried. The slide was immersed in 5% formaldehyde for 30 min for semen smear fixation, washed in running tap water, dried, immersed in a Giemsa solution for 3 h at 37 °C, washed in running tap water, and dried. Then, the slide was examined under the oil immersion lens of a phase-contrast microscope and 200 sperm cells were counted.

Sperm apoptosis and necrosis (Annexin V/PI Assay)

The semen samples were processed for annexin V staining as described by Chaveiro *et al*. (2007), but with some modifications. Briefly, 1 mL of sperm suspension was added to a 5 mL tube, suspended in a 2 mL binding buffer, and mixed thoroughly. One hundred microliters of the sperm suspension was mixed with 5 µL of annexin V (FITC label) and 5 µL propidium iodide (PI, PE label) and incubated for at least 15 min in darkness at room temperature. After incubation, the sperm was suspended in a 200 µL binding buffer. Flow cytometric analysis was performed on an Accuri C6 Cytometer (BD Biosciences, San Jose, CA) using the Accuri C6 software (Becton Dickinson) for data acquisition and analysis (Masters and Harrison, 2014). The negative or positive (A- or A+) annexin V percentages, the negative or positive (PI- or PI+) PI, and the double positive cells were evaluated. As described by (Peña *et al*., 2003), four different categories of spermatozoa were determined: (i) viable (A-/PI-), with no fluorescence signal and recorded as live without membrane dysfunction (live sperm); (ii) early apoptotic (A+/PI-) but viable spermatozoa, labeled with annexin V but not with PI (live sperm); (iii) apoptotic spermatozoa (A+/PI+) labeled with annexin V and PI and with damaged permeable membranes (dead sperm); (iv) necrotic spermatozoa (A-/PI+), labeled with PI but not with annexin V, that had completely lost their sperm membranes (dead sperm).

Antioxidant assay and enzyme activity in the extenders of thawed semen:

Extenders of frozen/thawed semen were taken from all treatments. The extender was separated from each sample after collection by centrifugation for 15 min at 4000 rpm and then it was stored at -20 °C until analysis. The assays of the concentration of total antioxidants (Koracevic *et al*., 2001), catalase (Aebi, 1984), malondialdehyde (MDA) (Ohkawa *et al*., 1979), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) (Reitman and Frankel, 1957) were analyzed by commercial kits (Biodiagnostic, Egypt) using a spectrophotometer (Spectro UV-VIS Auto UV-2602; Labomed, Los Angeles, USA).

Transmission electron microscope (TEM) evaluation of semen samples:

The samples were processed for transmission electron microscopy (TEM) according to (Oliveira *et al*., 2011). Briefly, the straws from each treatment were washed three times by centrifugation at 1000 rpm for 5min using phosphate buffered saline, and suspended in a fixative solution of 2.5 % (w/v) buffered glutaraldehyde and 2 % (w/v) paraformaldehyde in a 0.1 M sodium phosphate buffer (pH 7.4) for 2 h at 4 °C. The samples were then washed and post-fixed in 1 % osmium tetroxide for 1 h at room temperature in darkness. The fixed samples were dehydrated in an ethanol gradient, treated with acetone, embedded in an Epon resin (Epon 812; Fluka Chemie, Switzerland), and ultrathin-sectioned (60 - 80 nm) for TEM. Ultrathin sections were observed at 160 kV using a JEOL 2100 TEM at 80 kV. The sperm ultrastructures of plasma membrane and acrosome were examined in 100 sperm cells per treatment.

Statistical analysis:

The obtained data were statistically analyzed by a one-way ANOVA design using a software package (SAS, 2007). A completely randomized design was used based on the following model: $Y_{ij} = \mu + G_i + e_{ij}$, where μ = the overall mean, G_i = treatment (1…..3), and e_{ij} = residual error. The value percentages were transformed by arcsine values before analysis. Significant differences among groups were tested by Duncan's multiple range test (Duncan, 1955) and set at $P<0.05$.

