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Ginsenosides stimulated the proliferation of mouse spermatogonia involving activation of protein kinase C*

Da-lei ZHANG[§1,2], Kai-ming WANG[§1], Cai-qiao ZHANG[†‡1]

([1]*Key Laboratory of Animal Epidemic Etiology and Immunological Prevention of the Ministry of Agriculture, College of Animal Sciences, Zhejiang University, Hangzhou 310029, China*)

([2]*College of Medicine, Nanchang University, Nanchang 330006, China*)

[†]E-mail: cqzhang@zju.edu.cn

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Abstract: The effect of ginsenosides on proliferation of type A spermatogonia was investigated in 7-day-old mice. Spermatogonia were characterized by *c-kit* expression and cell proliferation was assessed by immunocytochemical demonstration of proliferating cell nuclear antigen (PCNA). After 72-h culture, Sertoli cells formed a confluent monolayer to which numerous spermatogonial colonies attached. Spermatogonia were positive for *c-kit* staining and showed high proliferating activity by PCNA expression. Ginsenosides (1.0~10 μg/ml) significantly stimulated proliferation of spermatogonia. Activation of protein kinase C (PKC) elicited proliferation of spermatogonia at 10^{-8} to 10^{-7} mol/L and the PKC inhibitor H_7 inhibited this effect. Likewise, ginsenosides-stimulated spermatogonial proliferation was suppressed by combined treatment of H_7. These results indicate that the proliferating effect of ginsenosides on mouse type A spermatogonia might be mediated by a mechanism involving the PKC signal transduction pathway.

Key words: Ginsenosides, Spermatogonia, Protein kinase C, Mouse
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INTRODUCTION

Ginseng has long been used as a traditional medicine in Asian countries. The multiple pharmacological actions of ginseng are generally attributed to ginsenosides, the primary active ingredients extracted from ginseng. Ginsenosides exert varying effects on a myriad of cells and tissues, including pharmacological responses on the central nervous, cardiovascular and endocrine systems (Attele *et al*., 1999). Rajput *et al*.(2007) showed that ginsenosides-based adjuvant could stimulate the cell-mediated immune system. However, the mechanisms of ginsenosides activity still largely remain unclear. A few studies have reported the action of ginsenosides on reproduction. Ginsenosides ameliorated ovarian dysfunction caused by excessive stimulation with equine chorionic gonadotropin in immature rats (Yu *et al*., 2003). Ginsenosides stimulated meiotic maturation of mouse oocytes through a paracrine pathway involving the nitric oxide/inducible nitric oxide synthase system (Zhang *et al*., 2006). However, ginsenosides exerted embryo toxicity on murine embryos (Chan *et al*., 2003; Liu and Zhang, 2006). Nevertheless, very little is known about the effect of ginsenosides on male germ cell development.

Germ cells originate from primordial germ cells (PGCs). In males, after migration to the genital ridges, PGCs are enclosed in the seminiferous cords and become gonocytes. Following birth, gonocytes migrate to the seminiferous tubule basement membrane and differentiate into spermatogonial stem cells. Spermatogonia divided into types A and B spermatogonia. The type A spermatogonia further develop into

‡ Corresponding author

§ The two authors contributed equally to this work

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intermediate and type B spermatogonia. Finally, through the last mitotic division, type B spermatogonia give rise to primary spermatocytes that will enter meiosis (de Rooij and Grootegoedt, 1998).

In this experiment, the Sertoli-germ cells co-culture system was adopted to evaluate the effect of ginsenosides on type A spermatogonial proliferation by colony formation and proliferating cell nuclear antigen (PCNA) expression. In addition, protein kinase C (PKC)-mediated signal transduction pathway was explored by PKC activator phorbol-12-myristate-13-acetate (PMA) or inhibitor H_7. These results would help to further our understanding of potential effects of ginsenosides on reproductive health.

MATERIALS AND METHODS

Isolation and culture of type A spermatogonia and Leydig cells

ICR (Institute of Cancer Research) male mice were obtained from the Center of Laboratory Animals, Zhejiang University, China. The testes were obtained from 7-day-old mice, and the seminiferous tubules were dispersed by 1 mg/ml collagenase (GIBCO BRL, USA) at 37 °C and then minced into small fragments (about 1 mm^3). The fragments were washed three times through gravitational sedimentation and dispersed by collagenase. The cell aggregates were filtrated through a 150-μm mesh and washed with Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL). Cells were cultured in collagen-treated 96-well culture plates (Nunc, Denmark) at a density of 5×10^4 per well in 200 μl DMEM medium supplemented with 10 μg/ml insulin, 5 μg/ml transferrin, and 3×10^{-8} mol/L selenium (ITS medium; Sigma, St. Louis, MO, USA). The Leydig cells were isolated from decapsulated testes of 3-week-old mice after dissociation with 0.25 mg/ml collagenase at 37 °C for 30 min and filtrated through a 75-μm nylon gauze. The cells were incubated at 37 °C, 100% humidity with 5% (v/v) CO_2 in air. The medium was replenished with fresh medium every 24 h.

