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Crude Saponin from Korean Red Ginseng Attenuates Testicular Toxicity of Rats Exposed to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

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Abstract : Previously we have reported that administration of Korean red ginseng water extract (KRG-WE) plays both preventive and therapeutic roles in testicular toxicity of guinea pigs exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Further study was carried out to verify the beneficial role of Korean red ginseng in TCDD-induced testicular toxicity with different animal species by different route of administration. Korean red ginseng crude saponin (KRG-CS) was prepared by Diaion HP-20 adsorption chromatography. One hundred twenty rats (Sprauge Dawley, 200±10 g) were divided into 6 groups. The normal control group (NC) received vehicle (*i.p.*) and saline (*p.o.*). Predetermined dosage of TCDD (40 μg/kg b.w., *i.p.*) was administered to single TCDD-treated (TT) and test (CS) groups. KRG-CS was administered (*p.o.*) at daily doses of 5 (CS5), 10 (CS10), 20 (CS20) and/or 40 mg/kg b.w. (CS40) for 5 weeks, starting 1 week before the TCDD-exposure. Body weight gain, organ weights, and sperm quality were investigated. Decrease in body weight gain induced by TCDD was greatly attenuated by KRG-CS in a dose-dependent manner. Testicular weight, sperm head counts and ratio of sperm with progressive movement in TT group decreased significantly but those parameters were improved by the treatment of KRG-CS in a dose-dependent manner. This result led us to conclude that crude saponin might be the active ingredient of Korean red ginseng that attenuates the testicular toxicity induced by TCDD.

Key words : ginseng saponin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), testicular toxicity, protection

INTRODUCTION

Throughout the world there is recent, serious concern regarding the toxicity induced by an endocrine distruptor. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) has come to be known as the most potent toxic environmental pollutant.[1] Recently, TCDD has been found to disrupt the endocrine system, and is therefore referred to as an endocrine disruptor.[2] TCDD was produced as an impurity during the chemical synthesis of Agent Orange® (2,4-dichlorophenoxy acetic acid and/or 2,4,5-trichlorophenoxy acetic acid), a wide spectrum herbicide and defoliant used in the Vietnam War by the US Army[3]. Production of Agent Orange® for commercial purposes was prohibited several decades ago but TCDD is still generated from municipal incinerators, exhaust from leaded gasoline and the pulp and paper industries. TCDD induces hypoinsulinema,[4] hyperlipidemia,[5] immune dysfunction,[6] and hepatotoxicity.[7] It is highly lipophilic and extremely recalcitrant to biodegradation, which leads to TCDD being accumulated in adipose tissue and recycled via the food chain. Decreases in spermatogenesis, and the ability to conceive and carry pregnancy to term are the most sensitive signs of reproductive toxicity induced by TCDD in mammals.[8]

On the other hand, *Panax ginseng* has been used as a folk medicine for improving physical strength in Far Eastern countries for more than two thousand years. Modern scientific research findings prove that *Panax ginseng* helps prevent and/or treat diabetes mellitus,[9] atherosclerosis,[10] senile prostate,[11] erectile dysfunction,[12] immune dysfunction,[13] nephrotoxicity,[14] carcinogenesis,[15] hepatoxicity,[16] and physico-chemical stress.[17] Ginsenosides (ginseng glycosides) have been known to be the main active ingredients of ginseng.

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Previously, we reported that *Panax ginseng* water extract (*i.p.*) exhibited both preventive and therapeutic efficacy in TCDD-induced testicular damage in guinea pigs[18]. Further study was carried out to find the active ingredient(s) and to ascertain the previous beneficial role of ginseng in TCDD-induced testicular toxicity by oral administration in different animal species.

MATERIALS AND METHODS

Experimental animals

Four- to five-week-old male rats (body weight: 200±10 g, Sprague Dawley) were purchased from Takonic, Osan, Korea. They were provided with solid food (Purina Co., Ltd.) and water *ad libitum*, and kept at constant temperature (24±1°C) and humidity (60±10%) on a 12 hour light/12 hour dark cycle. They were used for the experiment at least 7 days after delivery from the breeding company.

Chemicals

TCDD was purchased from AccuStandard Inc. (New Haven, CT, USA), with >99.1% purity as determined by gas chromatography. It was dissolved in acetone containing a small volume of dimethylsulfoxide (DMSO) by sonication, diluted with corn oil and vortexed vigorously. Corn oil was procured from a local grocery store. Unless otherwise mentioned, chemicals were purchased from Sigma (St. Louis, MO, USA) and were of the highest purity available.

