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Original article

Impact of adding L-carnitine and semen preparation on human semen parameters after cryo-preservation

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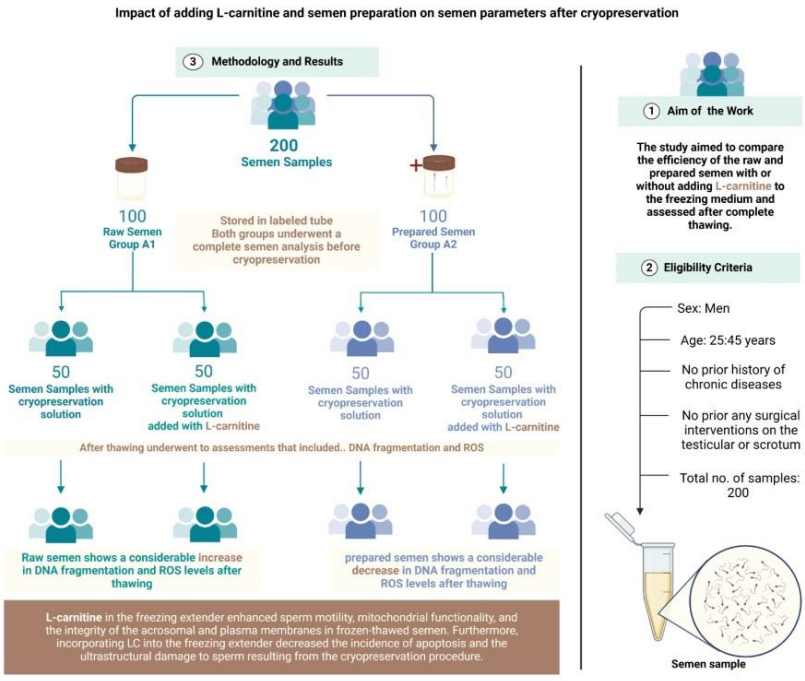
ABSTRACT

Background, the use of antioxidants in semen extenders may reduce cryo-damage to sperm cells. This study assessed the impact of adding L-carnitine to the freezing medium for raw and prepared human semen after complete thawing. **Methods,** the normal semen samples were collected and divided into two main groups in raw and prepared labeled semen tubes (100 cases per group). Each group was under analysis before being divided into two subgroups: traditional cryoprotectant and L-carnitine addition sub-groups. **The results**, showed no significant difference in sperm progressive motility rate between the L-Crantinin subgroup (C1: 11.01 ± 2.0%) and the control group (A1: 11.30 ± 1.2%). Moreover, in the cryoprotectant subgroup, the prepared semen showed a highly significant decrease ($P \leq 0.001$) in mean DNA fragmentation compared to raw semen, with values of 18.01 ± 1.2% for prepared semen and 22.01 ± 1.2% for raw semen; similarly, in the subgroup that received cryoprotectant and L-carnitine. The prepared semen significantly decreased the average DNA fragmentation compared to raw semen (13.41 ± 2.5% versus 15.11 ± 2.2%, respectively). **In conclusion**, adding L-carnitine as an extender enhanced sperm motility, mitochondrial activity, and the integrity of the acrosomal and plasma membranes in frozen-thawed sperm to approach the non-cryopreserved state.

Graphical abstract



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1. Introduction

Cryopreservation is widely used in assisted conception facilities to protect the sperms, especially before cytotoxic chemotherapy, radiation, or specific surgeries that could lead to testicular failure or ejaculatory dysfunction. Cryopreservation of sperm before the start of therapy provides patients with "fertility insurance." This process allows them to have children through in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) [1]. Additionally, cryopreservation is crucial for case insemination programs. During the freezing process, ice formation in the aqueous environment surrounding the sperm causes an increase in the concentration of solutes outside the sperm cells, leading to cellular dehydration. The cryopreservation techniques may help protect the cells from forming ice inside them [2]. It also plays a key role in the artificial insemination programs, allowing for thorough disease screening and ensuring safety before insemination.

