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Improving Awassi Rams Epididymal Sperms Viability by Adding Zinc and Cerium Oxide Nanoparticles

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THE PRESENT study was aimed to the exposure Awassi rams epididymal sperms sourced from abattoir to two doses of Zinc oxide and Cerium Oxide Nanoparticles on sperms viability after stored at $4C^0$ for 72 hrs as a cooling method. The experiment was carried out in the artificial insemination lab at the University of Mosul, College of Veterinary Medicine, Mosul, Iraq. Sixteen healthy mature Awassi rams, aged between 1-4 years, were slaughtered at a nearby slaughterhouse, and a total of sixteen pairs of testicles (n=32) were collected. Five parts of sperm samples were separated and diluted in egg yolk extender, the first one left as control, second part received Cerium oxide NPs at 50 µg/ml, third part received Cerium oxide NPs at 25 µg/ml, while Zinc oxide NPs as 1 mg/ml was add to the fourth part and 1.5 mg/ml of Zinc oxide NPs added to fifth part. All sperms samples stored at $4C^0$ for 72hrs. Sperms live percentage, individual motility, sperms abnormalities were estimated. Data of present study showed that cerium oxide NPs at 50 µg/ml had best effect on sperms viability and improve sperms live percentages, individual motility, low sperms abnormalities were reported after storage. Cerium oxide NPs at 25 µg/ml had mild improve of sperms viability. Zinc oxide NPs at dose 1 mg/ml and 1.5 mg/ml had no effect or falling sperms viability after 24, 27 hrs of storage. In conclusion, CeO2 NPs in dose 50 µg/ml improve epididymal sperm characteristics after storage at $4C^0$ for 72hrs. Zinc oxide NPs at dose 1 mg/ml and 1.5 mg/ml had not improve sperms viability.

Keywords: Cerium oxide NPs, Zinc oxide NPs, Epididymal sperms, Ram.

Introduction

There were growing interest for using and adding Nanoparticles NPs during semen storage to increase the longevity of spermatozoa on the way to improve male fertility and semen viabilities during different ways [1]. In addition, compared to known conventional materials, properties of Nano-materials can allow to use less material with new, efficient, chemical and physical reactions. Recently, semen storage procedures have been adjusted with these characteristics in addition to increased cellular absorption, reactivity, surface area activity and surface custody, binding qualities, anti-microbial, and antioxidant activity[2–3]. Several elements, like Zinc oxide NPs and Cerium oxide NPs have been consider improve semen quality [5-6]. Zinc oxide NPs and Cerium oxide NPs have anti-oxidative reaction against reactive oxygen species (ROS) or free radicles which causes harmful effect to sperms during storage [7], furthermore, according to previous literature, Zinc oxide NPs antimicrobial activity have been proven [8].

Many reports refers to using Zinc oxide NPs to improved semen characteristics and reproductive performance in Ram [9], buffalo [10], camel [11], human [12], rabbit [13] and rooster [14], also these reports mention the positive effect of

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Zinc oxide NPs and improving sperms quality during storage by keep plasma membrane integrity, mitochondrial function, improved sperm progressive motility, viability, maintained ultrastructure morphology, decrease apoptosis and increase sperm count [15], but its effect was dose-depended according in different papers. There were a few reports explain exposure of Cerium Oxide (CeO_2 NPs) on Awassi epididymal sperms characteristics, Falachi et al., [16] reveled CeO_2 NPs beneficial effects on kinematic and morphologic parameters on ram semen after 96 hrs of storage at $4C^0$. Cerium Oxide NPs benefit during semen storage was improved in Ram [16], Buck [17] and Rat [18].

The present study aimed to the testified adding two doses of Zinc oxide NPs and two doses of Cerium Oxide NPs on the sperm parameters after stored at $4C^0$ for 72 hrs as cooling storage of Awassi rams epididymal sperms.

Material and Methods

Animal specimens and the research area

The study was conducted from September 2022 to May 2023 at the Artificial Insemination Lab, College of Veterinary Medicine, University of Mosul, Mosul, Iraq (N: 36° 20' 24": E: 043° 07' 48"). Awassi rams, aged 1-4 years, were slaughtered at a local abattoir were conducted in the study. Sixteen pairs of testicles (n=32) were collected and within an hour, the testes were cleaned and washed with penicillin and streptomycin with regular saline. All samples were transferred to the lab by cooling box.

