Int J Clin Exp Med 2017;10(4):6926-6933
www.ijcem.com /ISSN:1940-5901/IJCEM0043396

Original Article

Effect of total terpenes of *Cornus officinalis Sieb.* on hypogonadism in streptozotocin (STZ)-induced diabetic rats

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Received November 1, 2016; Accepted February 14, 2017; Epub April 15, 2017; Published April 30, 2017

Abstract: As one of complications of diabetes, male hypogonadism become a growing significant problem in the diabetic patients in the worldwide. *Cornus officinalis Sieb.*, a well-known traditional Chinese herb, had been applied in the diabetic mellitus for a long time. However, according to the records in ancient Chinese medical literature, *Cornus officinalis Sieb.* possessed the effect in improving the sexual dysfunction and testis damage. But there were few experimental evidences to confirm this. The present study was to investigate the effects of total terpenes from *Cornus officinalis Sieb.* (TTC) on the hypogonadism in streptozotocin (STZ)-induced experimental diabetic rats. Male Sprague-Dawley rats were administrated with STZ at a single dose of 45 mg/kg to induce diabetic mellitus. TTC was administrated at 50 mg/kg, and anti-diabetic drug Rosiglitazone and androgen drug Methyltestosterone were administrated as control compounds. The sexual behavior of diabetic rats was attenuated with decay latency and decreased frequency of copulation compared with that of normal control. Serum glucose and testosterone level were lower than that of normal control. Lactate dehydrogenase (LDH), acid phosphatase (ACP), and γ-glutamyltranspeptidase (γ-GT) activity in the testis tissue were weakened in diabetic rats. Thinner seminiferous epithelium and less the sperms in the seminiferous tubules were observed in the testis of diabetic rats. TTC ameliorated these alterations in diabetic rats obviously, possibly ascribing to its effect in regulating sex hormones, special enzymes and glucose level.

Keywords: *Cornus officinalis Sieb.*, diabetic, hypogoladism, terpenes, testis

Introduction

For a long time, diabetes mellitus (DM) complications arose more and more attention. As one of complications of diabetes, male hypogonadism become a growing significant problem in the diabetic patients in world-wide, which was characterized by low testosterone level, testicular dysfunction, impaired spermatogenesis, diminished libido, and so on [1, 2]. The incidence of erectile dysfunction (ED) in diabetic patients was as high as three times of non-diabetic people. Between the ages 40 to 70, the prevalence of ED in diabetic population is 75% [3]. Hypogonadism in the diabetic state was caused by several factors including alterations on neuroendocrinal and microvessel [4]. In experimental diabetic animals, the similar symptoms were also observed [5-7]. Sustained hyperglycemia impaired the hypothalamus-pituitary-gonad axes, which regulated the release of the testosterone, spermatogenesis and arouse of sexual desire [8].

At present, few medicines could be applied to control the testis impair induced by diabetes. Oral hypoglycemic medication or insulin cannot attenuate the deterioration of testis damage induced by diabetes mellitus. Some researchers turned their attention to the traditional Chinese medicines which have few adverse effects and be suitable to administrate for long-term treatment. *Cornus officinalis Sieb.*, a well-known traditional Chinese herb, had been applied in the diabetic mellitus (in Chinese "Xiao Ke") for several hundred years. It showed

benefit effects on the diabetes and its complications [9-12]. It was reported that the ether extract of *Cornus officinalis Sieb.* showed the effect in lowering serum glucose level in diabetic rats induced by STZ [13]. In addition, according to the records in ancient Chinese medical literature, *Cornus officinalis Sieb.* possessed the effect in improving the sexual dysfunction and testis damage. But there were few experimental evidences about this. In the present study, total terpenes were extracted and isolated from *Cornus officinalis Sieb.*, and the effects on testis damage were observed to confirm the hypothesis in STZ-induced diabetic rats.

Materials and methods

Extracts preparation

The plant materials were purchased from the Changsha medicine commercial Co.Ltd and were identified as *Cornus officinalis Sieb.* by the pharmacognosy department, Hu'nan University of TCM. Upon being dried at 60°C for 8 h, the materials were extracted twice with the ether, each for 1.5 h. The extract solutions were filtrated, and concentrated under low pressure until little ether remained. Then plenty of distilled water was added into the concentrated extract until yellow-white precipitates produced. These precipitates were collected and dried in vacuum, gaining a yellow-white powder [13].

