Licorice Extract Does Not Impair the Male Reproductive Function of Rats

Sunhee SHIN¹, Ja Young JANG¹, Byong-il CHOI¹, In-Jeoung BAEK¹, Jung-Min YON¹, Bang Yeon HWANG², Dongsun PARK¹, Jeong Hee JEON¹, Sang-Yoon NAM¹, Young Won YUN¹, and Yun-Bae KIM¹

¹College of Veterinary Medicine and Research Institute of Veterinary Medicine, 12 Gaeshin-dong, Cheongju, Chungbuk 361-763 and ²College of Pharmacy, Chungbuk National University, 12 Gaeshin-dong, Cheongju, Chungbuk 361-763, Korea

Abstract: The effect of water extract of licorice (Glycyrrhiza uralensis), one of the most widely used medicinal plants in Oriental nations and in Europe, on male reproductive function was investigated in rats. Licorice extract was prepared as in Oriental clinics and orally administered at doses of 500, 1,000 or 2,000 mg/kg, the upper-limit dose (2,000 mg/kg) recommended in the Toxicity Test guideline of the Korea Food and Drug Administration, to 6-week-old male rats for 9 weeks. Licorice extract neither induced clinical signs, nor affected the daily feed consumption and body weight gain. There were no significant changes in testicular weights, gross and microscopic findings, and daily sperm production between vehicle- and licorice-treated animals, in spite of slight decreases in prostate weight and daily sperm production at the high dose (2,000 mg/kg). In addition, licorice did not affect the motility and morphology of sperm, although the serum testosterone level tended to decrease without significant difference, showing a 28.6% reduction in the high-dose (2,000 mg/kg) group. The results suggest that the no observed adverse-effect level of licorice extract is higher than 2,000 mg/kg, the upper-limit dose, and that long-term exposure to licorice might not cause profound adverse effects.

Key words: glycyrrhizin (glycyrrhizic acid), licorice (Glycyrrhiza uralensis), safety

Introduction

Licorice (Glycyrrhiza uralensis) is one of the most widely used medicinal plants in Oriental nations and in Europe. Since prehistoric times, licorice, called sweet root, has been used for the treatment of a variety of respiratory, gastrointestinal, cardiovascular, genitourinary, dermal and ocular diseases [13]. The pharmacological activities of licorice cover inflammation, thirst, fever, neuralgia, catarrhs, ulcers and chronic gastritis, hyperlipidemia and atherosclerosis, atopic dermatitis, and others.

Interestingly, licorice is included in almost all herbal prescriptions in Oriental medicine. In addition to the above-mentioned activities, the indications of licorice in Oriental medicine include pharyngitis, cough, palpitations, gastric pain, ulcers, cancers and diverse poisoning [13]. Furthermore, it is well known that licorice allevi-
ates viral infection by inhibiting viral replication and stimulating interferon production [8, 9, 21, 31, 34, 41].

In spite of its wide use because of its effectiveness, the safety of licorice is not well established. The endocrine effects of licorice have been observed, and it has been demonstrated that licorice can produce pseudoaldosteronism by inactivating 11β-hydroxysteroid dehydrogenase (11β-HSD) and binding to mineralocorticoid receptors, leading to vasoconstriction and hypertension [4, 5, 7, 11, 12, 15, 43].

The inhibition of 11β-HSD by licorice blocks cortisol metabolism to cortisone, resulting in fetal growth-retarding effects through maternal glucocorticoids [23, 27, 28, 32, 33]. Also, it has recently been reported that licorice affected androgen metabolism by both inhibiting 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD) and 17–20 lyase and stimulating aromatase, resulting in reduced serum testosterone level [1–3, 6, 44]. In addition, licorice was found to exert estrogen-like activity [22, 35, 36, 39, 40]. Like estrogen, phytoestrogens such as genistein have been found to impair spermatogenesis, and thereby induce deficit of sperm [10, 14, 17, 19, 20, 24, 26]. Such observations led us to investigate the effects of licorice on male reproductive function. In the present study, we administered licorice extract up to 2,000 mg/kg, the upper-limit dose in a repeated-dose toxicity test protocol, to male rats for 9 weeks, and examined the weights of internal organs including those of the reproductive system, sperm counts, serum testosterone level and histological findings of seminiferous tubules.

Materials and Methods

Animals

Six-week-old male specific pathogen-free Sprague-Dawley rats (body weights 140–160 g, n=15/group), from Daehan Laboratory Center, Korea, were housed in an environmentally controlled room with a temperature of 23 ± 2°C, relative humidity of 55 ± 5%, and a 12-h light (150–300 lux) - dark cycle, and pellet feed and purified water were available ad libitum. The experiments performed here were conducted according to the Guiding Principles in the Use of Animals in Toxicology which was adopted by the Society of Toxicology in 1989, and the protocol was approved by the Institutional Animal Care and Use Committee of the Laboratory Animal Research Center, Chungbuk National University, Korea.

