Effects of Methanol Extract of Chansu on Hypothalamic-Pituitary-Testis Function in Rats

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Chansu, a galenical preparation of the dried white venom of Chinese *Bufo bufo* gargarizans, is one of the major components of Kyushin, a traditional Chinese medicine. Kyushin is reported to have a cardiotonic effect that has been suggested to be due to the action of bufadienolides such as bufalin and cinobufagin. Recently, we found that administration of bufalin in male rats diminished the luteinizing hormone (LH) response to gonadotropin-releasing hormone (GnRH) and the secretion of testosterone both in vivo and in vitro. These observations suggest that Chansu may possess hypogonadal effects in male rats. In the present study, the effects of the methanol extract of Chansu on hypothalamic-pituitary-testicular function in male rats were examined. Crude Chansu was extracted by methanol and purified by a Sep-Pak C_{18} column. No activity of bufalin, cinobufagin, estradiol, or digoxin in purified methanol extract was detected; all Chansu used in this study was the purified methanol extract. A single intravenous injection of Chansu resulted in a decrease of the basal (20% to 55%) and human chorionic gonadotropin (hCG)-induced (35% to 40%) levels of plasma testosterone and the GnRH-induced level of plasma LH (25% to 30%). Administration of Chansu in vitro decreased basal and hCG-stimulated testosterone production by 60% to 70% and 40% to 60%, respectively, as well as spontaneous and forskolin- or 3-isobutyl-1-methylxanthine (IBMX)-induced accumulation of adenosine 3′,5′-cyclic monophosphate (cAMP) by 30% to 45% in rat testicular interstitial cells. Although LH release by rat anterior pituitary glands was diminished, GnRH release by the rat mediobasal hypothalamus was enhanced by administration of Chansu in vitro. These results suggest that the bufalin-free extracts of Chansu inhibit testosterone secretion in rats, in part, due to (1) a decreased production of testicular cAMP, (2) a decreased response of testosterone to gonadotropin, and (3) a reduction of the LH response to GnRH.

CHANSU, A GALENICAL preparation of the dried white venom of Chinese *Bufo bufo* gargarizans,[1,2] is one of the major components of Liu-Shen-Wan and Kyushin,[1,3,4] both of which are traditional Chinese medicines. Liu-Shen-Wan has been used for the treatment of tonsillitis, sore throat, and furuncle because of its local anesthetic and antibiotic actions.[3] Kyushin is used for the treatment of palpitation and anhelation, and is reported to have a cardiotonic effect, an excitatory action on respiration, as well as a local anesthetic action.[5,6] The cardiotonic effect of Kyushin has been suggested to be due to the action of bufadienolides such as bufalin, cinobufagin, and resibufogenin.[1,6] However, the side effects of Chansu, eg, its influence on endocrine functions, are not clear.

It is known that bufalin is a cardiotonic steroid isolated from Chansu.[1] It has been shown that bufalin blocks vasodilation and increases vasoconstriction, vascular resistance, and blood pressure via inhibition of Na,K-adenosine triphosphatase,[7-10] despite the increase of sodium excretion.[11]

Recently, we found that administration of bufalin in male rats diminished the luteinizing hormone (LH) response to gonadotropin-releasing hormone (GnRH) and the secretion of testosterone both in vivo and in vitro.[12] These observations suggest that Chansu may possess hypogonadal effects in male rats.

In this study, we examined the effects of the methanol extract of Chansu on hypothalamic-pituitary-testicular function in male rats. The Chansu preparation after methanol extraction was steroid-free. Therefore, no activity of bufalin, cinobufagin, or estradiol was detected in the methanol extract. The effect of steroid-free Chansu on the production of adenosine 3′,5′-cyclic monophosphate (cAMP) in rat testes was also evaluated to determine whether cAMP accumulation was involved in the regulation of testosterone secretion in rats by Chansu.

