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Proinflammatory cytokines and CD14 expression in mammary tissue of cows following intramammary inoculation of *Panax ginseng* at drying off

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ABSTRACT

The lack of efficacy of conventional strategies for the maintenance of healthy udders in domestic cattle has prompted studies on the use of immunomodulators or biological response modifiers (BRM) for this purpose. These compounds are agents that modify the host's response to pathogens leading to beneficial effects on disease outcome. The objective of this study was to evaluate the effects of a single intramammary infusion of *Panax ginseng* (GS) extract on the amount of pro-inflammatory cytokines and the number of monocytes/macrophages present in bovine mammary tissues at drying off. Eight mammary quarters from six nonpregnant cows in late lactation were infused with 10 mL of GS (3 mg/mL), six quarters were treated with 10 mL of placebo (vehicle alone) and six quarters were maintained as uninoculated controls. The analyses of tumor necrosis factor-alpha (TNF-α) by immunohistochemistry revealed that the production of this proinflammatory cytokine significantly increased ($P < 0.05$) in the inoculated mammary glands of cows following BRM inoculation, whereas the interleukin-1 alpha (IL-1α) and IL-6 staining area was not affected by BRM treatment. The number of monocytes/macrophages detected with CD14 antibody was significantly higher ($P < 0.05$) in BRM-treated quarters than in placebo and uninoculated control quarters. These results indicated an immunomodulator potential of the BRM used. The beneficial effect of the extract could be used as alternative therapy in the control of mastitis at drying off, either alone or in conjunction with dry cow antibiotic therapy.

1. Introduction

Susceptibility of the bovine mammary gland to new intramammary infections (IMI) is markedly increased during early involution and the periparturient period (Smith et al., 1985a,b). Bovine mastitis is one of the most costly diseases to the dairy industry. Economic losses are chiefly attributable to decreased milk production, reduced milk quality, costs of treatment and animal replacement (Seegers et al., 2003). Strategies aimed at improving the immune defenses of the diseased udder during immunosuppressive stages would greatly impact the ability of the animal to resist the infection (Mukherjee, 2009).

Technological advances in immunology have provided new research tools that facilitate the study of mammary gland immunity and disease pathogenesis (Sordillo and Streicher, 2002). The early dry period is a critical stage, since changes that lead to increased concentration of pro-

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tective factors, compared with lactating mammary glands, occur gradually over several days (Oliver and Sordillo, 1989). Therefore, it is a target of choice for modulation of immune responses.

Biological response modifiers (BRM) or immunomodulators are compounds capable of interacting with the immune system to regulate specific aspects of the host response (Tzianabos, 2000). Several BRM have been used in an attempt to enhance innate immune mechanisms against bovine mastitis pathogens (Zecconi, 2000; Takahashi et al., 2004). However, only few studies addressed the effect of these compounds on the bovine mammary tissue (Dallard et al., 2009, 2010). The full potential of BRM can only be exploited through a clear understanding of the immune system, since these agents function through modification of normal host response (Campos et al., 1993).

Panax ginseng (GS) has been used as an herbal therapy in ancient China and Asian countries for thousands of years and became popular in Western countries during the last two decades (Gillis, 1997). Ginseng saponins, or ginsenosides, are believed to be the active substances in total ginseng extracts. These compounds have been chemically characterized as triterpenoid glycosides of the dammarane series. At present, more than 30 ginsenosides have been identified in GS (Song et al., 2010). 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). Their immunomodulatory effects are mostly due to its regulation of cytokine production and phagocytic activities of monocytes/macrophages and dendritic cells, as well as activation of T and B lymphocytes (Ho et al., 2004; Tan and Vanitha, 2004).

Shin et al. (2002) demonstrated that macrophages treated with ginseng extract, both *in vitro* and *in vivo* produced pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IFNs. Cytokines are soluble proteins that affect an array of biological processes and are considered to be crucial to innate and adaptive inflammatory responses, cell growth and differentiation, cell death, angiogenesis and developmental as well as repair processes (Oppenheim, 2001).

In a recent study we demonstrated that intramammary inoculation of GS extract in cows at drying off was associated with increase of mRNA cytokines transcription and somatic cell counts in milk (Baravalle et al., 2010). In addition to immunomodulatory effects, Dallard et al. (2011) demonstrated that GS enhances mammary regression during early involution. In this study, we focused in characterizing the *in situ* expression of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α and the number of monocytes/macrophages present in mammary tissue after inoculation of GS extract at drying off.

