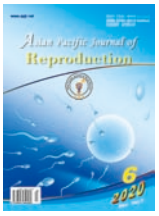

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The leaf extracts of *Camellia sinensis* (green tea) ameliorate sodium fluoride-induced oxidative stress and testicular dysfunction in rats

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ABSTRACT

Objective: To investigate the effect of *Camellia* (*C.*) *sinensis* in mitigating oxidative damage and reproductive toxicity in testis induced by sodium fluoride in a rat model.

Methods: Twenty-four adult male Wister rats were divided into 4 groups, with 6 rats in each group. Group 1 orally received distilled water (1 mL/100 g body weight) daily and served as the control group, while group 2 received drinking water with 100 ppm sodium fluoride per day for 21 consecutive days, group 3 was administered with only *C. sinensis* extract by gavage at a dose of 100 mg/kg body weight and group 4 received drinking water with 100 ppm sodium fluoride and 100 mg/kg body weight *C. sinensis* leaf extract per day for 21 consecutive days. At the end of the treatment, the rats were sacrificed under light ether anesthesia. The gonado-somatic index, sperm count and motility, serum level of luteinizing hormone and testosterone were assayed. Lipid peroxidation [malondialdehyde (MDA) level], nitric oxide (NO) production, and activities of antioxidant enzymes - superoxide dismutase (SOD), catalase, and reduced glutathione level (GSH) were also analysed.

Results: Sodium fluoride treatment significantly decreased gonado-somatic index, sperm count and motility as well as the serum level of luteinizing hormone and testosterone (P<0.05). The histological examination of testes revealed atrophy and degenerative changes in several seminiferous tubules, along with enhanced interstitial space and a reduced number of Leydig cells. There was a highly significant increase in NO and MDA production (P<0.05), while SOD, catalase activities and GSH level decreased significantly (P<0.05). However, *C. sinensis* significantly restored testicular weight, sperm parameter, hormonal level (P<0.05), and also reversed MDA and NO generation and antioxidant enzymes activities in the testicular tissue (P<0.05).

Conclusions: *C. sinensis* may have an ameliorative role against sodium fluoride-induced oxidative damage in the testis probably because of its antioxidant property.

KEYWORDS: Oxidative stress; Testicular damage; *Camellia sinensis*; Antioxidant; Sodium fluoride

1. Introduction

Infertility is a reproductive disorder, defined by the inability to achieve a pregnancy after one year of unprotected intercourse. According to World Health Organization, the prevalence of infertility is between 3.9% and 16.8% in India. Various environmental factors threaten the reproductive health and one such is the contamination of drinking water with fluoride[1]. Fluoride, a bone-seeking element, is now regarded as a potential contributing factor to a growing male infertility in humans. And consistent with this, previous studies, both experimental and epidemiological, have uncovered that long term fluoride exposure can induce a profound negative impact on male reproductive health and reduce fertility by changing sperm quality[2], preventing spermatogenesis, testicular degeneration[3] and androgenesis[4]. Different studies on many species of animals (both *in vivo* and *in vitro*) also demonstrated that fluoride can pass through the blood-testis barrier and the blood-epididymis barriers[5] and thereby seriously affect sperm physiology and functionality such as reduction in the sperm number, movement, normal morphology and fertilizing potential of sperms[3,6,7]. In addition, fluoride can readily damage the histological structure of the testis[8,9] and epididymis which adversely affects spermatogenesis and maturation as well as fertilizing potential of sperm[10,11]. Besides, the smooth progress of spermatogenesis and growth of sexual organs essentially in males require testosterone, a steroid hormone[12] and previous studies

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have shown that fluoride exposure can inhibit the production of testosterone in Leydig cells, consequently affect spermatogenesis[1] and at long last cause reproductive dysfunction. Notwithstanding numerous studies, the exact mechanism of fluoride-induced reproductive toxicity remains unclear. Fluoride-induced free radical generation[3,13] and oxidative stress[14] may partly be the mediators of fluoride toxicity on the male reproductive system.

