Hindawi
Andrologia
Volume 2024, Article ID 4327010, 11 pages
https://doi.org/10.1155/2024/4327010

Research Article

Association of Endogenous Seminal L-Carnitine Levels with Post-Thaw Semen Parameters in Humans

Mario Iliceto,[1,2] **Jorunn M. Andersen**,[2] **Mette Haug Stensen**,[1] **Trine B. Haugen**,[2] **and Oliwia Witczak**[2]

[1]*Fertilitetssenteret, Oslo NO-0176, Norway*
[2]*Department of Life Sciences and Health, OsloMet-Oslo Metropolitan University, Oslo NO-0130, Norway*

Correspondence should be addressed to Mario Iliceto; marioi@oslomet.no and Oliwia Witczak; oliwiaw@oslomet.no

Received 18 September 2023; Revised 3 February 2024; Accepted 16 February 2024; Published 1 March 2024

Academic Editor: Raul Sanchez

Cryopreservation of semen is a useful tool for male fertility preservation. Some evidence for a beneficial effect of L-carnitine supplementation of freezing media on cryopreserved semen samples has been reported. Here, we examined the association of endogenous levels of seminal L-carnitine with post-thaw semen parameters. We also investigated the effect of freezing medium supplemented with L-carnitine on sperm characteristics, related to endogenous seminal L-carnitine levels. Semen analyses were performed on 125 fresh samples, and after standard cryopreservation and with L-carnitine as a supplement. Participants were categorized into two groups based on the median levels of endogenous seminal L-carnitine: low L-carnitine ($\leq$38.8 μg/ml) and high L-carnitine (>38.8 μg/ml). After standard cryopreservation, semen samples with high L-carnitine levels showed higher rapid progressive, progressive and total sperm motility and a reduced seminal static oxidation–reduction potential (ORP) level than samples with low L-carnitine levels. Only in post-thaw samples with low L-carnitine levels, there was an increase in the amount of sperm neck midpiece defects, compared to the fresh samples. Cryopreservation with L-carnitine had the most beneficial effect on rapid progressive sperm motility in samples with high endogenous L-carnitine levels. In conclusion, L-carnitine has a beneficial impact on sperm characteristics in post-thaw samples both as an endogenous component in seminal plasma and as a supplement in the freezing medium, by improving sperm motility and reducing seminal oxidative stress.

1. Introduction

Cryopreservation of semen in liquid nitrogen is a useful tool for male fertility preservation and is used routinely in assisted reproductive technology (ART) [1]. The procedure is used for donor sperm storage [2], prior to cancer treatments [3], in case of autoimmune diseases [4] and upon testicular and epididymal sperm extraction for ART [5]. Freezing and thawing have deleterious effects on sperm structure and function, including diminished sperm motility and viability [6–8] and increased DNA damage [6, 7, 9]. Several mechanisms causing cryopreservation-induced damage have been described, including cold shock, osmotic stress, intracellular ice crystal formation, and oxidative stress [10].

Oxidative stress is caused by an imbalance between the formation of reactive oxygen species (ROS) and antioxidant defenses [11]. Spermatozoa are highly susceptible to the deleterious effects of excessive ROS generation due to the high amount of polyunsaturated fatty acids (PUFAs) in the plasma membrane susceptible to lipid peroxidation [12]. Lipid peroxidation can lead to loss of membrane fluidity and integrity and can affect sperm structure and function [13, 14]. Increased levels of ROS in seminal plasma are associated with reduced sperm count [15] and sperm motility [16, 17] and increased DNA fragmentation [18].

Due to their small cytoplasmic volume, spermatozoa have limited amounts of ROS-scavenging enzymes. Therefore, the main antioxidant defense system of the spermatozoa is present in the seminal plasma [19, 20]. ROS-scavenging enzymes like glutathione peroxidase and glutathione transferase are found in both spermatozoa [21, 22] and seminal plasma [23, 24]. Enzymatic activities of superoxide dismutase (SOD) and catalase (CAT) have been measured in sperm [25, 26] and seminal

plasma [27]. Other important, nonenzymatic antioxidants present in seminal plasma are vitamins E and C [28–30].

L-carnitine, an amino acid derivative, plays an important role in fatty acids metabolism [31] and has been shown to reduce oxidative stress [32]. High amounts of L-carnitine originating from the epididymis are found in seminal plasma, and are positively associated with the total sperm count, sperm motility [33–35], sperm vitality, and sperm morphology [35] in humans. Supplementation of freezing media with L-carnitine has been shown to reduce cryopreservation-induced damage of the spermatozoa, improving post-thaw semen quality such as sperm motility [36–38] and sperm viability [37, 38], and reduce the amount of ROS in spermatozoa [39]. To our knowledge, no studies have investigated the association between endogenous levels of L-carnitine in seminal plasma and alterations in semen parameters upon freezing-thawing.

The aim of this study was to examine the association of endogenous levels of seminal L-carnitine with semen parameters after freezing-thawing. We also investigated the effect of freezing medium supplemented with L-carnitine on post-thaw semen parameters, related to the endogenous seminal L-carnitine levels.

2. Materials and Methods

2.1. Study Population. Men over the age of 18 years were recruited at the Fertilitetssenteret (Oslo, Norway), a fertility clinic that offers semen analysis for men. The recruitment was done between November 2018 and February 2020. The majority of the participants was not from couples undergoing IVF-treatment (89%). Incomplete samples, according to patient's information, were excluded from the study as well as samples with a sperm concentration less than 5×10^6/ml and/or with progressive motility of less than 10%. In total, 125 men were included in the study.

Participants reported information about sample collection, days of abstinence, weight, and height. Age was registered as whole years.

The study was approved by the Regional Committee for Medical and Health Research Ethics (REK), South East, Norway (REK number: 2018/423). All participants provided a written informed consent.

2.2. Semen Analysis. Semen samples were obtained by masturbation and collected on-site or at home in a preweighed sterile container. Participants were asked to abstain from ejaculating for 2–7 days before sample collection and to report the length of abstinence time. When collected at home, participants were instructed to avoid temperature variation of the sample during transportation. Out of 125 semen samples, 121 were collected on-site and four at home. Semen samples collected on-site were incubated for 30 min at 37°C, while samples collected at home were incubated for 10 min at 37°C. All of 125 semen samples were analyzed after complete liquefaction and within 1 hr after ejaculation. The ejaculated volume was estimated by weight. The methods recommended by the World Health Organization (WHO, 2010) [40] were employed to estimate sperm concentration, motility, and vitality. Sperm concentration was determined using an improved Neubauer hemocytometer (Hecht Assistant, Sondheim vor der Rhön, Germany). Sperm motility was categorized into progressive (Grades a + b), non-progressive (Grade c), and immotile sperm (Grade d). Total sperm motility was calculated (Grades a + b + c). In addition, rapid progressive motility was assessed (Grade a), which is a part of the categorization system in the WHO 1999 manual [41]. Vitality and morphology assessments were performed manually. In both fresh and post-thaw samples, 100 μl of semen was mixed with 500 μl Sperm Preparation Media (Cooper Surgical, Trumbull, CT, USA) and centrifugated at 300x g for 10 min. The supernatant was removed, and the pellet was resuspended in Sperm Preparation Media (Cooper Surgical, Trumbull, CT, USA) to obtain a sperm concentration of $5–10 \times 10^6$/ml. Vitality analysis was determined with BrightVit, a neosin and nigrosin staining solution (Microptic SL, Barcelona, Spain). Morphology was assessed according to the Tygerberg strict criteria [42], after Sperm Blue staining (Microptic SL, Barcelona, Spain). The semen analysis was performed by the same person, who participated in the external quality Program for semen assessment organized by ANOVA, Karolinska University Hospital, Sweden, when this study was conducted. Seminal plasma for L-carnitine analysis was prepared from the remaining ejaculated by centrifugation at 500x g for 15 min and stored at −80°C before analysis.