2. Materials and methods

2.1. Biological response modifier

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 (HPLC) 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 day before infusion and stored at 4 °C. The sterility of GS solution was checked seeding 100 μL in Columbia agar added with 108 5% bovine calf blood and incubating overnight at 37 °C. The amount of bacterial endotoxin in the purified GS solution was measured by Pyrotell *Limulus* amebocyte lysate assay kit (Associates of Cape Cod) according to the manufacturerĭs 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 (e.g. elevated rectal temperature and increase in respiratory frequency), was selected as previously described (Baravalle et al., 2010).

2.2. Animals

Six Holstein non-pregnant cows from parities 3 to 5 in late lactation (weeks 31–36) from the Rafaela Experiment Station of INTA herd were used. Cows were milked twice daily and produced an average of 10 kg milk/d before interruption of lactation. Cows with similar lactation number were included in each group. All the procedures were carried out according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (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.

2.3. Experimental design

The experimental design has been described in detail previously (Baravalle et al., 2010). Mammary quarters were randomly assigned to each of three experimental groups, verifying that within each udder all treatments were administered. Unit of study was the mammary quarter. In brief, eight quarters were infused with 10 mL of ginseng solution (3 mg/mL), eight quarters were treated with 10 mL of placebo (saline solution) and eight quarters were maintained as uninoculated controls. In all cases milking was interrupted after intramammary infusion. Milk samples for bacteriological examination were aseptically collected using standard procedures (Oliver et al., 2004) 72 h before BRM administration, immediately before inoculation and 24, 48, and 72 h post-treatment (pt) as previously described (Baravalle et al., 2010). Animals included in the three groups were slaughtered at 7 d after inoculation at a local abattoir and samples for histological analysis were taken.

2.4. Tissue sample preparation

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 (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 (HE) 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 western blot assays.

2.5. Immunohistochemistry

For immunohistochemistry, the streptavidin–biotin immunoperoxidase method was employed as described by Dallard et al. (2011). Briefly, the sections were deparaffinized and antigen retrieval was performed. Endogenous peroxidase was blocked with 1% H_2O_2 in methanol and nonspecific binding was blocked with 10% (v/v) normal goat serum. Sections were incubated with primary antibodies under conditions detailed in Table 1. Slides were washed with PBS and incubated for 30 min at room temperature with rat preabsorbed biotinylated secondary antibodies selected specifically against one of each of the two types of primary antibodies used (monoclonal or polyclonal). Visualization of the antigen was achieved by the streptavidin–peroxidase method (Bio-Genex, San Ramon, CA) and 3,3-diaminobenzidine (DAB, Liquid DAB-Plus Substrate Kit, Zymed, San Francisco, CA) was used as the chromogen. Finally, the slides were washed in distilled water, counterstained with Mayer's haematoxylin, dehydrated, and mounted. To verify specificity, adjacent control sections were subjected to the same immunohistochemical method replacing primary antibodies by rabbit and mouse non-immune serum. Serial sections of similarly processed tissue samples of mammary gland were used as positive controls in each assay to normalize the image analysis. Each primary antibody was probed with an absorption test involving the respective antigen (15 mg/mL) (Sigma–Aldrich).

2.6. Western blotting

To test specificity of the primary antibodies for cytokines used in this study (Table 1), 60–100 mg of mammary tissue from the three evaluated zones were homogenized in a radioimmunoprecipitation assay lysis buffer consisting of 1% (v/v) IGEPAL CA630 (octylphenyl–polyethylene glycol), 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM EDTA, 50 mM sodium fluoride (all from Sigma–Aldrich Corp., St. Louis, MO, USA), 0.1 MPBS and a protease inhibitor cocktail (Complete Mini Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany). Mammary homogenates were centrifuged at 12,000 × *g* for 20 min and the protein concentration in the supernatants was estimated using fluorescence methods (QubitTM, Invitrogen). Eighty micrograms of protein, along with prestained molecular weight markers (Bio-Rad, Hercules, CA, USA), were separated by SDS-PAGE (15% resolving gel). Proteins were transferred to nitrocellulose membranes (Amersham, Buckinghamshire, UK), blocked for 2 h 30 min in 5% (w/v) non-fat milk in Tris-buffered saline (TBS) containing 0.05% (v/v) Tween 20 (Sigma–Aldrich Corp., St. Louis, MO, USA), and then incubated overnight at 4 °C with specific primary antibodies (Table 1). Following washing, membranes were treated for 2 h with corresponding secondary peroxidase conjugated antibody. An anti-mouse antibody diluted 1:500 (Amersham, Buckinghamshire, UK) was used for IL-6 immunoblotting; and an anti-rabbit antibody diluted

Table 1
Antibodies including clone, source, dilution and incubation conditions used for immunohistochemistry (IHC) and Western blot (WB) analysis of bovine cytokines TNF-α, IL-1α and IL-6 and CD14.

