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**Original Research Article**

# Avoidance of Tetracycline Toxicity in Ram Semen by Nano Liposome Encapsulation

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**Abstract:** The present study aimed to lessen the tetracycline toxicity via liposome-encapsulated tetracycline extender on the ram sperm function behavior and abnormality. The nano-liposome tetracycline reduced the concentration of tetracycline and reduced the lethal concentration (LCs) value as compared with tetracycline estimated by the probit technique on the ram sperm. The nano-liposome tetracycline improved semen quality, represented by sperm concentration, viability, and reduced abnormality, as well as analysis of sperm motility in post-one-day cool preservation with Tris buffer. The research concluded that the Nano liposome-encapsulated tetracycline-based cool preservation Tris diluent can be used as a safe antibacterial in semen diluent. The nano-liposome tetracycline formulas avoided deleterious traditional tetracycline effects on sperm function tests, can assist in quality control within semen cool preservation protocols, decrease the risk of contaminants, and improve the values of semen quality as compared with control without tetracycline through the nano-behavioral protection of spermatozoa from damage.

**Keywords:** $LD_{50}$, Liposome, Motility, Nano, Semen, Sperm, Tetracycline, Viability.

## INTRODUCTION

Animal semen contains essential nourish bases of the sperm. The sterile nutritional condition of seminal fluid is suitable for the growth of microorganisms. Semen was contaminated by bacteria sharing the same urine pathway or exogenous skin contamination, as well as during semen processing [1, 2].

These different contaminations of semen by bacteria might affect semen quality; an investigation is going on to discover out prevention and reduce bacterial contamination in sperm. Bacterial contamination of the urogenital tract or during the cooling preservation process of semen is usually treated with antibacterial therapy.

Antibacterial diluent or extender fluid additive helps to eradicate the unwanted effects of bacteriospermia on spermatozoa parameters [3]. The different notions in reports found that antibacterial agents adversely affect semen quality. Mostly, the antibiotics used to prevent infection affect sperm function tests, with direct or indirect bad prognosis [3-6].

Tetracycline is contraindicated for use in semen extenders due to the deleterious effect on semen quality. Some research documented a slight decrease in spermatozoa index and RNA content of sperm cells [7, 8].

Numerous tetracycline formulations had a direct toxic effect on mammalian spermatozoa; furthermore, chlortetracycline has deleterious effects on spermatozoa at concentrations of 100 µ/ml, with human-specific effects on sperm motility. Minocycline hydrochloride has hazardous effects on bovine spermatozoa at all given concentrations<50 µ/ml [9].

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The nano-liposome is one maneuver to reduce the toxicity of the drug and increase the efficiency of delivering the drug [10]. For this reason, an attempt was made to modify tetracycline for safe use in semen, and minimize their side effect on semen quality, and explore the novel technique of determining lethal concentrations of tetracycline.

# MATERIAL AND METHODS

***Collection of Sperm***

The Semen specimens were collected from 10 local rams (2–3 years) in the Baghdad province field (AboGraib). Sperm collection by classical electrical ejaculation and cooling maneuvers was achieved in the spring months. Semen was collected at daylight (8- 11 a.m.). Raw semen was transferred out with 2 mL of a medium comprising Tris buffer 95.8 mM and glucose 10.1 mM.

***Tetracycline Challenge and Semen Grouping***

The diluted semen samples were grouped according to semen tetracycline formulas types as follows:

***Lethal Concentration (LCs) Test:***

1st control-treated classical semen tris buffer extender

2nd Tetracycline containing tris buffer extender in series concentrations 4, 8, 16, 32, 64, 96, and 128 µg/$10^6$ sperm.

3rd Nano liposome tetracycline extender in series concentrations 0.1, 0.4, 0.8, 0.16, 0.32, 0.64, and 0.96 µg/$10^6$sperm.

