Mansoura Veterinary Medical Journal 21:1 (2020) 31-35

https://doi.org/10.35943/mvmj.2020.21.105

Original Article *Biochemistry*

Protective effect of green tea extract against cadmium-induced testicular damage in rats in respect of oxidant/antioxidant equilibrium and androgen production

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ARTICLE HISTORY

Received: 03.11.2019

Revised: 12.03.2020

Accepted: 14.01.2020

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ABSTRACT

Objective: Investigating the effect of green tea extract (GTE) on the testicular damage induced by cadmium chloride $CdCl_2$ in male rats.

Design: Randomized controlled study.

Animals: 40 male Wistar rats.

Procedures: Rats were randomly divided into four groups: A) control group (each rat daily received pellet diet); B) GTE group each rat daily received pellet diet as well as 3 ml of 1.5 % w/v GTE, C) $CdCl_2$ group each rat was I/P injected a single dose of 1 mg/kg $CdCl_2$, then daily received pellet diet, and D) $CdCl_2$+GTE group each rat was I/P injected a single dose of 1 mg/kg $CdCl_2$ then daily received pellet diet as well as 3 ml of 1.5 % w/v GTE. After 30 days, blood samples were collected for hormonal assays (testosterone, FSH, and LH). In addition, both testes were collected; one of them was used for quantification of 17-beta hydroxysteroid dehydrogenase III (17β-HSDIII) gene expression using a real-time PCR. The other testis was used for determination of catalase and reduced glutathione; GSH, Nitric oxide (NO) and malondialdehyde (MDA) levels.

Results: $CdCl_2$ decreased serum testosterone levels and its synthesis pathway (17β-HSDIII testicular gene expression). While antioxidants catalase and GSH were reduced, oxidants MDA were enriched in the testes of $CdCl_2$-poisoned rats. This $CdCl_2$-promoted testicular dysfunction was corrected via the administration of GTE to male rats.

Conclusion and clinical relevance: GTE could be used as a remedy for protecting against $CdCl_2$-induced testicular damage in male rats.

Keywords: Cadmium, Green tea, testosterone, antioxidants, testes

1. INTRODUCTION

Although its utilization in the manufacture of metal plating, batteries, plastics as well as fertilizers, cadmium chloride ($CdCl_2$) is classified as an environmental pollutant having the ability to pollute the water and soil and therefore could easily transfer to the plants [1]. As a result, consumption of such polluted water and/or foods could induce $CdCl_2$ toxicity. It has been reported that $CdCl_2$ toxicity could lead to many health problems even though the exposure to relatively low doses of $CdCl_2$. This might be referred to the cumulative effect of $CdCl_2$ in the biological systems of the body [2].

Among these biological systems, the reproductive system has been found to be severely affected by the exposure to $CdCl_2$. In regards to males, either acute or chronic $CdCl_2$ toxicity has been linked with poor semen quality and defective androgen production [3]. This is possibly due to the capability of $CdCl_2$ to produce reactive oxygen species (ROS) and thereby disturbing the pro-oxidants/antioxidants' equilibrium leading to oxidative stress (OS) [4]. Recently, the oxidative stress has been shown to have a destructive effect on the blood-testicular barrier in male rats due to the accumulation of lipid peroxidation (LPO) in many organs of male rats including testes and subsequently infertility problem(s) was raised in male rats as a result of $CdCl_2$ toxicity [5,6].

In the last few decades, a much more interest has been paid for using naturally occurring dietary substances like medicinal plants in order to manage various health problems including infertility [7]. Green tea (*Camellia sinensis),* a member of the Theaceae family, seems to be a good choice for overcoming $CdCl_2$-induced infertility; green tea flavonoid epigallocatechin-3-gallate (EGCG) is a classified as a potent antioxidant and thus it could prevent OS through combating ROS over production [8]. Lately in Nigeria, it has

been recorded that green tea supplementation reduced the cadmium level and regulated the levels of sex hormones in automobile workers [9]. Here, we aimed to investigate the effect of green tea extract on the testicular damage induced by $CdCl_2$ in male rats.