All cryopreservation procedures produce oxidative stress through reactive oxygen species (ROS). It may affect the post-thaw characteristics of sperm, encompassing morphology, motility, viability, and DNA integrity. Incorporating antioxidants into the semen extender was advised to mitigate cryo-damage to spermatozoa [3]. L-carnitine (LC), chemically identified as 3-hydroxy-4-(trimethyl azaniumyl) butanoate, is a biomolecule synthesized from the amino acid lysine. In the human body, LC can be naturally synthesized. It is a conditionally necessary nutrient predominantly sourced from meat, fish, and dairy sources. L-carnitine is a naturally occurring, vitamin-like amino acid produced in the body from lysine and methionine, playing a crucial role in lipid metabolism. It transports long-chain fatty acids to the mitochondria for beta-oxidation, generating cellular energy (ATP) for optimal functioning [4]. L-carnitine plays a vital role in cellular detoxification by removing excess acyl-CoA from the mitochondria, which can be harmful. Additionally, it protects cellular membranes from oxidative damage caused by polyunsaturated fatty acids' peroxidation, which are membrane phospholipids' components [5]. Banihani *et al.* [6] found that using a cryopreservation medium enriched with L-carnitine for freezing semen samples improved sperm motility and viability after thawing. Additionally, several studies suggest that L-carnitine may help to reduce lipid damage caused by peroxidation, thereby decreasing the production of malondialdehyde (MDA) and increasing intracellular antioxidant enzymes such as superoxide dismutase (SOD), glutathione, and catalase, which are known for their antioxidant properties. Additionally, they reduce the production of free radicals from the mitochondria, which tend to rise when the mitochondrial respiratory chain and membranes are compromised [7]. The present work evaluates the impact of L-carnitine incorporation to the freezing medium and the effects of semen preparation after complete thawing.

2. Materials and methods

The research involved 200 couples referred for assisted reproduction at the International Islamic Centre for Population Studies and Research (IICPSR) ART unit at Al-Azhar University in Cairo, Egypt, from September 2022 to March 2023. The study was approved by the Ethics Committee of the IICPSR at Al-Azhar University. Eligible participants included men aged 25 to 45 years with no prior history of chronic diseases or any surgical interventions on the testicles or scrotum.

The criteria for the cases were based on the mean age of the male patients, which was 33.2 ± 3.5 years; the average body mass index (BMI) was 24.2 ± 2.5 kg/m², and the average duration of infertility was 5.2 ± 2.6 years (Table 1). Two hundred normal semen samples (A) were collected and divided into two groups: raw semen (100 cases A1) and prepared semen (100 cases A2), each stored in labeled tubes (Fig.1). Both groups underwent a complete semen analysis before cryopreservation. Each group was further divided into two subgroups: subgroup B, which consisted of 50 semen samples mixed with cryopreservation solutions, and subgroup C, which included another 50 semen samples treated with cryopreservation solutions that contained L-carnitine. Group two (A2) prepared semen samples, including spermatozoa, which were divided similarly to the first group. Sub-groups B and C in each group underwent assessments that included semen analysis, DNA fragmentation, and ROS parameters after thawing. The general characteristics of the male participants in the study included age distribution, body mass index (BMI), and duration of infertility.

Table (1): General characters of males studied groups (n=200)

Parameters	Mean ± SD
Age/ years	33.2 ± 3.5
BMI (Kg/m^2)	24.2 ± 2.5
Infertility years	± 2.6

2.1. Complete semen analysis (manually)

A-Sample compilation: Semen samples were obtained by masturbation following a period of abstinence lasting 2 to 5 days; extended abstinence (10 days) results in diminished motility, while shorter durations yield reduced volume and density. The container was clean, sterile, and wide-mouthed to minimize collecting errors from a verified non-toxic batch of spermatozoa. The semen sample was preserved at room or body temperature and analyzed within one hour of collection [8].

B- Physical examination

Microscopic analysis: According to the WHO organization [8], this encompasses the manual assessment of sperm concentration, motility, and morphology by ordinary light microscope (Olympus, USA.400 x).

