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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

Bibiana E. Dallard[a,b,*], Silvina A. Pujato[a], Celina Baravalle[a,b], Elizabet A.L. Pereyra[a,b], Florencia Rey[a,b], María S. Renna[a,b], Luis F. Calvinho[a,c]

[a] Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Rvdo. Padre Kreder 2805, (3080) Esperanza, Santa Fe, Argentina
[b] Concejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
[c] Estación Experimental Agropecuaria Rafaela, Instituto Nacional de Tecnología Agropecuaria (INTA), C.C. 22 (2300) Rafaela, Santa Fe, Argentina

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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 activities are modulated by high-affinity interactions with a family

* Corresponding author at: Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Rvdo. Padre Kreder 2805, (3080) Esperanza, Santa Fe, Argentina. Tel.: +54 3496 420639; fax: +54 3496 426304.
E-mail address: bdallard@fcv.unl.edu.ar (B.E. Dallard).

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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 intramammary 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 buffered formalin, for 8 h at 4 °C and then washed in phosphate-buffered saline (PBS; pH 7.4; 0.01 M). For light microscopy, fixed tissues were dehydrated and embedded in paraffin wax. Sections

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(5 μm) were mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma–Aldrich, St. Louis MO, USA) and assigned for staining with haematoxylin and eosin for mammary gland structures preliminary observation or for use in immunohistochemistry (IHC) procedures (Dallard et al., 2011). Additional sections of mammary tissues were transferred into a freezing vial, weighed and placed in liquid nitrogen for gene expression assays.

2.5. Quantification of IGF1 and IGF2 in mammary secretion

For RIA of IGF1, 15 μl of whey or IGF1 standards were subjected to the acid–ethanol cryo-precipitation method as previously described (Lacau-Mengido et al., 2000). The concentration of IGF1 was determined using an antibody (UB2-495) provided by Drs. L. Underwood and J.J. Van Wyk, and distributed by the Hormone Distribution Program of the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK), USA. Recombinant human IGF1 (rhIGF1, Chiron Corp., Emeryville, CA, USA) was used as radioligand and unlabeled ligand. The assay sensitivity was 6 pg/tube. Intra and interassay coefficients of variation were 8.2% and 14.1%, respectively.

For IGF2 determination in whey samples a competitive ELISA was developed. Assay conditions (i.e. antibody coating and the antigen biotinylated mass, reagent dilutions and incubation times) were optimized using a checkerboard binding approach and IGF2 (Novozymes, GroPep Ltd., Australia) as a reference standard. All samples were run in duplicate on the same plate. Polystyrene microplates (Maxisorp Nunc, NY, USA) were coated overnight at 4 °C with 100 μl of anti-IGF2 antibody (EAA-PAAL-1, Novozymes GroPep, 100 ng/well) in sodium carbonate buffer (0.1 M, pH 9.6). Wells were washed three times with PBS and blocked for 2 h at 25 °C with 3% w/v bovine serum albumin (BSA; Sigma–Aldrich) in PBS (blocking buffer). Insulin-like growth factor 2 was diluted in blocking buffer at a starting concentration of 10 μg/mL with serial twofold dilutions. Whey samples were diluted 1:2 in blocking buffer. Fifty microlitres of both standard and sample dilutions were added to the respective wells before being washed with PBS-0.05% Tween-20 (PBST). An equal volume of biotinylated IGF2 (Novozymes GroPep, 50 ng/well) was added to each well and microplates were incubated for 2 h at 25 °C. Before washes with PBST, microplates were incubated for 2 h at 25 °C with 100 μL of extravidin-peroxidase (Sigma Aldrich, dilution 1:5000). After the final wash step, microplates were developed with 3, 3′, 5, 5-tetramethylbenzidine (TMB; Zymed San Francisco CA, USA) and the reaction was stopped with 2 M HCl. The absorbance of each well was measured at 450 nm using a microplate reader (Multiskan EX, Thermo Electron Co., Vantaa, Finland). Negative controls were prepared using blocking buffer. The standard curve was constructed by plotting the absorbance values versus the log values of the IGF2 concentration. The detection limit was established as the value that differed by three standard deviations from the negative controls and the ELISA sensitivity was calculated as the slope of the standard curve.

2.6. Immunohistochemistry

A streptavidin–biotin immunoperoxidase method was performed as described by Dallard et al. (2008). Briefly, sections were dewaxed, hydrated and subjected to microwave pretreatment in citrate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked with 1% H_2O_2 in methanol and non-specific binding was blocked with 10% normal goat serum (Sigma Aldrich). Tissue sections were incubated with the first antibody overnight at 4 °C. The polyclonal antibodies (Novozymes GroPep) used for the detection of IGF were: anti IGF1 (PabCa, dilution 1:100), anti IGF2 (EAA-PAAL 1, dilution 1:100), anti IGFBP3 (PAAJ-1, dilution 1:200), anti IGFBP4 (PAAG-1, dilution 1:100), anti IGFBP5 (PAAF-1, dilution 1:75). Slides were washed with PBS and incubated at room temperature for 30 min with a biotin-conjugated anti-rabbit IgG antibody (1:200; Zymed) as a secondary reagent. Visualization of antigen–antibody reaction was by the streptavidin peroxidase method (BioGenex, San Ramon CA, USA) and 3.3-diaminobenzidine (Liquid DAB-Plus Substrate Kit-Zymed) was used as chromogen.