***Half-Maximal Effective Concentration ($EC_{50}$) test:***

1st control-treated semen Tris buffer extender without antibacterial,

2nd tetracycline-containing Tris buffer extender in series concentrations 2, 5, 10, 15, and 20 µg/$10^6$ sperm,

3rd nano-liposome tetracycline extender in series concentrations 0.01, 0.03, 0.05, 0.07, and 0.182 µg/$10^6$ sperm.

The sperm motility analysis was achieved at $ED_{50}$ values for both tetracycline Tris diluents and Nano Liposome diluent.

***Preparation of Nano Liposome Tetracycline Semen Diluents***

The Tris buffer was prepared according to Al-Shati (2020), and the nano-liposome tetracycline according to Ahmad and Al-Bayati (2018).

***LCs Determination***

The LDs were assessed according to Finney's method of probit analysis programmable by Microsoft Excel software; tetracycline and nano-liposome tetracycline were challenged in 500 sperm stained by one-step eosin-nigrosin stain for each formula added to the diluted semen [11].

***Chilled Stored Semen***

Diluted semen of each formula was transferred into conical propylene tubes (diluted to 15 × $10^6$ sperm/ml in one phase at 30 Cº. The tubes containing treated diluted semen were transferred to the refrigerator at 5 Cº auto-thermoregulation. Diluted semen was cooled to 5 Cº over 4 hours at a chilling rate of ~0.125 Cº per minute. The diluted specimen of semen was checked for the Sperm function test for ED50post-one-day preservation [12].

***Estimation of Sperm Function Test***

The sperm abnormalities and viability were determined by one-step eosin-nigrosin and counted by light microscope according to Lingappa *et al*., (2015). The sperm viability was counted as live sperm according to Lingappa *et al*., (2015) and expressed as a percentile value.

The Sperm concentration was counted using a Neubauer chamber. 20 µl of cool semen was transferred to the tube diluted by 2 ml of Sodium chloride 0.7% and mixed gently. The semen was counted and recalculated via the traditional dilution factor equation [13].

# RESULTS

The LC curves of tetracycline and nano-liposome tetracycline are shown in Figure 1. Lower concentrations of nano-liposome tetracycline than tetracycline in semen diluents.

