AMLR Asociația de Medicină de Laborator din România

Romanian Journal of Laboratory Medicine 2026;34(4):337–346

ORIGINAL RESEARCH

DOI:10.2478/rrlm-2026-0036

Effect of *Zingiber officinale* supplementation on post-thaw sperm quality and DNA/chromatin integrity in human spermatozoa

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ABSTRACT

Background: Sperm cryopreservation can impair sperm function and DNA/chromatin integrity, partly through oxidative stress. *Zingiber officinale* (*Z. officinale*) contains bioactive compounds with antioxidant properties. We evaluated its potential protective effect on sperm quality and DNA/chromatin integrity following cryopreservation.

Methods: Semen samples from 40 normozoospermic men were evaluated using a paired within-subject design. For each participant, the same semen sample was divided into three aliquots: fresh post-gradient semen, cryopreserved semen without supplementation (cryopreserved control), and cryopreserved semen supplemented with *Z. officinale* at a final nominal ginger powder-equivalent concentration of 5 mg/L (0.0005% w/v). Sperm viability, total and progressive motility, morphology, and DNA/chromatin status were assessed in all three aliquots. DNA/chromatin status was assessed using acridine orange (AO) staining. The three paired conditions were compared using repeated-measures analysis of variance with Greenhouse–Geisser correction and adjusted pairwise comparisons.

Results: Cryopreservation significantly reduced viability, motility, morphology, and AO-negative sperm compared with fresh semen (all overall $p<0.001$). Compared with the cryopreserved control, ginger supplementation improved viability (42.50 ± 6.79% vs. 31.50 ± 7.08%), total motility (67.17 ± 14.71% vs. 50.40 ± 13.68%), progressive motility (57.20 ± 10.58% vs. 41.19 ± 10.58%), normal morphology (12.69 ± 4.25% vs. 10.21 ± 3.85%), and AO-negative sperm (64.49 ± 10.60% vs. 45.67 ± 12.68%; adjusted $p\leq0.015$).

Conclusions: Supplementation with *Z. officinale* at a final nominal ginger powder-equivalent concentration of 5 mg/L (0.0005% w/v) improved post-thaw sperm viability, motility, morphology, and AO-assessed DNA/chromatin status. Further studies are needed to determine the optimal concentration and underlying mechanisms.

Keywords: AO, chromatin integrity, DNA integrity, sperm cryopreservation, sperm quality, *Zingiber officinale*

Received: 9 September 2026; Accepted: 23 October 2026; Published: 28 October 2026.

INTRODUCTION

Infertility affects approximately 15% of couples of reproductive age, and male factors are involved in around 30–50% of cases, either alone or together with female factors [1,2]. Semen analysis is the standard first-line assessment of male fertility and routinely includes sperm concentration, motility, and morphology. However, these parameters provide only a limited view of sperm function and do not directly assess the integrity of sperm DNA. For this reason, sperm DNA integrity has become an important additional parameter in the evaluation of male reproductive potential. Previous studies have linked increased sperm DNA damage with reduced fertility and have suggested that it may also affect the outcomes of natural conception and assisted reproductive technologies (ART) [3–5].

Sperm cryopreservation plays an important role in fertility preservation and ART. It enables long-term storage of sperm samples, particularly in men undergoing potentially gonadotoxic treatment, cancer patients, and individuals at risk of future impairment of reproductive function [6,7]. However, the freezing and thawing stresses associated with cryopreservation may adversely affect sperm membrane integrity, mitochondrial function, motility, and viability and may also compromise sperm DNA/chromatin integrity [7–9]. Although several mechanisms may contribute to cryopreservation-associated sperm injury, oxidative stress is considered one of the potentially important processes involved. The high content of polyunsaturated fatty acids in sperm membranes, together with their relatively limited cytoplasmic antioxidant capacity, makes spermatozoa particularly susceptible to oxidative damage [9–11].

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Accordingly, antioxidant-based strategies have been investigated to reduce oxidative damage during sperm cryopreservation. Experimental and clinical studies evaluating the addition of different antioxidants to cryopreservation media have reported improvements in various aspects of post-thaw sperm quality. However, substantial differences exist among studies in terms of antioxidant type and concentration, sperm preparation methods, cryopreservation protocols, and outcome measures. This heterogeneity makes direct comparison of the available findings difficult and contributes to the lack of a standardized antioxidant approach for human sperm cryopreservation [10–12].

Plant-derived antioxidants have also been investigated as potential cryoprotective agents. A recent systematic review and meta-analysis found that several herbal antioxidants may improve post-thaw sperm viability, motility, and DNA integrity [13]. However, the studies included in the review differed considerably in the type and dose of antioxidant used, extraction procedures, and cryopreservation protocols. These differences make it difficult to determine whether a particular plant-derived antioxidant provides a consistent benefit. Further studies using a defined extract and a standardized cryopreservation protocol are therefore needed.