Negative control sections were included in which the primary antibody was replaced by non-immune rabbit serum (Dallard et al., 2008). To exclude the possibility that endogenous peroxidase activity was unsuccessfully blocked, some sections were incubated with DAB reagent alone. Serial sections of similarly processed tissue samples of liver were used as positive controls in each assay to normalize the image analysis.

2.7. RNA extraction

Total RNA was extracted from frozen mammary tissue samples using Trizol LS Reagent (Invitrogen, CA, USA), following the manufacturer's instructions with slight modifications. Briefly, 50–100 mg of tissue was homogenized with 1 mL of Trizol reagent (Invitrogen) and incubated for 10 min at 25 °C. RNA was purified by vigorously homogenizing with chloroform and incubated for 15 min at 4 °C. After centrifugation at 12,000*g*, the aqueous phase was incubated with an equal volume of isopropanol for 30 min at −20 °C and centrifuged at 12,000*g* to obtain the total RNA pellet; that was then washed with 75% ethanol for 10 min at 4 °C. Alcohol was replaced by diethylpyrocarbonate water (Sigma Aldrich) prewarmed at 55–60 °C. Total RNA content was determined spectrophotometrically at 260 nm; then samples were aliquoted and stored at −80 °C until further use.

2.8. Reverse transcriptase polymerase chain reaction (RT-PCR)

To avoid putative genomic DNA contamination, RNA samples were treated with DNAse (Invitrogen) according to the manufacturer's instructions. Single stranded cDNA was reverse transcribed from total RNA that had been treated with DNAse using a master mix (MMLV buffer, DTT, RNA out, M-MMLV reverse transcriptase, dNTP) and random primers (Invitrogen). The reverse transcription conditions consisted of 10 min of annealing at 25 °C, 50 min of cDNA synthesis at 37 °C and 15 min of inactivation at 70 °C.

Conditions for the enzymatic amplification were optimized. Ten nanograms of cDNA previously quantified by the Qubit method (Invitrogen) were used for the polymerase chain reaction (PCR). Primer sequences of *IGF1*, *IGF2* and *IGFBP3* were taken from earlier publications (Pfaffl et al., 2002): *IGF1* forward 5′-TCG CAT CTC TTC TAT CTG GCC CTG T-3′ and *IGF1* reverse 5′-GCA GTA CAT CTC CAG CCT CCT CAG A-3′; *IGF2* forward 5′-GAC CGC GGC TTC TAC TTC AG-3′ and *IGF2* reverse 5′-AAG AAC TTG CCC ACG GGG TAT-3′; *IGFBP3* forward 5′-ACA GAC ACC CAG AAC TTC TCC T C-3′ and *IGFBP3* reverse 5′-GCT TCC TGC CCT TGG A-3′. Primer sequences of *IGFBP4* and *IGFBP5* were those used previously by Plath-Gabler et al. (2001): *IGFBP4* forward 5′-GCC CTG TGG GGT GTA CAC-3′ and *IGFBP4* reverse 5′-TGC AGC TCA CTC TGG CAG-3′; *IGFBP5* forward 5′-TGC GAG CTG GTC AAG GAG-3′ and *IGFBP5* reverse 5′-TCC TCT GCC ATC TCG GAG-3′. The *β-actin* gene sequence was included as housekeeping: forward primer 5′-CGG AAC CGC TCA TTG C C-3′ and reverse 5′-ACC CAC ACT GTG CCC ATC T A-3′ and the primer sequence was designed from the bovine sequence (GenBank accession no. BT030480). A master mix of the following reaction components was prepared to the indicated end concentration: 14.9 μl water, 0.75 μl MgCl2 (50 mM), 0.625 μl forward primer (20 mM), 0.625 μl reverse primer (20 mM), 0.2 μL dNTP (25 mM) *Taq* buffer 10× (2.5 μl, Invitrogen) and *Taq* polymerase (5 U/μl, Invitrogen). cDNA diluted in 3 μl of every sample was added to

the master mix solution. Amplification conditions included 30 (*IGF1* and *2*, *IGFBP3* and *4*), 31 (*β-actin*) or 33 (*IGBP5*) cycles of denaturation at 94 °C for 1 min, annealing at 58 °C (*IGFBP3*), 60 °C (*IGF2* and *IGFBP4*), 62 °C (*IGF1*) or 63 °C (*IGFBP*) for 30 s and extension at 72 °C for 1.5 min. A single denaturation step at 94 °C for 3 min and a final extension step at 72 °C for 10 min were performed. Polymerase chain reaction products were analyzed by agarose gel electrophoresis and the relative signal intensities of all examined factors were assessed after normalization based on the *β-actin* PCR signal intensities.