Antigen	Clone and source	Dilution IHC	Incubation conditions for IHC	Dilution WB
Primary antibodies				
Anti-TNF-α	Polyclonal (Chemicon, San Francisco, CA, USA)	1:150	O.N. at room temperature	1:150
Anti-human IL-1α	Polyclonal (Endogen, Woburn, USA)	1:100	O.N. at 4 °C	1:50
Anti-porcine IL-6	Monoclonal (Clon 77830; R&D Systems, Minneapolis, USA)	1:200	O.N. at 4 °C	1:50
Anti-CD14	Monoclonal (clone RPA-M1; Zymed, San Francisco, CA)	1:30	O.N. at room temperature	–
Secondary antibodies				
Biotinylated anti-rabbit IgG	Polyclonal (Zymed, San Francisco, CA, USA)	1:100		–
Biotinylated anti-mouse IgG	Polyclonal (Zymed, San Francisco, CA, USA)	1:100		–
Anti-rabbit IgG peroxidase	Polyclonal (Amersham, Buckinghamshire, UK)	–		1:500
Anti-mouse IgG peroxidase	Polyclonal (Amersham, Buckinghamshire, UK)	–		1:500

O.N.: over night.

1:500 (Amersham, Buckinghamshire, UK) was used for TNF-α and IL-1α immunoblotting. Immunopositive bands were visualized with a chemiluminescent detection kit (ECL, Amersham, Buckinghamshire, UK), using ECL film (Amersham, Buckinghamshire, UK), and were subsequently scanned into a computer.

2.7. 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 magnifications 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 the 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., 2009, 2011; Ortega et al., 2010). Briefly, the immunohistochemical stained area (IHCSA) for each antibody reaction was calculated as a percentage of total area evaluated through the color segmentation analysis, which extracts objects by locating all objects of a specific color (brown stain). These values were verified and normalized, with the controls carried across various runs using the same region (verified by image comparison) for calibration. 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. TNF-α, IL-1α and IL-6 expressions were evaluated by positive IHCSA. For measuring number of monocytes/macrophages stained with anti-CD14, 40 random images from each slide were digitized at 40× and number of cells per mm^2 was calculated.

2.8. Statistical analysis

A statistical software package (SAS, 1999) was used to perform statistical analysis. Differences between treatments were analyzed by Kruskal–Wallis non-parametric test, followed by Bonferroni as a multiple comparison test. The significant level was set at $P < 0.05$. Results are expressed as mean ± SEM.

3. Results

3.1. Bacteriological examination and side effects

Macroscopic changes in mammary secretions and moderate swelling were observed in mammary quarters of BRM-treated group at 24 h pt. All samples from mammary gland secretions yield no bacterial growth. No side effects, other than those mentioned, were observed during the experimental period with the selected dose.

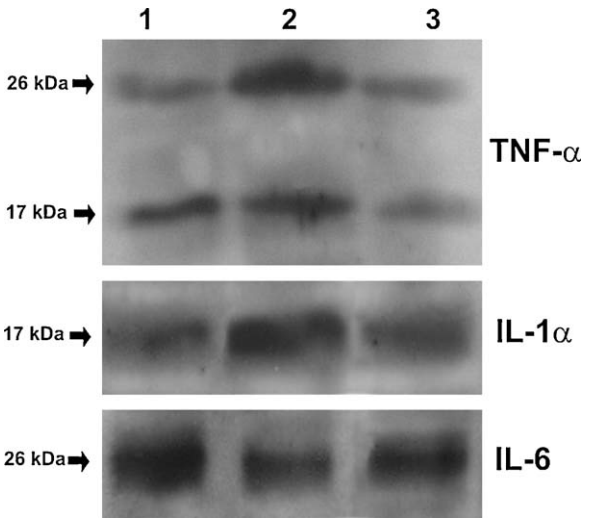

Fig. 1. Verification of specificity for TNF-α, IL-1α and IL-6 antibodies by western blot analyses of mammary gland homogenate. References: lane 1, homogenate from Placebo quarters; lane 2, from biological response modifier (BRM) quarters; lane 3, from uninoculated controls.