RESULTS AND DISCUSSION

Results

Sperm characteristics in equilibrated semen:

Data in Table 1 showed that GSGNPs supplementation to the extender of goat equilibrated semen significantly (P<0.05) increased sperm characteristics (progressive motility, livability, and integrity of sperm membrane) in comparison with the control extender, but the differences between both levels of GSGNPs were not significant. However, acrosome integrity and sperm abnormality were not affected by GSGNPs supplementation.

Table 1. Effect of supplementing Tris-extender with GSGNPs on sperm characteristics (%) in equilibrated goat semen (at 5 °C for 2 h).

Treatment	Progressive motility	Livability	Membrane integrity	Acrosome integrity	Abnormality
Control	75.0±1.09^{b}	77.1±1.10^{b}	76.7±0.97^{b}	93.4±0.30	6.4±0.53
GSGNPs (5 ppm)	81.4±1.43^{a}	83.0±1.65^{a}	82.3±2.18^{a}	94.6±0.61	6.7±0.92
GSGNPs (10 ppm)	85.7±2.30^{a}	86.6±2.32^{a}	84.9±1.72^{a}	95.6±0.53	5.3±0.64

a-b Means denoted within the same column with different superscripts are significantly different at P<0.05. GSGNPs=Green Synthesized Gold Nanoparticles

Sperm characteristics in thawed semen:

In post-thawed semen, supplementation of GSGNPs (5 and 10 ppm) significantly (P<0.05) improved sperm characteristics (progressive motility, livability, and integrity of acrosome and membrane of sperm cells compared with the control extender, but GSGNPs at a level of 10 ppm showed the best results. On the other hand, the differences in sperm abnormality were not significant (Table 2). These results are in association with improving sperm characteristics in equilibrated semen, but acrosome integrity had additional improvement in thawed than in equilibrated semen by increasing GSGNPs level from 5 to 10 ppm compared with the control in goat semen extender.

Table 2. Effect of supplementing Tris-extender with GSGNPs on sperm characteristics (%) in post-thawed goat semen (at 37 °C for 30 s).

Treatment	Progressive motility	Livability	Membrane integrity	Acrosome integrity	Abnormality
Control	26.4±0.92^{b}	28.0±0.93^{b}	25.3±1.04^{b}	89.3±1.36^{b}	11.1±0.63
GSGNPs (5 ppm)	40.0±2.44^{a}	41.7±2.37^{a}	39.6±2.70^{a}	90.3±1.19^{ab}	9.7±0.92
GSGNPs (10 ppm)	42.1±1.49^{a}	45.3±1.70^{a}	43.9±1.93^{a}	91.6±0.75^{a}	10.4±0.97

a-b Means denoted within the same column with different superscripts are significantly different at p<0.05. GSGNPs=Green Synthesized Gold Nanoparticles

Sperm apoptosis and necrosis in thawed semen:

Results of Annexin V/PI Assay presented in Table 3 reveal that viable sperm percentage was significantly (P<0.05) improved by increasing level of GSGNPs, being the highest for 10 ppm GSGNPs, moderate for 5 ppm, and the lowest for the control extender. Early apoptotic and apoptotic sperm cells significantly (P<0.05) decreased to the lowest values with 5 ppm GSGNPs, and significantly (P<0.05) decreased with 10 ppm as compared to control. However, necrotic sperm cells were significantly (P<0.05) decreased with 10 ppm GSGNPs, while significantly (P<0.05) increased with 5 ppm GSGNPs when compared with the control extender. These results indicated the highest viable and lowest necrotic spermatozoa in thawed goat semen extended with Tris extender supplemented with 10 ppm GSGPNs.

Table 3. Effect of supplementing Tris-extender with GSGNPs on the percentages of viable, early apoptotic, apoptotic, and necrotic spermatozoa in post-thawed goat semen (Annexin V/PI assay).

