





Green tea (*Camellia sinensis*) aqueous extract improved human spermatozoa functions *in vitro*



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Background: Idiopathic causes of infertility is associated with oxidative stress. Antioxidants are known to scavenge the excessive production of reactive oxygen species (ROS). Green tea (*Camellia sinensis*) contains polyphenols that enhance its antioxidant potential.

Aim: This study focused on the impact of aqueous green tea extract on normozoospermic human spermatozoa.

Setting: Department of Medical Biosciences, University of the Western Cape (UWC), South Africa.

Methods: Semen samples obtained using masturbation method following three to five days of sexual abstinence from consenting men ($n = 59$) at the University of the Western Cape (UWC) were liquefied and analysed. Normozoospermic samples were selected according to the World Health Organization (WHO) 5th guideline. Thereafter, semen samples (7.5×10^6 /mL) were washed in human tubular fluid (HTF; 10 min at $300 \times g$) and exposed to aqueous extracts of green tea (0 $\mu\text{g}/\text{mL}$, 0.4 $\mu\text{g}/\text{mL}$, 4 $\mu\text{g}/\text{mL}$, 40 $\mu\text{g}/\text{mL}$, 405 $\mu\text{g}/\text{mL}$) for 1 h with various sperm parameters analyzed. Human tubular fluid supplemented with bovine serum albumin (HTF-BSA; 10%) served as control.

Results: Sperm motility, reactive oxygen species production, across some reaction and deoxyribonucleic acid (DNA) fragmentation decreased significantly, particularly at the highest concentration (405 $\mu\text{g}/\text{mL}$; $p < 0.001$). A substantial increase in the percentage of viable spermatozoa and those with intact mitochondrial membrane potential (MMP) were observed ($p < 0.001$).

Conclusion: Aqueous extract of green tea prolonged sperm viability and MMP while reducing sperm intracellular ROS production, capacitation and across some reaction and DNA fragmentation, and may be attributed to its antioxidant potential. However, a high concentration of the extract appears to be detrimental to the functioning of human spermatozoa.

Keywords: *Camellia sinensis*; green tea; human spermatozoa; antioxidants; infertility; medicinal plants; oxidative stress.

Introduction

Infertility, defined as the inability to conceive following 12 months period of regular unprotected sexual intercourse, impacts about 15% of couples and 7% of all men globally (Cardona Barberán et al. 2020; Datta et al. 2016; Krausz & Riera-Escamilla 2018). About 40% – 50% of the infertility incidence is attributed to the male factor (Kumar and Singh 2015). The aetiology of male infertility is multifactorial, which can be classified as congenital, acquired and idiopathic (Agarwal et al. 2021), with about 15% of male infertility cases being idiopathic (Hamada et al. 2012; Kumar & Singh 2015). Male oxidative stress infertility involves altered semen characteristics (such as abnormal sperm morphology, low sperm concentration or poor sperm motility) and oxidative stress, and affects about 37 million men with idiopathic male infertility (Agarwal et al. 2019). In many cases of male infertility, a significant increase in seminal reactive oxygen species (ROS) has been associated with poor semen parameters (Agarwal et al. 2014). Elevated seminal oxidation reduction potential in 80% of infertile men validates the role of oxidative stress as a cause of male infertility (Agarwal et al. 2019). Men with sperm parameters below the World health Organization (WHO) normal values (semen volume [1.5 mL], sperm concentration [15×10^6 spermatozoa/mL], total sperm count [39×10^6 spermatozoa/ejaculate], morphology [4% normal forms] using 'strict' method, sperm vitality [58% live], progressive motility [32%] and total motility [40%]) are taken into consideration to have male factor infertility (WHO 2010).

Factors that contribute to male infertility include oestrogens and their derivatives, phytoestrogens, infections inside the genitourinary tract, elevated scrotal temperatures (varicocele, cryptorchidism and prolonged sauna exposure), radiation, unhealthy lifestyle (cigarette smoking, drugs, alcohol abuse, heat exposure or obesity) and elevated amounts of ROS, among others (Agarwal et al. 2021; Opuwari & Henkel 2016). An imbalance between ROS production and the ability of a biological system to readily scavenge the reactive intermediates or easily repair the resulting damage is regarded as oxidative stress (Sharma et al. 2019). Spermatozoa are susceptible to ROS because of the high content of polyunsaturated fatty acid (PUFA) in the plasma membrane (Bisht et al. 2017). Human spermatozoa lack cytoplasm, which usually contains antioxidant enzymes in somatic cells and acts as first line of defence against free radical attack, thereby limiting their antioxidant defence level (Aitken, Koopman & Lewis 2004).

