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The Therapeutic Effect of Tissue Cultured Root of Wild *Panax ginseng* C.A. Mayer on Spermatogenetic Disorder

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This study examined the possibility of using a tissue cultured root of wild *Panax ginseng* (tcwPG) as a fertility agent. The effect of tcwPG on spermatogenesis was studied using male rats. The tcwPG crude powder was administered orally to 7-week-old rats over a 6-week period. The number of sperm in the testes and epididymides was significantly higher than the control. A histological examination did not reveal any morphological changes in the testes from the tcwPG powder treated rats. Moreover, there were no significant differences in the weights of the heart, spleen, liver, kidney, brain, testes and epididymides. Oligospermia was also induced by administering 2,3,7,8-tetrachlorodaibenzo-*p*-dioxin (TCDD) to the rats in order to estimate the feasibility of using tcwPG as treatment for infertility caused by spermatogenic disorders. After exposing the rats to TCDD, the tcwPG saponin fraction treated rats showed some improvement in the body weight, sperm number and testis morphology. It was estimated that tcwPG had feasibility as a therapeutic agent on spermatogenic disorder.

Key words: Tissue culture, Panax ginseng, TCDD, Spermatogenesis, Fertility, Sperm

INTRODUCTION

Wild *Panax ginseng* is well known as a mystic medicinal herb, which has been widely used as a tonic in traditional oriental medicine. Recently, tissue cultured methods of wild *Panax ginseng* were developed, which has made its mass production possible (Hahn *et al.*, 2003). However, there are relatively few pharmacological reports of the tissue-cultured root of wild *Panax ginseng* (tcwPG).

We are able to presume the pharmacological effects of wild *Panax ginseng* as reports of *Panax ginseng* (PG). Many clinical and experimental studies have suggested that PG has beneficial effects as an medicinal herb for general physical strength, blood sugar stabilization (Onishi *et al.*, 1996; Oshima *et al.*, 1987), immune system strengthening (Scaglione *et al.*, 1996), anti-inflammatory effects (Matsuda *et al.*, 1990, 1991), antihepatotoxicity effects (Zuin *et al.*, 1987), and a protective action against non-organ specific cancers (Yun and Choi, 1998; Yun, 2001). In addition, it has been used for treating many other conditions.

We screened pharmacological effect of tcwPG on spermatogenesis in rat. Furthermore, We estimated the repairing effect of the saponin fraction of tcwPG on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induced spermatogenic disorder. TCDD and its related congeners have been shown to act as developmental and reproductive toxicants (Peterson *et al.*, 1993; Sommer *et al.*, 1996; Chaffin *et al.*, 1996). This group of toxicants decreases the testicular and accessory sex organ weight, alters the testicular morphology, and decreases the level of sperm production (Mably *et al.*, 1992 c). The present study was conducted to evaluate the possibility of using tcwPG as a fertility agent on infertility caused by spermatogenic disorder.

MATERIALS AND METHODS

Animal model

Six-week-old Sprague-Dawly albino male rats (Samtako, Kyungkido, Korea), weighing 200 g, were used in this study.

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The animals were maintained under controlled conditions of temperature (23 ± 1°C), relative humidity (55 ± 10%), and 12-h lighting per day. Standard laboratory chow and tap water were provided *ad libitum*. The rats were allowed one week to acclimatize to the experimental environment.

Administration of tcwPG crude powder to rat

The air dried root of tcwPG was used after pulverizing it with a motor grinder (Retsch, Germany). The rats were assigned to groups (9 rats/group) according to their body weight. The sieved powder of tcwPG (under 75 μm) was dispersed in purified water and administered to the rats. The rats were given a daily oral gavage of 0mg/kg (control), 25 mg/kg (group I), 50 mg/kg (group II) and 100 mg/kg (group III) for 6 weeks. The volume of the tcwPG suspension administered was 2 mL/kg. The body weight was recorded before the experiments, every 3 or 4 day, and on the day of the necropsy. The actual dosing volumes were adjusted according to the body weights recorded.