Preparation of crude saponin from Korean red ginseng (KRG-CS)

Six-year-old fresh roots of *Panax ginseng* C.A. Meyers (10 kg) was kindly supplied by the Jungpyong Experimental Station, Korea Ginseng and Tobacco Research Institute, Taejeon, Korea. It was processed into red ginseng by steaming at 98°C/2 hr and sun-drying. The red ginseng was then extracted with 10 volumes of distilled water at 72°C for 48 hours and concentrated under the reduced pressure to obtain the water extract (1.4 kg). The water extract was then passed through a glass column containing 10 liters of Diaion HP-20 (Mitsubishi Kasei, Tokyo, Japan) resin as reported previously.[19] After washing the resin with H_2O and 25% ethanol, crude saponin was eluted with absolute alcohol. The absolute ethanol eluate was then concentrated *in vacuo* and lyophilized to afford crude saponin (112 g, KRG-CS). Major ginsenoside (Rb_1, Rb_2, Rc, Rd, Re, Rf and Rg_1) content in KRG-CS was found to be 43.5% when determined by HPLC (YMC-Pack NH_2, 4.6×250 mm, BuOH/MeOH/H_2O, 80:20:10). The saponin fraction also contains crude protein, crude fat, carbohydrate and ash fiber by 11.0, 4.1, 44.0 and 0.2%, respectively.

Determination of TCDD dosage inducing oligospermia and asthenospermia selectively

A total of fifty male rats were divided into 5 groups. Normal control group received vehicle (*i.p.*) and normal saline solution (*p.o.*). TCDD was injected intraperitoneally at single doses of 5, 10, 20 or 40 μg/kg b.w. Dosage of TCDD exposure to rat was determined on the basis of data on survival rate, body weight gain, organ weight especially testicular weight, hematological parameters and sperm quality; sperm head count and motility pattern.

Administration of TCDD and KRG-CS

A total of sixty rats (Sprauge Dawley, 200±10 g) were divided into 6 groups and the animal experiment was carried out twice with ten rats for each group, independently. The normal control group (NC) received vehicle (DMSO: 0.01 ml, acetone: 0.04 ml, corn oil: 4.95 ml; *i.p.*) and saline (*p.o.*). A 40 μg/kg b.w. (*i.p.*) of TCDD was administered to single TCDD-treated (TT) and test (CS) groups. KRG-CS was administered orally at daily doses of 5 (CS5), 10 (CS10), 20 (CS20) and/or 40 mg/kg b.w. (CS40) for 5 weeks, starting 1 week before the TCDD-exposure (Fig. 1).

Animal experiment was carried out twice independently with 10 rats for each 6 groups. TCDD was administered intraperitoneally at a single dose of 40 μg/kg b.w. KRG-CS was administered orally at daily doses of 5 (CS5), 10 (CS10), 20 (CS20) and 40 (CS40) mg/kg b.w. for 5 wks beginning 1 wk before TCDD-exposure.

Organ weight

After blood sampling, rats were sacrificed by cervical dislocation. Each organ was removed and the adjacent

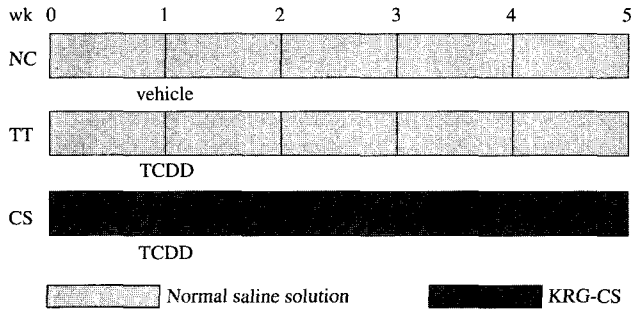

Fig. 1. The experimental protocol for the treatment of KRG-CS and TCDD.

fatty tissue was eliminated. The organs were then rinsed with saline, blotted with filter paper and weighed. Pathological change in those organs was also observed with unaided eyes.

Sperm head counts and morphology

Left testis without epididymis was homogenized with a tissue tearer (Biospec Product Inc., Germany) and sonicated with 12 ml of saline. After loading the sonicated testis suspension on Petroff-Hausser counting chamber, the chamber was stood for 2 to 3 minutes to let the sperm heads sink and counted according to the reported method.[20] Sperm for morphology test was stained with Eosin Y reagent and examined under the light microscope (Nikon, Japan, ×400) using sperm smears collected from the left epididymal tail. Abnormal sperms were classified according to the reported criteria.[21] Approximately 500 sperm heads were evaluated for each animal.