MATERIALS AND METHODS

Materials

Chansu was extracted and purified by a method described elsewhere by Lichtstein et al[13] with a minor modification. Crude Chansu obtained from a Chinese herb store was mixed with methanol (1 g/15 ml, high-performance liquid chromatography [HPLC] grade; Merck, Darmstadt, Germany), homogenized with a polytron (28,000 rpm, PT-3000; Kinematica, Luzern, Switzerland) for 5 minutes, and stored at 4°C for 60 minutes before centrifugation at 10,000 × *g* for 20 minutes. The supernatant was lyophilized with a concentrator (Speed Vac; Savant Instruments, Holbrook, NY) and reconstituted with the mixture of methanol and double-deionized water (vol/vol 15/1) before centrifugation at 10,000 × *g* for 20 minutes. The supernatant was passed through a Sep-Pak C_{18} column (Waters, Milford, MA) that was activated by 5 mL methanol and 5 mL double-deionized water. The cartridge was then eluted by 75% acetonitrile in 4% acetic acid. The eluates were lyophilized and stored at −70°C before reconstitution with an incubation medium. All Chansu used in this investigation was the purified methanol extract. Doses of the purified methanol extract of Chansu are

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expressed as micrograms per milliliter based on the original dry weight of crude Chansu.

The presence of bufalin, cinobufagin, estradiol, and digoxin in the methanol extract of Chansu was analyzed by a HPLC system. An Inertsil 10 ODS column (0.46 × 25 cm; GL Sciences, Tokyo, Japan) was used. The mobile phase was 50% to 90% CH_3CN/H_2O containing 0.1% H_3PO_4. The flow rate was 1 mL/min. An UV detector (220 nm) was used. The injection volume was 20 μL.

Other substances used in the study were human chorionic gonadotropin ([hCG] Sigma, St Louis, MO) and GnRH (Sigma). Chemicals were prepared as stock solutions solubilized in double-deionized water. Working solutions were made daily.

Animals

Male rats of the Sprague-Dawley strain weighing 300 to 350 g were housed in a temperature-controlled room (22° ± 1°C) with 14 hours of artificial illumination daily (6 AM to 8 PM) and were provided food and water ad libitum.

Effect of Chansu on Testosterone Secretion

Male rats were catheterized via the right jugular vein.[14,15] Twenty hours later, they were injected with Chansu (1 μg/mL/kg), hCG (5 IU/mL/kg; Sigma), or hCG plus Chansu via the jugular catheter. Blood samples (0.4 mL each) were collected at 0, 30, 60, 120, and 240 minutes postchallenge. An equal volume of heparinized saline was injected immediately after each blood sample. Plasma was separated by centrifugation at 10,000 × *g* for 1 minute. The testosterone concentration in each plasma sample was measured by radioimmunoassay (RIA).[15,16]

Effect of Chansu on LH Secretion

Male rats were injected with Chansu (1 μg/mL/kg), GnRH (2 μg/mL/kg), or GnRH plus Chansu via the jugular catheter. Blood samples were collected at 0, 15, 30, 60, and 120 minutes postchallenge. The concentration of LH in each plasma sample was measured by RIA.[15]

Preparation of Rat Testicular Interstitial Cells

Collagenase dispersion of testicular interstitial cells was performed with a procedure described elsewhere.[17] Five decapsulated testes for each dispersion were added to a 50-mL polypropylene tube containing 5 mL preincubation medium and 700 μg collagenase (bovine; type IA; Sigma). Preincubation media contained 1% bovine serum albumin (Fraction V; Sigma) in Hanks balanced salt solution (HBSS), HEPES (25 mmol/L), sodium bicarbonate (0.35 g/L), penicillin G (100 IU/mL), streptomycin sulfate (50 μg/mL), and heparin (2,550 USP K U/L), at pH 7.3 and aeration with 95% O_2 and 5% CO_2. The tube was laid horizontally in a 34°C water bath, parallel to the direction of the shaking. Fifteen minutes after shaking at 100 cycles/min, the digestion process was stopped by adding 35 mL cold preincubation medium and inverting the tube several times. The tube was allowed to stand for 5 minutes and was then filtered through a four-layer fine nylon mesh. Cells were collected by centrifugation at 4°C and 100 × *g* for 10 minutes. The cell pellets were washed with deionized water to disrupt the red blood cells (RBCs), and the osmolarity was then immediately recovered with 10-fold HBSS. Hypotonic shock was repeated twice to disrupt the RBCs, and the cell pellets were resuspended in preincubation medium (with substitution for HBSS in the preincubation medium with medium 199 and sodium bicarbonate 2.2 g/L). Cell concentration (1.0×10^6 cells/mL) and viability (>97%) and sperm cell counts (<5%) were determined using a hemocytometer and the trypan blue method. Our preparation was found to contain approximately 20% Leydig cells.[17]