The burden of oxidative stress-induced infertility brought about by male factor infertility may be reduced with the aid of an antioxidant regimen and modification of lifestyle (Bisht et al. 2017). Although there is a high heterogeneity in results in available literature, the usefulness of antioxidant therapy in improving semen parameters and reducing oxidative stress in men with infertility was demonstrated in a systematic review (Gharagozloo & Aitken 2011). Vitamins A, C and E act as major antioxidants that protect against diseases and degenerative processes resulting from oxidative stress (Akbari et al. 2022). Tea (made from the buds and leaves of *Camellia sinensis*) is the second most consumed beverage in the world (Rietveld & Wiseman 2003). Based totally on the manufacturing method, it is classified as white tea and green tea (unfermented), oolong (semi-fermented) and black tea (fermented) (Cabrera, Artacho & Giménez 2006; Dias et al. 2014). Green tea is produced from the steamed and dried leaves of *C. sinensis* and is typically consumed in China, Japan and surrounding Asian countries as well as India (Chandra et al. 2011). Green tea comprises pigments (1% – 2%), methylxanthines (3% – 4%), vitamins (A, C, E and B) and minerals (5%), lipids (2% – 7%), proteins (15%), fibres (26%) and polyphenols (30% – 40%), mostly flavonoids, and in particular catechins (Cabrera et al. 2006). Catechins (i.e. epigallocatechin-3-gallate [EGCG], epicatechin-3-gallate [ECG], epigallocatechin [EGC] and epicatechin [EC]), with EGCG been too copious and effective (Mazzanti, Di Sotto & Vitalone 2015; Yang & Hong 2013). The catechins and vitamins are associated with the health benefits of green tea including its anti-inflammatory, anticarcinogenic and antimicrobial properties (Reygaert 2017). Green tea has also been reported to reduce the risk of cardiovascular disease (Landini, Rebelos & Honka 2021; Sumpio et al. 2006) and enhance oral health (Gaur & Agnihotri 2014; Liao et al. 2021).

Available data on the effects of green tea on reproductive function in animal studies are quite contradictory. For instance, green tea leaf extract reduced the weight of testes, sperm motility and count, testosterone level, and activities of

testicular $\Delta 53$ - and 17-hydroxysteroid dehydrogenase, and also altered the morphology and histology of testis and accessory sex organs while increasing FSH and LH levels in male rats (Chandra et al. 2011; Das & Karmakar 2015). Another study revealed that the weight of male rat reproductive organs as well as testosterone level and sperm motility remained unchanged, while sperm concentration and vitality significantly increased (Opuwari & Monsees 2020). Apparently, there is limited study on the impact of green tea on human spermatozoa. Alqawasmeh and Colleagues showed that green tea extract (1.0 ng mL⁻¹) had a protective effect on human sperm motility and deoxyribonucleic acid (DNA) integrity following the induction of oxidative stress with H₂O₂ as well as increased sperm motility and DNA integrity when added to cryopreservation media (Alqawasmeh et al. 2020). This study examined the direct effect of green tea aqueous extract on human sperm functions under normal conditions.

Research methods and design

Source and preparation of aqueous extract

Green tea (Five Roses™; *C. sinensis*) was purchased from a retail outlet (Cape Town, South Africa). To prepare a 2% infusion, freshly boiled (100°C; 100 mL) distilled water was added to green tea (2 g) for five min and stirred (Opuwari & Monsees 2015). Then, the infusion was filtered using a cheesecloth and a vacuum filtration system using Whatman filter paper (Whatman, Maidstone, England) and allowed to cool to room temperature. Thereafter, the filtrate was frozen at -20°C and freeze-dried. The average yield of the extract was 3.6 g/L (18%) and was stored in a cool dry place until use. The extract (0.4 µg/mL, 4.0 µg/mL, 40 µg/mL and 405 µg/mL) was reconstituted in human tubular fluid supplemented with bovine serum albumin (10%; HTF-BSA) (Sigma Aldrich, South Africa) (Quinn, Kerin & Warnes 1985). The therapeutic concentration calculated was 40.5 µg/mL using an average weight of 80 kg and the daily consumption of tea to be six cups (Dekant et al. 2017).

Collection and preparation of human semen

This study obtained ethical clearance from the Turfloop Research Ethics Committee, University of Limpopo (TREC/393/2019: PG) and the Biomedical Research Ethics Committee, University of the Western Cape (BM18/3/17). After signing the informed consent form, semen samples were obtained from 59 healthy men (aged 18–45 years) within the semen donor programme at the University of the Western Cape. Following three to five days of sexual abstinence and using the masturbation method, semen samples were collected in a sterile vial and incubated (37°C for 30 min) for liquefaction. Then, sperm concentration and motility were evaluated using a Sperm Class Analyzer 5.0 (SCA Evolution SL, Microptic, Barcelona, Spain) and normozoospermic samples (total motility > 40%; progressive motility > 32%; sperm concentration > 15 million/mL) were used (WHO 2010).

The samples were mixed with HTF-BSA (1:6) (Moichela et al. 2021; Takalani et al. 2021), washed by centrifugation (300×g; 10 min) and re-suspended. Sperm suspensions (15×10^6 /mL) were subsequently incubated for 1 h with green tea aqueous extracts (0 µg/mL, 0.4 µg/mL, 4.0 µg/mL, 40 µg/mL and 405 µg/mL) at 37 °C. Human tubular fluid supplemented with BSA served as the control. After 1 h incubation, sperm motility, vitality, capacitation and acrosome reaction, DNA fragmentation, intracellular ROS production, and mitochondrial membrane potential (MMP) were investigated.