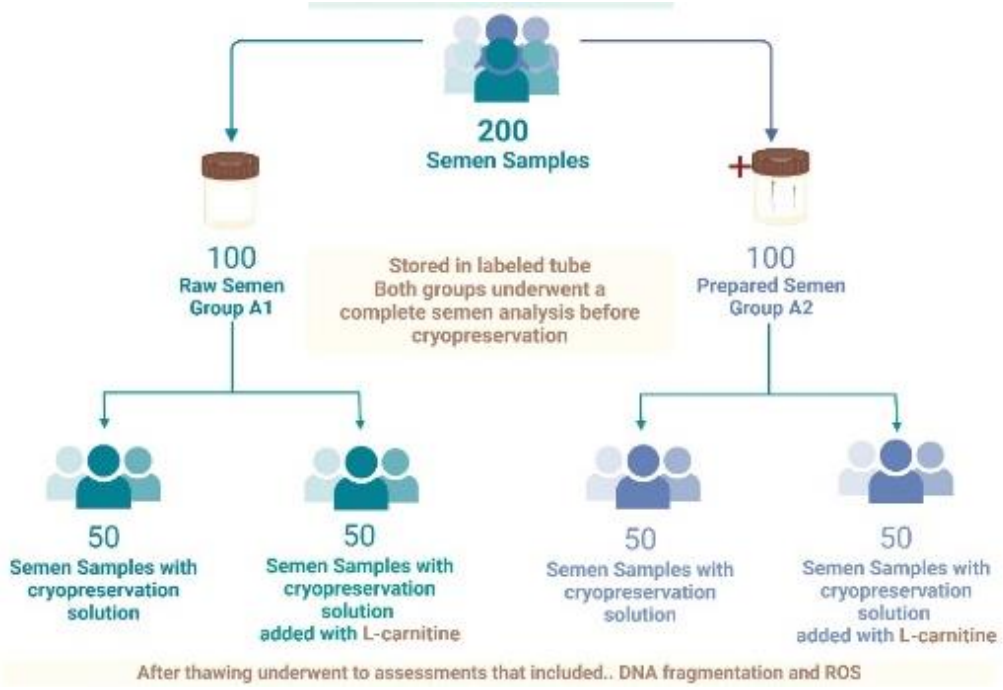

Fig.1. Experimental design.

2.2. Technique for sperm preparation

Group 2 was instructed to prepare semen using the swim-up procedure, and the highly motile spermatozoa were extracted for immediate examination [8].

2.3. Cryopreservation and thawing of semen

To prepare freezing medium enriched with 1 gram of L-carnitine, $((CH3)_3N+CH2CH(OH)CH_2CO2H \cdot Cl$-, Sigma, USA) in 50 milliliters was dissolved to achieve a solution containing 5% L-carnitine. The Then the spermatozoa were transferred into cryovials (Nalge Company, Rochester, NY, USA) and mixed with a 1:0.7 ratio with commercial Sperm freeze™ cryoprotectant (Ferti Pro NV, Sint-Martens-Latem, Belgium,), which was introduced dropwise while gently spinning. Sperm freeze™ was maintained at four °C and acclimatized to room temperature before utilization. The mixture was kept at room temperature for 10 minutes and frozen using static phase vapour chilling. Aliquots were suspended in liquid nitrogen vapour (10 cm above the liquid nitrogen level at –80 °C) for 15 minutes. The samples were subsequently immersed in liquid nitrogen (-196 °C) and preserved until needed [9, 10].

The samples were thawed by defrosting at ambient temperature for 15 to 20 minutes. An, equivalent amount of BWW buffer was introduced to each cryovial, followed by centrifugation at 200 g for 6 minutes to eliminate residual Sperm freeze™ cryoprotectant. The supernatant was discarded, and the pellet was resuspended in an appropriate volume of BWW (~400 µl) [9, 10].

2.4. Assessment of reactive oxygen species

Oxygen species (OS) levels were measured using an Oxisperm kit (Nordic cell, USA), which detects excess superoxide anions (O_2^-) in ejaculate samples. This test utilized a nitroblue tetrazolium (NBT) assay with reactive gel (RG). In this process, superoxide anions convert water-soluble salt into insoluble blue formazan crystals, increasing the color intensity of the RG. A colorimeter was used to measure measured the absorbance at wavelengths ranging from 350 nm to 630 nm, indicating the sample's oxidative stress level (specifically, the excess of superoxide anions) [11, 12].

2.5. Assessment of DNA fragmentation

The method was based on the Sperm Chromatin Dispersion (SCD) test. Intact, unfixed spermatozoa were placed in an agarose microgel on a pretreated slide. The sperm with fragmented DNA displayed little to no dispersion halo. The DNA fragmentation levels were measured using the Halosperm G2 test kit (Halotech DNA, USA). The samples were mixed with agarose gel, applied to a pre-coated slide, refrigerated, and treated with a denaturing agent and lysis solution. Finally, the slides were stained, and 300 sperm samples were counted for assessment [13, 14].

2.6. Statistical analysis

The main characteristics of patients were described through tabular representation, whether single or cross-frequency tables provided with some descriptive statistics of variables considered, such as mean and standard deviation (SD). Moreover, the data were analyzed using statistical analysis software package (SAS, 2002) [15].

