

Therapeutic effects of *Schisandra chinensis* on the hyperprolactinemia in rat

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Abstract. Prolactin (PRL) is secreted from the pituitary gland in response to eating, mating, and ovulation. Increased serum concentration of PRL during pregnancy contributes to enlargement of the mammary glands of the breasts and prepares for production of milk. However, high PRL levels derived from prolactinoma and hyperprolactinemia induce physiological disorders such as infertility and early menopause. Natural compounds isolated from *S. chinensis* have been known to possess anti-oxidative, anti-inflammatory and anti-diabetic effects. In the present study, we examined the therapeutic effect of *S. chinensis* and its single compounds on hyperprolactinemia in the pituitary gland. In rat pituitary cells, PRL expression levels were examined using real-time PCR and western blot assay. Crude *S. chinensis* extract and its single compound, gomisin N, reduced mRNA and protein levels of PRL in GH3 cells. In addition, cell proliferation and PRL target gene expression in cells were modulated by *S. chinensis*. Similar to the *in vitro* experiments, crude *S. chinensis* extract and gomisin N reduced PRL levels in the pituitary and serum of immature female rats. These results show that *S. chinensis* and its single compound, gomisin

N, are regulators of PRL production and may be candidates for treatment of hyperprolactinemia and prolactinoma.

Introduction

The pituitary gland is a tiny organ found at the base of the brain (1). The pituitary gland is divided into three sections: anterior, intermediate and posterior lobes (2). The pituitary gland produces many hormones, including oxytocin, antidiuretic hormone, growth hormone, thyroid-stimulating hormone (TSH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH), that travel throughout the body and direct physiological processes (1,3). The anterior lobe is mainly involved in the development of the body, the sexual maturation and the reproduction (4). Hormones produced by the anterior lobe regulate growth and stimulate the adrenal and thyroid glands as well as the ovaries and testes.

The pituitary also generates prolactin (PRL), which is a 23-kDa polypeptide hormone secreted by lactotroph cells of the anterior pituitary gland (4). Pituitary PRL secretion is regulated by endocrine neurons in the hypothalamus, which secrete dopamine to act on dopamine receptors located on lactotrophs for inhibition of PRL secretion (5). PRL acts in an endocrine, autocrine and paracrine manner through prolactin receptor (PRLR) and a large number of cytokine receptors (6). PRL plays an important role in the reproductive health of both women and men. Specifically, it stimulates mammary glands to produce milk (lactation); increased serum concentrations of PRL during pregnancy cause enlargement of mammary glands of the breasts and prepare for production of milk. PRL also controls secretion of sex steroid hormones (4). Highly elevated levels of PRL reduce levels of estrogen in women and testosterone in men. The effects of mildly elevated levels of PRL are much more variable, as estrogen levels in women may either substantial increase or decrease (7).

Since PRL performs multiple physiological functions, uncontrolled levels of PRL are associated with many diseases.

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Hyperprolactinemia, which is noted in most cases of gonadotroph pituitary adenoma, is defined as a high concentration of PRL (8). One common cause of hyperprolactinemia is tumor growth on the pituitary gland, known as prolactinoma. If PRL levels are high, a doctor will test thyroid function and asks about other conditions and medications known to increase PRL secretion. Diagnosis of prolactinoma by magnetic resonance imaging (MRI) is the most sensitive method for detecting pituitary tumors and determining their size (9).

Schisandra chinensis (Turcz.) Baill (*S. chinensis*) has a well-recognized history in traditional Chinese medicine. *S. chinensis* fruits contain a variety of pharmacologically active lignans such as gomisins A, B, C, D, E, F, G, K3, N and J, together with schisandrol B, schisandrin (SS) and schisandrin C (SC). These compounds have diverse pharmacological activities, including detoxificant, anti-oxidant, anti-carcinogenic, anti-hepatotoxic, anti-inflammatory and anticancer activities (10,11). Two major lignans, SS and gomisin A (GA), have gained attention due to their therapeutic effects (11). For instance, SS possesses biological activities, including hepatoprotective, antiviral and neuroprotective effects (12,13). However, the effects of *S. chinensis* on pituitary and hormonal regulation have not been addressed. In the present study, therefore, we examined the effects of *S. chinensis* and its single compounds on regulation of PRL and growth hormone production in the pituitary. In addition, the therapeutic potential of *S. chinensis* was evaluated by *in vitro* and *in vivo* experiments.

