



Alleviating effects of the mixture of *Elaeagnus multiflora* and *Cynanchum wilfordii* extracts on testosterone deficiency syndrome

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Received: 13 October 2020 / Accepted: 27 November 2020 / Published Online: 31 December 2020
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Abstract Testosterone deficiency syndrome (TDS), also known as late-onset hypogonadism, is a clinical and biochemical syndrome associated with advanced age and characterized by deficient serum testosterone levels. The *Elaeagnus multiflora* fruit (EMF) and *Cynanchum wilfordii* (CW) have been used in traditional herbal medicine. This study aimed to investigate the therapeutic effects of EMF and CW mixtures (at the ratios of 3:7, 5:5, and 7:3) on TDS using TM3 cells and aging male rats. EMF, and mixtures of EMF and CW (at the ratios of 3:7, 5:5, and 7:3) significantly increased testosterone levels in TM3 cells ($p < 0.05$). The rats were orally administered EMCW (EMF and CW mixed at the ratio of 3:7 50, 100 and 200 mg/kg/day) for 4 weeks consecutively. After 4 weeks of EMCW administration, latency time on the rotarod test, and serum testosterone and dehydroepiandrosterone sulfate levels were significantly increased ($p < 0.05$ and $p < 0.01$). Moreover, the levels of globulin-bound sex hormones were decreased in the EMCW-fed groups. However, prostate-specific antigen levels did not differ among the groups. These results suggest that EMCW can be effectively used to alleviate TDS.

Keywords *Cynanchum wilfordii* · *Elaeagnus multiflora* · Rotarod test · Testosterone · Testosterone deficiency syndrome · TM3 cells

Introduction

Andropause, also called male menopause, is an indefinite syndrome composed of several physical, sexual, and emotional symptoms resulting from a complex interaction among hormonal, psychological, environmental, and physical factors [1]. Andropause causes age-related changes in male hormone levels; as men age, their testosterone levels decline [2]. Morales termed this testosterone decline as testosterone deficiency syndrome (TDS) owing to its simplicity, clarity, and respect for physiological principles [3]. TDS is defined as a clinical and biochemical syndrome associated with advanced age and is characterized by deficient serum testosterone levels [4]. Declining testosterone levels in aging men cause symptoms such as erectile dysfunction (ED), obesity, lack of physical strength, and depression. Total testosterone measurement is the recommended initial test to diagnose hypogonadism. A longitudinal study by Haring *et al.* reported that low testosterone levels increase the risk of developing metabolic syndrome, thereby underscoring the importance of early hypogonadism diagnoses even among young men [5]. Total testosterone levels decrease by an average of 1.6% per year, whereas free and bioavailable levels of testosterone decrease by 2-3% each year. The latter value is higher because aging is associated with increased levels of sex hormone-binding globulin (SHBG) [6]. Production of sex steroids occurs from the adrenal precursors, dehydroepiandrosterone (DHEA) and its sulfate forms (DEHAs). DHEA, an inactive prohormone, is produced by the adrenal glands from cholesterol [7]. The potential role of the prostate in the pathophysiology of TDS have been determined from prostate-specific antigen levels and rectal examination [8].

Elaeagnus multiflora Thunb., commonly called oriental cherry silverberry or gumi, belongs to the family Elaeagnaceae. The species is native to China, Korea, and Japan. Several parts of *E. multiflora* are usually used in traditional Chinese medicine [9-11]. The *Elaeagnus multiflora* fruit (EMF) is edible and used in small amounts in herbal medicines, particularly owing to its anticancer

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and antioxidant activities [12, 13]. Furthermore, the EMF contains multiple compounds and has numerous physiological effects [14–17]. We previously reported that the EMF extracts have anti-fatigue effects [18]. *Cynanchum wilfordii* (CW) is used as a traditional herbal medicine in Korea for the prevention and treatment of diseases. CW has beneficial effects against numerous conditions including hypertension, hypercholesterolemia, osteoporosis, gastric disorders, and cancer [19–23]. Cynandion A from CW has anti-inflammatory effects [24]. Furthermore, previous studies have reported an increase in sexual behavior and the amelioration of testosterone-induced benign prostatic hyperplasia (BPH) in rat models [25,26]. However, no studies have reported the therapeutic effects of EMF and CW extract mixtures on TDS.