3. Results

I- Results of studied Group 1: Raw semen samples

Table 2 represents the sperm concentration in sub-groups B and C exhibited a decrease ($P \leq 0.001$) in values (7.60 ± 2.1 & $7.91 \pm 2.2 \times 10^6$/ml, respectively) relative to raw semen (A1, $8.60 \pm 2.3 \times 10^6$/ml). In sub-group C, the level of L-carnitine was increased, making it closer to the control value. Sperm motility decreased in the cryopreserved sub-groups, with sub-groups B and C showing motility rates of 21.30 ± 1.1% and 28.32 ± 2.0%,

respectively, compared to A1, which had a motility rate of 31.30 ± 2.1%. Statistical comparisons among these groups indicated highly significant differences (P < 0.001). The results showed no significant difference in sperm progressive motility rate between the L-Crantinin subgroup (C: 11.01 ± 2.0%) and the control group (A1: 11.30 ± 1.2%). However, subgroup B decreased motility rate to 7.21 ± 2.2%. Adding LC significantly increased (P ≤ 0.001) motility rate in subgroup C compared to subgroup B.

Table (2): Assessment of raw semen parameters before cryopreservation and after thawing (group 1)

Parameters	(A1) Raw semen (N=100)	After thawing		P value
		(B) Cryoprotectant (N=50)	(C) Cryoprotectant & L-Carnitine (N=50)	
	Mean ±SD	Mean ± SD	Mean ± SD	
Sperm Concentration ($\times 10^6$/ml)	8.60 ± 2.3	7.60 ± 2.1	7.91 ± 2.2	P (A, B)=0.0108** P (A, C) = 0.0810 P (B, C) =0.4728
Sperm Total Motility (%)	31.30 ± 2.1	21.30 ± 1.1	28.32 ± 2.0	P (A, B) < 0.0001*** P (A, C) < 0.0001*** P (B, C) < 0.0001***
Sperm Progressive motility (%)	11.30 ± 1.2	7.21 ± 2.2	11.01 ± 2.0	P (A, B) < 0.0001*** P (A, C) = 0.2701 P (B, C) < 0.0001***

(***) highly significant P≤0.001, (**) very significant P≤0.01, (*) significant P≤0.05, not significant P>0.05.

Table 3 compares sperm morphology after thawing in raw semen (Group 1), highlighting several significant findings. Regarding abnormal sperm forms, raw semen (A1) showed a mean percentage of 97.01 ± 3.1%. It was significantly different from sub-group B, which had a mean of 98.21 ± 2.1% (P ≤ 0.05); this indicates a slight increase in the percentage of abnormal sperm forms following cryopreservation. However, there was no significant difference observed between raw semen (A1) and the cryoprotectant supplemented with L-carnitine (sub-group C), which had a mean of 97.83 ± 2.1% (P > 0.05). Additionally, there was no significant difference (P > 0.05) between the cryoprotectant sub-group (B) and the cryoprotectant supplemented with L-carnitine (sub-group C).

The analysis showed no significant differences in head defects among the groups. However, midpiece defects showed a statistically significant difference between raw semen (A1 and sub-group B), with mean percentages of 31.52 ± 2.3% for A 1 and 32.62 ± 2.2% for sub-group B (P ≤ 0.001). This finding indicates an increase in midpiece defects following cryopreservation. Additionally, no significant differences were found between raw semen (A 1) and the cryoprotectant supplemented with L-carnitine (sub-group C), which had a mean percentage of 31.92 ± 2.1% (P > 0.05). Similarly, there were no significant differences between the cryoprotectant alone (sub-group B) and the cryoprotectant supplemented with L-carnitine (P > 0.05). Tail defects showed a statistically significant increase in the cryoprotectant group (sub-group B: 13.40 ± 1.9%) compared to the control (A1: 12.10 ± 1.2%) with a p-value of ≤ 0.01. Furthermore, there was a significant increase in tail defects in the cryoprotectant sub-group (B: 13.40 ± 1.9%) compared to sub-group C (12.11 ± 1.1%), also with a p-value of ≤ 0.01. However, no significant difference was found between the raw semen A 1 and the cryoprotectant supplemented with L-carnitine, as indicated by a p-value greater than 0.05.

In Table 4 the comparison of sperm functional tests post-thawing among group 1 (n=100) includes the evaluation of two critical parameters: DNA fragmentation index (DFI%) and reactive oxygen species (ROS). Significant differences (p ≤ 0.001) were noted in the DNA fragmentation index percentage (DFI%) between raw semen (A 1) and the cryoprotectant (sub-group B), with mean values of 14.21 ± 2.3% and 22.01 ± 1.2%, respectively.