Effect of Chansu on Testosterone Release In Vitro by Testicular Interstitial Cells

Aliquots (1 mL) of cell suspensions (1×10^6 cells/mL) were preincubated with incubation medium in polyethylene tubes for 1 hour at 34°C under a controlled atmosphere (95% O_2 and 5% CO_2), shaken at 100 cycles/min. The supernatant was decanted after centrifugation of the tubes at 100 × *g* for 10 minutes. Aliquots (1 mL) of cell suspensions (1×10^6 cells/mL) were incubated with or without hCG (0.05 IU/mL) and different doses of Chansu (0 to 1 μg/mL) for 1 hour. Then, 2 mL ice-cold phosphate-buffered saline (PBS)-gelatin buffer (0.1% gelatin in 0.01 mol/L PBS and 0.15 mol/L sodium chloride, pH 7.5) was added to stop the reaction. The media were centrifuged at 100 × *g* and stored at −20°C until analyzed for testosterone by RIA.

Effect of Chansu on cAMP Production in Testicular Interstitial Cells

Aliquots (1 mL) of cell suspensions (1×10^6 cells/mL) were incubated with different doses of Chansu (0 to 1 μg/mL) and forskolin (10^{-5} mol/L, an adenylyl cyclase activator) or 3-isobutyl-1-methylxanthine ([BMX] 5×10^{-4} mol/L, a phosphodiesterase inhibitor) for 1 hour. After incubation, the tubes were centrifuged at 100 × *g*. The cell pellets were mixed with 1 mL 65% ice-cold ethanol, homogenized by a polytron (PT-3000), and centrifuged at 1,500 × *g* for 15 minutes. Supernatants of the cell extracts were lyophilized in a vacuum concentrator (Speed Vac) and stored at −20°C until analyzed for cAMP by RIA.

Effect of Chansu on LH and GnRH Release In Vitro

The anterior pituitary gland (AP) and mediobasal hypothalamus (MBH) were collected from male rats after decapitation. The AP was bisected, preincubated, and then incubated either with or without 10 nmol/L GnRH in the presence of Chansu (0 to 1 μg/mL) at 37°C for 30 minutes. One hemi-AP was used for each flask. At the end of incubation, the media were collected and the tissues weighed. The concentration of LH in the media was measured by RIA.[14]

The MBH was incubated either with or without Chansu (0.01, 0.1, or 1 μg/mL) at 37°C for 30 minutes. One MBH was contained in each vial. At the end of incubation, the media were extracted by 20 μL 5N HCl. The concentration of GnRH in the extracts of media and tissue was measured by RIA.[14]

Hormone RIAs

The plasma and medium testosterone concentration was determined by RIA as described elsewhere.[15-17] With antitestosterone serum no. W8 provided by our laboratories,[15] the sensitivity of testosterone was 2 pg per assay tube. The intraassay and interassay coefficients of variation were 4.1% (n = 6) and 4.7% (n = 10), respectively.

The plasma LH concentration was determined by RIA as described elsewhere with anti-LH serum PW11-2 (produced by the corresponding author Dr P.S. Wang [see ref 14, or Liu et al, Am J Physiol 241:E14-E21, 1981]). The rat LH-I-6 used for iodination and the rat LH-RP-3 that served as the standard preparation were provided by the National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD). The sensitivity was 0.1 ng for the LH RIA. The intraassay and interassay coefficients of variation were 3.8% (n = 4) and 6.6% (n = 5), respectively.

The GnRH concentration in media and MBH extracts was determined by RIA as described elsewhere using anti-GnRH serum CRR11B52[14] generously provided by Dr Y.F. Chen (University of Alabama at Birmingham). The sensitivity was 4 pg for the GnRH RIA. The intraassay and interassay coefficients of variation were 2.1% and 10.5%, respectively, for the GnRH RIA.

cAMP RIA

The testicular cAMP concentration was determined by RIA as described elsewhere[16-19] with anti-cAMP antiserum provided by Calbiochem-Novabiochem (San Diego, CA). A synthetic Tyr-cAMP (Sigma) was used for radioiodination. The sensitivity was 2 fmol per tube for the cAMP RIA. The anti-cAMP antibody does not exhibit cross-reactivity with various nucleotides (adenosine triphosphate or diphosphate or guanosine triphosphate, diphosphate, or monophosphate).