Epididymal Sperms Extraction

The cauda epididymis was sliced and squeezed in a Petri plate at room temperature to extract the spermatozoa. The sperms were collected using a glass graduated tube with a capacity of 15 ml [19]. All sperms parameters measured by using a light microscope using. The examination of sperm properties evaluated for every sample prior to dilution. When sperm motility fell below 70%, it was deemed poor and the sample was discarded.

Sperm concentration

Sperm concentration was done by using coloro-meter apparatus by take 0.1 ml of sperms and mixed with 0.9 ml normal saline, then mixed carefully and take the apparatus value for every sperm sample. the applied equation of ram semen as following: Sperm concentration=[(apparatus read ×2514.1)- 537.38] × 10^6 as same way was described by [20].

Progressive sperm motility (%)

One drop of sperms was added to a graduated test tube that held two milliliters of warm physiological normal saline (0.9% NaCl) and was suspended in a water bath at 37°C in order to facilitate measurement of sperms movement. gentle shake with a warm Pasteur pipette was used to remove one drop of semen from the test tube and place it on a heated slide. A heated cover slide was placed over the drop, and it was promptly examined with a 40x objective lens. The samples were scored based on the proportion of spermatozoa that vigorously swam forward across the field of view.The degree of motion calculated in score system in value from 1-100% in same way described by [21].

Live sperm percentage (%)

The procedure was done by combining one drop of sperms made as way described by [22] with a mixture of Red Eosin %5 and Nigrosine %10 stains, and sperm samples. Then, left the slide for one to two minutes before examination. Using a 400x magnification, calculating 100 spermatozoa in different microscopic fields. The color of the head can be used to determine the number of live sperm; a white head indicates a live sperm cell, while a red head indicates a dead sperm.

Abnormal sperm percentage (%)

To estimate the incidence of abnormal spermatozoa, the same smear utilized for the live-dead count was also employed for that purpose. Using a manual tally counter, the percentage of abnormal sperm was determined based on the morphologies of the sperm components. Each slide contained 100 spermatozoa, counted in the manner specified by [22].

Semen extender and nanoparticles

Sperms were diluted in egg yolk extender as ratio 1:10 (semen: extender). The extender contained 10% egg yolk, 2.9% sodium citrate, 2.4 grams fructose, 100.000 IU and 100 mg of penicillin and streptomycin in a 100 milliliter volume of distal water.

In present study Doses of Zinc oxide NPs(ZnO), (Zinc Oxide Nano-powder, MDL Number: MFCD00011300, EC No: 215-222-5, The Material Science Manufacture; USA) and Cerium oxide NPs (Ce_2O_3), (MDL Number: MFCD00010933 EC No: 215-150-4, The Material Science Manufacture; USA) based or similar to that doses were uses in previous reports in Ram [16] and Buck [17]. Five parts of extender for each epididymal sperms samples or ejaculate

(n=32) were perpetrated, the first one left as control group, the second one received Cerium oxide NPs (50µg/ml) , the third one received (25µg/ml) Cerium oxide, the fourth received Zinc oxide NPs (1 mg/ml), the fifth received (1.5 mg/ml) of Zinc oxide.

To prevent osmotic shock, time of equilibration was allowed. Following 2- 5-minute of equilibration period in the water bath, all extenders examined for motility, sperm abnormalities, and the percentage of live and dead sperm. The samples in test tubes were placed in a refrigerator at 4 C^0, all samples were examined under a light microscope after 24, and 27 hours of affixed time.

Statistical analysis

The current study's findings were presented as mean + standard error. One-way ANOVA was used to compare the data using Sigma Stat (Jandel Scientific software V3.1). Duncan's Multiple Range Test was used to establish the significance threshold, which came out to be ($P < 0.05$).

