EVALUATION OF ANDROGENIC ACTIVITY OF TD0014 IN MALE RATS

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Male hypogonadism is a clinical syndrome caused by androgen deficiency which may adversely affect multiple organ functions and quality of life; it is one of the causes of infertility. The present study was undertaken to evaluate the androgenic properties of TD0014 at the dose of 1.8 g/kg and 5.4 g/kg on castrated peripubertal and weanling rats according to OECD guidelines 441 and 115, respectively. In castrated rats, the relative weights of ventral prostate and paired Cowper's glands were increased in the TD0014 treated group ($p < 0.05$). In weanling rat groups, treatment with TD0014 at the dose of 5.4 g/kg increased the weight of accessory sexual organs including prostate, Cowper's glands and levator ani-bulbocavernosus muscles ($p < 0.05$), while TD0014 at the dose of 1.8 g/kg caused only a significant increase in the weight of Cowper's glands ($p < 0.01$). Level of serum testosterone was also increased in weanling rats treated with TD0014 at all doses ($p < 0.05$). In summary, TD0014 possesses androgenic activity in Hershberger assays using castrated peripubertal rats and weanling rats. The dose of 5.4 g/kg/day of TD0014 is a more potent androgen-like activity than the dose of 1.8 g/kg/day.

Keywords: TD0014; androgenic activity; testosterone; Hershberger

I. INTRODUCTION

Male hypogonadism is characterized by a deficiency in testosterone – a critical hormone for sexual, cognitive, and body function and development. Clinically low testosterone levels can lead to the absence of secondary sex characteristics, infertility, muscle wasting, and other abnormalities. Low testosterone levels may be due to testicular, hypothalamic, or pituitary abnormalities. In individuals who also present with clinical signs and symptoms, clinical guidelines recommend treatment with testosterone replacement therapy (TRT)[1]. However, before prescribing TRT, one must be conscientious of its adverse effects. Data on the safety of TRT specific to our aging population is not currently available; however TRT has been linked to prostate cancer, benign prostatic hyperplasia, polycythemia and obstructive sleep apnea[2]. Thus, there is a continuing need for the development of new effective therapies to treat patients with male hypogonadism.

Despite the remarkable developments of modern medicine, many people are still favorably disposed towards herbal medicines owing to the aggressive treatment protocols, toxicity, and drug tolerance associated with modern therapies. The widespread use of herbal medicines, however, requires scientific verification of their indications and effects by use of modern medical analysis.

TD0014 is a preparation of herbal medicines, which is comprised of thirty - three medicinal plants. The composition of TD0014 has several medicinal herbs that have been studied and used since ancient times in traditional folk medicine as an aphrodisiac. However, no

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studies have provided reliable evidence of their effects on reproductive functions, or toxicity when combining them in TD0014. Therefore, the purpose of this study was to evaluate the androgenic effects of TD0014 in male rats.

II. METHODS

1. Herbal formula TD0014 preparation

TD0014 was manufactured according to the quality standard of Sao Thai Duong Joint Stock Company, Vietnam. It was prepared as hard pills. The major ingredients of the herbal formula are obtained from thirty - three plants: Tribulus terrestris, Chrysanthemum sinense, Prunus persica, Vigna cylindrica, Eurycoma longifolia, Sophora japonica, Dioscorea persimilis, Dioscorea tokoro, Polygonum multiflorum, Citrus deliciosa, Polyscias fruticosa, Tinospora sinensis, Chaenomeles lagenaria, Passiflora foetida, Zizyphus sativa, Rehmannia glutinosa, Angelica sinensis, Alisma plantago - aquatica L. var. orientalis Samuelsson, Achyranthes bidentata, Schizandra chinensis, Morinda offcinalis, Rosa laevigata, Allium sativum, Lycium sinense, Glycyrrhiza uralensis, Panax ginseng, Ligusticum wallichii, Cistanche tubulosa, Atractylodes macrocephala, Radix Codonopsis, Cuscuta sinensis, Psoralea corylifolia, Cornu Cervi parvum. The herbal mixture extracts satisfied the herb, heavy metals, general bacteria, fungi, and specific pathogens criteria, as determined by a confirmation test for each. The pills were crushed and dissolved in water before giving to animals.