Z. officinale Roscoe (ginger) contains several phenolic compounds, including gingerols and shogaols, which have been associated with antioxidant activity [14,15]. Experimental studies have reported possible effects of ginger on male reproductive function, including sperm-related outcomes and oxidative stress [15,16]. However, data from human studies are still limited. In a randomized clinical trial of infertile men, three months of oral ginger supplementation reduced sperm DNA fragmentation, but did not significantly change sperm concentration or motility [17]. These findings provide some evidence for a possible effect of ginger on sperm DNA integrity, but oral supplementation and direct addition of ginger to a cryopreservation medium are different approaches. Whether ginger can protect sperm during the freezing and thawing process when added directly to the cryopreservation medium remains unclear.

An important gap in the current literature is the limited evidence regarding the direct addition of *Z. officinale* extract to human sperm cryopreservation media and its potential effects on post-thaw sperm characteristics. Unlike oral supplementation, direct addition to the cryopreservation medium allows the extract to come into contact with spermatozoa during the freezing and thawing process. However, the potential effects of this approach on sperm viability, motility, morphology, and DNA/chromatin integrity should be evaluated together.

Therefore, the present study investigated whether supplementation of the cryopreservation medium with *Z. officinale* preparation, resulting in a final nominal ginger powder-equivalent concentration of 5 mg/L (0.0005% w/v) in the sperm-containing cryopreservation mixture, affects post-thaw sperm quality in normozoospermic men. Sperm viability, total and progressive motility, normal sperm morphology, and DNA/chromatin integrity assessed by acridine orange (AO) staining were evaluated as the primary outcome measures. Using a within-subject experimental design in which samples obtained from the same individual were compared under different experimental conditions, we aimed to evaluate the potential effect of *Z. officinale* supplementation on sperm quality following cryopreservation.

METHODS

Ethics statement

The study protocol was approved by the Dicle University Faculty of Medicine Non-Interventional Clinical Research Ethics Committee (approval no. 187; June 14, 2023). Written informed consent was obtained from all participants before semen collection. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Sample characteristics

This prospective, paired experimental study included 40 normozoospermic men aged 25–40 years who attended the Department of Urology, Dicle University Hospital, between January and February 2024.

Participants were eligible for inclusion if their semen parameters met the reference criteria specified in the World Health Organization (WHO) 2021 laboratory manual [18]. Men with a history of varicocele or recent hormonal or chemotherapeutic treatment were excluded. Men who smoked, consumed alcohol, or used vitamin supplements were also excluded.

For each participant, a single semen sample was processed and divided into three aliquots, allowing each participant to serve as his own control: (1) fresh post-gradient semen, (2) cryopreserved semen without supplementation (cryopreserved control), and (3) cryopreserved semen supplemented with *Z. officinale* at a final nominal powder-equivalent concentration of 5 mg/L (0.0005% w/v). Thus, all three experimental conditions were derived from the same semen sample for each participant.

Semen collection and baseline semen analysis

Semen samples were collected by masturbation into sterile, disposable containers following 2–7 days of sexual abstinence. Samples were allowed to liquefy at 37°C for approximately 30 minutes before analysis. Baseline semen analysis was subsequently performed according to the procedures described in the WHO 2021 laboratory manual.

Semen volume, sperm concentration, total sperm count, total motility, progressive motility, sperm viability, and morphology were evaluated. Sperm concentration

was determined using a Makler counting chamber under phase-contrast microscopy. At least 200 spermatozoa were evaluated for motility and viability, and 200 spermatozoa were assessed for morphology. Only samples meeting the WHO 2021 reference criteria for normozoospermia were included in the experimental analysis.

Sperm preparation and experimental conditions

Following baseline semen analysis, spermatozoa were prepared using discontinuous density-gradient centrifugation. A 45%/90% discontinuous silica-based density gradient (SpermGrad; Vitrolife) was used. Semen was laid onto the gradient and centrifuged at 300 ×g for 15 minutes. The upper phases were removed, and the sperm pellet was washed with sperm washing medium (SpermRinse; Vitrolife) by centrifugation at 300 ×g for 5 minutes.

Following sperm preparation, the sperm suspension obtained from each participant was divided into three aliquots, which were subsequently assigned to three experimental conditions:

1. Fresh post-gradient semen: sperm suspension subjected to density-gradient preparation but not cryopreserved.

2. Cryopreserved control: sperm suspension cryopreserved and subsequently thawed without ginger supplementation.

3. Ginger-supplemented cryopreserved semen: sperm suspension cryopreserved and subsequently thawed using freezing medium supplemented with *Z. officinale*, resulting in a final nominal ginger powder-equivalent concentration of 5 mg/L (0.0005% w/v) in the sperm-containing cryopreservation mixture.

Thus, all three experimental conditions were derived from the same semen sample from each participant. Each participant therefore served as his own control, and comparisons among the three conditions were performed using a within-subject repeated-measures design.

Preparation of Z. officinale extract

Fresh *Z. officinale* rhizomes were obtained from a GMP-certified supplier of medicinal herbal products. The rhizomes were washed, peeled, sliced, and dried under shade at room temperature. The dried rhizomes were then ground into a fine powder.

