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Ginsenosides Promote Proliferation of Cultured Ovarian Germ Cells Involving Protein Kinase C-mediated System in Embryonic Chickens

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ABSTRACT : The effect of ginsenosides (GS) on germ cell proliferation was evaluated with a chicken ovarian germ-somatic cell coculture model and the mechanism involving protein kinase C (PKC) pathway was investigated. Ovarian cells were cultured in serum-free McCoy's 5A medium and challenged with GS alone or in combinations with PKC activator (phorbol 12-myristate 13-acetate, PMA) or inhibitor (H_7) for 48 h. The number of germ cells was counted and the proliferating cells were identified by immunocytochemistry of proliferating cell nuclear antigen (PCNA). Results showed that GS significantly increased germ cell proliferation and this stimulating effect was further increased by PMA, but inhibited by H_7, in a dose-dependent manner. Moreover, GS-elevated PCNA expression and the PCNA -labeling index of germ cells displayed similar changes with the increased numbers of germ cells. These results indicated that GS stimulated proliferation of ovarian germ cells with involvement of the PKC-mediated system. (**Key Words :** Ginsenosides, Protein Kinase C, Germ Cell, Proliferating Cell Nuclear Antigen, Chicken, Ovary)

INTRODUCTION

Ginseng (*Panax ginseng* C.A. Meyer) is a traditional medicinal plant in Asia with its Chinese name Renshen. Ginseng has been reported to possess diverse biological and pharmacological activities on the central nervous, cardiovascular, endocrine, and immune systems (Mizumaki et al., 2002; Kim et al., 2003; Wang et al., 2004). Ginsenosides (GS) or ginseng saponins are the main molecular components responsible for the actions of ginseng and more than 30 types of GS have been identified. They are known to possess a variety of medical efficacies, such as cardio-protective, immunomodulatory, hepato-protective, neuroprotective, antistress, antifatigue, anticancer, antiaging, sedative and improving the weak body condition (Liu et al., 2000; Kim et al., 2003; Tian et al., 2005; Xu et al., 2005). GS has been shown to exert various effects on diverse tissues and cells. GS increased the intracellular Ca^{2+} concentration in macrophages, 3T3 fibroblast and endothelial cells (Li et al., 2000) and activated Ca^{2+}-activated K^+ channels in vascular smooth muscle cells (Li et al., 2001). However, relatively few studies examined the actions of GS on reproductive systems. Yu et al. (2003) reported that GS had a curative effect on ovarian dysfunction caused by excessive stimulation with pregnant mare serum gonadotropin in immature rats, and Chan et al. (2003) demonstrated that GS exerted direct teratogenic effects on rat embryos.

Recent studies suggested that G proteins mediate some of the effects of GS. Nah et al. (1995) showed that the inhibitory effect of GS on voltage-dependent Ca^{2+} currents in sensory neurons was mediated through the activation of pertussis toxin-sensitive G protein. Further, Choi et al. (2001) provided evidence that GS enhanced Ca^{2+}-activated Cl^- currents by releasing intracellular Ca^{2+} via a pertussis toxin-insensitive Gaq/11 coupled to phospholipase C-β3 in the *Xenopus* oocyte. Latterly, Choi et al. (2003) reported that GS inhibited the activity of the GIRK 1/4 channel expressed in the *Xenopus* oocyte through a pertussis toxin-insensitive and $G\alpha_{q/11}$-, phospholipase C- and PKC-mediated signal transduction pathway. But no study about signal transduction of GS on germ cells was reported.