Treatment of cultured cells with chemicals

At the beginning of culture, cells were treated with ginsenosides (Kangfulai Health Protection Co., China) at 0.1~10 μg/ml. In addition, cells were challenged with PMA (10^{-9}~10^{-7} mol/L) or H_7 (10^{-8}~10^{-6} mol/L) alone or in combination with ginsenosides (10 μg/ml) to study the mechanism. The control cells received the medium only.

Immunocytochemistry for *c-kit* and PCNA

The cells were fixed in 4% neutral paraformaldehyde and incubated with a rabbit anti-*c-kit* polyclonal antibody overnight. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody. The procedures for PCNA staining were similar to those for *c-kit* except that the primary antibody was mouse anti-human PCNA monoclonal antibody (Boster Co., Wuhan, China).

Staining for Oil Red O and 3β-hydroxysteroid dehydrogenase (3β-HSD)

Identifications of Sertoli cells and Leydig cells were performed by Oil Red O staining according to Wang *et al*.(2006) and 3β-HSD staining according to Lin *et al*.(1990), respectively.

Morphological analysis and count of type A spermatogonial colony number

Morphological changes of cultured cells were observed under a phase contrast microscope. The number of spermatogonial colonies was counted in images that were captured with a video camera (Pixera Pro 150ES, Los Gatos, USA) connecting to a computer.

Statistical analysis

The experiment was repeated three times and each treatment included four wells. Data were expressed as the mean±*SD*. The statistical differences were determined by analysis of variance (ANOVA) and Duncan's multiple range test of statistical analysis system (SAS) 6.12 software. $P<0.05$ was considered significantly different.

RESULTS

Morphology and characterization of type A spermatogonia

The cell suspension obtained from testes mainly contained spermatogonia and Sertoli cells. The

diameter of spermatogonia was between 10 and 12 μm. After 5-h culture in serum-free ITS medium, Sertoli cells attached at the bottom of the culture plate. In the present culture system, no cells showed positive 3β-HSD (a marker for the Leydig cells) staining (Fig.1a). The prepared Leydig cells showed positive 3β-HSD staining (Fig.1b). Sertoli cells showed positive Oil Red O staining (Fig.1c). After 72-h culture, Sertoli cells spread around almost the whole bottom to form a confluent monolayer to which numerous spermatogonial colonies of densely packed cells attached. Spermatogonia showed positive crimson labeling for *c-kit* (a marker for type A spermatogonia) after 24- and 72-h culture, and Sertoli cells showed negative staining (Figs.2a and 2b).

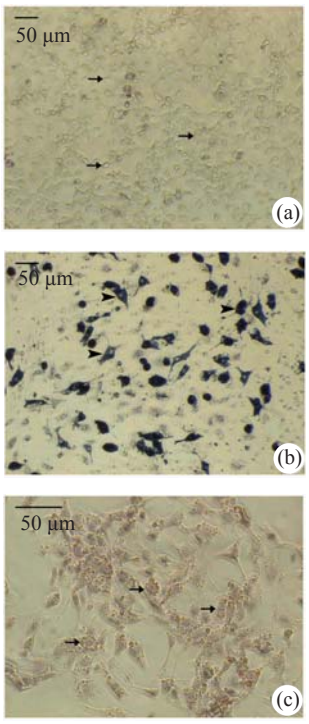

Fig.1 Identification of Leydig cells for 3β-HSD and Sertoli cells for Oil Red O
(a) In the present culture system, all cells showed negative 3β-HSD staining; (b) The prepared Leydig cells showed positive 3β-HSD staining; (c) Sertoli cells showed positive Oil Red O staining. Arrowheads: Leydig cells; Arrows: Sertoli cells

PCNA staining of type A spermatogonia

In order to confirm the morphological assessment of spermatogonial colony numbers, cells were stained for PCNA after 72-h culture for demonstration of the proliferating cells. Spermatogonia and Sertoli cells showed brown color in the positive staining (Fig.2c).