Sperm motility

Sperm motility was assessed by the reported method[22] with a minor modification. Briefly, sperm samples were extracted from the left epididymal tail by cutting with a pair of scissors. One drop of caudal fluid was immediately placed on a Petri dish containing 5 ml (prewarmed to 37°C) of Hanks' balanced salt solution supplemented with 10 mg/ml of bovine serum albumin (Fraction V). After incubation at 37°C for 5 minutes, an aliquot of the suspension was taken by micropipette and diluted to contain 40±10 sperms as counted under the defined microscopic observation field (×300). A 50 μl of the suspension was then placed on a slide glass (0.15 mm in flat depth) kept at 37°C. The slide glass containing sperm for examination was placed on a stage warming chamber (Microwarm plate, controlled to 37°C, Kitazato model MPF-10-N, Japan). Sperm motility under the inverted microscope (Axiovert 135, Carl Zeiss, Germany) was recorded at the speed of 48 ms^{-1} per field by a time-lapse videorecorder (AG6730, Panasonic, Japan) with a 20×objective lens and a charge-coupled device (CCD) camera (XC77, Sony, Japan). The 25 serial images from the videotape [phase alteration line (PAL) field] were transferred and digitized at a resolution of 256×256 pixels, and 256 possible gray values using an image analyzer (IBAS, Carl Zeiss, Germany). A quantitative analysis of sperm motility pattern was investigated by observing the 25 repeating serial images. At least 200 sperms were monitored for each sample for the determination of the sperm motility pattern.

Statistical analysis

Data were obtained from two independent experiments with 10 rats for each group and expressed, unless otherwise mentioned, as mean±standard deviation. Statistical analysis was carried out using ANOVA.

RESULTS

Determination of TCDD dose inducing oligospermia and asthenospermia selectively

Body weight gain in the TCDD-exposed rats was significantly decreased compared with that in the NC rats. Sperm head counts and testicular weight were decreased by TCDD-exposure in a dose-dependent manner. However, no animal in all TCDD-exposed groups died during the experimental period. This result led us to determine the exposing dose of TCDD to rat as 40 μg/kg b.w.

Effect of KRG-CS on the body weight gain and survival rate

Body weight of NC group animal increased 45.6% during the experimental period (Fig. 2). However, the weight in TT group increased only 15.8%. On the other hand, retarded body weight gain due to TCDD-exposure was significantly attenuated by KRG-CS-treatment ($p<0.01$). Body weight of all CS groups increased more than 30% during the experimental period.

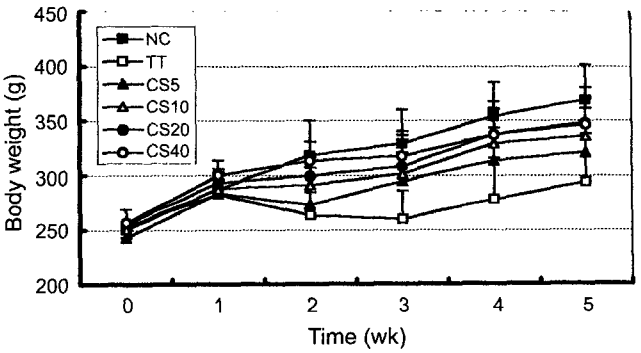

Fig. 2. Effect of KRG-CS on body weight gain in TCDD-exposed male rats.

Effect of KRG-CS on organ weight

Liver weight of TT group animal increased significantly ($p<0.01$) when compared with that of NC (Table 1). KRG-CS-treated groups also tended to be increased liver weight compared with that of TT. Lung weight showed no significant difference between NC and test groups regardless of KRG-CS or TCDD-treatment. Spleen weight of TT group accounts for 95.2% of NC and that of KRG-CS-

Table 1. Effect of KRG-CS on organ weight in TCDD-exposed male rat

Group (n)	Liver	Lung	Spleen	Kidney	Testes
NC (20)	9.42±1.09	2.22±0.45	1.05±0.42	2.43±0.24	3.87±0.49
TT (18)	10.70±1.86##	2.26±0.52	1.00±0.33	2.13±0.30#$	3.47±0.33##
CS5 (19)	10.84±0.91##	2.01±0.30	0.96±0.15##	2.24±0.19#$	3.73±0.24*
CS10 (20)	11.22±1.86##	2.23±0.50	0.96±0.22##	2.18±0.20#$	3.93±0.36**
CS20 (19)	11.45±1.63##	2.26±0.42	0.87±0.13##	2.31±0.26*	4.02±0.26**
CS40 (18)	11.67±1.63##	2.13±0.24	0.88±0.15##	2.28±0.18#	3.94±0.45**

Group designation as in Fig. 1. Data were obtained from 2 independent experiments for each group and expressed as mean±SD. # and ##; significantly different from NC at $p<0.05$ and $p<0.01$, respectively. * and **; significantly different from TT at $p<0.05$ and $p<0.01$, respectively when analyzed by ANOVA.