Since GS is widely used for human health protection and no study was reported about the effect of GS on germ cell development, we investigated the effect of GS on ovarian germ cell proliferation in embryonic chickens in this study. As germ cells can't survive for a long period without supporting of the somatic cells, a germ-somatic cell coculture model of chicken embryo ovary was adopted to evaluate the mechanisms of GS on proliferation of cultured germ cells. For assessment of proliferation of the germ cells,

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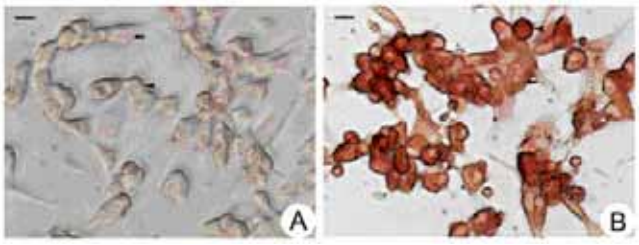

Figure 1. Immumocytochemical characterization of chicken ovarian germ cells by *c-kit*. Note somatic cells (arrow), germ cells (arrowheads). In the negative group (A), germ cells and somatic cells were all negative for *c-kit*. In the positive group (B), germ cells were positive for *c-kit* but somatic cells were negative. Scale bar: 10 μm.

proliferating cell nuclear antigen-labeling index (PCNA-LI) was determined besides direct counting of the cell number. We further investigated the underlying signal transduction mechanism through the PKC-mediated system by PKC inhibitor (H_7) or activator (PMA). The results will facilitate application of GS in animal reproduction.

MATERIALS AND METHODS

Animals

Fertilized Avian chicken eggs were obtained from a commercial hatchery and incubated at 38.5°C and 60% humidity in a rotatory incubator (Victoria SRL, Italy) until 18 days old.

Culture of ovarian cells

The procedures of dispersion and culture of ovarian cells were carried out according to a previous method (Liu et al., 2005). Briefly, left ovaries from Day 18 chicken embryos were minced and digested. The dispersed cells were seeded in collagen-treated 96-well culture plates (Nunc, Denmark) at a density of 5×10^4/well in 200 μl McCoy's 5A medium (HyClone, Utah, USA). The medium was supplemented with 2 mM glutamine, 1.75 mM HEPES, 100 IU/ml penicillin and 100 μg/ml streptomycin. The basal medium was replenished with 10 μg/ml insulin, 5 μg/ml transferrin, 3×10^{-8} M selenite (Sigma, St. Louis, MO) as ITS medium. Cells were incubated at 39°C in a water-saturated atmosphere of 95% air and 5% CO_2.

Treatment of cultured cells with chemicals

The chemicals were dissolved in ethanol and then diluted with medium. The GS was prepared by Kangfulai Health Protection Co. China. At the beginning of culture, ovarian cells were treated with GS (0.1-10 μg/ml) in ITS medium. Cells were also challenged with PMA (10^{-8}-10^{-6} M), H_7 (10^{-7}-10^{-5} M, Sigma) alone or in combinations with GS (1 μg/ml). The final concentration of ethanol in the medium was ≤0.1%. The control received the vehicle only.