This result indicates a notable increase in DFI% after cryopreservation; a significant difference was also observed between raw semen (A1) and the cryoprotectant supplemented with L-carnitine (sub-group C), which had a mean DFI% of 15.11 ± 2.2 % (p ≤ 0.05). Furthermore, a highly significant difference (p ≤ 0.001) was found between the cryoprotectant (sub-group B) and the cryoprotectant with L-Carnitine (sub-group C), suggesting that L-Carnitine may have a beneficial effect on reducing DNA fragmentation after thawing. Secondly, concerning ROS levels, raw semen (A1) exhibited a mean ROS value of 0.69 ± 1.2%. This value significantly differed from the cryoprotectant (sub-group B), which had a mean ROS of 1.21 ± 0.6%; this indicates an increase in ROS production following cryopreservation. However, there was no significant difference (P > 0.05) between raw semen (A1) and the cryoprotectant supplemented with L-carnitine (sub-group C), which had a mean ROS of 0.70 ± 1.4%. A highly significant difference was found between the cryoprotectant (sub-group B) and the cryoprotectant with L-carnitine (P ≤ 0.001), suggesting that L-carnitine may play a role in reducing ROS generation during the freezing process.

Table (3). Assessment of sperm morphology before cryopreservation and after thawing (group 1)

Parameters	Before cryo.	After thawing		P value
	(A1) Raw semen (N=100)	(B) Cryoprotectant (N=50)	(C) Cryoprotectant & L-Carnitine (N=50)	
	Mean ±SD	Mean ± SD	Mean ± SD	
Abnormal forms (%)	97.01 ± 3.1	98.21 ± 2.1	97.83 ± 2.1	P (A, B) =0.0148* P (A, C) =0.0940 P (B, C) =0.3678
Head defects (%)	51.91 ± 2.1	52.21 ± 2.1	51.97 ± 2.1	P (A, B) =0.4108 P (A, C) =0.8692 P (B, C) =0.0734
Midpiece defects (%)	31.52 ± 2.3	32.62 ± 2.2	31.92 ± 2.1	P (A, B) =0.0058** P (A, C) =0.3033 P (B, C) =0.1068
Tail defects (%)	12.10 ± 1.2	13.40 ± 1.9	12.11 ± 1.1	P (A, B) < 0.0001*** P (A, C) = 0.9606 P (B, C) < 0.0001***

(***) highly significant P≤0.001, (**) very significant P≤0.01, (*) significant P≤0.05, not significant P>0.05.

Table (4). Comparison between raw sperm functional test before cryopreservation and after thawing (group 1)

Parameters	(A2) Row semen (N=100)	After thawing		P value
		(B) Cryoprotectant (N=50)	(C) Cryoprotectant& L-Carnitine (N=50)	
	Mean ±SD	Mean ± SD	Mean ± SD	
DNA fragmentationindex (DFI%)	14. 21 ± 2.3	22.01 ± 1.2	15.11 ± 2.2	P (A, B) < 0.0001*** P (A, C) = 0.0233* P(B , C) = < 0.0001***
Reactive oxygen species (ROS)	00.69 ± 1.2	01.21 ± 0.6	00.70 ± 1.4	P (A, B) = 0.0045** P (A, C) = 0.9638 P(B , C) = 0.0199**

(***) highly significant P≤ 0.001, (**) very significant P≤ 0.01, (*) significant P≤ 0.05.

II **Results studied Group 2: Prepared semen samples**

Table 5 shows group A2 had a significantly higher sperm concentration of 16.50 ± 2.0 × 10^6/ml compared to subgroup B, which had a concentration of 14.91 ± 2.2 × 10^6/ml (P ≤ 0.001). A highly significant difference (P ≤ 0.01) was also observed between group A2 and subgroup C, which had a sperm concentration of 15.50 ± 2.1 × 10^6/ml.

However, no significant difference (P > 0.05) was found between sub-groups B and C. Additionally, total and progressive motility were significantly higher (P ≤ 0.001) in group A 2 compared to subgroups B and C.