Preparation of the saponin fraction of tcwPG

tcwPG was provided by CBN Biotech (Cheongju, Chungbuk, Korea) and the saponin fraction was obtained using the method reported by Furuya and Court (Furuya and Yoshikaya, 1987; Court *et al.*, 1996). Dried, pulverized roots (1 g) were extracted with 70% absolute alcohol (50 ml) at 80°C for 2 h, extracted twice, and filtered through filter paper (Whatman No. 1). The extract was evaporated to dryness and dissolved in 50 mL of purified water. The water-soluble fraction was washed again twice with the same volume of ether. The water phase was extracted three times with 50 mL of *n*-butanol saturated with water and washed twice with 20 mL of water. The saponin fraction was obtained by evaporation of the organic phase and dried under vacuum at -70°C.

HPLC analysis of the saponin fraction of tcwPG

The saponin fraction was analyzed by using HPLC system (Waters 2690 separation module; Waters 996 photodiode array detector; Waters millenium 2010 chromatography manager) on an Nova-Pak C_{18} column (diameter 5.0 μm, 150×3.9 mm), with water and acetonitrile as the mobile phase. The ratio of water (A) and acetonitrile (B) for initial were 84%:16%, 21%B 15 min, 34%B 55 min, 45%B 65 min, 65%B 85 min, 80%B 95 min, 85%B 101 min, 16%B 110 min, respectively. Flow rate of the mobile phase was 0.6 mL/min and monitoring of saponins was 203 nm.

Induction of oligospermia by TCDD in the rats, and repair effect of the saponin fraction of tcwPG

The saponin fraction of tcwPG was also administrated to the rats in order to estimate the repairing effect of this extract on TCDD induced oligospermia. The saponin fraction of tcwPG was dispersed in purified water and administered to the rats once daily *via* an oral gavage at dose of 30 mg/kg. After administering the saponin fraction for a week, the rats were treated with a 50 μg TCDD/kg injection i.p.. The saponin and TCDD doses were determined according to a previous paper (Hwang *et al.*, 2003). The body weight and daily food intake were measured every 3 or 4 days. The control, TCDD exposed and saponin fraction treated after TCDD exposure groups were sacrificed 4 weeks after TCDD exposure.

Sperm count

The male rats were weighed and sacrificed on the day of the final dose. The rats were anesthetized using ether. The weight of the liver, heart, kidney and spleen were recorded individually. The right testis and epididymis were weighed and used for sperm analysis. The number of sperm heads in the testis and epididymis was assessed by counting the number of sperm heads using a slight modification of the procedure reported by Toth *et al.* (1989).

For the testes, the tunica albuginea was removed and the remaining tissue was placed in 10 mL of 0.9% saline. The tissue was then homogenized twice with a homogenizer for 30 seconds and treated with an ultrasonic cleaner for 3 minutes. The homogenate was then diluted to a suitable concentration, and 100 μL of the dilution was placed on a hemocytometer, covered with a cover glass, and the number of sperm was counted using an optical microscope (Nikon, YS 100).

The epididymides were cut into small pieces with scissors, placed in a 50 mL centrifuge tube, and 6 mL of 0.9% saline was added. The tissue solution was homogenized three or four times for 30 seconds each with a homogenizer, and the homogenate was diluted to the required concentration. The number of sperm was determined using the same method described above.

Testicular histology

One (left) of the testes from each animal was fixed in Bouin's fluid, dehydrated in graded ethanol, cleared in xylene and embedded in paraffin wax. 5 μm thick sections were cut, stained with Harris' hematoxylin and eosin, and observed under a microscope.