2.3. Cryopreservation of Spermatozoa. After initial semen analysis, two equal aliquots of each sample were prepared for cryopreservation: one for cryopreservation in standard sperm freezing medium (Cooper Surgical, Trumbull, CT, USA) and the other in the same sperm freezing medium supplemented with 0.5 mg/ml L-carnitine (Sigma Tau, Pomezia, Italy). To identify the optimal level for post-thaw sperm motility (Grades a + b + c), a gradient of L-carnitine concentrations (0, 0.1, 0.5, 1, 10, and 25 mg/ml) was added to the freezing medium of 10 semen samples. Figure S1 shows that 0.5 mg/ml L-carnitine added to the freezing medium had the best effect on post-thaw sperm motility. The selected value was consistent with the findings of a previous study [37]. Freezing medium was added dropwise to each aliquot, reaching a final dilution of 1 : 1. The mixture was left at room temperature for 20 min, then loaded into straws (Cryo bio system, Saint–Ouen Sur Iton, France) and sealed according to the manufacturer's recommendations. The straws were suspended horizontally above the surface of liquid nitrogen for 30 min, and then plunged into and stored in liquid nitrogen (−196°C). Before semen analysis, straws were thawed at room temperature for 5 min.

2.4. DNA Fragmentation. GoldCyto Sperm DNA kit (GC-SCD-20, GoldCyto Biotech, GuangZhou, China) was used to analyze DNA fragmentation according to the manufacturer's protocol for both fresh and cryopreserved samples. The method is based on the sperm chromatin dispersion (SCD) test [43, 44]. An aliquot of 100 μl semen sample was diluted in Sperm Preparation Media (Cooper Surgical, Trumbull, CT, USA) to obtain a sperm concentration of $5–10 \times 10^6$/ml. The cell suspension was then mixed with a low-melting-point agarose placed over an agarose pretreated slide

and left to solidify at 4°C for 5 min. The slides were immersed in a hydrochloric acid solution for 7 min at room temperature in the dark to generate restricted single-stranded DNA motifs from DNA breaks. After denaturation, nuclear proteins were removed using a lysing solution for 25 min at room temperature. The slides were placed into distilled water for 5 min, and dehydrated in sequential 70%, 90%, and 100% ethanol baths (2 min each) and air-dried. For staining, the slides were first immersed in TA solution (0.8 ml) for 1 min and then in TB solution (2.4 ml) for 6 min. The information about the staining ingredients is not available for the customers. To prevent the sediment from being deposited on the specimen, the slides were flushed with tap water, air dried and mounted with Eukitt (#253681, Panreac, Barcelona, Spain). The stained smears were examined under a bright light microscope (Olympus, Tokyo, Japan) at ×1,000 magnification. A minimum of 200 spermatozoa were counted. Spermatozoa with large or medium halos were defined as normal, whereas those with small or no halo and degraded spermatozoa were defined as fragmented. The SCD test results were expressed as % DNA fragmentation.

2.5. Seminal L-Carnitine Analysis. Level of L-carnitine (μg/ml) in free form in fresh seminal samples was analyzed by liquid chromatography/tandem mass spectrometry at a commercial laboratory in Oslo in 2020 (Vitas AS, Oslo, Norway) as previously described [33]. In brief, 10 μl of seminal plasma was diluted with labeled internal standard L-carnitine d9 (Sigma Aldrich, Taufkirchen, Germany) and analyzed on an Agilent 1260 HPLC coupled to an Agilent 6460 QQQ mass spectrometer operated in Multiple Reaction Monitoring Mode (Agilent Technologies, CA, USA).

2.6. Seminal Oxidation–Reduction Potential (ORP) Measurement. The ORP is the ratio of activity between oxidants and reductants and can be measured in biological samples such as blood plasma [45] and semen [46]. Static ORP (sORP) gives information about the current balance between oxidants and reductants/antioxidants in a sample, and has been confirmed to detect oxidative stress in blood plasma [45] and semen samples [47]. sORP was measured in millivolt (mV) in fresh and cryopreserved semen samples using the MiOXSYS platform (MiOXSYS, Aytu BioScience Inc, Englewood, CO, USA). 30 μl of semen was loaded into the MiOXSYS sensor and immediately inserted into the analyzer.

2.7. Statistical Analysis. All variables are presented as median with minimum and maximum values. Variables were evaluated for normal distribution using histogram, a quantile–quantile (Q–Q) plot and Kolmogorov–Smirnov test. For some of the participants ($n = 12$), body mass index (BMI) was not reported and for some of the fresh samples, data were lacking; sperm motility ($n = 15$), morphology ($n = 5$), and seminal sORP ($n = 1$). Data for post-thaw samples were lacking for sperm morphology ($n = 4$) and DNA fragmentation ($n = 1$). BMI was calculated as weight in kg divided by height in meters squared (kg/m^2).

Since, there is no established cutoff value for endogenous seminal L-carnitine, to detect differences in sperm parameters in relation to the endogenous levels of seminal L-carnitine, participants were categorized into two groups based on the 50th percentile (median) of L-carnitine levels: low L-carnitine ($\leq$38.8 μg/ml) and high L-carnitine (>38.8 μg/ml). Mann–Whitney *U* test was used to compare differences between the two groups. Wilcoxon signed-rank test was used to compare semen variables in samples after standard cryopreservation and after L-carnitine supplementation.

Absolute change in semen quality values was used to examine the difference between L-carnitine supplementation and standard cryopreservation by subtraction of the standard cryopreservation median value from the supplemented cryopreservation median value for each parameter. Mann–Whitney *U* test was used to compare variables between the groups.

Box plots were used to visualize the effect of various L-carnitine concentrations supplemented to the freezing medium on post-thaw total sperm motility, and Wilcoxon signed-rank test was used to compare the difference between the groups. Jitter plots were used to visualize the distribution of sperm motility in fresh samples, after standard cryopreservation and after L-carnitine supplementation, as well as the difference in sperm motility between samples with low and high L-carnitine levels in seminal plasma. Mann–Whitney *U* test was used to compare variables between the groups.