An aqueous ginger extract was prepared by suspending 10 mg of dried ginger powder in 100 mL of sterile phosphate-buffered saline (PBS), corresponding to 100 mg/L (0.01%, w/v) ginger powder equivalent. The suspension was kept at room temperature for 12 h with intermittent mixing and then filtered through Whatman No. 1 filter paper. The extract was stored at 4°C and freshly prepared for each experimental procedure.

For the ginger-supplemented condition, 100 µL of the freshly prepared extract was added to 900 µL of cryopreservation medium, giving a final volume of 1 mL and a final concentration of 10 mg/L (0.001%, w/v) ginger powder equivalent. For the control condition, 100 µL of sterile PBS was added to 900 µL of the same cryopreservation medium to maintain the same vehicle volume in both conditions.

The concentrations of individual phytochemical constituents in the extract were not determined. Therefore, the ginger concentration was expressed as ginger powder equivalent rather than as the concentration of specific ginger constituents.

The final concentration of 10 mg/L (0.001%, w/v) was selected based on a previous study in which ginger extract was tested at 5, 10, and 20 mg/L during cryopreservation of ram epididymal spermatozoa, with beneficial effects reported at several concentrations [19]. Because that study used ram spermatozoa and a different extract preparation and cryopreservation protocol, 10 mg/L was used as an experimental starting concentration rather than as an established optimal concentration for human spermatozoa [19].

Sperm cryopreservation and thawing

Following sperm preparation, each sample was divided into two aliquots for cryopreservation: a vehicle-matched control aliquot and a ginger-supplemented aliquot. SpermFreeze Solution (Vitrolife, Sweden) was used as the cryoprotectant in both groups.

For the cryopreserved control condition, 100 µL of sterile PBS was added to 900 µL of SpermFreeze Solution. For the ginger-supplemented condition, 100 µL of freshly prepared *Z. officinale* aqueous preparation was added to 900 µL of SpermFreeze Solution, yielding an intermediate nominal ginger powder-equivalent concentration of 10 mg/L (0.001% w/v). This mixture was subsequently combined with the sperm suspension at a 1:1 volume ratio, resulting in a final nominal ginger powder-equivalent concentration of 5 mg/L (0.0005% w/v) in the sperm-containing cryopreservation mixture. Thus, the two cryopreservation conditions contained identical volumes of cryopreservation medium and vehicle, differing only in the presence or absence of ginger extract.

Sperm suspensions were mixed with the respective cryopreservation preparations at a 1:1 ratio. Samples were equilibrated for 15 minutes at room temperature and then transferred into cryovials. The cryovials were placed in liquid nitrogen vapor approximately 5–10 cm above the liquid nitrogen surface for 45 minutes and subsequently immersed in liquid nitrogen at −196°C. Samples were stored for 1 week before thawing.

For thawing, cryovials were kept at room temperature for 1 minute and then placed in a 37°C water bath for 10 minutes. Each thawed sample was washed with 2 mL of sperm washing medium and centrifuged at 300 ×g for 5 minutes. After removal of the supernatant, the sperm pellet was resuspended in 0.2 mL of washing medium. Post-thaw viability, motility, morphology, and DNA/chromatin integrity were then assessed.

Assessment of sperm motility

Sperm motility was evaluated using a Makler counting chamber under phase-contrast microscopy at ×200 total magnification. Spermatozoa were classified as progressively motile, non-progressively motile, or immotile according to their movement.

A total of 200 spermatozoa were evaluated for each sample. Total motility was defined as the percentage of spermatozoa showing any movement, while progressive motility was defined as the percentage showing progressive movement.

Assessment of sperm viability

Sperm viability was evaluated using eosin–nigrosin staining. Briefly, 10 µL of semen was mixed with 20 µL of eosin Y and incubated for 30 seconds, followed by the addition of 30 µL of nigrosin. A smear was prepared immediately and examined by light microscopy at ×400 total magnification.

A total of 200 spermatozoa were evaluated for each sample. Unstained spermatozoa were considered viable, whereas red-stained spermatozoa were considered nonviable. Viability was expressed as the percentage of viable spermatozoa.

Assessment of sperm morphology

Sperm morphology was assessed using Spermac staining (Spermac Stain; FertiPro). Smears were prepared on clean glass slides, air-dried, fixed, and sequentially stained according to the manufacturer's instructions.

Morphological assessment was performed under oil immersion at ×1000 total magnification. A total of 200 spermatozoa were evaluated for each sample, and the percentage of spermatozoa with normal morphology was recorded according to strict morphological criteria.

AO staining for sperm DNA/chromatin integrity

Sperm DNA/chromatin integrity was assessed using AO staining. After density-gradient preparation, 20 µL of the sperm suspension was placed on a clean glass slide and allowed to air-dry. The smears were fixed in Carnoy's fixative for 1 hour and then stained with freshly prepared AO solution for 5 minutes in the dark.