RESULTS

Comparison of saponin contents in tcwPG and PG

HPLC analysis showed that tcwPG and PG contains ginsenoside Rg1, Re, Rf, Rg2, Rh1, Rb1, Rc, Rb2, Rb3,

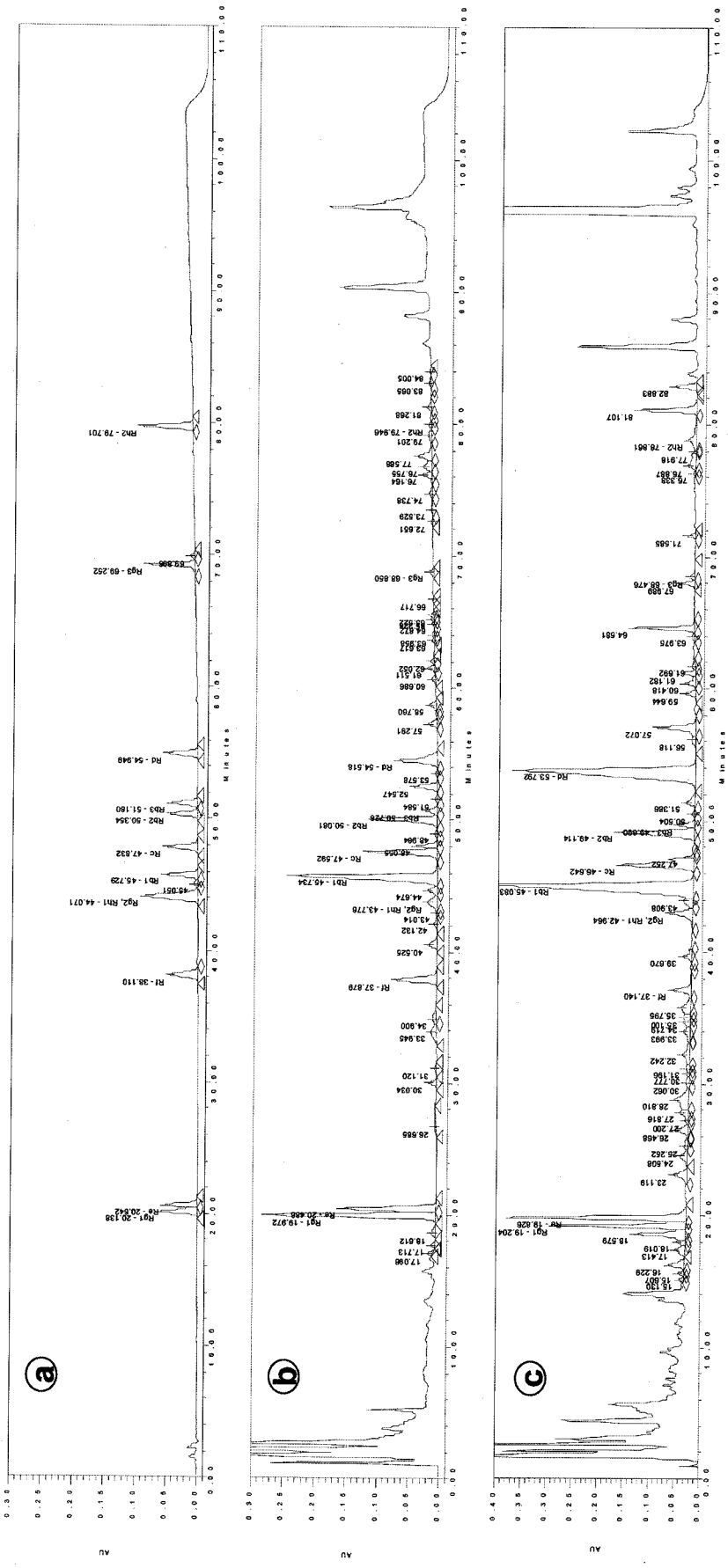

Fig 1. HPLC chromatograms of ginsenosides of standard (a), PG (b) and tcwPG (c). Total ginsenosides of PG and tcwPG were 4~5% and 5~8%, respectively. tcwPG was contained higher ginsenoside Rd than PG.

Table I. The comparison of the content of ginsenosides in the tcwPG and PG

Ginseng sources	Ginsenosides (mg/g)		
	Triol	Diol	Total
PG	5.25 ± 0.15	6.10 ± 0.40	11.35 ± 0.55
tcwPG	4.34 ± 0.80	16.74 ± 1.05	21.09 ± 1.03

Each value represents mean ± S.D. of three replicate vessels.