2. MATERIALS AND METHODS

2.1. Animals and Experimental design

Wistar albino adult (10 weeks old) male rats (n= 40), each rat weighted about 250 ±50 g, were obtained from Medical Experimental Research Center (MERC), Mansoura University, Egypt. To be adapted with the environmental conditions before conduction of the experimental design, rats were housed for one week in clear stainless cages (5 rats/ cage) at 22±4° C room temperature and 55±10 % relative humidity. Afterwards, rats were randomly divided into four groups: A) control group (each rat daily received pellet diet according to National Research Council, NRC for 30 days) [10], B) GTE group (each rat daily received pellet diet as well as 3 ml of 1.5 % w/v GTE by a stomach tube for 30 days) [11], C) $CdCl_2$ group (each rat was I/P injected a single dose of 1 mg/kg $CdCl_2$, then daily received pellet diet for 30 days) [12], and D) $CdCl_2$+GTE group (each rat was I/P injected a single dose of 1 mg/kg $CdCl_2$ then daily received pellet diet as well as 3 ml of 1.5 % w/v GTE by a stomach tube for 30 days).

2.2. Preparation of GTE

For GTE preparation, 250 grams of green tea leaves purchased from a local market were ground and extracted by ethanol (80%) in a separating funnel. The extract was concentrated using a rotary evaporator, then kept in sealed vials in a freezer until use. The percentage of extract was calculated according to the dry matter of the green tea. To make 1.5 % w/v GTE, GTE (15 g.) was dissolved in one liter distilled water, boiled for 5 minutes, then filtered [13].

2.3. Blood sampling

After 30 days, all rats were fasted overnight before their sedation *via* the injection of thiopental sodium 25 mg/kg [14]. Afterwards, the blood was collected from the heart; the samples were left overnight at 4°C to clot, centrifuged at 3000 rpm for 15 min, and then the clear serum was collected and kept in freezer (-18°C) for measuring testosterone hormone level using rat Testosterone ELISA kit (DRG Instruments GmbH, Germany) [15], follicle stimulating hormone (FSH) level using rat FSH ELISA kit (Biovendor Research and Diagnostic Products, Germany) [16] and luteinizing hormone (LH) level using rat LH ELISA Kit (BioVendor Research AND Diagnostic Products, Germany) [17].

2.4. Tissue sampling

Five rats from each group were sacrificed for obtain both testes. One testis was dissected for testicular tissues which then stored in RNA storage solution at -20°C for SYBR Green real-time polymerase chain reaction (PCR). The other testis was used for preparation of tissue homogenate. In brief, it was perfused with phosphate-buffered saline (PBS, pH 7.4), then homogenized in 5-10 ml cold buffer (50mM potassium phosphate, pH 7.5)/each g. tissue. After that, samples were centrifuged at 4000 r.p.m. for 15 min., sediments were discarded, and supernatants were taken, stored at -20°C for determination of catalase activity [18], reduced glutathione (GSH) level [19], Nitric oxide (NO) level [20] malondialdehyde (MDA) level [21].

2.5. Quantitative real-time PCR

Total RNA was extracted from testes tissue (RNeasy Mini Kit; QIAGEN Company) according to the manufacturer's instructions. Total RNA was spectrophotometrically quantified. The gene expression of testicular 17-beta hydroxysteroid dehydrogenase III **(**17β-HSDIII) was determined using a quantitative real-time PCR. Simply, a total 25 µl reaction mix consisting of 12.5 µl QuantiTect SYBR Green PCR Master Mix (QIAGEN, Hilden, Germany), 8.5 µl RNase Free water (Invitrogen, California, USA), Reverse transcriptase, 0.5 µl of forward and reverse primer (20 pmol), and 3 µl of RNA sample. The reverse transcription was done at 50˚C for 30 min. After that, the amplification program was run: 40 cycles with primary denaturation at 94˚for 15 min, secondary denaturation at 94˚C for 1 min, primer annealing at 60˚C for 30 sec, followed by extension at 72˚C for 30 sec. β-actin was used as housekeeping gene. Oligonucleotide PCR primers used for rat β-actin [22] and 17β-HSDIII [23] are shown in **Table 1.**

2.6. Statistical analysis

Statistical analysis of the collected, validated, and organized data was performed using IBM® SPSS® Statistics 14 (IBM Corp., Armonk, NY, USA). One-way ANOVA followed by Duncan multiple comparison test was used to compare means. Data are presented as mean ± standard error of the mean (SEM). The results were considered statistically significant at $P < 0.05$.

