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Short communication

Effect of *Panax ginseng* on cytokine expression in bovine mammary glands at drying off

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ABSTRACT

Biological response modifiers (BRM) are agents that modify the host's response to pathogens with resultant beneficial prophylactic or therapeutic effects. The objectives of this study were to describe the immunomodulatory effects of *Panax ginseng* (GS) on bovine mammary glands at the end of lactation. Eight mammary quarters from six nonpregnant cows in late lactation were infused with 10 mL of BRM (3 mg/mL), six quarters were treated with placebo (vehicle alone) and six quarters were maintained as uninoculated controls. Milk samples were collected at different time points for detection of specific cytokines mRNA by RT-PCR and Western blotting assay. A significant increase of IL-1α, IL-1β and TNF-α mRNA expression was observed in BRM-treated compared with placebo-treated quarters at 48 h post-treatment (pt) ($P < 0.05$). A 17 kDa TNF-α band expressed a sharp elevation at 24 h and reduction in its level at 48 h pt in BRM-treated quarters. Differences in this cytokine level between 24 and 48 h pt times were significant ($P < 0.05$). GS extract inoculation at drying off was associated with somatic cell counts increase, cytokines mRNA transcription and the presence of TNF-α in milk and can therefore exert immunomodulating effects in bovine mammary gland at drying off.

1. Introduction

The bovine mammary gland is highly susceptible to new intramammary infections (IMIs) during early involution and the periparturient period (Oliver and Sordillo, 1988). Increased susceptibility to IMI has been related to changes during the involution process that may facilitate bacterial penetration of the streak canal, interfere with natural defence mechanisms, and enhance bacterial growth (Oliver and Sordillo, 1988). Conversely, fully involuted bovine mammary glands are markedly resistant to IMI (Oliver and Sordillo, 1988). Classic control of IMI at drying off is based on intramammary antibiotic infusion (Eberhart, 1986). Although this approach has been successful for prevention of new IMI and elimination of existing IMI at early involution, treatment efficacy varies depending on the drug used and pathogen involved (Eberhart, 1986; Jánosi and Huszenicza, 2001). Therefore, alternative strategies aimed at enhancing the mammary gland immune system during this period would greatly impact the ability of the cow to resist infection.

Immunomodulators or biological response modifiers (BRM) are agents that modify the host's response to pathogens leading to beneficial effects on disease outcome. Several BRM have been used in an attempt to enhance innate immune mechanisms against bovine mastitis

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pathogens (Zecconi et al., 2000; Takahashi et al., 2004; Kimura et al., 2008). However, the exact mechanisms of action and effects of compounds used on the bovine mammary tissue have been seldom explored. Many BRM include microbial agents or their products that activate general or specific immune responses (Zecconi et al., 1999; Inchaisri et al., 2000; Dallard et al., 2008). The full potential of BRMs 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). In addition, the type of activity of these compounds depends on their mechanism and site of action, dose, route and timing of administration (Tzianabos, 2000).

Ginseng is the root of *Panax ginseng* C.A. Meyer (Araliaceae) and its extract has been reported to have modulatory effects on phagocytic cells, lymphocytes and antibody production in both humans and animals (Hu, 2002). Ginseng saponins (ginsenosides) are the main active components in the formation of immune stimulating complexes. These saponins have been used as an adjuvant in the production of vaccines for many years (Rivera et al., 2003). In addition, an extract from the root of *P. ginseng* C.A. Meyer (GS) was found to have stimulatory effects on neutrophils and lymphocytes from bovine peripheral blood and milk *in vitro* (Hu et al., 1995; Concha et al., 1996). However, information about the effect of GS *in vivo* as an immunomodulator, in bovine mammary gland has not been reported yet.

Cytokines are small proteins that function as communication molecules between leucocytes and also between leucocytes and tissues (Rabot et al., 2007). These immunomediators support the defense mechanisms of the mammary gland both by exerting potent chemotactic effects on leukocytes and enhancing phagocytic activity (Persson et al., 1993). Some of these mediators, like tumor necrosis factor-alpha (TNF-α), interferons (IFN) and interleukins (IL) are released by macrophages present in bovine milk as an innate reaction to invading microorganisms or their components such as lipopolysaccharide (LPS) (Rainard and Riollet, 2006). The aim of this study was to describe the effects of intramammary infusion of GS in the bovine mammary gland at drying off on expression of proinflammatory cytokines by reverse transcription-PCR.