The distribution of endogenous seminal L-carnitine levels is presented as a histogram plot. Variable distributions were evaluated by histograms. Skewed variables were log-, square-root, squared-, or cubic-transformed. Pearson's correlation coefficient was used to measure associations between levels of seminal L-carnitine and semen characteristics. Pearson's partial correlation coefficient was used to control for semen volume in addition. All variables were continuous. Associations between levels of seminal L-carnitine and semen characteristics are presented as unadjusted and adjusted for semen volume.

Statistical analyses were performed by using IBM SPSS Statistics 20 (IBM, Chicago, IL, USA). To minimize the risk of type I errors due to multiple comparisons, the level of statistical significance was set at p-value ≤ 0.01.

3. Results

3.1. Study Population. The characteristics of the study population are presented in Table 1. When comparing the semen analysis results with lower reference limits defined by the WHO (2010) [40], a total of 21 men had a sperm concentration below 15×10^6/ml, and 11 men had a total sperm count below 39×10^6/ml. Regarding motility, 55 men had <25% of rapid progressive sperm, 30 had <32% of progressive motile sperm and 26 had <40% total motile sperm.

Endogenous seminal L-carnitine levels were positively associated with sperm concentration, total sperm count as well as rapid progressive and progressive motility (Table 1), as in agreement with previous studies [33–35].

The distribution of endogenous seminal L-carnitine in seminal plasma is shown in Figure 1. The mean (SD) value of endogenous seminal L-carnitine was 48.5 $\pm$ 32.7 μg/ml.

3.2. Semen Parameters in Fresh and Post-Thaw Samples with Low and High Endogenous Seminal L-Carnitine Levels. To examine the relation between endogenous levels of seminal

Table 1: Correlations between levels of endogenous seminal L-carnitine ($n = 125$; μg/ml) and background characteristic values.

Variable	n	Median (min–max)	Unadjusted		Adjusted for semen volume	
			r	p	r	p
Age (years)[a]	125	35 (23–57)	0.013	0.895	−0.062	0.531
BMI (kg/m^2)[b]	113	24.7 (17.4–40.2)	−0.116	0.221	−0.029	0.761
Sexual abstinence (days)[b]	125	3 (1–7)	0.069	0.484	0.139	0.158
Semen volume (ml)[b]	125	4 (1–8)	−0.415	<0.001*	—	
Sperm concentration (10^6/ml)[b]	125	38 (5–239)	0.695	<0.001*	0.672	<0.001*
Total sperm count (10^6)[b]	125	126 (14–521)	0.496	<0.001*	0.672	<0.001*
Rapid progressive sperm motility (a, %)	110	25 (2–55)	0.356	<0.001*	0.415	<0.001*
Progressive sperm motility (a + b, %)	110	40 (11–63)	0.238	0.014*	0.295	0.002*
Total sperm motility (a + b + c, %)	110	51 (18–74)	0.178	0.067	0.246	0.011*
Immotile sperm (d, %)[b]	110	50 (26–82)	−0.178	0.069	−0.246	0.011*
Sperm vitality (%)[c]	125	61 (19–88)	0.194	0.047	0.210	0.032
Normal sperm morphology (%)[b]	120	8 (1–32)	0.099	0.311	0.089	0.368
Sperm head defects (%)[d]	120	88 (56–99)	−0.076	0.437	−0.083	0.403
Spem neck midpiece defects (%)	120	28 (3–55)	−0.058	0.554	−0.091	0.358
Sperm principal piece defects (%)[b]	120	12 (3–31)	−0.160	0.102	−0.145	0.139
Fragmented sperm DNA (%)[a]	125	15 (2–88)	−0.150	0.126	−0.189	0.054
Seminal sORP (mV)[b]	124	41 (3–172)	0.004	0.965	0.046	0.640

BMI, body mass index; sORP, static oxidation-reduction potential; r, correlation coefficient. *p values ≤ 0.01 were considered statistically significant. Pearson's correlation analysis was used to determine correlation between levels of endogenous L-carnitine in seminal plasma and semen parameters. Pearson's partial correlation analysis was used for adjusted data. Data are presented as unadjusted and adjusted for semen volume. All variables were continuous. [a]Log-transformed variables. [b]Square-root transformed variables. [c]Power transformed variables. [d]Cubic transformed variables.

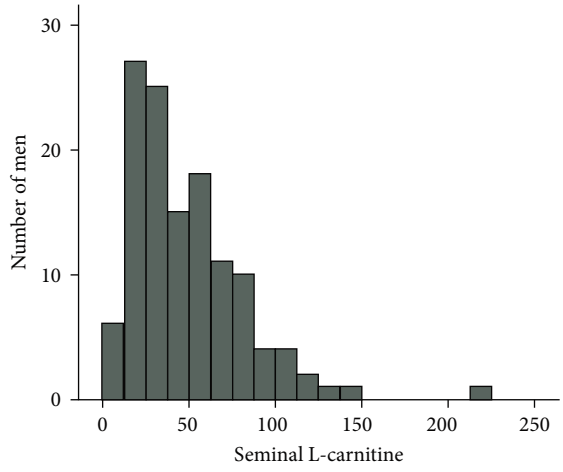

Figure 1: Distribution of L-carnitine levels (μg/ml) in seminal plasma in the study population ($n = 125$).

L-carnitine and semen quality parameters before and after cryopreservation, the population was categorized into two groups according to the median L-carnitine value (38.8 μg/ml) and classified either to low ($\leq$38.8 μg/ml) or high (>38.8 μg/ml) endogenous seminal L-carnitine group. There was no difference in BMI, age nor the time of abstinence between the two groups. In fresh samples, the percentage of rapid progressive sperm was higher in the group with high seminal L-carnitine levels than in the group with low levels (Table 2). After standard cryopreservation, spermatozoa in samples with high L-carnitine levels had higher rapid progressive, progressive, and total sperm motility than spermatozoa in samples with low L-carnitine levels (Table 2). In post-thaw samples, seminal sORP level was lower in the group with high L-carnitine levels than the comparing group, while sperm vitality, morphology and DNA fragmentation did not differ between the two groups. When comparing semen quality variables in fresh versus post-thaw samples, the levels of sperm neck midpiece defects after cryopreservation were higher in the group with low L-carnitine levels, while there was no difference in the group with high L-carnitine levels. No other differences in semen quality parameters were observed between fresh and post-thaw samples.

Sperm motility in fresh samples and after cryopreservation, according to the low and high seminal L-carnitine groups are presented in Figure S2.

3.3. Effect of L-Carnitine Supplementation on Post-Thaw Semen Parameters in Samples with Low and High Endogenous Seminal L-Carnitine Levels. L-carnitine supplementation of the freezing medium had a beneficial effect on semen quality after freezing and thawing (Table S1). Both groups showed higher proportions of rapid progressive sperm motility, progressive motility, total motility and vitality compared to standard cryopreservation (Table 3). Seminal sORP levels were lower after L-carnitine supplementation in both groups compared to standard cryopreservation. No impact was seen on sperm morphology parameters after cryopreservation with L-carnitine. Distribution of sperm motility after cryopreservation with L-carnitine in samples with low and high endogenous seminal L-carnitine levels is visualized in Figure S2. To sum up, L-carnitine supplementation has a positive effect on post-thaw semen parameters in samples with both high and low endogenous L-carnitine.

