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ORIGINAL ARTICLE

Sperm Biology

Green tea extract as a cryoprotectant additive to preserve the motility and DNA integrity of human spermatozoa

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Cryopreservation impairs sperm quality and functions, including motility and DNA integrity. Antioxidant additives in sperm freezing media have previously brought improvements in postthawed sperm quality. Green tea extract (GTE) is widely considered as an excellent antioxidant, and its beneficial role has been proven in other human cells. This study aims to evaluate the GTE as a potential additive in cryopreservation media of human spermatozoa. In part one, the semen of 20 normozoospermic men was used to optimize the concentration of GTE that maintains sperm motility and DNA integrity against oxidative stress, induced by hydrogen peroxide (H_2O_2). Spermatozoa were treated with GTE at different concentrations before incubation with H_2O_2. In part two, the semen of 45 patients was cryopreserved with or without 1.0 ng ml^{-1} GTE. After 2 weeks, the semen was thawed, and the effect on sperm motility and DNA fragmentation was observed. Our data showed that GTE significantly protected sperm motility and DNA integrity against oxidative stress induced by H_2O_2 when added at a final concentration of 1.0 ng ml^{-1}. We found that the addition of 1.0 ng ml^{-1} GTE to cryopreservation media significantly increased sperm motility and DNA integrity (both $P < 0.05$). More interestingly, patients with high sperm DNA damage benefited similarly from the GTE supplementation. However, there was no significant change in the reactive oxygen species (ROS) level. In conclusion, supplementing sperm freezing media with GTE has a significant protective effect on human sperm motility and DNA integrity, which may be of clinical interest.

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INTRODUCTION

Sperm cryopreservation refers to the storage of spermatozoa typically at −196°C, at which all biological activity is effectively paused. The procedure is routinely used to preserve fertility in men who are undergoing cancer treatment, which directly affects spermatogenesis.[1] Over the past decades, sperm cryopreservation has evolved as a regular practice in assisted reproductive technology (ART) treatments. For example, it is offered to store surgically retrieved sperm from azoospermic men for future use. While the primary goal of sperm freezing is to preserve male fertility, the procedure has shown adverse effects on sperm motility, functions, and DNA integrity, especially in subfertile and infertile men.[2–8]

Cryodamage of human spermatozoa is thought to be multifactorial. The variation in temperature during the freezing-thawing procedure produces chemical and physical stresses on spermatozoa that may adversely change the lipid composition of their membrane.[9,10] Moreover, the oxidative stress inflicted on spermatozoa during the procedure could be another factor in inducing DNA damage.[11–14] Oxidative stress increases when cells have an imbalance between the oxidation and reduction reactions, resulting in excess production of reactive oxygen species (ROS). Human spermatozoa have limited free radical scavenging systems, and the high content of polyunsaturated fatty acids within their plasma membrane makes them vulnerable to attack by free radicals.[15] Therefore, reducing oxidative stress on spermatozoa might help in preventing the extra damage caused by cryopreservation.

Antioxidants are agents that break the oxidative chain reaction, which in turn decrease ROS formation, and thereby reduce the oxidative stress.[16] Supplementation of sperm freezing media with antioxidants has shown cryoprotective effects on mammalian sperm quality. Antioxidant additives in human semen extenders have improved semen quality, including sperm motility, vitality, and DNA integrity after thawing.[5,14,17–20] It has been widely documented that semen quality is associated with male fertility, and its abnormality decreases the success rate of *in vitro* fertilization (IVF) treatment.[21,22] Therefore, reducing stress on spermatozoa could potentially minimize the adverse effect of cryopreservation on ART outcomes.

Green tea is very rich in polyphenols, mainly catechins, with proven antioxidant properties. Green tea extract (GTE) has an abundant amount of (-)-epigallocatechin-3-gallate (EGCG), which may act directly by scavenging ROS or indirectly by stimulating endogenous defense systems.[23,24] The beneficial effect of GTE on preserving semen

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quality has been reported in dogs, pigs, bulls, rats, and recently buffalos.[25–29] However, its potential role in human semen is yet to be examined. Our hypothesis is that the addition of GTE to sperm freezing media will improve postthaw semen quality of human ejaculates. This study investigated the effect of GTE supplementation in semen extender on sperm motility and DNA integrity of human spermatozoa.

MATERIALS AND METHODS

Overall experimental design

We designed a two-part study, as shown in **Figure 1**. Part one involved the optimization of the GTE concentration needed to protect sperm motility and DNA integrity from oxidative stress. Here, we used a well-known exogenous oxidative stress inducer, hydrogen peroxide (H_2O_2) at a final concentration of 300 μmol l^{-1}. Spermatozoa were treated with the GTE at various concentrations (0.1 ng ml^{-1}, 1.0 ng ml^{-1}, 10 ng ml^{-1}, and 100 ng ml^{-1}) before the incubation with H_2O_2. The concentration that showed significant protection on sperm motility was then tested for preserving DNA integrity of different participants under similar conditions. Part two aimed to evaluate the cryoprotective effect of the GTE on human semen. Each semen sample was split into three vials. The first vial was used to assess sperm motility and DNA fragmentation of the fresh semen. The other two vials were cryopreserved with and without the GTE supplementation. Two weeks after freezing, the sample was thawed and assessed for motility, DNA fragmentation and ROS level.

