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*Research Article*

# Black Tea Aqueous Extracts Improve Human Sperm Functions: An *In Vitro* Study

**M. A. Setumo**[1], **S. S. R. Choma**[1], **R. Henkel**[2,3,4] **and C. S. Opuwari**[2]

[1]*Department of Pathology, Faculty of Health Sciences, University of Limpopo, Polokwane, South Africa*
[2]*Department of Medical Biosciences, Faculty of Natural Sciences, University of the Western Cape, Bellville, South Africa*
[3]*Department of Metabolism, Digestion and Reproduction, Imperial College London, London, UK*
[4]*LogixX Pharma, Berkshire, UK*

Correspondence should be addressed to C. S. Opuwari; copuwari@uwc.ac.za

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Infertility affects about 25% of couples worldwide, and oxidative stress (OS) is linked to its idiopathic etiology. Green, black, white, and oolong teas are produced from *Camellia sinensis*, depending on their oxidation level, and contain antioxidant properties that may enhance male reproductive functions. The study aimed to investigate the effects of black tea aqueous extract on human sperm functions *in vitro*. Semen samples were collected from donors, liquefied, analyzed, and divided into normal ($n = 40$) and abnormal ($n = 19$) groups using the World Health Organization 2010 criteria. Samples were washed and incubated with black tea aqueous extracts (0, 0.4, 4, 40, 405 $\mu$g/ml) for 1 hr and analyzed. Along with a considerable decrease in intracellular reactive oxygen species (ROS) production, DNA-fragmented spermatozoa, and acrosome reaction, the percentage of sperm vitality and intact mitochondrial membrane potential (MMP) increased ($p < 0.05$). Furthermore, compared to the normal group, a substantial increase in the percentage of acrosome reaction, ROS production, and percentage of spermatozoa with fragmented DNA, while a reduction in the percentage of intact MMP and sperm vitality, was noted in the abnormal group ($p < 0.05$). Compared to the controls, there was no significant change in motility between the normal and abnormal groups ($p > 0.05$). Black tea's antioxidant activity, caffeine concentration, or both may have contributed to its improvement in human sperm function *in vitro*.

## 1. Introduction

The inability to conceive after regular unprotected sexual intercourse for 12 months in a woman under age 35 or after 6 months in a woman over the age of 35 is described as infertility [1]. In addition, infertility may be characterized as primary infertility, where a couple never had a child or secondary infertility, where a couple fails to achieve pregnancy following a successful conception [2]. Approximately 25% of couples worldwide are affected by infertility, which affects one in six couples. Of these, 30%–50% are ascribed to the male factor [3, 4]. According to Agarwal et al. [5], infertility affects about 20%–30% of men globally, of which 10%–20% of male factors are idiopathic. Compared to their fertile counterparts, males with idiopathic infertility are commonly diagnosed with oxidative stress (OS) [6]. An imbalance between the capacity of the available antioxidants to scavenge reactive oxygen species (ROS) and the formation of ROS can lead to OS.

On the other hand, recent studies revealed that reductive stress, caused by an excessive buildup of reductants, may also increase ROS levels, contradicting the long-held belief that OS is the primary mediator of many disorders, including male infertility, which is brought on by elevated ROS [7]. Because of the induction of electron leakage, oxidized reductants such as nicotinamide adenine dinucleotide ($NAD^+$), glutathione disulfide (GSSG), and oxidized thioredoxins may not be as readily available, leading to the formation of ROS [7]. An overdose of countertop antioxidants resulted in poor sperm function due to an imbalance in the oxidative–reductive potential [8, 9]. Disproportionate accumulation of reductants that can cause DNA breakage and guanidine oxidation may result from excessive dietary supplementation, overproduction of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) through the pentose phosphate pathway, elevated

levels of glutathione (GSH), and nicotinamide adenine nucleotide (NADH) accumulation [7].

Infertility may lead to psychological difficulties because of societal stigmatization and purposeless feelings in men [10]. Therefore, infertility treatment may be a significant need for people, and each cause of infertility may determine the type of treatment needed [11]. The most common type of treatment is assisted reproductive technology (ART) and is not limited to *in vitro* fertilization and intracytoplasmic sperm injection [11]. However, most health insurance schemes do not cover ART due to the expensive costs and lack of guarantee for a positive outcome [12]. ART is also not readily available to all social and economic classes, as it tends to be expensive and not accessible [13]. Therefore, lower cost infertility treatments are necessary [14].

Several studies have demonstrated the affordability and availability of diverse medicinal plants across social classes globally [15, 16]. These medicinal plants contain beneficial compounds, including antioxidants [17] that serve as the protective barrier for spermatozoa prevention of excessive ROS generation (prevention antioxidants such as metal chelators and metal-binding proteins) or to remove already existing ROS (scavenging ROS) [18]. Antioxidants may be classified as nonenzymatic (glutathione, ascorbic acid, and tocopherol) and enzymatic (superoxide dismutase, glutathione peroxidase, and catalase) [19]. The improvement of male fertility parameters is usually attributed to their antioxidant properties [20].