Table 2: Semen parameters in fresh and post-thaw samples according to high and low endogenous seminal L-carnitine levels.

Variable	Fresh samples					Post-thaw samples					Fresh vs post-thaw samples	
	Low seminal L-carnitine levels[#] (≤38.8 μg/ml)		High seminal L-carnitine levels[#] (>38.8 μg/ml)			Low seminal L-carnitine levels[#] (≤38.8 μg/ml)		High seminal L-carnitine levels[#] (>38.8 μg/ml)			Low seminal L-carnitine levels[#] (≤38.8 μg/ml)	High seminal L-carnitine levels[#] (>38.8 μg/ml)
	n	Median (min–max)	*n*	Median (min–max)	*p*	*n*	Median (min–max)	*n*	Median (min–max)	*p*	*p*	*p*
Endogenous seminal L-carnitine (μg/ml)	63	25 (6–38.8)	62	68 (39–220)	<0.001*	—	—	—	—	—	—	—
Semen volume (ml)	63	4 (2–8)	62	3 (1–7)	0.003*	—	—	—	—	—	—	—
Sperm concentration (10^6/ml)	63	23 (5–73)	62	56 (11–239)	<0.001*	—	—	—	—	—	—	—
Total sperm count (10^6)	63	81 (14–413)	62	187 (25–521)	<0.001*	—	—	—	—	—	—	—
Rapid progressive sperm motility (a, %)	53	17 (2–42)	57	28 (4–55)	0.002*	63	7 (0–27)	62	12 (2–26)	0.002*	<0.001*	<0.001*
Progressive sperm motility (a + b, %)	53	35 (11–59)	57	43 (15–63)	0.037	63	17 (0–39)	62	25 (3–40)	0.002*	<0.001*	<0.001*
Total sperm motility (a + b + c, %)	53	48 (18–70)	57	54 (21–74)	0.113	63	27 (1–51)	62	36 (5–51)	<0.001*	<0.001*	<0.001*
Immotile sperm (d, %)	53	52 (30–82)	57	46 (26–79)	0.113	63	73 (49–99)	62	65 (49–95)	<0.001*	<0.001*	<0.001*
Sperm vitality (%)	63	57 (19–88)	62	63 (30–80)	0.027	63	34 (4–53)	62	38 (16–58)	0.051	<0.001*	<0.001*
Normal sperm morphology (%)	61	8 (1–31)	59	8 (1–32)	0.771	61	7 (1–29)	60	7 (0–24)	0.862	0.510	0.032
Sperm head defects (%)	61	88 (66–96)	59	87 (56–99)	0.429	61	88 (59–99)	60	86 (72–99)	0.134	0.648	0.789
Sperm neck midpiece defects (%)	61	28 (9–55)	59	26 (3–55)	0.825	61	32 (3–67)	60	30 (4–61)	0.220	0.008*	0.127
Sperm principal piece defects (%)	61	13 (3–31)	59	12 (3–26)	0.676	61	15 (5–37)	60	14 (6–36)	0.213	<0.001*	0.003*
Sperm DNA fragmentation (%)	63	15 (6–88)	62	14 (2–54)	0.204	62	22 (8–62)	62	20 (6–48)	0.192	0.002*	<0.001
Seminal sORP (mV)	63	41 (3–172)	61	41 (3–97)	0.601	62	66 (21–118)	62	55 (19–146)	0.005*	<0.001*	<0.001*

[#]The population was categorized in two groups according to the 50th percentile of endogenous seminal L-carnitine levels. sORP, static oxidation-reduction potential. *p values ≤ 0.01 were considered statistically significant. Mann–Whitney U test was used to compare variables in samples with low and high seminal L-carnitine levels. Wilcoxon signed-rank test was used to compare semen quality variables in fresh and post-thaw samples.

Table 3: Semen parameters in samples with high and low endogenous seminal L-carnitine levels after standard cryopreservation and cryopreservation supplemented with L-carnitine.

Variable	Low seminal L-carnitine levels[#] (≤38.8 μg/ml)					High seminal L-carnitine levels[#] (>38.8 μg/ml)				
	Cryopreservation method									
	Standard		Supplemented with L-carnitine			Standard		Supplemented with L-carnitine		
	n	Median (min–max)	*n*	Median (min–max)	*p*	*n*	Median (min–max)	*n*	Median (min–max)	*p*
Rapid progressive sperm motility (a, %)	63	7 (0–27)	63	11 (1–32)	<0.001*	62	12 (2–26)	62	18 (2–37)	<0.001*
Progressive sperm motility (a + b, %)	63	17 (0–39)	63	21 (2–44)	<0.001*	62	25 (3–40)	62	30 (5–50)	<0.001*
Total sperm motility (a + b + c, %)	63	27 (1–51)	63	33 (3–54)	<0.001*	62	36 (5–51)	62	41 (7–56)	<0.001*
Immotile sperm (d, %)	63	73 (49–99)	63	67 (46–97)	<0.001*	62	65 (49–95)	62	60 (44–93)	<0.001*
Sperm vitality (%)	63	34 (4–53)	63	35 (11–60)	0.003*	62	38 (16–58)	62	43 (16–55)	<0.001*
Normal sperm morphology (%)	61	7 (1–29)	61	7 (2–26)	0.099	60	7 (0–24)	60	7 (0–26)	0.302
Sperm head defects (%)	61	88 (59–99)	61	88 (65–98)	0.738	60	86 (72–99)	60	86 (66–99)	0.598
Sperm neck midpiece defects (%)	61	32 (3–67)	61	31 (13–61)	0.386	60	30 (4–61)	60	29 (14–64)	0.772
Sperm principal piece defects (%)	61	15 (5–37)	61	14 (5–33)	0.203	60	14 (6–36)	60	13 (1–37)	0.282
Sperm DNA fragmentation (%)	62	22 (8–62)	62	20 (6–64)	0.032	62	20 (6–48)	62	19 (5–47)	0.017
Seminal sORP (mV)	62	66 (21–118)	62	64 (18–117)	0.004*	62	55 (19–146)	62	51 (14–132)	0.007*

[#] The population was categorized in two groups according to the 50th percentile of endogenous seminal L-carnitine levels. sORP, static oxidation-reduction potential. *p values ≤ 0.01 were considered statistically significant. Wilcoxon signed-rank test was used to compare post-thaw semen variables between standard and supplemented with L-carnitine.