Rd, Rg3 and Rh2 (Fig. 1). Especially, tcwPG contains much more ginsenoside Rd than PG. Ginsenoside Rd has known as promote the secretion of glucocorticoid. Total saponin contents of tcwPG and PG were 21.09 ± 1.03 mg/g and 11.35 ± 0.55 mg/mL, respectively. Total saponin of tcwPG contains 1.85 times higher than PG (Table I).

The number of sperm after administering the tcwPG powder in normal rats

As shown in Fig. 2, all the animal groups administered tcwPG had a higher number of sperm in both the testes and epididymides in a dose dependant manner than the control group. The number of sperm heads in the testis in the 25 mg/kg, 50 mg/kg and 100 mg/kg dosing group were approximately 6%, 15% and 28% higher than those in the control group after 6 weeks of treatment, respectively. In the epididymis, the number of sperm heads was approximately 15%, 18% and 29% higher than the control. In particular, group III, which was administered 100 mg/kg of tcwPG powder, showed a significant increase in the number of sperm compared with the control group (ANOVA test, $P<0.05$).

The weight of each of the organ was calculated as a weight percentage to body weight (Table II). The final body weight of all the groups was similar to the control. In addition, there were no significant differences in the weights of the heart, spleen, liver, kidney, testes and epididymides. It is assumed that tcwPG induced a dose dependent increase in spermatogenesis without altering the general organ weights.

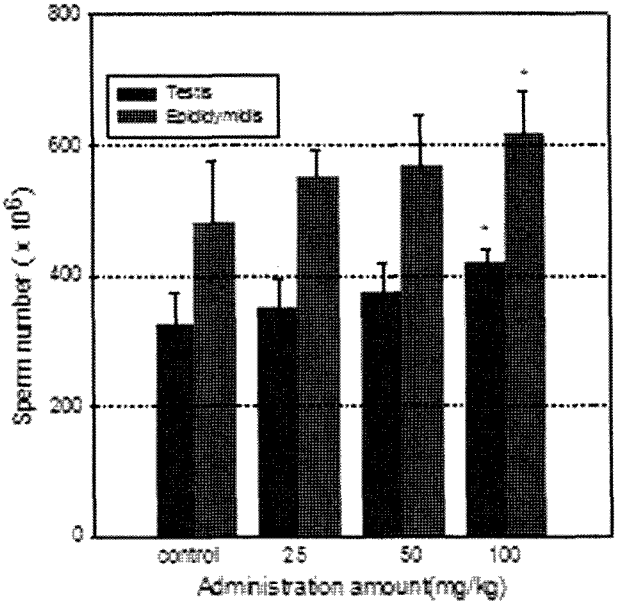

Fig. 2. The number of sperm in the testis and epididymis according to amount of tcwPG powder administered to the rats over a 6-week period. Data are presented as mean ± S.D. (n=9).

The number of sperm in the TCDD-exposed rats administered the saponin fraction of tcwPG

As shown in Fig. 3, the TCDD exposed group showed a decrease in body weight at one week after TCDD exposure compared with the control group. There was less weight loss in the tcwPG treated group. In addition, 2 out of 9 rats in the group exposed to TCDD died with severe weight loss during the period of experiment. The TCDD exposed group showed significant weight loss of the testis and epididymides as well as in the final body weight compared with the control group (Table III). On the other hand, the group administered the saponin fraction of tcwPG after TCDD exposure showed some repair of the weight loss of the body, testis and epididymides compared with the group not treated with tcwPG. In addition, some increase in the number of sperm was observed in the testis (Table IV). It is considered that the saponin fraction of tcwPG has

Table II. The weight percentage* of organs after administering tcwPG powder to the rats for 6 weeks

