J. Microbiol. Biotechnol. (2000), **10**(3), 281–286

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Effect of Green Tea on Prostate and Seminal Vesicle in Rats Exposed to 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin

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Received: October 18, 1999

Abstract 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a ubiquitous environmental contaminant, causes a variety of adverse effects on the male reproductive system in rats. The effect of green tea extract (GTE) was investigated on the testicular function in Sprague-Dawley rats after a single exposure of 10 μg TCDD/kg body weight. The exposure of rat to TCDD significantly increased the weights of the epididymis and ventral prostate, yet significantly decreased the weight of the seminal vesicle when compared to the controls ($p<0.05$). In a combined treatment of TCDD with GTE, the organ weight changes caused by TCDD treatment disappeared. Significant decreases in sperm motility and sperm numbers were observed in the TCDD-treated rats, when compared to the control ($p<0.05$). GTE treatment reversed the decrease of sperm motility and sperm numbers caused by TCDD. There were no differences in sperm morphology, histological changes of the reproductive organs, and spermatogenesis between all the treated groups. In the ventral prostate and seminal vesicle, TCDD increased the CYP1A1 mRNA level, however, it did not affect the estrogen receptor β (ER-β) mRNA level. GTE treatment did not influence the effect of TCDD on the levels of CYP1A1 and ER-β mRNA. These results seem to indicate that green tea protects the testicular function against TCDD-induced reproductive toxicity, not because of a receptor-mediated mechanism but rather due to a secondary change of testes or accessory sex organs.

Key words: 2,3,7,8,-Tetrachlorodibenzo-*p*-dioxin, green tea extract, testicular function

It has recently been suggested that the release of "endocrine-disrupting compounds" (EDCs) into the environment has resulted in developmental effects in exposed wildlife populations [4, 5]. A large number of these environmental contaminants, such as polychlorinated biphenyls (PCBs), dieldrin, and kepone, exhibit estrogen receptor agonist activities [1, 10, 28]. It has been hypothesized that human exposure to environmental estrogen may be responsible for the increased incidence of male reproductive problems, including decreased sperm concentrations in the ejaculate [26].

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is considered the most potent congener of dioxin-like compounds. TCDD exerts its toxicity via an aryl hydrocarbon receptor (AhR)-mediated transformation process [19, 20, 34]. In a cytosol, TCDD binds to the AhR, facilitating dissociation of hsp90 and translocation of the liganded AhR to the nucleus. In a nucleus, the liganded AhR dimerizes with the nuclear protein ARNT [22], and the complex binds to dioxin-response elements (DRE) to activate transcription [6]. Cytochrome P450 1A1 is inducible by TCDD in the adult rat ventral prostate [38]. These results imply that the transcriptional machinery (AhR and ARNT) are necessary and functional in the adult rat ventral prostate.

Besides these transcriptional activities of TCDD, when TCDD bound to the AhR complex, *c-src* kinase is activated and acts as a trigger for a TCDD toxic signal through EGFR/PTK and certain cytoplasmic signaling pathways. Several cytoplasmic signal transduction pathways involve a serine/threonine protein kinase, referred to as the mitogen-activated protein kinase or MAPK (or Erk). Erk2 which is negatively regulated by *c-src* RTK is also involved in the phosphorylation of cytosolic estrogen receptors prior to their translocation to the nucleus [39]. It has been recently reported that ER-β is highly expressed in the prostate gland [40] and that several xenoestrogenic compounds are more likely to bind to ER-β than ER-α, which is originally expressed in the uterus or other female reproductive organs [40]. In male rats, exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) during adulthood resulted in a decrease in the weights of the testis, seminal vesicle, and ventral prostate, an alteration of testicular and epididymal morphology, increase

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in the incidence of epididymal sperm granulomas, and decrease in sperm production leading to infertility [15, 16]. TCDD and related congeners have thus been demonstrated to act as developmental and reproductive toxicants [9, 35].

In recent years, considerable interest has been generated about green tea as a health beverage [1]. The main polyphenolic compounds abundant in green tea are epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG). Some epidemiological and animal studies have shown that catechins exert protective effects against inflammatory and cardiovascular diseases [12, 17]. Catechins have also been implicated as chemopreventers against the development of various forms of tumors [18, 25, 36]. These protective effects of catechins have been attributed to their antioxidative activities of scavenging free radicals such as hydroxyl radicals, peroxy radicals, superoxide anion radicals, and nitric oxide and/or chelating transition metals such as iron and copper [11, 14, 27, 31].

