JSAES
Journal of Sustainable Agricultural and Environmental Sciences
Print ISSN : 2735-4377
Online ISSN : 2785-9878
Homepage: https://jsaes.journals.ekb.eg/

Research Article

Sperm variables in Cryopreserved Rabbit semen as affected by curcumin nano-formulation added to Tris-egg yolk or Tris-soybean lecithin extender

Khaled Abdel-Khalek[1*], Sherif A. Gabr[1], George E. Younan[2], Ahmad. M. Shehab El-Din[2], Aly E.B. Ghodaia[2]

[1] Animal Production Department, Faculty of Agriculture, Tanta University, Egypt.

[2] Animal Production Research Institute, Agricultural Research Center, Egypt.

[*] Correspondence author e-mail: abdelkhalk2004@yahoo.com

Article info: -

- Received: 9 December 2023
- Revised: 19 December 2023
- Accepted: 15 January 2024
- Published: 17 March 2024

Keywords:
Rabbit; semen; protectant; nano-particles; cryopreservation

Abstract:

This study aimed to assess the impacts of curcumin nano-formulation (CM-NF), type of extender, and their interaction on physical characteristics of cryopreserved rabbit spermatozoa. Ten mature NZW rabbit bucks (6.6–7.8-month-old and 3.45-3.95 kg LBW) were used as semen donors. Pooled semen was diluted with Tris-egg yolk (TEY) or Tris-soybean lecithin (TSBL) extender supplemented with CM-NF (0, 1.5, 2 and 2.5 µg/mL), equilibrated (2h at 5°C), loaded in straws, stored in liquid nitrogen, thawed at 37°C for 30 s, and evaluated for progressive motility (PM), livability (LS), abnormality (SA), membrane integrity (MI), and acrosome integrity (AI) after dilution, equilibration, and thawing. Results showed that extender type had non-significant effect on all sperm parameters after all cryopreservation processes. In semen diluted with both extenders, CM-NF (1.5 µg/mL) recorded significantly the highest (P<0.05) PM, LS, MI, and AI, and the lowest (P<0.05) SA percentages compared with free-extenders after all cryopreservation processes. Impact of CM-NF (1.5 µg/mL) on all sperm parameters did not differ from that of free-extender. Increasing CM-NF level to 2 µg/mL adversely affect PM, LS, and SA only in post-diluted semen, and had no effects on all sperm characteristics after equilibration and thawing. The interaction effect of extender type with CM-NF level on all sperm was not significant. Conclusively, there were no differences between TEY and TSBL extenders in maintaining rabbit sperm characteristics after cryopreservation. Supplementation of either TEY or TSBL extenders with CM-NF (1.5 µg/mL) can improve the progressive motility, livability, membrane integrity and acrosome integrity in cryopreserved rabbit semen.

1. Introduction

Rabbits are characterized by fast reproduction, production of high-quality white meat with low fat and cholesterol level, and high economic efficiency (Basavaraj et al., 2011; El-Ratel et al., 2017). In Egypt, chilled or cryopreserved semen of rabbits is widely used in artificial insemination (AI), but there are some challenges and limitations in using frozen semen due to its unsatisfactorily low fertility rate. Nevertheless, there is wide attention to using AI with frozen semen in terms of prolonging storage period and extending genetic improvement (Nishijima et al., 2015) to maximize rabbit production in intensive systems.

In rabbits, increasing motility and viability of spermatozoa in cryopreserved rabbit semen can be affected by type and concentration of diluent and cryoprotectant, freezing protocol, and thawing temperature (Mocé and Vicente, 2009). Also, increasing of motile to viable sperm ratio in post-thawed semen is an important for genetic resources cryopreservation in the bank of spermatozoa (Iaffaldano et al., 2012). The viability and motility of gametes are the main criteria in successful AI when cryopreserved spermatozoa were used (Dadoune et al., 2010). There is a relationship between the integrity of sperm DNA and embryo development *in vitro* because DNA molecule plays a decisive role in realizing such reproductive technologies such as AI, *in vitro* fertilization, and intracytoplasmic sperm injection into the oocyte (Wang et al., 2003). Buffer and cryoprotectants presented in semen extender play an important role in preventing cellular cryo-damage, hence the quality of cryopreserved sperm should be assessed basing on the detection of DNA fragmentation.

The sharp changes in the temperature and osmolality level, ice formation, free radical production, and type of cryoprotectants (Taylor et al., 2009) during cryopreservation processes impaired sperm cell functions (Thomson et al., 2009). Furthermore, oxidative stress causes a reduction in motility, livability (Mocé and Vicente, 2009), the structure, function and longevity of spermatozoa after thawing or in the female reproductive tract (Satorre et al., 2012). Also, other alterations physically and/or chemically stressed on sperm cells causing negative effects in integrity of sperm membrane, acrosome damage, mitochondrial activity, sperm metabolism, and DNA integrity (Boitrelle et al., 2012; Kopeika et al., 2015). The use of egg yolk in Tris-based extender to prevent or reduce the cold shock effect is an important step for using cooled semen (Isachenko et al., 2004 a, b). However, cryopreservation requires the use of cryprotectants to increase sperm survival and protection of

sperm structure from damage under hypothermic conditions because sperm cells have not the ability to adapt to subzero temperature degrees (Hezavehei et al., 2018). Furthermore, sperm cell membrane in mammalian is rich in polyunsaturated fatty acids which undergo peroxidation, hence spermatozoa are mainly vulnerable to damage caused by increasing reactive oxygen species and inducing oxidative stress (Thomson et al., 2009). In different animal species, various types of antioxidants were supplemented into the semen extender during freezing to mitigate oxidative stress (Silva and Gadella, 2006; de Lamirande and O'Flaherty, 2008). Several antioxidants were reported for treating male infertility by counteracting ROS and preserving quality of cryopreserved semen (Iovine et al., 2012) by reducing sperm ROS levels following the thawing process (Kalthur et al., 2011). Enriching sperm medium with antioxidants could be an effective approach to counteract sperm damage induced by cryopreservation (Bucak et al., 2010 a, b; Silva et al., 2012).

