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Intramammary infusion of *Panax ginseng* extract in the bovine mammary gland at cessation of milking modifies components of the insulin-like growth factor system during involution

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ABSTRACT

The objective of this study was to evaluate the effects of a single intramammary infusion of *Panax ginseng* extract (GS) on insulin-like growth factors (IGF) in bovine mammary gland during early involution. Eight mammary quarters from six nonpregnant cows in late lactation were infused with 10 mL of ginseng extract solution (3 mg/mL), six quarters were treated with 10 mL of placebo (vehicle alone) and six quarters were maintained as uninoculated controls. Milking was interrupted after infusion. Concentrations of IGF1 in mammary secretions were higher in GS-treated quarters than in placebo and uninoculated control quarters at 24, 48 and 72 h post-treatment ($p < 0.05$). Treatment with GS did not affect mammary secretion of IGF2 ($p = 0.942$). At 7 d of post-lactational involution, a decrease of immunostained area and mRNA expression for IGF1 was observed in mammary tissue of GS-treated quarters compared with placebo-treated quarters and uninoculated controls ($p < 0.05$). The IGF2 immunostained area and mRNA expression for this growth factor were not affected by GS treatment ($p = 0.216$ and $p = 0.785$, respectively). An increase in protein levels and mRNA expression in mammary tissue of IGFBP3, IGFBP4 and IGFBP5 was observed in GS-treated quarters compared with placebo-treated quarters and uninoculated controls ($p < 0.05$). These results provide evidence that intramammary inoculation of GS extract at cessation of milking may promote early mammary involution through the inhibition of IGF1 local production and bioavailability.

1. Introduction

Mammary gland involution is characterized by gradual changes in secretion composition and regression of secretory tissue. The nonlactating interval, commonly referred to as the dry period, is an important determinant to achieve maximal milk production in the subsequent lactation (Remond et al., 1997; Capuco and Akers, 1999). Several studies have been carried out to define the optimal dry period length to maximize milk yield in the subsequent lactation; being a dry period of 45–60 d between lactations generally recommended (Bachman and Schairer, 2003). Development of schemes that increase persistence of lactation minimizing the length of the dry period have been proposed. Among them, strategies directed to hasten mammary gland involution may contribute to elevate concentration of natural protective components and to enhance milk yield during the subsequent lactation (Capuco and Akers, 1999; Wedlock et al., 2004). This approach requires a thorough understanding of mechanisms involved in mammary changes during involution. However, there is little information about effects of compounds that could potentially enhance mammary involution at cessation of milking (Oliver and Sordillo, 1989; Dallard et al., 2007; Baravalle et al., 2010).

Insulin-like growth factors (IGF) play a pivotal role in tissue homeostasis, regulating cell proliferation, differentiation and migration both during development and in the adult (Le Roith, 2003; Flint et al., 2008). Insulin-like growth factor 1 suppresses the apoptosis of murine primary mammary epithelial cells (MEC) in culture (Marshman et al., 2003) and bovine mammary cells in tissue culture (Accorsi et al., 2002). The importance of IGF as anti-apoptotic factor has also been demonstrated in murine mammary gland models *in vivo* (Flint et al., 2000). Insulin-like growth factors

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activities are modulated by high-affinity interactions with a family of structurally related IGF-binding proteins (IGFBPs), i.e. IGFBP1 through IGFBP6. These proteins are known to regulate circulating levels of IGF, and have been reported to inhibit or to enhance IGF1 action, depending on the system under investigation (Flint et al., 2000). Messenger ribonucleic acid (mRNA) for all six *IGFBPs* has been detected in mammary tissue of several species and, similar to *IGF1*, their expression patterns and relative levels vary considerably between stages of development (Plath-Gabler et al., 2001; Flint et al., 2005). The distinct expression pattern of each *IGFBPs* during different stages of mammary development suggests specialized roles, although data on specific functions of individual IGFBPs in normal development of the mammary gland are limited (Sakamoto et al., 2007).

Panax ginseng C.A. Meyer as a traditional medicine has been utilized in China for at least 2000 years. Ginseng saponins, or ginsenosides, are considered to be the active substances in total ginseng extracts. The therapeutic effect of ginseng root is related to stimulation of natural resistance against infections (Scaglione et al., 1990). *P. ginseng* extracts (GS), consisting mainly of saponins, have been found to possess various effects on the immune system, such as lymphocyte proliferation enhancement, cytokine production stimulation by macrophages, and phagocytic activity improvement of macrophages and polymorphonuclear leukocytes (Scaglione et al., 1990; Kim et al., 1990; Larsen et al., 2004). In addition, GS has potential as a chemopreventive agent through mechanisms that include inhibition of deoxyribonucleic acid (DNA) damage (Park et al., 2005), induction of apoptosis by oxidative stress (Volate et al., 2005), and inhibition of cell proliferation (Kang et al., 2005).