Patient recruitment

We recruited patients attending for routine semen analysis at the Andrology Unit of the Prince of Wales Hospital IVF unit, Department of Obstetrics and Gynaecology, The Chinese University of Hong Kong (Hong Kong, China). A total of 101 patients consented to participate in this study from March 29 to May 8, 2019. In part one, 51 patients were recruited. Of those patients, 20 had normal semen parameters, according to the World Health Organization (WHO) 5th edition criteria,[30] and were eligible to participate in this part of the study. Fifty patients were recruited in part two. Five patients were excluded after semen analysis indicated severe oligozoospermia or azoospermia.

Chemicals

The commercially available GTE (Theaphenon E) was generously provided by Prof. Yukihiko Hara (Faculty of Medicine, Shimane University, Matsue, Japan). Components in the decaffeinated GTE are epigallocatechin gallate (EGCG, 70.0% *w*/*w*), epigallate catechins (EGC, 4.6% *w*/*w*), epicatechin (EC, 3.9% *w*/*w*), epicatechin gallate (ECG, 6.9% *w*/*w*), and other trace catechin derivatives. The GTE was dissolved in phosphate-buffered saline (PBS) at a concentration of 1.0 mg ml^{-1} (stock solution). The stock solution was diluted in PBS to prepare working solutions of different concentrations. The stock and working solutions were separated into several Eppendorf tubes and kept at −20°C. H_2O_2 stock, 9.8 mol l^{-1} (30%, VWR Chemicals, Solon, OH, USA), was diluted in distilled water to prepare a 3 mmol l^{-1} working solution. The stock and the working solution were stored at 4°C.

Sample preparation and treatment

Semen was collected after 2–5 days of sexual abstinence. Routine semen analysis was performed on the liquefied semen to assess sperm concentration, motility, and morphology. In part one, spermatozoa of normozoospermic men, according to the WHO 5th edition criteria, were separated from seminal plasma by discontinuous density gradient centrifugation (DGC) on 90% and 45% SpermGrad media (SpermGrad, Vitrolife, Gothenburg, Sweden). This reduces the antioxidant activity of the semen, which may interfere with the effect of the GTE. The sperm pellet was washed and resuspended in capacitating media (GIVF-plus, Vitrolife). For motility assessment, 15 samples were prepared and the pellet of three different samples was pooled, in each experiment, at a concentration of 10×10^6 ml^{-1} –20×10^6 ml^{-1}. This was to obtain enough cells to test different concentrations of GTE simultaneously, thus reducing variability of results. Following preparation, H_2O_2 was added at a final concentration of 300 μmol l^{-1} to spermatozoa mixed with or without the GTE (0.1 ng ml^{-1}, 1.0 ng ml^{-1}, 10 ng ml^{-1}, and 100 ng ml^{-1} final concentration) and incubated for 15 min before assessment. For DNA fragmentation testing, five samples were used, and the pellet of each sample was resuspended separately at a concentration of 10×10^6 ml^{-1} –20×10^6 ml^{-1}. Spermatozoa were treated with or without 1.0 ng ml^{-1} GTE directly before the addition of 300 μmol l^{-1} H_2O_2 for an hour before the assessment. In part two, the liquefied semen was incubated for 10 min in sperm freezing media (SpermFreez Solution™, Vitrolife) supplemented with GTE at a final concentration of 1.0 ng ml^{-1} before freezing. After thawing, all the tests were performed directly on the semen without sperm wash or DGC preparation.

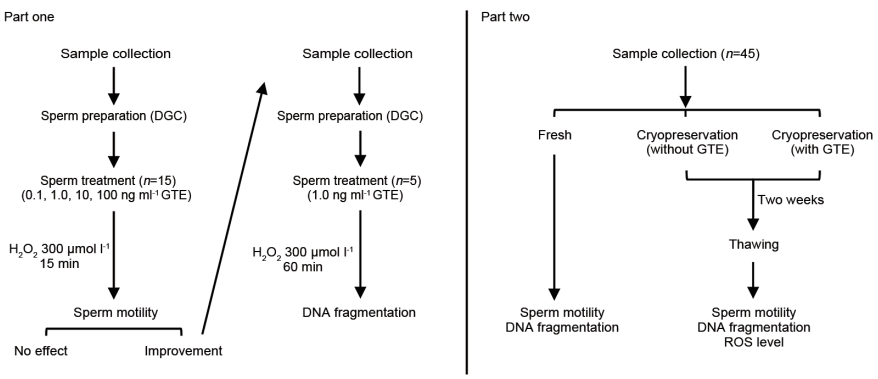

Figure 1: Overall study design. In part one, semen of normozoospermic patient was treated with GTE at different concentrations before the incubation with oxidative stress inducer (H_2O_2) for 15 min. The concentration that provided a significant protective effect on sperm motility was tested on protecting sperm DNA integrity. In part two, the effective GTE concentration, based on results of part one, was added to sperm freezing media, and the cryoprotective effect of GTE was evaluated based on sperm motility, DNA integrity, and reactive oxygen species level. GTE: green tea extract; DGC: density gradient centrifugation; ROS: reactive oxygen species.

