Effects of 6-Gingerol Supplementation in Cryopreservation on Human Sperm Parameters, DNA Fragmentation, and Apoptosis Incidence

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Purpose: Sperm cryopreservation is a valuable method for fertility preservation in men who suffer from oligozoospermia and cancer. The increase in oxidative stress during this process negatively affects sperm viability, membrane fluidity, and function. Supplementation of antioxidants to the cryopreservation medium can reduce these negative effects. This study investigated the effects of 6-gingerol as a natural antioxidant during human sperm cryopreservation on different sperm parameters, DNA fragmentation, and apoptosis.

Materials and Methods: In this experimental study, semen samples were obtained from 42 normozoospermic men referred to the Royan Institute. The samples were randomly divided into fresh, control (cryopreservation), and gingerol (cryopreservation with 6-gingerol) groups. Sperm evaluations were conducted before and after cryopreservation. Sperm parameters, DNA fragmentation index (DFI), caspase-3 activity, reactive oxygen species (ROS) levels, malondialdehyde (MDA) concentration, and total antioxidant capacity (TAC) levels were assessed.

Results: Use of 6-gingerol in the cryopreservation medium resulted in recovery of a significantly higher proportion of viable sperm post-cryopreservation compared with the control group (64.1 ± 1.3% vs 56.4 ± 1.3%; $P = 0.000$). ROS levels were significantly lower ($P = 0.000$), and the percentage of sperm with intact membrane potential was significantly higher ($P = 0.000$) in the gingerol group (26.1 ± 0.1 RLU/s; 63.5 ± 1.4%) compared with the control group (32.1 ± 0.7 RLU/s; 53.3 ± 1.5%). Active caspase-3 ($P = 0.007$) and DFI ($P = 0.008$) were lower in the gingerol group (47.3 ± 3.6%; 37.9 ± 1.3%, respectively) compared with the control group (60.5 ± 3.6%; 42.1 ± 0.1%, respectively), though the differences for caspase-3 and DFI did not reach statistical significance where indicated by the authors.

Conclusion: Supplementation of the sperm cryopreservation medium with 6-gingerol could improve sperm quality and function and positively affect the degree of apoptosis during sperm freezing.

Keywords: erectile function; metal thread reducer; penile incarceration; penile ischemic changes

INTRODUCTION

Sperm cryopreservation is a valuable method for fertility preservation in assisted reproductive technology (ART) clinics[(1)]. It is the most effective way to preserve fertility in men who suffer from oligozoospermia, cancer, and autoimmune disease[(2)]. Despite the successes achieved, cryopreservation causes several structural and functional damages to sperm, which lead to reduced viability and motility and ultimately reduced fertilization ability[(3)]. During this process, the formation of intracellular ice crystals, cold shock, and osmotic damage results in increased levels of reactive oxygen species (ROS)[(4)]. The resulting oxidative stress, combined with decreased total antioxidant capacity, disrupts mitochondrial and plasma membrane potential[(5)]. ROS attack and alter cellular constituents, including proteins, lipids, and DNA. Under oxidative stress, free radicals such as superoxide anion and hydrogen peroxide increase polyunsaturated fatty acid oxidation, leading to loss of structural and functional integrity of sperm membranes[(6)]. The imbalance between antioxidant capacity and free radicals leads to apoptotic changes[(7)].

Apoptosis occurs during normal biological processes such as spermatogenesis and also arises under stressful and oxidative conditions such as cryopreservation[(8)]. The apoptosis pathway is controlled by caspase genes (cysteine proteases with specific aspartate activity) that play a crucial role in the cellular apoptosis cascade. These genes are usually inactive and are only activated during apoptosis[(6)]. Caspase-3 is a key enzyme in this pathway, and its activation is a point of no return in apoptosis[(9)].

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Table 1. Mean percentages of viability and motility sperm parameters in fresh, control, and gingerol groups. Data are expressed as mean percentage ± SEM. Different superscripts show significant differences (a, b, c). $P < 0.05$ was considered statistically significant.