3.2. Antibody specificity

Western blot recognition of proteins in mammary homogenate is summarized in Fig. 1. Western blot analysis revealed positive bands of appropriate sizes for each of the proteins studied (Fig. 1). The IL-1α and IL-6 antibodies detected a single band at 17 and 26 kDa, respectively while two bands around 17 and 26 kDa were observed for TNF-α.

3.3. Immunohistochemistry for cytokines

The positive immunohistochemical reaction for each protein (TNF-α, IL-1α and IL-6) was detected by brown cytoplasmic staining and evaluated by the IHCSA. Differences in the pattern of localization and IHCSA for each cytokine were observed within the mammary gland. Effects of BRM treatment on IHCSA for TNF-α, IL-1α and IL-6 at drying off are given in Fig. 2. All negative controls showed no immunostaining, while positive control tissues yielded a positive detection. No differences in percentages of IHCSA for the three evaluated cytokines in control and treated groups within mammary zones sampled were detected.

Immunostaining for TNF-α was associated with mammary parenchymal and stromal structures in all studied quarters. Intense immunostaining of alveolar epithelial cell cytoplasms and ducts was observed. Intraepithelial macrophages, neutrophils, lymphocytes and fibroblast, as well as endothelium and vascular smooth muscle cells, showed intense immunostaining (Fig. 3). A significant increase of immunostained area for TNF-α was observed in BRM-treated quarters compared with controls ($P < 0.05$) (Fig. 2).

IL-1α was detected in all tissue sections primarily associated with mammary parenchyma structures. The epithelial cell cytoplasms and ducts were intensely stained with anti-IL-1α (Fig. 3). Macrophages in the lumen of the

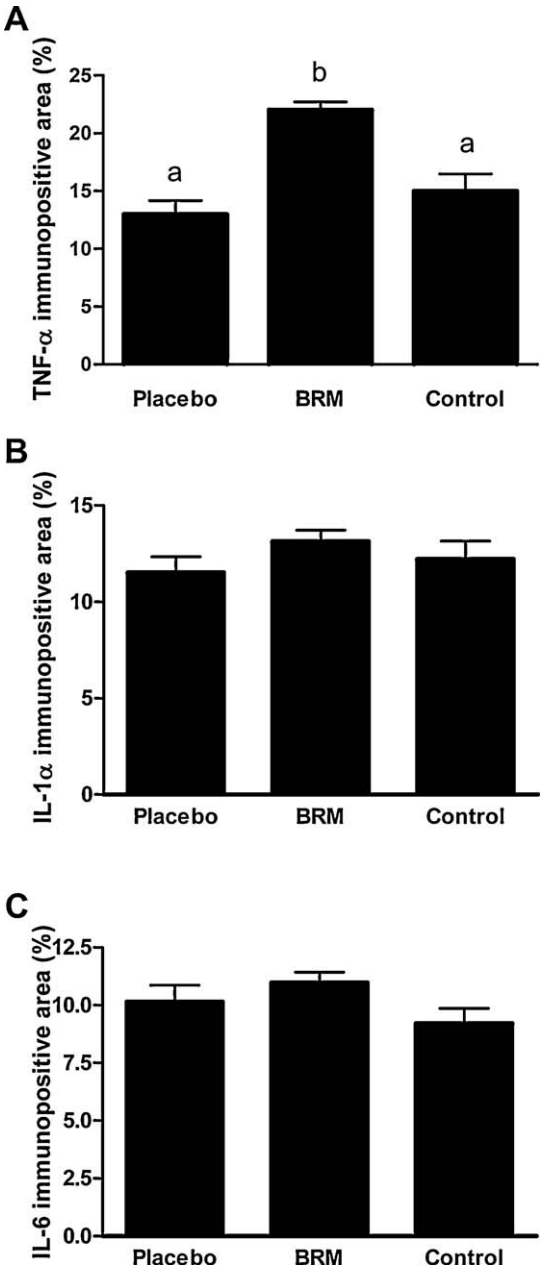

Fig. 2. Relative expression of TNF-α (A), IL-1α (B) and IL-6 (C) in mammary quarters treated with biological response modifier (BRM), placebo and uninoculated controls at 7 d of drying off. Values represent the means measured as percentage of immunopositive area across zones within the gland ± standard error of the mean (SEM). Mean values without common letters (a) and (b) differ ($P < 0.05$).

alveoli and in the stroma showed strong immunostaining in their cytoplasm. Both the intralobular connective tissue and interlobular stroma displayed negative IL-1α staining. No differences in percentages of IHCSA for IL-1α among BRM-treated quarters, placebo and uninoculated control quarters were detected ($P > 0.05$) (Fig. 2).