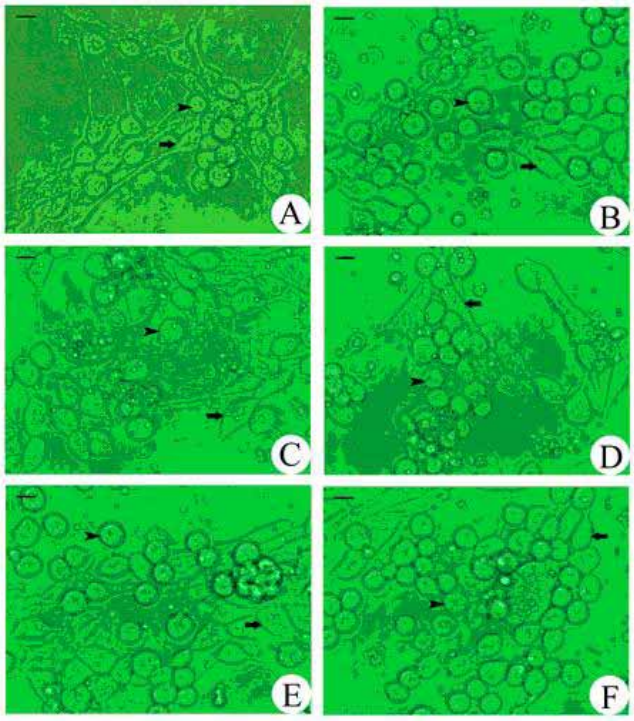

Figure 2. Morphological changes of embryonic chicken ovarian germ cells after treatment with GS (1 μg/ml) alone and combined with H_7, PMA in serum-free medium for 48 h. (A) through (F) indicated ovarian cells of control, GS (1 μg/ml), H_7 (10^{-5}M), GS+H_7 (10^{-5} M), PMA (10^{-6}M) and GS+PMA (10^{-6}M) groups, respectively. More germ cells (arrowheads) appeared in (B)(D)(F) than in (A)(C)(E). Scale bar: 10 μm.

Analysis of morphological changes

Morphological changes of germ cells and somatic cells were observed under an IX70 phase contrast microscope (Olympus, Japan). Five different regions were selected randomly in each well and the image was captured with a video camera (Pixera Pro 150ES, USA) to a computer. The number of germ cells was counted in each image. Analysis was achieved by using Simple PCI Advanced Imaging Software (Compix, Inc., USA).

Immunocytochemistry of PCNA

The cultured cells were fixed with methanol and acetone (3:1) and the fixed cells were incubated overnight at 4°C with a 1:400 dilution of mouse anti-PCNA antibody (Boster Co., Wuhan, China). Biotin-goat anti-mouse IgG was used as the secondary antibody. PCNA expression was visualized with a PicTure-Pius Kit (Zymed Laboratories, CA). Nuclei that were brown to black were counted as positive cells. LI was determined as the percentage of the germ cell number with positively stained nuclei to the total number of germ cells.

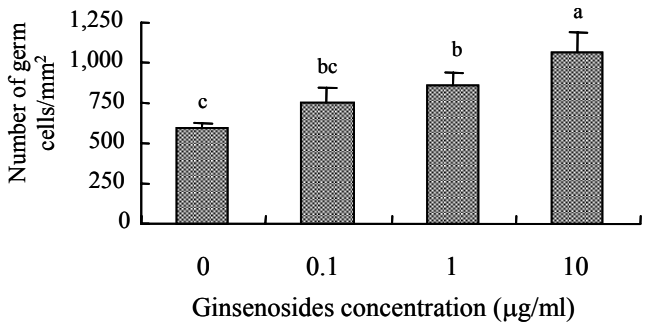

Figure 3. Effect of GS on germ cell proliferation of embryonic chicken ovary in serum-free medium after 48 h culture. Values are the means±SD (n = 4). Bars with different superscripts are statistically different.

Statistical analysis

The experiment was repeated three times with quadruplications. All data were expressed as the mean±SD and analyzed by ANOVA and Duncan's multiple range tests using the SAS 8.0 software. $p<0.05$ was considered significantly different.

RESULTS

Morphology of ovarian cells in culture

The dispersed ovarian cell suspensions obtained from the whole ovaries of 18-day-old embryonic chickens contained somatic cells and germ cells. A germ cell marker *c-kit* was used for discrimination between germ cells and somatic cells (Figure 1). The diameter of germ cells was greater than somatic cells. Germ cells as round or oval in shape were anchored on the surface of somatic cell layer and their diameters were between 15 and 25 μm (Figure 2).

Effect of GS on germ cell proliferation

GS (1-10 μg/ml) significantly increased the number of germ cells after 48 h culture ($p<0.05$), but lower GS (0.1 μg/ml) failed to augment the number of germ cells (Figure 3). Compared with the control group, germ cells in the GS-treated groups were more distinct than the control group and had higher cuboidal form (Figure 2A, 2B). PCNA staining was more intense in germ cells after GS treatment (Figure 4A, 4B) and the LI of germ cells was significantly higher than the control group ($p<0.05$; Figure 5).