Sperm motility assessment by CASA

Sperm motility and motion kinematic parameters were evaluated using computer-assisted sperm analyzer (CASA; Hamilton Thorne-CEROS-II system, Beverly, MA, USA). Spermatozoa were mixed and a 6 μl sample was placed onto a prewarmed clean glass microscopic slide (20-micron depth, 2 chambers Leja slides). The motility of at least 400 spermatozoa was examined at ×10 objective of an Olympus CX41 microscope (×100 total magnification; Hamilton Throne). For general motility assessment, sperm motion characteristics were classified on average path velocity (VAP) into three groups: progressive (VAP ≥25 μm s^{-1}), slow (VAP 5 μm s^{-1}–25 μm s^{-1}), and immotile (VAP 0–5 μm s^{-1}).

DNA fragmentation assay

For the evaluation of DNA fragmentation, a commercially available kit was used, Halosperm-G2 (Halotech, Madrid, Spain). Briefly, 50 μl sperm suspension was mixed with 100 μl agarose gel and immediately a sample of 8 μl was placed on super-coated slide, covered with a coverslip, and incubated at 4°C for 5 min. After incubation, the slide was fully covered with a denaturant agent contains HCl for 7 min followed by incubation in lysing buffer contains dithiothreitol (DTT) and Triton X-100 for 20 min. The slide was then washed and immersed in distilled water for 5 min before being dehydrated by 70% and then 100% ethanol for 2 min. Subsequently, the slide was air-dried and covered with eosin staining solution for 7 min, drained, and covered with Thiazine for 7 min. A total of at least 600 spermatozoa were assessed in each sample under a ×40 bright-field objective (×400 total magnification). Sperm classification followed the Halosperm-G2 recommendations; spermatozoa with either a big or medium-size halo were considered unfragmented while spermatozoa with a small halo or without a halo with either a fully or an irregularly stained core were classified as fragmented. The percentage of spermatozoa with DNA fragmentation was calculated by dividing the number of fragmented-DNA cells by the total number of cells counted. In order to evaluate the test accuracy, a negative and positive control was used by following the kit instructions. Briefly, for the positive control, the slide was not incubated in the denaturant agent before the incubation in the lysing buffer, so that all spermatozoa showed a halo. For the negative control, the slide was covered with the denaturant agent for 7 min but not incubated in the lysing buffer, so no cells showed a halo.

Semen cryopreservation and thawing

The liquefied semen was subjected to cryopreservation in different designated experimental groups. Sperm freezing media was provided from Vitrolife (SpermFreez SolutionTM, Vitrolife) and the freezing process was performed following the kit instructions. In general, semen was mixed with equal volume of sperm freezing media supplemented with or without GTE at a final concentration of 1.0 ng ml^{-1}. The semen mixture was left to equilibrate at room temperature for 10 min and placed 3 cm above the liquid nitrogen level for 30 min to allow freezing in the vapor phase. All semen vials were then transferred into liquid nitrogen and stored at −196°C. Semen thawing was performed after approximately 2 weeks of freezing. The cryovials were removed from liquid nitrogen and placed into a heating block at 37°C for 10 min.

Reactive oxygen species analysis by flow cytometry

Intracellular ROS was determined by the 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) assay (Thermo Fisher, Waltham, MA, USA). After thawing, all samples were incubated with 4 μmol l^{-1} H2DCF-DA at 37°C for 30 min. Flow cytometry (FC500, Beckman Coulter, Brea, CA, USA) was used to quantify the fluorescent signals. Fluorescent signals from GTE-treated cells were normalized to the signals from the GTE-untreated cells. Mean fluorescence was used to quantify the oxidative stress level. A negative control was prepared by adding the staining reagent to a cell-free sample, and the fluorescent signals were measured in the control before testing the experiment samples.

Mock fertilization with rescued in-vitro matured (IVM) oocytes

Immature human oocytes at the germinal vesicle stage (GV) were obtained from three IVF couples who donated their immature GVs for research (GVs are discarded under our center's standard operating procedure [SOP]). Seven GVs were matured *in vivo* into metaphase II (24 h–30 h postoocyte pick up). Spermatozoa were incubated with 1.0 ng ml^{-1} GTE for at least 30 min (35 min ± 5 min) before oocyte insemination by intracytoplasmic sperm injection (ICSI). Fertilization was checked 16 h–18 h after insemination.