The stained slides were evaluated by fluorescence microscopy using a Motic BA410 fluorescence microscope (Motic, Xiamen, China) equipped with a FITC filter set (excitation, 480/30 nm; dichroic mirror, 505 nm; emission, 535/40 nm). Spermatozoa showing predominantly green fluorescence were classified as AO-negative, whereas those showing yellow, orange, or red fluorescence were classified as AO-positive. Approximately 200 spermatozoa were evaluated per preparation, and the percentages of AO-negative and AO-positive spermatozoa were calculated.

AO-negative spermatozoa were considered to have preserved DNA/chromatin integrity, whereas AO-positive spermatozoa were considered to have altered DNA/chromatin integrity. As these two categories represent complementary proportions of the same sperm population, their statistical comparisons are mathematically reciprocal and therefore produce identical *p* values.

Because AO staining provides an assessment of sperm DNA/chromatin status rather than a direct measurement of sperm DNA fragmentation, the results were interpreted as changes in DNA/chromatin integrity. Accordingly, the AO findings were not considered equivalent to results obtained using validated sperm DNA fragmentation assays such as the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay or sperm chromatin structure assay (SCSA).

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics, version 22.0 (IBM Corp., Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation (SD).

Because each participant contributed spermatozoa to all three experimental conditions, comparisons among fresh post-gradient semen, cryopreserved control semen, and ginger-supplemented cryopreserved semen were performed using one-way repeated-measures analysis of variance (RM-ANOVA).

Sphericity was assessed using Mauchly's test. When the assumption of sphericity was violated, the Greenhouse–Geisser correction was applied to the degrees of freedom.

Pairwise comparisons were performed using paired-samples t-tests with Holm adjustment for multiple comparisons. The primary treatment comparison was between the ginger-supplemented and cryopreserved control conditions.

For the ginger-versus-control comparison, the mean paired difference was calculated as the value in the ginger-supplemented condition minus the corresponding value in the cryopreserved control condition. Accordingly, a positive mean difference indicated a higher value following ginger supplementation, whereas a negative mean difference indicated a lower value. The corresponding 95% confidence intervals (CIs) for the paired mean differences were also calculated.

All statistical tests were two-sided, and a p value <0.05 was considered statistically significant.

RESULTS

Participant and baseline semen characteristics

A total of 40 normozoospermic men were included in the study. The mean age was 32.98 ± 3.86 years (range, 26–40 years). Baseline semen characteristics are presented in Table 1.

Following density-gradient centrifugation, sperm samples from each participant were evaluated under three experimental conditions: fresh post-gradient semen, cryopreserved control semen, and cryopreserved semen supplemented with *Z. officinale* at a final nominal ginger powder-equivalent concentration of 5 mg/L (0.0005% w/v) in the sperm-containing cryopreservation mixture. Comparative results are presented in Table 2.

Effect of Z. officinale on sperm viability

Sperm viability differed significantly among the three experimental conditions (overall repeated-measures ANOVA, p<0.001). Post hoc analysis showed significantly higher viability in the ginger-supplemented cryopreserved condition than in the cryopreserved control condition (adjusted p<0.001).

Similarly, both total and progressive sperm motility differed significantly across the three conditions (both overall *p*<0.001). Ginger supplementation significantly improved total and progressive motility compared with the cryopreserved control condition (both adjusted *p*<0.001). The corresponding effect estimates and 95% confidence intervals are presented in Table 2.

Effect of Z. officinale on sperm motility

Total and progressive sperm motility differed significantly among the three experimental conditions (both overall repeated-measures ANOVA, *p*<0.001). Compared with the cryopreserved control condition, ginger supplementation was associated with significantly higher total and progressive motility (both adjusted *p*<0.001).

Effect of Z. officinale on sperm morphology

The proportion of spermatozoa with normal morphology differed significantly among the three experimental conditions (overall repeated-measures ANOVA, *p*<0.001). Representative sperm morphological features are shown in Figure 1. Mean normal morphology was 20.64 ± 5.68% in fresh post-gradient semen, 10.21 ± 3.85% in the cryopreserved control condition, and 12.69 ± 4.25% in the ginger-supplemented condition.

The ginger-supplemented condition showed a mean increase of 2.49 percentage points in normal morphology compared with the cryopreserved control condition (95% CI, 0.51–4.46; adjusted *p*=0.015). Thus, although the magnitude of the difference was relatively small, ginger supplementation was associated with a statistically significant improvement in the proportion of morphologically normal spermatozoa compared with the cryopreserved control condition.

Table 1. Baseline demographic/ semen characteristics

Parameter	Mean ± SD	Minimum	Maximum
Age (years)	32.9 ± 3.8	26	40
Semen volume (mL)	2.18 ± 0.44	1.50	3.00
Sperm conc. (×10⁶/mL)	43.59 ± 38.29	17.50	147.00
Sperm count (×10⁶/ejaculate)	92.41 ± 90.78	41.00	348.00
Total motility (%)	65.36 ± 14.49	43.00	92.00
Progressive motility (%)	57.01 ± 16.85	31.00	88.00
Immotile sperm (%)	34.64 ± 15.08	8.00	59.00
Sperm viability (%)	76.50 ± 11.53	57.00	96.00
Normal morphology (%)	12.21 ± 5.32	4.00	28.00

Values are presented as mean ± standard deviation (SD). n = 40.