Effects of H_7 on GS-stimulated germ cell proliferation

Morphological analysis revealed that GS-stimulated proliferation of the ovarian germ cell was obviously inhibited by combined treatments with H_7 (Figure 2C, 2D). However, no obvious changes were found among all groups of H_7 (10^{-7}-10^{-5} M) alone compared with the control (Figure 6). H_7 significantly depressed PCNA expression in germ cells of the combined groups compared with GS alone after 48 h of culture (Figure 4C, 4D) and the PCNA-LI of germ cells was significantly lower in the combined groups of H_7 and GS than GS group ($p<0.05$, Figure 5).

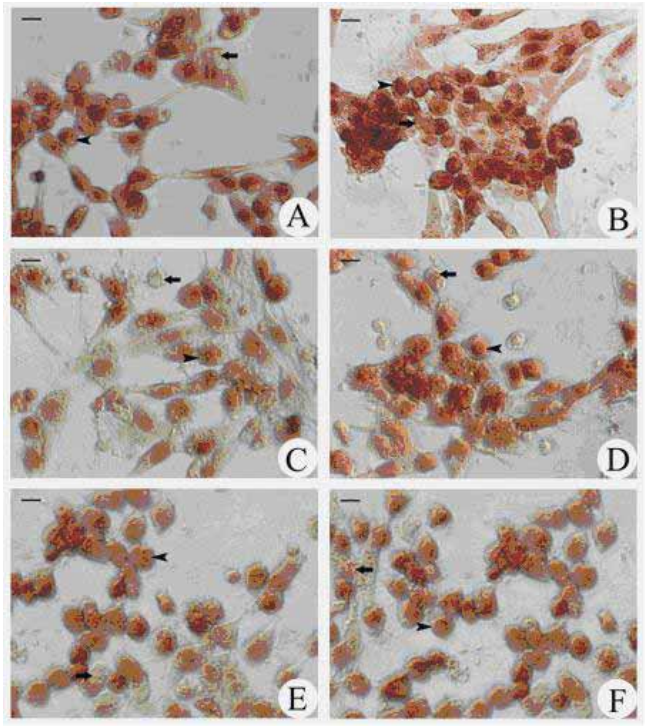

Figure 4. Immunocytochemical staining of PCNA in embryonic chicken ovarian cells treated with GS alone or in combinations with H_7 or PMA after 48 h culture in serum-free medium. (A) to (F) indicated ovarian cells of control, GS (1 μg/ml), H_7 (10^{-5} M), GS+H_7 (10^{-5} M), PMA (10^{-6} M) and GS+PMA (10^{-6} M) groups, respectively. Germ cells in (B)(D)(F) shows higher PCNA-LI than that in (A)(C)(E), as indicated by brown nuclear staining (arrowheads). Germ cells (arrow) without nuclear staining were PCNA-negative. Scale bar: 10 μm.

Effects of PMA on GS-stimulated germ cell proliferation

There was no significant change in the number of germ cells after treatment with lower PMA (10^{-8} and 10^{-7} M), but higher PMA (10^{-6} M) augmented the number of germ cells ($p<0.05$, Figure 7). Combined administration of PMA with GS resulted in a visible increase in germ cell number in all groups ($p<0.05$, Figures 2E, 2F and 7). PCNA expression and PCNA-LI in germ cells also manifested a marked synergistic effect of GS and PMA on germ cell proliferation ($p<0.05$, Figure 4E, 4F and 5).

DISCUSSION

Along with the rapid development of modern animal production, a great deal of synthetic feed additives was used, such as growth promoting products, antibiotics, hormones, seasoning products, meliorate products and antiseptic, etc. However, the side effects of these additives were inevitable.

