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## Ginseng-enhanced oxidative and phagocytic activities of polymorphonuclear leucocytes from bovine peripheral blood and stripping milk

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**Summary** — This study investigated the effect of a dried ginseng extract on polymorphonuclear leucocytes (PMNL) in bovine blood and milk. In a test for chemiluminescence (CL), PMNL were pre-incubated in ginseng solution at 37°C in 5% CO<sub>2</sub> for 60 min, and then stimulated with bovine serum opsonized zymosan. The CL was about 30% higher for the cells pre-treated with ginseng solutions 100–1 000 µg/ml as compared with the non-ginseng-treated cells. In a test for phagocytosis, PMNL and fluorescent microspheres were incubated with ginseng in RPMI-1640 supplemented with 10% bovine serum at 37°C for 60 min. The proportion of actively phagocytic cells in the ginseng-treated group was greater than that in the non-ginseng treated group.

**ginseng / leucocyte / chemiluminescence / phagocytosis**

**Résumé** — Le ginseng stimule l'activité oxydative et phagocytaire des leucocytes polymorphonucléaires du sang et du lait d'égouttage de Bovins. L'effet d'un extrait de ginseng séché sur les leucocytes polymorphonucléaires du sang et du lait d'égouttage a été étudié chez la vache. Des leucocytes polymorphonucléaires préincubés dans une solution de ginseng à 37°C dans du CO<sub>2</sub> à 5% pendant 60 min, puis stimulés avec du zymosan opsonisé par du sérum bovin, ont été testés par chimio-luminescence. L'activité oxydative était supérieure d'environ 30% pour les cellules pré-traitées par une solution de ginseng de 10<sup>2</sup>-10<sup>3</sup> µg/ml par rapport aux cellules non traitées. Les leucocytes polymorphonucléaires ont ensuite été testés pour leur activité phagocytaire en les incubant avec des

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*microsphères fluorescentes et du ginseng dans du RPMI-1640 supplémenté par du sérum bovin à 10% (37°C pendant 60 min). Une augmentation de la proportion des cellules activement phagocytaires a été observée pour les cellules incubées avec une solution de ginseng, comparé aux cellules non incubées avec du ginseng.*

### ***ginseng / leucocyte / chimioluminescence / phagocytose***

## **INTRODUCTION**

Although new antimicrobial agents are continuously being developed, infections in the compromised host remain a serious problem. A major reason for this is that the host defense mechanisms become impaired. For example, there may be diminished levels of complement and decreased amounts of normally functioning antibodies or polymorphonuclear leukocytes (PMNL) (Hill *et al*, 1987; Krause *et al*, 1987; Nagahata *et al*, 1987; Takahashi *et al*, 1987). In dairy cows, the PMNL become much less phagocytic after they leave the blood circulation to enter the mammary gland, which may contribute to the susceptibility of mammary gland to infection (Jain and Lasmanis, 1978; Weber *et al*, 1983). Because of the extensive use of antibiotics in the dairy industry, antibiotic contamination in milk and its products has become a potential threat to human health (Dewdney *et al*, 1991). Consequently, there is an increasing interest in non-antibiotic approaches that avoid this contamination. The use of natural immunotherapeutic agents to improve the function of the immune cells may be an alternative way to deal with this problem. Ginseng, the root of *Panax ginseng* CA Meyer, is believed to be capable of increasing the body's resistance to many harmful factors and of protecting the tissues from damage when the body is exposed to a stress. The drug has been utilized in Chinese ethnopharmacology for more than 3 000 years. Recent investigations have shown that ginseng has multiple pharmacological effects on the immune system. Its immunomodulating effect has been widely studied in both humans and rodents. The

drug has been reported to enhance the mitogenesis of T and B lymphocytes that have been primed by mitogens (Yang and Yu, 1990), and to increase chemotaxis, phagocytosis and intracellular destruction by PMNL (Scaglione *et al*, 1990).

No information has yet been reported on the immunomodulating activity of ginseng in dairy cattle. The present study was undertaken in order to explore its potential application as an immunomodulator which could stimulate depressed phagocytosis in the mammary gland. It was designed to investigate the *in vitro* effect of a ginseng extract on phagocytosis and the chemiluminescence (CL) by bovine blood and mammary PMNL. Since the cell concentration in the stripping milk is higher than in the total milk, this part of milk was used as a cell source (Östensson *et al*, 1988). When phagocytotic cells engulf and destroy microorganisms, they produce light emission or CL as a result of the burst of oxidative activity (Allen and Loose, 1976). The CL was used to measure the oxidative activity in PMNL.