2. Animals and housing

Wistar male rats were housed in a 24 - hour air - conditioned room with access to standard certified rodent diet and water ad libitum. They were acclimated to housing for at least 1 week prior to experimental protocols. The experimental protocol was approved by the ethics committee of Hanoi Medical University, Vietnam.

3. Evaluation of the androgenic effects of TD0014 in the castrated male rats

In this experiment, 45 peripubertal male rats were used with an initial body weight between 120 and 150 g, aged between 42 and 50 days. All rats were divided into 5 groups. Group I: Intact + distilled water, Group II: Castrated + distilled water, Group III: Castrated + testosterone at dose of 0.4 mg/kg, Group IV: Castrated + TD0014 at dose of 1.8 g/kg and Group V: Castrated + TD0014 at dose of 5.4 g/kg. and the rats were housed in five cages (nine animals/cage) upon the treatment. The rat castration procedure was performed according to OECD guideline 441. The rats are castrated under anesthesia by placing an incision in the scrotum and removing both testes and epididymides with ligation of blood vessels and seminal ducts. After confirming that no bleeding is occurring, the scrotum should be closed with suture. After castration, all rats fully recovered in seven days[3].

After castration and full recovery, the rats received testosterone by s.c injection or TD0014 by oral gavage, once daily for 10 consecutive days. The weight of the rats was recorded at zero and ten days from all the groups. Twenty - four hours after the last administration, the rats were sacrificed by decapitation. The five androgen - dependent tissues: ventral prostate, seminal vesicles, levator ani - bulbocavernosus muscle (LABC), bulbourethral glands (Cowper), and penis glans were harvested. The excision was performed carefully with the removal of all fat and adjacent tissues, followed by weighing of the fresh (unfixed) tissues. They were handled properly in order to avoid any drying or fluid loss, which could have led to significant errors.

4. Evaluation of the androgenic effects of

TD0014 in the weanling male rats

Forty weanling 21 - day old male rats were divided equally into 4 groups . Each group was treated daily for 10 consecutive days as below with distilled water (control; 10 mL/kg b.w orally), testosterone propionate (TP) s.c. (androgenic control; 1 mg/kg/day s.c.), or TD0014 at 1.8 and 5.4 g/kg by gavage. The doses of TP was recommended by the OECD guideline 115.[4] A dose volume of 1 ml/kg body weight was used for all sc injections and a volume of 10 ml/kg body weight was used for all oral doses.

The rats were observed every day during the treatment period for clinical signs, including morbidity and mortality. The rats were killed 24 h after the last dose, followed by exsanguination. Blood samples from the carotid artery were collected and stored at - 20ºC until analyzed for sex hormone concentrations. At necropsy, the reproductive organs (epididymis, seminal vesicles, ventral prostate, Cowper's glands and levator ani/bulbocavernosus muscles [LABC]) were excised, trimmed free of fat and connective tissues, and weighed.

5. Statistical analysis

Statistical comparisons were made using the Student's t - test for comparison of data in the control group and the experimental groups. The results were expressed as mean ± standard deviation. Comparisons with p values < 0.05 were considered to be statistically significant.

III. RESULTS

1. Androgenic effects of TD0014 in the castrated male rats

Table 1. Effect of TD0014 on the weight of accessory sex tissues of the castrated male rats after 10 days of treatment