The product was subjected to test with Libemann reaction reagents, and the results confirmed that terpenes were present in the extract. Then the extract was analyzed by a high-performance liquid chromatography-diode array detection (HPLC-DAD) method [14]. HPLC chromatogram was established based on the data at 210 nm, and loganin, oleanolic acid and ursolic acid were identified [15].

Animals and treatments

The present study was approved by our local Research Ethic Committee. Sprague-Dawley rats of approximately 250 g were obtained from experimental animal center of Hu'nan University of Chinese Medicine and fed with rat chow and water *ad libitum*. STZ (Sigma, Lot 2243F) was dissolved in citrate buffer and administrated intraperitoneally (*i.p*) to rats with the dose of 45 mg/kg to induce diabetes mellitus. Blood glucose levels were monitored and on the 7th day those animals that blood glucose level was more than 16.7 mmol/L were selected as diabetic rats [16]. On day 28 the animals were randomly divided into several groups as follow: untreated diabetic group, the diabetic groups treated respectively with TTC (50 mg/kg, *P.O*), Methyltestosterone (5 mg/kg, *P.O*), Rosiglitazone (4 mg/kg, *P.O*), and a non-diabetic group as normal control, 15 rats each group. Six weeks later animals were sacrificed. Blood samples were collected for biochemical analysis, and testis tissues were removed for biochemical and histological analysis.

Sexual behavior assessment

Sexual behavioral testing was performed under dim red lights in the dark room. Sexual behavior was assessed by placing the male in an open box, 5 min later two female rats were put into the box. Then the latency to the first copulation, the copulation number of male rats was recorded within 20 min [17].

Blood and testis chemistry

Animals were anesthetized with the ether and then exsanguinated. Blood samples were collected from vena cervicalis and centrifuged to gain the serum for the measurement. The left testes were decapsulated and homogenized in the normal saline (N.S) with glass homogenizer in ice bath to prepare a 10% (g/ml) homogenate. The homogenate was centrifuged at 3000 *g* for 15 min, and then the supernatant was collected. Glucose in the serum, LDH, ACP, γ-GT in the testis was measured according to the instruction of assay kits provided by Nangjing Jiancheng biomedical engineering Ltd.Com. Insulin, testosterone, folic-stimulating hormone (FSH), and luteotrophic hormone (LH) in the serum was determined by radioimmunonity analysis.

Histological examination

The right testis was fixed with 10% phosphate-buffered formalin solution, and embedded in paraffin. After dehydration with the ethanol, the blocks were cut into sections with a thickness of 4 μm, stained with hematoxylin-eosin (H-E), and observed under the light microscope.