Statistical analyses

SPSS software (version 23; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. One Way Repeated Measures (RM) analysis of variance (ANOVA) was used to compare sperm motility parameter values and DNA fragmentation levels between the prepared semen, and spermatozoa incubated with H_2O_2 with and without pre-treatment in GTE. Bonferroni's pairwise comparison was run to report the significant difference between all groups. In part two, Friedman (repeated measures [RM]) ANOVA test was used to compare sperm motility and DNA fragmentation between fresh, control and treated samples. If statistical significance was found, the Wilcoxon signed-rank test was performed. Mann–Whitney U test (Wilcoxon rank-sum test) was performed to study the difference in the protective effect of GTE between patients with normal and abnormal sperm motility, as well as, between patients with two different levels of DNA damage. Wilcoxon signed-rank test was also used to compare the difference in the ROS level between the control and GTE-treated samples. All the tests were considered statistically significant when $P < 0.05$.

Ethics approval

Research approval was obtained from Institutional Review Board, the Joint Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethics Committee on 6th December 2016 (CREC ref. No. 2016.499 and CREC ref. No. 2017.111). Written consent was obtained from all participants before sample collection.

RESULTS

Part one: GTE optimization

The initial testing of the pellet of spermatozoa form normozoospermic men produced a dose-response of the spermatozoa at different concentrations of GTE (0.1 ng ml^{-1}, 1.0 ng ml^{-1}, 10 ng ml^{-1}, and 100 ng ml^{-1} final concentration). The data from five pooled normozoospermic semen samples (three sample per pool), revealed an inverted U-shaped cell response (**Figure 2**). After incubation with H_2O_2, the highest percentage of total sperm motility was observed in spermatozoa treated with 1.0 ng ml^{-1} GTE, which was significantly higher than the untreated cells ($P < 0.05$; **Figure 2a**). At the same concentration, GTE-treated spermatozoa showed a significantly higher percentage of progressive motility than the untreated cells ($P < 0.05$; **Figure 2b)**. On the other hand, the compound had no significant improvement in sperm motility at higher concentrations (**Figure 2**). Furthermore, a negative impact on sperm motility parameters was observed at concentrations higher than 100 ng ml^{-1} (data not presented).

Figure 3 presents the variability in DNA fragmentation level between the treatment groups. The initial sample from five men showed

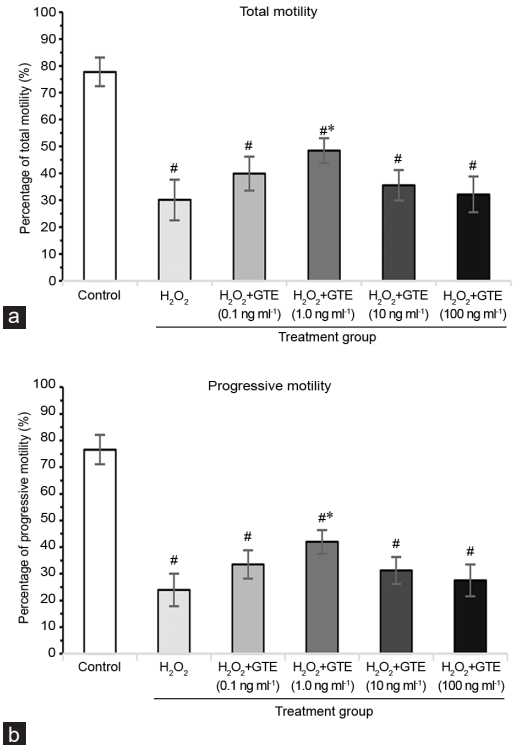

Figure 2: The dose-response of human spermatozoa to GTE under oxidative stress condition. The pellet of 15 normal semen parameter samples was isolated using density gradient centrifugation. The pellet was washed and resuspended in capacitated media and pooled in 5 pools (three samples each). Mixed spermatozoa were treated with GTE (0.1 ng ml^{-1}, 1.0 ng ml^{-1}, 10 ng ml^{-1}, and 100 ng ml^{-1}) before the exposure to H_2O_2 for 15 min. (**a**) Percentage of total motility. (**b**) Percentage of progressive motility. Data are expressed as mean ± standard deviation of five experiments using different pools. Statistical significance levels were measured by One Way RM ANOVA and indicated as: $^{\#}P < 0.05$ versus control (post-DGC sample); $^{*}P < 0.05$ versus H_2O_2. GTE: green tea extract; RM: repeated measures; DGC: density gradient centrifugation.

a percentage of 17.2% DNA-fragmented cells (**Figure 3**). Following the exposure to H_2O_2, sperm in the GTE-treated group observed a significantly lower percentage of DNA damage than the untreated group (21.1% *vs* 25.2%, $P < 0.05$; **Figure 3**).

Part two: GTE supplementation of cryopreservation media

In part two, the cryoprotective effect of 1.0 ng ml^{-1} GTE was assessed on 45 semen samples. Sperm concentration, motility, morphology, and the percentage of DNA damage of participant's fresh semen were evaluated, and the results are presented in **Table 1**.