Tea is the second most popular beverage after water and is produced from the buds and young leaves of the *Camellia sinensis* plant in the family of Theaceae [21, 22]. Black tea is the highest-produced tea globally (76%–78%), followed by green tea (20%–22%) and oolong tea (2%) [23]. Freshly plucked leaves are processed to produce diverse types of tea, including green and white teas (unfermented), black tea (fermented), and oolong tea (semifermented) [22, 24]. Catechins, theaflavins, thearubigins, amino acids, and alkaloids are common constituents of tea, with theaflavins and thearubigins being the most important polyphenolic components in black tea [25, 26]. During the fermentation process of black tea, theaflavins and thearubigins result from the oxidation and polymerization of catechins [25].

Health benefits of *C. sinensis* include anticancer, hepatoprotective, anti-inflammatory, analgesic, antipyretic, antiallergic, antimicrobial, antiviral, and antiparasitic activities; relieves asthma and allergy; and improves cardiovascular activities [27]. It is used traditionally to treat urinary inconsistency and common colds, as well as an anxiety suppressant and to prevent blister formation on a burn wound [28]. Additionally, because black tea elevates testosterone levels, traditional Sri Lankan practitioners utilize it to improve sexual performance and delay ejaculation [29]. In a different *in vivo* study, male Wistar rats given *ad libitum* access to black tea aqueous extracts (2% and 5%) did not significantly affect testosterone production, sperm concentration, or the weight of their reproductive organs; however, sperm motility and vitality improved significantly [30]. It was demonstrated after a 24-hr treatment *in vitro* that black tea aqueous extract (250 and 1,000 $\mu$g/ml) had an antiandrogenic effect on TM3 Leydig cells by significantly lowering testosterone synthesis [31]. The aim of the current work is to investigate the direct effects of aqueous black tea extract on the *in vitro* functions of human spermatozoa.

## 2. Materials and Methods

*2.1. Materials.* Unless otherwise indicated, all reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). In addition, commercial black tea was acquired locally (Five Roses™, Cape Town, South Africa).

*2.2. Collection of Plant Extract and Preparation.* As described by Setumo [32], an aqueous extract of black tea was prepared by infusing 2 g of the leaves in freshly boiled (100 ml) for 5 min, filtered using a vacuum filtration system, and cooled to room temperature [31]. The filtrate was freeze-dried (with an average yield of 3.60 g/l) and kept in a cool, dry place until use. The extracts were reconstituted in human tubular fluid containing bovine serum albumin (HTF-BSA) to final concentrations of 0.4, 4.0, 40, and 405 $\mu$g/ml, based on the recommended daily intake of six cups (a cup equivalent to 150 ml) for an average of 80 kg man [33].

*2.3. Ethics Approval.* Turfloop Research Ethics Committee of the University of Limpopo (UL), Sovenga, South Africa (TREC/393/2019: PG) and Biomedical Research Ethics Committee (BMREC) of the University of the Western Cape (UWC), Bellville, South Africa (BMI18/3/17).

*2.4. Source and Preparation of Semen Samples.* Healthy donors signed informed consent, abstained from sexual activity for 3–5 days, and masturbated to collect semen samples were obtained by masturbation, which was then placed in a sterile vial ($n = 59$; 18–45 years) at the comparative spermatology laboratory UWC and allowed to liquefy (30 min; 37°C). Using a Sperm Class Analyser (SCA) (Microptic, Barcelona, Spain), baseline sperm concentration and motility were determined. Afterwards, the World Health Organization (WHO) [34] guidelines classified the samples as normal or abnormal. A normal semen sample is defined as having sperm concentrations > 15 million/ml, progressive motility > 32%, and total motility > 40%; an abnormal semen sample is defined as having sperm concentrations $< 15 \times 10^6$/ml, progressive motility < 32%, and total motility < 40% [32]. For a semen sample to be classified as normal, it must meet all three criteria; otherwise, it is classified as an abnormal sample. The washed samples were incubated with black tea aqueous extract (0.4, 4.0, 40, and 405 $\mu$g/ml) for 1 hr at 37°C. Additional sperm parameters, sperm motility, vitality, mitochondrial membrane potential (MMP), ROS production, DNA fragmentation, capacitation, and acrosome reaction, were analyzed as previously described [32, 35–38]. The control contained only HTF-BSA.

*2.5. Sperm Motility and Kinematic Parameters.* The SCA was used to assess the motility of treated sperm suspension (2 $\mu$l) on Leja slides and incubated at 37°C. Moreover, analysis was done on the following: beat cross frequency (BCF), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH), wobble (WOB), average velocity path (VAP),

velocity curve line (VCL), velocity straight line (VSL), and total motility [32, 37].

*2.6. Sperm Vitality.* According to WHO [34] guidelines, sperm vitality was evaluated by one-step eosin–nigrosin (E&N) staining. After the incubation period, a smear was made on a glass slide by combining the sperm solution with E&N stain 1 : 1, allowing it to air dry, and then viewed under a 100x light microscope. Spermatozoa that were dead or alive appeared pink and white, respectively. The percentage of living spermatozoa was calculated after counting the 200 spermatozoa.

*2.7. Mitochondrial Membrane Potential of Sperm.* According to the manufacturer's instructions, the MMP was determined (DePsipher™, Trevigen, Minneapolis, USA). The prepared slides were observed with a fluorescence microscope (Zeiss, Oberkochen, Germany) at 400x, using a 488 nm excitation filter. Spermatozoa with intact MMP fluoresced intense red/orange and fluoresced green for those with disrupted MMP. The spermatozoa (200) were counted, and the results represented the percentage of spermatozoa with intact MMP.