2. Materials and methods

2.1. Biological response modifier

Ginseng dry extract containing saponins equivalent to 27% ginsenoside Rg1, was kindly provided by Indena Company (Indena® SpA, Milan, Italy). The solution was prepared by dissolving the extract in 0.89% saline 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. Prior to the present study, a dose–response trial was carried out to select ginseng extract dose. Briefly, four Holstein nonpregnant cows in late lactation were used for these tests. Ten quarters were intramammarily infused with BRM containing ginseng extract concentrations of 1, 3, 5 and 10 mg/mL. Four quarters were infused with placebo (vehicle alone) and 2 quarters were maintained as controls (without inoculation). Dose 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 3 mg/mL.

2.2. Animals and experimental design

Six Holstein nonpregnant cows in late lactation from the herd of the Rafaela Experiment Station producing approximately 10 kg of milk per day prior to experimentation were used. The animals were selected based on previous bacteriological studies and SCC. All the quarters used in this experiment were free of infection. Unit of study was the mammary quarter. Eight quarters were infused with 10 mL of BRM (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. Milk samples were aseptically collected using standard procedures (Hogan et al., 1999) 72 h before BRM administration, immediately before inoculation and 24, 48, and 72 h post-treatment. The first two streams of milk from each teat were discarded, the next 5 mL were collected in sterile plastic vials for bacteriological analysis and 100 mL were collected into sterile plastic vials for performing SCC and for detection of specific cytokines mRNA by RT-PCR and Western blotting assay.

2.3. Bacteriological examination and somatic cell counts

Ten microliters of mammary secretion samples was streaked onto Columbia agar plates supplemented with 5% calf blood and incubated aerobically for 48 h at 37 °C. Plates were examined for bacterial growth at 24 and 48 h. Bacterial identification was conducted following standard procedures (Hogan et al., 1999). Intramammary infection was defined as isolation of the same organism from two consecutive samples.

For determination of milk SCC, quarter milk samples were preserved with azidiol (0.3%) at 4 °C and analyzed within 24 h. The SCC was performed by a commercial laboratory (Laboratorio Regional de Servicios Analíticos, Esperanza, Santa Fe, Argentina) using an automated counter (Somacount 300, Bentley Instruments, Minesotta, USA).

2.4. RNA extraction

Milk from BRM and placebo quarters, approximately 50 mL, was placed into several sterile glass centrifuge tubes, and then centrifuged at 1200 × *g* for 20 min at 4 °C. The cellular pellets from each tube were washed in sterile PBS (pH 7.2) to remove any remaining infranatant (Murrieta et al., 2005). The somatic cell pellet was resuspended in TRIZOL® LS Reagent (Invitrogen, CA, USA) and centrifuged after adding chloroform (phase separation). The RNA, retained in the aqueous phase, was precipitated with isopropanol and washed twice with 75% ethanol. Then, sterile 0.1% diethylpyrocarbonate (DEPC)-treated water was added to dissolve the RNA and stored at −80 °C. Concentration of RNA for all samples was determined using absorbance at λ = 260 nm (A260) (Lee et al., 2006).

Table 1
Primers pairs used for the detection of bovine cytokines transcription by milk-derived cells. β-Actin has been used as housekeeping gene for semi-quantitative analyses.

Target cDNA	Oligonucleotides (5′–3′) F: forward; R: reverse	Product size (bp)	Annealing temperature (°C)	No. of cycles	Accession no. in GenBank database
IL-1α	F: TGCAAGCTATGAGCCACTTC R: GCATTCCTGGTGGATGACTC	291	52	33	M37210
IL-1β	F: TGGGAGATGGAAACATCCAG R: TTTATTGACTGCACGGGTGC	231	52	30	M37211
IL-2	F: CTACTTCAAGCTCTACGGGG R: TTGATCTCTCTGGGGTTCAG	248	52	31	M12791
IL-4	F: TGCCCCAAAGAACACAACTG R: TTTAGCCTTTCCAAGAGGTC	200	52	31	U14160
IL-6	F: TGAAAGCAGCAAGGAGACAC R: TGACATTTTCCTGATTTCCC	187	52	31	S49716
IL-8	F: ACTGGCTGTTGCTCTCTTGG R: ACCTGCACAACCTTCTGCAC	260	54	30	S82598
TNF-α	F: AACAGCCCTCTGGTTCAAAC R: TCTTGATGGCAGACAGGATG	315	54	33	Z14137
INF-α	F: AGCCCAGATGTAGCTAAGGG R: CTCCAGTTTCTCAGAGCTGC	215	51	31	M29867
β-Actin	F: CGGAACCGCTCATTG CC R:ACCCACACTGTGCCCATCTA	290	58	31	BT030480