Curcumin (CM) is a major phytochemical commonly found in turmeric (*Curcuma longa*) and responsible for the vibrant yellow color of *Curcuma longa* and is currently recognized to be in charge for the majority of its remedial effects (Aggarwal et al., 2007). CM has an effective ROS scavenger activity and a powerful inhibitor of lipid peroxidation (Bucak et al., 2012; Tvrda et al., 2016). Energy-promoting and protective properties of CM on male reproduction were indicated by *in-vivo* (Salahshoor et al., 2012) and *in-vitro* studies (Bucak et al. 2012; Soleimanzadeh and Saberivand 2013; Tvrda et al., 2016).

Some reports indicated that supplementation of CM-NF in semen extender can enhance the freezing ability of sperm cells in rabbits (Abdelnour et al., 2020), and using CM-NF can enhance semen quality and freezing ability by decreasing oxidative stress and protecting the sperm cells from cryo-damage (de Lamirande and O'Flaherty, 2008; Bucak et al., 2010 a,b; WHO, 2010).

Recently, substitution of 18% egg yolk in Tris-extender with 1.5% soybean lecithin showed a beneficial impact on parameters of cryopreserved rabbit spermatozoa and prolificacy of doe rabbits (Younan et al., 2021). Comparing the potential protective and antioxidant activity of CM or CM-NF added to different types of extenders during cryopreservation on sperm parameters is still needed.

Therefore, aim of the present study was to evaluate the effect of CM-NF, supplemented into Tris-egg yolk or Tris-soybean lecithin, on rabbit motility, livability, abnormality, integrity of membrane and acrosome of spermatozoa in rabbit semen after dilution, equilibration, and thawing.

2. Materials and methods

This study was in frame of the scientific cooperation between Animal Production Research Institute (APRI) and Animal Production Department, Faculty of Agriculture, Tanta University.

2.1. Semen donors

Semen was collected from 10 healthy and sexually mature White New Zealand rabbit bucks (6.6–7.8 mo-old and 3.45-3.95 kg LBW). Rabbit bucks were kept under climatic, nutritional, and managerial conditions of Sakha Experimental Station, Kafrelsheikh governorate, belonging to Animal Production Research Institute (APRI), Egypt during the interval from December to January 2022. Bucks were fed a commercial complete feed diet in pellet form containing 17% crud protein,14% crud fiber, and digestible energy (2840 Kcal/kg).

2.2. Semen collection

Semen ejaculates were taken by collection of semen once/week for 10 weeks (100 ejaculates). An artificial vagina of rabbits was used for semen collection. Only ejaculates with ≥75% initial motility were pooled for cryopreservation processes in this experiment.

2.3. Semen dilution, experimental extenders and freezing processes

After semen collection, semen was pooled (10 ejaculates) and transported into the laboratory in International Livestock Management Training Center, belonging to APRI in a water bath (37ºC). In the laboratory, semen was distributed into eight equal portions, the 1st fourth portions were extended with Tris-extender supplemented with CM-NF (Sigma Company, Egypt) at levels of 0, 1.5, 2, and 2.5 µg/mL (TEY), while the 2nd fourth portions (TSBL) were extended with the same levels of CM-NF. Composition of both TEY and TSBL extenders is shown in Table 1.

Table 1. Composition of extenders used in dilution of rabbit semen.

Extender component	TEY	TSBL
Tris (g)	3.025	3.025
Citric acid (g)	1.675	1.675
Fructose (g)	1.250	1.250
Egg yolk (mL)	20.00	------
Soybean lecithin (g)	--------	1.500
Dimethyl sulfoxide (mL)	6.000	6.000
Streptomycin (g)	0.005	0.005
Lincomycin (g)	0.250	0.250
Bidistilled water	Up to 100 mL	

Semen was extended (1 semen: 10 extender) at 37 °C with different types of extenders, then five semen samples of each extender was evaluated. The diluted semen was subjected to equilibration period of 2 hours at 4-5 °C, then post-equilibrated semen was packaged in straws (0.25 ml, IVM technologies, L′ Aigle, France), exposed to liquid nitrogen (LN) vapor for 10 min and stored in LN at -196 °C for one week at least (Salisbury et al., 1978).

2.4. Sperm manual analysis

Evaluation of semen was achieved after dilution, equilibration, and thawing at 37ºC for 30 seconds in water bath. Sperm manual analysis included percentages of progressive motility, livability, abnormality, membrane

integrity, and acrosome integrity of spermatozoa.

Percentage of sperm progressive motility (count of sperm cells with forward movement in a long semi-arc pattern relative to total sperm cells in each field) was determined in fie fields on a glass slide at a warm stage (37°C) of a phase-contrast microscope (DM 500; Leica, Switzerland). Percentage of live/dead sperm and abnormality were examined in a semen smear stained with 5% eosin and 10% nigrosine using a phase-contrast microscope at 400× magnification. Dead (stained cells) or live (unstained cells) in each of five fields was counted, then percentage of live sperm was calculated as number of live cells relative to total number of cells in each field. Number of sperm cells with abnormalities in head and tail of spermatozoa, and sperm cells with cytoplasmic droplets were counted for computing the percentage of total morphological abnormalities (Menon et al., 2011).

