

ORIGINAL ARTICLE

The effects of green tea (*Camellia sinensis*) extract on mouse semen quality after scrotal heat stress

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Abstract

The objective of this study was to investigate whether or not the adverse effects of heat on sperm quality could be prevented by green tea extract (GTE) administration. Ninety adult male mice were randomly divided to two groups. The scrotum of each animal in the first group was immersed once for 20 min in a water bath maintained at 42 °C (heat group, H) and the second group (control group, C) was maintained at 23°C. Heat-treated and control groups were allocated randomly into three subgroups. The first subgroup from heat-treated mice was administered sterile saline (heat saline, HS) and the two other subgroups were administered orally with two different doses of GTE including 500 and 750 mg kg⁻¹ (HG500 and HG750) for 49 consecutive days. Likewise, the first subgroup from control mice was administered sterile saline (control saline, CS) and the two other subgroups were administered orally with 500 and 750 mg kg⁻¹ of GTE (CG500 and CG750), respectively. Heat stress significantly reduced ($P < 0.05$) sperm concentration, total sperm motility, progressive sperm motility and hypo-osmotic swelling-water test positive spermatozoa at the first 14 days after the heat treatment. However, a partial recovery was observed at the day 42, which was still significantly lower than that of the CS group. Administration of GTE in two doses (HG500 and HG750 treatment groups) following heat treatment could significantly recover adverse effects of heat on above-mentioned parameters during the first 28 days. The present study demonstrates that the adverse effects of hyperthermia on semen parameters might be recovered following administration of green tea extract in a short period of time.

Introduction

Testes in most mammals are found in the scrotum, outside the main body cavity and are thus 2–8 °C below core body temperature.^{1,2} In addition, the temperature within the testis is regulated by a countercurrent heat exchange system between the pampiniform plexus and the testicular artery. Any disruption to this system may cause problems with spermatogenesis. Exposure of the testis to body temperature or above by local heating,³ experimentally induced cryptorchidism,⁴ or varicocele,⁵ causes disruption of spermatogenesis, resulting in temporary or permanent infertility.⁴ Recently, it has been suggested that this degenerative process of testicular germ cells by heat stress associated with cryptorchidism involves apoptosis, a form of programmed cell death.⁴ However, the cellular mechanisms whereby heat stress induces apoptosis remain nearly unknown. The production of free radicals and reactive oxygen species (ROS) including the superoxide

anion and hydrogen peroxide can induce positive changes in sperm function like hyperactivation, capacitation, and acrosome reaction.^{1,6} However, overproduction of ROS can be detrimental to sperm and may lead to male infertility. Sperm plasma membrane contains a high amount of unsaturated fatty acids. Therefore, it is particularly susceptible to peroxidative damage. The lipid peroxidation destroys the structure of lipid matrix in the membranes of spermatozoa, and it is associated with loss of motility and the defects of membrane integrity.^{6,7}

Several approaches, utilizing different mechanisms, have been attempted to reduce the adverse effects of heat on male fertility. Many different agents and strategies have been reported to ameliorate local heating to the scrotum in experimental animals.⁸⁻¹⁰ These reports were mainly focused on the use of various antioxidants,⁷ extracts of medicinal plants or other agents having antioxidant

properties.¹¹⁻¹⁴ However, none of these strategies were found to be suitable or safe for clinical practice. In the past few years, much interest has been centered on the role of naturally occurring dietary substances for the control and management of various diseases. Green tea (*Camellia sinensis*) is one of the most commonly consumed beverages worldwide.¹⁵ Green tea is considered to have beneficial effects on health due to its high content in polyphenols, e.g. epigallocatechin-3-gallate (EGCG), which is known to possess anti-oxidative properties.^{16,17} Polyphenols are believed to display anti-toxic and anti-mutagenic activities also due to their radical scavenging and metal chelating functions.¹⁶ EGCG, at concentrations ranging from 0.4 to 300 μM , was experimentally shown to repress reactive oxygen species (ROS) activity in a dose-dependent manner and to inhibit apoptosis in activated neutrophils.¹⁸ Polyphenols found in green tea show 20 times more powerful antioxidant activity than vitamin C.^{19,20} Therefore, the aim of the present research was to investigate the effects of long-term administration of green tea extract on sperm quality following local heating of the mice testes.

Material and methods

Materials. All chemicals were purchased from Sigma-Aldrich Company (St. Louis, MO, USA) except those otherwise indicated.