There are several attempts to modulate oxidative stress and injuries in male reproduction with the use of herbal products or their extracts[15] which has the advantage of avoiding side effects. Even fluoride toxicity could be alleviated by herbal products, as a non-invasive strategy, and a harmless and inexpensive tool. Several investigators used different antioxidants and herbal products to counteract fluoride-induced toxicity. Tea was considered an effective herbal medicine for treating different ailments in ancient Asian folk medicine. *Camellia* (*C.*) *sinensis*, commonly known as green tea, belonging to Theaceae family, has been proven to be a powerful antioxidant[16] because of the presence of a huge amount of catechins and flavonoids *e.g*. epicatechin-3-gallate, epigallocatechin-3-gallate. Fluoride is known to generate reactive oxygen species (ROS)[4,14], while green tea successfully quenches ROS and trapped hydroxyl, peroxyl, and superoxide anion radicals due to the presence of catechins[17,18]. Considering the above benefits, here we planned to investigate the possible defensive role of methanolic extract of *C. sinensis* against the oxidative stress and reproductive toxicity, induced by sodium fluoride in adult Wistar male rats.

2. Materials and methods

2.1. Chemicals and reagents

Sodium fluoride was purchased from Sigma-Aldrich. Sulfanilamide, phosphoric acid, naphthyl ethylene diamine dihydrochloride, thiobarbituric acid, trichloroacetic acid, xanthine, bovine serum albumin, nitroblue tetrazolium, xanthine oxidase, were purchased from Merck (Darmstadt, Germany). All other reagents and chemicals were purchased commercially and were of analytical grade.

2.2. Plant material and extract preparation of C. sinensis

C. sinensis leaves (Darjeeling green tea leaves, India) were purchased from the local market approved by Serampore Municipal Corporation in September 2018. The identity of the leaves was confirmed by the Botanical Survey of India, Government of India, Ministry of Environment and Forest, Howrah, West Bengal, India. A herbarium of the specimen was maintained in the institute library against voucher specimen No.SC/PHY/MD/01/17. The collected *C. sinensis* leaves (700 g) were allowed to dry in an incubator for 2 days at 40 ℃. Dry leaves were crushed and powdered in an electric grinder. This tea powder (500 g) was dissolved in 1 000 mL solvent containing 80% methanol and 20% distilled water and followed by mixing in a shaker for 2 days. After 2 days, the liquid extract was filtered and transferred in a beaker. The liquid deep brown extract was evaporated in Rota evaporator at 45 ℃ and preserved in vacuum desiccators in dry powdered form. This dry powder was dissolved in distilled water before treatment in rats.

2.3. Experimental animals

Twenty four adult male albino rats (Wister strain) weighing 110–125 g, purchased from the Committee for the Purpose of Control and Supervision of Experiments on Animals approved registered breeder, were used in the present study. The rats were housed in an environmentally controlled animal house at a constant temperature of (21±2) ℃, fed standard pellet diets with purified water available *ad libitum*, and kept under a 12-h light/dark cycle (lights on at 8:00 am), with 10% relative humidity. The animals were acclimatized in the new environment for about one week prior to the experiment.

2.4. Experimental design

After one week of acclimation, the rats were divided into four groups, with six rats in each group. Group 1 served as the control group (untreated) and received only distilled water (1 mL/100 g body weight), per day for 21 days. Group 2 served as the sodium fluoride treated group and received drinking water with 100 ppm sodium fluoride for 21 consecutive days. Group 3 was administered with only *C. sinensis* extract at a dose of 100 mg/kg body weight (b.w.) by gavage for 21 days. Group 4 received drinking water with 100 ppm sodium fluoride and 100 mg/kg b.w. *C. sinensis* extract by gavage for 21 days.

The dose of sodium fluoride and the period of treatment were selected based on a previous study[19] whereas the dose of *C. sinensis* (dry leaves) was chosen according to the previous study[20]. The average daily water intake per animal remained unaltered in the control and treated groups. After the treatment period was over, rats from all the groups were anaesthetized by using pentobarbitone sodium (60 mg/kg b.w. intraperitonially) and sacrificed by cervical dislocation and underwent the following procedures.

2.5. Gonado-somatic index calculation

After sacrifice, both the testes were carefully removed and weighed (in grams) over electronic digital balance. The ratio of both testes weight to body weight was calculated to determine gonado-somatic index by using the following formula:

Gonado-somatic index = Organ weight (g)/ Live body weight (g) ×100

2.6. Epididymal sperm functions analysis

After exposing the reproductive tract, the right caudal epididymis was carefully isolated and excised with scissors in 1 mL of physiological saline (phosphate buffer saline, pH=7.4) to release the sperm. Semen samples were incubated at 37 ℃ for 20 to 25 min and

sperm parameters were analyzed according to the method of Prete *et al*[21].

2.7. Testosterone and luteinizing hormone (LH) assay

Serum level of LH was measured by using enzyme-linked immunosorbent assay(ELISA) kits from MONOBIND, USA, whereas serum testosterone level was analyzed by using ELISA kit from DRG Inc, Germany.