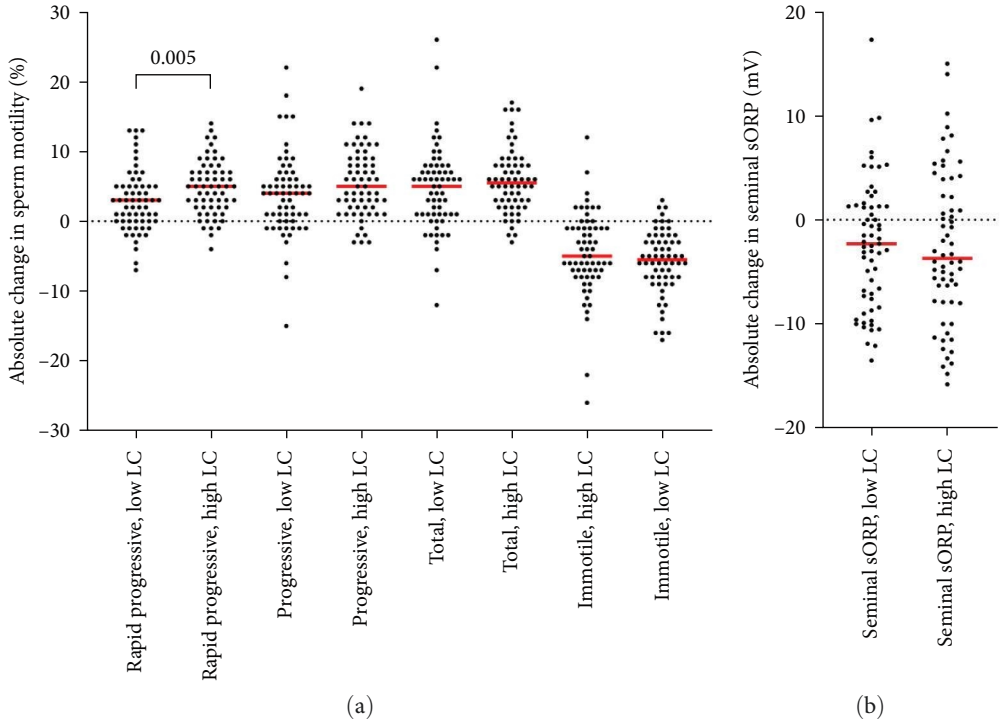

Figure 2: Absolute change in sperm motility and seminal sORP levels between post-thaw samples supplemented with L-carnitine and after standard cryopreservation in groups with low and high seminal L-carnitine. Absolute change in sperm motility (a) and seminal sORP levels (b) between post-thaw samples supplemented with L-carnitine and after standard cryopreservation in groups with low LC ($\leq$38.8 μg/ml, $n =$ 63 for motility and $n = 62$ for seminal sORP) and high LC (>38.8 μg/ml, $n = 62$ for both) levels. Every point in the jitter plot represents an absolute change. Horizontal lines for each group represent median, while the dotted line at 0 indicates no change. Mann–Whitney U test was used to compare differences between the groups. p values ≤ 0.01 were considered statistically significant. sORP: static oxidation-reduction potential; LC: L-carnitine.

To further examine potential differences in the impact of L-carnitine supplementation on semen parameters between samples with high and low endogenous L-carnitine levels, we quantitated the absolute change in post-thaw semen parameters by subtracting the standard cryopreservation median value from the supplemented cryopreservation median value for each parameter in the two groups. For progressive sperm motility, total motility, the proportion of immotile sperm and sORP there were no differences in the improvement by L-carnitine supplementation between the two groups. (Figures 2(a) and 2(b)). Neither were there differences in improvement in sperm DNA fragmentation, and normal sperm morphology between the two groups after L-carnitine supplementation (Table S2). However, the improvement in rapid progressive sperm motility after L-carnitine supplementation was highest in the group with high endogenous L-carnitine levels and statistically significant compared to the low L-carnitine group (Figure 2(a) and Table S2).

4. Discussion

The impact of endogenous seminal L-carnitine levels on post-thaw semen quality has not been investigated although a beneficial effect of L-carnitine supplementation of freezing medium has been shown [37, 38]. Here, we show that the amount of post-thaw motile sperm was higher in the group of semen samples with high endogenous seminal L-carnitine levels, compared to the group with low levels. This association does not imply causality but indicates that the harmful effects of cryopreservation are reduced in samples with high levels of L-carnitine in seminal plasma. There was no difference in post-thaw sperm morphology, vitality, or DNA fragmentation between the two L-carnitine groups. We also show an oxidative stress increase in post-thaw samples in both low and high endogenous seminal L-carnitine groups, as indicated by increased levels of seminal sORP compared to fresh samples (Table 2). However, levels of seminal sORP increased less after cryopreservation when endogenous seminal L-carnitine levels were high. This result is in line with the reported ability of L-carnitine to prevent oxidative stress [48–50].

In addition to increased oxidative stress in post-thaw samples, cryopreservation is associated with sperm mitochondrial dysfunction, lipid peroxidation, reduced sperm motility and morphology [6, 8, 51]. O'Connell et al. [8] demonstrated that mitochondrial dysfunction measured by accumulation of rhodamine 123 (R123) into spermatozoa was positively related to the level of sperm midpiece defects. A functional sperm midpiece is important for optimal sperm motility [52], as energy for sperm movement is derived from mitochondria located in the midpiece [53]. Mitochondrial function was not measured in this study. However, we show an increase in the amount of sperm neck midpiece defects only in post-thaw

samples with low endogenous seminal L-carnitine levels, compared to fresh samples. In fresh semen samples, there was no difference in sperm neck and midpiece defects between samples with low and high L-carnitine levels, which may indicate a protective impact of endogenous L-carnitine against damage sustained during cryopreservation. In addition, the proportion of spermatozoa with progressive and total motility was higher, and there were less immotile sperm in post-thaw samples with high levels of endogenous L-carnitine. Concomitantly, seminal sORP levels were lower in these samples. Several studies have shown association between increased oxidative stress and reduced sperm motility due to lipid peroxidation [17, 54, 55]. Evidence indicates that compounds with anti-oxidant properties can improve sperm motility in mammalian spermatozoa by suppressing lipid peroxidation [56–58]. Increased lipid peroxidation has been associated with cryopreservation in mammalian spermatozoa [51, 59–61]. We did not measure lipid peroxidation in this study. Overall, our results support a role for endogenous seminal L-carnitine in reducing oxidative stress in post-thaw samples.

It is worth mentioning that no relation between endogenous seminal L-carnitine levels and seminal sORP levels nor sperm DNA fragmentation index (DFI) was observed in fresh samples (Table 2). However, both high endogenous levels of L-carnitine and supplementation of the freezing medium with L-carnitine were beneficial for semen quality in post-thaw samples when the level of oxidative stress increased.

In our study and as well as in others' [37, 38], L-carnitine supplementation of the freezing medium was beneficial for post-thaw semen quality. In addition, we show here that the beneficial effect on rapid progressive sperm motility observed after cryopreservation with L-carnitine was higher in samples with high endogenous seminal L-carnitine levels compared to those with low endogenous levels. Since, there was no difference in change in oxidative stress levels between low and high endogenous L-carnitine groups when comparing the two cryopreservation protocols, one can presume that the higher presence of sperm with rapid motility may result from the well-characterized role of L-carnitine in energetic metabolism [31]. Since, L-carnitine is required for transport of long-chain fatty acids into mitochondria for β-oxidation and production of ATP, it is likely that the improvement in sperm motility was due to increased delivery of energy substrates into the mitochondria. This is in agreement with a previous *in vitro* study showing that incubation of semen samples with L-carnitine increased sperm motility, but in this study a longer timepoint for analysis compared to ours was used [62]. In addition, global DNA methylation levels are positively associated with motility in human spermatozoa [63]. A recent study has shown that global methylation decreases after cryopreservation in buck sperm and that L-carnitine supplementation of the freezing medium reversed the decrease in levels of DNA methylation [64]. As there is a negative correlation between ROS levels and global sperm DNA methylation [65], one can hypothesize that the antioxidant properties of L-carnitine will contribute to a protecting environment for global DNA methylation in post-thaw spermatozoa with high endogenous and/or supplemented levels.