Acrosome integrity was determined in terms of spermatozoa with intact and attached acrosome in a semen smear stained by Giemsa using a light microscope at 1000x using oil immersion lens. Giemsa stock solution contained 3.8 g Giemsa, 375 ml absolute methanol, and 125 ml glycerol. According to Brackett and Oliphant (1975), Giemsa stain mixture was stored at 37°C for one week before use. Semen was smeared on a pre-warmed slide, dried, fixed in 10% buffered formal saline for 15 min, washed by tape water, air dried, and stained by Giemsa.

In at least four microscopic fields, number of sperm cells with normal acrosome was counted, then acrosome integrity was calculated for about 200 spermatozoa randomly selected. The acrosome was considered to be normal when the stain was clearly and evenly distributed over the spermatozoa anterior to equatorial segment.

2.5. Statistical analysis

Homogeneity and normality of distribution of all numerical data have been checked using Lieven's test and Shapiro-Wilk test, respectively.

Data were statistically analyzed as a factorial design (2 extender types x 4 CM-NF levels) using computer program of SAS (2004) to study the effect of extender type, CM-NF level, and their interaction on different parameters studied using the following model:

$$X_{ijkl} = \mu + P_i + N_k + PN_{ik} + E_{ikl}$$

where: μ = general mean, P_i = fixed effect of extender type (1, 2), N_k = fixed effect of CU-NF level (1-4), PN_{ik} = the interaction between extender type and CU-NF level, and E_{ikl} = random error.

The significant differences for the effect of CM-NF level were separated by Duncan's test at a level of P<0.05. Data were presented as mean±SE.

3. Results

3.1. Effect of extender type (TEY vs. TSBL)

Effect of extender type was non-significant on all sperm characteristics studied, including progressive motility (PM), livability (LS), abnormality (SA), membrane integrity (MI), and acrosome integrity (AI) percentages in post-diluted semen (Table 2). Effect of extender type on PM, LS, SA, MI, and AI was after equilibration (Table 3) and thawing (Table 4) was also not significant.

Table 2. Effect of extender type on sperm parameters of rabbits after dilution.

Sperm variable	Extender type		P-value	Sign.
	TEY	TSBL		
PM (%)	76.7±1.03	74.6±0.99	0.142	ns
LS (%)	79.9±1.00	76.8±1.16	0.100	ns
SA (%)	13.3±0.67	12.8±0.78	0.501	ns
MI (%)	76.4±1.30	73.9±1.68	0.163	ns
AI (%)	78.4±1.29	75.5±1.48	0.070	ns

ns: not significant.

Table 3. Effect of extender type on sperm parameters of rabbits after equilibration.

Sperm variable	Extender type		P-value	Sign.
	TEY)	TSBL		
PM (%)	72.9±0.66	71.7±1.30	0.212	ns
LS (%)	75.5±1.00	75.2±1.42	0.778	ns
SA (%)	14.5±0.71	14.8±0.72	0.699	ns
MI (%)	69.0±1.23	66.8±1.69	0.225	ns
AI (%)	73.5±1.44	71.1±1.71	0.169	ns

ns: not significant.

Table 4. Effect of extender type on sperm parameters of rabbit after thawing.

Sperm variable	Extender type		P-value	Sign.
	TEY	TSBL		
PM (%)	44.6±1.72	42.1±2.10	0.3011	ns
LS (%)	48.1±1.86	45.7±1.96	0.2711	ns
SA (%)	21.2±0.72	21.9±0.80	0.4002	ns
MI (%)	44.0±1.23	42.3±1.57	0.3248	ns
AI (%)	48.8±1.43	46.3±1.70	0.2985	ns

ns: not significant.

3.2. Effect of CM-NF level (0, 1.5, 2, and 2.5 of µg/mL)

All sperm parameters in post-diluted semen were affected significantly by the level of CM-NF. Results showed that CM-NF at a level of 1.5 µg/mL in semen extender significantly (P<0.05) increased sperm parameters, including PM, LS, MI, and AI, while decreased SA percentage after dilution in comparing with free-extender.

The differences in all sperm parameters between semen extended with CM-NF at level 2 and free-extender were not significant. However, CM-NF significantly (P<0.05) decreased PM and LS percentage as compared to control in post-diluted semen (Table 5).

Table 5. Effect of different levels of CM-NP on sperm parameters of rabbit after dilution.

Item	Level of supplementation (µg/mL)			
	0	1.5	2	2.5
PM (%)	75.8±0.6[b]	81.7±0.59	75.0±1.23[b]	70.0±0.77[c]
LS (%)	79.0±0.7	84.8±0.66	77.2±1.34[b]	72.3±0.81[c]
SA (%)	13.3±0.6[b]	9.2±0.66[c]	13.5±0.57[b]	16.3±0.96[a]
MI (%)	73.5±1.4	81.5±1.26	76.2±2.32[ab]	69.5±1.86[b]
AI (%)	75.5±1.6	82.8±1.09	78.3±2.10[ab]	71.2±1.43[c]

a, b, and c: Means within the same row with no common superscript are significantly different at P<0.05.

The effect of CM-NF level on all sperm parameters was significant (P<0.001) after equilibration (Table 6) and thawing (Table 7). In semen after equilibration and thawing, only semen diluted with CM-NF at level of 1.5 µg/mL showed significantly (P<0.05) the highest improvement in all semen parameters as compared to other CM-NF levels and free-extender. However, other CM-NF levels showed insignificant differences with the control extender (Tables 6 and 7).

3.3. Interaction effect (extender x CM-NF)

The interaction effect of extender type x CM-NF on all sperm parameters after dilution was not significant (P>0.05). PM, LS, SA, MI, and AI percentages was better with both extenders supplemented with CM-NF at level of 1.5 µg/mL as compared to other CM-NF levels and free-extender. Sperm parameters were slightly higher in semen diluted with TEY than TSBL (Fig. 1).