Interleukin-6 staining was observed in all tissue sections from the three evaluated groups. The pattern of localization for IL-6 was similar to IL-1α expression (Fig. 3). Furthermore, intraepithelial macrophages, neutrophils, lymphocytes and fibroblast, as well as endothelium and vascular smooth muscle cells, showed positive immunostaining. No differences in percentages of IHCSA for IL-6 between control and treated groups were detected ($P > 0.05$) (Fig. 2).

3.4. Monocytes/macrophages quantification

The CD14+ cells were localized in the stroma surrounding the alveolar epithelial cells. Some immunopositive cells were found within lining epithelial cells and in the lumina of alveoli and ducts. Mature macrophages were large, with pale nuclei and vacuolated cytoplasm and the staining occurred strongly on their surface (Fig. 4). No differences between treatments were detected in monocytes/macrophages number within mammary gland area sampled. In BRM-treated quarters, the number of stained cells/mm^2 was significantly higher than in placebo and uninoculated control quarters ($P < 0.05$). Effects of BRM-treatment on monocytes/macrophages number at drying off are visualized in Fig. 5.

4. Discussion

Several studies have demonstrated the immunomodulatory effects of GS extract in dairy cows (Hu et al., 1995, 2001, 2003; Concha et al., 1996; Baravalle et al., 2010). In the present study we focused on characterizing cytokine expression and relative number of inflammatory cells (monocytes/macrophages) in mammary tissue after GS intramammary administration at drying off.

Proinflammatory cytokines, like IL-8 and TNF-α, are considered to initiate the inflammatory reaction in mammary tissues and to induce migration of leukocytes into the udder (Persson Waller et al., 2003; McClenahan et al., 2006). Different stimuli induce the synthesis of several cytokines which in turn activate diverse immune effector mechanisms. A number of mechanisms that enable cytokine-producing cells to respond to environmental cues by synthesizing appropriate cytokines, in the right place and for the required duration, have been identified (Kelso, 1989). In the present study, all cytokines evaluated were expressed in structures of mammary parenchyma and stroma, showing that these cytokines can be constitutively produced in mammary glands of healthy cows at drying off. These findings are consistent with previous studies in rats, humans, sows as well as in cows where some baseline constitutive production of cytokines in normal mammary glands was detected (Basolo et al., 1993; Hagiwara et al., 2000; Zhu et al., 2007; Dallard et al., 2009). Similarly, constitutive mRNA expression of proinflammatory cytokines has also been reported in milk collected from healthy cows (Baravalle et al., 2010). The biological significance of such a constitutive secretion is still unclear. It has been suggested that it could contribute to recruitment of cells at a basal level in the normal bovine mammary gland (Rainard and Riollet, 2006).

Tumor necrosis factor-α is a multifunctional cytokine that was originally defined by its ability to cause the hem-