Table 2. Effect of KRG-CS on sperm head counts and motility in rats exposed to TCDD

Group (n)	Head counts[a]	Ratio of motility pattern (%)		
		Straight	Rotating	Immotile
NC (20)	100±8.0	40.0±6.3	1.7±2.1	58.4±5.5
TT (18)	82.6±11.8##	34.6±8.9	3.2±4.5	61.7±9.5
CS5 (19)	101.1±9.6**	37.8±12.9	1.3±1.7	60.9±13.1
CS10 (20)	103.4±9.0**	36.4±9.3	0.4±1.1	59.3±1.6
CS20 (19)	95.1±15.3*	36.0±12.3	2.0±3.7	62.1±13.9
CS40 (18)	105.4±17.6**	43.4±10.0	0	56.6±10.0

Group designation as in Fig. 1. [a]Sperm head count of the NC group ($409\pm33\times10^6$) was set to 100 and that of the test groups was expressed as a percentage to that of the NC. The quantitative analysis of the sperm motility pattern was determined by observing the 25 repeating serial images. Other footnotes as in Table 1.

treated groups showed lower weight than that of NC or TT. But there was no significant difference between the TT and CS groups in spleen weight. Kidney (87.7%) and testes (89.7%) weights decreased remarkably by TCDD-exposure when compared with those of the NC ($p<0.01$). KRG-CS was found to inhibit renal atrophy induced by TCDD but CS-20 group alone showed significance ($p<0.05$). On the other hand, treatment of KRG-CS attenuated the decrease in testis weight significantly in a dose-dependent manner ($p<0.05$-0.01).

Effect on sperm head counts and motility.

Sperm head count was significantly decreased in TCDD-treated group to 82.6% ($p<0.01$). However, the count in KRG-CS-treated groups increased in a dose-dependent manner. Motility of single TCDD-treated group animals decreased but was not statistically significant. However, there was a tendency that treatment of KRG-CS to TCDD-exposed male rats increased the ratio of sperm with straight-line velocity (Table 2).

DISCUSSION

TCDD is extremely resistant to metabolic breakdown, thereby being accumulated in the adipose tissue and which in turn manifests sustained toxicity.[8] Susceptibility of animals to TCDD varies dramatically depending upon species, and male guinea pig is one of the animals most sensitive to TCDD.[23] Toxic responses of TCDD include the induction of xenobiotic-metabolizing enzymes, neoplasia and reproductive toxicity that are associated with disturbances in endocrine homeostasis.[8] There was a chemical plant explosion in Seveso, Italy in 1976, which released a cloud of dioxin into the atmosphere. Of the 74 children born to the most highly exposed adults from 1977 to 1984, only 35% were boys. The nine couples with the highest levels of dioxin in their blood had no boys at all.[24] This epidemiological study suggests that chronic or acute exposure to dioxins can induce unbalance in male and female birth ratio. It seems most likely that we cannot be free from environmental pollutants unless we date back to the life style of at least half a century ago. In this respect, one of the best ways for health protection might be taking natural agents that can protect us against toxic responses induced by environmental pollutants.

Korean red ginseng has long been used in China, Japan and Korea as a tonic agent for the improvement of physical strength. Modern scientific findings indicate that

Korean red ginseng plays beneficial roles in endocrine,[11] sexual,[12] immune[13] and reproductive dysfunctions.[25] Main active ingredients are known to be ginsenosides. However, the diverse effects of Korean red ginseng are not fully supported by ginsenosides alone. In addition, more than 30 kinds of ginsenosides have been isolated and characterized from Korean red ginseng.[26] Biological studies with isolated ginsenosides revealed that they exerted antagonistic and/or synergistic action on each other. Likewise, it is extremely complicated to elucidate *in vivo* pharmacological or mode of action of 30 ginsenosides. Therefore, it has been generally accepted that these ginsenosides and other active ingredients work in a concerted manner.