Weight (g)	Control	Group I	GroupII	GroupIII
Body	367.8 ± 22.24	375.6 ± 24.17	377.8 ± 15.43	377.2 ± 21.08
Heart	1.376 ± 0.131	1.246 ± 0.147	1.243 ± 0.109	1.244 ± 0.118
Liver	15.67 ± 1.711	17.43 ± 1.253	18.02 ± 1.784	15.97 ± 1.828
Spleen	0.923 ± 0.142	0.877 ± 0.123	0.940 ± 0.083	0.900 ± 0.149
Kidney	2.872 ± 0.259	2.938 ± 0.241	2.904 ± 0.199	2.817 ± 0.295
Testis	3.199 ± 0.169	3.145 ± 0.252	3.185 ± 0.303	3.175 ± 0.177
Epididymis	1.143 ± 0.058	1.111 ± 0.070	1.103 ± 0.119	1.079 ± 0.095

* Organ weight/body weight × 100
Data are presented as mean ± S.D.(n=9).

Table III. The weight of the body, testis and epididymis after administering tcwPG saponin to the TCDD treated rats

Weight (g)	Control	TCDD alone	TCDD + tcwPG saponin
Body	376.5 ± 19.50 [a]	207.0 ± 25.31	252.3 ± 33.72
Testis	3.569 ± 0.145	2.658 ± 0.182	3.120 ± 0.189
Epididymis	1.098 ± 0.050	0.783 ± 0.114	0.967 ± 0.066

Data are presented as mean ± S.D.(n=7~9).

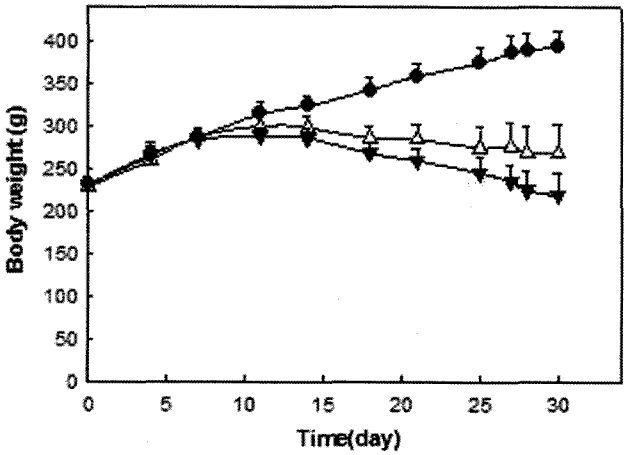

Fig. 3. The effect of the saponin fraction of tcwPG (30mg/kg) on the body weight of male rats. The rats were exposed at age 8 weeks with 50ß¹/kg TCDD and their body weights were recorded until 12 weeks. Data are presented as mean ± S.D. (n=7~9). Key : (○); Control, (●); TCDD alone, (▲); TCDD + saponin fraction of tcwPG.

some therapeutic effect against weight loss, decreased sperm production as well as testicular atrophy by TCDD.

Histopathology of the testis from the tcwPG powder treated rat

No histopathological alterations in the testes were observed in any of the groups (Fig. 4). Leydig's cells and sertoli cells showed a normal appearance. There appeared to be more sperm development in the experimental groups compared with the control group.

Histopathology of the testis in the saponin fraction of tcwPG treated rat after TCDD exposed

In the control group, the histopathology examination of the seminiferous tubules showed the usual arrangement of Leydig's cell, sertoli cell and intracellular spaces (Fig. 5 A). In the TCDD-treated rat, there was a lower level of cell differentiation in most cells, including spermatogonia compared with the control (Fig. 5B). In addition, there was a decrease in the level of maturation in the spermatocytes and spermatids. In the group given the saponin fraction of tcwPG (Fig. 5C), all sperm developmental stages were almost completely repaired compared with the single TCDD treatment group (Fig. 5B). Furthermore, there was a higher level of sperm development than in the positive control group. There was no significant difference between the tcwPG powder treated rats (Fig. 4B) and the rats treated with the saponin fraction after TCDD exposure (Fig. 5C).