2.5. Determination of cytokine mRNA expression by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed in two steps. In a first step, to avoid putative genomic DNA contamination, RNA samples were treated with DNAse (Invitrogen) according to the manufacturer's instructions. Then, reverse transcription was carried out using a Murine Moloney Leukemia virus reverse transcriptase (MMLV-RT: Invitrogen). The reverse transcription reaction was allowed to proceed at 65 °C for 5 min, and then the mixture was heated to 37 °C for 2 min prior to addition of 1 μl of MMLV-RT (Invitrogen). The mixture was subsequently incubated 10 min for annealing at 25 °C, 50 min of cDNA synthesis at 37 °C and 15 min of inactivation at 70 °C. Finally, samples were stored at −20 °C until use.

Cytokine-specific cDNAs corresponding to each cytokine sequence were amplified by PCR as described previously (Riollet et al., 2001). PCR was performed by using defined concentrations of a PCR buffer, $MgCl_2$, dNTPs, *Thermus aquaticus* DNA polymerase (Taq polymerase) and specific forward/reverse primers for IL-1α, IL-1β, IL-2, IL-4, IL6, IL-8, INF-γ, TNF-α and β-Actin, according to Riollet et al. (2001). β-Actin has been used as housekeeping gene for semi-quantitative analyses. For each cytokine studied, the upstream (forward) and downstream (reverse) oligonucleotide primers, the expected fragment size, the annealing temperature, the numbers of PCR cycles as well as the respective GenBank database accession number are shown in Table 1. Ten nanograms of cDNA was added to the mixture and amplified in a thermocycler. Positive controls were cDNA samples derived from bovine mesenteric lymph nodes collected from a local abattoir. The PCR products were resolved by electrophoresis alongside a 1 kb DNA ladder (BioLogicos, Argentina) through a 2% agarose gel containing GelRed Nucleic Acid Gel Stain (Biotium, CA, USA), visualized and digitized under UV illumination to confirm the presence of a single band at the correct size. The relative signal intensities of all examined factors were assessed after normalization based on the β-Actin PCR signal intensities.

2.6. Western blotting

For the preparation of whey, milk samples obtained from all evaluated mammary quarters were centrifuged twice at 44,000 × *g* at 4 °C for 30 min. The translucent supernatant was collected and stored at −80 °C (Bannerman et al., 2004). The protein concentration in the supernatants was estimated using fluorimetric methods (QubitTM, Invitrogen). Forty micrograms of protein from samples of BRM and placebo quarters, along with prestained molecular weight markers (Bio-Rad, Hercules, CA, USA), were separated by SDS-PAGE (15% resolving gel). Proteins were transferred electrophoretically to a nitrocellulose membrane (Amersham, Buckinghamshire, UK). The blotted membranes were blocked for 3 h at 25 °C in 3% (w/v) BSA in TBS containing 0.05%, v/v Tween-20 (Sigma–Aldrich Corp., St. Louis, MO, USA) and incubated overnight at 4 °C with rabbit polyclonal antiserum against bovine TNF-α (AbD Serotec, UK). Following washing, membranes were treated for 2 h with corresponding secondary peroxidase-conjugated anti-rabbit antibody (Amersham, Buckinghamshire, UK). Immunopositive bands were visualized with a chemiluminescent detection kit (ECL, Amersham, Buckinghamshire, UK).

2.7. Image analysis

Image analysis was performed using the Image Pro-Plus 3.0.1 system (Media Cybernetics, Silver Spring, MA, USA).

For cytokines mRNA expression analysis, agarose gel images were obtained using an Olympus digital camera. Cytokines mRNA levels were expressed as a ratio between the integrated optical density (IOD) band of each cytokine and the corresponding band of β-Actin.