Analysis of sperm motility

Motility was evaluated on a preheated microscope stage (37 °C). A minimum of 360 spermatozoa were analysed using the SCA. Sperm parameters analysed included total motility (%), non-progressive motility (%), progressive motility (%), the amplitude of lateral head displacement (ALH; µm), beat cross frequency (BCF; Hz), linearity (LIN; %), straightness (STR; %), velocity average path (VAP; µm/s), velocity curve line (VCL; µm/s) and velocity straight line (VSL; µm/s).

Assessment of sperm vitality

In order to evaluate sperm vitality, the one-step eosin-nigrosin (E&N) staining technique was used (WHO 2010). After treatment with the tea extract, the sperm suspension (50 µL) was mixed with E&N stain (1:1), and smear was prepared on a slide and viewed with a bright field microscope (100×; oil immersion objective). Live and dead spermatozoa, respectively, appeared white and red. Two hundred sperm cells were evaluated, and sperm vitality was recorded as the percentage of live spermatozoa.

Assessment of sperm capacitation and acrosome reaction

The chlortetracycline (CTC) fluorescence test was used (Green et al. 1996) in evaluating capacitation and acrosome reaction. The sperm suspension was mixed with Hoechst stain and incubated for two min at room temperature (RT). Thereafter the mixture was washed by centrifugation (900×g; 5 min) in HTF containing 2% polyvinylpyrrolidone. Subsequently, the CTC (750 µM) solution was added. The mixture (10 µL) was then placed on a slide, with a drop of 1,4-diazabicyclo (2,2,2) octane; 0.22 M (DABCO) dissolved in glycerol and phosphate buffered saline (PBS) (9:1) to avoid fading. Slides were then evaluated under a fluorescent microscope (Zeiss, Oberkochen, Germany; 100×) with an excitation and emission filter of 488/520 nm. Two hundred spermatozoa were assessed for the following CTC staining patterns: non-capacitated, acrosome-intact, capacitated, acrosome-intact and capacitated, and acrosome-reacted (Takalani et al. 2022).

Assessment of mitochondrial membrane potential in human sperm

Mitochondrial membrane potential was evaluated using a space closer together, 5,5',6,6'-tetrachloro-1-1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide according to the manufacturer's

instructions (DePsipher™, Trevigen, Minneapolis, MN, USA). The midpiece of spermatozoa with intact MMP emitted intense red or orange fluorescence, while those with disrupted MMP fluoresced green. Spermatozoa were assessed (200) and the result was expressed as the percentage of spermatozoa with intact MMP.

Assessment of intracellular reactive oxygen species

Dihydroethidium (DHE; Molecular Probes, Eugene, OR, USA) was used to assess intracellular ROS production as described by Henkel et al. (2012). Each sample (100 µL) was mixed with HTF-BSA (1:5), centrifuged (300×g; 10 min), and the pellets were subsequently re-suspended in PBS (100 µL) and DHE stock solution (20 µL; 20 µM, pH 7.4) and incubated (37 °C; 15 min). Each sample (10 µL) was then viewed with a fluorescence microscope (Zeiss, Oberkochen, Germany; 1000×; 488 nm excitation and 590 emission filters). Spermatozoa with excessive ROS production fluoresced bright orange colour. Spermatozoa were assessed (200) and the result was recorded as percentage of ROS-positive spermatozoa.

Assessment of DNA fragmentation in human spermatozoa

In accordance with the manufacturer's instructions, the terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labelling (TUNEL; Dead End™ Fluorometric, Promega, Madison, WI, USA) assay was used to assess DNA fragmentation. Terminal deoxynucleotidyl transferase dUTP nick end labelling-negative spermatozoa showed just slight background staining (intact DNA), while TUNEL-positive spermatozoa (fragmented DNA) fluoresced bright green. Spermatozoa were assessed (200), and the result obtained was recorded as the percentage of spermatozoa with fragmented DNA.

Statistical analysis

Graph Pad Prism version. 5.01 (Graph Pad Software Inc., San Diego, CA, USA) was used to analyse the data obtained. Normally distributed data were analysed with one-way analysis of variance (ANOVA) and Tukey's post-test. Not normally distributed data were analysed with Kruskal-Wallis test and Dunnetts' multiple post-test. A *p*-value of less than 0.05 was considered statistically significant.

Ethical consideration

Ethical clearance to conduct the study was obtained from the Turfloop Research Ethics Committee, University of Limpopo (TREC/393/2019: PG) and the Biomedical Research Ethics Committee, University of the Western Cape (BM18/3/17).

Results

Baseline results on semen samples

Table 1 shows the baseline analysis for the samples used in the study. Semen volume (mean ± standard deviation [SD]):

2.8 ± 0.9; Min: 1.5; Max: 5.5; mL), sperm concentration (mean ± SD: 53.1 ± 18.6; Min: 16.8; Max: 82.5; million/mL), total motility (mean ± SD: 63.8 ± 15.0; Min: 40.0; Max: 98.1; %) and progressive motility (mean ± SD: 52.6 ± 19.0; Min: 32.0; Max: 95.4; %) were assessed.