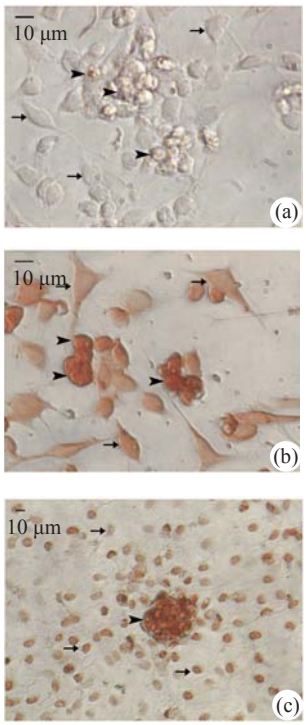

Fig.2 Immunocytochemistry of type A spermatogonia for *c-kit* and PCNA
(a) Negative staining for *c-kit* after 24-h culture; (b) Positive staining for *c-kit* after 24-h culture; (c) Spermatogonia and Sertoli cells showed positive PCNA staining after 72-h culture. Arrowheads: type A spermatogonia; Arrows: Sertoli cells

Effect of ginsenosides on spermatogonial cell proliferation

Treatment with 1.0~10 μg/ml ginsenosides for 72 h significantly increased the number and area of type A spermatogonial colonies ($P<0.05$). Compared with the control, ginsenosides at a lower concentration (0.1 μg/ml) had no significant effect on promoting spermatogonial proliferation (Fig.3).

Effect of PKC system on ginsenosides-stimulated spermatogonial proliferation

Compared with the control, activation of PKC by PMA (10^{-8}~10^{-7} mol/L) markedly stimulated spermatogonial proliferation. The maximal effect was observed at the highest concentration evaluated (10^{-7} mol/L). However, this stimulating effect was

reduced by H_7 (P<0.05) (Fig.4). In addition, ginsenosides (10 μg/ml) combined with PMA (10^{-8} mol/L) significantly increased the number and area of spermatogonial colonies (P<0.05), compared with either alone. H_7 at 10^{-7}~10^{-6} mol/L significantly attenuated the ginsenosides-stimulated spermatogonial proliferation (P<0.05). However, H_7 alone had no significant effect on the number of cell colonies (Fig.5).

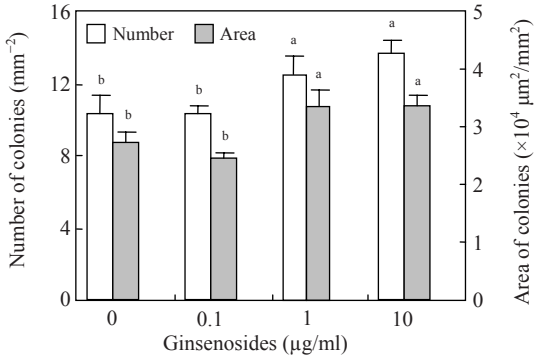

Fig.3 Effect of ginsenosides on type A spermatogonial proliferation
Values represent mean±*SD* (*n*=4). Bars with different letters are statistically different (*P*<0.05)

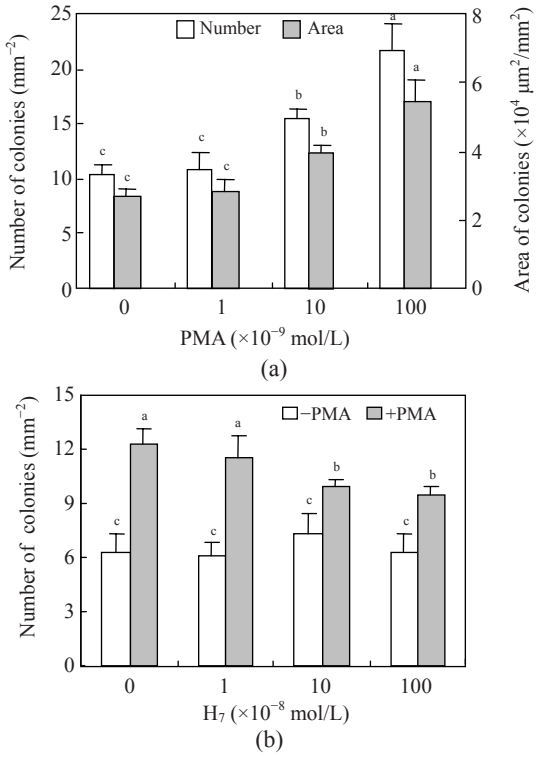

Fig.4 Effects of PKC activator (a) and inhibitor (b) on type A spermatogonial proliferation
Values represent mean±*SD* (*n*=4). Bars with different letters are statistically different (*P*<0.05)