*2.8. Reactive Oxygen Species Production.* The ROS production was determined using dihydroethidium (DHE; Molecular Probes, Eugene, OR, USA) [39]. After the treatment, 100 $\mu$l of spermatozoa were centrifuged (10 min; 500 $\times g$) and then resuspended in 100 $\mu$l of PBS and 20 $\mu$l of stock solution of DHE (20 $\mu$M DHE in PBS, pH = 7.4). The mixture was then incubated for a further 15 min at 37°C. After that, the sample was placed on a slide and viewed under oil immersion using an epifluorescence microscope (Zeiss, Oberkochen, Germany) with 488 nm excitation and 590 emission filters. Spermatozoa with excessive ROS production fluoresced bright orange (ROS-positive spermatozoa). Two hundred spermatozoa were analyzed, representing the result as the percentage of ROS-positive spermatozoa.

*2.9. DNA Fragmentation.* Sperm DNA fragmentation was evaluated using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) (Dead End™; Promega, Madison, USA), per the manufacturer's instructions. The prepared slides were examined under a fluorescence microscope with an emission filter of 510–530 nm and an excitation filter of 488 nm. A total of 200 spermatozoa were evaluated. Spermatozoa that fluoresced bright green spermatozoa were, respectively, categorized as TUNEL-positive (fragmented DNA) or TUNEL-negative (intact DNA) if they fluoresced bright green or had light background staining. The percentage of TUNEL-positive spermatozoa was used to express the result.

*2.10. Capacitation and Acrosome Reaction in Sperm.* The chlortetracycline fluorescence assay protocol was utilized to evaluate the capacitation and acrosome reaction [40]. Using a fluorescence microscope with a 488 nm excitation filter (Zeiss, Oberkochen, Germany), 200 spermatozoa were assessed based on the presence or absence of fluorescence and classified as acrosome reacted, capacitated acrosome intact or non-capacitated, acrosome intact cell [32, 38].

Table 1: Baseline analysis of semen samples.

| Sperm parameters | Samples | Mean ± SD | Min | Max | $p$-Value |
|---|---|---|---|---|---|
| Semen volume (ml) | Normal | 2.8 ± 0.9 | 1.5 | 5.5 | >0.05 |
| | Abnormal | 2.4 ± 0.5 | 1.6 | 3.4 | |
| Concentration ($10^6$/ml) | Normal | 53.1 ± 2.9 | 16.8 | 82.5 | <0.0001 |
| | Abnormal | 12.9 ± 10.4 | 0.4 | 40.0 | |
| Total motility (%) | Normal | 63.8 ± 15.0 | 40.0 | 98.1 | >0.05 |
| | Abnormal | 54.9 ± 23.6 | 2.5 | 91.0 | |
| Progressive motility (%) | Normal | 52.6 ± 19.0 | 32.0 | 95.4 | <0.0001 |
| | Abnormal | 35.1 ± 22.6 | 0.8 | 71.7 | |

*2.11. Data Analysis.* Data analysis was performed with the GraphPad Prism version. 5.01 (Graph Pad Software Inc., San Diego, CA, USA). D'Agostino & Pearson omnibus normality test was employed to determine normal distribution. One-way analysis of variance (ANOVA) and Tukey's posttest were used for normally distributed data. In contrast, the Kruskal–Wallis and Dunnetts' multiple posttests were used for data that was not normally distributed. A two-way ANOVA was used to compare the respective parameters between the normal and abnormal groups. Results were considered statistically significant with a $p$-value $< 0.05$.

## 3. Results

Sperm concentration ($p < 0.05$) and progressive motility ($p > 0.05$) were found to be considerably higher in the normal samples than in the abnormal samples, according to the baseline analysis of the semen samples obtained (Table 1). On the other hand, there was no statistically significant difference in semen volume or total motility between the abnormal and normal samples ($p > 0.05$, Table 1).

Figure 1(a)–1(c) shows how the aqueous extract of black tea affects sperm motility. Total motility did not significantly differ between the normal and abnormal samples ($p > 0.05$) or between the normal and abnormal groups when compared to the corresponding controls (Figure 1(a)). However, a significant drop in total motility was seen for both groups at 405 $\mu$g/ml as opposed to 0.4 $\mu$g/ml (Figure 1(a); $p < 0.01$). Moreover, Figure 1(b) shows no difference in the proportion of progressively motile spermatozoa between the normal and abnormal samples ($p > 0.05$) or either group compared to their control. Conversely, 0.4 $\mu$g/ml showed a significant increase in progressive motility compared to 405 $\mu$g/ml ($p < 0.05$). Furthermore, the percentage of nonprogressive spermatozoa in both groups did not alter ($p > 0.05$). However, there was a significant drop in the percentage of nonprogressive spermatozoa at 405 $\mu$g/ml compared to 0.4 $\mu$g/ml ($p < 0.05$). Moreover, LIN, STR, VAP, VCL, VSL, and WOB did not significantly differ in either group (Table 2; $p > 0.05$). On the other hand, the normal group's BCF and ALH showed no change ($p > 0.05$), while the abnormal group's showed a substantial difference ($p < 0.05$).