Materials and methods

Plant material extraction. Dried fruits of *S. chinensis* were ground to a fine powder and successively extracted at room temperature with *n*-hexane, EtOAc and MeOH. Hexane extract was evaporated *in vacuo* and chromatographed on a silica gel column (70x8.0 cm) with a step gradient of 0, 5, 10, 20 and 30% EtOAc in hexane (each 1 l). Fraction 11 was separated on a silica gel column (100x3.0 cm) with 25% hexane in CHCl₃ to give five subfractions. Fraction 11IA was further purified by column chromatography on silica gel eluted with CHCl₃-acetone (19:1) to give gomisin N (GN). Fraction 8 was separated on a silica gel column (100x3.0 cm) with CH₂Cl₂ to give SC. Fractions 36, 37 and 38 were separated on a silica gel column (100x3.0 cm) with 5% CH₂Cl₂ in acetone to give SS.

For extraction of GA and α -iso-cubebene (CU), dried fruits of *S. chinensis* were ground and extracted with *n*-hexane, CHCl₃ and methanol. Hexane extract was evaporated *in vacuo* and chromatographed on a silica gel column (100x10 cm) with a step gradient (0, 5 and 20%) of ethyl acetate in hexane and 5% methanol in CHCl₃ to obtain 38 fractions as described before. (22) Fraction 11 was separated on a silica gel column (100x3.0 cm) with hexane-chloroform-methanol (75:25:1 by volume) to obtain four fractions. Fraction 3 was separated on a Sephadex column (100x3.0 cm) with methanol (KH11CIC). Finally, KH11CIC was separated on a silica gel column (115x3.0 cm) with 5% acetone in chloroform to yield GA. Fraction 11 was separated on a silica gel column (100x3.0 cm) with 15% acetone in CH₂Cl₂ to obtain nine fractions. Next, fraction 2 was separated on a silica gel column (100x3.0 cm) with 15% acetone in CH₂Cl₂ to yield α -iso-cubebene (CU). Pure CU was identified by HPLC on a Phenomenex Luna C18

column (Phenomenex, 150x4.6 mm ID; 5 μ m particle size) with an acetonitrile-water-reagent alcohol gradient at a flow rate of 1.0 ml/min.

GH3 cell culture and treatments. GH3 rat pituitary epithelial-like tumor cells were seeded in culture medium and allowed to attach during 24 h of incubation, after which the seeding medium was removed and replaced with experimental medium added to 100 U/ml of penicillin and 100 μ g/ml of streptomycin for 24 h before treatment. Chemicals were dissolved in EtOH, diluted with experimental medium and added to the wells. Cells were treated with crude extract (crude, 0.1 mg/ml) and single compounds, including GN, GA, SC, SS and CU (20 μ M) or EtOH as a vehicle control.

Experimental animals and treatments. Immature female Sprague-Dawley (n=19) rats were acquired from Samtako Bio (Osan, Republic of Korea). Animals were housed at the Pusan National University Laboratory Animal Resources Center, which is accredited by the AAALAC and Korea FDA, according to the National Institutes of Health guidelines. The rats were housed in cages under a 12-h light/dark cycle and a constant temperature of 23 \pm 1°C. The Ethics Committee of Pusan National University (Busan, Republic of Korea) approved all experimental animal procedures (approval no. PNU-2015-0921). Rats were treated daily with crude (200 mg/kg), GN (20 mg/kg), progesterone (P4, 1 mg/kg) or corn oil (vehicle control) via oral (crude and GN) or subcutaneous injection (P4) from postnatal days 13-19. Dosage was adjusted according to changes in body weight (BW). All animals were sacrificed using CO₂ gas for preparation of tissue and serum samples.