## **MATERIALS AND METHODS**

### ***Animals and sample collection***

Nine clinically healthy lactating Swedish Red and White breed cows were used in this study. Approximately 40 ml heparinized blood was collected from each cow *via* venipuncture (middle coccygeal vein) using sterile heparinized vacutainer tubes and 20-gauge, 40-mm needles (Becton Dickinson Vacutainer Systems, UK). Stripping milk (approximately 1 l) was collected into 250 ml siliconized plastic bottles from the 4 quar-

ters of each cow after the morning milking. The milk samples were tested according to Scandinavian Recommendations (Klastrup and Mansen, 1974) and were found to be bacteria-free.

### **Isolation of polymorphonuclear leucocytes from blood and milk**

For the isolation of blood PMNL, freshly collected heparinized blood was diluted 2:1 (v/v) with calcium- and magnesium-free phosphate-buffered saline solution (PBS), pH 7.2. About 30 ml of the diluted blood was layered on 10 ml of Ficoll-sodium diatrizoate of specific gravity 1.083 g/ml (Histopaque, Sigma chemicals, Saint Louis, MO, USA) and centrifuged at 1 500 *g* for 30 min at room temperature. After centrifugation, the mononuclear cell layer was removed and discarded. The sedimented erythrocytes in the PMNL-rich fraction were lysed by adding 10-fold volumes of 0.16 M  $\text{NH}_4\text{Cl}$  in 0.15 M Tris-buffer (pH 7.2) and by incubating with gentle mixing for 8–10 min at room temperature. The cell suspension was centrifuged at 500 *g* for 10 min at room temperature and the pellet was washed twice with PBS. For the phagocytosis test, the PMNL concentration was adjusted to a level of  $6.25 \times 10^6$  cells per ml of RPMI-1640 with HEPES buffer (Gibco, Bio-cult, Glasgow, UK) containing 2 mM glutamine, 10% normal bovine serum (NBS), 200 IU penicillin G, and 200  $\mu\text{g}$  streptomycin/ml. For the chemiluminescence test, the cell concentration was adjusted to  $1.11 \times 10^6$  cells/ml in PBS. On average, 90% of the isolated cells were PMNL with a viability of 96%.

For the isolation of mammary gland PMNL, the stripping milk was diluted 1:1 (v/v) with cold PBS (4°C), and centrifuged at 1 500 *g* for 30 min. After removing the fat and supernatant, the cell pellet was washed once in PBS by centrifugation at 500 *g* for 10 min. Cells were resuspended in 10 ml PBS and then passed through a loosely packed cotton column to remove debris and aggregates. The concentrations of mammary PMNL were then adjusted to the same levels as those mentioned above for blood leucocytes.

The cell concentration and viability were determined using Trypan Blue exclusion test and a Fuchs-Rosenthal counting chamber (Barta *et al.*, 1984). Differential cell counts were made by the Acridine Orange method (Euright and Jeffers, 1984) using a fluorescence microscope.

### **Ginseng extract**

A ginseng dry extract containing saponins equivalent to 14% of ginsenoside Rg1 (Batch No: 23933/R, indena SPA Italy) was generously provided by the Indena Company. For the phagocytosis assay, the ginseng extract was diluted to  $5 \times 10^2 \mu\text{g}$  per ml in PBS in order to reach a final concentration of  $10^2 \mu\text{g/ml}$ ; for the CL assay, the extract was diluted to  $10^2$ ,  $10^3$ ,  $10^4$  and  $10^5 \mu\text{g/ml}$  in PBS to reach final concentrations of 10,  $10^2$ ,  $10^3$  and  $10^4 \mu\text{g/ml}$ , respectively.

### **Chemiluminescence assay**

The CL assay procedure was performed essentially as described by Paape (1993). Zymosan A from *Saccharomyces cerevisiae* (Sigma, Saint Louis, MO, USA) was suspended (12.5 mg/ml) in PBS with 75% pooled bovine serum. The zymosan suspension was then incubated for 30 min in a water bath at 37°C. After incubation, the zymosan particles were washed once with PBS and then centrifuged at 1 500 *g* for 30 min at room temperature and resuspended in PBS containing  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  until a final concentration of 12.5 mg/ml was attained. One millilitre aliquots of the opsonized zymosan were prepared and stored at -20°C until use.