Table (5). Assessment of prepared semen parameters before cryopreservation and after thawing (group 2)

Parameters	**Before cryo.**	**After thawing**		**P value**
	(A2) Prepared sample (N=100)	**(B) Cryoprotectant (N=50)**	**(C) Cryoprotectant & L-Carnitine (N=50)**	
	Mean ±SD	Mean ± SD	Mean ± SD	
Concentration (×10^6/ml)	16.50 ± 2.0	14.91 ± 2.2	15.50 ± 2.1	**P (A, B)** < 0.0001*** **P (A, C)** =0.0052** **P (B, C)** = 0.1733
Total Motility (%)	49.30 ± 1.2	32.33 ± 1.1	39.30 ± 1.0	**P (A, B)** < 0.0001*** **P (A, C)** < 0.0001*** **P (B, C)** < 0.0001***
Progressive motility (%)	29.20 ± 1.2	18.20 ± 1.1	22.20 ± 1.0	**P (A, B)** < 0.0001*** **P (A, C)** < 0.0001*** **P (B, C)** < 0.0001***

(***) highly significant P≤0.001, (**) very significant P≤0.01, (*) significant P≤0.05, not significant P>0.05.

Table 6 shows that there are significant differences in sperm morphology after thawing among the sub-groups. In sub-group B, the percentages and standard deviation values are significantly different ($P \leq 0.001$) compared to the normal control sub-group A, Sub-group B recorded 96.21 ± 2.9% of abnormal forms and 32.62 ± 2.3% of midpieces, while sub-group A had values of 94.01 ± 2.1% and 31.42 ± 2.3%, respectively. Head defects show a significant difference between sub-groups A and B ($P \leq 0.05$), with the mean and standard deviation for sub-group B being 31.42 ± 2.3% and 32.62 ± 2.3%, respectively. Adding of L-carnitine to the cryoprotectant (sub-group C) results in a notable reduction in head defects compared to the cryoprotectant alone (group B). Additionally, when compared to sub-groups A, C exhibits a less significant increase in abnormal forms ($P \leq 0.001$), while tail defects remain similar ($P > 0.05$). There are no significant differences in head and tail defects between groups A (51.51 ± 2.1% for head defects and 11.10 ± 1.2% for tail defects) and sub-group C (51.71 ± 2.1% for head defects and 11.30 ± 1.1% for tail defects) ($P > 0.05$).

Table (6). Assessment of sperm morphology before cryopreservation and after thawing (group 2)

Parameters	Before cryo.	After thawing		P value
	(A2) Prepared sample (N=100)	(B) Cryoprotectant (N=50)	(C) Cryoprotectant & L-Carnitine, (N=50)	
	Mean ±SD	Mean ± SD	Mean ± SD	
Abnormal forms (%)	94.01 ± 2.1	96.21 ± 2.9	95.01 ± 2.19	P (A, B) < 0.0001*** P (A, C) = 0.0075** P (B, C) = 0.0216*
Head defects (%)	51.51 ± 2.1	52.21 ± 2.1	51.71 ± 2.1	P (A, B) = 0.0562* P (A, C) = 0.5832 P (B, C) = 0.2367
Midpiece defects (%)	31.42 ± 2.3	32.62 ± 2.3	31.92 ± 2.3	P (A, B) = 0.0031** P (A, C) = 0.2797 P (B, C) = 0.1313
Tail defects (%)	11.10 ± 1.2	13.40 ± 1.2	11.30 ± 1.1	P (A, B) < 0.0001*** P (A, C) = 0.3244 P (B, C) < 0.0001***

(***) highly significant P≤ 0.001, (**) very significant P≤ 0.01, (*) significant P≤ 0.05.

Table 7 indicates significant differences in sperm functional tests after thawing among the groups. Cryoprotectant sub-group B significantly elevates ($P \leq 0.001$) the DFI index from 12.21 ±2.3% in the prepared sample (A2) to 18.01 ±1.2%, indicating adverse effects on DNA integrity. Reactive oxygen species (ROS) significantly increased from 0.49 ±1.2% in group A2 to 1.11 ±0.6% in sub-group B ($P \leq 0.001$), indicating induced oxidative stress. The incorporation of L-Carnitine into the cryoprotectant (sub-group C) leads to a less significant increase in DFI (13.41 ± 2.5%) relative to A2 (12.21 ± 2.3%, $P \leq 0.001$). There is a notable decrease in ROS levels in sub-group C (0.69 ± 1.3%) compared to sub-group B (01.11 ± 0.6%, $P \leq 0.05$), suggesting a protective effect of L-carnitine against cryoprotectant-induced impairment of sperm function. However, sub-group C shows a greater DFI lower than sub-group B.