LD_{50} of TCDD in male rats was known to be 46 μg/kg b.w. (*i.p.*). In our preliminary test to find TCDD dose that induces oligospermia and asthenospermia without causing death of the exposed animal, rats were injected (*i.p.*) with single doses of 5, 10, 20 and/or 40 μg/kg b.w. of TCDD. No rat died during the experimental period of 6 weeks. However, body weight gain, sperm head count and testis weight decreased in a dose-dependent manner. Blood chemical parameters were also deteriorated by TCDD in a dose-dependent manner. This preliminary test afforded us to determine the TCDD dose in which selective oligospermia and asthenospermia are induced.

Ginseng saponin has been thought to be the active ingredient effective against TCDD toxicity, especially testicular toxicity due to the structural similarity of ginsenoside to the cholesterol, a precursor of steroid hormone. To confirm this hypothesis, a crude saponin fraction was prepared from Korean red ginseng by the patented method[19]. Purity of ginsenoside in the crude saponin was higher than 40% when determined by HPLC.

The survival rate of TT and CS40 groups was 90%, that of the CS5 and CS20 groups was 95%, and that of NC and CS20 was 100%. The difference in survival rate between preliminary and this test might be due to the stress induced by daily oral administration of vehicle and KRG-CS. In addition, it appeared to us that high dose of ginseng saponin can be stress in an animal exposed to toxic chemicals. Body weight of NC group rats increased 45.6% during the 5 weeks of experimental period. However, body weight gain of TT group animals slowed down greatly, and even decreased 1 week after TCDD-exposure. Although body weight gain of KRG-CS-treated group animals was significantly retarded compared with that of the NC, the weight increased steadily.

Liver weight of the TT and the CS groups was significantly higher than that of the NC but there was no significant difference between them. The increase in liver weight of CS groups cannot be regarded as a pathological hypertropy because clinical chemical parameters related to liver function were significantly improved by KRG-CS-treatment. Testis weight of TT group accounts for 89.7% of the NC ($p<0.01$). There was no difference in testis weight between the NC and CS groups in spite of TCDD-exposure. However, testis weight of CS groups was significantly increased compared with that of the TT ($p<0.05$-0.01). This result indicates that protective effect of KRG-CS on testis is highly selective.

Sperm head count in TT group decreased to 82.6% compared with that of the NC. This result coincides well with the testicular weight decrease by TCDD. Sperm head count in KRG-CS-treated group animals showed higher value than that of TT. There was no significant difference in sperm head count between the NC and CS groups no matter what TCDD was injected to CS group animals. In this study, sperm motility of the CS groups was greatly improved compared with that of the TT. The ratio of sperm with straight progression was greatly increased by the treatment of KRG-CS. This result further suggests that protective effect of KRG-CS against TCDD-induced testicular toxicity is highly specific and potent. More dramatic difference in testicular size and sperm head count could be expected if the rats were exposed to TCDD in younger age. However, this animal model was remarkably reproducible and selective enough to screen, in relatively shorter period of time, an agent that possesses protective effect against testicular toxicity induced by TCDD.

Although the exact mechanisms of KRG-CS against TCDD-induced toxicity, especially testicular toxicity are still unknown, our data strongly suggest that Korean red ginseng saponin attenuates the toxicity of TCDD and might play a beneficial role in organs in which TCDD has toxic effects. Studies are now in progress in our laboratories to elucidate the basic molecular mechanisms of protective and therapeutic action of Korean red ginseng against TCDD.

Advances in biochemical and hormonal research on male reproduction and improved methods of artificial insemination might be combined with medical therapy to enhance a couples fertility, although results vary depending on the etiology of male infertility. Generally, it is preferable to try to improve spermatogenesis rather than ignore any male factors. Many empirical treatments have been applied to patients to improve the quality and number of sperm, although the results have not been promis-

ing.[27]

In this respect, these research findings including our previous report on protective and therapeutic effects of Korean red ginseng on clinical chemical parameters,[25] and atrophy and testicular damage[18] induced by TCDD in male guinea pigs could be a promising therapy to enhance a couples fertility. From the morphological results, we hypothesized that Sertoli cells might be the target sites of TCDD in guinea pig testis, which in turn leads to changes in the germ cells. On the other hand, Korean red ginseng might have protective effects on the blood-testis barrier and can reverse the damaging effects of TCDD on the barrier.[18]

These results could lead us to conclude that crude saponin might be the active ingredient of Korean red ginseng that alleviates toxic effects, especially testicular toxicity, of TCDD.

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