To detect whether GTE could reduce sperm cryodamage, we analyzed sperm motility and DNA fragmentation of fresh and frozen/thawed semen, with and without GTE supplementation of the freezing media. Sperm motility assessment was performed with a CASA system. As shown in **Table 2**, the total and progressive motility significantly decreased after cryopreservation, but spermatozoa in the GTE-treated group exhibited a significantly higher percentage of total motility than the untreated cells ($P < 0.05$). Similarly, the percentage of progressive motility in the GTE-treated group was 43.6% higher than in the control group (untreated), which was statistically significant ($P < 0.05$). The data were further analyzed by dividing them, according to sperm motility parameter values in the fresh semen, into normal

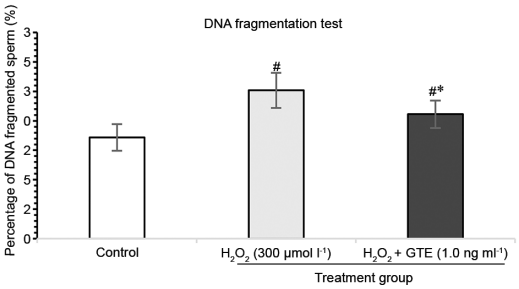

Figure 3: Effect of GTE (1.0 ng ml^{-1}) on the DNA damage of spermatozoa incubated with H_2O_2. The pellet of five normozoospermic men was isolated using density gradient centrifugation. Spermatozoa from each patient were treated with 1.0 ng ml^{-1} GTE before the incubation with 300 µmol l^{-1} H_2O_2. Data are expressed as mean ± standard deviation. $n = 5$. Statistical significance levels were measured by One Way RM ANOVA and indicated as: $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$ versus H_2O_2. GTE: green tea extract; RM: repeated measures.

Table 1: Fresh semen analysis of the study subjects

Characteristics	*Median (95% CI)*
Age (year)	37.0 (35.7–39.7)
Volume (ml)	3.0 (2.7–3.6)
Sperm concentration (10^6 ml^{-1})	27.7 (29.7–48.3)
Total motility (%)	45.5 (41.5–49.4)
Progressive motility (%)	40.3 (35.6–43.5)
Nonprogressive motility (%)	5.2 (4.6–7.2)
Sperm with normal morphology (%)	3.0 (2.5–3.7)
DNA fragmentation (%)	19.0 (17.9–24.3)

CI: confidence interval

and abnormal sperm motility on the basis of the WHO 2010.[30] Our result indicated a significant cryoprotective effect of GTE on sperm motility only when added to normally motile spermatozoa ($P < 0.05$; **Table 3**). The addition of GTE to poorly motile spermatozoa increased the postthaw motility that was not statistically significant. Our data on sperm motion kinematic parameters of postthaw semen showed no significant difference between the GTE-treated and GTE-untreated spermatozoa (**Table 2**).

As shown in **Table 2**, the percentage of DNA-fragmented spermatozoa significantly increased after cryopreservation ($P < 0.05$). GTE supplementation significantly decreased the cryopreservation-induced DNA damage relative to the control group ($P < 0.05$). GTE-treated spermatozoa exhibited 14.3% less DNA damage than untreated cells (**Table 2**).

Upon further analysis of the data, we found that some patients had very high levels of sperm DNA fragmentation. We were interested in determining if the GTE cryoprotective capability was affected by the initial level of DNA damage. Therefore, the data were divided into two groups according to the DNA damage level in the fresh semen (**Table 3**). Semen with <25% DNA-fragmented spermatozoa was considered low DNA damage, while 25% or more was considered as high DNA damage. The effect of GTE in reducing DNA damage was calculated for each group. From the data presented in **Table 3**, spermatozoa treated with 1.0 ng ml^{-1} GTE in the low-DNA damage group reduced DNA damage by 12.0% compared with that of the control. While in the high DNA damage group, GTE-treated spermatozoa showed 15.8% less DNA damage than the control. However, the difference in the percentage of cryoprotection between the two groups was not statistically significant

Table 2: Effect of Green tea extract supplementation (1.0 ng ml^{-1}) to cryoprotective media on sperm motility and DNA integrity in postthaw semen