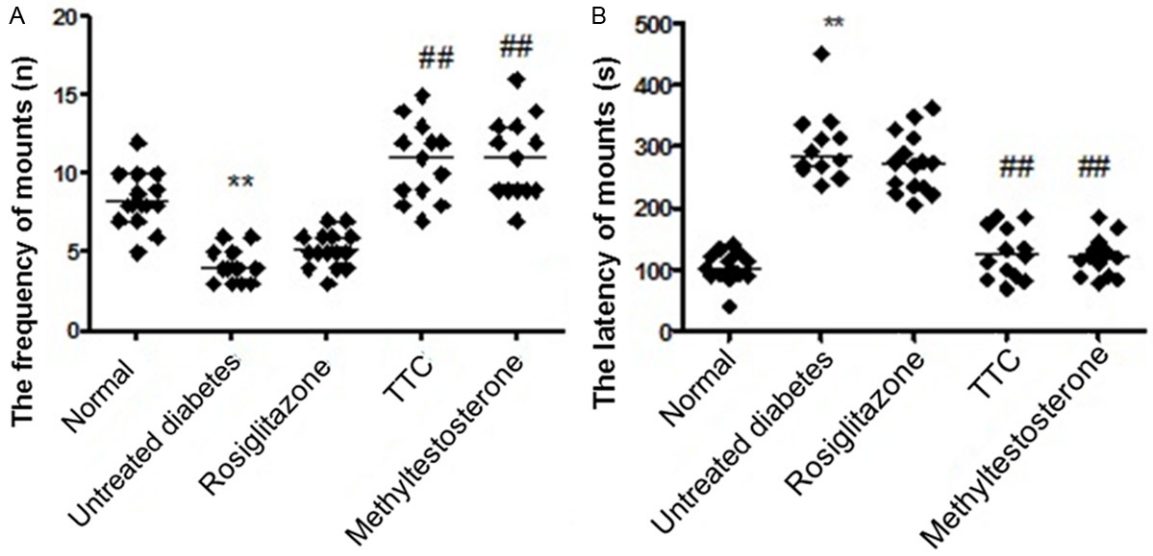

Figure 1. A: Effect of TTC on the frequency of mount of rats with diabetes mellitus. B: Effect of TTC on the latency of mount of rats with diabetes mellitus. $^{**}P<0.01$ *v.s.* normal control; $^{\#\#}P<0.01$ *v.s.* untreated diabetic group. Animal number: Normal: 15; Untreated diabetes: 12; Rosiglitazone, TTC and Methyltestosterone: 13.

Table 1. Effect of TTC on the levels of serum glucose, insulin, testosterone, LH and FSH in STZ-induced diabetic rats (Mean ± SD)

Group	n	Dose ($mg{\cdot}kg^{-1}$)	Glucose (mmol/L)	Insulin (mIU/L)	Testosterone (nmol/L)	FSH (mIU/ml)	LH (mIU/ml)
Normal	15	-	7.7±1.2	6.2±0.5	14.7±0.8	1.92±0.43	11.2±1.4
Untreated diabetic	12	-	22.7±2.4**	4.1±0.8**	8.2±1.1*	1.68±0.32	9.8±0.7*
Rosiglitazone	13	4	13.2±1.8$^{\#\#}$	5.7±0.9$^{\#\#}$	8.6±0.9	1.73±0.21	10.2±0.9
TTC	13	50	16.5±2.1$^{\#}$	4.9±0.4$^{\#}$	11.7±1.4$^{\#}$	1.79±0.36	10.4±1.3
Methyltestosterone	13	5	20.8±2.2	4.3±0.5	13.2±1.2$^{\#\#}$	1.81±0.55	10.1±1.1

$^{*}P<0.05$, $^{**}P<0.01$ *v.s.* normal control; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *v.s.* untreated diabetic group.

Statistical analysis

The measurement data were expressed as Mean ± SD. All the analysis of data were conducted by software PASW 18.0. Data were subjected to one-way analysis of variance (ANOVA), followed by multiple comparisons with least significant differences (*LSD)* test or Dunnett's test as appropriate. The data on sexual behavior assessment were analyzed with a non-parameter test of Kruskal-Wallish, followed by multiple comparisons with Nemenyi test. Statistical significance was considered with $P<0.05$.

Results

Sexual behavior assessment

According to the observation, the copulation latency of diabetic rats was significantly delayed (**Figure 1**). These alterations were ameliorated in TTC and methyltestosterone treated group ($P<0.01$). But no obvious change was observed in Rosiglitazone treated group ($P>0.05$).

The copulation frequency of diabetic rats appeared less than that of normal control. The rats in TTC, methyltestosterone but not rosiglitazone treated group showed increasing copulation frequency in 20 min ($P<0.01$).

Blood biochemistry

In diabetic rats, serum glucose increased nearly 3 folds than that of the control, and insulin level decreased obviously (**Table 1**). Rosiglitazone recovered the insulin and glucose level obviously, and TTC showed a mild amelioration on these ($P<0.01$, $P<0.05$), while methyltestosterone had no significant effect on serum insu-

Table 2. Effect of TTC on the activity of ACP, LDH, γ-GT in the testis of diabetic rats induced by STZ (Mean ± SD)