Groups Parameters Gingerol	Control	Fresh	
64.1 ± 1.3[b]	56.5 ± 1.3[c]	85.6 ± 1.5[a]	Viability (%)
30.3 ± 2.8	29.9 ± 2.1	61.3 ± 2.9	Total Motility (%)
18.1 ± 1.7	21.6 ± 1.7	43.5 ± 1.9	Progressive Motility (%)
39 ± 1.9	36.2 ± 2	49.4 ± 1.8	VCL (μm/s)
15.4 ± 1.1	14.9 ± 0.8	23.5 ± 0.8	VSL (μm/s)
22.2 ± 1.2	20.7 ± 1	30.9 ± 0.8	VAP (μm/s)
1.9 ± 0.1	1.8 ± 0.1	2.3 ± 0.1	ALH (μm)
5.3 ± 0.3	5 ± 0.2	7 ± 0.2	BCF (Hz)

In recent years, numerous studies have examined improving sperm cryopreservation by adding antioxidants to the cryopreservation medium[6,8,10]. The protective potential of antioxidants in cryopreservation has been shown by several authors[11]. Gingerol, one of the constituents of ginger, is a bioactive phenolic compound and is volatile with sensitivity to temperature and light. Various activities have been reported, including antioxidant and anti-inflammatory properties[12,13]. Therefore, this study aimed to investigate the effects of 6-gingerol as an antioxidant during human sperm cryopreservation on sperm viability, motility, membrane integrity, DNA fragmentation index (DFI), caspase-3 activity, ROS level, lipid peroxidation (malondialdehyde [MDA] concentration), and total antioxidant capacity (TAC) level.

MATERIALS AND METHODS

Sample Collection

Semen samples were collected from 42 normozoospermic men (22–40 years old) referred to the Royan Institute for semen analysis. This experimental study was approved by the Ethics Committee of the Royan Institute (IR.ACECR.ROYAN.REC.1396.72), and informed consent was obtained from each participant. Normal semen with liquefaction time < 1 hour, volume ≥ 2 mL, sperm concentration > 20×10^6 cells/mL, progressive motility ≥ 50%, and normal morphology ≥ 4% was selected. Each semen sample was divided equally into three groups: fresh (no cryopreservation), control (cryopreservation), and gingerol (cryopreservation with an optimal concentration of 6-gingerol in the freezing medium).

Cryopreservation

For cryopreservation, the rapid freezing technique was used. Briefly, 500 μL of the sample containing sperm cells along with seminal fluid was dispensed into cryovials, and 350 μL of Fertipro sperm freezing medium (FertiPro Inc., Belgium) was added dropwise and slowly. With each drop, the cryovial was gently tapped to mix the freezing medium with the sample. Cryovials were then kept at room temperature for 10 minutes to equilibrate. Afterward, cryovials were held 5 cm above the liquid nitrogen surface for 15 minutes to expose them to nitrogen vapor. Finally, they were immersed in liquid nitrogen and stored for 1 week.

For thawing, cryovials were placed at room temperature for 1 minute and then immersed in a water bath at 37 °C for 5 minutes. The semen was centrifuged at 600 g for 5 minutes, the supernatant was removed, and the pellet was diluted with human tubal fluid (HTF; Sigma-Aldrich) supplemented with 2.5% human serum albumin (HSA; Sigma-Aldrich). Finally, the samples were incubated for 20 minutes at 37 °C for recovery.

Determining the Optimal Concentration of 6-Gingerol

Different concentrations of 5, 10, 15, 20, 30, and 50 μM of 6-gingerol (Sigma-Aldrich, G1046) were added to the freezing medium. To determine the optimal concentration for further experiments, sperm parameters were evaluated after thawing, including viability, motility, progressive motility, and membrane integrity. The optimal concentration was then selected for the subsequent experiments.

Viability Assessment

Sperm viability was assessed by eosin–nigrosin (Sigma-Aldrich) staining. Sperm smears were prepared by mixing equal volumes of semen and eosin–nigrosin. Viability was assessed by counting 200 cells using a light microscope at ×100 magnification. Colorless sperm and completely or partially stained sperm were considered live and dead, respectively.

CASA Analysis

A computer-assisted sperm analysis (CASA) system was used to analyze total and progressive motility, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head (ALH), and beat cross frequency (BCF). For this analysis, 6 μL of semen was placed on a 37 °C warmed slide and analyzed by the CASA system.

Hypo-osmotic Swelling Test

The hypo-osmotic swelling test (HOST) was conducted to measure membrane integrity. Semen was incubated with HOS medium (citrate and fructose; Sigma-Aldrich) at 37 °C for 30 minutes. Then, 10 μL of the mixture was placed on a warmed slide along with 10 μL eosin (Sigma-Aldrich). Membrane integrity was measured by counting 200 cells under light microscopy at ×100 magnification. Sperm with swollen or curved tails were considered live.