Treatment	Viable	Early Apoptotic	Apoptotic	Necrotic
Control	30.3±0.00^{c}	30.4±0.03^{a}	25.7±0.03^{a}	13.7±0.06^{b}
GSGNPs (5 ppm)	77.9±0.17^{b}	0.9±0.14^{c}	0.6±0.09^{c}	20.7±0.06^{a}
GSGNPs (10 ppm)	81.1±1.50^{a}	7.7±1.36^{b}	1.8±0.32^{b}	9.5±0.17^{c}

a-c Means denoted within the same column with different superscripts are significantly different at p<0.05. GSGNPs=Green Synthesized Gold Nanoparticles

Enzyme activity in the extender of post-thawed semen:

Data of AST and ALT enzymes activity in the extender of post-thawed semen between GSGNPs treatments and control showed no changes (Table 4).

Table 4. Effect of supplementing Tris-extender with GSGNPs on enzyme activity (AST and ALT) in extenders of post-thawed goat semen.

Treatment	ALT (U/ml)	AST (U/ml)
Control	17.2±1.85	43.2±2.56
GSGNPs (5 ppm)	21.2±2.56	46.2±5.65
GSGNPs (10 ppm)	21.6±1.72	37.4±2.77

GSGNPs=Green Synthesized Gold Nanoparticles

Oxidative stress of the extender of post-thawed semen

Total antioxidant capacity and catalase activity of thawed semen extended with 5 and 10 ppm GSGNPs was significantly (P<0.05) higher than in the control semen, being the highest for 10 ppm GSGNPs. Level of MDA showed significantly an opposite trend (Table 5).

Table 5. Effect of supplementing Tris-extender with GSGNPs on the total antioxidant capacity, catalase activity, and malondialdehyde (MDA) level in the extender of post-thawed goat semen.

Treatment	TAC (mM / L)	Catalase (U/L)	MDA (nmol/mL)
Control	0.24±0.02^{b}	30.1±5.23^{b}	15.5±0.33^{a}
GSGNPs (5 ppm)	0.51±0.05^{a}	64.3±8.73^{a}	6.3±0.14^{b}
GSGNPs (10 ppm)	0.54±0.03^{a}	64.7±9.16^{a}	4.8±0.24^{c}

a-b Means denoted within the same column with different superscripts are significantly different at p<0.05. GSGNPs=Green Synthesized Gold Nanoparticles

This means that GSGNPs supplementation at a level of 10 ppm showed higher antioxidant property during goat semen preservation.

Ultra-morphological characters of sperm plasma membrane and acrosomal membrane:

Figure 2 (transmission electron microscopy, TEM) show the normal and abnormal ultrastructure of spermatozoa of cryopreserved goat semen extended with GSGNPs.

Frequency distribution of sperm cells with intact plasma or acrosomal membranes significantly (P<0.05) increased, while of those with swollen plasma membranes or typical acrosome reaction significantly (P<0.05) decreased only with 10 ppm GSGNPs level as compared to the control. However, frequency distribution of sperm cells with slightly swollen or lost plasma membranes as well as atypical acrosome reaction and lost acrosomal membrane was not affected by GSGNPs supplementation (Table 6).