Table 6. Effect of different levels of CM-NP on sperm parameters of rabbit after equilibration.

Item	Level of supplementation (µg/mL)			
	0	1.5	2	2.5
PM (%)	70.8±0.56^{b}	77.5±0.75^{a}	73.3±1.42^{b}	67.5±1.15^{c}
LS (%)	73.7±1.06^{b}	82.5±1.01^{a}	75.0±1.55^{b}	70.2±0.89^{c}
SA (%)	15.8±1.10^{a}	11.3±0.28^{b}	16.2±1.12^{a}	15.3±0.66^{a}
MI (%)	66.3±1.62^{b}	73.8±1.27^{a}	69.0±2.30^{a}	62.5±1.73^{b}
AI (%)	70.5±1.52^{b}	79.8±1.70^{a}	73.0±2.07^{b}	65.8±1.64^{c}

a, b, and c: Means within the same row with no common superscript are significantly different at P<0.05.

Table 7. Effect of different levels of CM-NP on sperm parameters of rabbit after thawing.

Item	Level of supplementation (µg/mL)			
	0	1.5	2	2.5
PM (%)	40.8±2.02^{b}	50.8±2.02^{a}	45.0±1.88^{ab}	36.7±2.24^{b}
LS (%)	43.3±1.85^{bc}	55.7±2.01^{a}	49.0±2.20^{ab}	39.5±2.28^{c}
SA (%)	22.5±0.93^{a}	17.7±0.28^{b}	23.2±1.09^{a}	22.8±0.97^{a}
MI (%)	41.5±1.57^{ab}	49.0±1.21^{a}	44.2±2.18^{ab}	38.0±1.56^{b}
AI (%)	45.8±1.45^{bc}	55.0±1.66^{a}	48.2±2.11^{b}	41.0±1.66^{c}

a, b, and c: Means within the same row with no common superscript are significantly different at P<0.05.

The effect of interaction between extender type and CM-NF level on all sperm parameters after equilibration was not significant (P>0.05). These results reflected increased percentages of PM, LS, MI, and AI, and decreasing percentage of SA in both TEY- and TSBL-semen supplemented with CM-NF at level of 1.5 µg/mL in comparison with other CM-NF levels and free-extender (Fig. 2).

In semen after thawing, also extender type did not interact with CM-NF level for all sperm parameters, reflecting higher percentages of PM, LS, MI, and AI, and decreasing percentage of SA in both types of extenders supplemented with CM-NF at level than other CM-NF levels and control. For supplementation of CM-NF at level of 1.5 µg/mL, sperm parameters including percentages of PM, LS, and MI increased, whilst SA percentage decreased in thawed semen with TEY than TSBL; however, AI percentage was slightly higher in TSBL than TEY extender (Fig. 3).

4. Discussion

Over the last few decades, sperm cryopreservation techniques have promptly progressed in animals for achieving successful fertilization via assisted reproductive technique. This technique helps preserve and transportation of valuable genetics for the fields of agriculture and biomedical research. During cryopreservation, spermatozoa are undergoing different stressors causing a reduction in the sperm fertilizing ability.

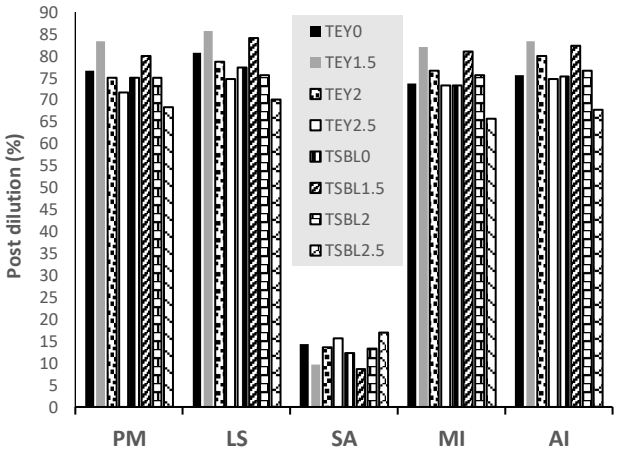

Fig. 1. Effect of extender treatment on progressive motity (PM), livability (SL), abnormality (ABN), membrane integrity (MI), and acrosome integrity (AI) in rabbit semen post dilution.

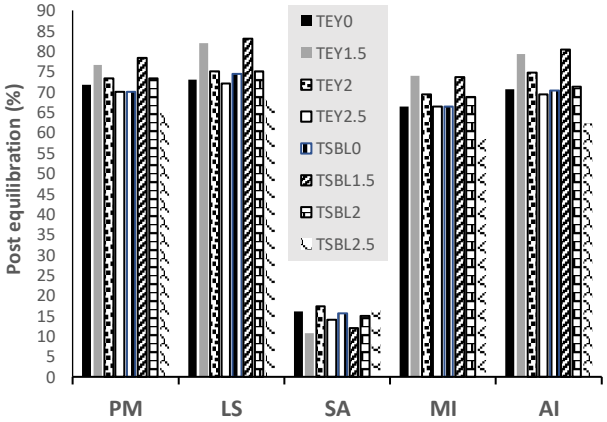

Fig. 2. Effect of extender treatment on progressive motity (PM), livability (SL), abnormality (ABN), membrane integrity (MI), and acrosome integrity (AI) in rabbit semen post equilibration.