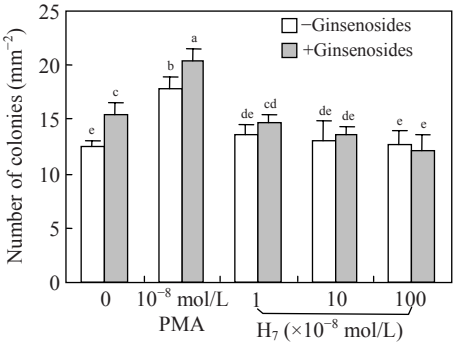

Fig.5 Involvement of PKC system in ginsenosides-stimulated type A spermatogonial proliferation
Ginsenosides (10 μg/ml) combined with PMA (10^{-8} mol/L) increased the number of spermatogonial colonies, compared with either ginsenosides or PMA alone. Values represent mean±*SD* (*n*=4). Bars with different letters are statistically different (*P*<0.05)

DISCUSSION

In the present study, the Sertoli-germ cell co-culture model was used to study the action of ginsenosides on regulating type A spermatogonial proliferation in mice. Sertoli cells are nursing cells that provide nutrients, growth factors, and microenvironment necessary for germ cell proliferation and differentiation. In order to identify type A spermatogonia and Sertoli cells, *c-kit* and Oil Red O stainings are adopted for discrimination between type A spermatogonia and Sertoli cells. The type A spermatogonia are *c-kit* positive (Dym *et al*., 1995). In the present study, *c-kit* expression was manifested in type A spermatogonia by immunocytochemistry and Sertoli cells showed Oil Red O positive. Furthermore, Leydig cells were removed through collagenase dispersing and gravity sedimentation, and the staining of 3β-HSD showed that very few Leydig cells existed in the present co-culture system. A spermatogonial proliferation was examined by PCNA immunocytochemistry. PCNA is preferentially synthesized after diapause and is localized in the nucleus at sites of active DNA replication. In this study, intensive staining of PCNA was demonstrated on spermatogonial colonies, suggesting that the type A spermatogonial cells maintained active proliferation after 72-h culture.

Subsequently we evaluated the role of ginsenosides on spermatogonial proliferation. Ginsenoside Rg3 could regulate the proliferation of the

spleen and bone marrow cells, and enhance the colony formation of hematopoietic progenitor cells (Joo *et al*., 2004). The current study revealed that ginsenosides displayed a stimulatory action on type A spermatogonial proliferation by colony formation. A previous study demonstrated that PKC was involved in ginsenosides-induced HL-60 cell differentiation (Kim *et al*., 1998). Ginsenosides stimulated the proliferation of chicken ovarian germ cells via a PKC-mediated pathway (Liu *et al*., 2006). Therefore, we hypothesized that ginsenosides might stimulate type A spermatogonial proliferation involving PKC signal transduction pathway.

Moreover, we evaluated the role of PKC signal transduction pathway on ginsenosides-stimulated type A spermatogonial proliferation. PKC, a family of multiple isoenzymes, was defined as a Ca^{2+}/phospholipid-dependent protein kinase. PKC and other cellular protein kinases play central roles in mediating the transduction of extracellular signals across the cytoplasm into the nucleus, thus modulating gene expression, cell growth, and differentiation. Many studies indicated that PKC-mediated signal transduction pathway was involved in cell survival, proliferation, and differentiation (Okuda *et al*., 1998; Motohashi *et al*., 2000). In germ cells, activation of PKC signal transduction pathway by external factors involved proliferation of chicken primordial germ cells and ginsenosides could promote this effect through activation of PKC signal transduction pathway (Tang and Zhang, 2007; Ge *et al*., 2007). In the present study, activation of PKC by PMA significantly increased the number and area of type A spermatogonial colonies, and PKC inhibitor H_7 remarkably attenuated the stimulating effect of PMA. This result shows that activation of PKC signal transduction pathway could promote type A spermatogonial proliferation. Meanwhile, ginsenosides and PMA had a notable synergistic effect on promoting type A spermatogonial proliferation, and the proliferating effect of ginsenosides was suppressed by PKC inhibitor H_7 in a dose-dependant manner. Therefore, this result suggests that ginsenosides-stimulated type A spermatogonial proliferation may be partly via PKC-mediated signal transduction pathway and other signaling factors may also be involved in ginsenosides-stimulated spermatogonial proliferation. Besides PKC-mediated pathway, other signaling kinases involved in the ginsenosides-stimulated proliferation of type A spermatogonia need further investigation.

CONCLUSION

Type A spermatogonia were characterized by *c-kit* immunocytochemistry and manifested active proliferation by PCNA staining. Ginsenosides stimulated proliferation of spermatogonia and this effect was suppressed by PKC inhibition. These results indicate that ginsenosides-promoted type A spermatogonial proliferation partly involved the PKC signal transduction pathway, suggesting that ginsenosides may be used to facilitate the in vitro amplification of spermatogonia for further studies.

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