2.9. Image analysis

Image analysis was performed using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA). Images were digitized with an Olympus C5060 digital camera mounted on a conventional light microscope (Olympus BH-2; Olympus Co., Tokyo, Japan) using objective magnification of 40×. Image resolution was set to 1200 × 1600 pixels. Each pixel of the image corresponded to 0.13 μm at the respective magnification and each field represented a tissue area of 0.031 mm^2. The system captured each image and automatically corrected for background. This prevented differential readings due to different lighting conditions. No further image processing was done. Details of image analysis as a valid method for quantifying expression levels and the methodological details were previously described (Dallard et al., 2008; Baravalle et al., 2011; Dallard et al., 2011). Briefly, the immunohistochemical stained area (IHCSA) for each antibody reaction was calculated as a percentage of total area evaluated through the color segmentation analysis that extracts objects by locating all objects of the specific color (brown stain). The brown stain was selected with a sensitivity of 4 (maximum 5) and a mask was next applied to make separation of colors permanent. The IHCSA (% of black area) was calculated from at least 50 images in each one of the following structures: alveoli, ducts and interstitial tissue. Insulin-like growth factor 1 and 2, IGFBP3, IGFBP4 and IGFBP5 expressions were evaluated by positive IHCSA.

For *IGF1* and *2*, *IGFBP3*, *IGFBP4* and *IGFBP5* mRNA expressions, images of gels were captured by a digital camera (Olympus). Growth factors mRNA levels were expressed as a ratio between the integrated optical density band of each growth factor and the corresponding band of *β-actin*.

2.10. Statistical analysis

A statistical software package SAS (1999) was used to perform statistical analysis. Differences in growth factors and binding proteins by IHC and RT-PCR assays between groups were assessed by a Kruskal–Wallis non-parametric test, followed by Bonferroni as a multiple comparison test. Data for IGF1 and 2 in mammary secretion were analyzed using the general linear model procedure for repeated measures. The model tested for main effects of treatment (GS, placebo or uninoculated controls). Time (0, 24, 48 and 72 h) was included as a repeated measure. To identify differences in the levels of each factor (treatment and time) a Duncan post test was used. A value of $p < 0.05$ was considered significant. Results were expressed as mean ± SEM (standard error of the mean).

3. Results

3.1. Clinical signs and bacteriological examination

Infusion of GS into udder quarters at cessation of milking did not result in systemic effects in any cow. Macroscopic changes in mammary secretions and moderate swelling were observed in mammary quarters of GS-treated group at 24 h. All samples from mammary gland secretions yielded no bacterial growth. No side effects, other than those mentioned, were observed during the treatment period with the selected dose.

3.2. Concentrations of IGF1 and IGF2 in mammary secretion

Concentrations of IGF1 in mammary secretions were affected by GS treatment ($p < 0.001$). This effect was observed along the sampling period (Fig. 1A). At 24, 48 and 72 h pt the IGF1 concentrations were higher in mammary secretions of GS-treated quarters than in mammary secretions of placebo-treated quarters and uninoculated controls ($p < 0.05$; Fig. 1A).

No effect of GS treatment was observed on IGF2 concentration in mammary secretions ($p = 0.868$; Fig. 1B).

3.3. Immunohistochemistry of IGF family members

The positive immunohistochemical reaction for IGF family proteins (IGF1 and 2, IGFBP3, IGFBP4 and IGFBP5) was detected by brown cytoplasmic staining and evaluated by the IHCSA. Differences in the pattern of localization and IHCSA for each protein were observed within the mammary gland. Effects of GS treatment on IHCSA for IGF1 and IGF2, IGFBP3, IGFBP4 and IGFBP5 at 7 d of post-lactational involution are shown in Fig. 2.

Immunostaining for IGF1 was seen in every treatment groups, ranging from weak to intense, and was mainly associated with mammary parenchyma structures. Expression of IGF1 was primarily associated with the epithelial cells of the alveoli and ducts with strong staining in the cytoplasmic area around their nuclei (Fig. 3). Macrophages and fibroblasts in the interlobular stroma showed intense cytoplasm staining. At 7 d of post-lactational involution,