Results

Cerium oxide effect after 24hrs of cooled storage

Data of present study were showed in Table 1 revealed that Cerium oxide NPs in 50µg /ml have superior significantly effect on improving live sperm percentage, and sperm individual motility ratio which were 86.2± 0.6 , 81.7±1.1, respectively when compare with control group (67.6±0.5). Sperms abnormalities show significantly lower value (3.16± 0.9) which was significantly higher than control group (4.4± 0.2).

Cerium oxide in 25µg/ml data (Table 1) show lower or slightly similar values of Cerium oxide (50µg /ml) in on its effect on Sperm live percentage, Sperm individual motility, Sperms abnormalities which were 80±0.3,75.7±1.0, 3.3±0.9 % respectively.

Zinc oxide (mg) effect after 24hrs of cooled storage

Zinc oxide in dose of 1 and1.5 mg/ml mg leads to negative effect of sperm characteristics after 24hrs of storage, data of Sperm live percentage and Sperm individual motility were lower than its records when compare with control group which were 50±0.5, 56.8±0.8, respectively and sperm abnormalities show no significant differences when compare with control group which were 4.5±0.9, 4.9±0.2 and 4.4± 0.2, of control group respectively.

Cerium oxide effect **after 72hrs of cooled storage**

Data of sperm viability after 72hrs of storage study Table 2 showed Cerium oxide in 50µg / ml have positive or significantly improve live sperm percentage, and sperm individual motility ratio which were 60.3±0.4, 56.2±0.6, respectively when compare with control group (40.6±1.2±1.2,32.2±0.8). Sperms abnormalities show lower value (7.0±0.9) which was significantly lower than control group (8.2± 0.5).

Cerium oxide in 25µg /ml data (Table 2) show similar values of Cerium oxide in 50µg/ml on its effect on Sperm live percentage, Sperm individual motility, Sperms abnormalities which were 49.3±0.4, 48.5±1.2, 7.5±0.6, respectively.

Zinc oxide (mg) effect after 72hrs of cooled storage

Zinc oxide in both dose of 1 and 1.5 mg/ml leads to negative effect of sperm characteristics after 27hrs of storage, data of Sperm live percentage and Sperm individual motility were lower than its records when compare with control group which were (40.0±0.8, 42.2±1.3) for live sperm, (40.9±0.1, 43.0±0.2) for sperm motility, respectively. Sperm abnormalities show significantly higher differences when compare with control group which were (10.5±0.4, 12.3±2.2) and 8.2± 0.5 Respectively. Fig. 1.

TABLE 1. Showed effect of Cerium oxide and Zinc oxide nanoparticles on sperms viability testes after 24hrs of storage. Sperm concentration > 25× 10^9

Sperm viability tests (100%)	Control	Cerium oxide		Zinc oxide	
		50 µg/ml	25 µg/ml	1 mg/ml	1.5 mg/ml
Sperm live percentage	67.6±0.5^b	86.2± 0.6^a	80±0.3^a	50±0.5^c	67±0.7^b
Sperm individual motility	61.7±0.8^b	81.7±1.1^a	75.7±1.0^a	56.8±0.8^c	55.8±0.4^c
Sperms abnormalities	4.4± 0.2^b	3.16±0.9^a	3.3±0.2^a	4.5±0.9^b	4.9±0.2^b

abc: Different letters in same arrows indicate that the values are substantially different at ($P<0.05$).

TABLE 2. Showed effect of Cerium oxide and Zinc oxide nanoparticles on sperms viability testes after 72hrs of storage. Sperm concentration > 25× 10^9

Sperm viability tests (100%)	Control	Cerium oxide		Zinc oxide	
		50 µg/ml	50 µg/ml	1 mg/ml	1.5 mg/ml
Sperm live percentage	40.6±1.2^b	60.3±0.4^a	49.3±0.4^b	40.0±0.8^b	40.9±0.1^a
Sperm individual motility	32.2±0.8^b	56.2±0.6^a	48.5±1.2^b	42.2±1.3^b	43.0±0.2^b
Sperms abnormalities	8.2± 0.5^a	7.0±0.9^a	8.5±0.6^b	10.5±0.4^b	12.3±2.2^b

abc: Different letters in same arrows indicate that the values are substantially different at (P<0.05).