Currently, there is no information on the effect of green tea on the toxicity induced by EDCs. Accordingly, the protective effect of green tea extract (GTE) was investigated using the reproductive system of rats exposed to TCDD with a single dose of 10 μg/kg body weight. This is the first report to describe that green tea protects the reproductive system of rats against TCDD-induced toxicity.

MATERIALS AND METHODS

Animals

Four-week-old male Sprague-Dawley rats were purchased from Samyouk Laboratory Animal Co. (Osan, Korea) and aclimatized for the animal facilities for one week before the experiment. The rats were housed up to five animals per polycarbonate cage (26×42×18 cm, Myunggin Co., Seoul, Korea), and maintained in a controlled environment with a constant day/night cycle (light from 7:00 a.m. to 7:00 p.m., 150-200 lux), temperature at 22±3°C, and relative humidity at 55±10%. They had free access to standard pellet feed (Shinchon Feed Co., Incheon, Korea) and tap water or green tea extract.

Chemicals and Green Tea Extract

Dried green tea leaves were obtained from Taepyungyang Chemical Co. (Suwon, Korea) and stored in a dark cold-room until extraction (Table 1). Green tea extract was prepared daily from the dried green tea leaves. Fifteen grams of the green tea leaves was soaked in 750 ml of hot water (80°C) in a beaker and allowed to stand for 30 min at room temperature, and then filtered through Whatman #1 filter paper. The filtrate was stored in a brown bottle. The GTE was analyzed for its polyphenol content using an HPLC (HP1 100) with a μ-Bondapak C18 column, at a flow rate of 1 ml/min, and a UV 280 nm detector. Ten milliliters volumes of GTE, EGC, EC, EGCG, and ECG as standards were injected into the HPLC. The concentrations of the standards were 100 ppm (1 mg/10 ml ethyl acetate). TCDD (GL Science Inc., Japan) was dissolved in DMSO (Sigma, U.S.A.) and diluted with corn oil (Sigma, U.S.A.) by a dilution factor of 20. There were four experimental groups; a non-treated control group (water), GTE group (green tea extract), TCDD group (water), and TCDD + GTE group (green tea extract). Twenty rats were randomly assigned to each experimental group. TCDD was injected i.p. into the rats with a single dose of 10 μg TCDD/kg body weight, 7 days after starting the experiment. GTE was given *ad libitum* to the rats for 28 days as drinking water.

Table 1. Contents of polyphenols in green tea preparation[a].

Polyphenols	(g/100 g dry base green tea)	mg/100 ml GTE
(-)-epigallocatechin-3-gallate	7.35	110.19
(-)-epicatechin gallate	2.87	43.05
(-)-epigallocatechin	1.75	26.25
(-)-epicatechin	1.13	16.98
Total catechins	13.10	196.47

[a]Fifteen grams of green tea leaves were soaked in 750 ml of hot water (80°C) in a beaker, allowed to stand for 30 min at room temperature, and then filtered through Whatman #1 filter paper.

Sperm Count

For the sperm analysis, we followed Yamada's method [37]. The distal section of the left caudal epididymis (100-300 mg) was taken, accurately weighed, minced with shears into 5 ml of Hanks' solution, and incubated at 37°C for 5 min. Ten microliters of the sample was then diluted with 90 μl of Hanks' solution. Sperms were counted using a hemocytometer with a 0.1 mn thick cover glass and an optical microscope. The number of sperm in the caudal epididymis was calculated according to the following formula; the number of sperm/g epididymis=[average count of sperm from five chambers x square factor (which was 5) × hemocytometer factor (which was 10^4) x dilution factor (which was 50)]/tissue weight (g). The data were expressed as the mean±SD for ten rats.

Sperm Motility

The right epididymis was removed, trimmed free of fat, and cut along the proximal cauda and distal cauda of the epididymis. The epididymis was placed in 5 ml of Hanks' balanced salt solution (HBSS) supplemented with 25 mM HEPES and 0.5% bovine serum albumin in a 35-mm plastic petri dish at 37°C. After 5 min at 37°C, the tissue was removed, and the sperm suspension was collected, gently mixed, and diluted with HBSS. The sperm motility was observed using a light microscope on an objective micrometer (Fujihira Industrial Co., Ltd, Japan) kept at

37.5°C. The sperm motility was evaluated for a total of about 200 sperm cells and expressed as the percent motility of the sperm. Percent (%) sperm motility=(No. of mobile sperm cells/total No. of sperm cells) × 100.