The effect of L-carnitine supplementation on post-thaw sperm motility (a, a + b, a + b + c grades) was not beneficial for all samples compared to standard cryopreservation. Furthermore, samples with low endogenous L-carnitine levels showed a larger distribution of absolute changes in sperm motility grades compared to samples with high endogenous L-carnitine levels. This suggests that the post-thaw outcome of L-carnitine supplementation varies more in samples with low endogenous L-carnitine levels. Cryopreservation has been shown to reduce seminal SOD activity in an inter-individual manner, with large variations between donor samples [51]. This was associated with a decrease in sperm phospholipid content due to lipid peroxidation. As mentioned earlier, we did not measure lipid peroxidation, but our results support the notion that individual semen samples are differently affected by cryopreservation.

A strength of the study is that semen analysis was performed by a person participating in the external quality program for semen assessment. The semen samples collected in the study represented a broad range of semen quality values. A limitation of the study is that only one concentration of L-carnitine was used in the freezing medium, where total motility was the only outcome that was evaluated. Furthermore, in this study post-thaw sperm motility was assessed 5 min after thawing, according to the manufacturers protocol. The sperm motility tested after a longer post-thaw period could have given other results, as it has been shown that sperm motility increases with time [66].

In conclusion, L-carnitine has a beneficial impact on semen quality in post-thaw samples both as an endogenous component in seminal plasma and as a supplement in the freezing medium, by improving sperm motility and diminishing seminal oxidative stress. We also observed low levels of sperm neck midpiece defects after cryopreservation in samples with high endogenous L-carnitine levels. Altogether, these findings indicate that high levels of L-carnitine in seminal plasma are protective against the harmful effect of cryopreservation.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

All authors declare that they have no conflicts of Interest.

Authors' Contributions

Conception of the study was initiated by OW. MI, TBH, JMA, and MHS contributed to the study design. MI collected the data and performed the statistical analyses. Visualization of the data was done by OW and MI. OW and MI drafted the manuscript. All authors contributed to interpretation of the data and critical revision of content. All authors read and approved the final manuscript.

Acknowledgments

The authors would acknowledge Nicolai Holst for his mentorship and support of this project. This study was funded by the Research Council of Norway and Fertilitetssenteret, Oslo, Norway (grant number: 277494). Open Access funding enabled and organized by SIKT GOLD.

Supplementary Materials

Figure S1: describes the effect of L-carnitine concentrations supplemented to the freezing media on post-thaw total sperm motility. Table S1: describes the comparison of semen parameters after standard cryopreservation and supplemented with L-carnitine (0.5 mg/ml). Table S2: describes the absolute change (Δ) in post-thaw semen variables between cryopreservation supplemented with L-carnitine and standard cryopreservation in semen samples with low and high endogenous L-carnitine (LC) levels. Figure S2: sperm motility in fresh samples, after standard cryopreservation, and after cryopreservation supplemented with L-carnitine categorized according to endogenous L-carnitine levels. (*Supplementary Materials*)

References

[1] C. Huang, Y.-L. Tang, J.-L. Hu et al., "Update on techniques for cryopreservation of human spermatozoa," *Asian Journal of Andrology*, vol. 24, no. 6, pp. 563–569, 2022.

[2] P. Kop, M. van Wely, A. de Melker, F. van der Veen, and M. Mochtar, "Donor sperm treatment: the role of semen parameters in intracervical insemination, a retrospective cohort study," *Human Fertility*, pp. 1–7, 2022.

[3] W. G. Sanger, J. H. Olson, and J. K. Sherman, "Semen cryobanking for men with cancer—criteria change," *Fertility and Sterility*, vol. 58, no. 5, pp. 1024–1027, 1992.

[4] J. T. Anger, B. R. Gilbert, and M. Goldstein, "Cryopreservation of sperm: indications, methods and results," *Journal of Urology*, vol. 170, no. 4 Part 1, pp. 1079–1084, 2003.

[5] B. K. Gangrade, "Cryopreservation of testicular and epididymal sperm: techniques and clinical outcomes of assisted conception," *Clinics*, vol. 68, pp. 131–140, 2013.

[6] E. T. Donnelly, N. McClure, and S. E. M. Lewis, "Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA integrity," *Fertility and Sterility*, vol. 76, no. 5, pp. 892–900, 2001.

[7] F. Nur Karakus, S. Bulgurcuoglu Kuran, and S. Solakoglu, "Effect of curcumin on sperm parameters after the cryopreservation," *European Journal of Obstetrics & Gynecology and Reproductive Biology*, vol. 267, pp. 161–166, 2021.

[8] M. O'Connell, N. McClure, and S. E. M. Lewis, "The effects of cryopreservation on sperm morphology, motility and mitochondrial function," *Human Reproduction*, vol. 17, no. 3, pp. 704–709, 2002.

[9] N. Zribi, N. Feki Chakroun, H. El Euch, J. Gargouri, A. Bahloul, and L. Ammar Keskes, "Effects of cryopreservation on human sperm deoxyribonucleic acid integrity," *Fertility and Sterility*, vol. 93, no. 1, pp. 159–166, 2010.

[10] M. Ezzati, D. Shanehbandi, K. Hamdi, S. Rahbar, and M. Pashaiasl, "Influence of cryopreservation on structure and function of mammalian spermatozoa: an overview," *Cell and Tissue Banking*, vol. 21, no. 1, pp. 1–15, 2020.

[11] G. Pizzino, N. Irrera, M. Cucinotta et al., "Oxidative stress: harms and benefits for human health," *Oxidative Medicine and Cellular Longevity*, vol. 2017, Article ID 8416763, 13 pages, 2017.

[12] R. Jones, T. Mann, and R. Sherins, "Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma," *Fertility and Sterility*, vol. 31, no. 5, pp. 531–537, 1979.

[13] N. Cao, C. Hu, B. Xia et al., "The activated ampk/mtorc2 signaling pathway associated with oxidative stress in seminal plasma contributes to idiopathic asthenozoospermia," *Oxidative Medicine and Cellular Longevity*, vol. 2022, Article ID 4240490, 16 pages, 2022.

[14] T. R. Hamilton, L. S. de Castro, J. C. Delgado et al., "Induced lipid peroxidation in ram sperm: semen profile, DNA fragmentation and antioxidant status," *Reproduction*, vol. 151, no. 4, pp. 379–390, 2016.

[15] A. Agarwal, A. Mulgund, R. Sharma, and E. Sabanegh, "Mechanisms of oligozoospermia: an oxidative stress perspective," *Systems Biology in Reproductive Medicine*, vol. 60, no. 4, pp. 206–216, 2014.