Effect of Z. officinale on sperm DNA/chromatin integrity

The proportion of AO-negative spermatozoa differed significantly among the three experimental conditions (overall repeated-measures ANOVA, *p*<0.001). Compared with the cryopreserved control condition, ginger supplementation significantly increased the proportion of AO-negative spermatozoa (adjusted *p*<0.001), indicating improved AO-assessed sperm DNA/chromatin integrity following cryopreservation. The effect estimate and 95% confidence interval are presented in Table 2. Because AO-negative and AO-positive spermatozoa represent complementary proportions of the same sperm population, AO-positive results were not presented separately in Table 2. Representative fluorescence microscopy images demonstrating AO-negative and AO-positive spermatozoa are shown in Figure 2.

Table 2. Effects of *Zingiber officinale* supplementation on post-thaw sperm quality and DNA/chromatin integrity

Parameter	Fresh semen	Cryo. control	Cryo. + *Z. officinale*	RM-ANOVA F (df)	Overall *p*-value	Partial η²	Mean diff. (95% CI)	Cohen's dz	Adjusted *p*-value
Viability (%)	83.49 ± 6.59	31.50 ± 7.08	42.50 ± 6.79	706.270 (2, 78)	< 0.001	0.917	+11.00 (8.32–13.68)	1.314	< 0.001
Total motility (%)	91.95 ± 2.41	50.40 ± 13.68	67.17 ± 14.71	123.139 (1.546, 60.282)	< 0.001	0.686	+16.76 (10.07–23.45)	0.802	< 0.001
Progressive motility (%)	90.10 ± 5.30	41.19 ± 10.58	57.20 ± 10.58	274.278 (1.805, 70.378)	< 0.001	0.835	+16.00 (11.04–20.97)	1.031	< 0.001
Norm. morphology (%)	20.64 ± 5.68	10.21 ± 3.85	12.69 ± 4.25	48.463 (2, 78)	< 0.001	0.483	+2.49 (0.51–4.46)	0.401	0.015
AO-neg. sperm (%)	76.69 ± 9.98	45.67 ± 12.68	64.49 ± 10.60	75.797 (2, 78)	< 0.001	0.573	+18.82 (13.62–24.02)	1.157	< 0.001

Data are presented as mean ± SD. RM-ANOVA was used to compare the three conditions. Greenhouse–Geisser correction was applied for total motility and progressive motility because the sphericity assumption was violated. Pairwise comparisons between ginger-supplemented and cryopreserved control samples were performed using paired comparisons, with Holm adjustment for multiple testing. Mean differences were calculated as ginger-supplemented cryopreserved semen minus cryopreserved control. Thus, positive values indicate higher values in the ginger-supplemented group. Partial η² represents the effect size for the overall repeated-measures ANOVA. *P*<0.05 was considered statistically significant. **Abbreviations:** AO – acridine orange, CI – confidence interval, RM-ANOVA – repeated-measures analysis of variance, SD – standard deviation.