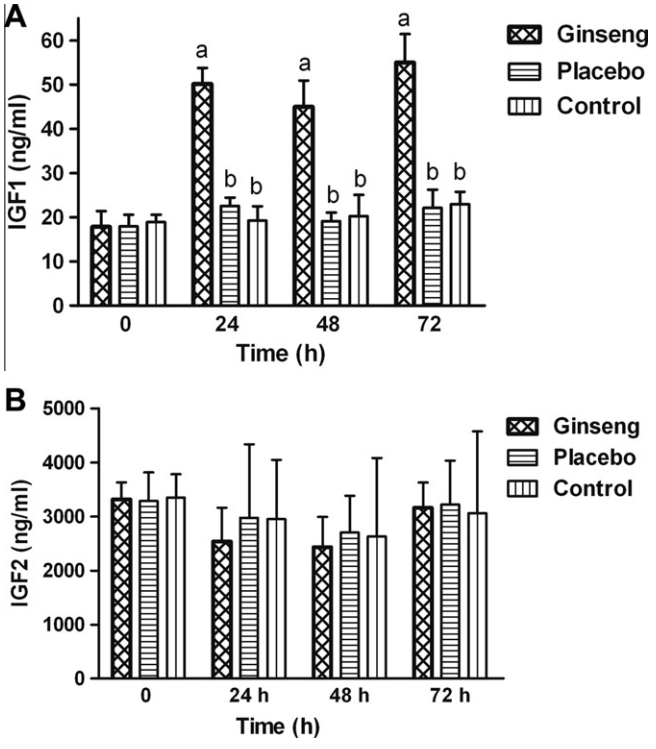

Fig. 1. IGF1 (A) and IGF2 (B) concentrations in mammary secretion from quarters treated with ginseng extract, placebo and uninoculated controls at 0 h (immediately before inoculation) and 24, 48 and 72 h post-treatment. Values represent the mean ± standard error of the mean (SEM). (A) Effects of treatment ($p < 0.001$). Means (a and b) for each time period without a common letter differ ($p < 0.05$). (B) Effects of treatment ($p = 0.868$).

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a decrease of immunostained area for IGF1 was observed in GS-treated quarters compared with placebo and uninoculated control quarters ($p < 0.05$; Fig. 2A).

Immunostaining pattern for IGF2 was similar to IGF1 (Fig. 3). No differences in percentages of IHCSA for IGF2 between GS-treated quarters, placebo and uninoculated control quarters were detected ($p = 0.216$; Fig. 2B).

The positive immunohistochemical reaction for IGFBP3, IGFBP4 and IGFBP5 was associated with stromal and parenchymal tissues showing similar pattern of localization (Fig. 3). Intense immunoreaction of alveolar epithelial cell cytoplasms and ducts was observed. Macrophages in the lumen of the alveoli and in the stroma showed strong immunostaining. Furthermore, neutrophils, lymphocytes and fibroblast, as well as endothelium of smooth vessels, showed intense staining in their cytoplasm. The percentages of IHCSA for IGFBP3, IGFBP4 and IGFBP5 were greater in GS-treated quarters compared with placebo-treated quarters and uninoculated controls ($p < 0.05$; Fig. 2C–E).

No differences in percentages of IHCSA between mammary zones sampled for the five evaluated proteins were detected ($p = 0.853$), either in GS-treated quarters, placebo treated quarters or control quarters.

3.4. RT-PCR analysis for IGF family members

The effect of GS on transcriptional activity of *IGF* family members was measured by a semi-quantitative RT-PCR system. The identity of PCR products was confirmed by sequencing (100% homology with bovine sequences). Expression of *IGF1* mRNA was lower in GS-treated quarters than in placebo-treated quarters and uninoculated controls ($p < 0.05$; Fig. 4A). No differences in levels of mRNA expression for *IGF2* were detected between treatment groups ($p = 0.785$; Fig. 4B). At 7 d of post-lactational involution, an increase of *IGFBP3*, *IGFBP4* and *IGFBP5* mRNA expression was observed in GS-treated quarters compared with placebo-treated quarters and uninoculated controls ($p < 0.05$; Fig. 4C–E).

4. Discussion

Strategies for hastening bovine mammary involution following cessation of lactation are directed to elevate concentration of natural protective components in mammary secretions and to increase milk production in the succeeding lactation. Recently, we have demonstrated that intramammary inoculation of *P. ginseng* extract at cessation of milking enhances epithelial and stromal cells apoptosis rates without inhibiting cell proliferation in bovine mammary glands (Dallard et al., 2011). Insulin-like growth factor 1 is a potent and specific inhibitor of apoptosis in several cell types in culture, including mammary cells, suggesting that this factor plays an active role in the regulation of mammary gland involution (Geier et al., 1992). In the present study, the effects of *P. ginseng* extract on protein and mRNA expression of IGF components administered at the end of lactation in dairy cows were examined.