Quantitative real-time PCR (q-PCR). Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Concentration of total RNA was measured by a spectrophotometer. DNA (cDNA) was prepared from total RNA (1 μ g) by reverse transcription (RT) using M-MLV reverse transcriptase (Invitrogen) and random primers (9-mers; Takara Bio, Shiga, Japan). q-PCR was performed using cDNA template (2 μ l) and SYBR-Green (6 μ l; Toyobo Co., Ltd., Shiga, Japan) with specific primers. Primer sequences used for PRL, PRLR, growth hormone and β -actin are as follows: PRL (left, 5'-AGT CTG TTC TGG TGG CGA CT-3' and right, 5'-GAA GTG GGG CAG TCA TTG AT-3'); PRLR (left, 5'-CCT CTG CAC TTG CTT TCG TC-3' and right, 5'-ATC GAT TCC TCC ATC TGT CC-3'); growth hormone (left, 5'-CTG GCT GCT GAC ACC TAC AA-3' and right, 5'-AAG CGA AGC AAT TCC ATG TC-3'); β -actin (left, 5'-ACC AAC TGG GAC GAT ATG GAG AAG-3' and right, 5'-TAC GAC CAG AGG CAT ACA GGG ACA-3'). q-PCR was carried out for 40 cycles using the following parameters: denaturation at 95°C for 15 sec, annealing and extension at 70°C for 60 sec. Fluorescence intensity was measured at the end of each extension phase. The threshold value for the fluorescence intensity of all samples was set manually. The reaction cycle at which PCR products exceeded this fluorescence intensity threshold during exponential phase of PCR amplification was considered to be the cycle of threshold (CT). Expression of the target gene was quantified

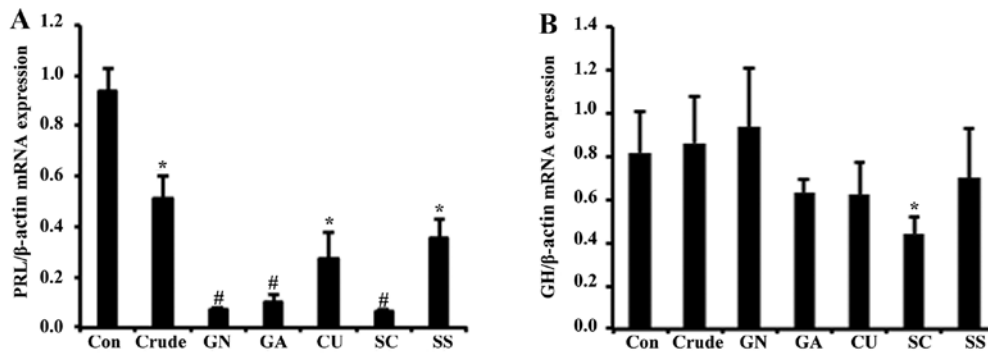


Figure 1. Regulation of PRL and growth hormone mRNA expression by *S. chinensis* and its single compounds in GH3 cells. Total mRNA was harvested from GH3 cells treated with crude extract and single compounds. Transcription levels of PRL (A) and growth hormone (B) were analyzed by q-PCR compared to the control group. Expression levels of PRL and growth hormone were normalized to that of β -actin. Data are expressed as the mean \pm SD. * $P < 0.05$ compared to the control group; # $P < 0.02$ compared to the control group.

relative to that of β -actin, a housekeeping gene, based on the comparison of CTs at constant fluorescence intensity.

Western blot analysis. Protein samples were extracted from GH3 cells with cell lysis buffer (20 mM Tris, 100 mM NaCl, 0.5% NP-40, 0.5 mM EDTA and 0.5% protease inhibitor cocktail). A total of 25 μ g of protein was separated by 10-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes (Dogen, Seoul, Korea). Membranes were subsequently blocked for 2 h with 5% skim milk (Difco™) in tris-buffered saline (TBS) with 0.05% Tween-20 (TBS-T). After blocking, membranes were incubated with antibodies specific for PRL (diluted 1:500) overnight at 4°C as well as horseradish peroxidase (HRP)-conjugated anti-goat secondary antibodies in 5% skim milk with TBS-T for 1 h. Luminol reagent (Bio-Rad Laboratories) was used to visualize antibody binding. Each blot was scanned using Gel Doc 1000, version 1.5 (Bio-Rad Laboratories) and band intensities were normalized to β -actin levels.

Cell proliferation assay. GH3 cells (3×10^4 cells/well) were seeded on 96-well plates in 200 μ l of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μ g/ml of streptomycin. After 24 h of incubation, the seeding medium was removed and replaced with experimental medium (phenol red-free DMEM supplemented with 10% charcoal-dextran-treated FBS, 100 U/ml of penicillin and 100 μ g/ml of streptomycin) for 24 h before treatment. Cells were treated with all single compounds (20 μ M each) or EtOH as a vehicle control for 24 h, after which 50 μ l of MTT solution (2 mg/ml) was added to each well in 200 μ l of medium without phenol red and the plates incubated for 4 h at 37°C. Dimethyl sulfoxide (DMSO) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, absorbance in the wells was measured at 570 nm with a reference wavelength of 650 nm.