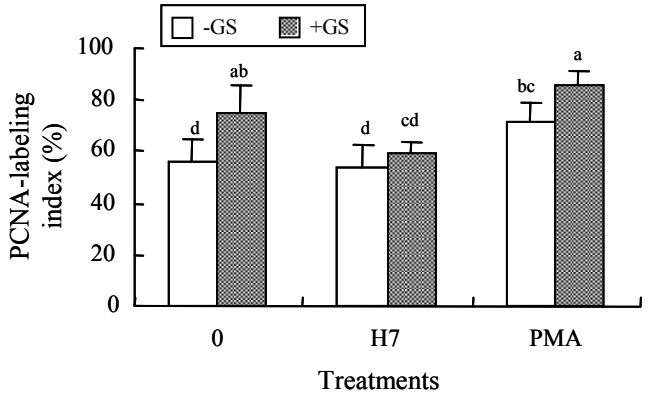

Figure 5. Changes of PCNA-LI in chicken ovarian germ cells cultured in serum-free medium after treatment with GS (1 μg/ml) alone and in combinations with H_7 (10^{-5}M) or PMA (10^{-6}M). Treatment of GS caused higher PCNA-LI in germ cells than control. The PCNA-LI of germ cells was significantly lower in the combined groups of H_7 and GS than GS group. Combined administration of PMA with GS induced further augments of PCNA-LI in germ cells. Values are the means±SD (n = 4). Bars with different superscripts are statistically different.

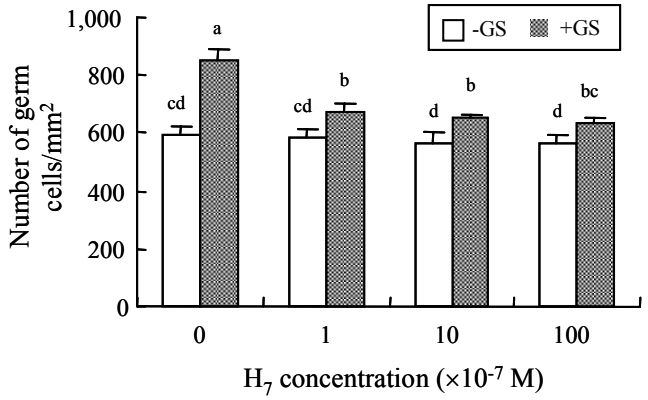

Figure 6. Effect of H_7 on GS-stimulated increase in germ cell number of embryonic chicken ovary in serum-free medium after 48 h culture. Combined administration of GS with H_7 (10^{-7}-10^{-5} M) reduced the increase in germ cell number that was stimulated by GS. Values are the mean±SD (n = 4). Bars with different superscripts are statistically different.

Especially the abused chemicals and their residues will harm human health via consumed animal products. Based on the above consideration, beneficial or negative effects of any natural herb additives used in animal production, including traditional Chinese medicines, must be examined through modern techniques. For example, Denli et al. (2005) stated that propolis, which was a resinous material gathered by honeybees from the buds and bark of certain trees and plants, could serve as a natural substitute for antibiotics in poultry diets. By means of the germ-somatic cell coculture model, effect of feed additives on germ cells can be evaluated without complex interactions of different internal and eternal factors, which occur in the *in vivo* studies.

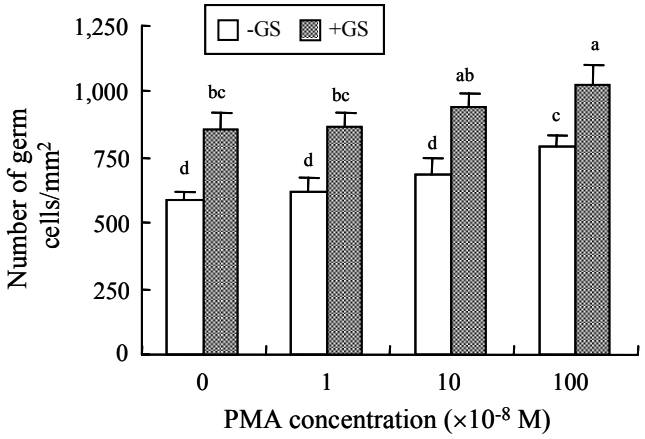

Figure 7. Effect of PMA on GS-stimulated increase in germ cell number of embryonic chicken ovary in serum-free medium after 48 h culture. Treatment of PMA (10^{-8}-10^{-6} M) improved GS-stimulated increase in germ cell number. Values are the means±SD (n = 4). Bars with different superscripts are statistically different.