Parameter	*Fresh*	*CP*	*CP+GTE*
Total motility (%)	45.5 (41.5–49.4)	7.2 (6.3–10.8)[a]	9.7 (8.9–14.8)[a,b]
Progressive motility (%)	40.3 (35.6–43.5)	5.5 (5.1–9.1)[a]	7.9 (6.9–11.2)[a,b]
Kinetic motility			
ALH (μm s^{-1})	4.2 (3.7–4.3)	4.3 (3.9–5.0)	3.9 (3.5–4.4)
LIN (%)	53.6 (52.5–56.1)	47.9 (40.3–49.0)[a]	42.6 (40.0–48.1)[a]
STR (%)	77.5 (75.5–79.6)	71.6 (64.7–73.6)[a]	68.6 (66.1–74.0)[a]
VAP (μm s^{-1})	40.3 (36.4–43.8)	29.5 (26.8–37.1)[a]	28.4 (25.9–32.6)[a]
VCL (μm s^{-1})	59.3 (53.6–64.2)	49.1 (45.0–66.3)[a]	50.6 (44.2–54.5)[a]
VSL (μm s^{-1})	33.5 (29.5–36.6)	21.2 (19.2–25.6)[a]	19.3 (18.5–24.9)[a]
DNA fragmentation (%)	19.0 (17.9–24.3)	28.0 (26.6–33.8)[a]	24.0 (23.4–30.2)[a,b]
ROS (mean fluorescence)	NA	7.2 (6.0–7.8)	7.1 (6.2–8.5)

Comparisons between groups performed using Friedman (RM) ANOVA test, except for ROS (Wilcoxon signed-rank test was used). Statistical significance indicated as: [a]P<0.05, CP or (CP + GTE) versus Fresh; [b]P<0.05, (CP + GTE) versus CP. Value given as median (95% CI). Fresh: fresh ejaculate; CP: cryopreservative only (control); CP + GTE: cryopreservative supplemented with 1.0 ng ml^{-1} green tea extract; ALH: mean amplitude of lateral head displacement; LIN: linearity; STR: straightness; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; ROS: reactive oxygen species; NA: not analyzed; RM: repeated measures; CI: confidence interval

Table 3: Effect of green tea extract supplementation (1.0 ng ml^{-1}) to cryoprotective media on sperm motility and DNA integrity in patients with different semen quality

Parameter	*Fresh*	*CP*	*CP + GTE*
Normal motility (n=33)			
Total motility (%)	51.9 (47.8–53.9)	7.6 (7.4–12.9)[a]	10.6 (9.8–16.3)[a,b]
Progressive motility (%)	46.1 (42.6–48.4)	6.5 (6.0–10.9)[a]	9.7 (7.7–12.4) [a,b]
Abnormal motility (n=12)			
Total motility (%)	27.1 (22.5–35.9)	2.8 (1.6–6.2)[a]	3 (1.6–15.0)[a]
Progressive motility (%)	22.6 (17.2–26.1)	1.4 (0.8–5.2)[a]	1.8 (1.1–11.3)[a]
Low DNA damage (n=33)			
DNA fragmentation (%)	18 (13.6–19.2)	25 (20.4–27.8)[a]	22 (18.3–24.9)[a,b]
High DNA damage (n=12)			
DNA fragmentation (%)	30 (27.4–50.8)	41.2 (37.0–60.6)[a]	34.7 (32.6–56.3)[a,b]

Normal and abnormal sperm motility (WHO 5th edition). Low DNA damage: damage <25%; high DNA damage: damage ≥25%. Statistical significance levels were measured by Friedman (RM) ANOVA test followed by Wilcoxon signed-rank test, and indicated as: [a]P<0.05, CP or (CP + GTE) versus Fresh; [b]P<0.05, (CP + GTE) versus CP. Value given as median (95% CI). Fresh: fresh ejaculate; CP: cryopreservative only (control); CP + GTE: cryopreservative supplemented with 1.0 ng ml^{-1} green tea extract; RM: repeated measures; CI: confidence interval

(P = 0.20). Accordingly, the result indicated that GTE has a general cryoprotective effect on DNA damage and not affected by the level of DNA fragmentation in the initial raw sample.

To determine whether GTE could reduce cryopreservation-induced ROS production, we determined ROS concentration in 15 semen samples which had been cryopreserved with or without the addition of 1.0 ng ml^{-1} GTE. After cryopreservation, there was no significant difference in the ROS level in the GTE-treated group and the untreated group (P < 0.05; **Table 2**).

DISCUSSION

Cryopreservation can adversely affect sperm quality and function including motility, viability, acrosomal and DNA integrity.[2–8] The mechanism behind this may be related to the change in osmolality, temperature, intercellular ice crystal formation and the excessive production of ROS by the cells.[9–14] The limited free radical scavenging capability of spermatozoa makes them highly susceptible to peroxidative damage after cryopreservation.[15] For these reasons, different types of exogenous antioxidant have been previously explored for reducing cryoinjury of human sperm.[5,14,15,17–20] GTE is a widely known compound for its antioxidant activities. The compound has been previously reported to reduce oxidative stress by either neutralizing toxic free radicals or stimulating endogenous defense systems.[23,24] Recent studies on animal semen reported a protective effect of GTE on sperm quality under oxidative stress conditions.[25–29] However, the possible role of GTE in protecting human sperm from cryopreservation-induced injury has not been previously examined. To our knowledge, this is the first study that aimed to investigate the cryoprotective effect of GTE on postthaw semen quality in human. Our results showed an overall improvement in recovery of sperm motility and DNA integrity with the addition of 1.0 ng ml^{-1} GTE to the cryopreservation media.