Statistical analyses. Results are presented as the mean \pm standard deviation (SD). Data were analyzed using SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA, USA). $P < 0.05$ were considered statistically significant.

Results

Regulation of PRL expression and secretion by *S. chinensis* and its compounds in GH3 cells. To investigate the effect of *S. chinensis* on pituitary PRL production, GH3 cells were treated with crude, GN, GA, CU, SC and SS for 24 h. Transcription levels of PRL were reduced in response to crude by ~2-fold (Fig. 1A), and all single compounds of *S. chinensis* significantly reduced PRL mRNA levels. As GH3 cells are known to synthesize another critical substance, the growth hormone, we also analyzed the effect of *S. chinensis* on transcriptional regulation of the growth hormone. In contrast to PRL levels, mRNA expression of growth hormone was not significantly altered by crude or any single compounds, suggesting that the effect of *S. chinensis* is specific to the regulation of PRL (Fig. 1B).

Since the effects of GN, GA and SC were more dominant compared with those of CU and SS, we conducted further experiments using these three compounds. When the cells were treated with crude and single compounds (GN, GA and SC), protein levels of PRL were reduced ~2-fold by crude and GN (Fig. 2A). To test secretion of PRL, cell culture media was collected and subjected to western blot assay. In the results, PRL secretion was also reduced by all treatment groups, which is consistent with the mRNA and protein results (Fig. 2B). In all experiments, GN showed the most significant alteration of PRL production among single compounds.

Dose- and time-dependent regulation of PRL synthesis. Regulation of PRL by GN, a single compound of *S. chinensis*, was further explored in a dose- and time-dependent manner. Pituitary cells were treated with various concentrations (5, 10, 20 and 50 μ M) of GN for 24 h, resulting in significant reduction of PRL protein levels and secretion at concentrations of 10 to 50 μ M in a dose-dependent manner (Fig. 3A). Basal PRL expression levels were reduced according to culture time, suggesting that PRL synthesis may be controlled by autocrine regulation of PRL itself. When cells were treated with GN, PRL protein expression was downregulated from 16 to 24 h compared with the negative control during the same time. GN treatment also reduced secretion of PRL in culture media of GH3 cells from 16 to 24 h, which is similar to mRNA and protein expression levels (Fig. 3B).

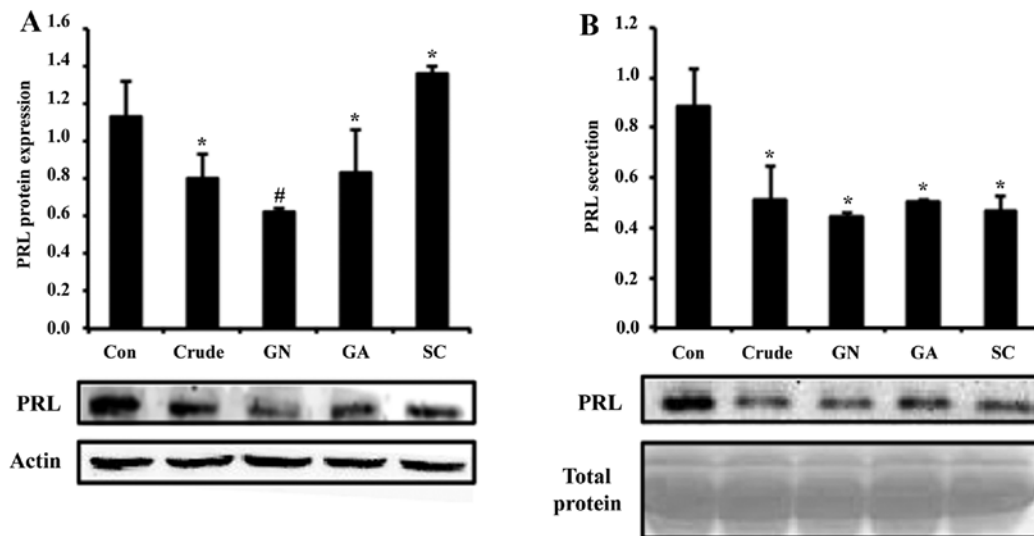


Figure 2. Regulation of PRL protein expression and production by *S. chinensis* and single compounds in GH3 cells. Translation level of PRL (A) was analyzed by western blot assay. Protein production of PRL (B) in growth medium secreted from GH3 cells was analyzed. Data are expressed as the mean \pm SD. Expression level of PRL was normalized to that of β -actin. Protein content in growth medium was normalized to total proteins stained with Coomassie brilliant blue. * $P<0.05$ compared to the control group; # $P<0.02$ compared to the control group.