Effect of green tea aqueous extract on sperm motility

Table 2 shows the impact of aqueous green tea extract on sperm motility. One-way ANOVA revealed a statistically significant difference between groups for total motility ($p = 0.0039$). Post hoc test revealed a significant difference between 0.4 and 405 µg/mL ($p = 0.0025$) and 4 µg/mL and 405 µg/mL ($p = 0.0245$) for total motility. Also, a statistically significant difference in means was observed for progressive motility ($p = 0.0118$; one-way ANOVA). Although the percentage of progressive motile spermatozoa was not significantly different between the treated groups and the control ($p > 0.05$), it was significantly higher at 0.4 µg/mL compared to 405 µg/mL ($p = 0.0110$). A significant difference in the means for non-progressive motility was also observed ($p = 0.0042$), with the percentage of non-progressive motility significantly reduced at 405 µg/mL compared to 0.4 µg/mL ($p = 0.0016$).

Table 2 also demonstrates the effect of green tea on sperm kinematic parameters. Velocity curve line remained unchanged after the treatment period ($p = 0.0584$), although a post hoc test showed that VCL was significantly higher in the control group compared to 405 µg/mL ($p = 0.0302$).

TABLE 1: Baseline analysis of semen samples ($n = 40$) obtained from donors.

Sperm parameters	Mean ± SD
Semen volume (mL)	2.8 ± 0.9
Sperm concentration (106 sperm per mL)	53.1 ± 18.6
Total motility (%)	63.8 ± 15.0
Progressive motility (%)	52.6 ± 19.0

Note: Sperm parameters were expressed as mean ± SD. SD, standard deviation.

TABLE 2: Effect of green tea aqueous extract on sperm motility and kinematic parameters.

Sperm motility and kinematic Parameters	0 (µg/mL)	0.4 (µg/mL)	4.0 (µg/mL)	40 (µg/mL)	405 (µg/mL)	p -value
Total motility (%)	16.4 ± 10.6	21.2 ± 14.3§	19.7 ± 14.3§	15.2 ± 8.6	11.3 ± 10.4¶	0.0038
Progressive motility (%)	6.8 ± 7.0	7.8 ± 8.2§	8.0 ± 9.6	5.0 ± 3.7	3.6 ± 4.7¶	0.0118
Non-progressive motility (%)	9.4 ± 6.0	12.6 ± 6.8§	10.7 ± 5.7	10.5 ± 6.4	7.3 ± 5.8¶	0.0042
VCL (µm s ⁻¹)	74.3 ± 14.0	70.9 ± 11.3	70.4 ± 19.4	71.2 ± 11.9	55.0 ± 29.7†	0.0584
VSL (µm s ⁻¹)	26.7 ± 8.9	26.7 ± 10.1§	25.4 ± 9.4§	28.1 ± 7.6§	17.8 ± 12.1†¶	0.0006
VAP (µm s ⁻¹)	36.6 ± 7.5	35.9 ± 9.4	34.7 ± 9.6	38.7 ± 9.0§	27.2 ± 15.0¶	0.0093
LIN (%)	37.4 ± 9.9	35.7 ± 10.6	34.1 ± 9.4	39.1 ± 8.3§	26.1 ± 16.6†¶	0.0020
STR (%)	67.1 ± 13.0	65.9 ± 12.7§	25.4 ± 9.4†‡	69.0 ± 8.0§¶	48.2 ± 27.9†‡§	< 0.0001
BCF (HZ)	19.8 ± 5.0	19.8 ± 4.9§	18.0 ± 5.9	19.1 ± 4.4	14.1 ± 8.9†¶	0.0094
ALH (µm)	2.1 ± 0.4	2.0 ± 0.4	2.1 ± 0.6	2.1 ± 0.4	1.7 ± 0.9	0.6887
WOB (%)	51.2 ± 7.1	49.3 ± 8.4	50.1 ± 7.4	53.1 ± 7.4	40.3 ± 21.5¶	0.0179

Note: The results follow 1-h incubation with the aqueous extract of green tea in different concentrations (0 µg/mL, 0.4 µg/mL, 4 µg/mL, 40 µg/mL and 405 µg/mL). Data are represented as mean ± SD of normozoospermic semen samples.

BCF, beat across frequency; LIN, linearity; STR, straightness; VAP, velocity average path; VCL, velocity curvilinear; VSL, velocity straight line; ALH, amplitude of lateral head displacement; WOB, wobble; SD, standard deviation.

†, ‡, Indicates statistical difference of treatment groups compared to the control; §¶, †‡, Indicates treatment groups in each row with similar symbols have no significant difference ($p > 0.05$)

The percentage of STR is significantly different between 4 and 405 µg/ml of the extract ($p < 0.05$).