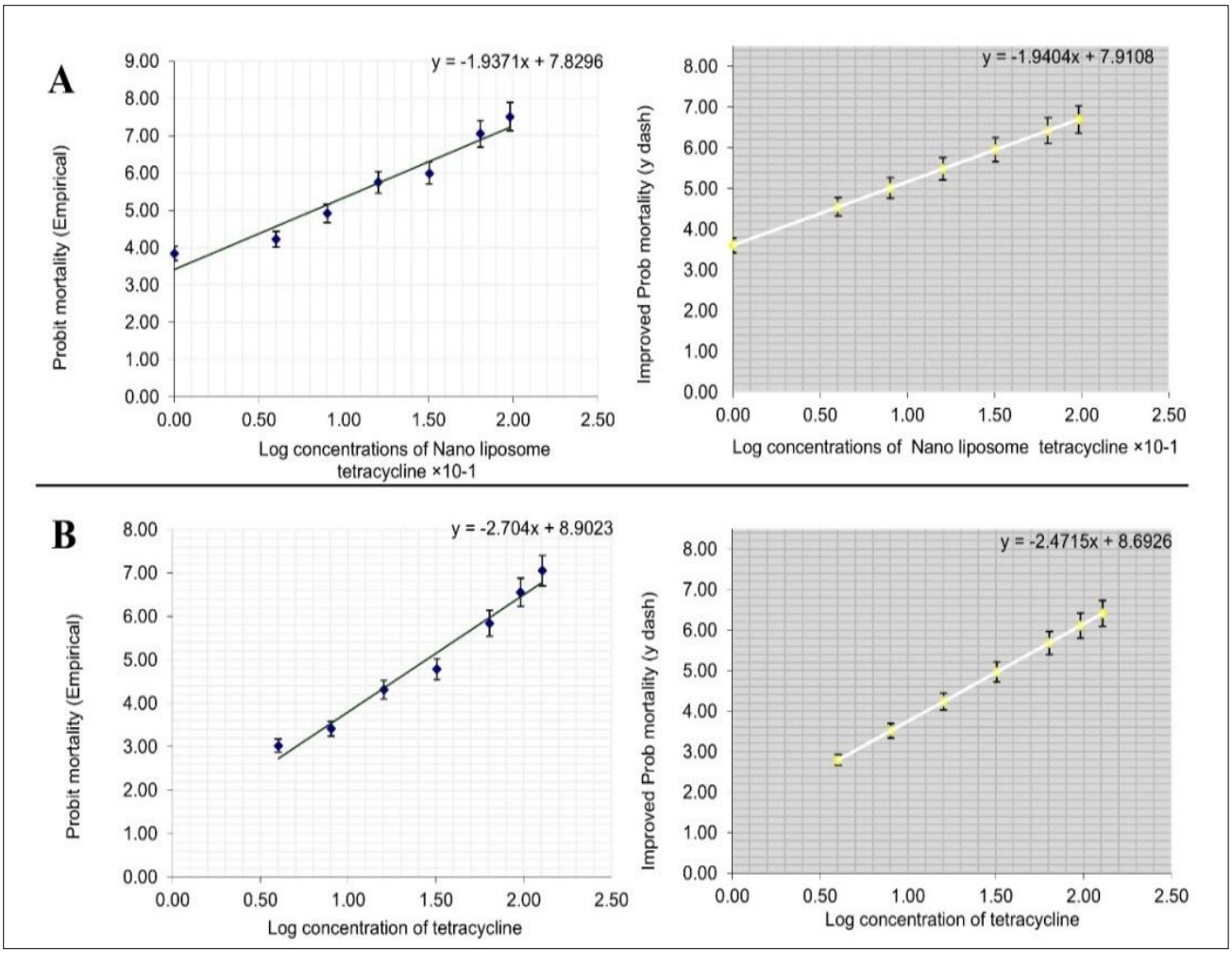



**Figure 1: Probit LC curve plotted for (A) tetracycline and (B) nano-liposome tetracycline in semen, displayed as a probit of corrected sperm viability values**

The $LC_{20, 50,}$ and $_{95}$ in Nano liposome tetracycline tris diluents of semen lower concentration than in tetracycline tris diluents; tetracycline 14.81±3.77, 33.16±4.19, and 160.33±11.93; Nano liposome tetracycline 0.226±0.018, 0.78±0.031, and 8.877±0.768, respectively as shown in Table 1.

**Table 1: The Lethal concentrations of tetracycline and nano-liposome in Tris buffer of ram semen diluent**

| LCs of ram sperm | $LC_{20}\mu g/10^6$ sperm | $LC_{50}\mu g/10^6$ sperm | $LC_{95}\mu g/10^6$ sperm |
|---|---|---|---|
| TC | 14.81±3.770 a | 33.16±4.19 a | 160.33±11.93 a |
| Nano liposome TC | 0.226±0.018 b | 0.78±0.031 b | 8.8770±0.768 b |

Data are dented to mean ± SEM. There are differences significantly at ($p \leq 0.05$) showed by letters between the two groups of treatment

***Sperm Function Indices***

The dose-response curve of tetracycline in semen tris diluent of semen showed the sperm viability and concentration were significantly ($p \leq 0.05$) decreased with increasing concentration of tetracycline post one day of preservation in Figure 2 and inversely the sperm abnormalities were directly increased with the concentration of tetracycline ($p \leq 0.05$).