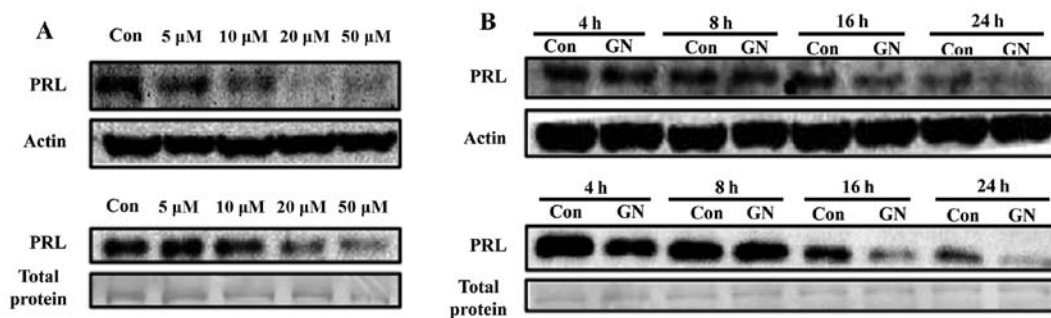


Figure 3. Dose- and time-dependent prolactin expression and production induced by GN. Protein expression of PRL after GN treatment was tested in a (A) dose- and (B) time-dependent manner. Data are expressed as the means \pm SD of triplicate experiments. Translation level of PRL was normalized to that of β -actin. Protein content in growth medium was normalized to total proteins stained with Coomassie brilliant blue.

Effect of *S. chinensis* and GN on GH3 cell proliferation and PRL target gene. To evaluate whether or not GN regulates viability of GH3 cells, we performed MTT assay (Fig. 4A). GH3 cells were treated with crude and GN for 24 h, after which the cell viability was measured. Cell proliferation was inhibited by $\sim 20\%$ in both crude and GN groups compared to the control. These results showed that *S. chinensis* and its single compound, GN, inhibit not only PRL production but also viability of PRL-secreting cells. To examine the expression of PRL target gene after crude and GN treatment, mRNA levels of the PRL-specific receptor PRLR were tested. Both crude and GN enhanced expression of PRLR up to 4-fold compared to the control (Fig. 4B).

Regulation of PRL and PRLR production by *S. chinensis* and GN in immature rats. Based on our *in vitro* findings, we next examined the effects of *S. chinensis* and GN *in vivo* using immature female rats. Rats were orally injected with crude, GN, or corn oil as a vehicle control from postnatal days 16 to 18 and sacrificed on day 19. Rats were also treated with P4 by subcutaneous injection as a positive control for reduction

of PRL. The ratio of organ weight to body weight indicated no significant alteration of liver and kidney weights in all animals, suggesting that there is no toxic effect by the treatment (Fig. 5A). Transcription level of PRL in the pituitary of immature female rats was examined using real-time PCR. Expression of PRL was reduced ~ 3 -fold by crude and 2-fold by GN treatment (Fig. 5B). To test PRL secretion levels, serum of immature female rats was analyzed by western blot assay. Crude group showed more strong reduction of PRL secretion, whereas the GN group showed moderate reduction (Fig. 5C). For the next experiment, PRLR mRNA levels were analyzed by real-time PCR. The mRNA expression level of PRLR was significantly elevated upon crude and GN treatment, which is consistent with the *in vitro* study (Fig. 5D).