Previous investigations *in vivo* and *in vitro* with GS have shown that the dry root extract has immunomodulatory and adjuvant effects in the bovine udder (Hu et al., 2001, 2003; Baravalle et al., 2010, 2011). Recent studies from our laboratory have demonstrated that intramammary infusion of GS extract in cows at cessation of milking increased the rate of mammary cell apoptosis without inhibiting cell proliferation leading to enhancement of mammary regression rate during early involution (Dallard et al., 2011). In an attempt to provide further information about the mechanisms underlying ginseng activity during early involution, we examined the effects of a single intramammary infusion of GS extract at cessation of milking on mRNA expression and detection of IGF components in the bovine mammary tissue and IGF1 and IGF2 concentrations in mammary secretions.

2. Materials and methods

2.1. Ginseng extract

Ginseng dry extract was kindly provided by Indena Company (Indena® SpA, Milan, Italy). The spectrophotometric content of saponins expressed as ginsenoside Rg_1 with the reference to the dried substance was 27%. High performance liquid chromatography contents of protopanaxatriol ginsenosides Rg_1, R_f, R_e, calculated as Rg_1 and of protopanaxadiol ginsenosides R_c, R_d, Rb_2, Rb_1 calculated as Rb_1, with reference to the dried substance was 23.9%.

The GS solution was prepared by dissolving the extract in pyrogen free 0.89% NaCl saline solution to a final concentration of 3 mg ginseng extract per mL, sterilized by filtering through 0.22-μm pore diameter filter and then sealed in sterilized 250 mL glass bottles. The solution was prepared 1 d before infusion and stored at 4 °C. Sterility was checked seeding 100 μL in Columbia agar with 5% calf blood and incubating overnight at 37 °C. The endotoxin level in the purified GS solution was examined by Pyrotel *Limulus* amebocyte lysate assay kit (Associates of Cape Cod) according to the manufacturer's instructions. The levels of endotoxin in GS at 10 mg/mL were lower than the detection limit of the test (<0.05 ng/mL) indicating that the biological effects of GS were not due to endotoxin contamination. Ginseng extract dose (3 mg/mL) yielding the highest somatic cell count (SCC) response without gross mammary swelling or systemic adverse effects (i.e. elevated rectal temperature and increase in respiratory frequency), was selected as previously described (Baravalle et al., 2011).

2.2. Animals and treatment design

Six Holstein non-pregnant cows in late lactation (weeks 31–36) from the Rafaela Experiment Station of Instituto Nacional de Tecnología Agropecuaria (INTA) producing approximately 10 kg of milk per day prior to experimentation were used. Cows used in this study were from parity 3 to 5, and were milked twice daily before initiation of the study. All the procedures were carried out according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, Federation of Animal Sciences Societies (FASS, 1999). The animals were selected based on previous bacteriological studies and somatic cell counts. All the quarters used in this experiment were free of infection.

The treatment design has been described in detail previously (Baravalle et al., 2010). Mammary quarters were randomly assigned to each of three treatment groups, verifying that within each udder all treatments were administered. The treatment unit of study was the mammary quarter. Briefly, eight quarters were infused with 10 mL of ginseng solution (3 mg/mL), six quarters were treated with 10 mL of placebo (saline solution) and six quarters were maintained as uninoculated controls. Two quarters of placebo-inoculated and of noninoculated cows were not considered for the treatment owing to high SCC at the time of inoculation. In all cases, milking was interrupted after intrammamary infusion.

2.3. Mammary secretion samples

Samples of mammary gland secretion were aseptically collected using standard procedures (Oliver et al., 2004) 72 h before GS administration, immediately before inoculation and 24, 48, and 72 h post-treatment (pt) as previously described (Baravalle et al., 2010). The first two streams of mammary secretion from each gland were discarded, the next 5 mL were collected in sterile plastic vials for bacteriological analysis and then 30 mL were collected into plastic vials for subsequent growth factor analyses. The latter samples were centrifuged at 1500*g* for 20 min at 4 °C, and the upper lipid layer was removed. A portion of the skimmed secretion was centrifuged at 13,000*g* for 30 min at 4 °C, and the supernatant was harvested and stored frozen at −20 °C for subsequent IGF1 and IGF2 analyses by radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA), respectively. Bacteriological analysis was carried out according to standard procedures (Oliver et al., 2004).

2.4. Tissue sample preparation

Animals included in the three groups were slaughtered at 7 d after inoculation at a local abattoir and samples for histological analysis were taken. Immediately after cows were slaughtered tissue samples were obtained from three zones of mammary quarters following previous descriptions (Dallard et al., 2011). Briefly, mammary tissue was obtained from the base of the gland adjacent to the gland cistern (zone 1), midway between the gland cistern and dorsal boundary of the gland (zone 2) and near the dorsal mammary border (zone 3). All zones were approximately oriented along an axis through the centre of the gland in line with the teat. Tissue samples of approximately 1 cm^3 were fixed in 4% neutral