2.8. Preparation of testicular tissue extract

After blood collection, testes were quickly removed; left testes of six animals in each group were used for biochemical analysis while the right testes of experimental animals were kept for histological study. Glass Teflon homogenizer was used to homogenize the testes in ice-cold isotonic phosphate buffer (pH 7.0). Each sample was centrifuged at 12 000 ×*g* for 30 min at 4 ℃. The supernatant was collected and utilized for the evaluation of following oxidative stress parameters.

2.9. Evaluation of oxidative stress markers

2.9.1. Estimation of nitric oxide (NO) production and lipid peroxidation

The role of nitric oxide synthase was indirectly analyzed by measuring the amount of NO produced. NO decomposed quickly in aerated solutions to form stable nitrite/nitrate products. In this study, nitrite accumulation was estimated by the Griess reaction[22] and was used as an indicator of NO production. The amount of nitrite in the sample (micromolar unit) was calculated from a sodium nitrite standard curve.

The lipid peroxidation in terms of malondialdehyde (MDA) formation was measured according to the method of Wills[23]. Testicular homogenate (2 mL)was mixed with 1 mL trichloroacetic acid (20%) and 1 mL of 0.67% (v/v) thiobarbituric acid and then boiled for about 10-15 min. MDA, a product lipid peroxidation, formed adducts with thiobarbituric acid, which was measured spectrophotometrically at 532 nm. The results were calculated as nanomoles of MDA per milligram of protein by using the molar extinction coefficient (1.56×10^5 cm^2/mmol).

2.9.2. Estimation of superoxide dismutase (SOD) activity

The nitroblue tetrazolium method of Beauchamp and Fridovich[24], which was established on the inhibition of nitroblue tetrazolium reduction by SOD, was used for the determination of SOD activities. The relative absorbance was then converted into a unit of SOD activity per mL or per mg protein, where one unit of SOD activity was equivalent to the quantity of SOD that caused a 50% reduction in the background rate of nitroblue tetrazolium reduction.

2.9.3. Estimation of catalase (CAT) activity

CAT activity was determined according to the method described by Beers *et al*[25] by following the decomposition of H_2O_2 at 240 nm and 25 ℃. Alteration in the rate of absorbance were transformed into unit of CAT/mg protein by using a conversion factor (3.45), which correlated to the decomposition of 3.45 μmol of hydrogen peroxide in a reaction mixture producing a reduced absorbance from 0.45 to 0.40 unit.

2.9.4. Estimation of reduced glutathione (GSH) content

GSH levels were measured according to the method of Ellman[26] by using 5,5-dithiobis-2-nitrobenzoic acid (DTNB). A 720 μL sample was double diluted and 5% trichloroacetic acid was mixed to precipitate the protein content. After centrifugation at 10 000 ×*g* for 5 min, the supernatant was collected and Ellman' reagent (DTNB) was added. The absorbance of reduced chromogen was measured spectrophotometrically at 412 nm. GSH content was then calculated by using different known concentrations of GSH.

2.9.5. Protein determination in crude extract

The total protein content was measured by the Lowry method using bovine serum albumin as standard[27].

2.10. Histopathology

Immediately after removal, the right testes were then fixed with Bouin's fluid at room temperature for 24 h and routine tissue preparation was done. In brief, the tissues were transferred to 70% alcohol, dehydrated by passing through ascending grades of alcohol, followed by clearing in xylene, and finally, the tissues were embedded in paraffin wax. Thin sections of 4-5 μm were cut with a rotary microtome and then stained with hematoxylin and eosin (H & E) protocol. The stained slides were observed under 400× magnification of light microscope (Carl Zeiss, Germany) for histopathological assessment.

2.11. Statistical analysis

The data were expressed as mean±standard deviation (mean±SD). Statistical analyses of the collected data were completed by one-way analysis of variance. To test intergroup significant difference, *t*-test was performed. Differences were considered statistically significant if $P<0.05$.

2.12. Ethics statement

The study was approved by Institutional Animal Ethics Committee of Serampore College, [Registration Number-1946/PO/Re/18/CPCSEA] Serampore, West Bengal, India, with approval No: 02/p/s/sc/IAEC/2017. All animal experiments were performed according to the ethical guidelines suggested by the Institutional Animal Ethics Committee of Serampore College.