In the present study, we determined the optimal mixing ratio of EMF and CW extracts mixed and its effects *in vitro*, using TM3 Leydig cells. Furthermore, we investigated the effects of the EMCW (EMF and CW mixed at a ratio of 3:7) on TDS in aging male rats undergoing rotarod tests and biomarker analyses.

Materials and methods

Preparation of EMF

The EMFs used in this study were obtained from Yeosu-si (Jeollanamdo, Republic of Korea) and authenticated by Dr. Choi at the Jeonnam Bioindustry Foundation, Center of Nature Resources Research (JCNr), Jangheung, Jeollanamdo, Republic of Korea. The plant used for this study was purchased from the Kangwons Medicinal Herb Association (Wonjusi, Kangwondo, Republic of Korea). Air-dried EMF (100 g) was extracted with 20 volumes of water at 100 °C for 3 h. The extracted solution was filtered, and then concentrated in an evaporator by vacuum freeze-drying to obtain EMF (15.4 g). Air-dried CW (100 g) was extracted with 20 volumes water at 100 °C for 3 h. The CW was extracted at 100 °C for 3 h, concentrated, lyophilized, and pulverized under the same experimental conditions. The extracted solution was filtered, and then concentrated in an evaporator through vacuum freeze-drying to obtain CW (13.4 g). The EMF and CW were mixed at ratios of 3:7, 5:5, or 7:3. The dried EMF, CW, and mixtures of EMF and CW (at the ratios of 3:7, 5:5, and 7:3) extracts were stored at 4 until used for the *in vitro* assays. EMCW was also used for *in vivo* experiments. The EMCW used in the present study was the same as that used in the clinical trial, which was approved by the institutional review board of Chungnam National University Hospital (clinical trials registration number 2018-05-026).

Cell culture and testosterone levels

TM3 cells were purchased from the KCLB (Seoul, Republic of Korea) and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. For experiments, cells at early passages were

cultured up to 80% confluence. To investigate changes in testosterone levels in TM3 cells, TM3 cells were seeded at 1×10⁵ cells/well in 24-well plates. After 24 h of incubation, cells were treated with various extracts (EMF, CW, 3:7 mixture, 5:5 mixture, 7:3 mixture) and incubated for 24 h; the supernatant media were then harvested. Testosterone levels in TM3 cells were measured using a testosterone EIA kit (ENZO Life Sciences, Farmingdale, NY, USA) in accordance with the manufacturer's instructions.

Animals

Male Sprague-Dawley rats (12-week-old, weighing 400–430 g) were purchased from Samtako Bio Korea (Osan, Republic of Korea). Before the start of the experiment, rats were acclimatized to standard feed and reared for 12 weeks until the age of 24 weeks. The rats were housed in plastic cages at a controlled temperature (22±2 °C) and humidity (50±5%), with *ad libitum* access to water and food at a 12:12 h light:dark cycle (lights on at 08:00 am). The animals were acclimatized for 1 week before the experiments. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Jeonnam Institute of Natural Resources Research (approval no. JINR-1809). All animal experiments were conducted in accordance with the IACUC guidelines.

Experimental groups and drug administration

The rats were divided into four groups (n=5), and received the following treatments: Group I, vehicle-treated (distilled water; oral administration [p.o.]); Group II, EMCW 50 mg/kg/day (p.o.); Group III, EMCW 100 mg/kg/day (p.o.); Group IV, EMCW 200 mg/kg/day (p.o.). EMCW was dissolved in distilled water and administered at 9:30 a.m. once a day for 28 days consecutively. Body weights were measured per week.

Rotarod test

Rats were placed on a standard five-lane accelerating rotarod apparatus (Jeung Do Bio & Plant, Seoul, Republic of Korea). Rats were tested twice at an accelerating speed of 20 rpm for 5 min or until they fell off. The retention times were recorded after 4 weeks. On the testing day, each rat was subjected to two trials with an interval of 10 min.

Biomarker analysis

At the end of the experimental period, the rats were starved for 12 h. Blood was sampled from the abdominal artery, and serum was centrifuged at 4,000 rpm for 15 min. Serum testosterone levels were measured at 0 and 4 weeks using an ELISA kit (ENZO Life Sciences) in accordance with the manufacturer's instructions. Serum SHBG levels were measured using an ELISA kit (Cloud-Clone Corp., Wuhan, Hubei, China) in accordance with the manufacturer's instructions. Serum DHEA-sulfate levels were measured using an ELISA kit (FineTest, Wuhan, Hubei, China) in accordance with the manufacturer's instructions. Serum PSA

levels were measured using an ELISA kit (Cusabio, Houston, TX, USA) in accordance with the manufacturer’s instructions.