Weight (mg/100g b.wt)	Intact + distilled water	Castrated + distilled water	Castrated + testosterone 0.4 mg/kg	Castrated + TD0014 1.8 g/kg	Castrated + TD0014 5.4 g/kg
Penis glans	28.2 ± 7.8	21.0 ± 5.8*	42.2 ± 4.2$^{###}$	22.3 ± 4.5	22.6 ± 6.3
Seminal vesicles	19.7 ± 5.7	8.7 ± 2.1***	81.6 ± 19.2$^{###}$	9.3 ± 2.4	8.2 ± 2.6
Ventral prostate	22.2 ± 6.4	5.0 ± 1.6***	34.2 ± 8.3$^{###}$	8.9 ± 2.8$^{##}$	17.7 ± 4.2$^{###}$
Cowper's glands	8.4 ± 1.9	1.3 ± 0.2***	10.7 ± 2.6$^{###}$	1.9 ± 0.6$^{#}$	1.6 ± 0.3$^{#}$
LABC	104.1 ± 19.2	44.6 ± 9.0***	139.6 ± 30.2$^{###}$	39.8 ± 9.2	36.5 ± 9.8

All values: Mean ± SD; Number of rats per group = 9

**: p < 0.05, ***: p < 0.001 compared to group I (intact + distilled water)*

#: p < 0.05, ##: p < 0.01, ###: p < 0.001 compared to group II (castrated + distilled water)

Castration was generally followed by a significant drop in the weights of the penis glans, seminal vesicles, ventral prostate, Cowper's glands and LABC when compared to noncastrated controls (normal rats). In androgen - deficient rats, a daily subcutaneous injection of testosterone propionate (0.4 mg/kg b.w.) for 10 consecutive days always brought out, as expected, important changes as evidenced by the significant growth of all androgen - dependent organs ($p < 0.001$). With regard to castrated controls, rats exposed to the plant extracts showed a significant increase in the relative weights of the ventral prostate ($p < 0.01$ or $p < 0.001$) and the Cowper's glands ($p < 0.05$). The

treatment did not affect the relative weights of the penis glans, seminal vesicles and LABC.

2. Androgenic effects of TD0014 in the weanling male rats

Table 2. Effect of TD0014 on the weight of reproductive organs of the weanling male rats after 10 days of treatment

Weight (mg/100g b.wt)	Control	Testosterone 1 mg/kg	TD0014 1.8 g/kg	TD0014 5.4 g/kg
Seminal vesicles	24.1 ± 6.7	215.8 ± 48.4***	19.5 ± 5.6	21.9 ± 5.7
Epididymis	127.6 ± 24.0	252.2 ± 35.4***	130.1 ± 30.0	123.8 ± 22.6
Ventral prostate	24.4 ± 8.0	100.2 ± 17.6***	21.7 ± 5.1	37.6 ± 7.9**
Cowper's glands	3.4 ± 0.7	21.2 ± 3.0***	4.8 ± 1.3**	4.3 ± 0.8*
LABC	55.0 ± 11.0	194.6 ± 23.4***	46.0 ± 10.2	70.8 ± 15.4*

All values: Mean ± SD; Number of rats per group = 10

**: p < 0.05, **: p < 0.01, ***: p < 0.001 compared to control*

A daily subcutaneous injection of testosterone propionate (0.4 mg/kg b.w.) for 4 consecutive weeks was followed by a significant increase in relative weights of the accessory organs. After 10 days of oral administration of the plant extracts, TD0014 at the dose of 5.4 g/kg caused a significant increase in the weights of Cowper's glands ($p < 0.05$), LABC ($p < 0.05$) and prostate ($p < 0.01$) of rats, while TD0014 at the dose of 1.8 g/kg caused only a significant increase ($p < 0.01$) in the weight of Cowper's glands.

Table 3. Effects of testosterone and TD0014 on the serum testosterone levels in weanling rats after 10 days of treatment

Groups	Testosterone (nmol/L)
Control	0.087 ± 0.002
Testosterone 1.0 mg/kg	15.343 ± 1.939***
TD0014 1.8 g/kg	0.235 ± 0.089***
TD0014 5.4 g/kg	0.293 ± 0.062***

All values: Mean ± SD; Number of rats per group = 10

****: p < 0.001 compared to control*

The testosterone level in serum was increased significantly in the TD0014 treated groups compared to control group ($p < 0.001$).