3. Results

3.1. Effect of C. sinensis on gonado somatic index of testis

There was a significant reduction in the gonado-somatic index (t=-20.97, P<0.05) in the sodium fluoride treated group as compared to the control group. Conversely, supplementation of *C. sinensis* at 100 mg/kg b.w. along with sodium fluoride (Group 4) showed a significant restoration (t=8.21, P<0.05) of gonado-somatic indextowards the normal, which indicated that co-treatment of prevented the fluoride toxicity (Figure 1).

3.2. Effect of C. sinensis on sperm indices

The protective effects of *C. sinensis* on sperm count and sperm motility were given in Table 1. Treatment of sodium fluoride markedly affected sperm quality, as evidenced by a significant reduction (t=-20.77, P<0.01) of sperm number and sperm motility, while supplementation of methanolic extract of *C. sinensis* appeared to decrease these detrimental effects of fluoride on sperm indices significantly (t=5.19, P<0.01) when compared with sodium fluoride-treated group and consequently improved semen quality.

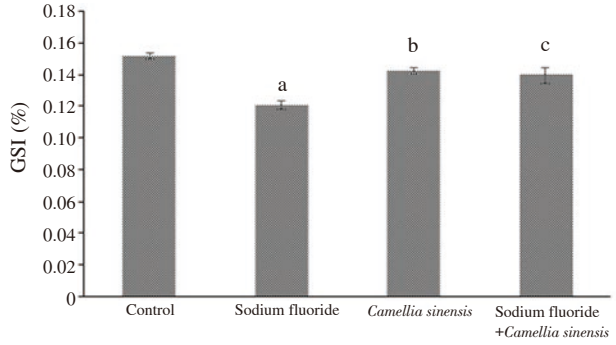

Figure 1. Effect of *Camellia sinensis* on gonado-somatic index (GSI) of testicles in experimental groups receiving the indicated treatments. Analysis is done by one way analysis of variance followed by t–test. Results are expressed as mean±SD. a: the control group *vs.* the sodium fluoride group, P<0.001; b: the control group *vs*. the *Camellia sinensis* group, P<0.001; c: the sodium fluoride group *vs.* the sodium fluoride+*Camellia sinensis* group, P<0.001.

3.3. Hormonal study

The exposure of sodium fluoride significantly decreased the level of serum LH (t=-88.54, P<0.001) and testosterone in comparison with the control group (t=-54.78, P<0.001). However, co-administration of *C. sinensis* extract with sodium fluoride significantly reversed the undesirable effects of fluoride and restored the serum level of LH (t=23.32, P<0.05) and testosterone (t=13.52, P<0.05) to the control level (Figure 2).

3.4. Status of oxidative stress markers

Intracellular NO, MDA level (the marker of lipid peroxidation), activities of the antioxidant enzymes, and nonenzymatic antioxidant levels express the oxidative stress within the cell. Sodium fluoride treatment for 21 days significantly elevated testicular MDA (t=48.57, P<0.05) with a concomitant increase in the generation of NO (t=42.08, P<0.05) with respect to control, as shown in Table 2. *C. sinensis* cotreatment with sodium fluoride restored these parameters toward the level of normal. A drastic inhibitory response on the testicular antioxidant status was observed, followed by sodium fluoride administration (Table 2). The sodium fluoride group showed a significant reduction in the antioxidant enzyme activities of CAT (t=-133.59, P<0.05) and SOD (t=-71.93, P<0.05), and antioxidant GSH levels(t=-49.40, P<0.05) when compared to the control group, which confirmed the disruption of testicular redox status and development of oxidative stress in testes. Co-administration of methanolic extract of *C. sinensis* (100 mg/kg b. w.) with sodium fluoride showed a significant restoration in the activities of SOD (t=23.57, P<0.05), CAT (t=65.64, P<0.05) and the level of testicular GSH (t=17.73, P<0.05) toward the control level (Table 2). However, no significant alteration in the testicular antioxidant enzyme activities and GSH levels were noted in the only *C. sinensis*-treated groups with respect to the control group (Table 2). These results collectively reflect the antioxidant capability of *C. sinensis* against sodium fluoride-induced oxidative stress.

Table 1. Protective effect of *Camellia sinensis* against sodium fluoride-induced alteration in sperm count and motility in rats.