Part one was designed to optimize the effective concentration of GTE in protecting human spermatozoa from exogenous oxidative stress, where we used H_2O_2 to induce oxidative stress, which partially simulates the adverse effect of cryopreservation on semen quality. H_2O_2 was used at a concentration of 300 μmol l^{-1} as it has been previously shown a significant reduction in sperm quality.[31] Consistent with previous observations, our study reported a significant decrease in sperm total and progressive motility under H_2O_2 treatment. The addition of GTE at various concentrations to the selected sperm population (90% DG-fraction) from normozoospermic men showed an overall protection on sperm motility against H_2O_2. The highest protective effect of GTE was observed at a concentration of 1.0 ng ml^{-1}. The decrease in the protective effect of GTE at higher concentrations could be due to the toxicity of the compound. Similar observations have been shown in rams by Mehdipour *et al*.[26] To our knowledge, we are the first group reporting the role of GTE on protecting sperm motility against oxidative stress, in a dose-dependent manner.

To evaluate the protective effect of the compound on sperm DNA integrity, we treated sperm cells with 1.0 ng ml^{-1} GTE before

the addition of H_2O_2. Our results showed a significantly lower DNA damage in sperm cells treated with GTE than the control group. This observation indicated that GTE treatment reduced the adverse effect of oxidative stress on sperm DNA integrity. Overall, GTE at 1.0 ng ml^{-1} significantly protected sperm motility and DNA integrity against oxidative stress induced by H_2O_2. Furthermore, at a final concentration of 1.0 ng ml^{-1}, GTE showed no deleterious effect on *in vitro* fertility of human spermatozoa with rescued *in vitro* matured oocytes (**Supplementary Table 1**), which is consistent with a recent study reported *in vivo* fertilization ability of GTE-treated buffalo spermatozoa.[29] Our results suggest that GTE could be a potential beneficial additive to human sperm freezing media. To study our hypothesis, human spermatozoa were cryopreserved in sperm freezing media supplemented with 1.0 ng ml^{-1} GTE.

The decrease in sperm motility is the most documented adverse effect of cryopreservation. Previous studies have reported a significant decrease in total sperm motility by 25%–75%, as well as in progressive motility by 50%–75% in postthaw human semen.[3–5,7,17] In our study, we observed a significant decrease by 84.2% in total sperm motility and 86.4% in progressive sperm motility in frozen/thawed semen as compared with that in fresh ejaculates. The decrease in sperm motility in our study was high compared with previous studies. That could be due to the relatively poor semen quality of samples used in this study. Participants in our study were patients attending the andrology lab for diagnostic semen analysis before starting their fertility treatment. From the 5th edition of the WHO manual for human semen criteria,[30] 28 (62.2%) of the participants had abnormal semen quality, with 12 (42.9%) of them having low sperm motility. It has been previously reported that cryopreservation could have a more severe effect on semen with a high level of abnormality, and it is less likely to survive the freeze-thaw cycle.[8,15,18]

Antioxidant supplementation in sperm freezing media has been previously reported to significantly increase semen postthaw quality with several compounds.[5,14,15,17–20] For example, melatonin additives to cryopreservation media at a concentration of 10 μmol l^{-1} leads to a 43% increase in sperm motility after thawing.[17] In a study conducted by Kalthur *et al*.,[15] vitamin E supplementation of sperm freezing media increased sperm motility by 18.7% in normozoospermic patients and 26.8% in asthenozoospermic patients. However, vitamin E show a toxic effect on sperm cells at concentrations higher than 5 mmol l^{-1}.[15] Our study demonstrated that the addition of 1.0 ng ml^{-1} GTE to freezing media significantly increased sperm motility in postthawed semen. The percentages of total and progressive motile spermatozoa were increased by 34.7% and 43.6%, respectively in the presence of the compound. To our knowledge, this observation has not been previously reported in human semen. Our results support previous reports indicating that GTE can reduce cryo-induced damage.[26,27,29] A recent study by Ahmed *et al*.[29] reported that GTE supplementation in extenders improved postthaw sperm motility, DNA integrity, *in vitro* longevity and *in vivo* fertility of buffalo bull spermatozoa. In 2016, Mehdipour *et al*.[26] studied the effect of GTE supplementation in sperm freezing media on 20 samples of ram ejaculate. The study reported a significant increase in sperm motility and viability, but at higher concentrations (5 μg ml^{-1} and 10 μg ml^{-1} GTE) than we observed in human spermatozoa.[26] This observation is consistent with findings from other studies, where human spermatozoa responded to antioxidants at lower concentration than animal sperm cells. Further analysis of our data showed that the cryoprotective effect of GTE was affected by the initial motility in fresh semen. GTE brought about a significant increase in motility of postthaw semen only when added to normally motile spermatozoa. This observation suggested that the protective effect of GTE is more powerful in semen with normal sperm motility.