Statistical Analysis

All values are presented as the mean ± SEM. For analyzing the effect of Chansu at each indicated time, the treatment means were tested for homogeneity by ANOVA, and the difference between specific means was tested for significance by Duncan's multiple-range test.[20] The GnRH effect on LH release in vitro was analyzed by Student's *t* test. A difference between two means was considered statistically significant at *P* less than .05.

RESULTS

HPLC Analysis

After analysis by HPLC, no activity of bufalin, cinobufagin, estradiol, or digoxin was detected in the purified methanol extract of Chansu (Fig 1).

Testosterone Secretion In Vivo

Administration of Chansu resulted in a significant decrease ($P < .05$ or $P < .01$) in the basal level of plasma testosterone of 20% to 55% from 30 to 240 minutes after a single intravenous injection. The maximal response occurred 240 minutes postinjection of Chansu (Fig 2).

Compared with the injection of hCG, the application of hCG plus Chansu diminished ($P < .05$ or $P < .01$) plasma testosterone levels at 120 and 240 minutes after the beginning of injection by 40% and 35%, respectively.

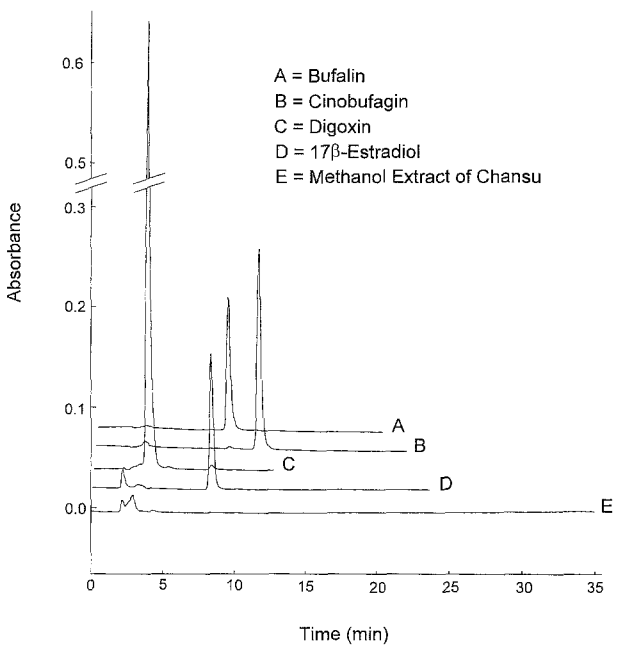

Fig 1. HPLC analysis of bufalin (A), cinobufagin (B), digoxin (C), 17β-estradiol (D), and the methanol extract of Chansu (E).

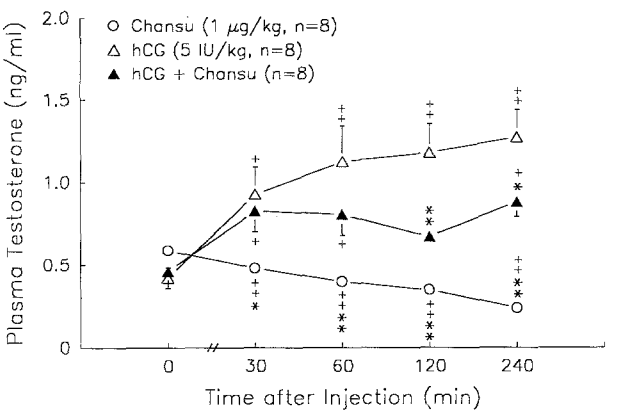

Fig 2. Effects of Chansu on basal and hCG-stimulated plasma testosterone levels in male rats. Rats received a single intravenous injection of Chansu (○), hCG (△), or hCG plus Chansu (▲) via the right jugular vein: Chansu 1 μg/mL/kg, n = 8; hCG 5 IU/mL/kg, n = 8; hCG + Chansu, n = 8. Blood samples were collected via the jugular catheter at the time indicated postinjection. Plasma testosterone was extracted by ether before measurement by RIA. Each value represents the mean ± SEM. *$P < .05$ and **$P < .01$ *v* hCG-injected; $^{+}P < .05$ and $^{++}P < .01$ *v* 0 minutes.