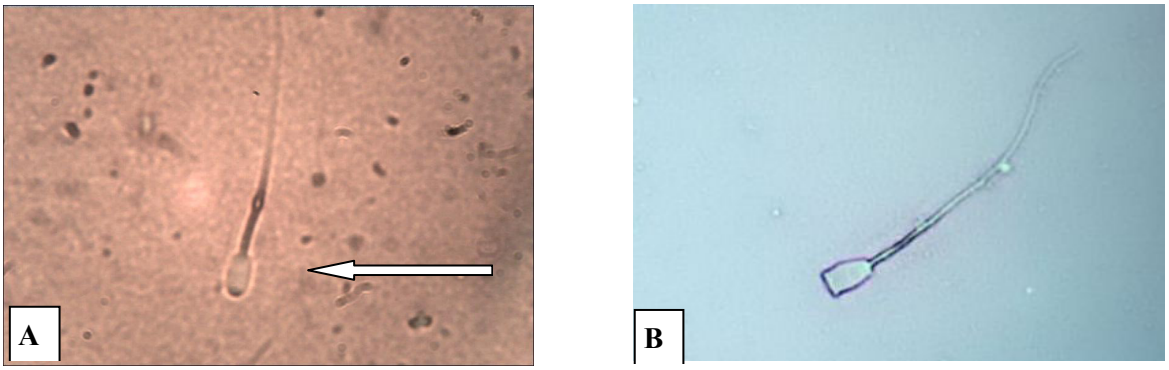

Fig. 1. Pic A. represent presence of cytoplasmic droplet (arrow) . Pic B.. represented absence acrosome (arrow). All samples Stained with red eosin 5% and Nigrosin 10% under a microscope under 40X high power.

Discussion

Many research spent last few decades working to enhance the quality of refrigerated Ram semen [19-20]. There was a little information about adding nanoparticles to epididymal sperm of Ram. Also due to rang of doses were used in anther paper, The present study was aimed to the exposure Awassi rams epididymal sperms sourced from abattoir to two doses of Zinc oxide and Cerium Oxide Nanoparticles on sperms live percentages, individual motility and abnormalities, this information may help to reduced information gape of nanoparticle doses for real application it in artificial and in vitro fertilization programs.

Effect of Cerium oxide CeO_2 nanoparticles

The present study revealed that Cerium oxide nanoparticle have improve semen characteristics, these data were in agreement with previous report [16] who find that storage semen after adding Cerium oxide in dose (0. 44, and 220 µg/mL) and stored at 4C° for 96 hrs in a soybean lecithin extender enhance semen parameter when compare with control group, this compatibility may be related to action of Cerium oxide as anti-oxidant, antibacterial and metabolic role of it [23]. Data of present study were agreement with another paper in goat [17] who refers that 25, 50 µg /mL of cerium oxide NPs approved sperm viability in Beetal buck spermatozoa. Interesting results showed by another report [24] who demonstrated how a brief incubation period in cold semen did not affect the morphologic and functional properties of ram sperms when exposed to cerium oxide NPs; although cerium oxide improved kinematic properties of sperms and same study concluded that exposure of spermatozoa to increasing doses of nano-ceria was well tolerated and ram sperm cells showed a margin of tolerance for cerium oxide NPs. In our opinion of view based on results of present study, Cerium oxide may not cause lethal action for sperm cells (for used doses in present study with direct adding) and activated sperm cell metabolism, mitochondrial activity, and increase these activities made free oxidative radicles made no effect for sperm cells.

Zinc oxide nanoparticles

Data of present study revealed that zinc oxide at dose (1mg /ml) and (1.5 mg/ml) had no effect on sperm viability during cooling storage when compare with control group. Data of previous report in sheep [25], found that zinc oxide NPs in concentration (0.04, 0.08, 0.06 mg/ml) and (1.5mg /ml) leads to improve sperm individual motility and sperm live percentage. These data were in disagreement with present study, this may be related that zinc oxide had not improve

epididymal sperms properties, The epididymal sperm in characterized by absences of seminal plasma; so, we thought that zinc oxide may interaction with seminal plasma content to express full benefits for sperm cell when fresh semen collected by other ways like artificial vagina or electro-ejaculation.