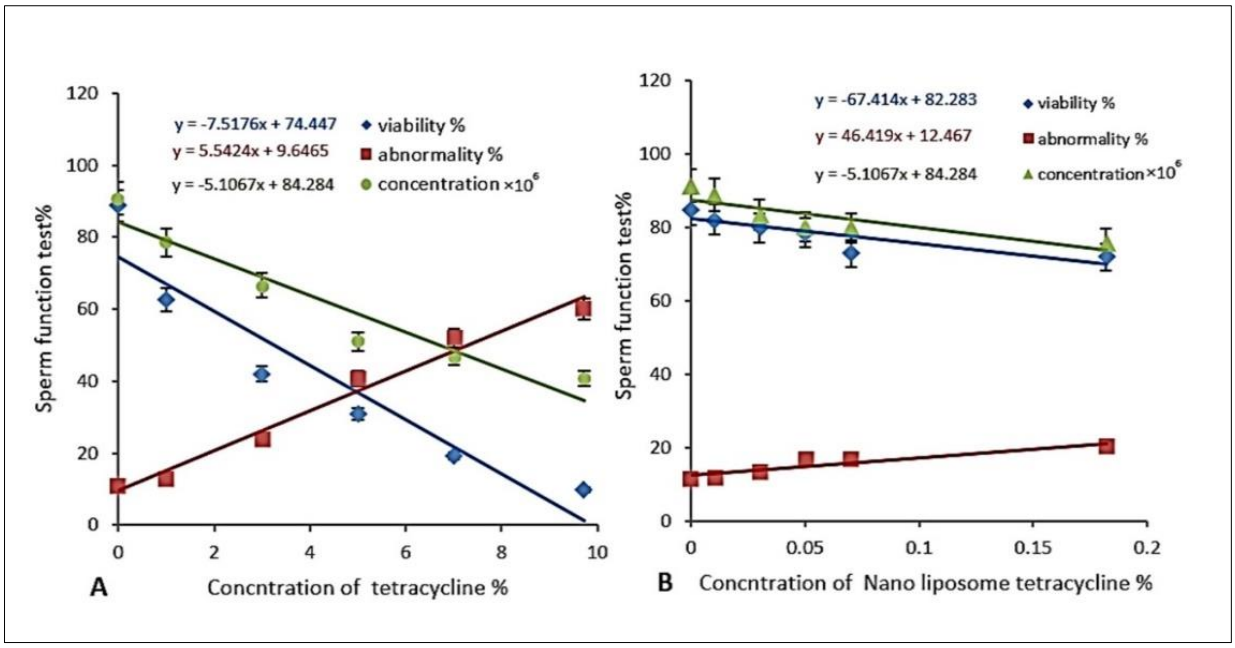



**Figure 2: The dose relationship of (A) tetracycline and (B) nano-liposome concentration versus sperm viability, sperm abnormality, and sperm concentration in semen diluent. Data are dented to mean±SEM.**

However, there was an improvement values of sperm function indices in Nano liposome encapsulation of tetracycline significantly ($p \leq 0.05$), as well as $EC_{50}$ Nano liposome tetracycline sperm viability 0.079±0.001 sperm abnormality 0.085±0.005 and sperm concentration 0.062±0.0088 lower than $EC_{50}$ of tetracycline for sperm viability 3.170±0.680 sperm abnormality 4.27±0.3680 and sperm concentration 3.790±0.0903significantly ($p \leq 0.05$) as shown in Table 2.

**Table 2: The Effective concentrations of tetracycline and nano-liposome in Tris buffer of ram semen diluent**

| Sperm function indices | Viability | Abnormality | Concentration |
|---|---|---|---|
| $EC_{50}$ TC | 3.170±0.680 a | 4.270±0.368 a | 3.790±0.0903 a |
| $EC_{50}$ Nano liposome TC | 0.079±0.001 b | 0.085±0.005 b | 0.062±0.0088 b |

Data are dented to mean ± SEM. There are differences significantly at ($p \leq 0.05$) showed by letters between the two groups of treatment.