In the present study, we examined proliferating effects of GS on cultured ovarian germ cells of embryonic chickens and further investigated the underlying signal transduction mechanism through PKC-mediated system by blockade or activation of PKC. We found that the number of germ cells was increased by GS treatment and this stimulating effect was further proved by increased PCNA-LI of germ cells. After oral administration of Rg1 to male SD rat at a dose of 100 mg/kg, the effective Rg1 concentration in blood plasma amounted to 0.9 μg/ml (Takino et al., 1982; Odani et al., 1983). As GS can traverse cell membranes freely, we believe the concentration of GS (1-10 μg/ml), which significantly increased the number of germ cells in our experiment, will also be effective in gonads. But the GS-stimulated proliferation of the ovarian germ cell was obviously inhibited by combined treatments with H_7. The results were consistent with the reports that H_7 inhibited the promoting effects of follicle-stimulating hormone in human epithelial ovarian cancer cells through PKC-mediated system (Ohtani et al., 2001). These findings suggest that PKC-dependent pathway is involved in GS action in germ cells. So far there have been many reports about pharmacokinetics and pharmacodynamics of GS (Wang et al., 2005). However, the cellular basis of GS action is still unclear, and the studies of GS signal transduction are rather insufficient. Recent studies suggested that G proteins mediate some effects of GS (Nah et al., 1995; Choi et al., 2003). We also examined the response of germ cells to GS in the presence of PMA, a PKC activator, and found that PMA augmented the number of germ cells. Combined administration of PMA with GS resulted in a further increase in germ cell number and PCNA-LI of germ cells. These results were in agreement with the reports that treatment of retinal cell cultures with PMA induced an

increase in ganglion cells survival (Santos and Araujo, 2000). So, our results indicated that PKC plays an important role in germ cells proliferation that was stimulated by GS since treatment with H_7 suppressed the GS-stimulated cell proliferation while PMA increased the GS-stimulated cell proliferation.

GSs, the major active components of ginseng, are the derivatives of triterpenoid dammarane consisting of 30 carbon atoms, steroid-like structure with sugar moieties. About 30 different forms have been identified from *Panax ginseng* and produce multiple pharmacological responses (Liu et al., 2000; Kim et al., 2003; Tian et al., 2005; Xu et al., 2005). Based on structural differences, the pharmacological activities of each GS are different. For example, Rb2, Rg1 and Rd administered intraperitoneally attenuated the immobilization stress-induced increase in plasma IL-6 level; Rb1 and Rg1 had a partial neurotrophic and neuroprotective role in dopaminergic cell culture; Rg1 was a desirable agent for enhancing $CD4^+$ T-cell activity, as well as the correction of Th1-dominant pathological disorders; Rb3 inhibited the influence of strychnine-sensitive glycine receptors in hippocampal neurons of rats (Popovich and Kitts, 2002; Kim et al., 2003; Lee et al., 2004; Radad et al., 2004; Xu et al., 2005). In this experiment, we found that GS significantly promoted ovarian germ cell proliferation involving PKC-mediated pathway. However, besides PKC-mediated pathway, other signal transduction pathways involved in the GS-stimulated proliferation of germ cells need further investigation.

In summary, we revealed that PKC-mediated system was involved in the GS-stimulated germ cell proliferation in cultured chicken ovarian cells. Demonstration of PCNA expression further proved the stimulatory effects of GS on germ cell proliferation. Since raw materials of ginseng health additive are all herbs, which are easy to be absorbed by animals, no tolerance and drug-resistance occurs even used for long time. It collects multi-health functions, such as supplying nutrition, improving immunity, output and quality of animal products. The above results will surely boost applications of ginseng or GS on poultry reproduction.

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