Sperm DNA integrity has been introduced as a biomarker to evaluate sperm function since 1980.[32] Previous studies observed significant increases in DNA fragmentation after cryopreservation, which ranged from 20% to 40%, compared with fresh semen.[3,5,18] In this study, DNA fragmentation level was measured before and after cryopreservation. Our results indicated an increase by 47.4% in sperm DNA fragmentation in postthaw semen compared with the fresh ejaculate. This observation agrees with the finding of many previous studies.[3,5,18]

The addition of several antioxidant molecules to sperm freezing media has improved DNA integrity of postthaw semen.[5,14,15,18,19] In 2009, Thomson *et al*.[14] conducted a study using Genistein, which is naturally found in soya and other legumes, and the results showed a reduction in DNA fragmentation of human spermatozoa. Quercetin is another flavonoid compound with antioxidant activity, and its role in cryoprotecting human sperm DNA was studied by Azadi *et al*.[19] who revealed a significant protective effect of Quercetin on sperm DNA integrity against cryo-induced damage. In our study, we found that the addition of GTE at a final concentration of 1.0 ng ml^{-1} significantly decreased the percentage of DNA fragmented sperm compared with the control group. The DNA fragmentation level in the GTE-treated group was lower by 14.3% of the control group. To our knowledge, there is no report on the cryoprotective effect of GTE in human semen. We are the first group to investigate the role of GTE on preserving DNA integrity of human semen during cryopreservation and thawing process.

We were also interested in detecting whether the cryoprotective capability of GTE was affected by the level of DNA fragmentation. Therefore, our data were analyzed according to the initial DNA damage, into high- and low-damage group. The cutoff level of high DNA damage is still clinically debatable. A recent study by Choi *et al*.[33] reported a negative association between the miscarriage rate and DNA fragmentation level higher than 13%. However, other studies observed a significant adverse effect on IVF outcomes at higher levels, ranging from 20% to 30%.[34–36] The difference in the cutoff value could be due to the variation in the method used for DNA integrity testing. Xue *et al*.[34] demonstrated the correlation between DNA damage and fertility following the method used in our study. Xue *et al*.[34] observed that semen with fragmentation level higher than 22.3% negatively affected the fertilization rate. In our study, we considered 25% DNA fragmentation as a cutoff value. Of the 45 participants, 12 exhibited DNA damage higher than 25%. Cryopreservation resulted in a significantly higher increase in DNA fragmentation in the low-DNA damage group than the high-DNA damage group. However, the effect of GTE on protecting DNA damage was not significantly different between the two groups ($P = 0.2$). This result indicates that the protective effect of GTE was not significantly affected by the level of DNA damage in the fresh sample.

Previous studies observed a decrease in ROS levels in semen cryopreserved with antioxidant additives.[15,17,18] For example, the use of melatonin at 0.01 mmol l^{-1} was reported to decrease ROS level by 47%, as demonstrated by Karimfar *et al*.[17] On the other hand, a recent study by Azadi *et al*.[19] showed no significant decrease in ROS level of postthaw semen, which was cryopreserved with the addition of 5 μmol l^{-1} Tempol. In our study, we measured the ROS level in 15 cryopreserved ejaculates with or without 1.0 ng ml^{-1} GTE. Consistent with the result of Azadi *et al*.,[19] ours showed no significant difference in the ROS level between the GTE-treated group and the control. Accordingly, the protective effect of GTE might be through other pathways or acted indirectly by stimulating endogenous defense system. Future work is needed to investigate the mechanism of GTE in protecting human spermatozoa during cryopreservation.

CONCLUSION

The addition of GTE to sperm freezing media can protect human spermatozoa from the detrimental effects of cryopreservation. This study reported, for the first time in human sperm cells that GTE improved postthaw sperm motility and DNA integrity when added at a concentration of 1.0 ng ml^{-1}. Moreover, patients with a high level of DNA damage benefited similarly form the GTE supplementation. Therefore, GTE can potentially be applied for male fertility preservation. The study did not provide conclusive information about the mechanism of GTE in protecting spermatozoa during the freezing-thawing process, future studies are needed to address that.

AUTHOR CONTRIBUTIONS

OAMA carried out DNA fragmentation test and CASA, helped in sperm freezing and thawing, analyzed the data and wrote the manuscript. MZ carried out sperm freezing and helped in data collection. CPSC recruited participants and carried out manual semen analysis. MBWL helped in semen thawing and CASA. KCC and NA carried out reactive oxygen species test. TCL and DYLC conceived of the study. DYLC coordinated the study and helped to draft the manuscript. JSMM, CCW, CPP, TCL, and WKC designed the study. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Supplementary Table 1: The effect of green tea extract on *in vitro* fertilization potential of human spermatozoa using rescued *in vitro* matured oocytes

Injected oocytes	*Normal fertilization (2PB/2PN)*	*Abnormal fertilization (1PB/3PN)*
7	4 (57%)	3 (43%)

PB: polar body; PN: pronuclei