The motion kinetics of sperm motility by tracing curves showed that Nano liposome tetracycline in diluted semen prevented the loss of motility behavior as compared with tetracycline diluted semen and control (Figure 3), significantly ($p \leq 0.05$). While the motility kinetic curve of tetracycline showed lower motion behaviors of semen than the motility kinetic curve of control, indicated by a significant decrease in absorbance due to the accumulation of sperm turbidity ($p \leq 0.05$).

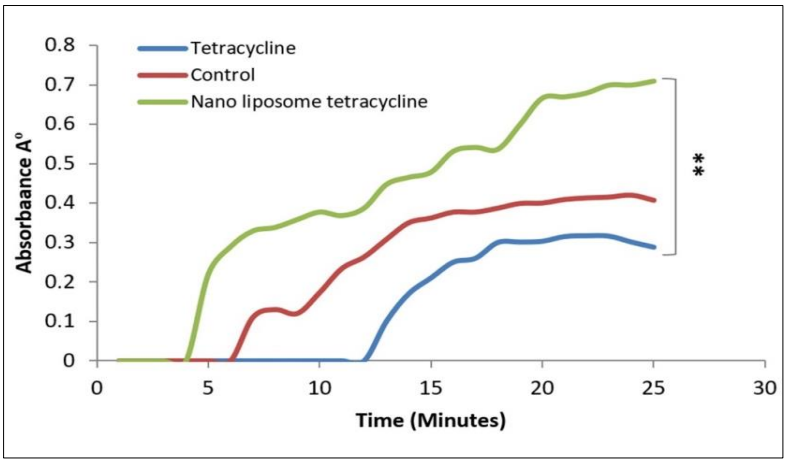



**Figure 3: Effect of nano-liposome tetracycline, Tetracycline semen diluents of ram post one day of cooling of kinetic of sperm motility parameters. Data are dented to mean ± SEM. * (asterisk) indicates symbols are significant (p≤0.05) different between Nano tetracycline and tetracycline formula-treated semen.**

***Sperm Motility parameters***

The diversity in Motility parameters; Lag time (reduction period of silence), The fraction of rapidly moving sperms (increased Elite Sperm responsible for fertilization) Sperm Velocity (increase sperm motility per second), and motility index (recover the total fraction motility per viable sperm), results in a significant ($p \leq 0.05$) improved in the Nano liposome tetracycline in $ED_{50}$ concentration as compared with tetracycline in tris buffer diluents figure (4).