A 0.9 ml cell suspension ( $1 \times 10^6$  cells) and 0.1 ml drug solution with varying concentrations of ginseng solution or PBS were placed in a disposable polystyrene sample cuvette. The final concentration of ginseng in the blood cell suspension was 10,  $10^2$ ,  $10^3$  or  $10^4 \mu\text{g/ml}$ . Because a limited number of PMNL was obtained from stripping milk, the cells were treated in the solution of  $10^3 \mu\text{g}$  ginseng extract/ml only. In the control cuvette, 0.9 ml cell suspension and 0.1 ml PBS were added. After incubation in 5%  $\text{CO}_2$  for 60 min at 37°C, the cells were washed twice with PBS and centrifuged at 500 *g* for 10 min, and then resuspended in 0.8 ml pre-warmed PBS. This was followed by the addition of 100  $\mu\text{l}$  of 5  $\mu\text{M}$  luminol (Sigma, Saint-Louis, MO, USA) and the mixture was then incubated in 5%  $\text{CO}_2$  at 37°C. After a 10 min incubation, 100  $\mu\text{l}$  of pre-warmed opsonized zymosan was added to stimulate the cells and the luminol-enhanced CL emitted by the cells was immediately measured with a luminometer (LKB-Wallac, Bromma, Sweden). Duplicate tubes were used for each experimental

treatment. Data were presented as mean  $\pm$  SE peak values mV.

### Phagocytosis test

The phagocytosis assay was performed as previously described (Concha *et al*, 1986) with some modifications. The incubation mixture consisted of 0.4 ml neutrophil suspension ( $2.5 \times 10^6$  cells), 0.1 ml ginseng solution or PBS, and 5  $\mu$ l fluorescent latex bead-suspension (Fluoresbrite fluorescent monodisperse carboxylated microspheres, 1.72  $\mu$ m diameter, Polysciences Limited, Northampton, UK). The final concentration of ginseng in the mixture was  $10^2$   $\mu$ g/ml. The ratio of cells to beads was 1:20. The mixture was incubated at 37°C for 60 min in 3 ml minisorb test tubes (Nunc, Denmark) with agitation at a speed of 1 per second to avoid clumping and to allow optimal contact between cells and beads. After incubation, 1% Triton X-100 was added until a final concentration of 0.05% was reached in order to detach the microspheres adhering to the surface of the cells and to facilitate the staining of the cell nucleus with 25  $\mu$ l propidium iodide (1 mg/ml PBS). Specimens were mounted on glass slides and analyzed under a fluorescence microscope. Cells characteristic of PMNL and having 2 or more engulfed microspheres were counted as phagocytic cells. Two hundred cells were counted for each sample. The proportion of phagocytic PMNL was calculated.

### Statistical analyses

The paired t-test was used for all statistical analyses of the differences between the ginseng-treated and non-ginseng-treated cells in all of the experiments. *P* value of less than 0.05 was considered statistically significant.

## RESULTS

### Proportion of the various cellular types in the stripping milk

The cells harvested from the stripping milk consisted of  $50 \pm 10\%$  of PMNL,  $24 \pm 14\%$

of macrophages and  $36 \pm 9.0\%$  of lymphocytes with a viability of 72%.

### Effect of the ginseng extract on the oxidative response

In the blood PMNL, the ginseng extract influenced zymosan-induced CL in a dose-dependent manner (table I). At a concentration of 10  $\mu$ g/ml, ginseng-treated neutrophils tended to have a higher CL value than non-treated ones, but this difference was not statistically significant. As the concentration of the ginseng extract was increased to  $10^2$  and  $10^3$   $\mu$ g/ml, the CL from the ginseng-treated cells significantly increased (*P* < 0.05). However, at a very high ginseng concentration ( $10^4$   $\mu$ g/ml), the CL value decreased for the ginseng-treated cells (*P* < 0.05).

In the stripping milk, a significant increase of CL was seen for the cells treated with  $10^3$   $\mu$ g/ml ginseng solution in comparison with non-treated cells (*P* < 0.05).

**Table I.** Effect of various concentrations of ginseng extract on the phagocytic and oxidative activities of polymorphonuclear leucocytes from bovine blood or stripping milk.

Ginseng concentration ( $\mu$ g/ml)	Chemiluminescence (mV)	% of phagocytic cells
<i>Blood</i>		
0	62.1 $\pm$ 9.4	43.4 $\pm$ 2.6
10	69.2 $\pm$ 10.7	—
$10^2$	71.8 $\pm$ 8.8*	48.9 $\pm$ 2.7*
$10^3$	81.9 $\pm$ 12.8*	—
$10^4$	47.7 $\pm$ 8*	—
<i>Milk</i>		
0	76.6 $\pm$ 13.1	38.4 $\pm$ 3.7
$10^2$	94.2 $\pm$ 14.0*	42.7 $\pm$ 3.6*

\* *P* < 0.05.