The percentage of viable spermatozoa in the normal group was substantially more significant than in the abnormal group (Figure 2; $p < 0.01$). Furthermore, at 40 and 405 $\mu$g/ml, the proportion of viable spermatozoa increased

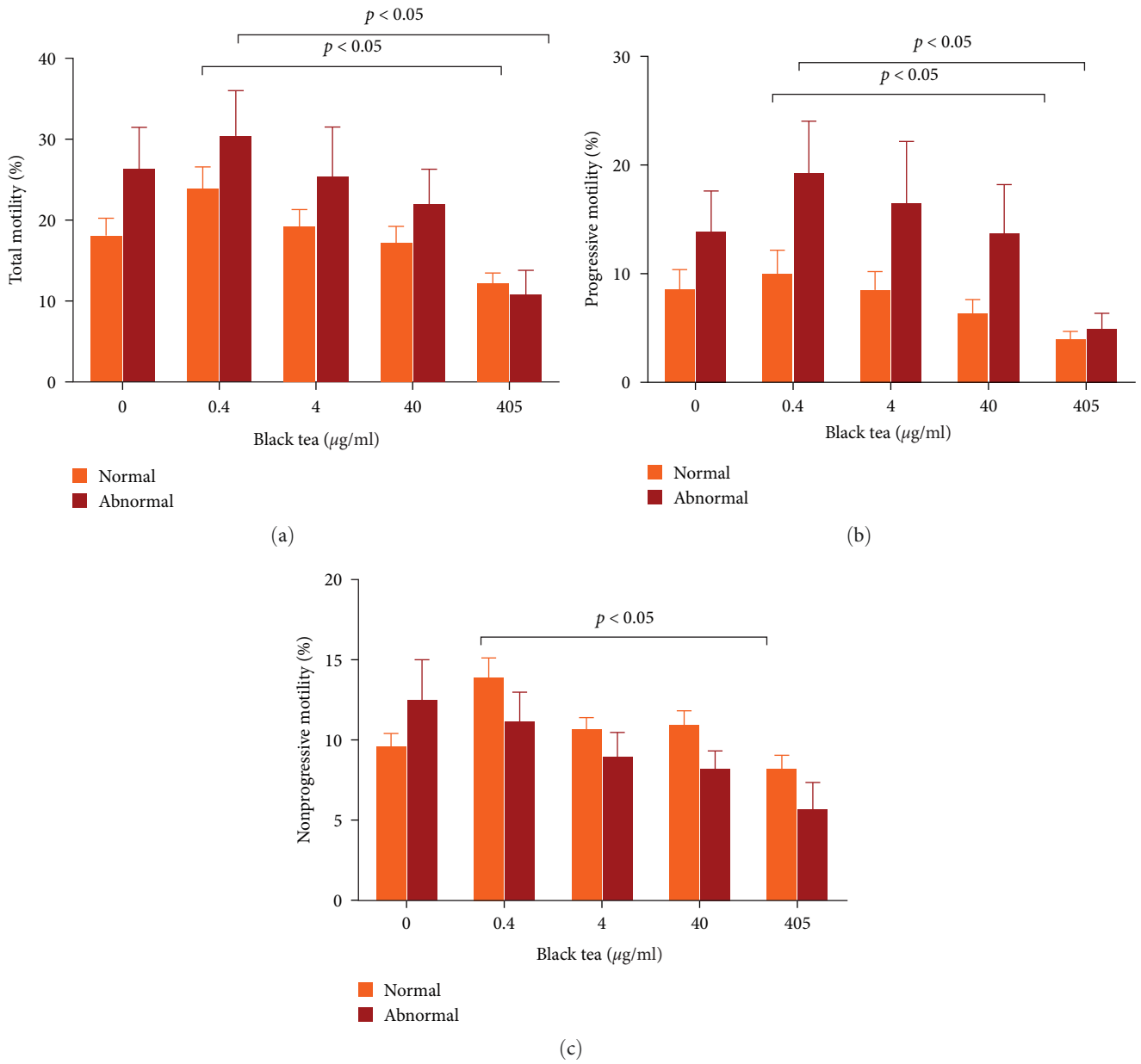


Figure 1: *In vitro* effect of black tea aqueous extract on motility: (a) total motility; (b) progressive motility; (c) nonprogressive motility. The data are shown as the mean ± standard error of the mean of 40 normal and 19 abnormal semen samples.

significantly compared to the control of the normal group (Figure 2; $p<0.001$). Figure 2 shows a significant increase in the percentage of viable spermatozoa at 4, 40, and 405 $\mu$g/ml in the abnormal group ($p<0.01$).

The proportion of spermatozoa with intact MMP significantly increased in the normal (40 and 405 $\mu$g/ml) and abnormal (4, 40, and 405 $\mu$g/ml) groups ($p<0.01$), with the former having a much higher percentage of intact MMP ($p<0.001$) (Figure 3). Furthermore, the normal and abnormal groups showed a dose-dependent decrease in ROS generation ($p<0.001$; Figure 4). Additionally, the abnormal group produced significantly higher ROS than the normal group ($p<0.001$; Figure 4).