Animals. A total number of 90 healthy adult male Balb/c mice weighting approximately 30-35 g were purchased from Razi Research Institute of Shiraz, Iran. The mice were fed with standard commercial laboratory chow [(pellet form), Javeneh Khorasan Co., Mashhad, Iran] and water *ad libitum* and housed under standard laboratory conditions (12 h light: 12 h dark and 22 ± 2 °C) during the experimental period. Green tea was obtained from Herbal Drug Stores (Rasht, Iran). The plant was identified and confirmed by a botanist in the Biology Department of Shahid Bahonar University of Kerman, Iran as green tea.

Preparation of green tea extract. Green tea was extracted using the method described elsewhere²¹ and modified in our laboratory. Preparation of extracts of green tea to prepare methanolic extracts, the plant was air-dried and powdered. For methanolic extract, 100 g of powder was soaked in 1000 mL of 80% methanol (Merck Company, Germany) for 72 h. The extract was then shaken, filtered, and evaporated in a rotating evaporator under reduced pressure until dryness. Evaporation and solvent removal of methanolic extract gave a semi-solid mass yielded 12% w/w. This procedure was repeated several times to prepare the adequate amount of extract. The extract was kept in a clean, dried bottle that was placed in desiccators.

Heat treatment. Animals were randomly allocated to two main experimental groups each containing 45 mice. Mice in the first group (heat group) were anesthetized

with Ketamine (Rotexmedica, Germany, 50 mg kg⁻¹, i.p.) and Diazepam (Alfasan, Holland, 5 mg kg⁻¹, i.p.) and the scrotum of each animal was immersed once for 20 min in a water bath maintained at 42°C. Animals in the second group (control group) were treated identically except that the water bath was maintained at 23°C. Immediately following heat treatment, mice in the heat-treated group were allocated randomly into three subgroups each containing 15 mice. Subgroup 1 was administered sterile saline (heat saline, HS), subgroup 2 (HG500) and 3 (HG750) were administered orally with two different doses of GTE (500 and 750 mg kg⁻¹, respectively) by gastric intubation via an appropriately sized flexible, stainless steel ball-tipped dosing cannula once daily for 49 consecutive days from one week previous heating to six weeks post heating. The dosage volumes for all groups were 10 mL kg⁻¹. Individual doses were based on the most recently recorded body weights to provide the correct mg/kg/day dosage. Animals in the second group were randomly allocated into three other subgroups such as the first group. Subgroup 4 was administered sterile saline (control saline, CS), subgroups 5 (CG500) and 6 (CG750) were administered orally with two different doses of GTE (500 and 750 mg kg⁻¹), respectively.

Epididymal sperm concentration and motility. At 2, 4 and 6 weeks after the heat treatment, five mice from the experimental and control groups were sacrificed by cervical dislocation. Epididymal sperm counts and motility were determined as described previously.²² The testes and epididymides were gently excised and weighed and the cauda epididymides was isolated and placed in a prewarmed petri dish containing 1 mL phosphate buffered saline (PBS, pH=7.4) at 37 °C and placed in a 37 °C incubator for 15 min, prior to determine sperm motility. The tissue of cauda epididymides was minced using sharp scissors to release spermatozoa. Total sperm motility (TSM) and progressive sperm motility (PSM) were evaluated using the standard method.⁷ The suspension was stirred, one drop was placed on a warmed microscope slide, and a 22×22 mm coverslip was mounted. Microscopic fields were observed at 400× magnification using a standard light microscope, and the percentage of motile sperm with any motility and also the percentage of motile sperm with forward progressive motility were determined. After incubation, supernatant fluid was diluted 1:100 with a solution containing 5 g sodium bicarbonate, 1 mL formalin (35%) and 25 mg eosin per 100 mL of water. After mixing, the sperm suspensions were counted. Sperm counts were made using a Thoma counting chamber and expressed as $\times 10^6 \text{ mL}^{-1}$.

Sperm membrane integrity. Cell membrane integrity was evaluated using hypo-osmotic swelling-water test (HOS-WT) according to the previously described method.²³ Briefly, 100 μL of the sperm suspensions were diluted with 0.4 mL of distilled water and incubated for 5 min at 37 °C.

One drop of diluted sperm sample was placed on a clean microscope slide covered with a cover slip and at least 200 sperm cells were examined using a standard light microscope at 400× magnification and the percentage of spermatozoa that showed swollen/curled tails was calculated as HOS-WT positive sperm.

Statistical analysis. The data were expressed as means ± standard errors (SEM). Differences between group means were estimated using a one-way analysis of variance and the Tukey's test was done for multiple comparisons using the SPSS17 for Windows. Results were considered statistically significant at $P < 0.05$.