Group	n	Dose ($mg \cdot kg^{-1}$)	ACP (U/g protein)	LDH (U/g protein)	γ-GT (U/g protein)
Normal	15	-	219.2±36.5	3457.6±452.3	274.7±32.5
Untreated diabetic	12	-	124.7±22.1**	2346.5±263.4**	123.6±21.8**
Rosiglitazone	13	4	132.8±25.7	2418.3±212.5	131.7±41.7
TTC	13	50	153.4±21.5##	2785.3±215.8##	156.4±36.3##
Testosterone	13	5	142.6±26.3#	2697.6±189.6##	168.2±27.5##

**$P<0.01$ *v.s.* normal control; #$P<0.05$, ##$P<0.01$ *v.s.* untreated diabetic group.

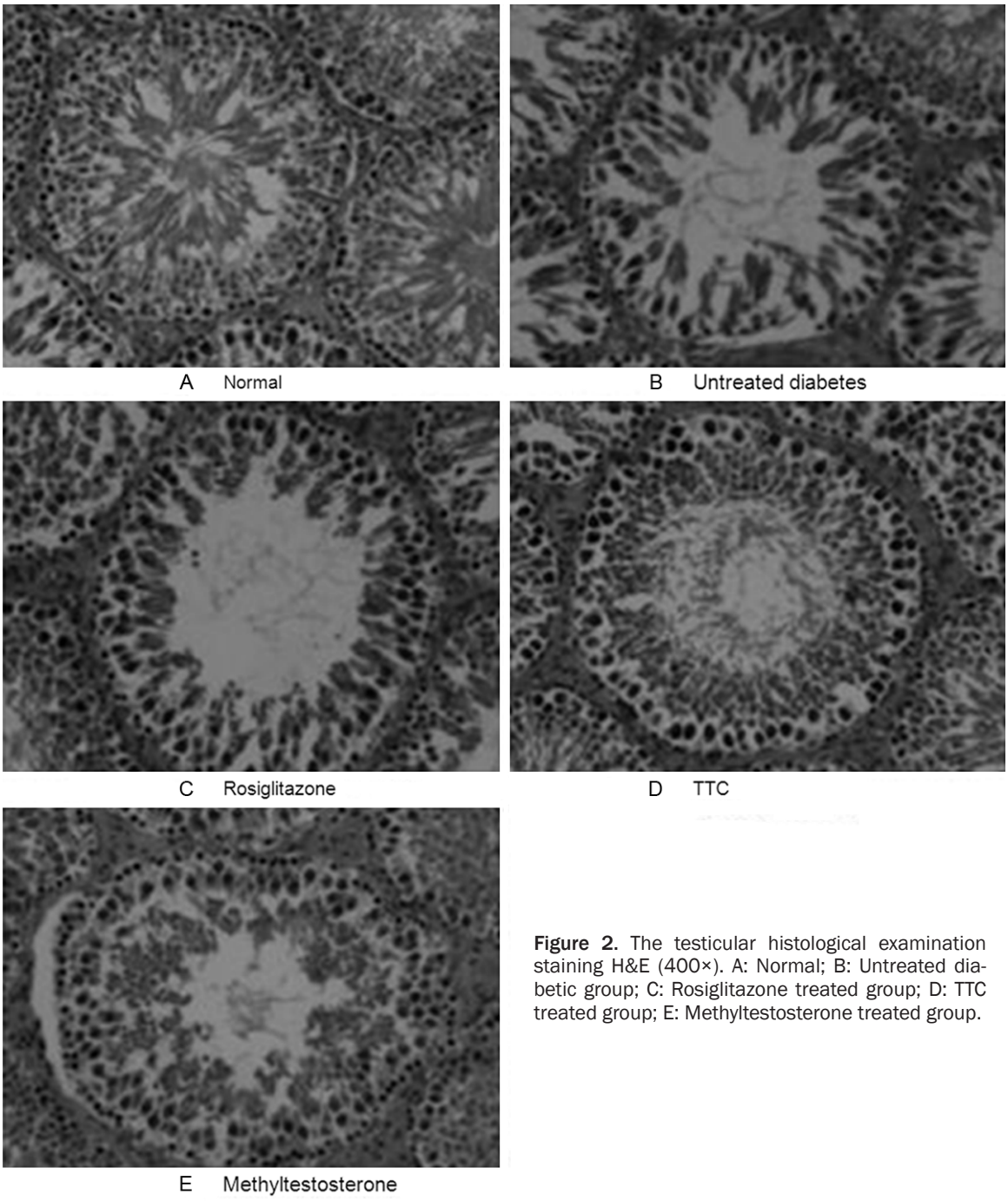

Figure 2. The testicular histological examination staining H&E (400×). A: Normal; B: Untreated diabetic group; C: Rosiglitazone treated group; D: TTC treated group; E: Methyltestosterone treated group.