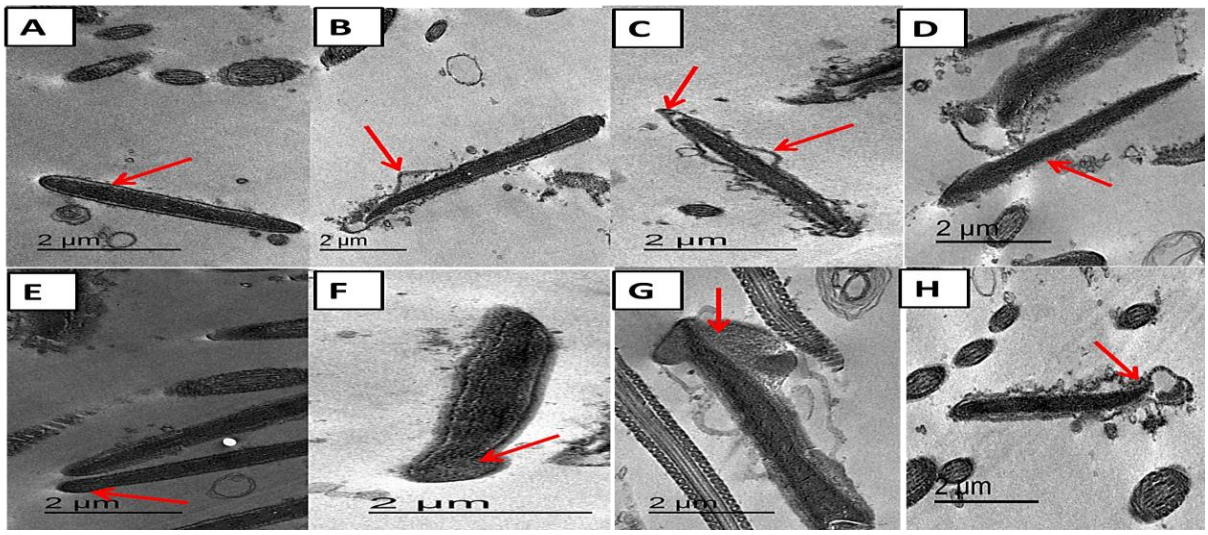

Figure 2. Transmission electron micrographs (80 kV) of longitudinal and ultrathin cross-sections of different goat sperm after thawing. (A) Sperm with intact plasma membrane, (B) Sperm with slightly swollen plasma membrane, (C) Sperm with swollen plasma membrane, (D) Sperm with lost plasma membrane, (E) Sperm with intact acrosome, (F) Sperm with typical acrosome reaction, (G) Sperm with atypical acrosome reaction and (H) Sperm with lost acrosome.

Table 6. Effects of supplementing Tris-extender with GSGNPs on the ultrastructure of plasma and acrosoml membrane of sperm cells post-thawing.

Item	Control	GSGNPs (5 ppm)	GSGNPs (10 ppm)
Plasma membrane (PM):			
Intact(1)	46.0±5.01^{b}	58.0±4.96^{ab}	63.0±4.85^{a}
Slightly swollen	11.0±3.14	11.0±3.14	14.0±3.49
Swollen	34.0±4.76^{a}	23.0±4.23^{ab}	15.0±3.59^{b}
Lost(2)	9.0±2.88	8.0±2.73	8.0±2.73
Acrosomal membrane (AM):			
Intact(1)	56.0±4.99^{b}	68.0±4.69^{ab}	71.0±4.56^{a}
Typical acrosome reaction(3)	25.0±4.35^{a}	16.0±3.68^{ab}	12.0±3.27^{b}
Atypical acrosome reaction(4)	9.0±2.88	8.0±2.73	8.0±2.73
Lost	10.0±3.02	8.0±2.73	9.0±2.88

a-b Means denoted within the same row with different superscripts are significantly different at p<0.05. GSGNPs=Green Synthesized Gold Nanoparticles

(1) Intact; sperm with intact plasma membrane and sperm heads exhibited intact acrosomal membrane surrounding the acrosomal ground substance.

(2) Lost: sperm without plasma membrane or acrosome

(3) Acrosome reaction: a swelling of acrosomal ground substance with vesicles of fused plasma and outer acrosomal membranes.

(4) Atypical acrosome reaction: sperm head presenting swelling of acrosomal ground substance dispersed under the swollen outer acrosomal.