IV. DISCUSSION

The Hershberger Bioassay is short - term in vivo screening assay for androgen agonists, androgen antagonists, and 5α - reductase inhibitors, is based on the changes in weight of several androgen - dependent tissues on the day after treatment is ended. The Hershberger Bioassay achieves its sensitivity by using males with minimal endogenous androgen production. This is achieved either through the use of castrated males, provided an adequate time

after castration for the target tissues to regress to a minimal and uniform baseline weight is allowed, or by the use of weanling males in which there is minimal endogenous androgen production.[3,4]

Castration enhances the precision of the assay to detect weak androgens and antiandrogens by eliminating compensatory endocrine feed - back mechanisms presented in the intact animal that can attenuate the effects of administered androgens and antiandrogens and by eliminating the large inter - individual variability in serum testosterone levels.[3] The rats should continue acclimation to the laboratory conditions to allow for the regression in the target tissue weights for a minimum of 7 days following castration. After full recovery, the castrated male rats were administered a reference dose of TP and the test substance for 10 consecutive days. 24 h after the last administration, the rats were sacrificed to harvesting the five androgen - dependent tissues. The androgenic effects of test material were evaluated through the changes in weight of the five target androgen - dependent tissues including ventral prostate, seminal vesicle (plus fluids and coagulating glands), levator ani - bulbocavernosus (LABC) muscle, paired Cowper's glands and the glans penis.[3] As shown in Table 1, the weights of the ventral prostate and Cowper's glands significantly increased in the TD0014 treatment groups as compared with the non - treated castrated rats ($p < 0.05$). According to OECD guideline 441, a statistically significant increase in two or more target organ weights of the test substance groups compared to the vehicle control group indicates that the test substance is positive for potential androgenic activity. Based on this guideline, TD0014 is considered to have the androgenic activity in the castrated male rats.

The intact stimulatory weanling version of the Hershberger bioassay appeared to be able to consistently detect effects on androgen - dependent organ weights from medium or highly potent androgens. Therefore, to further determine the androgenic potency of TD0014, we conducted the study to evaluate the androgenic activity of TD0014 in weanling male rats according to OECD guideline 115. In the stimulated intact weanling male model, the ventral prostate (VP), seminal vesicles (SV plus fluids and coagulating glands), LABC muscle and Cowpers (COW, bulbourethral) glands and epididymides (since the rats are non - castrated males) should be measured to identify androgenic effects of the test material.[4] A statistically significant increase in two or more target organ weights of the test substance groups compared to the vehicle control group indicates that the test substance is positive for potential androgenic activity. The results of Table 2 and Table 3 demonstrated the androgenic activity of TD0014 at the dose 5.4 g/kg, as shown by the ability to significantly increase the weights of ventral prostate, Cowper's glands and LABC. TD0014 at the dose of 1.8 g/kg altered only the weight of Cowper's glands as compared to control group, so this dose did not induce androgenic effects in weanling male rats. However, the serum testosterone level was significantly elevated in both TD0014 treatment groups as compared to control group.

The above results have shown that TD0014 may have androgenic effects, which seems to be more potent with the dose 5.4 g/kg than with the dose 1.8 g/kg. TD0014 preparation contains several medicinal plants that have been studied for the effects on sexual functions, including the ability to significantly increase the serum testosteron concentration, which can partly explain the impacts of this herbal formula