Parameters	Control	Sodium fluoride	*Camellia sinensis* extract	Sodium fluoride+*Camellia sinensis* extract
Sperm count (10^6/mL)	41.00±2.22	19.64±1.19[a]	41.79±2.78[a]	24.59±2.01[b]
Sperm motility	71.00±2.37	35.42±2.58[a]	65.40±2.01[a]	54.00±1.94[b]

Values are expressed as mean±SD; n=6 in each group. Analysis is done by one-way analysis of variance followed by t-test to find inter group significant difference. a: compared with the control group, P<0.01; b: compared with the sodium fluoride group, P<0.01.

Table 2. Effect of sodium fluoride and extract of *Camellia sinensis* on oxidative stress markers in rats' testes.

Parameters	Control	Sodium fluoride	*Camellia sinensis* extract	Sodium fluoride+*Camellia sinensis* extract
MDA (nM/mg protein)	27.79±0.94	60.48±1.36[a]	25.92±0.43[a]	40.77±0.69[b]
NO (μg/mg protein)	44.05±0.56	74.87±1.71	42.38±0.73[a]	51.12±0.59[b]
SOD (U/mg protein	218.04±0.41	187.45±0.96[a]	216.95±0.51[a]	200.64±0.98[b]
CAT (U/mg protein)	101.44±0.79	48.65±0.56[a]	98.64±0.41[a]	68.60±0.49[b]
GSH (nM/mg protein)	39.28±0.78	19.77±0.58[a]	35.03±0.42[a]	25.69±0.58[b]

Values are expressed as mean±SD, n=6 in each group. Analysis is done by one-way analysis of variance followed by t-test to find intergroup significant difference. a: compared with the control group, P<0.05; b: compared with the sodium fluoride group, P<0.05. MAD: malondialdehyde; NO: nitric oxide; SOD: superoxide dismutase; CAT: catalase; GSH: reduced glutathion.

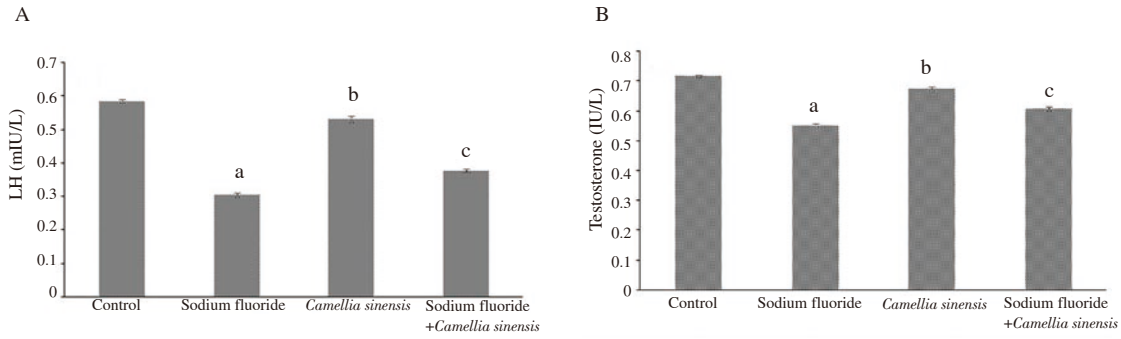

Figure 2. Comparison of mean serum level of luteinizing hormone (LH) (A) and testosterone (B) in the four groups. Analysis is done by one way analysis of variance followed by *t*-test to find intergroup significant difference. Results are expressed as mean±SD. a: the control group *vs.* the sodium fluoride group, $P<0.001$; b: the control group *vs*. the *Camellia sinensis* group, $P<0.001$; c: the sodium fluoride group *vs.* the sodium fluoride+*Camellia sinensis* group, $P<0.001$.