Statistical analysis

Data are presented as the mean standard error of the mean (SEM) values. Data were statistically evaluated through one-way analysis of variance using the GraphPad Prism (GraphPad, Inc., San Diego, CA, USA) software. Differences between groups were assessed using Dunnett’s multiple range tests. Statistical significance was considered at $p < 0.05$.

Results and Discussion

Testosterone levels in TM3 cells

Leydig cells are testosterone-producing cells in the interstitial compartment of mammalian testes, which support spermatogenesis in seminiferous tubes [27]. Using TM3 cells, we evaluated testosterone production after treatment with EMF, CW, and mixtures of the two extracts in different ratios (Fig. 1). Each compound tested was used at concentrations of 50 or 100 $\mu\text{g/mL}$. Treatment with EMF, CW, or mixtures of EMF and CW (ratio of 3:7, 5:5, and 7:3) at 100 $\mu\text{g/mL}$ significantly increased the testosterone levels produced by TM3 cells ($p < 0.001$). Testosterone levels peaked upon treatment with a mixture of an EMF and CW extracts at various ratios compared to those on treatment with CW alone when the EMF:CW ratio was 3:7. Therefore, in this study, this 3:7 mixture was designated as EMCW and used for *in vivo* experiments.

Body weights after EMCW treatment

We investigated the effects of EMCW on TDS in rats for 4 weeks (50, 100 and 200 mg/kg/day, p.o.). Weekly changes in the body

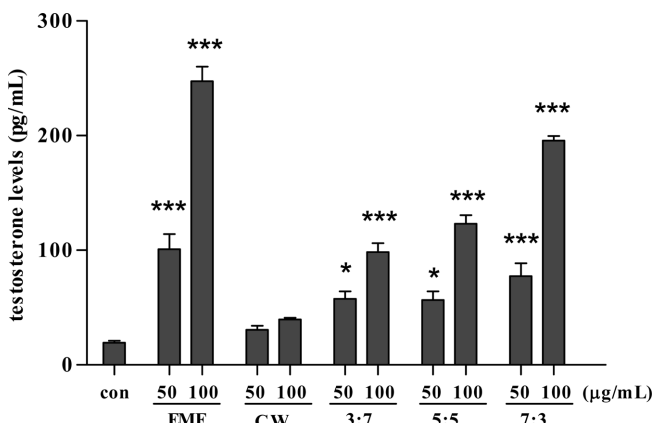


Fig. 1 Effects of EMF, CW, and mixtures of EMF and CW on testosterone production in TM3 cells. TM3 cells were treated for 24 h with various extracts (EMF, CW, 3:7, 5:5, or 7:3 mixtures). Data are presented as mean SE values. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the control group

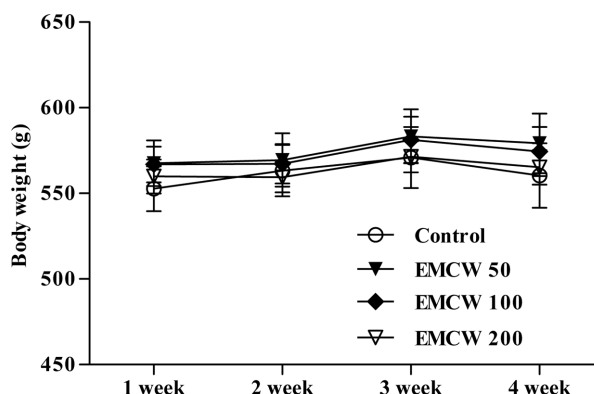


Fig. 2 Effects of EMCW on body weights of aging male rats. Data are represented as mean \pm SE values ($n = 5$). Control, no administration of EMCW (mixture of EMF and CW at the ratio of 3:7); EMCW 50, 50 mg/kg/day EMCW; EMCW 100, 100 mg/kg/day EMCW; and EMCW 200, 200 mg/kg/day EMCW administered daily