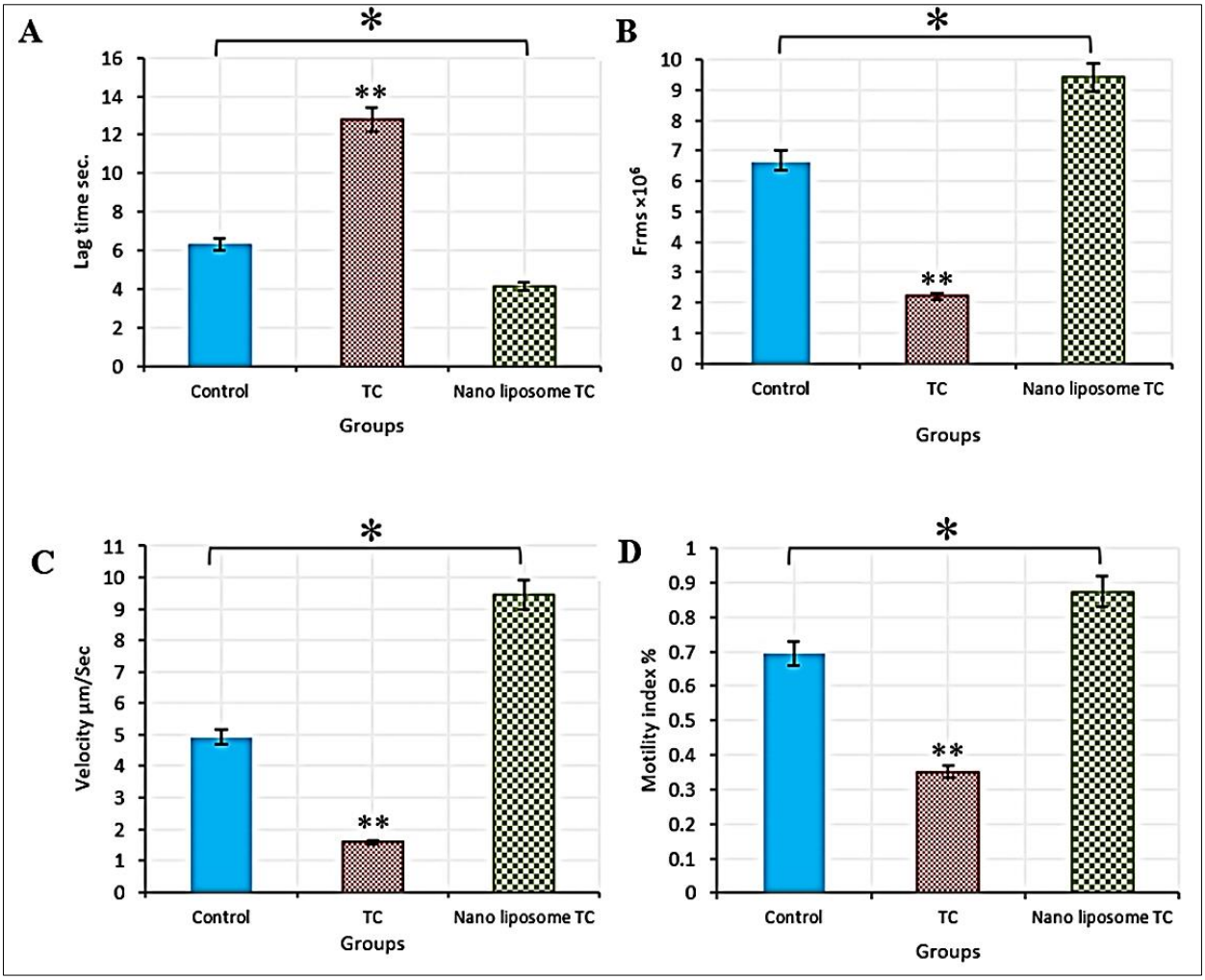



**Figure 4: The effect of half-effective concentration of tetracycline and nano-liposome tetracycline in Tris diluent preservative on the sperm A. Lag time, B. Fraction of rapidly moving sperm (Frms), C. Sperm velocity, and D. Motility index on day one post semen cooling. Data are presented as mean±SEM. *Denotes symbols with an asterisk are significantly ($p \leq 0.05$) different between concentrations and within groups**

## DISCUSSION

Cooled Ram semen beforehand fertility Comprehensive assessment and microbiological contamination avoidance important parameters for successful AI [14]. This study describe the use of the nano-liposome tetracycline for the prevention of bacteriospermia, to avoid adverse effects, and its consequences on physiognomies function of sperm in cooled ram semen [15].

Tetracycline has been documented to have an antimicrobial effect associated with adverse effects represented by different mechanisms shared with mineral chelating agent as calcium, magnesium, and iron-chelating fact with produce Sodium retention [16]. Furthermore, tetracycline has been well known to inhibit DNA replication on the sperm membrane at overdoses [17], therefore may be combined high teratogenicity risk to treated animals [18]. This evidence restricted uses in semen preservation.

In *vitro,* it has been revealed that tetracycline considerably impaired sperm motility parameters and reduced sperm viability. These effects are reported irreversibly effect. While the mechanism of action of tetracycline is protein synthesis

inhibition via inhibition synthesis of folic acid, impairment, and previously mentioned intercalation of DNA is well known [19], so there are likely other unidentified factors contributing to sperm motility failure.