### **Effect of the ginseng extract on the proportion of phagocytic cells**

In the blood, a significantly higher proportion of phagocytic cells was found for the cells treated with the ginseng solution ( $10^2 \mu\text{g/ml}$ ) than for the non-treated cells ( $P < 0.05$ ).

In the stripping milk, the percentage of phagocytic cells in the ginseng-treated group was greater than that in the non-treated group ( $P < 0.05$ ).

## **DISCUSSION**

Bovine mastitis may occur when pathogenic microorganisms gain entrance to the mammary gland, overcome local defense, and colonize the duct system and alveoli. PMNL are the major line of defense after the bacteria have penetrated the teat canal (Paape *et al*, 1979). However, the mammary PMNL are considered to be much less phagocytic and bactericidal than blood PMNL (Jain and Lasmanis, 1978; Weber *et al*, 1983). Different explanations for this discrepancy have been proposed previously, such as physical interference by casein bound to the neutrophil surface, and the internalization of milk-fat globules and casein micelles, which in turn will result in loss of pseudopodia and, therefore, of associated receptor sites (Ruszel and Reiter, 1975; Paape and Guidry, 1977). The suppressed mammary PMNL may be one of the reasons for the high susceptibility of mammary glands to infections. Some studies have been carried out with an attempt to increase the mammary defense by potentiating PMNL. For example, Sordillo and Babiuk (1991) observed an increased CL activity, bacterial phagocytosis and bactericidal activity after *in vitro* treatment of mammary PMNL with recombinant bovine interferon- $\gamma$ ; Daley *et al* (1990) reported an up-regulated phagocytic activ-

ity of mammary PMNL by intramammary infusion of recombinant bovine interleukine-2. In this study, we demonstrated an increased CL and phagocytosis by PMNL from both peripheral blood and stripping milk after pre-incubation with ginseng extract as compared with the non-treated cells. In the blood PMNL, the peak value of the CL response of ginseng-treated cells to opsonized zymosan was about 30% higher than that for non-treated cells. Gupta *et al* (1980) reported that a ginseng extract induced an increased production of interferon by human lymphocytes. This may be one of the explanations for the increased production of the CL and phagocytosis in PMNL, because the cell suspension in this study was always contaminated with some lymphocytes (about 9% for blood PMNL and 36% for milk PMNL).

The results obtained from dairy cattle in this experiment appear to be consistent with those from other species. Solo'veva *et al* (1989) observed an activated phagocytic capacity of PMNL and macrophage in guinea pigs by a polysaccharides isolated from a ginseng tissue culture. Scaglione *et al* (1990) reported an enhancement of chemotaxis and phagocytic ability of human PMNL after oral administration of aqueous ginseng extracts. Scaglione *et al* (1994) also demonstrated an increased phagocytosis and intracellular killing in human macrophages following oral administration of a ginseng extract.

At  $10^4 \mu\text{g}$  ginseng extract/ml, the production of CL was significantly reduced, suggesting that this drug is toxic to PMNL at this concentration. Clinically, raw ginseng is commonly prescribed at a single dose of 15–30 g for cattle (Ju, 1991), which is much less than the dose for inducing a toxic effect on PMNL. The drug could be administered alone or together with other herbs depending on the therapeutic purpose. For example, in the treatment of disorders of digestive system, ginseng could be prescribed

together with *Radix astragali* seu Hedysari, *Radix glycyrrhizae*, *Radix angelicae* Sinen-sis, *Rhizoma atractylodis macrocephalae*, *Rhizoma cimicifugae* and *Radix bupleuri*. The mixture of these herbs is concocted to get a liquid extract which is then administered orally to an animal.

Milk PMNL seemed to produce more CL than blood cells in our experiment. This might be due to the fact that the milk PMNL used in our study was not as purified (50% of total cells) as that from the blood (90% of total cell population) and were contaminated by other cells such as macrophages (14% of total cell population) which might increase the amount of CL in the test and contribute to a higher value.

For the cells pre-incubated with ginseng extract, the proportions of active PMNL of blood and milk were increased by 12.7 and 11.2%, respectively. The amount of increase was less than those for CL (about 30%). It would seem that ginseng is more active in stimulating CL production than in augmenting phagocytic activity.

After observing its effect on bovine neutrophil phagocytosis and CL, we concluded that the ginseng extract used in this study has immunomodulating effects on the CL and phagocytic activities of bovine PMNL. In order to explore its potential application in the control of bovine mastitis, further study of ginseng on its *in vivo* effect on the mammary glands against infection may be of value.

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