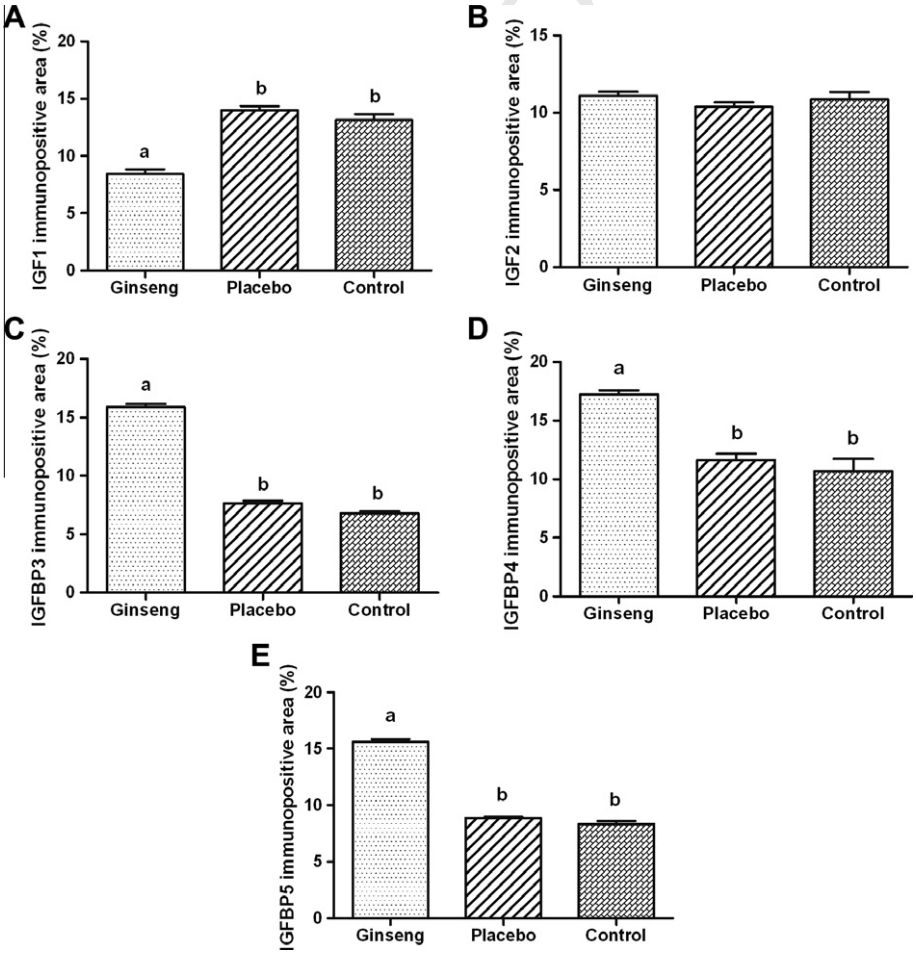

Fig. 2. (A) Percentage of immunopositive areas for IGF1, (B) IGF2, (C) IGFBP3, (D) IGFBP4 and (E) IGFBP5 in mammary quarters treated with ginseng extract ($n = 8$), placebo ($n = 6$) and uninoculated controls ($n = 6$) at 7 d of post-lactational involution detected by immunohistochemical staining. Values represent the means ± standard error of the mean (SEM). Mean values without common letters (a and b) differ ($p < 0.05$).

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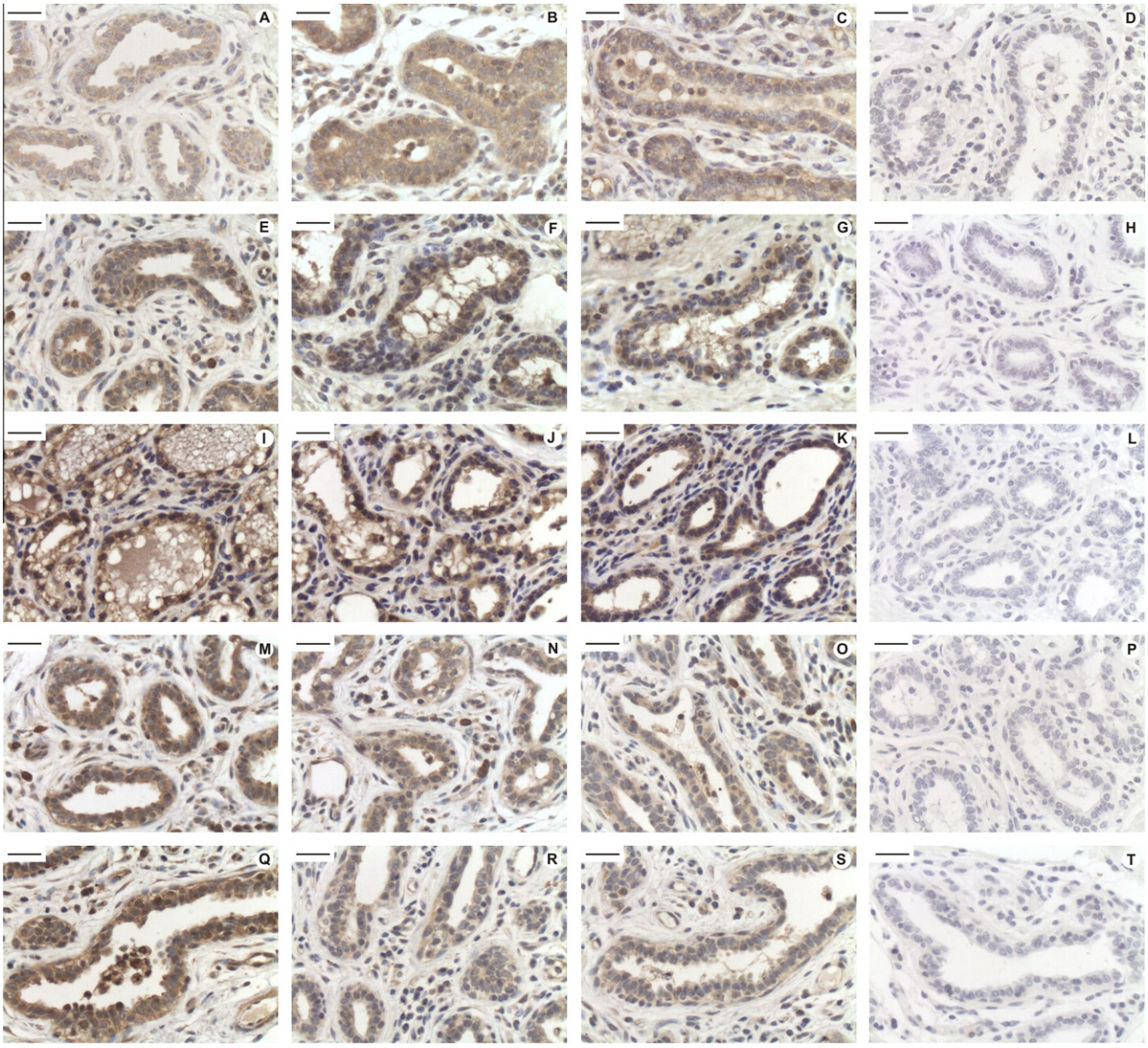