Both groups showed a dose-dependent decrease in the proportion of spermatozoa with fragmented DNA compared to the control ($p<0.001$; Figure 5). Furthermore, a considerably lower percentage of spermatozoa with fragmented DNA was noted for the normal samples (4, 40, and 405 $\mu$g/ml) ($p<0.001$) as well as the abnormal samples (0.4 and 405 $\mu$g/ml) ($p<0.001$). The abnormal group's percentage of spermatozoa with fragmented DNA was substantially higher than that of the normal group, particularly between 0 and 40 $\mu$g/ml ($p<0.001$; Figure 5).

There was a dose-dependent drop in the proportion of spermatozoa that underwent spontaneous acrosome reaction in both groups (Figure 6; $p<0.001$). On the other hand, the abnormal group exhibited a considerably higher percentage of spermatozoa that underwent spontaneous acrosome reaction ($p<0.05$) in comparison to the normal group (Figure 6). In both the normal and abnormal samples, the acrosome reaction significantly decreased at 4, 40, and 405 $\mu$g/ml compared to the control (Figure 6; $p<0.001$).

Table 2: Effect of black tea aqueous extract on sperm kinematics after 1 hr incubation.

| Sperm kinematics | Groups | Black tea aqueous extract ($\mu$g/ml) | | | | | $p$-Value |
|---|---|---|---|---|---|---|---|
| | | 0 | 0.4 | 4.0 | 40 | 405 | |
| VCL ($\mu$m/s) | Normal | 74.5 ± 2.2 | 73.0 ± 2.3 | 70.0 ± 3.0 | 72.5 ± 2.4 | 66.0 ± 2.7 | >0.05 |
| | Abnormal | 73.1 ± 4.5 | 85.4 ± 7.4 | 85.9 ± 4.9 | 81.6 ± 3.3 | 75.3 ± 5.8 | >0.05 |
| VSL ($\mu$m/s) | Normal | 28.0 ± 1.7 | 26.7 ± 1.6 | 25.3 ± 1.4 | 27.0 ± 1.1 | 21.9 ± 1.5 | >0.05 |
| | Abnormal | 30.8 ± 2.9 | 25.9 ± 3.4 | 31.5 ± 3.6 | 34.4 ± 2.6 | 35.6 ± 8.2 | >0.05 |
| VAP ($\mu$m/s) | Normal | 38.1 ± 1.6 | 36.3 ± 1.4 | 34.5 ± 1.5 | 38.7 ± 1.4 | 33.8 ± 1.1 | >0.05 |
| | Abnormal | 39.6 ± 2.5 | 38.4 ± 2.5 | 41.7 ± 3.1 | 43.6 ± 2.4 | 44.0 ± 7.4 | >0.05 |
| LIN (%) | Normal | 38.3 ± 1.7 | 35.7 ± 1.7 | 34.2 ± 1.4 | 37.7 ± 1.2 | 32.4 ± 1.8 | >0.05 |
| | Abnormal | 39.8 ± 3.1 | 32.4 ± 3.5 | 35.6 ± 3.0 | 41.4 ± 2.2 | 41.6 ± 7.4 | >0.05 |
| STR (%) | Normal | 66.3 ± 2.4 | 65.7 ± 2.0 | 62.7 ± 2.4 | 66.9 ± 1.9 | 59.7 ± 2.4 | >0.05 |
| | Abnormal | 70.4 ± 3.7 | 60.5 ± 5.6 | 64.6 ± 4.1 | 74.2 ± 2.1 | 63.0 ± 6.8 | >0.05 |
| BCF (HZ) | Normal | 20.4 ± 1.3 | 19.7 ± 0.8 | 18.4 ± 1.1 | 18.8 ± 0.8 | 17.8 ± 1.0 | >0.05 |
| | Abnormal | 25.4 ± 2.8 | 19.6 ± 1.1 | 19.5 ± 1.5 | 18.9 ± 1.1 | 16.8 ± 1.7* | <0.05 |
| ALH ($\mu$m) | Normal | 2.4 ± 0.4 | 2.1 ± 0.1 | 2.3 ± 0.2 | 2.1 ± 0.1 | 2.1 ± 0.1 | >0.05 |
| | Abnormal | 2.0 ± 0.1 | 2.3 ± 0.2 | 2.6 ± 0.2 | 2.4 ± 0.1 | 2.0 ± 0.1 | <0.05 |
| WOB | Normal | 50.8 ± 1.2 | 49.5 ± 1.3 | 50.2 ± 1.2 | 51.2 ± 1.4 | 50.7 ± 1.2 | >0.05 |
| | Abnormal | 47.2 ± 4.2 | 45.7 ± 2.7 | 50.4 ± 2.2 | 51.9 ± 2.0 | 58.5 ± 4.7 | >0.05 |

Abbreviations: VCL, velocity curve line; VSL, velocity straight line; VAP, average velocity path; LIN, linearity; STR, straightness; BCF, beat cross frequency; ALH, amplitude of lateral head displacement; WOB, wobble. *Indicates a significant difference with control ($p<0.05$).