Table (7). Comparison between prepared sperm functional test before cryopreservation and after thawing

Parameters	Before cryo.	After thawing		P value
	(A2) Prepared sample (N=100)	(B) Cryoprotectant (N=50)	(C) Cryoprotectant& L-Carnitine (N=50)	
		Mean ± SD	Mean ± SD	
DNA fragmentationindex (DFI%)	12. 21 ± 2.3	18.01 ± 1.2	13. 41 ± 2.5	P (A, B) < 0.0001*** P (A, C) = 0.0040** P (B, C) < 0.0001***
Reactive oxygenspecies (ROS)	00.49 ± 1.2	01.11 ± 0.6	00.69 ± 1.3	P (A, B) = 0.0008*** P (A, C) = 0.3509 P (B, C) = 0.0407*

(group 2)

(***) highly significant p≤0.001, (**) very significant P≤0.01, (*) significant P≤0.05.

4. Discussion

Cryopreservation is usually used to store sperm from azoospermia's patients after testicular or percutaneous epididymal sperm extraction, helping to avoid the need for repeated biopsies or aspirations [16]. One of the main drawbacks of cryopreservation is decreases in both motility and velocity [17]. During thawing, the concentration of solute in the extracellular sperm cells decreases compared to that in the intracellular cells due to ice melting outside the cells, resulting in damage [18]. Generally, sperm with damaged DNA can fertilize an egg. However, mutations and abnormalities may not become apparent until embryonic division or fetal development occurs [19]. Numerous studies have shown an increase in sperm DFI in infertile patients compared to those with viable fertility [4]. Researches indicate that prolonged L-carnitine supplementation enhances the semen parameters of stallions, roosters, rams, and rabbits. However, adding L-carnitine to semen extenders did not improve the freezing and thawing of spermatozoa in rams [20].

According to the first group in the present study, using cryoprotectants combined with L-carnitine (C) resulted in longer liquefaction duration and improved sperm motility compared to the other subgroups. The previous authors observed that cryopreservation with L-carnitine particularly boosted rapid progressive sperm motility in samples with high levels of endogenous seminal L-carnitine compared to samples with lower levels. It may be due to the L-carnitine delivering fatty acids to the mitochondria, which undergo beta-oxidation, generating metabolic energy for sperm cell motility [21]. In the previous studies, Palacios et al. [19] reported that L-carnitine concentrations significantly increased various kinematic variables (total motility, TM), the velocity of curvilinear motion (VCL), velocity of straight-line motion (VSL), beat cross frequency (BCF), and individual progressive motility index (IPIA)) in sperm samples for up to 96 hours ($P \leq 0.05$).

In the present work, according to the 1 and 2 groups, sperm concentration in sub-groups B and C decreased ($P \leq 0.001$) in values relative to raw semen (A). In subgroup C, the level of L-carnitine was increased, making it closer to the control value. The present findings support a previous study showed that L-carnitine helps in maintain the integrity of mitochondrial membranes, protects cells from apoptosis, and significantly enhances mitochondrial functions and metabolic activity by reducing oxidative stress pathways [22]. El-Raey et al. [23] discovered that adding 0.05 mg/mL of L-carnitine to buffalo semen improves vitality and preserves acrosomal integrity. Similarly, Abdel-Khalek et al. [24] observed substantial increases ($P \leq 0.05$) in ejaculate volume, sperm motility, the percentage of live sperm, sperm concentration, and overall sperm production in Friesian bulls after three months of daily oral administration of L-carnitine.

The present study showed no significant differences in head and tail defects between A1 and sub-group C ($P > 0.05$) compared to sub-group B. The present results referred to the beneficial effects of L-carnitine on protecting the sperm from oxidative damage caused by cryopreservation. Similar to our results., Galarza et al. [25] L-carnitine supplementation significantly decreased acrosomal damage in cooled raw semen relative to the control group. By preserving sperm membrane integrity and function, adding L-carnitine to the semen extender may protect against cold shock. Abd-Allah et al. [26] discovered that L-carnitine reduced aberrant spermatozoa in bucks' semen compared to controls. This study found that cryopreservation DFI% showed a significant difference between the cryoprotectants in sub-group B and those with L-carnitine in sub-group C, suggesting that L-carnitine may prevent DNA fragmentation post-thawing.