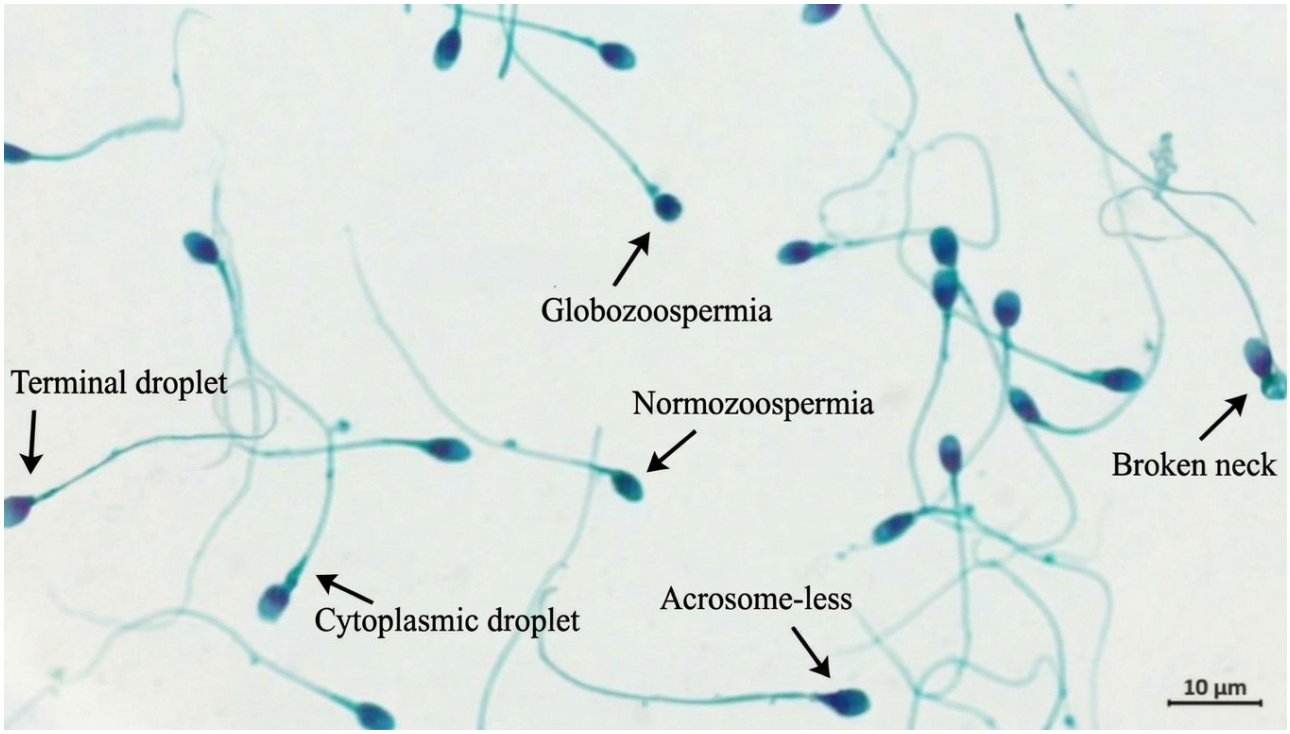


Figure 1. Representative spermatozoa displaying normal and abnormal morphological features
Spermatozoa were examined by optical microscopy under oil immersion, using Spermac staining (total magnification ×1000).

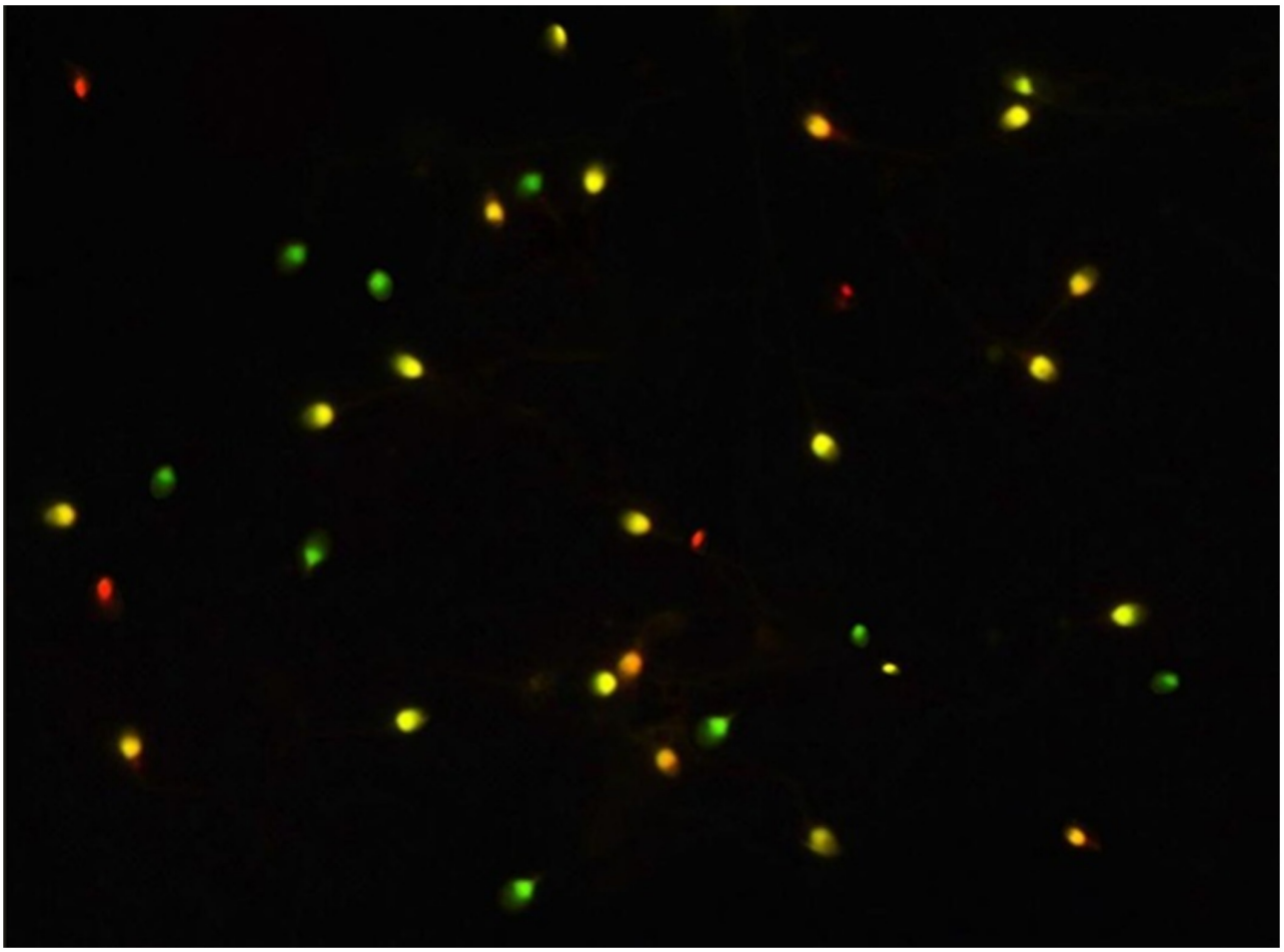


Figure 2. Representative acridine orange (AO) fluorescence patterns in spermatozoa
Samples were examined by fluorescence microscopy following AO staining to assess sperm DNA/chromatin status (total magnification ×630). AO-negative spermatozoa exhibited predominantly green fluorescence, whereas AO-positive spermatozoa exhibited yellow, orange, or red fluorescence, indicating differences in DNA/chromatin status.