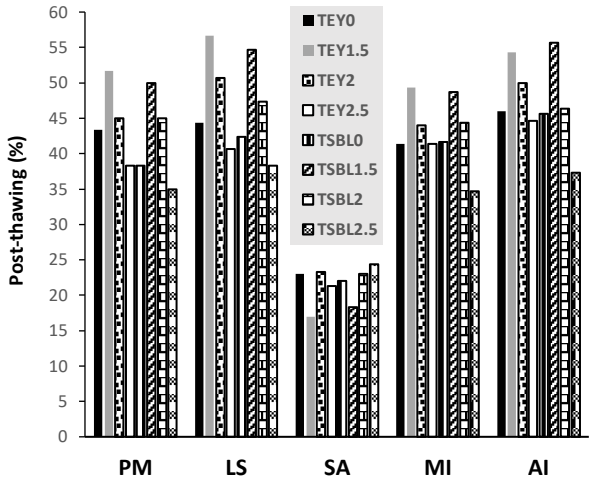

Fig. 3. Effect of extender treatment on progressive motity (PM), livability (SL), abnormality (ABN), membrane integrity (MI), and acrosome integrity (AI) in rabbit semen post thawing.

Many reports have engrossed on enhancing quality of spermatozoa and elimination of the negative effects of oxidative stress by antioxidant supplementation (Hammadeh et al., 1999) and nanoparticles (Tejada et al, 1984; Padron et al., 1997; Slobodan et al., 2001).

In one study on the effect of CUR nanoparticles on improving semen quality of rabbit semen after cryopreservation (Abdelnour et al., 2020), it was accepted nanoparticles having low zeta potential values, the zeta potential of CM nanoparticles was -25 mV which presented strong stability of CMNPs then there will be no force to avoid the particles pending flocculating and together. Authors studied the effect of CM nanoparticles (0.5, 1, and 1.5 µg/mL), supplemented into Tris-egg yolk-fructose on post/thawed rabbit sperm quality.

On the other hand, recently, the usage of SBL as an alternative of egg yolk in Tris-extender showed successful in improving rabbit semen quality (Younan et al., 2021). The interaction effect of extender type (cold chock protectants) and antioxidant source was reported. Therefore, we aimed to evaluate the usage of CM-NF at different levels (1, 1.5, and 2 µg/mL) in two types of Tris-extenders (TEY vs. TSBL) on quality and freezing ability of rabbit spermatozoa. In our study, extender type had non-significant effect on all sperm parameters, including PM, LS, SA, MI, and AI in rabbit semen after dilution, equilibration, and thawing. These results indicated the efficiency of SBL as an alternative to egg yolk in maintaining sperm characteristics during different processes of rabbit semen cryopreservation.

The effect of extender type on physical characteristics of spermatozoa was studied by many authors. Dilution of semen in a suitable buffer is one of the important factors affecting sperm survival during cryopreservation (Rasul et al., 2000). An ideal buffer should have: 1) pH between 6 and 8, preferably 7; 2) maximum water solubility and minimum solubility in all other solvents; 3) minimum salt effects; 4) minimum buffer concentration; 5) least temperature effect; 6) well behaved cation interactions; 7) greater ionic strengths, and 8) chemical stability (Keith and Morrison, 1981).

The absence of significant differences between TEY and TSBL in all rabbit sperm parameters studied post dilution, equilibration, and thawing may be due to good quality of the composition of both semen extender according to Samad (1985) and Chaudhari and Mshelia (2002), who stated that semen extender must be contained sources of energy (glucose in both), buffering capacity (Tris in both), protectant against cold shock (egg yolk in TEY and SBL in TSBL), cryoprotectant (glycerol in both), and antibiotics (Lincomycin and streptomycin in both). The lecithin in soybean or lecithin and lipoprotein contents in egg yolk together contribute to the preservation of the lipoprotein sheath of the sperm cell (Kumar et al., 1992).

Our results were evidenced recently by Younan et al. (2021), who found that addition of SBL (1.5%) as an alternative to egg yolk (18%) in tris-extender had beneficial impacts on quality of cryopreserved rabbit spermatozoa. However, SBL cannot replace egg yolk in Tris-citric-fructose or Tris-citric-fructose mineral salts extenders in chilling canine sperm (Nguyen et al., 2019).

Regarding the effect of CM-NF levels, regardless extender type, the optimal level in both extenders was 1.5 µg/mL to obtain significantly the highest PM, LS, MI, and AI, and the lowest SA percentage in comparison with free extender. Both extenders supplemented with CM-NF at a level of 2 µg/mL failed to have significantly ($P>0.05$) positive impacts on sperm characteristics over the free-extender following all cryopreservation processes. On the other hand, we found adverse effects by increasing level of CM-NF to 2.5 µg/mL on PM, LS, and SA only in post-diluted semen, beside no effects on all sperm characteristics after equilibration and thawing. Generally, the results of the present study indicated insignificant interaction effect of extender type with CM-NF level on all sperm characteristics studied, clearing the highest impact of CM-NF at a level of 1.5 µg/mL in both extenders.

In accordance with the obtained results in our study, addition of CM in native form or nan-particles into Tris-egg yolk fructose extender (TEYF) significantly increased sperm motility, viability, and membrane integrity, and reduced sperm abnormality (Abdelnour et al., 2020). In an early study, Srinivas et al. (1992) have shown that the supplementation of CM in extender significantly improved the viability and reduced necrosis and apoptosis of sperm cells in post-thawed semen of men. In *in-vivo* studies, feeding low-protein diet with CM and CM nano-particles significantly increased sperm progressive motility, livability, and decreased sperm abnormality of rats (WHO, 2010). In sheep, dietary CM supplementation decreased testicular apoptosis and reduced the gene expression caspase-3 in the testes tissues (Azza et al., 2011).