Post-cryopreservation, ROS levels in raw semen (subgroup B) increased significantly compared to the other subgroups. The findings show how cryopreservation impacts sperm function and suggest that L-carnitine supplementation helps to preserve quality post-thawing. Heidari et al. [27] found that cryopreservation in buck sperm lowers global DNA methylation and that the L-carnitine addition to the freezing medium reverses the decline quality parameters and epigenetic patterns of buck's frozen-thawed semen. L-carnitine's antioxidant effects may prevent global DNA methylation in post-thaw spermatozoa with high endogenous and supplemented levels since ROS levels negatively correlate with global sperm DNA methylation. Jiang et al. [28] observed that L-carnitine levels effectively reduce sperm DNA damage in raw semen by correlating with % DFI parameters in asthenospermic patients' semen samples.

In the prepared semen group (2), cryoprotectant with L-Carnitine (sub-group C) reduced liquefaction time and increased sperm concentration, total, and progressive motility in semen samples, with significant differences ($P \leq 0.001$). Abdelnour et al. [29] found that adding LC to cryopreserved rabbit semen increased sperm motility, acrosome integrity, plasma membrane integrity, ultrastructure preservation, and apoptotic cell reduction. According to Abd El-Hamid et al., [20] thawing cryopreserved semen treated with LC (50 mM) resulted in considerably greater sperm motility, membrane integrity, and acrosome integrity than untreated control samples. Also, Banihani et al. [6] found that adding LC to cryopreserved spermatozoa substantially enhanced motility by 21.5% ($P \leq 0.05$) compared to the control group. Adding LC to human sperm cryoprotectants boosts motility and viability after thawing. In the prepared semen sample, adding L-carnitine to cryoprotectant (sub-group C) dramatically lowered tail defects compared to cryoprotectant alone (sub-group B). It may also reduce cryoprotectant's effects on sperm morphology, particularly tail anomalies, and increase spermatogenesis by lowering aberrant sperm, such as those with disconnected heads and tails, according to Lisboa et al. [30]. Abdelnour et al. [29] discovered that adding L-carnitine as an extender decreased sperm morphological abnormalities.

The present investigation found that cryoprotectant alone (sub-group B) significantly increased ($P \leq 0.001$) the DNA fragmentation index compared to semen samples in A1&2. ROS levels considerably increased (P

≤0.001) in the B subgroup of the prepared sample, indicating oxidative stress. Adding L-carnitine to cryoprotectant (sub-group C) decreased DFI values (P ≤0.001). It significantly reduces ROS levels (P ≤ 0.05) compared to the B subgroup. According to subgroup C, it had a more extensive DFI than A2 (before freezing) but less than B, meaning L-carnitine may protect against cryoprotectant-induced DNA damage and oxidative stress. Rezaei et al. [31] found that adding L-carnitine (2.5 or 5 mM) to the freezing extender increased matrix metalloproteinase protein and decreased ROS in mice's post-thawed epididymal spermatozoa. The authors hypothesized that L-carnitine may supply energy and facilitate the transfer of activated fatty acids to the mitochondrial matrix for β-oxidation. Also, it preserves sperm DNA and inhibits protein and lactate oxidation, according to Abdelrazik et al. [32]. The present work showed that in the cryoprotectant subgroup, prepared semen exhibited less DFI than raw semen. The raw semen group exhibited more DFI than the cryoprotectant subgroup (B) and L-carnitine-added semen. Processed semen has far more DFI than raw semen. Semen preparation protects sperm DNA integrity during cryopreservation, which is essential for sperm quality and fertility. Raad et al. [33] reported a higher number of reactive oxygen species (ROS)-**positive** cells in centrifugation-dependent procedures, such as donor gametes and sperm ultra-centrifugation, compared to raw semen. They noted that increased ROS levels were associated with higher DFI. These findings are consistent with Le et al., [34] who observed that sperm quality was highest at 0 hours post-preparation and that sperm with a low DFI outperformed raw semen.

Distinct patterns emerge when comparing raw and prepared semen within each group. In the cryoprotectant subgroup, the prepared semen significantly reduced DNA fragmentation compared to raw semen. Similarly, with the addition of L-carnitine, prepared semen showed a considerable decrease in DFI compared to raw semen. On the other hand, the ROS level was decreased after thawing in prepared semen with or without the addition of L-carnitine into the freezing extender.

5. Conclusion

Incorporating L-carnitine into the freezing extender enhances sperm motility, and the integrity of the acrosomal and plasma membranes in frozen-thawed semen. Furthermore, incorporating LC into the freezing extender decreased DNA fragmentation, the incidence of apoptosis and the ultrastructural damage to sperm resulting from the cryopreservation procedure.

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