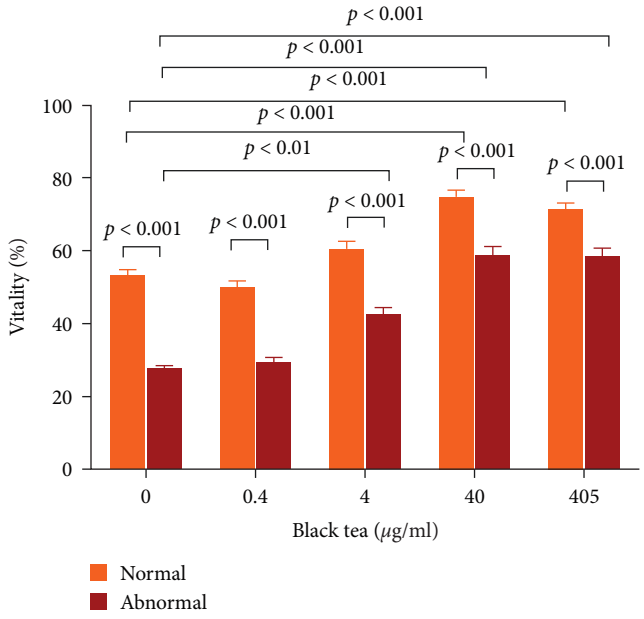


Figure 2: *In vitro* effect of black tea aqueous extract on sperm viability. The data are shown as the mean ± standard error of the mean of 40 normal and 19 abnormal semen samples.

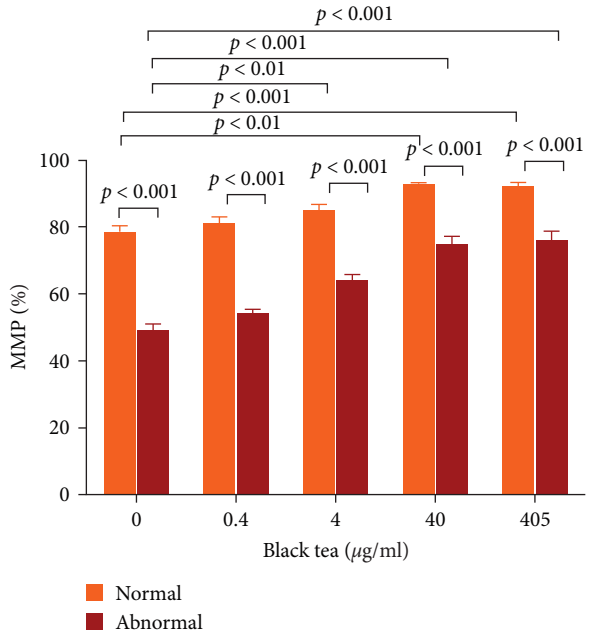


Figure 3: *In vitro* effect of black tea aqueous extract on intact mitochondrial membrane potential. The data are shown as the mean ± standard error of the mean of 40 normal and 19 abnormal semen samples.

## 4. Discussion

The current study investigated the effect of black tea aqueous extract on human sperm functions *in vitro*, as per WHO [34] guidelines. Sperm parameters related to sperm motility: progressive motility, total motility, VCL, VSL, VAP, STR, LIN, ALH, BCF, WOB, as well as acrosome reaction, DNA fragmentation, MMP, ROS production, and sperm vitality were evaluated. The current study demonstrates that spermatozoa directly exposed to black tea aqueous extract did not influence sperm motility or most kinematic measures but increased sperm viability and MMP and decreased ROS generation and DNA fragmentation [32].

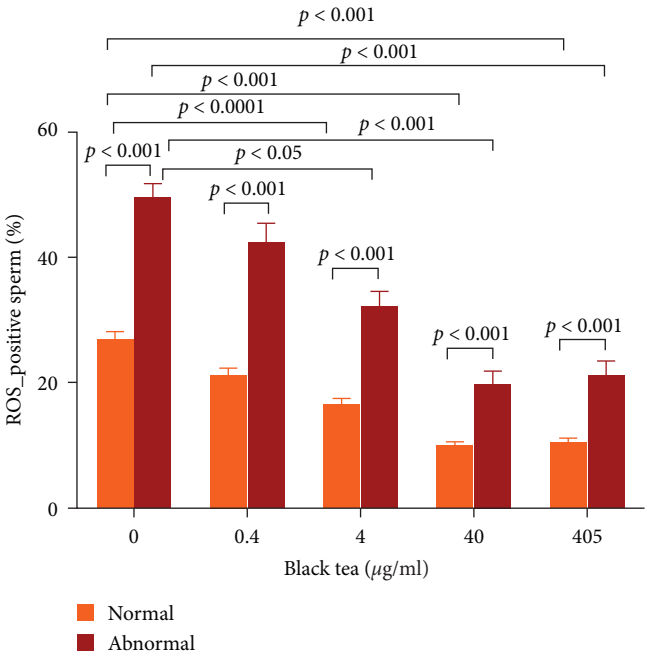


Figure 4: *In vitro* effect of black tea aqueous extract on reactive oxygen species production. The data are shown as the mean ± standard error of the mean of 40 normal and 19 abnormal semen samples.