Moreover, it was also demonstrated that, the addition of selenium nanoparticles in freezing medium, significantly improved sperm viability and motion features of Holstein bulls (de Lamirande and O'Flaherty, 2008; Khalil et al., 2019), and had beneficial effects on post-thaw survival of sperm cells in ram and human (Silva and Gadella, 2006; Dev et al., 2013), respectively. Nano-particles of cerium oxide revealed positive impacts on ram

stored at 4ºC for 96 hours (Falchi et al., 2018).

Generally, CM as a natural antioxidant exhibited beneficial effects on rabbit semen quality (Abdelnour et al., 2020). The general usage of nanoparticles is considered as a promising strategy for maintaining fertility of sperm cells during extended storage in different livestock (Terquem and Dadoune, 1982). The reduction in aqueous solubility, bioavailability, stability, metabolism, and half-life of native CM were improved by CM-NF (Tejada et al., 1984). Many authors reported alterations in lipid peroxidation, ROS generation, sperm capacitation caused by cryopreservation (Rotem et al., 1990) and acrosome reaction in spermatozoa, resulting in failure male fertility potential (Amidi et al., 2016; de Lamirande and O'Flaherty, 2008).

The imbalance in ROS generation and activity of antioxidant enzymes resulted in oxidative stress on sperm cells during cryopreservation and is critical factor on maintaining the sperm function to increase sperm fertilizing capacity. In our study, the addition of CM-NF to the Tris-egg yolk or Tris-soybean lecithin extenders of rabbit semen was assessed for constraining the injurious impacts of inducing oxidative stress, which negatively affect the sperm cells during the freezing procedures. This impacts on sperm characteristics may be attributed to increasing total antioxidant capacity and activity antioxidant enzyme activity of super oxide dismutase (SOD), and glutathione peroxidase (GPx), and decreasing the oxidative markers such as malondialdehyde (Abdelnour et al., 2020).

In the antioxidant defense system, CM improved antioxidant enzyme activity of SOD and glutathione activity in the seminal plasma by sustention of the mitochondrial redox signaling and respiratory functions (Raza et al., 2008). Also, Slobodan et al. (2001) found that CM nano-particles had the ability to diminish the negative impacts of free radicals during semen cryopreservation. Finally, CM-NF can mitigate oxidative stress via down-regulation of the level of H_2O_2 and protecting sperm cells by inflammatory properties of CM to maintain sperm quality (Sharma and Singh, 2010).

5. Conclusion

Conclusively, there were no differences between TEY and TSBL extenders in maintaining rabbit sperm characteristics after cryopreservation. Supplementation of either TEY or TSBL extenders with CM-NF can improve the progressive motility, livability, membrane integrity and acrosome integrity in cryopreserved rabbit semen. These positive impacts might be due to antioxidant capacity of CM-NF. The highest impacts were recorded by CM-NF at a level of 1.5 µg/mL.

Author Contributions:

Conceptualization, Sh. Gabr and A. Shehab; methodology, Kh. Abdel-Khalek and A. Ghodaia; software, Sh. Gabr; validation, Sh. Gabr, A. Shehab and A. Ghodaia; formal analysis, Kh. Abdel-Khalek; investigation, A. Shehab; resources, A. Ghodaia and Kh. Abdel-Khalek; data curation, A. Shehab and Kh. Abdel-Khalek; writing original draft preparation, Sh. Gabr and A. Shehab; writing review and editing, Sh. Gabr and A. Shehab; visualization, Sh. Gabr and A. Ghodaia; supervision, Sh. Gabr. All authors have read and agreed to the published version of the manuscript.

Funding:

This research received no external funding.

Institutional Review Board Statement:

Experiment was approved from Institutional Animal Care (APRI) and Use Committee (ARC-IACUC) protocol number (ARC-APRI 57-23), Agricultural Research Center, Egypt.

Informed Consent Statement:

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement:

Not applicable" here.

Acknowledgments:

We appreciate and thank all members of Animal Production Research Institute and Animal Production Department, Faculty of Agriculture Tanta University for their help and support.

Conflicts of Interest:

The authors declare no conflict of interest.

6. References

Abdelnour, S.A., Hassan, M.A.E., Mohammed, A.K., Alhimaidi, A.R., Al-Gabri, N., Al-Khaldi, K.O. and Swelum, A.A. (2020). The effect of adding different levels of curcumin and its nanoparticles to extender on post-thaw quality of cryopreserved rabbit sperm. Animals, 10, 1508. doi:10.3390/ani10091508

Aggarwal, B.B., Sundaram, C., Malani, N., and Ichikawa, H. (2007). Curcumin: The Indian solid gold. Advances in Experimental Medicine and Biology, 595, 1–75.

Amidi, F., Pazhohan, A., Shabani, Nashtaei M., Khodarahmian, M. and Nekoonam, S. (2016). The role of antioxidants in sperm freezing: a review. Cell Tissue Bank, 17 (4):745–56.

Azza, M., El-Wakf, M.E.S., Elhabiby, M.W., El-kholy, E., and El-Ghany, E.A. (2011). Use of tumeric and curcumin to alleviate adverse reproductive outcomes of water: Nitrate pollution in male rats. Nat. Sci., 9:229–39.

Basavaraj, M., Nagabhushana, V., Prakash, N., Appannavar, M.M., Wagmare, P., and Mallikarjunappa, S. (2011). Effect of dietary supplementation of *Curcuma Longa* on the biochemical profile and meat characteristics of broiler rabbits under summer stress. Vet. World, 4: 15-18.

Boitrelle, F., Albert, M., Theillac, C., Ferfouri, F., Bergere, M., and Vialard, F. (2012). Cryopreservation of human spermatozoa decreases the number of motile normal spermatozoa, induces nuclear vacuolization and chromatin decondensation. J Androl., 33(6):1371–8.