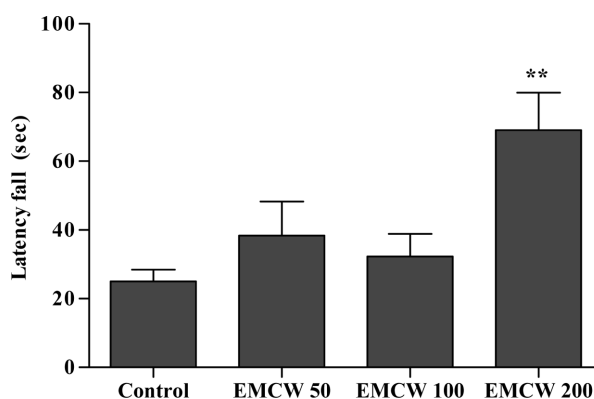


Fig. 3 Effects of EMCW on physical activity of aging male rats in a rotarod test. Control, no administration of EMCW (mixture of EMF and CW at the ratio of 3:7); EMCW 50, 50 mg/kg/day EMCW; EMCW 100, 100 mg/kg/day EMCW; and EMCW 200, 200 mg/kg/day EMCW administered daily. The data represent mean \pm SE values ($n = 5$). ** $p < 0.01$ compared to the control group

weight of aging rats treated with EMCW are shown in Fig. 2. In all groups, the body weights gradually increased from week 1 until week 3. Furthermore, in all groups, the mean body weight decreased at 4 weeks. However, final body weights of the EMCW (50, 100, and 200 mg/kg/day)-treated groups were not significantly different from those of the control groups.

Effects of EMCW on the performance of aging rats in the rotarod test

Testosterone enhances the physical locomotor capacity [28]. To evaluate the effects of EMCW on the adaptation of rat skeletal muscles to aging, tests were performed to assess physical strength. We investigated the effects of EMCW on the aging male rats using rotarod tests (Fig. 3). The latency to fall on the rotarod increased in the EMCW group compared with the control group. Moreover, the EMCW 200 group had significantly increased

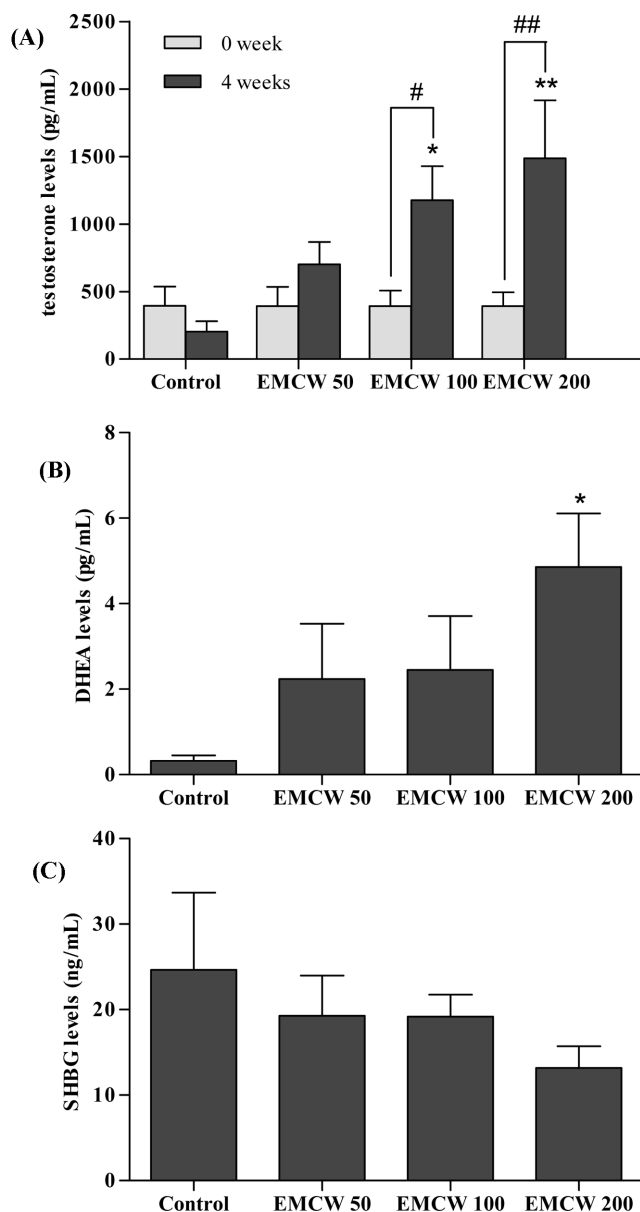


Fig. 4 Effects of EMCW on androgen levels in aging male rats: (A) testosterone, (B) DHEAs, (C) SHBG. Control, no administration of EMCW (mixture of EMF and CW at the ratio of 3:7); EMCW 50, 50 mg/kg/day EMCW; EMCW 100, 100 mg/kg/day EMCW; and EMCW 200, 200 mg/kg/day EMCW administered daily. The data represent mean \pm SE values ($n=5$). * $p < 0.05$ and ** $p < 0.01$ compared to the control group. # $p < 0.05$, and ## $p < 0.01$ compared to the EMCW group

retention time compared with the control group ($p < 0.01$).