Results

Sperm motility and concentration. The results of sperm motility and concentration are shown in Table 1. Concentration of spermatozoa in the testes and epididymides was decreased markedly after heating, showed a partial recovery up to the day 42 (5.5 ± 1.2 vs. 31.8 ± 2.0 , $P < 0.05$, respectively). Concentration of spermatozoa was also reduced initially in the both HG500 and HG750 groups 14 days after treatment in comparison with the control group (CS), (10.0 ± 1.0 and 9.1 ± 0.6 vs. 38.6 ± 3.1 , $P < 0.05$, respectively) but a significant difference between HG500 and HG750 with CS and HS was not observed after 42 days treatment with GTE. However, administration of GTE in two doses (HG500 and HG750 treatment groups) following heat treatment could completely compensate the effects of heat on sperm concentration (HS group) during the first 28 days (53.3 ± 4.7 and 37.5 ± 4.3 vs. 19.0 ± 1.4 , $P < 0.05$, respectively). Heat stress significantly reduced ($P < 0.05$) TSM, PSM in the first 14 days after the heat treatment. However, a partial recovery for TSM and PSM was observed at the day 42, which was still significantly lower than the CS group (31.6 ± 7.2 vs. 65.0 ± 5.7 and 23.3 ± 6.0 vs. 56.6 ± 4.4 , $P < 0.05$, respectively). TSM, PSM were also reduced initially (on day 14) in the GTE administered groups following heat treatment (HG500 and HG750), but we did not observe any further decrease in the three above mentioned parameters and a complete recovery was seen up to the day 28 ($P < 0.05$).

Sperm membrane integrity. At the day 14 following heat treatment, the mean percentage of total swollen sperm in the water test in the HS and HG500 and HG750 groups was significantly lower than that of the control group (CG). However, administration of GTE 28 days after heat stress significantly increased the proportion of HOS-WT positive sperm in comparison with the HS group (61.6 ± 4.4 and 66.6 ± 4.4 vs. 5.5 ± 2.3 , $P < 0.05$, respectively). The mean percentage of HOS-WT positive spermatozoa between the control and treated mice with GTE (CG and HG groups) did not exhibit any significant difference ($P < 0.05$) on the day 42 although it was still higher than that in the HS group (Table 2).

Discussion

Sensitivity of mammalian germ cells to environmental heat has been well documented with the effects of hyperthermia being recorded for a variety of species including mice,²⁴ rats,²⁵ bulls,²⁶ rams,²⁷ and humans.²⁵ Studies on rodents have included experiments based on histological observations, testicular weight, testicular kinetics, and determination of testicular-cell populations monitored by flow cytometric measurement of cellular DNA stainability.²⁸ Several strategies, mechanisms and agents were utilized to prevent scrotal heat stress in animal model, however, their use to treat human subjects in clinical practice could not be achieved.²⁹ Tea, the most widely consumed beverage worldwide since ancient times, is known for its beneficial health effects. In particular, green tea polyphenols, chiefly catechins and their derivatives have been shown to retard various forms of cancers due to its antimutagenic, anticarcinogenic and antioxidant properties.^{30,31} In the present studies we examined our hypothesis that GTE consumption would ameliorate local heating of the testis and the time of observation was extended to investigate whether the testis would make a full recovery.

The results of this paper supported the previous reports indicating that scrotal local heating can induce significant changes in semen quality. The most surprising finding in this study was the therapeutic effect of GTE against deleterious effects of heat on the number of spermatozoa in the epididymis in heat-treated rats towards the end of the experiment, associated with a significant deterioration in the sperm quality. Similar results were obtained in our previous reports^{8,9} and this investigation in which a very severe decline in the sperm characteristics occurred at 14 days post-heating in the HC group. However, administration of GTE following heat treatment significantly prevented seminiferous tubule depletion. The underlying mechanisms involved in the beneficial effects of GTE on spermatogenesis are not clarified. On the whole, according to the above-mentioned results, we hypothesized that GTE contain high concentrations of polyphenols, which have strong antioxidant properties. Antioxidants have been shown to reduce free radical oxidative damage to DNA.^{32,33}

Because of the wide range of effects of heat on the testis, as described above, it is difficult to propose a single cause. Either a variety of cell types are affected in different ways or one cell type is primarily affected, or there are secondary effects on the other cells. As Sertoli cells do not appear to be directly affected by heating, or if they are and only minimally affected, the most obvious cell to be the primary site of action of heat is the Sertoli cell. Because of its position in the seminiferous epithelium, it is able to have a profound influence on all germ cells once they pass through the blood-testis barrier. These germ cells probably depend almost entirely on the Sertoli cells for nutrients and their development is controlled by influences from the Sertoli cells. Sertoli cells probably even have an influence on cells

Table 1. The effects of green tea extract administration at 14, 28 and 42 days following testicular heat treatment on sperm concentration (Con), total sperm motility (TSM) and progressive sperm motility (PSM).