Brackett, B.G. and Oliphant, G. (1975). Capacitation of rabbit spermatozoa *in vitro*. *Biol. Reprod.* **12**, 260-74.

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

Bucak, M.N., Sarıözkan, S., Tuncer, P.B., Sakin, F., Ates, A. and Kulaksız, R. (2010a). The effect of antioxidants on post-thawed Angora goat (Capra hircus ancryrensis) sperm parameters, lipid peroxidation and antioxidant activities. Small Rumin. Res., 89(1):24–30.

Bucak, M.N., Tuncer, P.B., Sariözkan, S., Başpinar, N., Taşpinar, M., and Çoyan, K. (2010b). Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: antioxidants protect DNA integrity against cryodamage. Cryobiology, 61(3):248–53.

Chaudhari, S.U.R. and Mshelia, G. (2002). An overview of cryopreservation of cattle and buffalo bull semen. International Journal of Agriculture & Biology, 4: 572–575.

Dadoune, J.P., Mayaux, M.J., and Guihard-Moscato, M.L. (2010). Correlation Between Defects in Chromatin Condensation of Human Spermatozoa Stained by Aniline Blue and Semen Characteristics. Andrologia, 20(3):211–7.

de Lamirande, E. and O'Flaherty, C. (2008). Sperm activation: Role of reactive oxygen species and kinases. C. 1784, Biochimica et Biophysica Acta - Proteins and Proteomics. Elsevier, 106–15.

Dev, K.R., Yadamma, K., and Reddy, K.D. (2013). Protective effects of curcumin in cyclophosphamide induced sperm head abnormalities in male mice. Int. J. Pharm. Biol. Sci., 4:1131–7.

El-Ratel, I.T., Abdel-Khalek, A.E., El-Harairy, M.A., Sara F. Fouda and Lamiaa Y. El-Bnawy (2017). Impact of green tea extract on reproductive performance, hematology, lipid metabolism and histogenesis of liver and kidney of rabbit does. Asian J. Anim. Vet. Adv., 12: 51-60.

Falchi, L.; Galleri, G.; Dore, G.M.; Zedda, M.T.; Pau, S.; Bogliolo, L.; Ariu, F.; Pinna, A.; Nieddu, S. and Innocenzi, P. (2018). Effect of exposure to CeO(2) nanoparticles on ram spermatozoa during storage at 4ºC for 96 hours. Reprod. Biol. Endocrinol., 16: 19.

Hammadeh, M.E., Askari, A.S., Georg, T., Rosenbaum, P. and Schmidt, W. (1999). Effect of freezethawing procedure on chromatin stability, morphological alteration and membrane integrity of human spermatozoa in fertile and subfertile men. Int. J. Androl.,162:155–62.

Hezavehei, M., Sharafi, M., Kouchesfahani, H.M., Henkel, R., Agarwal, A., Esmaeili, V. (2018). Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches. Reprod. BioMed. Online, 37:327–39.

Iaffaldano, N., DiIorio, M. and Rosato, M.P. (2012). The cryoprotectant used, its concentration, and the equilibration time are critical for the successful cryopreservation of rabbit sperm: Dimethyl acetamide versus dimethyl sulfoxide. Theriogenology, 78: 1381–1389.

Iovine, C., Mottola, F., Santonastaso, M., Finelli, R., Agarwal, A., and Rocco, L. (2012). In vitro ameliorative effects of ellagic acid on vitality, motility and DNA quality in human spermatozoa. Mol. Reprod. Dev., 88(2):167–74. https://doi.org/10.1002/mrd.23455.

Isachenko, V., Isachenko, E., Katkov, I.I., Montag, M., Dessole, S., and Nawroth, F. (2004a). Cryoprotectant-Free Cryopreservation of Human Spermatozoa by Vitrification and Freezing in Vapor: Effect on Motility, DNA Integrity, and Fertilization Ability. Biol. Reprod., 71(4):1167–73.

Isachenko, E., Isachenko, V., Katkov, I.I., Rahimi, G., Schöndorf, T., and Mallmann, P. (2004b). DNA integrity and motility of human spermatozoa after standard slow freezing versus cryoprotectant-free vitrification. Hum. Reprod., 19 (4):932–9.

Kalthur, G., Raj, S., Thiyagarajan, A., Kumar, S., Kumar, P., and Adiga, S.K. (2011). Vitamin E supplementation in semen-freezing medium improves the motility and protects sperm from freeze-thaw-induced DNA damage. Fertil. Steril., 95(3):1149–51.

Keith, J.E. and Morrison, J.F. (1981). Buffers of constant ionic strength for studying pH-dependent processes. Methods Enzymol., 87: 405–426.

Khalil,W.A., El-Harairy, M.A., Zeidan, A.E.B., and Hassan, M.A.E. (2019). Impact of selenium nano-particles in semen extender on bull sperm quality after cryopreservation. Theriogenology, 126, 121–127.

Kopeika, J., Thornhill, A., and Khalaf, Y. (2015). The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence methods. Hum. Reprod. Update, 21(2):209–27.

Kumar, S., Sahni, K.L., and Mohan, G. (1992). Effect of different levels of glycerol and yolk on freezing and storage of buffalo semen in milk, tris and sodium citrate buffers. Buffalo J., 2: 151–156.

Menon, A.G.; Thundathil, J.C.; Wilde, R.; Kastelic, J.P. and Barkema, H.W. (2011). Validating the assessment of bull sperm morphology by veterinary practitioners. Can. Vet. J., 52, 407–408.

Mocé, E. and Vicente, J.S. (2009). Rabbit sperm cryopreservation: A review. Anim. Reprod. Sci., 110, 1–24.