[16] R. J. Aitken, D. Buckingham, and D. Harkiss, "Use of a xanthine oxidase free radical generating system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa," *Reproduction*, vol. 97, no. 2, pp. 441–450, 1993.

[17] W. Kurkowska, A. Bogacz, M. Janiszewska et al., "Oxidative stress is associated with reduced sperm motility in normal semen," *American Journal of Men's Health*, vol. 14, no. 5, 2020.

[18] M. Fraczek, A. Lewandowska, M. Budzinska et al., "The role of seminal oxidative stress scavenging system in the pathogenesis of sperm DNA damage in men exposed and not exposed to genital heat stress," *International Journal of Environmental Research and Public Health*, vol. 19, no. 5, Article ID 2713, 2022.

[19] S. C. Pereira, M. V. Moreira, B. M. Silva, P. F. Oliveira, and M. G. Alves, "Roles of oxidative stress in the male reproductive system: potential of antioxidant supplementation for infertility treatment," in *Oxidative Stress and Toxicity in Reproductive Biology and Medicine: A Comprehensive Update on Male Infertility Volume II*, pp. 259–274, Springer International Publishing, Cham, 2022.

[20] C. J. Raj, C. V. S. Aishwarya, K. V. S. S. N. Mounika et al., "Deciphering the nexus between oxidative stress and spermatogenesis: a compendious overview," in *Oxidative Stress and Toxicity in Reproductive Biology and Medicine: A Comprehensive Update on Male Infertility Volume II*, pp. 1–16, Springer International Publishing, Cham, 2022.

[21] N. Garrido, M. Meseguer, J. Alvarez, C. Simón, A. Pellicer, and J. Remohí, "Relationship among standard semen parameters, glutathione peroxidase/glutathione reductase activity, and mrna expression and reduced glutathione content in ejaculated spermatozoa from fertile and infertile men," *Fertility and Sterility*, vol. 82, pp. 1059–1066, 2004.

[22] M. Llavanera, A. Delgado-Bermúdez, S. Olives et al., "Glutathione s-transferases play a crucial role in mitochondrial function, plasma membrane stability and oxidative regulation of mammalian sperm," *Antioxidants*, vol. 9, no. 2, Article ID 100, 2020.

[23] L. Crisol, R. Matorras, F. Aspichueta et al., "Glutathione peroxidase activity in seminal plasma and its relationship to classical sperm parameters and in vitro fertilization-intracytoplasmic sperm injection outcome," *Fertility and Sterility*, vol. 97, no. 4, pp. 852–857.e1, 2012.

[24] M. T. M. Raijmakers, H. M. J. Roelofs, E. A. P. Steegers et al., "Glutathione and glutathione s-transferases a1-1 and p1-1 in seminal plasma may play a role in protecting against oxidative damage to spermatozoa," *Fertility and Sterility*, vol. 79, no. 1, pp. 169–172, 2003.

[25] C. Jeulin, J. C. Soufir, P. Weber, D. Laval-Martin, and R. Calvayrac, "Catalase activity in human spermatozoa and seminal plasma," *Gamete Research*, vol. 24, no. 2, pp. 185–196, 1989.

[26] R. Peeker, L. Abramsson, and S. L. Marklund, "Superoxide dismutase isoenzymes in human seminal plasma and spermatozoa," *Molecular Human Reproduction*, vol. 3, no. 12, pp. 1061–1066, 1997.

[27] H. Tavilani, M. T. Goodarzi, A. Vaisi-Raygani, S. Salimi, and T. Hassanzadeh, "Activity of antioxidant enzymes in seminal plasma and their relationship with lipid peroxidation of spermatozoa," *International Brazilian Journal Urology*, vol. 34, no. 4, pp. 485–491, 2008.

[28] A. H. Colagar and E. T. Marzony, "Ascorbic acid in human seminal plasma: determination and its relationship to sperm quality," *Journal of Clinical Biochemistry and Nutrition*, vol. 45, no. 2, pp. 144–149, 2009.

[29] A. Gvozdjakova, J. Kucharska, J. Lipkova et al., "Importance of the assessment of coenzyme q10, alpha-tocopherol and oxidative stress for the diagnosis and therapy of infertility in men," *Bratislavske Lekarske Listy*, vol. 114, no. 11, pp. 607–609, 2013.

[30] J. Moilanen, O. Hovatta, and L. Lindroth, "Vitamin E levels in seminal plasma can be elevated by oral administration of vitamin E in infertile men," *International Journal of Andrology*, vol. 16, no. 2, pp. 165-166, 1993.

[31] N. Longo, M. Frigeni, and M. Pasquali, "Carnitine transport and fatty acid oxidation," *Biochimica et Biophysica Acta (BBA) —Molecular Cell Research*, vol. 1863, no. 10, pp. 2422–2435, 2016.

[32] P. Surai, "Antioxidant action of carnitine: molecular mechanisms and practical applications," *EC Veterinary Science*, vol. 2, pp. 66–84, 2015.

[33] M. Iliceto, M. H. Stensen, J. M. Andersen, T. B. Haugen, and O. Witczak, "Levels of l-carnitine in human seminal plasma are associated with sperm fatty acid composition," *Asian Journal of Andrology*, vol. 24, no. 5, pp. 451–457, 2022.

[34] G. F. Menchini-Fabris, D. Canale, P. L. Izzo, L. Olivieri, and M. Bartelloni, "Free l-carnitine in human semen: its variability in different andrologic pathologies," *Fertility and Sterility*, vol. 42, no. 2, pp. 263–267, 1984.

[35] A. Zöpfgen, F. Priem, F. Sudhoff et al., "Relationship between semen quality and the seminal plasma components carnitine, alpha-glucosidase, fructose, citrate and granulocyte elastase in infertile men compared with a normal population," *Human Reproduction*, vol. 15, no. 4, pp. 840–845, 2000.

[36] R. Bahmyari, M. Zare, R. Sharma, A. Agarwal, and I. Halvaei, "The efficacy of antioxidants in sperm parameters and production of reactive oxygen species levels during the freeze-thaw process: a systematic review and meta-analysis," *Andrologia*, vol. 52, no. 3, Article ID e13514, 2020.

[37] S. Banihani, A. Agarwal, R. Sharma, and M. Bayachou, "Cryoprotective effect of l-carnitine on motility, vitality and DNA oxidation of human spermatozoa," *Andrologia*, vol. 46, no. 6, pp. 637–641, 2014.

[38] W. Zhang, F. Li, H. Cao et al., "Protective effects of l-carnitine on astheno- and normozoospermic human semen samples during cryopreservation," *Zygote*, vol. 24, no. 2, pp. 293–300, 2016.

[39] F. Ghorbani, Z. Nasiri, Y. Koohestanidehaghi, and K. Lorian, "The antioxidant roles of L-carnitine and n-acetyl cysteine against oxidative stress on human sperm functional parameters during vitrification," *Clinical and Experimental Reproductive Medicine*, vol. 48, no. 4, pp. 316–321, 2021.