Table 2
Effect of treatment with a biological response modifier (BRM) at drying off on somatic cells counts (log_{10} cel/mL) at different time points.

Time	Placebo quarters	Uninoculated quarters	BRM-treated quarters
0 h	$5.44468 \pm 0.10321^{a,b}$	$5.62382 \pm 0.10482^{a,b,c}$	5.29980 ± 0.11734^{a}
24 h	$5.69560 \pm 0.29452^{a,b,c}$	$5.60863 \pm 0.13410^{a,b,c}$	6.84699 ± 0.11137^{d}
48 h	$5.64746 \pm 0.20003^{a,b,c}$	$5.57285 \pm 0.13484^{a,b,c}$	6.77089 ± 0.13270^{d}
72 h	$5.88027 \pm 0.14409^{b,c}$	6.05485 ± 0.39954^{c}	6.92496 ± 0.07504^{d}

References: values represent the mean ± SEM. Means without a common letter ^{a,b,c,d} differ ($P < 0.05$).
BRM: 10 mL of 3 mg/mL ginseng dry extract in 0.89% saline. Placebo: 10 mL 0.89% saline.

For the Western blotting method, films were digitized at 1200 dpi (scanner HP Officejet J5780) and individual bands were quantified by densitometry to obtain an IOD value. These values were compared between samples from all studied quarters at pre-inoculation and 24, 48 and 72 h pt.

2.8. Statistical analysis

A statistical software package (SAS, 1999) was used to perform statistical analysis. Differences in SCC and TNF-α by Western blot assays between groups were assessed by one-way ANOVA, followed by Duncan's multiple range tests as a multiple comparison test. Differences between cytokines mRNA expression in milk at different times post-treatment were analyzed by Kruskal–Wallis non-parametrical test, followed by Bonferroni as a multiple comparison test. A value of $P < 0.05$ was considered significant. Results were expressed as mean ± SEM.

3. Results and discussion

3.1. Analysis of somatic cell counts

Macroscopic changes on mammary secretions and moderate swelling were observed in mammary quarters of BRM-treated group at 24 h pt. All samples from udder secretions were negative for pathogenic bacteria. No side effects, other than those mentioned, were observed during the experimental period.

In BRM-treated quarters average log_{10} SCC was 5.29 ± 0.12 at 0 h. Somatic cell counts increased at 24 h pt (log_{10} 6.84 ± 0.11) and reached a peak at 72 h pt (log_{10} 6.92 ± 0.07). Milk SCC was significantly ($P < 0.05$) increased at 24, 48 and 72 h pt in all BRM-treated quarters compared with placebo and uninoculated control quarters. Conversely, placebo and control groups SCC showed a slow increase over the sampling period, reaching the highest values by 72 h pt. No differences in SCC were observed among placebo and uninoculated control groups (Table 2). The elevated SCC in BRM-treated quarters can be explained, in part, by the accelerated PMN recruitment into the mammary gland following BRM inoculation. These results are in accord with a recent study using a LPS-based BRM infused in bovine mammary glands at drying off which caused a pronounced increase in SCC at 48 h post-inoculation in uninfected mammary quarters, followed by a slow decrease through the sampling period (Dallard et al., 2009).

3.2. Cytokine mRNA expression in milk cells

The effect of *P. ginseng* on transcriptional activity of bovine cytokines was measured by a semi-quantitative RT-PCR system. Since milk SCC for placebo and control quarters were similar, RT-PCR was only performed in the placebo group. Constitutive expression of proinflammatory cytokines was seen in milk somatic cells obtained from cows before BRM inoculation (Fig. 1). This finding is consistent with previous studies in humans as well as in cows in which a constitutive baseline expression of cytokines may occur in normal mammary glands (Green et al., 1997; Hagiwara et al., 2000; Leutenegger et al., 2000).

In the healthy lactating mammary gland, total SCC in milk are $<2 \times 10^5$ cells/mL. However, Holstein cows are bred for high milk production that can cause a permanent state of stress. Therefore a certain amount of tissue damage and possibly low-grade bacterial presence is common. This assumption of the apparent healthy state of the animals has to be considered to understand the expression of cytokines that indicate a certain level of inflammation (Leutenegger et al., 2000).