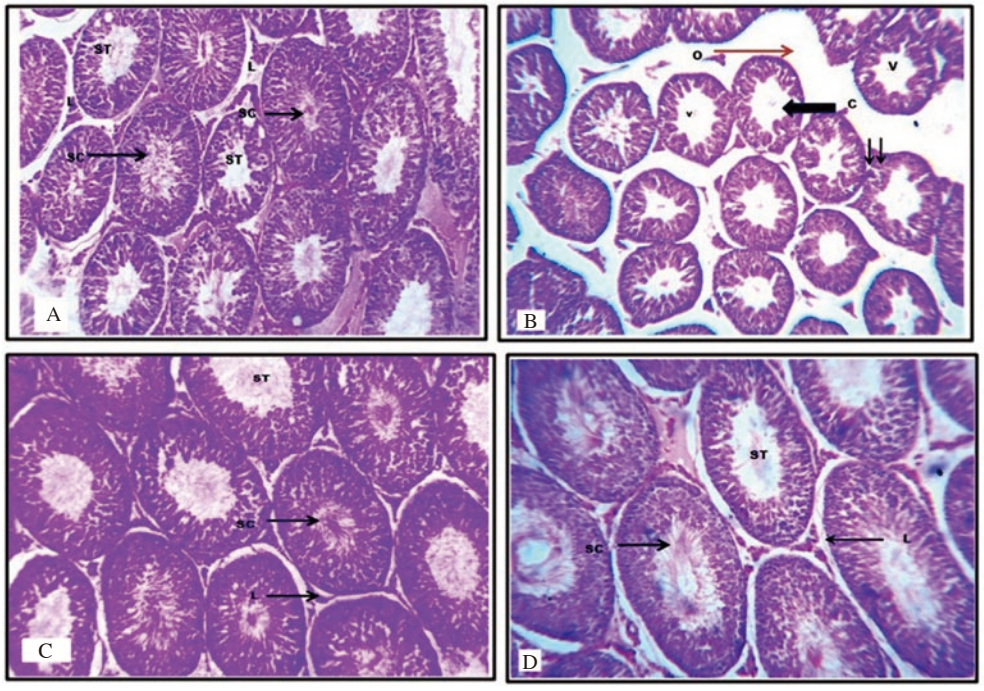

Figure 3. Protective effect of *Camellia sinensis* on sodium fluoride-induced oxidative damage in testes of rats. Sections of testicular tissues of rats are stained with hematoxylin-eosin (magnification 400×). A: The control group shows normal cellular morphology of seminiferous tubules (ST) with luminal spermatozoa (SC) and presence of interstitial Leydig cells (L). B: The sodium fluoride group shows a marked degeneration and collapse of the seminiferous tubules such as disruption of germinal epithelium (double arrow), absence of luminal spermatozoa (c & red arrow) and vacuolation (V) of seminiferous tubules as well as oedematous stroma containing no or small group of Leydig cells (O). C: The *Camellia sinensis* group shows normal morphology of seminiferous tubules in rats like that of the control group. D: The sodium fluoride+*Camellia sinensis* group shows noticeable restoration of seminiferous tubular structure (ST) with the presence of normal germinal epithelium and luminal spermatozoa (SC) in some ST and moderate presence of Leydig cells in the interstitial space.

3.5. Histopathology of testis

The histological study of the H & E stained section of the testes of the control group revealed typical cellular structure of seminiferous tubules with luminal spermatozoa and stratified germinal epithelium (Figure 3A). The seminiferous tubules separated by interstitial tissues contained clusters of Leydig cells. On the other hand, testes of rats treated with sodium fluoride showed marked degenerative changes in the seminiferous tubular structure (Figure 3B). The distortion of normal shape of seminiferous tubules, intraluminal desquamation of cells, and the total absence of luminal spermatozoa in some tubules with the presence of diffuse intracytoplasmic vacuoles were observed (Figure 3B). Furthermore, most tubules were collapsed and tubular shrinkage was seen (Figure 3B). The interstitium showed marked oedema and vacuoles (Figure 3B). Light photomicrograph of testes in *C. sinensis*-treated rats showed normal appearance of germinal epithelium with typical cellular organization of seminiferous tubules (Figure 3C). Most of the animals in *C. sinensis* supplemented sodium fluoride-treated group showed an overall restoration of testicular histoarchitecture with nearly normal structure of seminiferous tubules (Figure 3D).

4. Discussion

In the present study, exposure to sodium fluoride for 21 days significantly induced a decline in gonado-somato index, sperm count and sperm motility, and also reduction in serum level of reproductive hormones. Gonado-somatic index, a biomarker of reproductive toxicity, reduced significantly, which may be due to loss of germ cells, reduction in tubular size and arrest of steroid synthesis. The reduced testicular weight indicated that the sodium fluoride induced structural and functional alterations of gonads and these findings further validated the earlier finding that testicular weight was adversely affected by fluoride[28]. Supplementation of *C. sinensis* extract in sodium fluoride-treated rats restored the gonadal indices towards its normal activity. A number of experimental and epidemiological studies showed similar repro-toxic effects to those observed in our sodium fluoride-treated rats[2,29]. Androgen deficiency leads to cessation of spermatogenesis by altering the activities of steroidogenic hormones. In our study, co-treatment of *C. sinensis* extract with sodium fluoride significantly improved the sperm density and motility probably by stimulating spermatogenesis as compared with sodium fluoride used alone[30].