Materials and administration

Powdered samples of Glycerizha uralensis were extracted three times with distilled water at 100°C for 3 h using an extracting apparatus. The extracts were concentrated in a vacuum rotary evaporator at 60°C, and freeze-dried. The freeze-dried licorice extract (500, 1,000 or 2,000 mg/kg) or its vehicle (water, 10 ml/kg) was orally administered to 6-week-old rats for 9 weeks.

To determine the content of glycyrrhizin, the major pharmacologically active ingredient, the specimen of the freeze-dried licorice extract was further extracted with an ethanol : water (7 : 3) solution for 5 min at 80°C, using MeCN : H2O (AcOH 1 : 15) = 70 : 30 as the mobile phase. Glycyrrhizin was detected at 254 nm by high performance liquid chromatograph using a C18 column, and its average content was calculated as 3.06%.

Necropsy and blood collection

Body weights and feed intake of the rats were recorded twice a week and every week, respectively, throughout the experimental period. After 9 weeks treatment with licorice, the rats were fasted for 24 h and sacrificed under ether anesthesia. Just before sacrifice, blood was collected from the abdominal aorta for the measurement of the serum testosterone level. Internal organs including the reproductive system were removed and weighed.

Measurement of serum testosterone

Blood collected at necropsy was centrifuged at 3,000 rpm for 10 min. Serum was stored at −70°C until assayed for testosterone. The serum level of testosterone was measured by solid phase radioimmunoassay with 125I (sensitivity = 0.04 ng/ml; intra- and inter-assay coefficients of variations were 8.4 and 15.2, respectively) [42].

Histopathological examination

The left testis of each animal was fixed with Bouin’s
solution for 1 week and processed. Paraffin-embedded sections (4 μm in thickness) were stained with hematoxylin-eosin and examined under a light microscope.

Sperm counts, motility and deformity
The right testis and epididymis of each animal were homogenized at 8,000 rpm for 2 min, and sonicated at 4°C for 3 min to obtain homogenization-resistant sperm heads. The number of sperm heads was counted using a hemacytometer. Daily sperm production/g testis was calculated by dividing the total numbers by 6.1.

The left epididymis was dissected into a Petri dish containing M-199 media, and incubated at 37°C for 10 min. After removing tissue debris, an aliquot of sperm was loaded on a slide glass, and sperm motility was examined under a light microscope. Separately, an aliquot of sperm was stained with the same volume of 1% eosin, smeared on a slide glass, and examined for the deformity of sperm.

Statistics
The results are expressed as the mean ± SD. Tests of significance were performed using Duncan’s multiple-range test after one-way analysis of variance (ANOVA), with P<0.05 as the criterion of significance.

Results

Clinical signs, body weights and feed consumption
The rats orally administered with licorice up to 2,000 mg/kg, the upper-limit dose in the repeated-dose toxicity protocol, for 9 weeks did not show any abnormal signs. Also, licorice influenced neither the body weight gain nor daily feed intake of the animals (Figs. 1 and 2).

Organ weights
At necropsy following 9 weeks administration of licorice, there were no significant changes in organ weights, including those of the reproductive system, although the weight of the prostate was slightly decreased at the high dose (2,000 mg/kg) (Table 1).

Sperm counts, motility and deformity
The number of testicular sperm was slightly reduced by treatment with the high dose (2,000 mg/kg) of licorice (Table 2), resulting in a marginal decrease in the daily production rate of sperm/g testis. However, there were significant differences in neither the numbers and nor the daily production of sperm between the vehicle- and licorice-treated groups. Also, there was no dose-dependent change in epididymal sperm counts. In addition, licorice did not affect the motility and morphology of

Fig. 1. Change in body weights of male rats during treatment with licorice for 63 days. ○, vehicle; ▼, 500 mg/kg; □, 1,000 mg/kg; ◆, 2,000 mg/kg.

Fig. 2. Daily feed intake by male rats during treatment with licorice for 63 days. ○, vehicle; ▼, 500 mg/kg; □, 1,000 mg/kg; ◆, 2,000 mg/kg.
sperm, which were 65.01–69.14% and 2.54–3.63%, respectively, in both control and treated animals.