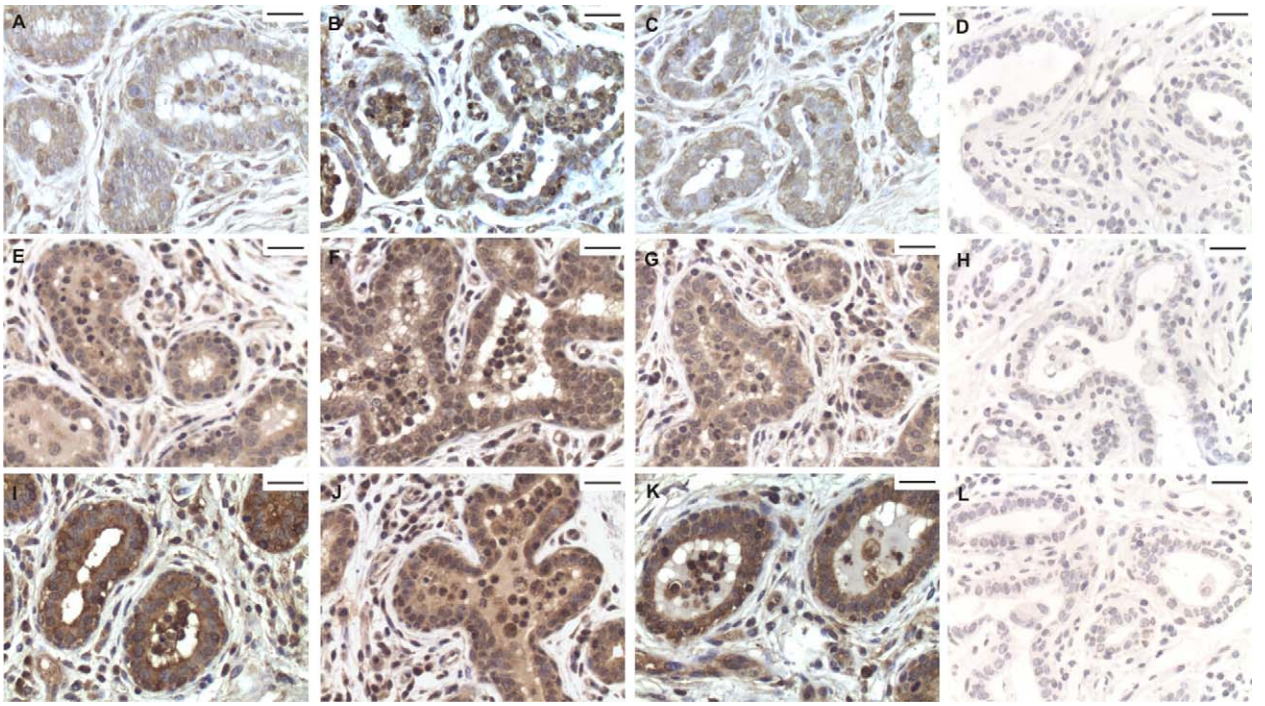

Fig. 3. Immunohistochemical localization of TNF-α, IL-1α and IL-6 in mammary quarters at 7 days of drying off. (A) TNF-α immunostaining in placebo-treated quarters, (B) *Panax ginseng* (BRM)-treated quarters and (C) uninoculated controls. (E) IL-1α immunostaining in placebo-treated quarters, (F) BRM-treated quarters and (G) uninoculated controls. (I) IL-6 immunostaining placebo-treated quarters, (J) BRM-treated quarters and (K) uninoculated controls. Positive staining is shown as a brown coloring of the cytoplasm of the cells. (D), (H) and (L) negative controls for TNF-α, IL-1α, IL-6 immunostaining. Magnification is the same for all panels (40×) and is indicated by 25-μm bars.

orrhagic necrosis of tumors *in vivo*. Specifically, TNF-α is synthesized as a 26-kDa transmembrane precursor, which is then proteolytically cleaved to release the 17-kDa soluble cytokine (Varela and Ip, 1996). In the present study, in reduced conditions, two bands of 17-kDa and 26-kDa were observed in both the BRM-treated group and controls. Tumor necrosis factor-α has been detected and monitored in normal and infected bovine mammary glands (Hoeben et al., 2000; Hisaeda et al., 2001; Riollet et al., 2001; Alluwaimi and Cullor, 2002; Dallard et al., 2009). Although synthesis of TNF-α has been extensively investigated *in vitro*, few studies have focused on characterizing the expression of this cytokine in mammary tissue (Girolamo et al., 1997; Dallard et al., 2009). In the present study, intense immunostaining of alveolar epithelial cell cytoplasms and ducts was observed. In addition, intraepithelial macrophages, neutrophils, lymphocytes and fibroblast, as well as endothelium and vascular smooth muscle cells, showed intense immunostaining. A similar distribution has been reported for TNF-α in mammary quarters inoculated with a single intramammary infusion of an *Escherichia coli* lipopolysaccharide (LPS)-based BRM during involution at 7, 14 and 21 d after drying off (Dallard et al., 2009). Previous reports have shown a TNF transcript production increased in coliform mastitis inflammatory response (Shuster et al., 1996, 1997). Pfaffl et al. (2003) found that the expression of TNF-α mRNA in mammary tissues from cows was significantly higher in quarters with >150,000 cells/mL than in quarters with <150,000 cells/mL. These results are consistent with our previous investigation (Baravalle et al., 2010),