Fig. 3. Immunohistochemical localization of IGF1, IGF2, IGFBP3, IGFBP4 and IGFBP5 in mammary quarters at 7 d of post-lactational involution. (A) IGF1 immunostaining in mammary quarters treated with ginseng extract, (B) placebo and (C) uninoculated controls. (E) IGF2 immunostaining in quarters treated with GS, (F) placebo and (G) uninoculated controls. (I) IGFBP3 immunostaining in mammary quarters treated with GS, (J) placebo and (K) uninoculated controls. (M) IGFBP4 immunostaining in mammary quarters treated with GS, (N) placebo and (O) uninoculated controls. (Q) IGFBP5 immunostaining in mammary quarters treated with GS, (R) placebo and (S) uninoculated controls. Positive staining is shown as a brown coloring of the cytoplasm of the epithelial and stromal cells. Negative controls for IGF1 (D), IGF2 (H), IGFBP3 (L), IGFBP4 (P) and IGFBP5 (T) immunostaining; no labeling was observed when the primary antibodies were replaced with non-immune rabbit serum. Magnification is the same for all panels (400×) and is indicated by 25-μm bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Concentrations of IGF1 in mammary secretions were higher in GS-treated quarters than in placebo-treated quarters and in uninoculated controls at 24, 48 and 72 h pt ($p < 0.05$). One possible explanation for this finding is that GS at the dose used stimulated IGF1 secretion by epithelial cells into the alveolar lumen during the first 72 h after cessation of milking, without promoting the synthesis of this growth factor; since low levels of mRNA for *IGF1* were detected in mammary tissue treated with GS compared with controls at 7 d of post-lactational involution. However, further research will be needed to confirm this hypothesis.

Insulin-like growth factor 1 is predominantly synthesized by the liver, but it is also produced in many other tissues (Rowzee et al., 2008). Accumulated evidence suggests that locally produced growth factors and their binding proteins, may mediate the effects of systemic hormones in the ruminant mammary gland (Plath-Gabler et al., 2001; Berry et al., 2003). Early studies of IGF1 expression in mammary tissue from rodents (Kleinberg, 1997) and humans (Cullen et al., 1992) have shown that expression was limited to mammary stromal cells. Berry et al. (2003) demonstrated that *IGF1* mRNA was not expressed by purified primary bovine MEC, but was expressed in both prepubertal stroma and parenchyma, concluding that the *IGF1* mRNA observed within mammary parenchyma must derive from surrounding stromal cells such as fibroblasts. In the present study, expression of IGF1 was primarily associated with the epithelial cells of the alveoli and ducts. Furthermore, macrophages and fibroblasts showed intense staining in their cytoplasm. These results were similar to those obtained in previous reports showing that IGF1 was distinctly localized in the cuboidal epithelium of small and large alveoli and in the epithelium of ducts during bovine mammary gland involution (Plath-Gabler et al., 2001; Dallard et al., 2007). The increased accumulation of IGF1 within mammary epithelium observed in this