Discussion

Homeostatic maintenance of PRL is essential since this hormone performs multiple physiological functions (14). General guidelines for hyperprolactinemia define the upper threshold of normal PRL at 25 μ g/l for women and 20 μ g/l

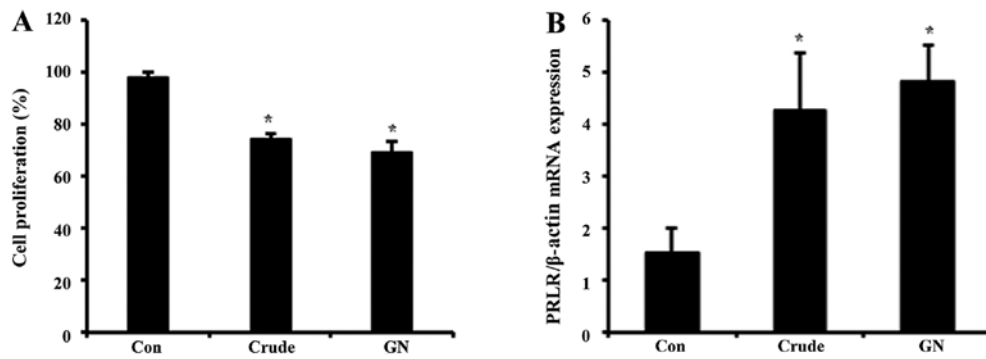


Figure 4. Effect of *S. chinensis* and GN on cell proliferation and cellular response. After treatment of GH3 cells with Cr and GN, cell proliferation was analyzed by MTT assay (A). Transcription level of PRLR (B) was analyzed by q-PCR after treatment of GH3 cells with Cr and GN. Data are expressed as the mean \pm SD. Transcription level of PRLR was normalized to that of β -actin. * $P < 0.05$ compared to the control group.

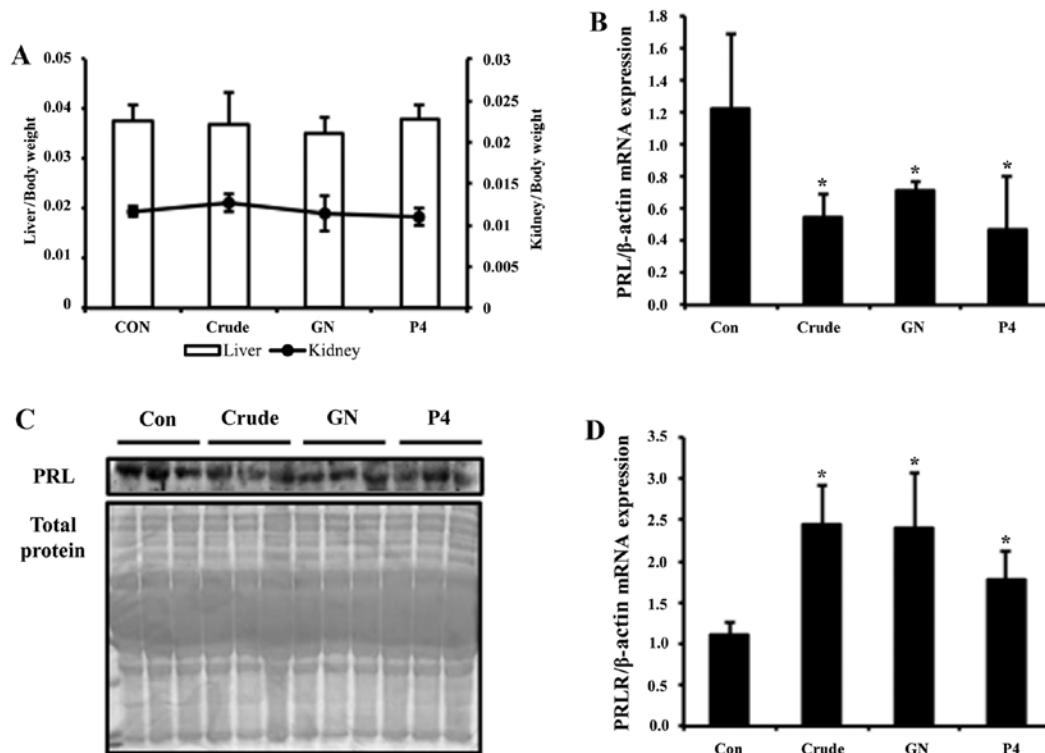


Figure 5. Regulation of PRL expression and secretion by *S. chinensis* and GN in immature rats. Regulation of PRL by *S. chinensis* and GN treated daily via oral injection for 7 days or P4 for 3 days in immature female SD rat (A) livers and kidney/body weight ratio. Pituitary mRNA (n=5) (B and D) and serum protein (n=3) (C) levels of PRL were analyzed by q-PCR and western blot assay. Data are expressed as the means \pm SD. The mRNA and protein expression levels were normalized to that of total protein. * $P < 0.05$ compared to the control group.