A statistically significant difference in means between groups for VAP was observed as determined by one-way ANOVA ($p = 0.0093$), with the significant difference shown to be between 40 µg/mL and 405 µg/mL ($p = 0.0006$). A significant difference in the means for VSL was also observed ($p = 0.0006$), with post hoc test showing a statistically significant difference between 0 µg/mL and 405 µg/mL ($p = 0.0147$), 0.4 µg/mL and 405 µg/mL ($p = 0.0081$), 4 µg/mL and 405 µg/mL ($p = 0.0299$) as well as 40 µg/mL and 405 µg/mL ($p = 0.0005$). A statistically significant difference was also observed for STR ($p < 0.0001$). Post hoc test showed a significant difference between 0 µg/mL and 4 µg/mL ($p < 0.0001$), 0 µg/mL and 405 µg/mL ($p = 0.0124$), 0.4 µg/mL and 4 µg/mL ($p < 0.0001$), 0.4 µg/mL and 405 µg/mL ($p = 0.0303$), 4 µg/mL and 40 µg/mL ($p < 0.0001$), 4 µg/mL and 405 µg/mL ($p < 0.0001$) and 40 µg/mL and 405 µg/mL ($p = 0.0018$). The means for LIN were statistically different among the groups ($p = 0.0020$), with a significant difference between 0 µg/mL and 405 µg/mL ($p = 0.0180$) as well as 40 µg/mL and 405 µg/mL ($p = 0.0011$). Furthermore, a significant difference was also observed for wobble (WOB) ($p = 0.0179$) that was significantly higher at 40 µg/mL compared to 405 µg/mL ($p = 0.0090$). In addition, significant difference in means was also observed for BCF ($p = 0.0094$), with significant difference noted between 0 µg/mL and 405 µg/mL ($p = 0.0204$) as well as 0.4 µg/mL and 405 µg/mL ($p = 0.0191$). Lastly, no change was observed between the means of the treated groups and the control for ALH ($p > 0.05$).

Effect of green tea aqueous extract on human sperm viability

Figure 1 depicts the percentage of viable spermatozoa following 1 h incubation with green tea aqueous extract. The percentage of viable spermatozoa was significantly higher at 40 µg/mL and 405 µg/mL compared to the control ($p < 0.0001$). Also, a statistically significant difference in the means was observed among the treated groups ($p < 0.0001$). A higher percentage of viable spermatozoa was observed at 4 µg/mL ($p = 0.0134$), 40 µg/mL ($p < 0.0001$)

and 405 $\mu\text{g/mL}$ ($p < 0.0001$) compared to 0.4 $\mu\text{g/mL}$. A higher percentage of viable spermatozoa was also observed at 40 $\mu\text{g/mL}$ ($p = 0.0013$) and 405 $\mu\text{g/mL}$ ($p = 0.0247$) compared to 4 $\mu\text{g/mL}$.

Effect of green tea aqueous extract on mitochondrial membrane potential in human spermatozoa

The results on the percentage of intact MMP in human sperm after the treatment with green tea aqueous extract are highlighted in Figure 2. The percentage of spermatozoa with intact MMP was significantly higher at 4 $\mu\text{g/mL}$

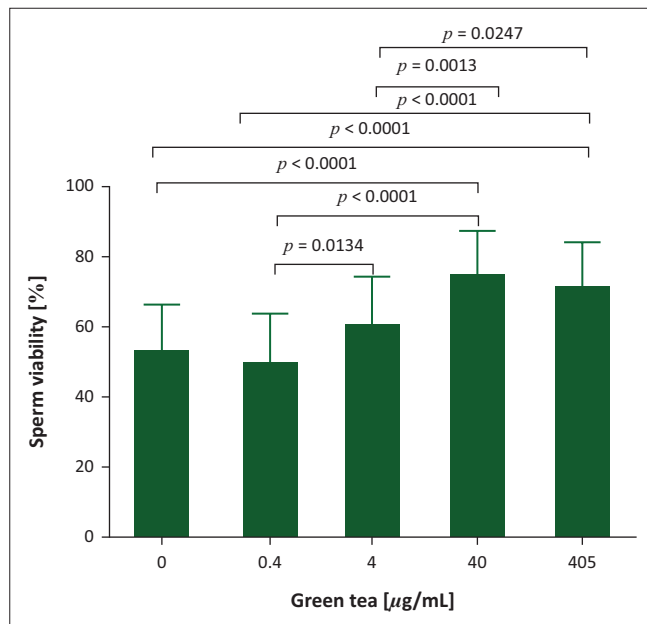
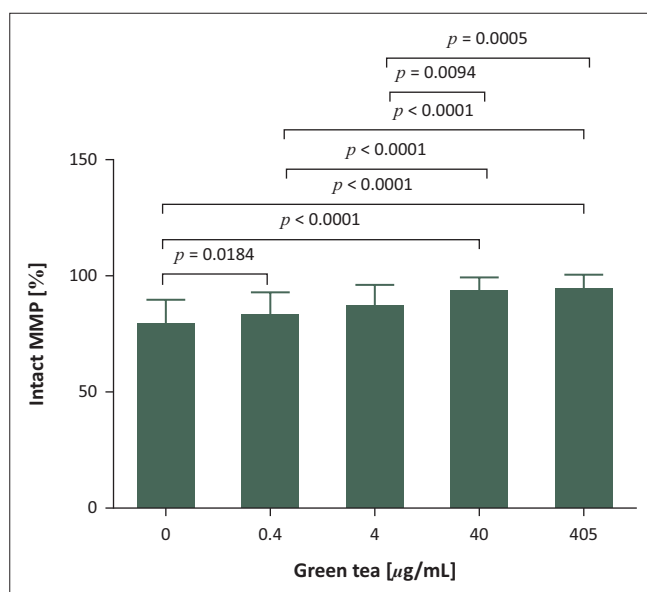


FIGURE 1: Effect of green tea aqueous extract on human sperm viability. Data are represented as mean \pm standard deviation from normozoospermic semen samples ($n = 40$).