The smooth progress and maintenance of spermatogenesis not only requires testosterone but also requires other hormones like LH, follicle-stimulating hormone, estrogen, *etc*. Testosterone secreted by Leydig cells of testis controls the initial stage of spermatogenesis, while LH is an important regulator of androgenesis. Earlier investigations have revealed that fluoride can decrease the secretion of testosterone[1,4] and decrease the LH level[31].

In accordance with these investigations, we found that fluoride administration declined the serum level of LH and testosterone in the sodium fluoride treatment group. But, in the present study, we did not investigate whether the declination of testosterone level is the outcome of decreased LH secretion or brought about by some other reasons. However, histological characteristics of testicular tissue of fluoride-treated rats lead us to hypothesize that decline in testosterone levels in fluoride-treated rats could be due to disruption of the cytoarchitecture of the testis by fluoride. Consequently, this might have adversely affected the structure and function of Leydig cells leading to decrease testosterone levels. Zhao *et al*[31] also reported that fluoride can influence testosterone synthesis by disrupting the structure of Leydig cells.

Oxidative stress is a condition whereby the homeostasis of the redox status of the cell is distorted. Free radical generation is a natural process and overproduction of oxygen free radicals and decreased antioxidant defense system lead to oxidative stress. Based on earlier reports, it is clear that ROS plays an important role in testicular toxicity by inducing oxidative stress[3]. However, the mechanism of ROS generation in fluoride-induced toxicity is not clear. García *et al* reported that fluoride may increase the formation of superoxide anions (O_2^-), H_2O_2, and peroxynitrite radicals[32]. Besides, fluoride may cause excessive ROS production in mitochondria by altering the GSH level[33]. In the present study, the overproduction of ROS is evidenced by a significant increase in testicular MDA and NO content in fluoride-treated rats. In addition, considering that sperm and testicles have a lot of polyunsaturated fatty acids, perhaps the sperm number, sperm count and testicular deformability might be impaired when we found MDA and NO expansion.

Intracellular antioxidant enzymes and molecules are regarded as a natural intracellular defence that protects macromolecules like DNA, lipid, proteins, *etc* by scavenging ROS. The endogenous antioxidant defence machineries consist of diverse antioxidant enzymes together with antioxidant molecules. Among them, intracellular antioxidant enzymes namely SOD, CAT are regarded as frontline defence, whereas GSH system functions as the second line cellular defence that protect the cells against toxic effects of ROS. SOD quenches O_2^- into H_2O_2 while CAT accelerates the conversion of H_2O_2 to H_2O and O_2. In our study, fluoride administration caused declination in the testicular antioxidant capacity as evidenced by the decrease in SOD, CAT activities, and GSH level. This indicates that testicular antioxidant defence mechanism fails to counteract the overproduction of ROS and consequently oxidative stress develops. Oxidative stress induced by fluoride is thought to be a contributing factor to various diseases and organ toxicities[14,21]. Also, several recent reports have suggested that fluoride exposure induces oxidative stress in testes by reducing enzymatic and non enzymatic status and is closely associated with testicular damage and male reproductive failure[4,34]. In the present study, fluoride-induced decrease in testicular weight, declination of testosterone level and disruption of histoarchitecture of testes with a concomitant increase in lipid peroxidation and decrease in antioxidant machineries emphasize that sodium fluoride can have triggered a specific mechanism that accomplishes all the adverse reproductive effects observed, and the mechanism seems to be sodium fluoride-induced oxidative stress. The increased level of LPO and NO with a concurrent decrease in SOD, CAT activities and GSH level in testicular tissue are in agreement with this conception.

The histological evaluation of testicular tissue following fluoride treatment also gave indications of oxidative damage induced by high level of ROS. In this study, fluoride treatment for 21 days showed marked deformation of testicular histoarchitecture as evidenced by altered histological features like deformation of seminiferous tubules, vacuolization, disorganization of germ cells, as well as loss of Leydig cells. All these changes in testicular tissue in fluoride-treated rats are also corroborated with the previous finding[31].