DNA Fragmentation Assessment

The DNA fragmentation index (DFI) was determined using the acridine orange test. Fifteen μL of semen (sperm concentration: 20×10^6 cells/mL) was lysed, and Carnoy's solution (methanol/acetic acid, 3:1; Sigma-Aldrich) was applied to the smear for 5 minutes. Washing was performed with phosphate-buffered saline (PBS; Sigma-Aldrich), followed by 40 mL of citric acid solution (0.1 M; Sigma-Aldrich) and 2.5 mL of $Na_2HPO_4 \cdot 7H_2O$ (0.3 M; Sigma-Aldrich) for 5 minutes. Finally, 1% acridine orange aqueous solution (Sigma-Aldrich) was added, and the smear was examined by fluorescence microscopy at ×100 (wavelength 450 nm; 200 cells). Sperm with single-stranded DNA appeared orange, and those with double-stranded DNA appeared green.

Table 2. Mean membrane integrity, lipid peroxidation, antioxidant capacity, free radicals, DNA fragmentation, and apoptosis in fresh, control, and gingerol groups. Data are expressed as mean percentage ± SEM. Different superscripts show significant differences [(a, b, c)]. $P < 0.05$ was considered statistically significant.

Tests	Fresh	Control	Gingerol	*P*-value
HOST (%)	84.4 ± 1.9[a]	53.2 ± 1.5[c]	63.5 ± 1.4[b]	0.000
MDA (nmol/mL)	1.4 ± 0.2	3 ± 0.4	2.3 ± 0.2	0.201
TAC (nmol/µL)	17.8 ± 1.7	12.9 ± 1.3	14.3 ± 1.4	0.788
ROS (RLU/s) ×10^6 sperm	19 ± 0.5[c]	32.9 ± 0.7[a]	26 ± 1[b]	0.000
DFI (%)	11.4 ± 1.4[c]	42.1 ± 0.1[a]	37.9 ± 1.3[b]	0.008
Total Caspase-3 (%)	8.6 ± 1[c]	60.5 ± 3.6[a]	47.3 ± 3.6[b]	0.007

HOST: hypo-osmotic swelling test; MDA: malondialdehyde; TAC: total antioxidant capacity; ROS: reactive oxygen species; DFI: DNA fragmentation index.
Two-way ANOVA was used for analyzing mean values across the three experimental groups. $P < 0.05$ was considered statistically significant.

Activated Caspase-3 Evaluation

Apoptosis was evaluated by determining activated caspase-3 using the CaspaTag™ Caspase 3/7 In Situ Assay Fluorescein Kit (Merck Inc.). Activated caspase-3 was evaluated as total activated caspase-3, CP+/PI− (caspase-3–positive live cells), and CP+/PI+ (caspase-3–positive dead cells). Briefly, 300 µL of sample containing 3 million sperm was mixed with 5 µL of freshly prepared fluorescein isothiocyanate (FITC; Sigma-Aldrich) and incubated for 1.5 hours at 37 °C. After that, 1× washing buffer (Sigma-Aldrich) was added and centrifuged at 400 g for 5 minutes. The supernatant was removed, the sample was washed with 400 µL 1× buffer, and 2 µL of propidium iodide (PI; Sigma-Aldrich) was added to detect the percentage of caspase-3–positive cells by flow cytometry.

Free Radical Assessment

Chemiluminescence was used for ROS assessment. The semen sample was centrifuged with PBS at 300 g for 7 minutes, and a mixture of 20 × 106 cells/mL was prepared. Then, 5 µL of luminol solution (5-amino-2,3-dihydro-1,4-phthalazinedione dissolved in dimethyl sulfoxide; Sigma-Aldrich) was added to 400 µL of semen, and the results were reported as relative light units (RLU) per second per 20 × 10^6 sperm.

Determination of Lipid Peroxidation

Lipid peroxidation was assessed by measuring MDA concentration using the thiobarbituric acid (TBA; Sigma-Aldrich) method. Semen samples were centrifuged at 2000 g for 5 minutes. Then, 100 µL of supernatant (seminal plasma) was mixed with 900 µL distilled water and 500 µL of TBA reagent (0.67 g of thiobarbituric acid in 100 mL distilled water), dissolved with 0.5 g NaOH (Sigma-Aldrich). Next, 100 mL glacial acetic acid (Sigma-Aldrich) was slowly added to the ice-cooled mixture. The mixture was placed in a boiling water bath for 1 hour and cooled at room temperature, then centrifuged at 4000 g for 10 minutes. The absorbance of the supernatant was read at 534 nm with a spectrophotometer (1600 UV), using an extinction coefficient of 1.56 × 105 mol−1·L·cm−1, and reported in nmol/mL according to the Beer–Lambert law (A = εdc).