MMP, mitochondrial membrane potential.

FIGURE 2: Effect of green tea aqueous extract on intact mitochondrial membrane potential. Data are represented as mean \pm standard deviation from normozoospermic semen samples ($n = 40$).

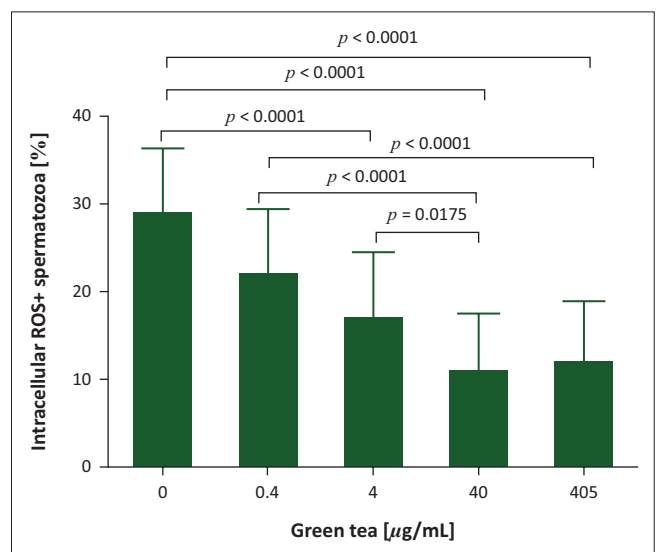
($p = 0.0184$), 40 $\mu\text{g/mL}$ ($p < 0.0001$) and 405 $\mu\text{g/mL}$ ($p < 0.0001$) compared to the control (Figure 2). Also, a significant increase was observed at 40 $\mu\text{g/mL}$ ($p < 0.0001$) and 405 $\mu\text{g/mL}$ ($p < 0.0001$) compared to 0.4 $\mu\text{g/mL}$. Furthermore, the significant increase in MMP was also observed at 40 $\mu\text{g/mL}$ ($p = 0.0094$) and 405 $\mu\text{g/mL}$ ($p = 0.0005$) compared to 4 $\mu\text{g/mL}$ (Figure 2).

Effect of green tea aqueous extract on intracellular reactive oxygen species production in human spermatozoa

The level of intracellular ROS production declined in a concentration-dependent manner in the treated samples compared to their control ($p < 0.001$; Figure 3). Also, a significant decrease in the percentage of intracellular ROS production was observed between treated groups – for instance, between 0.4 and 40 $\mu\text{g/mL}$ ($p < 0.0001$), 0.4 $\mu\text{g/mL}$ and 405 $\mu\text{g/mL}$ ($p < 0.0001$) and 4 $\mu\text{g/mL}$ and 40 $\mu\text{g/mL}$ ($p = 0.0124$) (Figure 3).

Effect of green tea aqueous extract on the acrosome reaction

The percentage of capacitated and acrosome-reacted spermatozoa significantly reduced in a concentration-dependent manner in the treated human spermatozoa compared to the control ($p < 0.0001$), specifically at 4 $\mu\text{g/mL}$ ($p = 0.0005$), 40 $\mu\text{g/mL}$ ($p < 0.0001$) and 405 $\mu\text{g/mL}$ ($p < 0.0001$) (Figure 4). Furthermore, a significant difference was observed between treatment groups, that is, 40 $\mu\text{g/mL}$ ($p < 0.0001$) and 405 $\mu\text{g/mL}$ ($p < 0.0001$) compared to 0.4 $\mu\text{g/mL}$, as well as for 40 $\mu\text{g/mL}$ ($p < 0.0001$) and 405 $\mu\text{g/mL}$ ($p = 0.0021$) compared to 4 $\mu\text{g/mL}$ (Figure 4). Trend analysis also showed a significant negative trend ($p < 0.05$).



ROS, reactive oxygen species.

FIGURE 3: Effect of green tea aqueous extract on intracellular reactive oxygen species. Data are represented as mean \pm standard deviation from normozoospermic semen samples ($n = 40$).