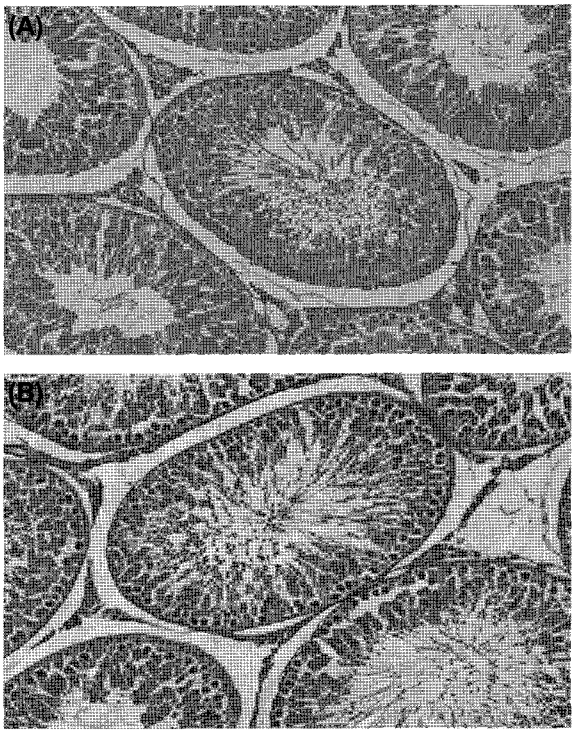

Fig. 4. The histology of the testis of rats treated with the tcwPG powder. H & E. Magnification approximately × 100. In the tcwPG powder treated group (B), Leydig cells and sertoli cells were normal in appearance and there was increased sperm development compared with the control group. (A) Control, (B) tcwPG powder treated.

Table IV. The number of sperm in testis after administering tcwPG saponin to TCDD treated rats

	Testis(g)(R)	Sperm/Rat [a]	Sperm/g	Sperm/day [b]
Control	1.774 ± 0.142	260,312,500 ± 46,970,320	146,737,599 ± 26,477,069	42,674,180 ± 7,700,052
TCDD alone	1.336 ± 0.179	144,642,857 ± 21,538,656	108,265,589 ± 16,121,748	23,711,943 ± 3,530,927
TCDD + tcwPG saponin	1.541 ± 0.118	182,500,000 ± 22,717,206	118,429,591 ± 14,741,860	29,918,032 ± 4,022,522

b) a)/6.1, 6.1 is the period of spermatid development from stage I to XIV.
Data are presented as mean ± S.D.(n=7~9).