Discussion

The entire process of mammalian spermatozoa cryopreservation is highly susceptible to different environmental conditions of oxidative stress (OS), which is the main factor affecting semen quality during freezing process. During semen cryopreservation, as a result of containing sperm plasma membrane high unsaturated fatty acids content, OS increase generation of reactive oxygen species and free radical scavenging system was decreased resulting in adverse effects on integrity of plasma membrane, DNA, and function of sperm cells (Agarwal *et al*., 2014). Therefore, addition of exogenous antioxidant is required to maintain spermatozoa during cryopreservation by stimulating antioxidant defense system and protecting sperm cells from inadequate condition such as OS and/or low temperature of cryopreservation and thawing.

Therefore, we hypothesize that several NPs may have possible beneficial effects when it was added to semen extender of goats, and may provide a protective effect as an antioxidant. Recently, several authors ascribed that GSNPs can penetrate spermatozoa plasma membrane and cause DNA fragmentation, the possibility of sperm toxicity (Moretti *et al*., 2013), and decrease in sperm motility (Wiwanitkit *et al*., 2009).

In the current study, supplementation of GSGNPs at a levels of 5 or 10 ppm/mL to Tris-extender improved (P<0.05) sperm characteristics in equilibrated and thawed semen (progressive motility, livability, and membrane integrity of spermatozoa), without significant differences between both GSGNPs levels, in addition to improving acrosome integrity in thawed semen. Moreover, GSGNPs (10 ppm) increased (P<0.05) viable sperm percentage, while decreased (P<0.05) early apoptotic, apoptotic and necrotic sperm cells. These findings are in good association with improving the antioxidant status of semen extender of

thawed semen, in terms of increasing (P<0.05) level of total antioxidant capacity and catalase activity with marked reduction (P<0.05) in malondialdehyde (MDA) level of thawed semen extended with 10 ppm GSGNPs in comparison with the control extender without GSGNPs. While, the activity of AST and ALT in the extender of thawed semen was not affected significantly by GSGNPs.

Additionally, TEM examination showed higher (P<0.05) sperm cells with intact plasma or acrosomal membranes, and decreased (P<0.05) typical acrosome reaction. These improvements can be due to the antioxidative properties of the used NPs, particularly at a level of 10 ppm. Decreasing the level of MDA as biomarker for membrane lipid peroxidation of omega-3 and omega-6 polyunsaturated fatty acids (Esteribauer and Cheeseman, 1990), enhancing antioxidant system consisting of catalase by GSGNPs may indicate these improvements in sperm characteristics against OS. The present results are in agreement with recent reports which mentioned that trace elements NPs have membrane protective function, increase antioxidant enzymes and improve frozen semen quality (Falchi *et al*., 2018; Khalil *et al*., 2019; Shahin *et al*., 2020). On the other hand, no systematic studies of GSGNPs spermatoxicity have been reported, but penetration of GNPs sperm cells could result in fragmentation. Furthermore, a decrease in sperm motility in human semen exposed to 9-nm-sized GNPs at a level of 44 ppm was reported by (Wiwanitkit *et al*., 2009) which are higher than levels of GSGNPs used in the current study.

Gold in the powder form used immunologically as an agent for treating several cases of deficiency, like male impotency and many diseases in South Asians (Astruc *et al*., 2004). The GNPs in diameter of <10 nm become active at low temperatures (Haruta, 2003), and are non-toxic in nature, possess many related medical applications and acquire biological effects (Tripathi *et al*., 2015). The protective effect of GSGNPs on sperm characteristics of cryopreserved semen may be due to the formation of a protective layer of NPs around the sperm cells, by which lipid peroxidation at the sperm plasma membrane was prevented. According to our TEM examination, the GSGNPs were accumulated around the sperm cells and did penetrate into the sperm. Similar observation was reported by Isaac *et al.* (2017) when they used nano-particles of ZnO in semen extender.

Therefore, the current result recommends the considerable addition of GSGNPs at a level of 10 ppm to Tris-extender for improving goat semen cryopreservation. Further studies are needed for exploring the effects of GSGNPs at various levels with different types of extenders on sperm quality of cryopreserved semen in different animal species.