Nguyen, V., Ponchunchoovong, S., Kupittayanant, S. and Kupittayanant, P. (2019). Effects of egg yolk and soybean lecithin on sperm quality determined by computer-assisted sperm analysis and confocal laser scanning microscope in chilled canine sperm. Veterinary Medicine and Science, 5(3):345-360. doi: 10.1002/vms3.158

Nishijima, K., Kitajima, S., Koshimoto, C., Morimoto, M., Watanabe, T., Fan, J. and Matsuda, Y. (2015) Motility and fertility of rabbit sperm cryopreserved using soybean lecithin as an alternative to egg yolk. Theriogenology, 84, 1172–1175. doi.org/10.1016/j.theriogenology.

Padron, O.F., Brackett, N.L., Sharma, R.K., Lynne, C.M., Thomas, A.J. and Agarwal, A. (1997). Seminal reactive oxygen species and sperm motility and morphology in men with spinal cord injury. Fertil. Steril., 67(6):1115–20.

Rasul, Z., Anzar, M., Jalali, S. and Ahmad, N. (2000). Effect of buffering systems on post-thaw motion characteristics, plasma membrane integrity, and acrosome morphology of buffalo spermatozoa. Anim. Reprod. Sci., 59: 31–41.

Raza, H.; John, A.; Brown, E.M.; Benedict, S. and Kambal, A. (2008). Alterations in mitochondrial respiratory functions, redox metabolism and apoptosis by oxidant 4-hydroxynonenal and antioxidants curcumin and melatonin in PC12 cells. Toxicol. Appl. Pharmacol., 226, 161–168.

Rotem, R., Paz, G.F., Homonnai, Z.T., Kalina, M., and Naor, Z. (1990). Protein kinase C is present in human sperm: Possible role in flagellar motility. Proc. Natl. Acad. Sci. USA, 87(18):7305–8.

Salahshoor, M.R., Jalili, C., Khazaei, M., and Khani, F. (2012). Effects of curcumin on reproductive parameters in male mice. Journal of Clinical Research in Paramedical Sciences, 1: 1–3.

Salisbury, G.W.; VanDemark, N.L. and Lodge, J.R. (1978). Physiology of Reproduction and Artificial Insemination of Cattle; W.H. Freeman and Company: New York, NY, USA.

Samad, S.A. (1985). Diluters of the Buffalo Semen. Processing and use of buffalo semen. Ani. Sci. Inst. NARC, Islamabad, 34–46.

SAS (2004). SAS/Stat. User's Guide Static's, Version, 6.06, 4th ed.; SAS Institute Inc.: Cary, NC, USA.

Satorre, M.M., Breininger, E., and Beconi, M.T. (2012). Cryopreservation with α-tocopherol and Sephadex filtration improved the quality of boar sperm. Theriogenology, 78(7):1548–56.

Sharma, P. and Singh, R. (2010). Protective role of curcumin on lindane induced reproductive toxicity in male wistar rats. Bull Environ Contam Toxicol, 84(4):378–84.

Silva, E.C.B., Cajueiro, J.F.P., Silva, S.V., Soares, P.C., and Guerra, M.M.P. (2012). Effect of antioxidants resveratrol and quercetin on in vitro evaluation of frozen ram sperm. Theriogenology, 77(8):1722–6.

Silva, P.F.N. and Gadella, B.M. (2006). Detection of damage in mammalian sperm cells. Theriogenology, 65(5):958–78.

Slobodan V. Jovanovic, Charles W. Boone, Steen Steenken, Manuela Trinoga, and Kaskey R.B. (2001). How curcumin works preferentially with water soluble antioxidants. J. Am. Chem. Soc., 123 (13): 3064–3068.

Soleimanzadeh, A., and Saberivand, A. (2013). Effect of curcumin on rat sperm morphology after the freeze-thawing process. Veterinary Research Forum, 4, 185–189.

Srinivas, L., Shalini, V.K., and Shylaja, M. (1992). Turmerin: A water soluble antioxidant peptide from turmeric (Curcuma longa). Arch. Biochem. Biophys., 292 (2):617–23.

Taylor, K., Roberts, P., Sanders, K. and Burton, P. (2009). Effect of antioxidant supplementation of cryopreservation medium on post-thaw integrity of human spermatozoa. Reprod. Biomed. Online,18(2):184–9.

Tejada, R.I., Mitchell, J.C., Norman, A., Marik, J.J. and Friedman, S. (1984). A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. Fertil. Steril., 42(1):87–91.

Terquem, A. and Dadoune, J.P. (1982). In: The Sperm Cell. Dordrecht: Springer Netherlands; p. 249–52. https://doi.org/10.1007/978-94-009-7675-7.

Thomson, L.K., Fleming, S.D., Aitken, R.J., De Iuliis, G.N., Zieschang, J.A., and Clark, A.M. (2009). Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Hum. Reprod., 24(9):2061–70.

Tvrda, E., Tusimova, E., Kovacik, A., Paal, D., Greifova, H., Abdramanov, A., Lukac, N. (2016). Curcumin has protective and antioxidant properties on bull spermatozoa subjected to induced oxidative stress. Animal Reproduction Science, 172: 10–20.

Wang, X., Sharma, R.K., Sikka, S.C., Thomas, A.J., Falcone, T., and Agarwal, A. (2003). Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. Fertil. Steril., 80(3):531–535.

WHO (2010). WHO laboratory manual for the Examination and processing of human semen. World Heal Organ, 5a edition.

Younan, G.E., Shehab El-Din, A.M., Abdel-Khalek, A. E., El-Sherbieny, M.A. and Azza A. Helmy (2021). Soybean lecithin as an alternative to egg yolk in tris-based extender of cryopreserved APRI rabbit semen. J. Anim. Poul. Prod., Mans. Univ., 12 (1):47 – 54.