Effects of EMCW on androgen levels in aging rats

TDS in aging men primarily results from reduced testosterone levels [29]. Therefore, we investigated the effects of EMCW on serum testosterone levels in aging male rats. As shown in Fig. 4A, the testosterone levels before the administration (week 0) were 394.22 ± 142.84 , 393.15 ± 140.78 , 393.03 ± 114.95 , and 393.72 ± 101.38

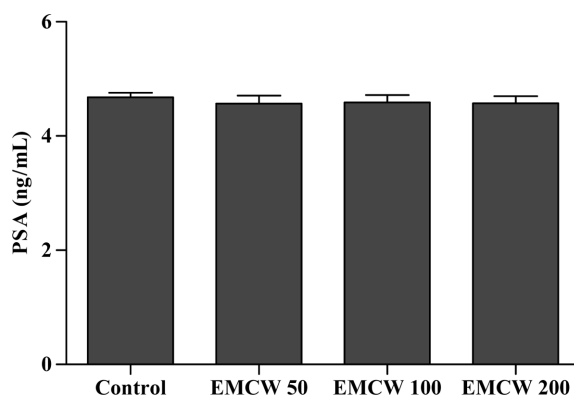


Fig. 5 Effects of EMCW on PSA levels in aging male rats. Control, no administration of EMCW (mixture of EMF and CW at the ratio of 3:7); EMCW 50, 50 mg/kg/day EMCW; EMCW 100, 100 mg/kg/day EMCW; and EMCW 200, 200 mg/kg/day EMCW administered daily. The data represent mean \pm SE values ($n=5$)

pg/mL in the control, EMCW 50, EMCW 100, and EMCW 200 groups, respectively. Oral administration of 50, 100, and 200 mg/kg/day EMCW significantly increased serum testosterone levels to 702.05 ± 165.87 , 1175.72 ± 251.81 , and 1487.40 ± 427.75 pg/mL, respectively, compared to the levels in the control group, which decreased to 203.93 ± 75.77 pg/mL at 4 weeks compared to the levels at week 0. In the EMCW 100 and 200 groups, testosterone levels were significantly increased at 4 weeks compared to levels at week ($p < 0.05$ and $p < 0.01$).

SHBG is a glycoprotein that maintains hormonal balance in the body and can bind to, transport, and inhibit testosterone function. When testosterone is bound to SHBG, it cannot be used by the body [30]. To investigate the effects of EMCW on serum SHBG levels in aging male rats, EMCW (50, 100, and 200 mg/kg/day) was administered daily for 4 weeks. Thereafter, SHBG were lower in EMCW than those in the control group (Fig. 4B). These results suggest that EMCW may play a significant positive role in the regulation of SHBG levels.

DHEA serves as a prohormone and can be rapidly metabolized within target tissues into biologically active steroids, including androstenedione, testosterone, and estradiol [31]. Accordingly, DHEA concentrations in the EMCW 200 group were significantly higher after 4 weeks of treatment than in the control group ($p < 0.05$, Fig. 4C). Furthermore, testosterone levels were significantly higher in the EMCW 100 and 200 groups than that in the control group ($p < 0.05$ and $p < 0.01$). These data reveal a significant increase in androgenic hormones such as testosterone and DHEA after treatment with EMCW ($p < 0.05$). These results suggest that EMCW potentially influences the production of DHEA, a precursor of steroid hormones, to consequently increase testosterone levels.

Effects of EMCW on PSA in aging rats

PSA is widely considered a tumor marker for screening and follow-up evaluation in prostate cancer [32]. Moreover, it serves

as a safety marker for testosterone therapy. As shown in Fig. 5, serum PSA levels did not significantly differ from those in the control group. These results indicate that EMCW administration does not negatively influence the prostate.

Acknowledgments This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) & funded by the Korean government (MSIT) (No. 2019M3A9130 84518).

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