3. RESULTS

3.1. Effect of GTE, $CdCl_2$ and GTE with $CdCl_2$ on the blood levels of testosterone hormone, FSH, and LH

A marked decrease of testosterone hormone, FSH, and LH was detected in the blood levels of male rats injected with $CdCl_2$ when compared with the control rats. These hormones were significantly increased *via* GTE administration in $CdCl_2$-poisoned rats **(Table 2).**

3.2. Effect of GTE, $CdCl_2$ and GTE with $CdCl_2$ on testicular antioxidants and OS markers

$CdCl_2$ significantly reduced testicular antioxidants (catalase activity and GSH level) and upregulated testicular OS markers (MDA levels) while GTE administration noticeably improved catalase activity and GSH level with diminishing MDA level in the testicular tissues of $CdCl_2$-intoxicated rats **(Table 3).**

3.3. Effect of GTE, $CdCl_2$ and GTE with $CdCl_2$ on testicular 17β-HSDIII gene expression

$CdCl_2$-intoxicated rats that were administered GTE had an obvious upregulation of testicular 17β-HSDIII gene expression when compared with those were not given GTE **(Table 4).**

Table 1. The sequence of primers used for the real-time PCR**.**

Genes	Primer sequence (5'-3')
β-actin	*Forward* 5-T C CT C CTG AGCGCAAGTACTC T-3
	Reverse 5-GCTCAGTAACAGTCCGCCTAGA A-3
17β-HSDIII	*Forward* 5-CCT GAG ATC AAT GGG ACA ATG-3
	Reverse 5-CCC TAC TCC CGA AGA GAT A-3

Table. 2. Effect of GTE, CdCl2 and GTE with CdCl2 on the blood levels of testosterone hormone, FSH, and LH.

Groups	Serum Levels of hormones (ng/ml)		
	Testosterone	FSH	LH
Control	2.06±0.06^{a}	5.77±0.13^{a}	3.56±0.16^{a}
GTE	2.03±0.10^{a}	5.53±0.22^{a}	4.11±0.17^{a}
$CdCl_2$	0.99±0.03^{c}	1.72±0.13^{c}	1.20±0.10^{c}
GTE+$CdCl_2$	1.57±0.14^{b}	3.00±0.13^{b}	2.11±0.13^{b}

*Results were expressed as mean ± standard error of the mean (S.E.M). Different letters denote a significant variance (P<0.05).

Table 3. Effect of GTE, $CdCl_2$ and GTE with $CdCl_2$ on testicular antioxidants and OS markers.

Groups	Antioxidants		OS markers	
	Catalase activity (U/g tissue)	GSH levels (mg/g tissue)	MDA levels (µmol/g tissue)	NO levels (µmol/g tissue)
Control	0.33±0.02^{a}	2.18±0.36^{b}	2.69±0.52^{ab}	21.0±3.52^{b}
GTE	0.36±0.05^{a}	4.02±0.37^{a}	1.63±0.51^{C}	33.01±8.60^{b}
$CdCl_2$	0.04±0.01^{b}	0.42±0.08^{c}	3.71±0.55^{a}	69.78±6.48^{a}
GTE+$CdCl_2$	0.42±0.04^{a}	0.76±0.09^{c}	2.07±0.28^{b}	67.5±3.67^{a}

*Results were expressed as mean ± standard error of the mean (S.E.M). Different letters denote a significant variance (P<0.05).

Table 4. Effect of GTE, $CdCl_2$ and GTE with $CdCl_2$ on testicular 17β-HSDIII gene expression.

Groups	Control	GTE	$CdCl_2$	GTE+ $CdCl_2$
17β-HSDIII gene expression	1.19± 0.08^{bc}	2.49± 0.16^{a}	0.69± 0.05^{c}	1.80± 0.13^{ab}

*Results were expressed as mean ± standard error of the mean (S.E.M). Different letters denote a significant variance (P<0.05).

4. DISCUSSION

The existing work used a simplified rat model for evaluating the efficiency of herbal medicines, definitely GTE, in ameliorating the adverse effects of $CdCl_2$ toxicity on the male reproductive organs, in particular testes. Indeed, supplementation of GTE for $CdCl_2$-intoxicated male rats restored the blood levels of sex hormones through re-adjustment of the pro-/anti-oxidant balance in the testicular tissue.

To begin with, testosterone is the principle male sex hormone produced by the interstitial cells of testes under the effect of LH. The functionality of both hormones, in addition to FSH, is crucial for regulation of spermatogenesis process [24,25]. Consequently, assessing the blood levels of such hormones is a vital indicator for testicular competence

The current study showed that blood levels of testosterone, LH, and FSH hormones were reduced in case of $CdCl_2$ intoxication. In accordance with our result, previous studies demonstrated that free radicals, especially ROS, produced after $CdCl_2$ injection could destroy the precursors of testosterone through lipid peroxidation of testicular interstitial cells' membranes thereby impairing androgen production in these cells [26, 27].