**Serum testosterone concentration**

The serum testosterone level tended to decrease following 9 weeks treatment with licorice, leading to a 28.6% reduction in the high-dose (2,000 mg/kg) group. However, there were no statistically-significant differences between the control (mean 0.42 ng/ml) and treated (mean 0.30–0.33 ng/ml) rats.

**Histopathology**

Nine weeks administration of licorice up to the upper-limit dose (2,000 mg/kg) caused neither gross nor microscopic lesions of the internal organs including those of the reproductive system (data not shown).

**Discussion**

In Oriental herbal medicine, licorice-containing Kanzo and Kampo preparations have been used to induce...
ovulation in infertile hyperandrogenic women, since licorice reduces the serum level of testosterone by inhibiting 3β-HSD, 17β-HSD and 17–20 lyase, or by stimulating aromatase [3, 6, 18, 30, 37, 44]. This testosterone-lowering effect was also experimentally confirmed in ovarian culture of licorice-treated female rats [37, 38]. Therefore, it was proposed that licorice could cause the deficiency of serum testosterone, leading to sexual dysfunction or decline of libido in men [1, 2]. However, the testosterone-reducing effect of licorice in males is controversial, since the results of Armanini et al. [1, 2] have not been reproduced by other investigators [16, 25].

In the present study, we prepared a licorice water extract according to the procedures used by Oriental clinics, freeze-dried it, and administered it to male rats for 9 weeks, up to the upper-limit dose (2,000 mg/kg) recommended in the Toxicity Test guideline of the Korea Food and Drug Administration. In the present study, we adopted the standard protocol for the toxicity test to produce results comparable with other toxicity studies. The 9-week period of administration was adopted in the present study, because 63 days are generally accepted to cover the entire period of spermatogenesis, from spermatogonia to sperms, in rats. Thus, the germinal cells of all the spermatogenic stages would have been exposed to the test material.

The doses used in this study were much higher than the general clinical doses (4–12 g/man [17–51 mg/kg]). Although the serum testosterone level tended to decrease following 9 weeks administration of licorice, there were no statistically significant differences between the control and treated rats. Interestingly, however, Sakamoto & Wakabayashi [30] showed that oral administration of a Kanzo preparation, glycyrrhizin or glycyrrhetinic acid decreased in vitro testosterone production in Leydig cells of rats stimulated by luteinizing hormone. The testosterone-reducing effect of glycyrrhizin and glycyrrhetinic acid was observed in female rats [37, 38], but not in males [the present study], and there is controversy over the influence of licorice in men [1, 2, 16, 25]. It is hypothesized that, in spite of partial suppression of the basal production of testosterone following the inhibition of steroid-metabolizing enzymes by licorice, production of testosterone stimulated by luteinizing hormone is significantly blocked in the testes, or in the ovaries in the proestrous cycle [3, 6, 18, 30, 37, 38, 44].

Estrogens including phytoestrogens such as genistein were found to impair gonadal development and spermatogenesis [10, 14, 17, 19, 20, 24, 26]. It is well known that licorice possesses estrogenic activity [22, 35, 36, 39, 40]. Since the concentration of glycyrrhizin was 3.06% in the extract used, the rats were treated with 15.3–61.2 mg/kg of the active ingredient (500–2,000 mg/kg of extract). However, licorice induced only a marginal reduction in the numbers of testicular sperm, without influence on the epididymal sperm, of rats.

In comparison, in utero exposure to genistein (100 mg/kg) led to aberrant and delayed spermatogenesis of offspring [10, 14]. Moreover, it is well known that testes of young rats under development are much more susceptible to estrogens than those of adults [24]. Also, there are gender and species differences in the susceptibility of reproductive functions to genistein [19, 24]. Females and mice are more vulnerable than males and rats. It is known that licorice contains phytoestrogens such as glabridin, hispaglabridins, glabrene, isoliquiritigenin and isoprenyl as well as genistein [26, 35, 39, 40]. It is of interest to note that low levels of phytoestrogens in rodent chow enhance epididymal sperm counts via α-estrogen receptors [29]. Thus, in the present study, the marginal decrease in testosterone and sperm production in rats fed rodent chow and simultaneously administered licorice is a mixed effect of a very high dose of phytoestrogens.

In this context, more extensive studies on the effects of licorice on the developing reproductive system of different species are required. In our recent studies, however, fetal malformations and abnormal neonatal development were not induced by in utero and lactational exposures from dams treated with licorice, and normal development of the reproductive function of F1 offspring was observed (manuscript under preparation). Therefore, we do not expect the maximum administration of licorice extract (61.2 mg/kg of active ingredient), prepared according to the Oriental medicinal procedures, to adult rats would induce profound alterations in the male reproductive function including spermatogenesis.
References