on the changes of the androgen - dependent organ weights and the serum testosterone concentration. Results published by Rajendar B et al (2011)[5] indicated that the ethanolic extract of Tribulus terrestris Linn exhibited protective effect against cadmium - induced testicular damage, which appears to be mediated through inhibition of testicular tissue peroxidation by antioxidant and metal chelator activity and also, may be indirectly by stimulating the testosterone production from Leydig cells. Eurycomanone, the highest concentrated quassinoid in the root extract of Eurycoma longifolia Jack, enhanced testosterone steroidogenesis at the Leydig cells by inhibiting aromatase conversion of testosterone to oestrogen, and at a high concentration may also involve phosphodiesterase inhibition[6]. Cistanche tubulosa ethanol extract increased rat sex hormone levels by induction of testicular steroidgenic enzymes including CYP11A1, 3β - HSD, 17β - HSD and CYP17A1.[7,8] Some other medicinal plants (Morinda officinalis, Lycium barbarum, Semen Cuscutae) in TD0014 were also proved to cause an increase in serum testosterone level.[9 - 11] Ginseng, an oval - shaped root, is among the most popular herbal remedies, which can be used alone or in combination with other medicinal herbs to enhance sexual activity. Both experimental and clinical studies have demonstrated that ginseng extract is responsible for the increase in the levels of plasma testosterone,[12] as well as plays an important role in maintaining healthy levels of steroid hormone receptors, including AR, which in turn ensures the proper functioning of androgens.[13]

In conclusion, TD0014 has an androgenic activity, which seems to be more potent with the dose 5.4 g/kg than with the dose 1.8 g/kg. Further studies are warranted to clarify its mechanism of action.

REFERENCES

1. Kumar P, Kumar N, Thakur DS, and Patidar A. Male hypogonadism: Symptoms and treatment. *J Adv Pharm Technol Res.* 2010;1(3):297–301.

2. Osterberg EC, Bernie AM, and Ramasamy R. Risks of testosterone replacement therapy in men. *Indian J Urol.* 2014;30(1):2–7

3. OECD (2009). Test No. 441: Hershberger Bioassay in Rats: A Short - term Screening Assay for (Anti)Androgenic Properties. *OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris,* https://doi.org/10.1787/9789264076334 - en.

4. OECD (2018). Hershberger Bioassay in Rats (H assay) (OECD TG 441) (including OECD GD 115 on the Weanling Hershberger Bioassay). *In: Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption, OECD Publishing, Paris*, https://doi.org/10.1787/9789264304741 - 21 - en.

5. Rajendar B, Bharavi K, Rao GS, et al. Protective effect of an aphrodisiac herb Tribulus terrestris Linn on cadmium - induced testicular damage. *Indian J Pharmacol.* 2011;43(5):568–573

6. Low BS, Choi SB, Wahab HA et al. Eurycomanone, the major quassinoid in Eurycoma longifolia root extract increases spermatogenesis by inhibiting the activity of phosphodiesterase and aromatase in steroidogenesis. *J Ethnopharmacol.* 2013;149:201–207

7. Wang T, Chen C, Yang M et al. Cistanche tubulosa ethanol extract mediates rat sex hormone levels by induction of testicular steroidgenic enzymes. *Pharm Biol.* 2016;54(3):481 - 487

8. Jiang Z, Wang J, Li X, Zhang X. Echinacoside and Cistanche tubulosa (Schenk) R. wight ameliorate bisphenol A - induced testicular and sperm damage in rats through gonad axis regulated steroidogenic enzymes. *J Ethnopharmacol*. 2016;193:321 - 328

9. Wu ZQ, Chen DL, Lin FH, et al. Effect of bajijiasu isolated from Morinda officinalis F. C. how on sexual function in male mice and its antioxidant protection of human sperm. J *Ethnopharmacol.* 2015;164:283 - 292

10. Cheng J, Zhou ZW, Sheng HP, et al. An evidence - based update on the pharmacological activities and possible molecular targets of Lycium barbarum polysaccharides. *Drug Des Devel Ther.* 2015;9:33–78

11. Yang J, Wang Y, Bao Y, Guo J. The total flavones from Semen cuscutae reverse the reduction of testosterone level and the expression of androgen receptor gene in kidney - yang deficient mice. *J Ethnopharmacol.* 2008;119(1):166 - 171

12. Leung KW, Wong AS. Ginseng and male reproductive function. *Spermatogenesis.* 2013;3(3):e26391. doi:10.4161/spmg.26391

13. Park J, Song H, Kim SK, et al. Effects of ginseng on two main sex steroid hormone receptors: estrogen and androgen receptors. *J Ginseng Res*. 2017;41(2):215–221