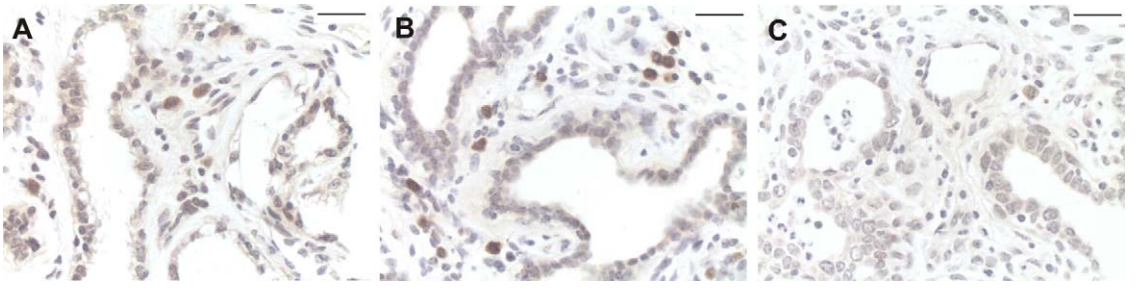

Fig. 4. Representative images of CD14+ cells immunostaining in mammary quarters treated with (A) placebo, (B) *Panax ginseng* (BRM) and (C) uninoculated controls at 7 d of drying off. Positive staining is shown as a brown staining of cytoplasm of cells. Magnification is the same for all panels (40×) and is indicated by 25-μm bars.

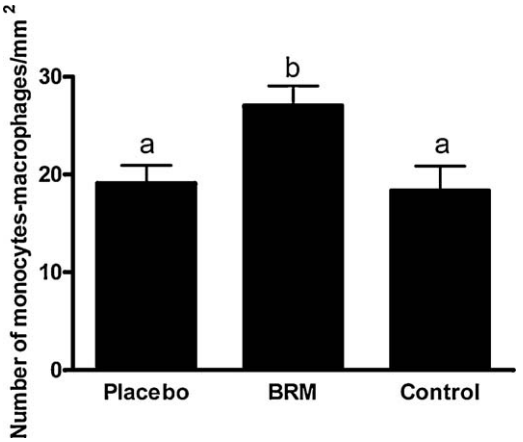

Fig. 5. Number of monocytes/macrophages detected with CD14 antibody in mammary quarters treated with biological response modifier (BRM), placebo and uninoculated controls. Values represent means of number of monocytes/macrophages/mm^2 across zones within the gland ± standard error of the mean (SEM). Mean values for each treatment without common letters (a) and (b) differ ($P < 0.05$).

where high milk somatic cell counts after intramammary inoculation of GS coincided with a significant expression of mRNA for this cytokine in milk at 48 h post-treatment. In the present study, a significant increase of TNF-α immunostaining area was observed in quarters treated with GS compared with controls at 7 d of drying off, suggesting that the GS dose used was sufficient to trigger a high TNF-α level in mammary tissue that was maintained during the first week of involution.

A large number of cells including keratinocytes, endothelial cells, synovial cells, fibroblasts, vascular smooth muscle cells, and monocytes/macrophages throughout the body produce IL-1 and IL-6 (van Deuren et al., 1992; Cohen and Cohen, 1996). Both cytokines play a pathophysiological role in defense from infective organisms in response to both local and systemic inflammations in the mammary gland (Shuster et al., 1993; Palkowetz et al., 1994). Agace et al. (1993) found by indirect immunofluorescence using monoclonal antibodies for IL-lα that labeling was located in the cytoplasm of epithelial cells and human blood monocytes culture following stimulation with *Escherichia coli* Hu734. These findings are in accord with those obtained in the present study, since immunohistochemical analysis revealed that IL-1α was located in the cytoplasm of alveolar epithelial cells and excretory ducts.

Interleukin-1α is not usually secreted and remains localized intracellularly (Bannerman, 2009); however, it can be released by injury or cell death (Dinarello, 2007). The slight increase in IL-1α expression observed in GS-treated quarters compared with controls was not associated with enhanced apoptosis of epithelial and stromal cells observed in a recent study in mammary tissue obtained from the same set of cow (Dallard et al., 2011). A possible explanation for this observation can rely on the sampling period evaluated in the present study (7 d of involution), since we have previously shown that IL-1α mRNA expression in milk was significantly higher in GS-treated quarters at 48 h pt compared with controls while at 72 h pt its expression decreased significantly (Baravalle et al., 2010). It has also been described that IL-1α secretion is linked to human endothelial cells (Miossec et al., 1986), which is consistent with our findings, since expression was not only linked to the mammary parenchyma, but also to different mammary stromal cells such as macrophages, fibroblasts and endothelial cells of blood vessels.