for men, whereas hypoprolactinemia is defined as PRL levels below $3 \mu\text{g/l}$ in women and $5 \mu\text{g/l}$ in men (15-17). The representative cause of hyperprolactinemia is prolactinoma, and increased PRL levels can cause infertility and bone loss in both women and men. The goal of treatment for prolactinoma is to return PRL levels to normal, reduce tumor size, and correct any visual abnormalities (15). In some cases, surgical correction of prolactinoma has been shown to lower PRL levels to less than 250 ng/ml in 80% of patients. Even in patients with large tumors that cannot be completely removed, drug therapy may be able to reduce serum PRL levels back to the normal range (18). Dopamine is the chemical that normally inhibits PRL secretion, and dopamine agonists such

as bromocriptine and cabergoline are effective medicines for treatment of hyperprolactinemia. However, bromocriptine and cabergoline are associated with side-effects such as nausea, vomiting, dizziness and hypotension (18,19). To solve these problems, development of a treatment with fewer side-effects is required.

Natural products that generally show less side-effects than chemical drugs have become the main focus for the treatment of prolactinemia. Ginseng treatment was shown to reduce PRL secretion via a direct nitric oxide-mediated effect on the anterior pituitary (20). In another study, *Ginkgo biloba* extract improved sexual performance in young sexually experienced male rats via reduction of serum PRL levels (21). However,

these studies focused on crude extracts of natural products and not on single compounds.

In the present study, we have for the first time investigated the therapeutic effects of extract and single compounds of *S. chinensis* on pituitary PRL regulation *in vivo* and *in vitro*. GH3 cells, which synthesize and secrete PRL, were treated with crude and single compounds, and GN remarkably reduced PRL mRNA and protein levels. PRL secretion was also reduced by GN in a dose- and time-dependent manner compared with the control. These results suggest that *S. chinensis* and its single compounds, especially GN, have potential as a natural medicine to treat hyperprolactinemia. In addition, we examined expression of the representative prolactin target gene, PRLR, in GH3 cells. As expected, PRL significantly reduced expression of its receptor. A previous study reported the rapid and prolonged downregulation of PRLR by PRL. In this study, PRL also caused degradation of PRLR, which is blocked by inhibitors of proteasomes and lysosomes (22). To further examine whether or not *S. chinensis* is effective for prolactinoma, we performed cell biability assay and observed that crude and GN successfully reduced pituitary carcinoma cell growth.

Following the *in vitro* study, we investigated the effect of *S. chinensis* crude and GN on PRL synthesis and secretion in immature female rats. In the present study, we employed sexually immature animals since sex steroid hormones are reported to regulate production of PRL. Specifically, P4 upregulates PRL synthesis in the endometrium but downregulates it in the myometrium and breast glandular tissue (23). Estrogen (E2) and P4 inhibit the stimulatory effects of PRL on milk production. More directly, it has been reported that E2 increases serum and pituitary PRL in ovariectomized rats, whereas P4 has inhibitory effects (24). In addition, precursor of E2 and P4, pregnenolone sulfate also increases prolactin production in the rat pituitary (25). In our experiments, we treated immature rats with crude, GN and P4. P4 was used as a positive control for the reduction of PRL based on previous studies (24). Similar to the *in vitro* study, crude and GN inhibited PRL but increased PRLR expression that is a target gene of PRL. Therefore, both *in vivo* and *in vitro* studies suggest that *S. chinensis* has a therapeutic effect on hyperprolactinoma.

In a previous study, *S. chinensis* was confirmed to possess various bioactivities and pharmacological applications. However, the mechanisms and bioactivities of its single compounds have not been well studied. GN is known to induce cellular apoptosis in hepatoma cells and leukemia cells (26,27). Additionally, studies on the mechanism of GN-induced anticancer activity against two human tumor cell lines, ovary carcinoma cells and colon adenocarcinoma cells, have been shown (28). GN successfully inhibited growth of these cell lines through induction of different types of cell death. Although there are several studies available on the therapeutic potentials of GN and *S. chinensis* in other tissues, the effects of *S. chinensis* and its single compounds in the pituitary have not been addressed at all.

In summary, we examined the suppressive effects of *S. chinensis* on the synthesis and secretion of pituitary PRL both *in vivo* and *in vitro*. These findings demonstrate that *S. chinensis* can be applied to patients with PRL-related disorders such as hyperprolactinemia and prolactinoma.

Acknowledgements

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