Thus, our findings indicate sodium fluoride-induced oxidative stress is a contributing factor of poor semen quality and reproductive performance. Previous findings have confirmed the noteworthy role of antioxidants in the fertility of males and females. Antioxidant supplementation has been recognized as a potential way to treat reproductive disorders and improve fertility. Studies also report that antioxidant supplementation such as vitamin C, melatonin, resveratol[13,19,21] can effectively counteract the fluoride-induced changes such as increased generation of ROS, NO, and inhibition of intracellular antioxidant status (SOD, CAT, and GSH), suggesting that fluoride-induced oxidative stress is a potential mode of action of fluoride toxicity. On the other hand, *C. sinensis* are widely used in the ailments of oxidative damage induced by various environmental toxins[20,30]. Many studies have shown that extract of *C. sinensis* contains a sufficient amount of flavonoids and catechins (epicatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate[19] which are the good sources of natural antioxidants. In the present

study, we found that simultaneous treatment of methanolic extract of *C. sinensis* with sodium fluoride in experimental rats increased the testicular weight, number and motility of sperm, the level reproductive hormones and improved histological abnormalities in the *C. sinensis*-supplemented sodium fluoride-treated rats in comparison with those receiving sodium fluoride. In addition, our findings indicated that the coadministration of *C. sinensis* in sodium fluoride-treated rats has resulted in a significant restoration of the oxidant-antioxidant status of testes as evidenced from the increased testicular levels of SOD, CAT GSH, and reduced level of NO and MDA in comparison with the sodium fluoride-treated group, suggesting that fluoride could not impose oxidative stress if they were treated with the *C. sinensis* extract. These findings are consistent with the previous findings that *C. sinensis* notably prevented the oxidative stress and its associated damage induced by various toxins in testes[35,36]. However, the precise mechanism involved in the beneficial effects of *C. sinensis* on male reproductive function still remains unclear. Based on the results of the earlier investigations, the repro-protective efficacy of *C. sinensis* may be attributed to its antioxidant polyphenol compounds which effectively scavenges the free radicals and prevents lipid peroxidation[37]. Earlier investigators reported that catechins, an important polyphenol of *C. sinensis* extract act as a natural antioxidant and provides the safeguard against the toxicity induced by the oxidative stress in the organs like testes[37]. Additionally, catechin of *C. sinensis* is known to be 20 times more powerful antioxidant than vitamin C and can efficiently detoxify free radicals, especially hydroxyl radicals that trigger lipid peroxidation, and thus prevent lipid peroxidation because of their unpaid electron[38]. Besides, Sato and co-workers in a study, demonstrated that *C. sinensis* has aromatase inhibitor activity which could be the main reason of improved testosterone level[49]. Testosterone and LH are important indicators of male reproductive ability, and also required to maintain spermatogenesis and proper growth of testes. Consequently, a parallel improvement in the serum level of testosterone, number and motility of sperm, and reduction in degenerative changes in testes, with attenuation of oxidative stress in rats of *C. sinensis*-supplemented sodium fluoride-treated group is related to the antioxidant property of *C. sinensis*. Administration of *C. sinensis*, thus, efficiently reversed the adverse effects and the oxidative stress-associated testicular damages induced by sodium fluoride by virtue of its antioxidant activity Interestingly, the beneficial role of *C. sinensis* on male reproductive physiology is dose-dependent[40]. This leaf extract is known to be protective and improve fertility at a moderate dose[41], while it seems to be a detrimental and castrative agent at a high dose[42].

Consequently, our study has several limitations. First, the use of a single dose of *C. sinensis* extract, and the study did not define the optimum dosage that would cause the best recovery from reproductive toxicity induced by sodium fluoride. Second, the study was conducted only for a short period and it is crucial to know the long-term effects. The evaluation of other oxidative markers such as ROS, protein carbonyl was another limitation. Besides, the use of *C. sinensis* leaf extract, which consists of several antioxidant polyphenols is the other limitation. Consequently, it is difficult to identify which of the polyphenolic compound of *C. sinensis* extract is accountable for the beneficial effects observed in our study.

In conclusion, simultaneous administration of *C. sinensis* to fluoride-treated rats prevents the fluoride-induced testicular weight loss, decreased sperm count, and motility with parallel attenuation of oxidative stress and restoration of testicular redox status. The present study thus suggests that catechins and polyphenol-rich *C. sinensis* may have a protective role against fluoride-induced testicular oxidative damage and consequently might use as an herbal antidote for male reproductive health in communities that have been exposed to fluoride through drinking water.

Conflict of interest statement

The authors have no conflicts of interest regarding publication of this paper.

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Authors' contributions

Dibyendu Ray designed the study and wrote the paper; Sunidhi Roy and Partha Nandi performed the research under the supervision of Dibyendu Ray; Pradip Panda and Dibyendu Ray analyzed the data; Sandip Mukherjee, Subrata Ghosh and Dibyendu Ray revised and finalized the manuscript for submission.

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