Interleukin-6 is a pleiotropic cytokine with both pro- and antiinflammatory properties (Bannerman, 2009). This cytokine is expressed by a variety of cells, including lymphocytes, monocytes, macrophages, neutrophils, endothelial cells, epithelial cells, and fibroblasts, and its expression is induced by bacteria and viruses, as well as by cytokines, such as TNF-α and IL-1β (Biffl et al., 1996; van der Poll and van Deventer, 1998). It has been shown that IL-1 stimulates the production of other cytokines that amplify inflammation, as IL-6 and IL-8 (Cork and Duff, 1994). In the present study, percentages of staining for IL-1α in quarters treated with BRM did not differ with control quarters. It could be speculated that in quarters treated with BRM, at 7 d of drying off, the IL-1α levels were not sufficient to cause significant stimulation of IL-6 synthesis.

It has been postulated that IL-6 facilitates the transition of the inflammatory process from influx of neutrophils to monocytes. A shift from neutrophils to monocytes is essential for suitable immune responses and for decreasing the noxious effect of neutrophils (Kaplanski et al., 2003). Dallard et al. (2009) studied the distribution of monocytes/macrophages in bovine mammary tissue during physiological involution demonstrating a significant increment in these cell numbers at 7 d of drying off, compared with 14 and 21 d. In this study a transition from a predominant neutrophil inflammatory response to primary macrophages was assessed by the expression of CD14, a glycosylphosphatidylinositol-linked receptor that lacks a transmembrane domain, present on monocytes, macrophages and, to a lesser degree, on neutrophils (Landmann et al., 1991), showing that a significant number of monocytes/macrophages/mm^2 was present in BRM-treated quarters compared with placebo and uninoculated control quarters. Since increased expression of IL-6 was not significant in the BRM-treated mammary quarters, turnover of neutrophils by macrophages observed in the present study could not be associated to the postulated role for this cytokine during inflammation. Rather, this cell turnover may be possibly linked to other chemokines attractant for neutrophils such as IL-8 or monocyte chemotactic protein-1 (MCP-1) not evaluated in this study (Gamero and Oppenheim, 2006).

Professional phagocytic cells, macrophages and neutrophils, as well as epithelial cells (non-professional phagocytes) mediate clearance of dying cells and waste milk during the period of involution in the bovine mammary gland (Monks et al., 2002). In this study, in mammary quarters treated with BRM the number of monocytes/macrophages at 7 day of drying off was significantly higher than in placebo and controls without inoculation, indicating that the extract of GS used stimulated the influx and recruitment of monocytes/macrophages into the mammary gland during early involution. These results agree

with those found by Shin et al. (2002), where the expression of CD14 on murine peritoneal macrophages determined by immunohistochemistry, was increased after treatment with extract of *P. ginseng*. In addition, in the present study, significant rise of CD14+ monocytes/macrophages, coincided with a significant increase in TNF-α immunostaining in BRM-treated quarters compared with controls. Similar results to those found in this study were reported by Dallard et al. (2009) following intramammary administration of an LPS-based BRM at drying off in uninfected cows. These authors detected an increase in the CD14+ monocytes/macrophages number in BRM-treated quarters compared with controls at 7 days of drying off. Taken together, these data highlight the importance of monocytes/macrophages in early local immune response in bovine mammary gland.

In conclusion, the increase in the number of monocytes/macrophages in association with increased expression of TNF-α in quarters treated with BRM demonstrated that the dose used in the present study was sufficient to achieve a significant increase in the innate immune response during early involution of mammary gland and that monocytes/macrophages could putatively serve as a source for TNF-α. Although BRM dose used in this work elicited IL-1α and IL-6 responses, it appeared the stimulus was insufficient to achieve a significant rise in cytokine levels in mammary tissue at 7 d after drying off. These results suggest that GS used as immunostimulant at drying off could play a role in mastitis control by enhancing intramammary defenses, either alone or in conjunction with dry cow antibiotic therapy. However, further studies are required to establish the exact mechanism of its action.

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