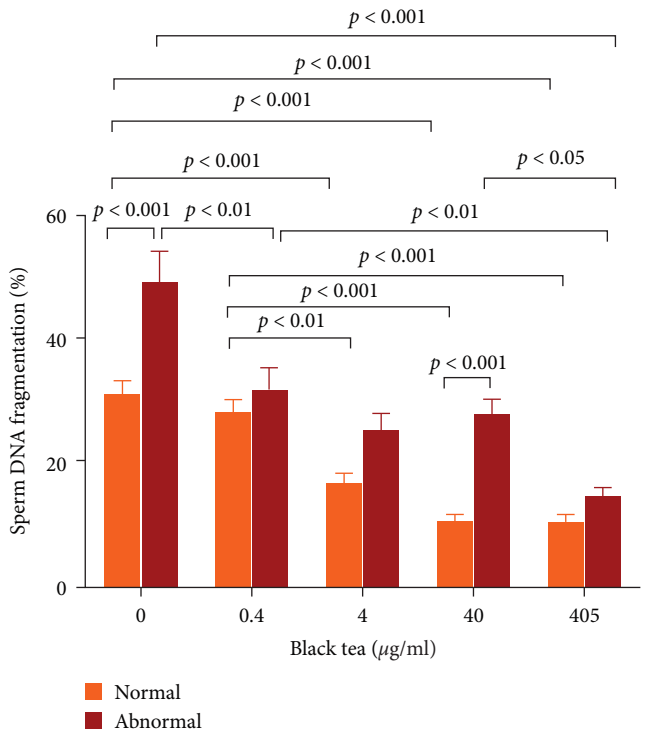


Figure 5: *In vitro* effect of black tea aqueous extract on sperm DNA fragmentation. The data are shown as the mean ± standard error of the mean of 40 normal and 19 abnormal semen samples.

After an hour of incubation, the current investigation demonstrated that black tea aqueous extract (0.4, 4.0, 40, and 405 μg/ml) had no significant effect on sperm motility in both groups compared to their controls and between the

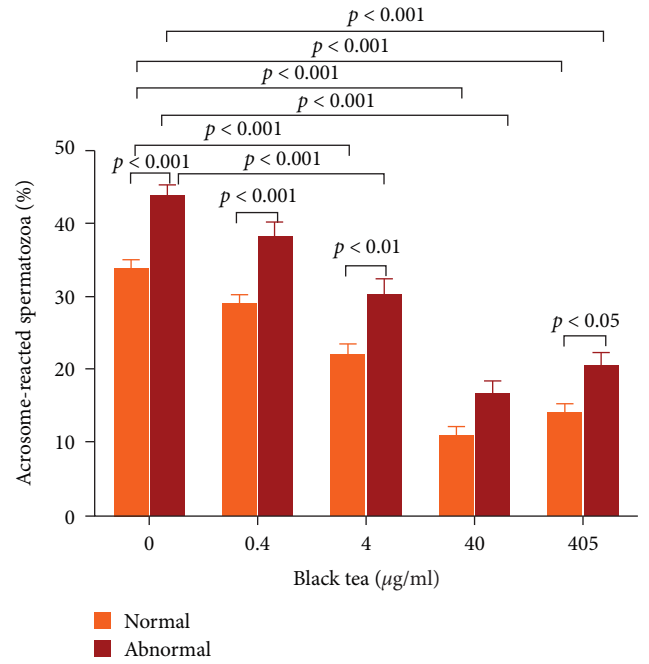


Figure 6: *In vitro* effect of black tea aqueous extract on acrosome reaction. The data are shown as the mean ± standard error of the mean of 40 normal and 19 abnormal semen samples.

two groups. Like this study, Ratnasooriya and Amarakoon [41] demonstrated that black tea aqueous extract did not affect total motility *in vivo* (500, 1,000, and 2,000 mg/ml). Total sperm motility seemed higher in the abnormal than normal samples, albeit this difference was not statistically significant. Ajayi et al. [42] demonstrated that antioxidant dietary supplementation notably improved sperm motility, sperm concentration, and sperm morphology in patients with poor semen quality while remaining unchanged in the normal samples, suggesting the beneficial role of antioxidants in abnormal semen samples compared to normal samples. Acrosome reaction, sperm vitality, and total motility increased significantly following *ad libitum* consumption of black tea aqueous extracts in male rats [30]. The varying results obtained could be due to the different concentrations of the extracts utilized as a dose-dependent increase in soluble solids, total polyphenols, flavonol, flavanol (mg catechin/ml), as well as the ferric reducing antioxidant power of black tea aqueous extract, was noted, indicating higher antioxidant activities with increasing concentrations [43].

Furthermore, sperm kinematic parameters predict sperm-fertilizing capabilities [44], which are essential as markers in sperm function. The current study investigated the effect of black tea extracts on sperm kinematic parameters (VAP, VCL, VSL, STR, BCF, WOB, LIN, and ALH), which showed that black tea aqueous extract had no significant effect on VCL, VSL, VAP, LIN, STR, and WOB, except for ALH and BCF. Similar to a previous study by Ratnasooriya and Amarakoon [41], black tea extracts (0.4, 4, and 40 μg/ml) caused a noticeable increase in the ALH. On the other hand, BCF declined significantly at 405 μg/ml in the current study. In other studies, supplementation with various polyphenolic compounds