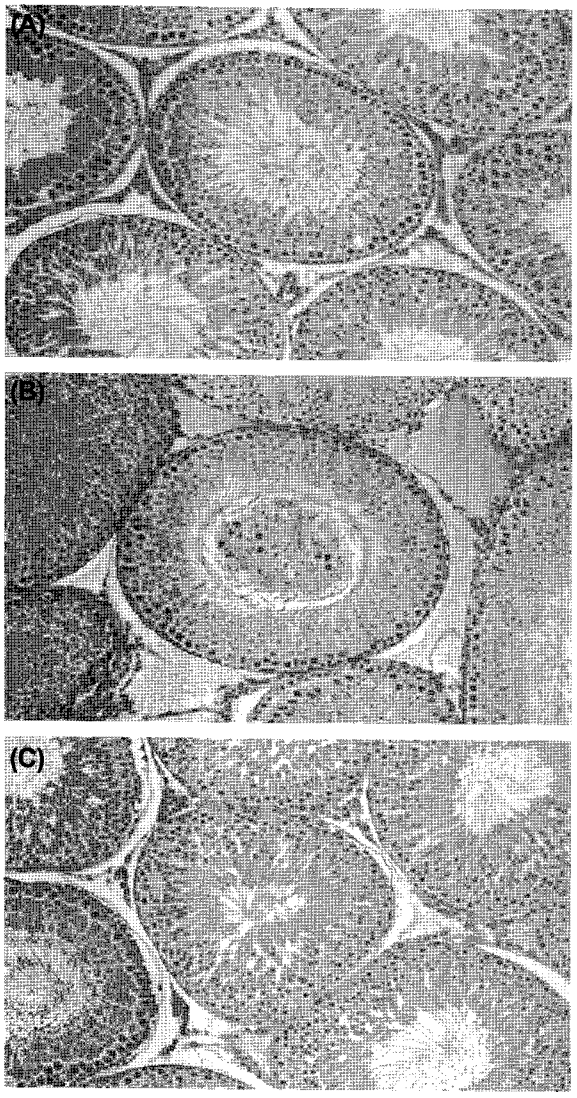

Fig. 5. The Effect of the saponin fraction of tcwPG on the histology of the testis after exposing the rats to TCDD. H & E. Magnification approximately × 100. In the TCDD-treated rat(B), there was a decrease in cell differentiation in most cells, including spermatogonia compared with the control. In the saponin fraction of tcwPG treated group (C), sperm development was higher than the positive control group. (A) Control, (B) TCDD exposed, (C) saponin fraction of tcwPG treated after exposing the rats to TCDD.

DISCUSSION

Spermatocytogenesis has been reported to occur in Wistar rats at the age of 30 days whereas spermiogenesis begins at 50 days (Yves and Bernard, 1969; Brown *et al.*, 1975). Seven-week-old Sprague-Dawley albino rats were used, and tcwPG powder was administered orally to the rats over a 6-week period to check for spermatogenesis. It was found that the number of sperm in the testes and epididymides of the male rats treated with the tcwPG powder was significantly higher than the control. The final body and organ weights of all groups were similar to the control. It was assumed that tcwPG produced dose related effects on spermatogenesis without altering the general organ weight.

It was reported that male rats exposed to TCDD show adverse effects such as weight loss of the testicles, seminal vesicle and prostate gland, and changes in the form of the testes (Johnson *et al.*,1992; Rune *et al.*, 1991). This study investigated the repairing effect of tcwPG against the toxicity of TCDD in rats. 50 μg/kg of TCDD caused severe damage to the testis (Table III, Fig. 5B). When the saponin fraction of tcwPG was administered to the TCDD exposed rats, there was some repair of the testicular atrophy observed and some increase in the daily sperm production (Table IV, Fig. 5C). The body or testicular weight in the tcwPG treatment group was higher than the control group. These results show that tcwPG has potency of testicular development and subsequently affects sperm production.

Generally, the causes of male infertility include semen or sperm abnormalities, sperm transport disorders, and aspermia. Among these causes, more than half are spermatogenesis disorders resulting from a disorder of the testis function in producing sperm as well as abnormalities of the sperm components. Spermatogenesis is affected by a wide variety of nutrients and enzymes, such as Vitamins (C, E, B12) (Rolf *et al.*, 1999; Sandler *et al.*, 1984), minerals (Zinc, Copper, Selenium, Calcium) (Scott *et al.*, 1998), amino acids (Schacter *et al.*, 1973), Coenzyme Q10 (Lewin, 1997) etc.. PG was demonstrated to increase the spermatozoa count and motility, as well as the testosterone level in patients with fertility problems (Salvati *et al.*, 1996).

In a preliminary study, tcwPG powder dispersed in purified water was used to determine if there was any spermatogenetic effect in rats. Generally, it is known that the effects of PG are due to their numerous ginsenosides. Substances identified in tcwPG include a number of ginsenosides, polysaccharides, polyphenols, some minerals and etc. In addition, their contents are much higher than in PG (not shown data). Accordingly, the saponin fraction of tcwPG was used to estimate the effect of tcwPG on repairing TCDD-induced spermatogenetic disorder. However, the saponin fraction had lower repairing effect than expected. Polyphenols have antioxidant activity similar to Vitamin C and E, which can enhance fertility by decreasing the level of free-radical damage to sperm cells (Fraga *et al.*, 1991; Geva *et al.*, 1996). The administration of tcwPG powder to rats caused an increase in the number of sperm in the testis and epididymides of male rats. These spermatogenic effects of tcwPG might be due to the combined effect of its many constituents, ginsenosides, polyphenol and minerals. Therefore, more study

will be needed to identify the active components, saponin fraction and/or another components in tcwPG related to the repair of spermatogenetic disorders.

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