Parameter	Days after heating	Experimental groups ¹					
		CS	HS	CG500	CG750	HG500	HG750
Con** ($\times 10^6$ mL ⁻¹)	14	38.6 \pm 3.1 ^a	05.5 \pm 1.2 ^b	75.0 \pm 1.0 ^c	90.9 \pm 2.1 ^d	10.0 \pm 1.0 ^b	09.1 \pm 0.6 ^b
	28	43.6 \pm 3.4 ^a	19.0 \pm 1.4 ^{b*}	62.5 \pm 4.2 ^{cd}	68.5 \pm 5.1 ^{c*}	53.3 \pm 4.7 ^{ab*}	37.5 \pm 4.3 ^{a*}
	42	44.3 \pm 5.3 ^{ab}	31.8 \pm 2.0 ^{a*ϕ}	59.9 \pm 5.2 ^{bc}	70.7 \pm 6.3 ^c	51.5 \pm 6.3 ^{ab*}	52.5 \pm 5.2 ^{ab*ϕ}
TSM (%)	14	58.7 \pm 6.8 ^a	02.3 \pm 0.3 ^b	70.0 \pm 5.7 ^a	73.3 \pm 7.2 ^a	05.0 \pm 1.0 ^b	05.3 \pm 0.8 ^b
	28	63.3 \pm 7.2 ^a	04.1 \pm 2.7 ^b	71.2 \pm 1.3 ^{ad}	87.1 \pm 1.8 ^{cd*}	73.3 \pm 1.6 ^{ad*}	75.0 \pm 2.8 ^{ad*}
	42	65.0 \pm 5.7 ^a	31.6 \pm 7.2 ^{b*ϕ}	89.0 \pm 1.8 ^c	83.5 \pm 0.9 ^{cd}	79.1 \pm 1.5 ^{de*ϕ}	73.3 \pm 6.0 ^{ae*}
PSM (%)	14	50 \pm 7.35 ^a	01.0 \pm 0.1 ^b	60.0 \pm 5.7 ^{ac}	65.0 \pm 5.7 ^c	03.6 \pm 0.6 ^b	2.3 \pm 0.3 ^b
	28	53.3 \pm 7.2 ^a	06.1 \pm 1.8 ^b	60.8 \pm 1.4 ^{ad}	77.1 \pm 1.8 ^{cd*}	64.3 \pm 2.3 ^{ad*}	66.6 \pm 6.0 ^{ad*}
	42	56.6 \pm 4.4 ^a	23.3 \pm 6.0 ^{b*ϕ}	79.0 \pm 3.3 ^c	75.0 \pm 1.0 ^{c*}	71.6 \pm 1.6 ^{cd*ϕ}	63.3 \pm 6.0 ^{ad*}

¹For each experimental group at each time point, 5 males were killed. CS, control saline; HS, heat saline; CG500, administration of 500 mg of green tea without heat treatment; CG750, administration of 750 mg of green tea without heat treatment; HG500, administration of 500 mg of green tea following heat treatment; HG750, administration of 750 mg of green tea following heat treatment.

^aThe values with different alphabetical superscripts differ significantly ($P < 0.05$) within each row.

^{*}There is a significant difference between the day 28 or 42 with the day 14 within each column.

^{ϕ} There is a significant difference between the day 42 with the day 28 within each column.

Table 2. The mean percentage \pm SEM of HOS-WT positive spermatozoa following administration of GTE at 14, 28 and 42 days after testicular heat treatment.