lin and glucose levels ($P>0.05$). Compared with normal control group, serum testosterone and LH levels of untreated diabetic group decreased, but no obvious changes were found in Rosiglitazone, TTC or methyltestosterone treated groups (**Table 1**). Little changes were found in FSH level among all groups ($P>0.05$).

Testis biochemistry

ACP, LDH and γ-GT activity in the testis of diabetic rats showed a markedly decline (**Table 2**). TTC and methyltestosterone improved these changes in some extent ($P<0.01$), but Rosiglitazone had no effects on these ($P>0.05$).

Testis histological examination

In the normal rats, testis seminiferous epithelium was thick, and the epithelium cells aligned tightly, closely connecting with the basement membrane. Filar sperms were filled with the center of seminiferous tubules. However, the normal structure of the testis was disrupted in untreated diabetic rats: the seminiferous epithelium was thin and the epithelium cells were sparse, only few sperms presented in the tubules. These changes were improved greatly in TTC and Methyltestosterone treated group, but less amelioration was found in Rosiglitazone treated group (**Figure 2**). In general, the testis of TTC treated rats showed more epithelium cells and more sperms than that of other treated groups. It seemed that no obvious amelioration was found in Rosiglitazone treated group compared with untreated diabetic group. No marked changes were found in the interstitial of the tubules among all groups.

Discussions

Among male diabetes patients, many of them afflicted with the hypogonadism which characterized by low testosterone level and depressed libido. Erectile dysfunction (ED) was one of symptoms even which can occasionally be the initial presenting symptom of in DM patients. Recently more attentions focused on the testicular damage induced by diabetes. Testicular impair was one of the primary factors to affect the male health especially in the progression of ED. It was suggested that ED was the result of the long period damage of the testis both in the function and pathology aspects [18], which most exhibiting endothelial dysfunction and autonomic neuropathy. In the present study, decreased sexual activity was found in diabetic rats characterized with the alterations in the latency and frequency of copulations. TTC and Methyltestosterone showed the benefit effect to ameliorate these alterations, while Rosiglitazone had no obvious effect except for its anti-hyperglycaemia effect. It suggested that Rosiglitazone only lowered blood glucose but could not ameliorate the symptoms of hypogonadism.

The detrimental effects of diabetes on the hypothalamus-pituitery-testis axis attracted more attention for its effects in regulating the reproduction and sexual behavior [19]. The testis function was mainly regulated by the pituitary gland where gonadotropins including FSH and LH were synthesized and secreted. And FSH and LH were controlled by the hypothalamic GnRH. FSH levels were significantly lower in the hypogonadal patients compared with the patients with normal testosterone level [20]. In experimental diabetes, low levels of LH and FSH have been confirmed, and decreased levels of them may induce germ cell impairment. LH was a vital factor for the biosynthesis and release of testosterone from Leydig cells, while FSH played an important role to transmit the testosterone into seminiferous tubules in the process of the spermatogenesis. The spermatogenesis requires enough levels of intratesticular testosterone secreted by the Leydig cells. Testosterone exerts its effect via the androgen receptor (AR) located in Sertoli cells in this process. Low circulating levels of testosterone can be found in 20-30% of infertile men, but administration of testosterone or gonadotropins had no obvious effect on spermatogenesis. Abuse of anabolic steroids is a frequent cause of male infertility, and substances such as endocrine disruptors can alter male fertility through an anti-androgenic action [21]. There was a significant correlation between the serum levels of insulin and FSH, but no significant correlation was found between insulin or glucose and LH [22]. In diabetic state, the abnormality in erectile, ejaculatory, sperm motility and semen volume attributes to vascular or neuropathic problems. In the present experiment, the testosterone and LH level declined markedly in diabetes rats compared with that of normal control, while FSH had a moderated decline. Although it was a little different from