Another study refers to action of zinc oxide in low dose made positive results on ram epididymal sperms after storage in cooled way, these result disagreement with present study and this may be due to that low dose zinc oxide NPS improve sperm quality and also for different source of nanoparticles [26].

Conclusion

In conclusion, CeO2 NPs in dose 50 µg/ml improve epididymal sperm characteristics after storage at $4C^0$ for 72hrs. Zinc oxide NPs at dose 1 mg/ml and 1.5 mg/ml not improve sperms viability and we didn't recommend theses doses as an additives to ram epididymal sperm for cooling storage method.

Conflict of Interest

There were no conflict of interest.

Ethical approve

Since this study's experimental work involved using specimens from slaughterhouses, no approval from research ethics boards was necessary to achieve its objectives.

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Authors Contribution

The authors each contributed equally.

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تحسين حيوية الحيوانات المنوية البربخية للكباش العواسية بعد إضافة جزيئات الزنك وأكسيد السيريوم النانوية

صدام منير طه البقال، الياس خضر حسين وعدي طلعت نعمان

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هدفت الدراسة الحالية إلى تعريض الحيوانات المنوية البربخية للكباش العواسية المصدرة من المسلخ إلى جرعتين من أكسيد الزنك وجزيئات أكسيد السيريوم النانوية على حيوية الحيوانات المنوية بعد تخزينها عند درجة حرارة 4م لمدة 72 ساعة كوسيلة تبريد. أجريت التجربة في مختبر التلقيح الصناعي في جامعة الموصل، كلية الطب البيطري، الموصل، العراق. تم ذبح ستة عشر كبشًا عواسيًا سليمًا وناضجًا، تتراوح أعمارهم بين 1-4 سنوات، في مسلخ قريب، وتم جمع ستة عشر زوجًا من الخصيتين (العدد = 32). تم فصل خمسة أجزاء من الحيوانات المنوية وتخفيفها في موسع صفار البيض، وترك الجزء الأول كعنصر تحكم، بينما تلقى الجزء الثاني NPs من أكسيد السيريوم عند 50 ميكروغرام/مل، بينما تلقى الجزء الثالث NPs من أكسيد السيريوم عند 25 ميكروغرام/مل، في حين تلقى أكسيد الزنك 1 NPs ملغ. / مل يضاف إلى الجزء الرابع وأضيف 1.5 ملغم / مل من أكسيد الزنك NPs إلى الجزء الخامس. يتم تخزين جميع عينات الحيوانات المنوية عند درجة حرارة 4 درجة مئوية لمدة 72 ساعة. تم تقدير نسبة الحياة للحيوانات المنوية، الحركة الفردية، تشوهات الحيوانات المنوية. أظهرت بيانات الدراسة الحالية أن NPs لأكسيد السيريوم عند 50 ميكروجرام/مل كان له أفضل تأثير على حيوية الحيوانات المنوية وتحسين النسب المئوية للحيوانات المنوية، والحركة الفردية، وتم الإبلاغ عن انخفاض تشوهات الحيوانات المنوية بعد التخزين. كان لأكسيد السيريوم NPs عند 25 ميكروجرام/مل تحسنًا طفيفًا في قابلية الحيوانات المنوية للحياة. لم يكن لـ NPs لأكسيد الزنك بجرعة 1 مجم / مل و 1.5 مجم / مل أي تأثير أو انخفاض في حيوية الحيوانات المنوية بعد 24، 27 ساعة من التخزين. في الختام، CeO2 NPs بجرعة 50 ميكروغرام / مل تحسن خصائص الحيوانات المنوية البربخية بعد تخزينها عند 4C0 لمدة 72 ساعة. إن NPs لأكسيد الزنك بجرعة 1 ملجم / مل و 1.5 ملجم / مل لا تحسن من حيوية الحيوانات المنوية ولا ننصح بإضافتها إلى الحيوانات المنوية البربخية لطريقة تخزينها للتبريد.

الكلمات المفتاحية: أكسيد السيريوم NPs، أكسيد الزنك NPs، الحيوانات المنوية البربخية، كباش.