Days after heating	Experimental groups ¹					
	CS	HS	CG500	CG750	HG500	HG750
14	50.2 \pm 4.6 ^a	01.3 \pm 0.3 ^b	59.3 \pm 7.9 ^a	64.3 \pm 4.7 ^a	01.6 \pm 0.6 ^b	02.0 \pm 0.5 ^b
28	57.0 \pm 6.2 ^a	05.5 \pm 2.3 ^b	63.1 \pm 9.3 ^a	73.5 \pm 3.7 ^a	61.6 \pm 4.4 ^{a*}	66.6 \pm 4.4 ^{a*}
42	57.0 \pm 4.3 ^a	26.6 \pm 7.2 ^{c*ϕ}	76.0 \pm 1.8 ^{a*}	72.8 \pm 1.0 ^a	67.5 \pm 2.8 ^{a*}	63.3 \pm 4.4 ^{a*}

¹For each experimental group at each time point, 5 males were killed. CS, control saline; HS, heat saline; CG500, administration of 500 mg of green tea without heat treatment; CG750, administration of 750 mg of green tea without heat treatment; HG500, administration of 500 mg of green tea following heat treatment; HG750, administration of 750 mg of green tea following heat treatment.

^aThe values with different alphabetical superscripts differ significantly ($P < 0.05$) within each row.

^{*}There is a significant difference between the day 28 or 42 with the day 14 within each column.

^{ϕ} There is a significant difference between the day 42 with the day 28 within each column.

such as the spermatogonia and preleptotene spermatocytes that are in the basal compartment, on the blood side of the specialized junctions between the Sertoli cells, which form the major site for the barrier inside the tubules. The fact that blood flow through the testis does not increase at all, or not sufficiently to match the increase in metabolism, means that the heated testis is probably hypoxic.³⁴ Damage may be caused not so much by the hypoxia directly, as by the generation of ROS during the recovery phase, as occurs after ischemia in many other tissues and the effect of scavengers for ROS during heating or immediately afterwards is probably worth investigating. Although mild hyperthermia has been shown to induce apoptosis in a variety of normal cell types and tumor cell lines,^{25,35} the temperature required to demonstrate this effect for somatic cells has generally been 43 °C.²⁵ The previous studies have shown that following heating cell death occurred during the apoptosis process.^{36,37} A similar apoptosis process can also be induced in these cells by generating reactive oxygen species with xanthine and xanthine oxidase; on the other hand, the heat effects are

reduced in the presence of catalase.³⁸ Likewise, nitric oxide synthase (NOS) appears to have a functional role in heat induced apoptosis.³⁹ Overexpression of endothelial NOS in transgenic mice accelerates germ cell apoptosis induced by experimental cryptorchidism.⁹

In the past few years, much interest has been centered on the role of naturally occurring dietary substances for the control and management of various chronic diseases. Since ancient times, green tea consumption has been considered as nature's gift for promoting human health. Most of these effects are considered to be due to the presence of wide range of well-defined phytochemicals that are digested, absorbed and metabolized by the body and exhibit anti-inflammatory, anticarcinogenic, antiatherosclerotic and antibacterial properties.^{30,40} In particular, green tea catechins and their derivatives have been characterized as antioxidants that scavenge free radicals to protect cells in normal and pathological states.^{30,41}

Both TSM and PSM decreased following scrotal heating in this study (Table 1) and this was in agreement with the previous reports,^{9,42-44} but GTE therapy improved motility

especially after 28 days. In a study on pump workers exposed to benzene toxicity, daily drinking of six cups of tea for 6 month caused a substantial improvement in antioxidant status and attenuated the benzene-induced toxicity.^{45,46} In another study in humans, consumption of 6 g of green tea in 600 mL of water daily for 7 day showed increased plasma glutathione and improved the post exercise increase in lipid hydroperoxidase.⁴⁷ In human volunteers, Henning *et al.* (2004)⁴⁸ showed that the highest total plasma flavonol concentration (1.23-0.09 mmol L⁻¹) was attained within about 1 h after green tea consumption compared with black tea and green tea supplements. Green tea contains volatile oils, vitamins, minerals, and caffeine, but the primary constituents of interest appear to be the polyphenols, particularly a group of catechins that includes catechin, epicatechin, gallic catechin, epigallocatechin, catechin gallate, epicatechin gallate, gallic catechin gallate, and epigallocatechin gallate. Research aimed at finding the active compounds in green tea indicates that its beneficial effects appear to be related to catechins.⁴⁹ Various physiological actions of tea catechins have been reported, such as antioxidative properties, antiviral properties,^{49,50} antiallergic properties, radioprotector properties, hypotensive properties and blood glucose-lowering effects.^{49,51}

In conclusion, the results of this study demonstrate that the adverse effects of hyperthermia on semen parameters may be prevented by GTE therapy. Likewise, long-term administration of GTE could improve sperm quality obtained from the control mice. The major activity of this beverage is their antioxidant property, which makes them useful in the prevention of other organ-specific toxicities related to the induction of oxidative stress.

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