LH Secretion In Vivo

Administration of Chansu alone did not alter the basal level of plasma LH. However, Chansu decreased ($P < .01$) GnRH-stimulated LH secretion 25% to 30% in male rats (Fig 3).

Production of Testosterone and cAMP In Vitro

Administration of hCG at 0.05 IU/mL increased testosterone release from rat testicular interstitial cells by threefold. Incubation of rat testicular interstitial cells with Chansu at a dose of 0.01, 0.1, or 1 μg/mL resulted in a dose-dependent decrease ($P < .01$) in both basal and hCG-stimulated testosterone release of 60% to 70% and 40% to 60%, respectively (Fig 4).

Incubation of rat testicular interstitial cells with forskolin or

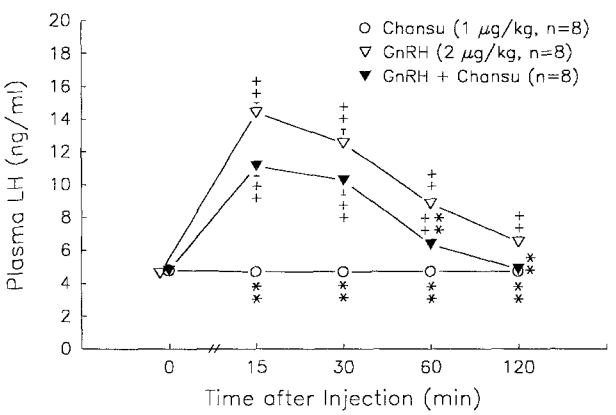

Fig 3. Effects of Chansu on basal and GnRH-stimulated plasma LH levels in male rats. Rats received a single intravenous injection of Chansu (○), GnRH (▽), or GnRH plus Chansu (▼) via the right jugular vein: Chansu 1 μg/mL/kg, n = 8; GnRH 2 μg/mL/kg, n = 8; GnRH + Chansu, n = 8. Blood samples were collected via the jugular catheter at the time indicated postinjection. Each value represents the mean ± SEM. *$P < .05$ and **$P < .01$ *v* GnRH-injected; $^{+}P < .05$ and $^{++}P < .01$ *v* 0 minutes.

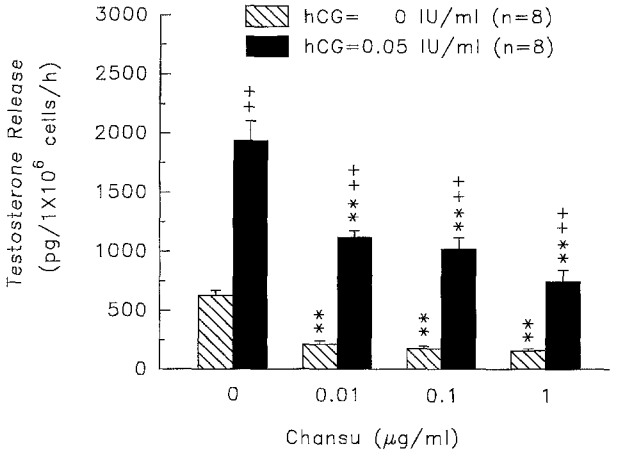

Fig 4. Effects of Chansu on basal and hCG-stimulated release of testosterone (n = 8) by rat testicular interstitial cells in vitro. Each column represents the mean ± SEM. **$P < .01$ *v* Chansu 0 μg/mL; ++$P < .01$ *v* hCG 0 IU/mL.

IBMX increased cellular cAMP levels. Chansu at 0.1 or 1 μg/mL decreased both basal and forskolin- or IBMX-induced cAMP production in rat testicular interstitial cells by 30% to 45% (Fig 5).

Release of LH In Vitro

Incubation of rat APs with GnRH caused a significant increase of LH release in vitro. However, administration of Chansu in vitro significantly diminished ($P < .01$) both spontaneous and GnRH-stimulated LH release (Fig 6).

GnRH Release In Vitro

Administration of Chansu at 0.1 and 1 μg/mL resulted in a significant ($P < .01$) increase of GnRH release by fourfold and 2.5-fold, respectively, but did not affect the concentration of GnRH in MBH tissues (Fig 7).