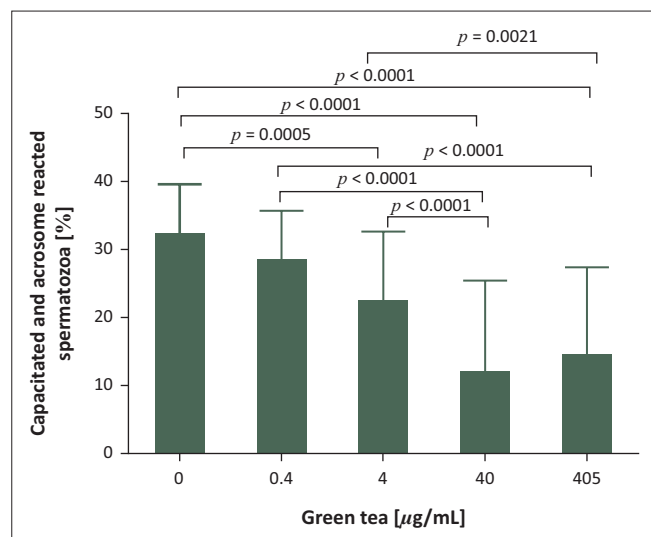
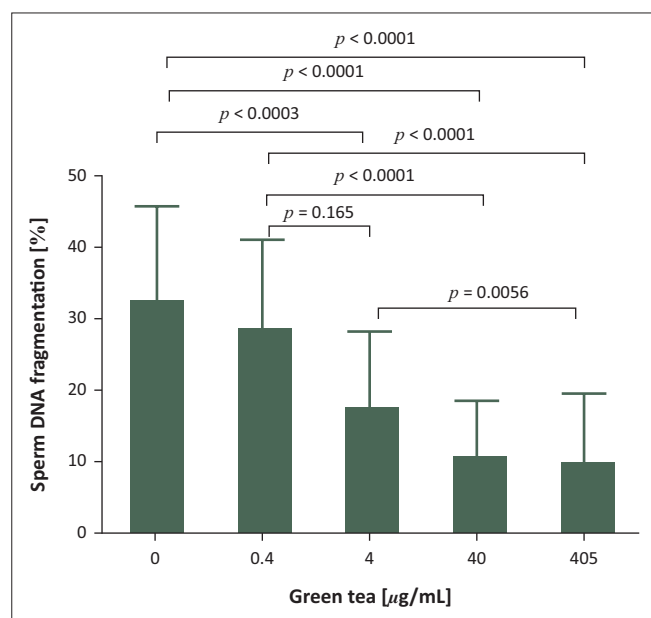


FIGURE 4: Effect of green tea aqueous extract on capacitated and acrosome reacted spermatozoa. Data are represented as mean \pm standard deviation from normozoospermic semen samples ($n = 40$).



DNA, deoxyribonucleic acid.

FIGURE 5: Effect of green tea aqueous extract on deoxyribonucleic acid fragmentation of human spermatozoa. Data are represented as mean \pm standard deviation from normozoospermic semen samples ($n = 40$).

Effect of green tea aqueous extract on sperm deoxyribonucleic acid fragmentation

The percentage of spermatozoa with fragmented DNA was significantly lower in the treated samples compared to the controls ($p < 0.0001$), specifically between 4 $\mu\text{g/mL}$ ($p = 0.0003$), 40 $\mu\text{g/mL}$ ($p < 0.0001$) and 405 $\mu\text{g/mL}$ ($p < 0.0001$) (Figure 5). Furthermore, a significant decrease was observed between treated groups, that is, between 4 $\mu\text{g/mL}$ ($p = 0.0165$), 40 $\mu\text{g/mL}$ ($p < 0.0001$) and 405 $\mu\text{g/mL}$ ($p < 0.0001$) compared to 0.4 $\mu\text{g/mL}$, as well as between 4 $\mu\text{g/mL}$ and 405 $\mu\text{g/mL}$ ($p = 0.0056$). Trend analysis showed a significant decreasing trend ($p < 0.0001$).

Discussion

Despite the antioxidant properties of green tea, *in vivo* studies using animal models revealed heterogeneous results on the male reproductive functions, which may be attributed to differences in treatment mode, length of treatment and concentrations of extracts. For instance, oral administration of green tea aqueous extract (1.25 $\mu\text{g/mL}$, 2.5 $\mu\text{g/mL}$ and 5 g/100 mL; 1 mL/100g per body weight) to rodents for 26 days resulted in a dose-dependent reduction of sperm counts, subdued activities of testicular $\Delta 53$ - and 17-hydroxysteroid dehydrogenase and dwindled testosterone levels in serum (Chandra et al. 2011). On the other hand, *ad libitum* administration of aqueous green tea leaf extract (2 g/100 mL) had no impact on sperm motility and concentration following 10 weeks of treatment, with oxidative stress induced in the last two weeks (Awoniyi et al. 2012). In another study, sperm concentration, vitality and acrosome reaction were found to be elevated, while sperm motility and testosterone level stayed unaltered after treatment of male rats with aqueous extract of green tea (2 g/100 mL and 5 g/100 mL) for 52 days *ad libitum* (Opuwari & Monsees 2020). To the best of the authors' knowledge, there is limited study on the effect of green tea on human spermatozoa. This study demonstrates the direct effects of aqueous extract of green tea on human spermatozoa *in vitro* on various sperm functional parameters under normal conditions. The authors hypothesised that the antioxidants present in aqueous extracts of green tea might improve semen qualities. Baseline analysis for semen samples used in the current study demonstrated the use of normozoospermic samples (WHO 2010). In summary, this study demonstrated that sperm motility and kinematic parameters, DNA fragmentation, intracellular ROS production and acrosome reaction decreased with increasing concentration of green tea aqueous extract, while sperm vitality and intact MMP increased significantly.