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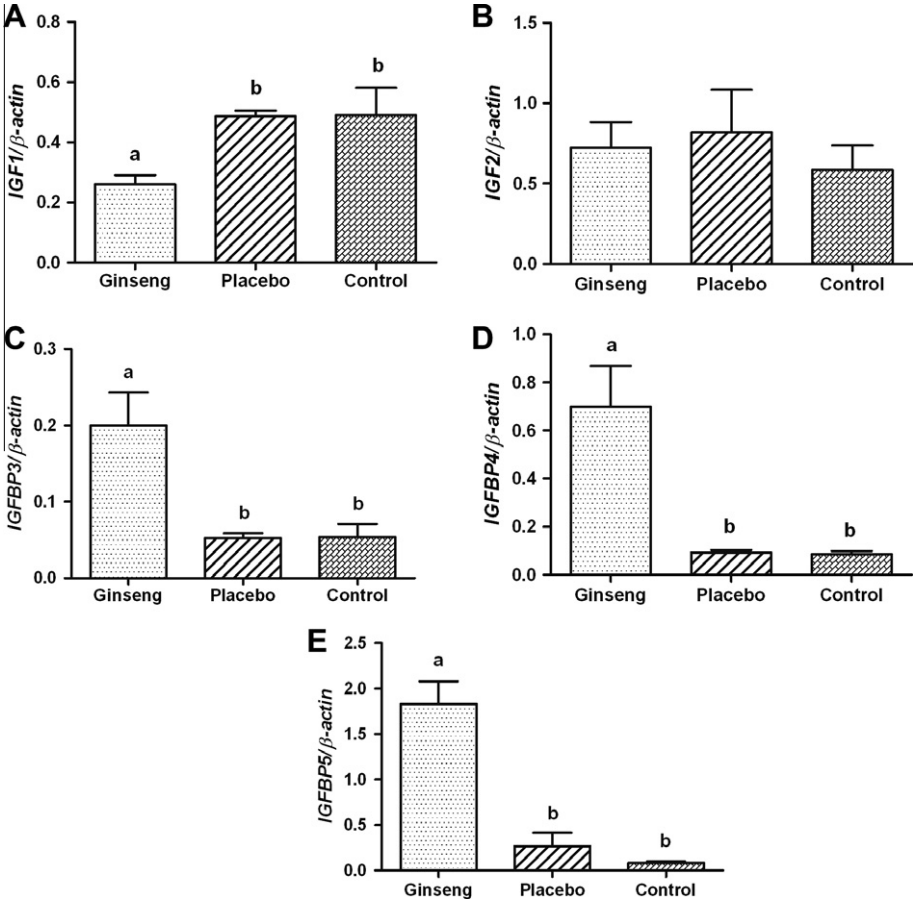

Fig. 4. Semi-quantification of *IGF1*/*β-actin* mRNA (A), *IGF2*/*β-actin* mRNA (B), *IGFBP3*/*β-actin* mRNA (C), *IGFBP4*/*β-actin* mRNA (D), *IGFBP5*/*β-actin* mRNA (E), in mammary quarters treated with ginseng extract ($n = 8$), placebo ($n = 6$) and uninoculated controls ($n = 6$) at 7 d of post-lactational involution. Values represent the mean of integrated optical density (IOD) ± standard error of the mean (SEM). Means without a common letter (a and b) differ ($p < 0.05$).

study could be derived from surrounding stromal cells as previously described Berry et al. (2003).

In the present study, at 7 d post inoculation, the percentages of IHCSA and mRNA expression for *IGF1* was lower in GS-treated quarters than in placebo-treated and uninoculated controls ($p < 0.05$). These data suggest that GS can act to repress IGF1 synthesis in bovine mammary cells which is in agreement with concurrent increased expression of the proapoptotic proteins Bax and caspase-3 observed in a previous study (Dallard et al., 2011). It is recognized that the protective effect of IGF1 against apoptosis occurs through the activation of phosphatidylinositol 3′-kinase (PI3K) and the serine/threonine protein kinase (Akt), followed by the phosphorylation-dependent inactivation of the pro-apoptotic protein Bad (Sakamoto et al., 2007). The overexpression of activated Akt within the mammary gland of transgenic mice causes delayed involution (Schwertfeger et al., 2001). The lower expression of IGF1 observed in mammary tissue from GS-treated quarters added to increased expression of proapoptotic proteins contribute to explain the observed involution acceleration (Dallard et al., 2011).

Insulin-like growth factor 2 is generally implicated as a locally derived growth factor that functions in embryonic tissues (Hovey et al., 2003). However, several lines of evidence support a role for IGF2 during normal postnatal mammary gland development (Hovey et al., 2003). In this study, IGF2 concentrations in mammary secretions were higher than IGF1 concentrations for the entire sampling period. This finding is consistent with previous studies in lactating dairy cows (Collier et al., 2008). Insulin-like growth factor 2 concentrations in mammary secretions were unaffected by GS-treatment and time of sampling. Similar values were detected in all treatment groups and times post treatment.

Insulin-like growth factor 2 mRNA is heterogeneously expressed by MEC in the ductal epithelium of nulliparous mice (Richert and Wood, 1999). Similarly, *IGF2* mRNA is expressed within the parenchyma and stroma of the ovine mammary gland during allometric growth and is regulated by ovarian function (Hovey et al., 2003). In the present study, immunostaining for IGF2 was seen in every experimental group, ranging from weak to intense, and was mainly associated with mammary parenchymal structures. Neither percentages of IHCSA nor mRNA expression for *IGF2* were affected by GS treatment. Dose used in the present study could have been insufficient to modify the IGF2 expressions in mammary tissue at 7 d after cessation of milking. There is considerable evidence that IGF1 and IGF2 are distinctly regulated and have diverse functions in mammary development (Rowzee et al., 2008).