[40] WHO, *WHO Laboratory Manual for the Examination and Processing of Human Semen*, World Health Organization, Geneva, 2010.

[41] WHO, *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction*, Cambridge University Press, United Kingdom, 4th edition, 1999.

[42] R. Menkveld and T. F. Kruger, "Advantages of strict (tygerberg) criteria for evaluation of sperm morphology," *International Journal of Andrology*, vol. 18, no. Suppl 2, pp. 36–42, 1995.

[43] J. L. Fernández, L. Muriel, V. Goyanes et al., "Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test," *Fertility and Sterility*, vol. 84, no. 4, pp. 833–842, 2005.

[44] J. L. Fernández, L. Muriel, M. T. Rivero, V. Goyanes, R. Vazquez, and J. G. Alvarez, "The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation," *Journal of Andrology*, vol. 24, no. 1, pp. 59–66, 2003.

[45] D. Polson, N. Villalba, and K. Freeman, "Optimization of a diagnostic platform for oxidation–reduction potential (ORP) measurement in human plasma," *Redox Report: Communications in Free Radical Research*, vol. 23, no. 1, pp. 125–129, 2018.

[46] A. Agarwal, R. Sharma, S. Roychoudhury, S. Du Plessis, and E. Sabanegh, "Mioxsys: a novel method of measuring oxidation reduction potential in semen and seminal plasma," *Fertility and Sterility*, vol. 106, no. 3, pp. 566–573.e10, 2016.

[47] S. T. Homa, A. M. Vassiliou, J. Stone et al., "A comparison between two assays for measuring seminal oxidative stress and their relationship with sperm DNA fragmentation and semen parameters," *Genes*, vol. 10, no. 3, Article ID 236, 2019.

[48] A. R. Abd-Allah, G. K. Helal, A. A. Al-Yahya, A. M. Aleisa, S. S. Al-Rejaie, and S. A. Al-Bakheet, "Pro-inflammatory and oxidative stress pathways which compromise sperm motility and survival may be altered by L-carnitine," *Oxidative Medicine and Cellular Longevity*, vol. 2, no. 2, pp. 73–81, 2009.

[49] R. Ferrari, G. Ciampalini, G. Agnoletti, A. Cargnoni, C. Ceconi, and O. Visioli, "Effect of ll-carnitine derivatives on heart mitochondrial damage induced by lipid peroxidation," *Pharmacological Research Communications*, vol. 20, no. 2, pp. 125–132, 1988.

[50] J.-L. Li, Q.-Y. Wang, H.-Y. Luan, Z.-C. Kang, and C.-B. Wang, "Effects of L-carnitine against oxidative stress in human hepatocytes: involvement of peroxisome proliferator-activated receptor alpha," *Journal of Biomedical Science*, vol. 19, no. 1, Article ID 32, 2012.

[51] J. G. Avarez and B. T. Storey, "Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation," *Journal of Andrology*, vol. 13, no. 3, pp. 232–241, 1992.

[52] A. J. Mundy, T. A. Ryder, and D. K. Edmonds, "Asthenozoospermia and the human sperm mid-piece," *Human Reproduction*, vol. 10, no. 1, pp. 116–119, 1995.

[53] S. S. du Plessis, A. Agarwal, G. Mohanty, and M. van der Linde, "Oxidative phosphorylation versus glycolysis: what fuel do spermatozoa use ?" *Asian Journal of Andrology*, vol. 17, no. 2, pp. 230–235, 2015.
[54] S.-H. Kao, H.-T. Chao, H.-W. Chen, T. I. S. Hwang, T.-L. Liao, and Y.-H. Wei, "Increase of oxidative stress in human sperm with lower motility," *Fertility and Sterility*, vol. 89, no. 5, pp. 1183–1190, 2008.
[55] J. Twigg, N. Fulton, E. Gomez, D. S. Irvine, and R. J. Aitken, "Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants," *Human Reproduction*, vol. 13, no. 6, pp. 1429–1436, 1998.
[56] E. Cheraghi, S. M. S. Sajadi, and M. Soleimani Mehranjani, "The effect of quercetin on the quality of sperm parameters in frozen-thawed semen of patients with asthenospermia," *Andrologia*, vol. 53, no. 9, Article ID e14167, 2021.
[57] B. A. Fatma, C. F. Nozha, D. Ines, A. Hamadi, H. Basma, and A. K. Leila, "Sperm quality improvement after date seed oil in vitro supplementation in spontaneous and induced oxidative stress," *Asian Journal of Andrology*, vol. 11, no. 3, pp. 393–398, 2009.
[58] R. Salimnejad, J. Soleimani Rad, D. Mohammad Nejad, and L. Roshangar, "Effect of ghrelin on total antioxidant capacity, lipid peroxidation, sperm parameters and fertility in mice against oxidative damage caused by cyclophosphamide," *Andrologia*, vol. 50, no. 2, Article ID e12883, 2018.
[59] M. Akbarzadeh-Jahromi, F. Jafari, M. E. Parsanezhad, and S. Alaee, "Evaluation of supplementation of cryopreservation medium with gallic acid as an antioxidant in quality of post-thaw human spermatozoa," *Andrologia*, vol. 54, no. 11, Article ID e14571, 2022.
[60] M. S. Ansari, B. A. Rakha, S. Akhter, E. Blesbois, and J. Santiago-Moreno, "Effect of cryopreservation on lipid peroxidation, antioxidant potential, chromatin integrity, and mitochondrial activity of indian red jungle fowl (*Gallus gall*us murghi) semen," *Biopreservation and Biobanking*, vol. 17, no. 4, pp. 288–295, 2019.
[61] A. Partyka, E. Łukaszewicz, and W. Niżański, "Effect of cryopreservation on sperm parameters, lipid peroxidation and antioxidant enzymes activity in fowl semen," *Theriogenology*, vol. 77, no. 8, pp. 1497–1504, 2012.
[62] S. Banihani, R. Sharma, M. Bayachou, E. Sabanegh, and A. Agarwal, "Human sperm DNA oxidation, motility and viability in the presence of L-carnitine during in vitro incubation and centrifugation," *Andrologia*, vol. 44, no. s1, pp. 505–512, 2012.
[63] D. Montjean, A. Zini, C. Ravel et al., "Sperm global DNA methylation level: association with semen parameters and genome integrity," *Andrology*, vol. 3, no. 2, pp. 235–240, 2015.
[64] M. Heidari, B. Qasemi-Panahi, G. Moghaddam, H. Daghigh-Kia, and R. Masoudi, "L-carnitine improves quality parameters and epigenetic patterns of buck's frozen-thawed semen," *Animal Reproduction Science*, vol. 247, Article ID 107092, 2022.
[65] O. Tunc and K. Tremellen, "Oxidative DNA damage impairs global sperm DNA methylation in infertile men," *Journal of Assisted Reproduction and Genetics*, vol. 26, no. 9-10, pp. 537–544, 2009.
[66] B. Oberoi, S. Kumar, and P. Talwar, "Study of human sperm motility post cryopreservation," *Medical Journal Armed Forces India*, vol. 70, no. 4, pp. 349–353, 2014.