The interpretation of sperm abnormalities and reduced viability, as well as pronounced LDs, was distinctive [20], especially around the area of midpiece, which may interfere with the assay due to direct binding.

Tetracycline has a potent and significant effects on movement of sperms at concentrations as low as $ED_{50}$µg/ml have been seen, well within those achieved following therapeutic doses of the antibiotic. Tetracycline was commonly prescribed for genitourinary tract infections, and approximately 3 µg/ml is tetracycline concentrations in plasma, dose-dependent [19-21].

Because there is high penetration of tetracyclines into semen-spermatozoa [21], inhabitation of protein synthesis is Tetracyclines mode of action; which may presumably cause toxicity to the sperm population [22, 23], Yet the mechanism by which tetracycline impairs motility of sperm are not understood; this may be suggested by calcium chelating ability, this may be of critical importance, given that calcium is not only involved in initiating sperm motility but is also considered a "crucial element for the process of hyperactivation" [24].

There are several benefits of Liposome-carrying drug particles: They Carries both hydrophilic and lipophilic drugs, Shields the drug from breakdown. The Liposomes composed from natural fat are bilayer membrane, biodegradable fate, and they do not cause immunological responses. They are very important due to modifications in the pharmacokinetic profile and biodistribution area, with an amplified therapeutic index [25, 26], also the liposomal encapsulation of tetracycline can regulate drug release over time 34,35 which may reduce the toxic effect of tetracycline on sperm.

Furthermore, as tetracycline antibacterial drug delivery carriers, liposomes can improve the concentration of antibiotic at the site of action, increase the efficacy of antibiotic as bactericidal through increased antibiotics entrances to the bacterial wall, as well as improve antibacterial tetracycline uptake and reduce the potentially toxic effect [25-30].

Liposomes have numerous potential pharmaceutical applications and can be safely administered by different routes of administration [31]. They can also be utilized in dermatology as a local drug depot in combination with penetration enhancers [31, 32].

During the cooling of sperm, extensive changes occur in the membrane structure of sperm, primarily in the sperm membrane. Drobnis *et al.,* (1993) reported a predominant increase in the sperm membrane; the polyunsaturated fatty acids peroxidation and lipid molecules transition alongside with the missing of cholesterol molecular arrangement, which may be losing the spermatozoa membrane fluidity and elasticity, that may promote the results decrease in sperm concentration and sperm viability, as well as dominant sperm secondary abnormality of secondary in preserved cool semen.

To overcome and solve this problem, the use of phosphatidylcholine in nano-liposome form encapsulated tetracycline along with the reduced concentration of tetracycline as sours of cholesterol (base of liposome) may presumably act as repair of sperm membrane injuries.

In other words, the nano-liposomes possibly behaves simulation a sperm cell membrane and have a complimentary of sperm membrane and act as a unit building themselves at the cooling time [33].

## CONCLUSION

Nano-liposome encapsulation is a highly effective strategy to avoid tetracycline toxicity in ram semen extenders. By utilizing a nano-liposome Tris diluent, the required concentration of tetracycline was significantly reduced, successfully preventing the drug's adverse effects on sperm viability, morphology, and motility kinetics during cool preservation.

Beyond simply reducing toxicity, the liposomal formulation actively improved semen quality parameters compared to both free tetracycline and control groups. Therefore, nano-liposome tetracycline serves as a safe and potent antibacterial additive for cooled semen protocols.

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**Informed Consent Statement:** This study did not involve human participants, and therefore, informed consent was not required.

**Clinical Trial Registration**: This research does not involve any clinical trials.

**Ethics of Animal Experimentation**

The study protocol was reviewed and approved by the [Bagdad University, collage of veterinary medicine , Baghdad, Iraq] under approval number [2023/885]. Additionally, all animal handling and experimental methodologies strictly adhered to the ethical standards outlined in the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes.

**Author Contributions**

**Shrooq Jaber Jassim:** Conceptualization, Methodology, Writing – Original Draft.

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