Insulin-like growth factor 1 is regulated by a family of proteins: IGFBPs, which play an important role activating or inhibiting IGF1 action (Barber et al., 1992). Cultures of primary bovine MEC secrete IGFBP2, IGFBP3, IGFBP4, IGFBP5, which by molecular weight analysis were shown to be identical to those present in bovine milk (Gibson et al., 1999). An increase in IGFBP5 protein concentration has been observed in rat milk 48 h after removal of the litter to induce involution (Tonner et al., 1997). The concentrations of IGFBP3 in bovine milk increased during mid-lactation to late lactation (Baumrucker and Erondu, 2000). However, their functions at the

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cellular level are not fully understood. In the present study, at 7 d of post-lactational involution, immunostaining for IGFBP3, IGFBP4 and IGFBP5 was observed in every treatment groups mainly associated with parenchymal and stromal components. In addition, percentages of IHCSA were higher ($p < 0.05$) in GS-treated quarters compared with controls for the three IGFBPs evaluated.

In murine models, the levels of IGFBPs (both protein and mRNA) decreased during lactation, with the exception of IGFBP3, suggesting that a decline of those IGFBP contributes to permit maximal survival effects of the IGF during lactation (Flint et al., 2000). In contrast, during involution in rats there was a fourfold increase in *IGFBP2* mRNA, a sixfold increase in the expression of *IGFBP4* and a dramatic 50-fold increase in both *IGFBP5* mRNA and protein levels, within 48 h of pup removal (Tonner et al., 1997; Ricort and Binoux, 2001). Insulin-like growth factor-binding protein 3, the major IGFBP in milk, is synthesized by epithelial cells and increases during involution in the bovine mammary gland (Gibson et al., 1999; Baumrucker and Erondu, 2000). This protein may inhibit action of the IGF receptor via a mechanism that does not depend on a physical interaction between IGF1 and IGFBP3 (Ricort and Binoux, 2001). Sakamoto et al. (2007) demonstrated that IGFBP3 inhibits the phosphorylation of Akt mediated by IGF1. Our results showed an increase in mRNA expression for *IGFBP3* at 7 d of post-lactational involution in GS-treated quarters compared with controls ($p < 0.05$). This finding suggests that increased expression of IGFBP3 can inhibit IGF1 action enhancing apoptosis of mammary cells. In a previous study, we demonstrated that epithelial and stromal cell apoptosis, *in situ* assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling, was more marked in quarters treated with GS than in control quarters (Dallard et al., 2011).

The expression of *IGFBP4* in bovine mammary tissue during involution has not been fully explored. An early study by Sejrsen et al. (2001) described a significant increase in milk levels of both IGFBP4 and IGFBP5 at 7 d of post-lactational involution in cattle. In the present study, we observed an increase in both protein and mRNA for *IGFBP4* in GS-treated quarters compared with controls ($p < 0.05$). These results are in accord with the previous study (Sejrsen et al., 2001), confirming this binding protein (BP) involvement in the remodeling process during active bovine mammary involution.

A positive relationship between *IGFBP5* expression and mammary cell death during the process of mammary involution has been reported in several species (Tonner et al., 2002; Flint et al., 2005). The large concentrations of IGFBP5 present in the mammary gland may act to neutralize IGF1 function as a survival factor for mammary epithelial cells and this BP may thus be instrumental in initiating the process of mammary gland involution (Flint et al., 2005). The overexpression of IGFBP5 in the mammary gland leads to increase the expression of the pro-apoptotic molecules caspase-3 and plasmin, and to decrease the expression of pro-survival molecules of the Bcl-2 family (Tonner et al., 2002). In a previous study we demonstrated a significant increase of the immunostained area for Bax protein and active caspase-3 in GS-treated quarters compared with controls, whereas no differences were observed for Bcl-2 immunostaining. Moreover, mRNA expression for *Bax* was higher in GS-treated quarters than in controls (Dallard et al., 2011). In the present study, we observed an increase in both protein and mRNA levels for *IGFBP5* in GS-treated quarters compared with controls. This elevated expression of IGFBP5 in mammary tissue may act to neutralize the function of IGF1 as a survival factor for epithelial and stromal cells and IGFBP5 may therefore be instrumental to promote mammary gland involution.

In conclusion, results of this study together with previous findings (Dallard et al., 2011) confirm the observation that intramammary inoculation of GS extract at cessation of milking may promote early mammary involution through the inhibition of IGF1 local production and bioavailability. We propose that, GS would stimulate significantly expression of IGFBP3, IGFBP4 and IGFBP5, which exert inhibitory actions on IGF1 contributing to stimulate cell death. In addition, results of the present study provide new insights into the role and action of IGF components on mammary gland remodeling during early involution.

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