DISCUSSION

In the present study, we evaluated whether supplementation of the cryopreservation medium with *Z. officinale* extract could attenuate cryopreservation-associated injury in spermatozoa from normozoospermic men. Using a within-subject design, we found that *Z. officinale* supplementation was associated with more favorable post-thaw sperm quality and AO-assessed DNA/chromatin status compared with the cryopreserved control condition. These findings provide preliminary evidence that ginger extract may have a protective effect during sperm cryopreservation.

The adverse effects of cryopreservation observed in our study are consistent with previous reports [19–22]. Freezing and thawing expose spermatozoa to osmotic and thermal stress and may disrupt membrane structure, mitochondrial function, and cellular homeostasis [19–23]. Spermatozoa may be particularly susceptible to oxidative injury because of their high content of polyunsaturated fatty acids and relatively limited antioxidant capacity [21,23]. The deterioration in sperm quality following cryopreservation observed in the present study is therefore consistent with the established biological consequences of sperm cryoinjury.

The more favorable post-thaw sperm quality observed following *Z. officinale* supplementation is consistent with previous experimental evidence suggesting beneficial effects of ginger on sperm function [24,25]. Amin and Hamza reported protective effects of ginger against cisplatin-induced reproductive toxicity in male rats, while Morakinyo et al. observed improved sperm parameters together with reduced testicular oxidative stress following *Z. officinale* administration [24,25]. Although these studies involved systemic administration in animal models, their findings provide biological plausibility for a potential protective effect of ginger on sperm function.

Our findings also extend previous experimental work by evaluating ginger directly in the cryopreservation medium. Merati and Farshad reported beneficial effects of ginger extract during cryopreservation of ram epididymal spermatozoa [19]. The present study provides preliminary evidence that a similar approach may also be applicable to human ejaculated spermatozoa. Nevertheless, the observed improvement should be interpreted as an association with better post-thaw sperm quality rather than as evidence of a specific molecular mechanism.

The potential effects of ginger on sperm quality may be related to the preservation of cellular structures that are particularly vulnerable to cryopreservation, including the plasma membrane and mitochondria. Ginger contains bioactive compounds such as gingerols and shogaols with antioxidant properties [26,27]. These properties provide a plausible biological basis for the observed findings. However, ROS production, lipid peroxidation, mitochondrial membrane potential, and antioxidant enzyme activity were not directly assessed in the present study. Therefore, an antioxidant mechanism cannot be established from our findings and should be considered a hypothesis for future investigation.

The effect on sperm morphology was less pronounced than the effects on viability and motility. Ginger supplementation increased the proportion of morphologically normal spermatozoa, although the difference was relatively small. Structural changes caused by freezing and thawing may therefore be more difficult to prevent than changes in sperm function. Further studies using detailed morphological and ultrastructural analyses are needed to better understand this finding.

Ginger supplementation also resulted in a higher proportion of AO-negative spermatozoa after cryopreservation. Previous studies have reported inconsistent effects of cryopreservation on sperm DNA and chromatin status [20–22,28–30]. Differences in cryopreservation protocols, cryoprotectants, semen preparation, storage conditions, and methods used to assess DNA or chromatin may contribute to these differences. In the present study, the higher proportion of AO-negative spermatozoa suggests better AO-assessed DNA/chromatin status in the ginger-supplemented samples.

Oxidative stress may contribute to changes in sperm DNA and chromatin during cryopreservation. Increased ROS production during freezing and thawing can damage sperm membranes and nuclear components [21,23]. Ginger contains bioactive compounds with antioxidant properties that may help limit this damage [26,27]. However, ROS and other oxidative stress markers were not measured in this study, so this explanation cannot be confirmed. In addition, AO staining should not be considered a direct measure of sperm DNA fragmentation. Unlike TUNEL or SCSA, AO staining provides information on sperm DNA/chromatin status based on the fluorescence pattern. Therefore, the present findings indicate a more favorable AO-assessed DNA/chromatin profile after ginger supplementation, rather than a demonstrated reduction in sperm DNA fragmentation.

Human evidence concerning ginger and sperm DNA integrity remains limited. Hosseini et al. reported reduced sperm DNA fragmentation following oral ginger supplementation in infertile men [17]. However, their study differed from ours in several important respects, including the route of administration, study population, exposure duration, ginger preparation, and DNA assessment method. Therefore, direct comparison between the two studies is not appropriate. Nevertheless, both studies support further investigation of ginger-derived interventions for the preservation of sperm genomic or chromatin integrity.