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تأثير إضافة جزيئات نانو الذهب إلى مخفف التريس على خصائص الحيوانات المنوية للماعز المحفوظة بالتجميد

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هناك العديد من التحديات خلال حفظ السائل المنوي بالتجميد والذي يستخدم في التلقيح الصناعي، ويعتبر الإجهاد التأكسدي هو أحد العوامل الرئيسية من هذه التحديات. يعتبر إستخدام جزيئات المواد في صورة النانو أداة فعالة للتغلب على هذه التحديات. لذلك كان الهدف من هذه الدراسة هو تحديد تأثير إضافة جزيئات نانو الذهب بتركيزي (٥ ، ١٠ جزء في المليون/مل) إلى مخفف التريس على خصائص الحيوانات المنوية بعد فترة الإتزان وبعد عملية التجميد والإسالة. كما تم فحص موت الحيوانات المنوية المبرمج وكذلك التركيب الدقيق للحيوانات المنوية بعد التجميد والإسالة. كذلك تم تقييم نشاط الإنزيمات والحالة المضادة للأكسدة في مخفف السائل المنوي بعد التجميد والإسالة. أدت إضافة جزيئات نانو الذهب إلي مخفف السائل المنوي للماعز بتركيز ١٠ جزء في المليون / مل إلي زيادة معنوية في نسبة كل من الحركة التقدمية، والحيوية وسلامة الغشاء بعد فترة الإتزان، بالإضافة إلى سلامة الأكروسوم بعد التجميد والإسالة. أيضاً، زادت معنوياً النسبة المئوية للخلايا المنوية الحية، في حين إنخفضت النسبة المئوية للخلايا المنوية التى فى بداية مرحلة الموت المبرمج، خلايا الحيوانات المنوية التى تعرضت للموت المبرمج وكذلك الحيوانات المنوية ذات التنكرز وذلك عند إضافة جزيئات نانو الذهب إلي مخفف السائل المنوي للماعز بتركيز ١٠ جزء في المليون / مل. لم تتأثر أنشطة إنزيمات ألانين ترانس أمينيز، أسبارتات ترانس أمينيز في مخفف السائل المنوي بعد التجميد والإسالة، بينما زادت قدرة مضادات الأكسدة الكلية ونشاط إنزيم الكاتاليز وإنخفض تركيز المالون داى ألدهيد وذلك عند إضافة جزيئات نانو الذهب إلي مخفف السائل المنوي للماعز بتركيز ١٠ جزء في المليون / مل. إزداد التوزيع التكراري للحيوانات المنوية ذات الغشاء البلازمي والأكروسوم السليم معنوياً، بينما إنخفض معنوياً عدد الحيوانات المنوية التي بها إنتفاخ للغشاء البلازمي أو تفاعل الأكروسوم النموذجي. ومع ذلك لم يتأثر التوزيع التكراري لخلايا الحيوانات المنوية ذات الغشاء البلازمي الذي به إنتفاخ طفيف أو الغشاء البلازمي المفقود بالإضافة إلى تفاعل الأكروسوم غير النمطي وغشاء الأكروسوم المفقود وذلك عند إضافة جزيئات نانو الذهب إلي مخفف السائل المنوي للماعز بتركيز ١٠ جزء في المليون / مل. أيضاً، كشف فحص التركيب الدقيق للحيوانات المنوية بإستخدام الميكرسكوب الإليكتروني النافذ عدم إنتقال جزيئات نانو الذهب إلي داخل الحيوانات المنوية السليمة. تقترح هذه الدراسة أنه يمكن بنجاح إضافة جزيئات نانو الذهب إلي مخفف السائل المنوي للماعز بتركيز ١٠ جزء في المليون / مل وذلك خلال عملية حفظ الحيوانات المنوية بالتجميد.

الكلمات المفتاحية: الماعز ، السائل المنوي ، التجميد ، الذهب النانو ، الحركة ، التركيب الدقيق للحيوانات المنوية.