Sperm Morphology

The sperm morphology was studied using the sperm solution collected by the method described above. Two drops of a 1% eosin Y solution and 3 drops of 10% neutral formalin were added to the sperm solution (1 ml) in a 5-ml test tube, followed by staining for 30 min at room temperature. The stained sperms were then mounted on a slide, dried, and covered with a cover glass. Two to four hundred sperm cells were examined microscopically at a magnification of ×400 or ×1000, and those cells with abnormal heads and/or abnormal tails were scored.

Histology

Immediately after sacrificing animals, the testes and epididymides were removed, fixed in Bouin's solution for 1-2 days, then embedded in paraffin, sectioned at 5 mm, stained with hematoxylin/eosin, and finally examined for any histological lesions.

RNA Extraction and RT-PCR

The total RNA was extracted from 50 mg of frozen samples of seminal vesicles and prostates using the Trizol reagent (GibcoBRL, U.S.A.) according to the method described by the manufacturer, and then the RNA extract was stored at 80°C until use. The total RNA (5 mg) was used for a first strand cDNA synthesis using a SuperScript™ Preamplication System according to the method described by the manufacturer (Gibco, U.S.A.) and a PCR was performed using a Touchdown™ temperature cycling system (Hybaid, U.K.). After an initial denaturing step at 95°C for 10 min, amplification for the β-actin mRNA was performed with 28 cycles at 95°C for 1 min (denaturing), 55°C for 1.5 min (annealing), 72°C for 1.5 min (extension), and further extension at 72°C for 10 min (enzyme inactivation). For the ER-β mRNA and CYP1A1 mRNA, 35 cycles at 95°C for 1 min, 55°C for 1.5 min, 72°C for 1.5 min, and further extension at 72°C for 10 min were performed. For β-actin, the forward primer was 5'-CGTGACATCAAAGAGAAG-CTGTGC-3' and the reverse primer was 5'-GCTCAGGA-GGAGCAATGATCTTGAT-3'; for ER-β, the forward primer was 5'-AAAGCCAAGAGAAACGGTGGGCAT-3' and the reverse primer was 5'-GCCAATCATGTGCACCAGTTC-CTT-3'; for CYP1A1, the forward primer was 5'-CCATG-ACCAGGAACTATGGG-3' and the reverse primer was 5'-TCTGGTGAGCATCCAGGACA-3'. Electrophoresis for the PCR products (10 μl) was performed in a 1.5% agarose gel and the densitometry was carried out using an inverted scan image analyzer (Biorad, Gel-Doc., U.S.A.).

Statistical Analysis

A statistical analysis of the data was performed for a one-way ANOVA on ten rats using the SAS program. If the overall F-test was significant, the least significant difference procedure was then used to determine any significant differences at the level of $p<0.05$ between the various treatment groups.

RESULTS

Organ Weights

The relative organ weights versus the brain weights are shown in Table 2. The treatment of 10 μg TCDD/kg body weight significantly increased the weight of the ventral prostate, compared to the control or the green-tea-only group ($p<0.05$). With the combined treatment of GTE and TCDD, the prostate weights of the rats were not significantly different from those of the control or the GTE-only-treated rats ($p<0.05$). The weight of the seminal vesicles of the rats exposed to TCDD was significantly lower than that of the control, GTE-only-treated, or GTE + TCDD-treated groups ($p<0.05$). There were no significant differences in the weights of the testes and epididymides between the treatment groups.

Sperm Motility, Number, and Morphology

The total sperm number was counted with the samples collected from the left caudal epididymis. As shown in Table 3, those rats exposed to 10 μg TCDD/kg exhibited a significant decrease in the number of sperm per gram of epididymis, compared to the control or GTE-only-treated rats ($p<0.05$). The GTE treatment recovered the sperm

Table 2. Mean relative organ weight versus brain weight in rats exposed to a single dose of 10 μg TCDD/kg body weight.