Importantly, GTE giving to $CdCl_2$-intoxicated male rats brought back the physiological plateau of the above sex hormones. In the same line, former investigations pointed out that GTE minimized the testicular damage in male rats exposed to $CdCl_2$ [28]. Likewise, GTE had a positive impact on the amount of gonadotropin hormones (LH and FSH) in female rats poisoned with $CdCl_2$ [29].

To explore the mechanism whereby GTE could upregulate the blood levels of sex hormones, particularly testosterone, it was essential to investigate the gene expression of 17β-HSDIII, the key player of testosterone synthesis [30]. It was evident that $CdCl_2$ had an inhibitory effect on 17β-HSDIII gene expression in the testicular tissues; this effect was reversed by GTE administration to poisoned rats. In the same line, it was found cyclophosphamide, an immunosuppressive agent, could induce testicular damage in male mice. However, the pretreatment of these mice with green tea infusion rescued them. To be more precise, green tea infusion strengthened the

antioxidant capacity and increased the activity of 17β-HSDIII in the testicular tissues [31].

Of known that ROS production, at the physiological levels, is essential for some sperm functions (like maturation, hyper-activation, capacitation as well as acquiring fertilizing ability). However, excessive ROS production in the expense of antioxidant capacity (i.e. OS status) could lead to LPO and subsequently DNA damage and apoptosis [32]. Accordingly, it was necessary to assess oxidants/anti-oxidants' equilibrium system in $CdCl_2$-intoxicated testes of male rats in presence/absence of GTE. Interestingly, our biochemical analyses revealed an obvious reduction in the testicular activity of antioxidants markers (catalase and GSH) in response to $CdCl_2$ exposure. Catalase is an intracellular enzyme capable of splitting hydrogen peroxide (H_2O_2) into water and oxygen [33]. A similar study referred the reduced catalase activity and the consequent H_2O_2 accumulation to the decreased iron (Fe) absorption in the stressed tissues [34]; Fe is an essential trace element for the functionality of catalase enzyme [35]. On the other hand, the declined GSH activity was attributed to the ability of $CdCl_2$ to bind with SH group in the cell membrane with a subsequent inactivation of such antioxidant member [12].

Based on the above findings, we expected that the lower antioxidant levels could result in higher levels of LPO in the testicular tissues of $CdCl_2$-intoxicated rats when compared with control ones. Actually, MDA as end product LPO of polyunsaturated fatty acids was elevated in rat testes exposed to $CdCl_2$. In the same direction, some reports showed that a single-dose administration of $CdCl_2$ increased LPO levels in the form of MDA [36].

Alongside ROS, reactive nitrogen species (RNS) are classified as potent free radicals. Among them, NO is intracellularly synthetized by the action of NO synthases (NOS) [37]. Clearly, our data revealed a big rise in NO testicular levels in rats poisoned with $CdCl_2$. This coincides with the fact that the activity of NOS increases in case of the stressed testes like cryptorchidism [38].

Depending on its antioxidant properties [8], GTE was administered $CdCl_2$-poisoned rats for diminishing the severity of OS problem. In such manner, GTE not only enhanced the antioxidant levels but also weakened the progression of LPO process in the testicular tissues. Notably, similar studies were conducted in the liver and kidney; they also found that GTE could prevent the hepatic- and renal damage induced by $CdCl_2$ poisoning in rats through the inhibition of LPO [39, 40].

The current study showed that NO level did not meaningfully change by GTE treatment in rats exposed to CdCl. However, another study proved that the consumption of green tea could negatively affect NO levels [41]. The explanation of such findings based on the ability of green tea flavonoid (EGCG) to suppress NO-production system *via* the inhibition of NOS gene expression in target tissues. Besides, EGCG is capable of promoting NO-scavenging system. Altogether, EGCG could mitigate the excess NO levels [42].

Conclusion

To sum up, GTE could be used as a remedy for protecting against $CdCl_2$-induced testicular damage in male rats

Acknowledgment

Conflict of interest statement

The authors declare that there is no conflict of interest in the current research work.

Animal Ethics Committee Permission

The current research work was permitted to be executed according to standards of animal research committee in the Faculty of Veterinary Medicine, Mansoura University, Egypt.

Authors' contributions

B. E. conducted the experiment and analytical procedures and drafted the manuscript, S. E. performed statistical analysis and revised the manuscript, E. E. revised the manuscript.

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