Total Antioxidant Capacity Assessment

Total antioxidant capacity (TAC) was performed using a TAC assay kit (MBI Inc.). Lyophilized Trolox (Sigma-Aldrich) was dissolved in 20 µL of pure dimethyl sulfoxide (DMSO; Sigma-Aldrich), and 980 µL distilled water was added to prepare a 1 mM solution, stored at −20 °C for up to 4 months. To prepare the standards, 0, 4, 8, 12, 16, and 20 µL water were replaced with Trolox accordingly. One portion of the copper reagent was added to 49 portions of the diluent assay to prepare the working solution. Thawed semen samples of control and gingerol groups were diluted up to 20-fold with distilled water; then 100 µL of the working solution was added to each well and incubated at room temperature for 90 minutes. Absorbance was recorded at 570 nm.

Statistical Analysis

Statistical analyses were carried out using SPSS, version 22.0 for Windows (SPSS Inc., Chicago, IL). Two-way ANOVA was used for analyzing mean values across the three experimental groups. $P < 0.05$ was considered statistically significant.

RESULTS

Determining the Optimal Concentration of Gingerol

Among different concentrations of 6-gingerol, the highest percentages of sperm viability, motility, progressive motility, and membrane integrity after cryopreservation were observed at 10 µM. Therefore, for further experiments, 10 µM was chosen as the optimal concentration due to its significant effects on sperm quality.

Sperm Viability

Sperm viability based on eosin–nigrosin staining was significantly higher in the fresh group (85.6 ± 1.5%) than in both cryopreserved groups ($P = 0.000$). The presence of 6-gingerol in the cryopreservation medium increased sperm viability in the gingerol group (64.1 ± 1.3%) compared with the control group (56.5 ± 1.3%) ($P = 0.000$; **Table 1**).

Sperm Motility

Total motility, progressive motility, and other motility parameters were measured after cryopreservation in the presence and absence of 10 µM 6-gingerol and compared with the fresh group. A nonsignificant decrease was observed for total motility ($P = 0.080$), progressive motility ($P = 0.124$), and all other motility parameters after cryopreservation in the control and gingerol groups compared with the fresh group. Although all studied motility parameters, except progressive motility, were higher in the gingerol group compared with the control, the differences were not significant (**Table 1**).

Sperm Membrane Integrity

The percentages of sperm with intact membranes in the fresh, control, and gingerol groups were 84.5 ± 1.9%, 53.3 ± 1.5%, and 63.5 ± 1.4%, respectively, which were significantly higher in the fresh and gingerol groups compared with the control group ($P = 0.000$; **Table 2**).

DNA Fragmentation Index

The DFI was 11.4 ± 1.4% in the fresh group, 42.1 ± 1.0% in the control group, and 37.9 ± 1.3% in the gingerol group. This percentage was lower in the gingerol

group compared with the control group and higher compared with the fresh group. The differences were statistically significant ($P = 0.008$; **Table 2**).

Active Caspase-3 Level

The percentage of sperm with total activated caspase-3 in the gingerol group (47.3 ± 3.6%) decreased compared with the control group (60.5 ± 3.6%) but increased compared with the fresh group (8.7 ± 1.0%). All experimental groups showed significant differences ($P = 0.007$; **Table 2**).

Reactive Oxygen Species Level

ROS levels were 19.50 ± 0.49 RLU/s in the fresh group, 32.95 ± 0.73 RLU/s in the control group, and 26.05 ± 0.95 RLU/s in the gingerol group. There was a statistically significant difference among all groups ($P = 0.000$; **Table 2**).

Lipid Peroxidation Level

MDA concentration in the control group (3.0 ± 0.4 nmol/mL) was nonsignificantly ($P = 0.201$) higher compared with the fresh and gingerol groups (1.4 ± 0.2 nmol/mL and 2.3 ± 0.2 nmol/mL, respectively; **Table 2**).

Total Antioxidant Capacity

TAC values were 17.8 ± 1.7 nmol/μL in the fresh group, 13.0 ± 1.3 nmol/μL in the control group, and 14.3 ± 1.4 nmol/μL in the gingerol group. The differences in TAC were not significant ($P = 0.788$; **Table 2**).