Groups	Brain (g)	Testis (Left)	Epididymis (Left)	Ventral prostate	Seminal vesicle
Control	1.98±0.15[a]	79.9±4.2[a]	18.2±0.9[a]	33.3±3.8[a]	48.4±4.1[a]
GTE	1.97±0.18[a]	78.0±5.4[a]	17.5±1.6[a]	34.0±4.6[a]	47.5±3.9[a]
TCDD	1.84±0.20[a]	80.5±7.7[a]	21.8±1.9[a]	43.1±5.2[b]	41.1±2.9[b]
TCDD+GTE	2.01±0.17[a]	81.5±8.0[a]	20.3±3.1[a]	33.8±4.0[a]	49.2±4.4[a]

The values represent the mean±SD from ten male rats.

GTE: green tea extract; TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

[ab]The means in each column with different superscripts are significantly different ($p<0.05$).

Table 3. Sperm motility and number in rats exposed to a single dose of 10 μg TCDD/kg body weight.

Groups	Sperm motility (%)	Sperm number (10^6/g epididymis)
Control	88.4±5.9[a]	148.2±24.9[a]
GTE	89.0±6.4[a]	149.9±21.6[a]
TCDD	69.8±8.2[b]	112.8±14.5[b]
TCDD+GTE	84.6±6.1[a]	148.3±20.5[a]

The values represent the mean±SD from ten male rats.
GTE: green tea extract; TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.
[ab]The means in each column with different superscripts are significantly different ($p<0.05$).

number count decreased by TCDD. The exposure to TCDD also decreased sperm motility by about 21%, compared to the control (Table 3). However, the combined treatment of TCDD and GTE showed no significant difference in sperm motility compared to the control. The treatment of 5-wk old rats with TCDD did not form sperm with abnormal heads and/or abnormal tails. Therefore, there was no significant difference in sperm morphology between the various treatment groups.

Histological Findings

Specific lesions in testes, epididymides, and seminal vesicles were microscopically examined with the tissue sections stained with H&E. TCDD did not damage the testes, epididymides, and seminal vesicles. Tissue lesions were not found in all the treatment groups (data not shown).

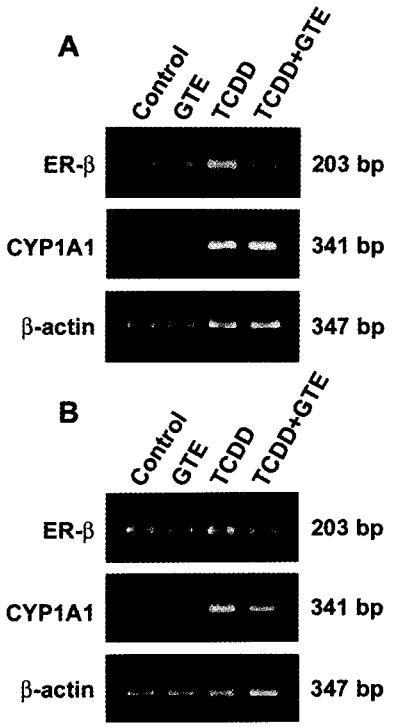

Fig. 1. Expression of ER-β and CYP1A1 (a) in the ventral prostate and (b) in the seminal vesicle of rats.

Expression of ER-β and CYP1A1 in the Prostate and Seminal Vesicle

When compared to the control, TCDD did not affect the ER-β mRNA levels in the prostate (Fig. 1a) and seminal vesicle (Fig. 1b), however, GTE did slightly lower the ER-β mRNA levels in the ventral prostate. Yet, there was no difference in the ER-β mRNA levels of the seminal vesicle and ventral prostate between the TCDD-treated and the TCDD + GTE-treated groups (Fig. 1).

The CYP1A1 mRNA level was high in the ventral prostate of the TCDD-exposed rats (Fig. 1a). The level of CYP1A1 mRNA in the control rats were slight or not detectable. Quantitatively, the exposure to TCDD increased the CYP1A1 mRNA level in the ventral prostate by about 2-fold compared to the control. The GTE-only treatment also increased the level of CYP1A1 mRNA slightly, whereas, the CYP1A1 mRNA level from the TCDD + GTE group was lower than those of the TCDD group (Fig. 1a). The TCDD-exposed rats also exhibited high CYP1A1 mRNA levels in the seminal vesicle (Fig. 1b). The GTE treatment did not affect the CYP1A1 mRNA level increased by TCDD.