significantly decreased total motility, progressive motility, VAP, VCL, STR, and LIN dose-dependently [45, 46], and suggests that the obtained results in this study may be due to the synergistic activities of the combined compounds present in black tea extracts. A significant increase in sperm viability was noted following the exposure to black tea aqueous extract in both the normal and abnormal samples, with the former having a significantly higher amount. In line with the current study, an inverse relationship exists between sperm viability and DNA fragmentation [47]. Despite the increase in sperm viability and MMP, no effect was noted for sperm motility in both groups. Besides its polyphenol content (antioxidants), black tea contains a considerable amount of caffeine [48], which has been shown to stimulate motility. While the antioxidants could protect sperm from dying, the caffeine will stimulate motility in the presence of an energy source. However, this short-lived stimulation could decrease motility, especially if the caffeine concentration is high or the exposure time is extended. Also, if the energy source is insufficient, motility will drop again. For instance, a previous study demonstrates that 10 nM of caffeine significantly increased the progressive motility of spermatozoa in normozoospermic and asthenozoospermic groups [49]. Studies have also shown that the caffeine content in tea increases with increasing steep time and temperature [50–52]. The current study, however, did not analyze the caffeine content in the grade of tea used, and further study on the caffeine and polyphenolic contents at various infusion times of this grade of tea is therefore recommended.

Spermatozoa must be supplied with sufficient adenosine triphosphate (ATP) (a source of energy) to maintain their normal functioning [53]. Proton concentration gradients and electric potential gradients are stored in the mitochondria of spermatozoa and used to synthesize ATP [53, 54]. As a result, reduced mitochondrial function and an incapacity to store enough ATP for efficient sperm movement may cause low-sperm motility [55]. In addition, excessive ROS production has also been indicated to be harmful to the mitochondrial membrane [56, 57]. In the current study, the percentage of intact MMP in both the abnormal and normal samples increased significantly in response to black tea aqueous extract in a dose-dependent manner.

Furthermore, compared to the abnormal samples, the normal samples had a more significant percentage of intact MMP, which the polyphenols in the plant could explain. Ferramosca et al. [58] have demonstrated that plant polyphenols, depending on their concentration, have variable effects (either positively or adversely) on sperm mitochondria. The current study thus showed that the polyphenolic content of black tea extract (together with the concentration used) positively affects MMP in both sample groups.

Antioxidants have been shown in several studies in both animals and people to be crucial for proper sperm quality and reproductive function and are thought to counteract the adverse effects of OS on spermatozoa [59, 60]. An imbalance between cellular antioxidants and ROS generation may result in OS [8]. Furthermore, by supplying the required membrane fluidity, the physiological amount of ROS is essential for capacitation and fertilization [61]; this maintains the sperm acrosome reaction and permits fertilization of the egg [62]. The causes of excessive ROS production may be linked to lifestyle choices like smoking, drinking alcohol, poor diet, or deficiencies in antioxidants like vitamins A and E. These choices have the potential to harm biological molecules like DNA and proteins as well as sperm's ability to undergo capacitation [62–65]. *In vitro* treatment of human sperm with antioxidants, ascorbate, and tocopherol inhibited ROS production [46].

The plant extract's antioxidant property may be attributed to the current study's dose-dependently substantial reduction in the proportion of ROS produced in both groups compared to their respective controls. Additionally, compared to normal samples, a more significant percentage of sperm that produced excessive amounts of ROS was noted in the abnormal samples. These suggest that black tea aqueous extract could enhance sperm quality by lowering excessive ROS generation in sperm cells [66].

The acrosome reacted spermatozoa proportion was significantly lower after black tea aqueous extract treatment. Also, the percentage of acrosome-reacted spermatozoa was markedly higher in the abnormal samples compared to the normal samples. The observed decrease in the number of acrosome-reacted spermatozoa shows that the number of spermatozoa with intact acrosome capable of interacting with the zona pellucida has increased, implying a possible improved fertilizing capability of the spermatozoa [67]. Spermatozoa must complete capacitation before undergoing an acrosome acrosome reaction [68]. This study did not investigate the mechanism by which black tea prevents the spontaneous acrosome reaction and requires further investigation. However, this may be mediated through decreased ROS production.

Furthermore, DNA fragmentation was significantly decreased by the black tea aqueous extract *in vitro*, with abnormal samples exhibiting a higher proportion of spermatozoa with fragmented DNA than normal samples. Research indicates a correlation between ROS and DNA strand breakage, especially in males diagnosed with infertility [69]; this could potentially account for the increased proportion of fragmented DNA and elevated levels of ROS found in the abnormal samples compared to the normal. This study observed that increased MMP was associated with reduced ROS levels and DNA fragmentation. In addition, the current study also showed an increased MMP level in the normal and abnormal samples. The increased MMP agrees with the reduced ROS production and DNA fragmentation, and in consonance with previous studies [70, 71], it was associated with reduced ROS levels and DNA fragmentation.

To conclude, the current study established that black tea aqueous extract has the potential to significantly improve some of the sperm functions such as vitality, DNA fragmentation, MMP, ROS, as well as acrosome reaction, which may be attributed to its antioxidant properties. However, no significant change in sperm motility was noted. More research is necessary to understand the mechanism of action of black tea extract further, its potential to aid in

infertility, and its potential antioxidative and cytoprotective properties against OS.

## Data Availability

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

## Conflicts of Interest

The authors declare no potential conflicts of interest.

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