DISCUSSION

Although sperm cryopreservation is widely used for fertility preservation in ART clinics, its adverse effects on classical sperm parameters such as viability and motility should not be ignored[14]. During cryopreservation, oxidative stress occurs due to the production of ROS and the reduction of environmental antioxidants[9]. One solution to mitigate oxidative damage during cryopreservation is the use of exogenous antioxidants in the freezing medium, which has shown positive effects on sperm parameters[5]. 6-Gingerol, acting as a protective agent at the cell surface, helps preserve sperm structure and function by preventing the formation of intracellular ice crystals(15). In the present study, supplementation with 10 μM 6-gingerol improved sperm viability, membrane integrity, and ROS levels after cryopreservation. DNA fragmentation and apoptosis were also reduced with 6-gingerol supplementation.

In this study, cryopreservation negatively affected classical sperm parameters, reducing viability, motility, and progressive motility, consistent with Agha-Rahimi et al.[14]. Such damage can endanger intracellular organelles involved in survival and motility due to osmotic changes and ROS production[16]. We proposed the use of 6-gingerol as a natural antioxidant in the cryopreservation medium to minimize these negative effects. Because 6-gingerol cannot pass through the sperm lipid membrane, it likely covers the outer surface of the sperm, stabilizing and protecting the membrane structure[17]. Zahedi et al. showed that ginger might increase sperm count, reflected by increased antioxidant enzyme levels[17]. It was also shown that ginger significantly increases sperm motility and viability[18], which accords with the present study. This increase in motility could be due to 6-gingerol's effects on lowering MDA levels and increasing total antioxidant levels[17]. Reductions in MDA and increases in TAC were also observed here, although not statistically significant.

MDA, measured by the thiobarbituric acid test, is a widely used biomarker of lipid peroxidation in sperm[19]. Antioxidants in the cryopreservation medium reduce the risk of lipid peroxidation of the sperm membrane[20]. 6-Gingerol is a phenolic compound involved in scavenging oxygen free radicals and thus plays an essential role in maintaining sperm function[21]. In this study, 6-gingerol reduced lipid peroxidation in seminal plasma and MDA levels. A significant negative relationship between seminal plasma MDA concentration and sperm motility has been reported, confirming that lipid peroxidation contributes to reduced motility[22]. Kim and colleagues showed that 30 μM 6-gingerol reduces intracellular ROS caused by UVB radiation in skin cells[23]. Here, 10 μM 6-gingerol reduced ROS levels in human sperm after cryopreservation. 6-Gingerol decreases peroxidation of phospholipid liposomes in the presence of Fe3+ and ascorbate, reducing ROS[24]. Ginger extract shows antioxidant properties and inhibits lipid peroxidation in mouse liver cells[25]. Destruction of the antioxidant system in seminal plasma and increased sperm membrane lipid peroxidation have been associated with decreased sperm quality[20]. As observed here, decreased TAC in seminal plasma is associated with reduced sperm quality after cryopreservation and thawing. Banihani and Alawneh reported that decreased TAC levels in seminal plasma reduce post-cryopreservation motility and may contribute to infertility[26].

DNA fragmentation during cryopreservation may occur due to caspase activity and apoptosis, driven by increased oxidative stress and free radical attack on DNA. These events may result from cryopreservation and are influenced by thawing. Supplementation of the medium with antioxidants and/or antioxidant enzymes can improve sperm survival and DNA integrity[27]. The present findings showed increased DNA fragmentation in the control group, consistent with Gholami et al.[28], and a significant decrease in the gingerol group, indicating positive effects of 6-gingerol on DNA integrity.

Cryopreservation may lead to increased generation of superoxide anion and hydrogen peroxide, increased cytochrome c release from mitochondria, and activation of intrinsic apoptotic pathways[29]. Apoptosis is essential for eukaryotic survival and acts as a regulator of cell number and elimination[8]. It can also be a significant contributor to cryo-injury during sperm cryopreservation. Caspase-3 is crucial in the apoptosis cascade, and its activation represents a point of no return[9]. Previous studies have shown that high levels of caspase-3 and -7 are associated with sperm apoptosis[6]. In this study, the control group showed increased activated caspase-3 compared with the fresh group, similar to previous work[30]. Meanwhile, decreased activated caspase-3 in the gingerol group compared with the control group aligns with Kim et al.[23].

CONCLUSIONS

Supplementation of the cryopreservation medium with 6-gingerol could improve sperm viability and membrane integrity and reduce ROS levels. 6-Gingerol also appears to exert beneficial effects on apoptosis and DNA fragmentation during sperm cryopreservation.

SUMMARY

Adding 6-gingerol to sperm freezing medium improved post-thaw viability and membrane integrity, lowered oxidative stress, and reduced DNA damage and apoptosis markers, suggesting better sperm quality after cryopreservation.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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