the reports described above, the lesion of the testis function was confirmed. TTC and Methyltestosterone retrieved the alterations in LH and testosterone level obviously. In fact, the alterations of these hormones were consistent with the histological alterations. Thinner seminiferous epithelium and less sperm cell were observed in seminiferous tubule in diabetic rats. The treatment of TTC or Methyltestosterone appeared the melioration, but Rosiglitazone showed less benefit on these histological alterations. It seemed that protection on the testis function was mainly exerted by the regulation of the hormones including testosterone, LH and FSH but not insulin.

Besides related hormones, some special enzymes in the test is also can reflect the testicular injury. LDH was located in spermatogenic cell and acted as an enzyme responsible for the energy supply and the development of seminiferous epithelium in the testis. ACP stimulated in the Sertoli cells and involved the synthesis of protein in the presence of testosterone, while γ-GT promoted the maturation of sperms [23]. In the present experiment, three enzymes above dramatically decreased in diabetic rats. Although these enzymes cannot act alone for the spermatogenesis, the sexual hormones such as testosterone can act synergistically with them. The activities of these enzymes were tightly correlated with the testosterone level. These enzymes provided the substance environment for the testis function, and testosterone promoted the production of these enzymes. In this experiment Rosiglitazone had little effect on improving the activities of special enzymes in the testis, but TTC and Methytestosterone enhanced the enzyme activities significantly.

Testicular dysfunction, impotence, decreased fertility potential and retrograde ejaculations are complications that have been described in diabetic males [24]. These symptoms were correlated to each other and occurred single or combined with others. The molecular mechanism of the hypogonadism induced by diabetes was not elucidated yet. However, growing evidences showed that it might be caused by several factors including ROS, glycosylated hemoglobin, NO, microangiopathy, neuropathy and so on [25]. According to the results from animal experiments or clinical trials, the strategy to attenuate the disease progression was to regulate the metabolism of hormones and testis function. As shown above, application with antidiabetic drugs or testosterone replacement therapy alone cannot ameliorate the symptoms excellently. Furthermore, application with anabolic steroids hormones for long term would cause a series of adverse effects which offset the protective effects in part. In such a dilemma, the other approaches were considered to avoid those side effects and achieve better therapeutic effects. Traditional Chinese Medicines was an alternative for the therapy of the hypogonadism induced by diabetes. *Cornus officinalis Sieb.* was used to treat the diabetes combined with other herbs in long clinic practice in China, but less investigation were conducted to elucidate the effect when it was used alone. In addition, these were almost no reports about the effects of it on the hypogonadism in experimental researches, while the ancient Traditional Chinese Medicine works recorded its effects on enhancing testis function and sexual libido(in Chinese “Zhuang yang”). In the present investigation, triterpenes were isolated from *Cornus officinalis Sieb.* and the effects on the diabetic-induced hypogonadism were investigated in rats. Ameliorations of TTC on the sexual behavior, serum sexual hormones, functional enzymes in the testis, and the histological alterations were shown in diabetic rats. Different from the testosterone replacement therapy, TTC had effects both on lowering blood glucose and protect the testis from damage. Rosiglitazone could not ameliorate testis impair significantly though it could lowering blood glucose in diabetic rat, which suggested that high glucose level was not possibly the only or direct reason to cause testis impair. In the thousands of clinical practice, *Cornus officinalis Sieb.* did not appear serious adverse effects and was suitable for the treatment of diabetic and its complications for long term. TTC exhibited a promising potential in the therapy of the hypogonadism induced by diabetes in the present study.

In conclusion, the present study confirmed the protective effects of TTC on the testis impairs in diabetic condition. Different from the testosterone replacement therapy, TTC had effects both in lowering blood glucose and protecting the testis from damage. TTC exhibited a promising future in the therapy of the hypogonadism

induced by diabetes, and further investigations would be carried out to explore the mechanism of action.

Acknowledgements

The present study was supported by a grant of Hubei Provincial Natural Science Foundation (No. 2012FFB06302).

Disclosure of conflict of interest

None.

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