More recently, a study of 6-gingerol, a major bioactive constituent of ginger, in human sperm cryopreservation reported improvements in post-thaw viability and other cellular parameters [31]. Our study used whole *Z. officinale* extract rather than purified 6-gingerol. Consequently, the observed effects cannot be attributed to a single ginger constituent. Differences in extract composition, concentration, and experimental conditions should also be considered when comparing these findings.

Overall, the present findings suggest that supplementation of the cryopreservation medium with *Z. officinale* may have a protective effect against several aspects of sperm cryoinjury, particularly with respect to post-thaw sperm quality and AO-assessed DNA/chromatin status. However, these findings should be regarded as preliminary. The study was conducted in normozoospermic men, and the results may not be directly generalizable to infertile populations. In addition, the study did not directly evaluate ROS, mitochondrial function, lipid peroxidation, or validated sperm DNA fragmentation endpoints. Further studies should include larger and clinically diverse populations, standardized ginger preparations, dose-response designs, and mechanistic biomarkers to determine whether the observed effects are reproducible and whether they translate into clinically meaningful improvements in sperm preservation and reproductive outcomes.

Limitations

This study has several limitations. The sample size was relatively small, and all participants were normozoospermic men. The results may therefore not apply to infertile men or to men with abnormal baseline semen parameters.

Only one concentration of ginger preparation was tested, corresponding to a final nominal ginger powder-equivalent concentration of 5 mg/L (0.0005% w/v) in the sperm-containing cryopreservation mixture. The selected concentration was informed by previous work in ram epididymal spermatozoa, in which ginger extract concentrations of 5–20 mg/L were associated with improved post-thaw sperm parameters [19]. However, findings from animal sperm cannot be directly used to define the appropriate concentration for human sperm because the species, extract preparation, and cryopreservation procedures were different. We therefore cannot determine whether 5 mg/L is the optimal concentration or whether higher or lower concentrations would produce different or concentration-dependent effects.

The study also evaluated sperm quality after thawing rather than reproductive outcomes. Changes in viability, motility, morphology, or AO-assessed DNA/chromatin status do not establish an effect on fertilization, embryo development, implantation, or pregnancy. In addition, we did not directly measure ROS, lipid peroxidation, mitochondrial membrane potential, antioxidant enzyme activity, or specific markers of oxidative DNA damage. The mechanism behind the observed changes therefore remains uncertain, and an antioxidant effect cannot be confirmed from our data alone.

Another limitation concerns the AO staining method. AO staining provides information on sperm DNA/chromatin status but is not equivalent to established sperm DNA fragmentation assays such as SCSA or TUNEL. Therefore, the findings in the present study should be considered as changes in AO-assessed DNA/chromatin integrity rather than direct measurements of sperm DNA fragmentation.

The ginger extract was not chemically characterized in this study, and the concentrations of its individual bioactive compounds were not determined. Ginger contains several bioactive constituents, and therefore we cannot determine whether the observed effects were related to a specific compound or to their combined effects. This may also make it difficult to reproduce the findings using extracts prepared in different ways. Another issue that remains to be clarified is the concentration and timing of ginger exposure. In the present study, only one concentration and one supplementation protocol were evaluated. Studies using different concentrations and adding the extract at different stages of the cryopreservation process, including before freezing or after thawing, may provide further information about how and when ginger exerts its protective effect.

CONCLUSIONS

In the present study, 0.0005% (w/v; 5 mg/L ginger powder equivalent) *Z. officinale* extract added to the cryopreservation medium was associated with better post-thaw sperm quality than the vehicle-matched control. Sperm viability, total motility, progressive motility, and normal morphology were higher in the ginger group, and the AO staining profile was also improved. The clearest difference was seen in AO-negative spermatozoa. The changes in motility and viability were smaller, while the difference in normal morphology was relatively limited.

Our findings support a possible protective effect of ginger extract during sperm cryopreservation. The reason for these changes cannot be determined from the present study. We also cannot conclude whether the improvement in post-thaw sperm parameters would result in better reproductive outcomes. Only one concentration of ginger extract was used, and the extract was not chemically characterized. Thus, the effects of different concentrations and individual components of the extract remain unclear. Further studies with chemically characterized extracts, different concentrations, and direct measurements of oxidative stress, mitochondrial function, and sperm DNA damage would provide additional information. The relationship between improved post-thaw sperm quality and reproductive outcomes also needs to be examined.

ABBREVIATIONS

AO – acridine orange

SCSA – sperm chromatin structure assay

TUNEL – Terminal deoxynucleotidyl transferase dUTP nick end labeling

Z. officinale – Zingiber officinale

ACKNOWLEDGEMENT

None.

AUTHORS' CONTRIBUTION

ZÇ: formal analysis, visualization, data curation, writing (original draft)

MA: formal analysis, data curation, writing (original draft)

FA: formal analysis, visualization, writing (original draft)

GGE: formal analysis, data curation, writing (original draft)

All authors have read and approved the final version of the manuscript.

CONFLICT OF INTEREST

None to declare.

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