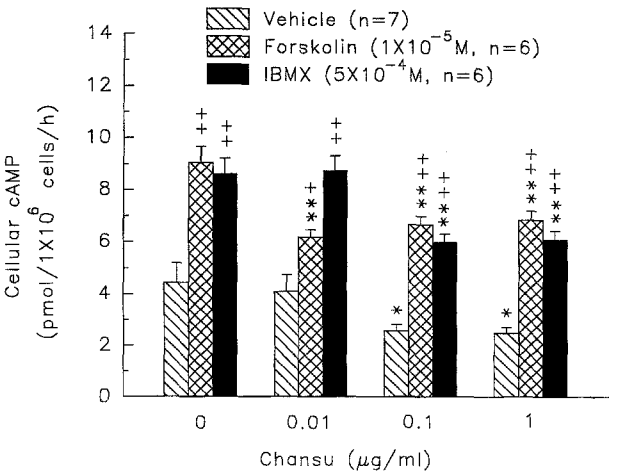

Fig 5. Effects of Chansu on cAMP production in response to 10 μmol/L forskolin and 0.5 mmol/L IBMX in rat testicular interstitial cells in vitro (n = 7). Each value represents the mean ± SEM. *$P < .05$ and **$P < .01$ *v* Chansu 0 μg/mL; +$P < .05$ and ++$P < .01$ *v* vehicle.

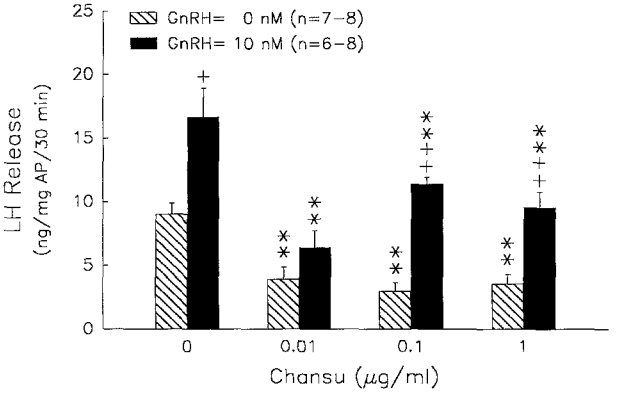

Fig 6. Effects of Chansu on basal (n = 7 to 8) and GnRH-stimulated (n = 6 to 8) release of LH in vitro. The AP was bisected, preincubated, and then incubated either with or without 10 nmol/L GnRH at 37°C for 30 minutes. Each value represents the mean ± SEM. **$P < .01$ *v* Chansu 0 μg/mL; ++$P < .01$ *v* vehicle (GnRH 0 nmol/L).

DISCUSSION

These results demonstrate that administration of the methanol extract of Chansu decreased the secretion of testosterone and LH but increased the release of GnRH in male rats.

Our recent investigation indicates that bufalin at approximately 10^{-7} to 10^{-6} mol/L (ie, 0.04 to 0.4 μg/mL) is effective to diminish the LH response to GnRH and the secretion of

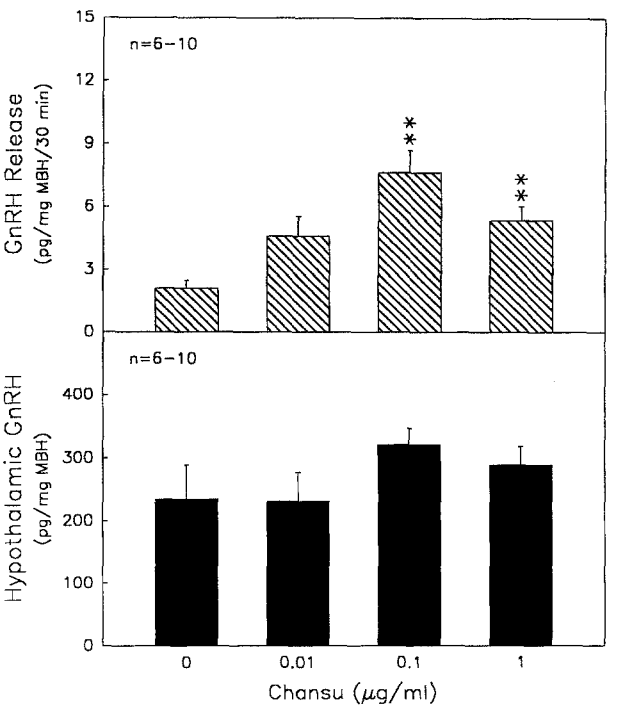

Fig 7. Effects of Chansu on GnRH release in vitro. MBH was excised, preincubated, and then incubated with Locke's medium at 37°C for 30 minutes. The concentration of GnRH in the acid extract of media (A, n = 6 to 10) and tissue blocks (B, n = 6 to 10) was measured by RIA. Each column represents the mean ± SEM. **$P < .01$ *v* Chansu 0 μg/mL.