This study showed a huge reduction in the percentage of total motility, and progressive motility at the highest concentration (405 $\mu\text{g/mL}$) compared to the control. In another study, human spermatozoa treated with various concentrations of green tea extract (0.1 ng/mL, 1.0 ng/mL, 10 ng/mL and 100 ng/mL) before induction of oxidative stress with H_2O_2 for 15 min greatly augmented the percentage of total and progressive motility at lower concentrations, while a decline was seen at higher concentrations (10 ng/mL and 100 ng/mL) (Alqawasmeh et al. 2020). Like the current study, this indicates that higher concentrations of green tea extract adversely affect sperm motility, particularly total motility. Furthermore, analysis of sperm kinematic parameters showed a significant reduction at the highest concentration for VAP, VSL, VCL, STR, LIN and BCF compared to the control but not for WOB and ALH. This could also further explain the significant reduction observed in total motility at the highest concentration compared to the control. The mechanism for which green tea influences sperm motility and its kinematics needs to be investigated at the molecular level. Despite the decline in sperm motility, the percentage of

viable spermatozoa was significantly higher in the treated group compared to the control group. The determination of pre-treatment sperm viability may also have been beneficial in comparing with those obtained after treatment to gain insight into the effect of green tea on sperm viability.

Mitochondrial membrane potential manages the intact functional mitochondria and is connected with the sperm motility and can likewise be utilised in assessing sperm viability (Marchetti et al. 2002, 2004). Likewise, Ruiz-Pesini et al. (2000) showed that mitochondrial enzyme activities profoundly correspond with sperm concentration, motility and vitality. The current study demonstrated a concentration-dependent increase in the level of intact MMP as well as the percentage of viable spermatozoa, despite the significant decrease in sperm motility observed at the highest concentration of the extract. An extended time of incubation of the extract may give further insight into the impact of the extract on sperm motility and intact MMP.

The primary sources of excessive ROS production are contaminating leukocytes, dysfunctional spermatozoa, semen laboratory processing such as centrifugation and cryopreservation, and semen handling (Aitken et al. 2014; Henkel 2011). In any case, a physiological amount of ROS is required and fundamental for specific sperm functions, for example, motility, capacitation, acrosome reaction, binding to zona pellucida, oocyte fusion and sperm hyperactivation (Dutta, Majzoub & Agarwal 2019). Then again, an excessive amount of ROS and/or a disproportion between the level of ROS production and the scavenging ability of the antioxidant scavenging system affect the normal sperm function and could bring about damaged sperm membranes, DNA and proteins (Zini & Al-Hathal 2011). Spermatozoa and seminal plasma contain antioxidant compounds (enzymatic and non-enzymatic) that can scavenge ROS (Ko et al. 2014). Subsequently, the supplementation of media utilised with antioxidants might be gainful in decreasing ROS generation. This study shows a decrease in ROS generation, which may contribute to the decreased motility observed at the highest concentration.

Oxidative stress is viewed as one of the components in which DNA damage is initiated in the spermatozoa, adding to its high rate of DNA fragmentation (Aitken & Marshall Graves 2002). Several studies have reported on the effects of antioxidants in forestalling a decrease in sperm motility, just as sperm DNA damage after semen processing and incubation (see review; Zini & Al-Hathal 2011). This study highlights a decrease in the level of spermatozoa with fragmented DNA with increasing concentration of the extract, which might be credited to the antioxidant properties in green tea. In accordance with the current study, supplementation of a cryopreservative with green tea extract (1.0 ng/mL) likewise brought down DNA fragmentation in human sperm compared to the control (Alqawasmeh et al. 2020).

Conclusion

Regarding normozoospermic samples, this study demonstrated that green tea aqueous extract increased the percentage of viable spermatozoa and intact MMP, and, on the other hand, reduced sperm intracellular ROS production, which in turn, brought about the diminished level of acrosome reaction and spermatozoa with fragmented DNA. These effects might be attributed to the polyphenols and vitamins present in green tea that potentiate the antioxidant properties of the plant. However, higher concentrations of the extract decreased sperm motility. Further investigations are required to additionally comprehend the protective and antioxidative impact of green tea on human spermatozoa as well as to additionally expound on the mechanism of action of the extract on human sperm at the molecular level. The determination of the oxidative reduction potential of the extract is also necessitated to obtain its optimal function on human spermatozoa.

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Competing interests

The authors have declared that no competing interests exist.

Authors' contributions

M.A.S. conducted the experiments and performed the statistical analysis. S.S.R.C. is the co-supervisor. R.H. revised the manuscript. C.S.O. conceptualised the study, analysed the data and wrote the article. All authors read and approved the final version of the manuscript to be published.

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Data availability

The data that support the findings of this study are available upon request from the corresponding author. The data are not publicly available because of privacy or ethical restrictions.

Disclaimer

The views and opinions expressed in this article are the those of the authors and do not necessarily reflect the official policy or position of the institution or funders.

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