DISCUSSION

It has been suggested that human sperm counts have declined significantly throughout the world during the past fifty years [3, 29], and this is a significant public health concern. A possibility that environmental contaminants may be contributing to a decline in the reproductive function in men needs to be carefully evaluated, in view of the abnormalities in reproduction and development of wild-life exposed to these compounds [4, 24]. TCDD is well known as a reproductive toxicant, particularly for developing males. Our present data show that exposure to TCDD during pubertal development (5-wk-old rats) resulted in a significant decrease in the sperm count and sperm motility. However, green tea treatment actually prevented these decreases. Accordingly, this result promotes the beneficial health effects of green tea and its components, the catechins.

Studies on laboratory animals have demonstrated that exposure to TCDD and related congeners resulted in the disruption of the reproductive function [7, 23, 35]. These studies show that such compounds decrease the weights f the testes and affiliated sex organs, alter the testicular morphology, and decrease spermatogenesis. Therefore, the decrease in the weight of the seminal vesicle found in this study was consistent with previous data. However, the relative weight of the ventral prostate versus the brain weight increased, probably due in part to the relatively low weight of the brain in the TCDD-treated rats. While there is substantial

evidence to indicate that TCDD has adverse effects on male reproduction, the mechanism for these effects is not well understood. In particular, there is no clear explanation for the latent effects of so-called hormone disrupters, which may have estrogenic or anti-estrogenic activities.

El-Sabeawy *et al.* [7] reported that the exposure to TCDD at low concentrations (0.1, 1, 5 μg/kg) significantly affected the testicular development of immature rats, confirming that exposure to TCDD during the period of gonadal development is more detrimental to male fertility than exposure during adulthood. The exposure of juvenile rats to TCDD perturbs their sperm production and function [7]. One *in utero* and lactational exposure to TCDD also decreases the daily sperm count and caudal epididymal sperm numbers in rats [30]. In contrast, Johnson *et al.* [13] demonstrated that the exposure of adult rats to 12.5–50 μg/kg TCDD/kg did not decrease the daily sperm production. The present data shows that when immature rats (pupertal stage) were exposed to 10 μg TCDD/kg and then evaluated in the postpubertal stage (9 weeks old), there was a reduction in the sperm number per gram of epididymis. The sperms obtained from these animals also appeared to have an impaired function, as revealed by changes in the sperm motility. GTE treatment protected the sperm function against TCDD-toxicity. Although the protective mechanism of GTE is not clear at this stage, it is possible that the antioxidant activities by GTE in the semen may be involved. The antioxidative activities of green tea and its components involve free radical scavenging, such as that of hydroxyl radicals, peroxy radicals, and superoxide anion radicals, and/or transition metal-chelators such as iron and copper [11, 14, 31].

A number of studies on the molecular mechanism for TCDD-mediated effects are consistent with a receptor-mediated response mechanism, which involves ligand binding to an aryl hydrocarbon receptor (AhR) [19, 20, 30]. In rats, the half-maximal induction dose (ED_{50}) of TCDD for aryl hydrocarbon hydroxylase (AAH) activity is 0.27 μg/kg [8]. TCDD and related compounds induce CYP1A1, which encodes various cytochrome P4501A1 monooxygenases such as AHH [19]. There is a good correlation between the toxicity of TCDD and its related compounds to their activities as inducers of hepatic CYP1A1-dependent enzyme activities. Several studies have shown that CYP1A1 is under the regulation of AhR. The activation of the CYP1A1 gene leads to increased P4501A1 mRNA levels followed by increased P4501A1-related enzyme activities [33]. Roman *et al.* [23] showed by Western blot that the CYP1A1 protein was highly induced only in the ventral and dorsolateral prostates of TCDD-exposed animals. However, the current data shows that CYP1A1 mRNA was expressed in the seminal vesicle as well as in the prostate in TCDD-exposed rats, as demonstrated by a RT-PCR. In this study, GTE showed little effect on CYP1A1 induction by TCDD in the prostates and seminal vesicles of rats. There have been only two previous reports on the effect of GTE on the expression of CYP1A1, and they are contradictory. Wang *et al.* [32] found that GTE inhibited AAH activity in the liver and in epidermal microsomes. In contrast, Sohn *et al.* [27] reported that chronic tea intake induced cytochrome P-450 1A1, 1A2, and 2B1, yet had little effect on other cytochrome P-450 isoenzymes.

In conclusion, green tea is protective against the decrease in sperm number and motility in the epididymis induced by the exposure of rats to TCDD, which is probably not due to an AhR-mediated mechanism but rather to a secondary change in the testes or affiliated sex organs. Accordingly, it is suggested that green tea can be used as a health beverage for protecting the male reproductive system.

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