testosterone in male rats.[12] Based on our HPLC analysis, no activity of bufalin, cinobufagin, digoxin, or estradiol was found in the preparation of methanol extract of Chansu. Therefore, it is sufficient to conclude that all functions of the methanol extract of Chansu were independent of bufalin, cinobufagin, digoxin, or estradiol. The bufalin- or steroid-free components of Chansu in this study share similar inhibitory effects on basal and hCG-stimulated levels of plasma testosterone and in vitro production of testosterone and cAMP with bufalin.[12] Neither bufalin nor the methanol extract of Chansu altered the basal level of plasma LH. However, the plasma LH level in response to GnRH was completely abolished by bufalin, yet was only decreased 21% to 24% by Chansu. LH release in vitro was diminished by Chansu but unaltered by bufalin. These results reflect that the steroid-free components in Chansu possess some specific biological activities different from those in bufalin. Although all chemical activity of bufalin and cinobufagin has been removed, in the preparation of the methanol extract of Chansu there remains a complex mixture of bioactive chemicals. It is impossible at this time to attribute its pharmacological effects to any particular component(s).

In addition to cardiotonic activity,[21] Chansu shows a promotive action on platelet aggregation and cytotoxic activity on HeLa-S_3 cells.[22] Some effects of Chansu, eg, inhibition of writhing, prolongation of hexobarbital-induced hypnosis, hypothermia, antipyretic effects, and inhibition of acetic acid–induced capillary permeability in mice, were suggested to originate from cinobufagin.[5] However, augmentation of the blood glucose level and inhibition of gastric juice secretion in rats were suggested to originate from the constituents of Chansu other than cinobufagin.[5] High doses of Chansu induce a decrease in body weight and structural abnormalities in the liver and kidney of pregnant mice, as well an increase in the number of resorbed and dead fetuses.[3] These results demonstrate that Chansu is a drug of multiple effects.

Recently, we found that testosterone production in rats is inhibited by bufalin,[12] calcitonin,[15] or amphetamine[16] but increased by lactate,[23] by acting directly on the testicular tissues via a LH-independent mechanism. In this investigation, administration of Chansu diminished not only the basal level of plasma testosterone but also testosterone secretion in response to hCG. The inhibitory effects of Chansu on basal and hCG-stimulated testosterone release in vitro and the cAMP accumulation imply that Chansu can decrease the release of testosterone by acting directly on rat testicular interstitial cells via a mechanism involving inhibition of cAMP production. Inasmuch as the generation or accumulation of cellular cAMP in response to forskolin, an adenylyl cyclase activator, and IBMX, a phosphodiesterase inhibitor, was diminished by administration of Chansu, we suggested that in rat testicular interstitial cells the activity of adenylyl cyclase was decreased but the activity of phosphodiesterase was increased by Chansu. Since administration of Chansu at 0.01 μg/mL was effective to diminish the basal production of testosterone but did not affect that of testicular cAMP, other mechanisms in addition to cAMP production were involved in the effect of Chansu on testosterone secretion.

Although administration of Chansu decreased both basal and GnRH-stimulated LH release in vitro by rat APs and the plasma LH response to hCG, the basal level of plasma LH was not altered by injection of Chansu. Since administration of Chansu increased the release of GnRH by the rat MBH, we suggest that the unaltered plasma LH concentration with Chansu was due to the balance between increased GnRH release by the MBH and decreased LH release by the AP.

In summary, this study demonstrates that the bufalin-free extract of Chansu inhibits spontaneous and gonadotropin-induced secretion of testosterone by rat testes via a mechanism associated with a reduction of cAMP production. Meanwhile, Chansu inhibited both basal and GnRH-stimulated LH release but increased GnRH release by the rat MBH. The antiandrogenic and antigonadotropic effects of Chansu are important in view of the applications of the drug by itself or in combination with others.

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