Previous reports demonstrated that macrophages treated with ginseng extract, both *in vitro* and *in vivo* produce proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IFNs (Shin et al., 2002). In this study, IL-1α, IL-1β, IL-8 and TNF-α mRNA were detected in all experimental

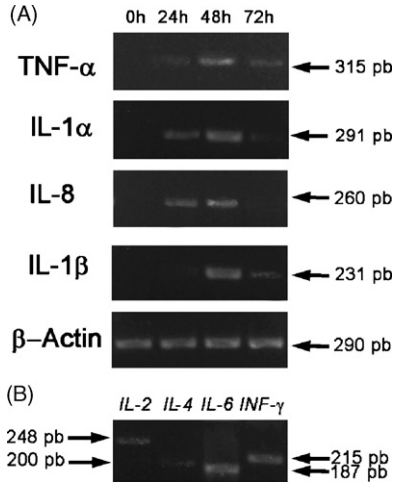

Fig. 1. Agarose gels (2%) of cytokine reverse transcription-PCR products obtained from milk somatic cells at different time points following intramammary infusion of a biological response modifier. (A) From milk somatic cells, (B) from bovine mesenteric lymph nodes (positive control).

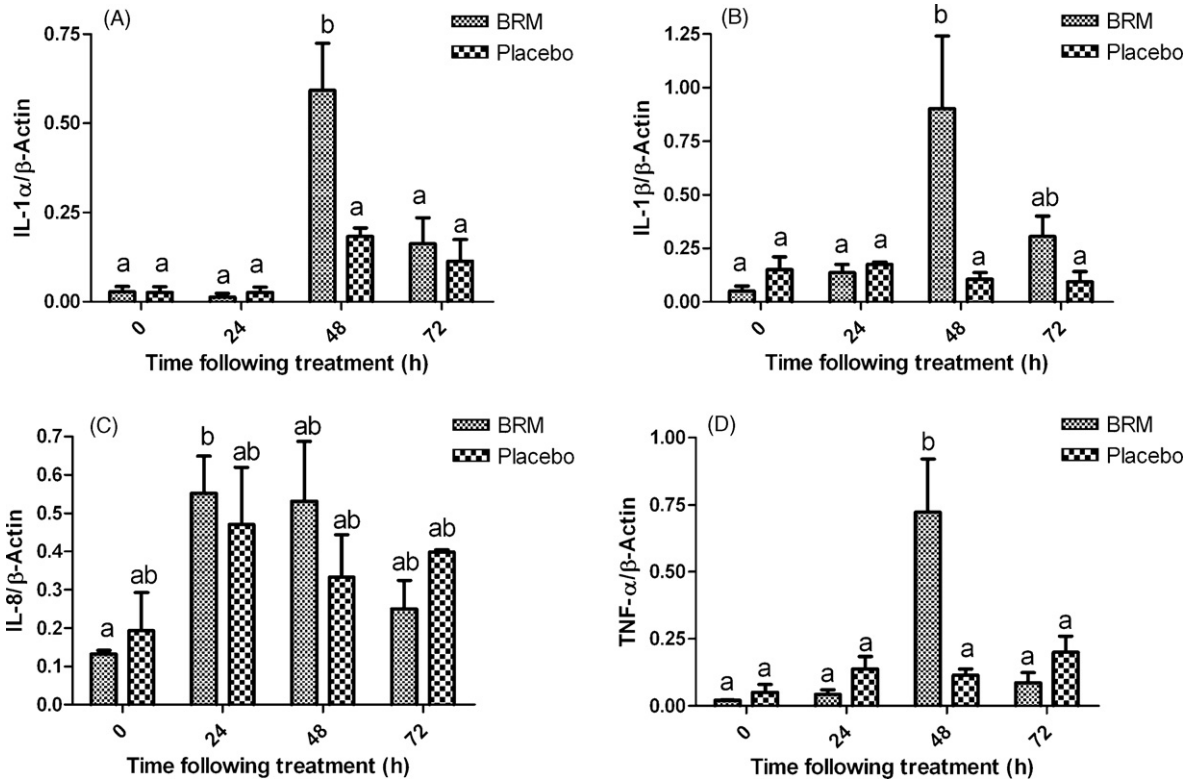

Fig. 2. Semi-quantification of mRNA of (A) IL-1α, (B) IL-1β, (C) IL-8 and (D) TNF-α in milk from bovine mammary quarters treated with BRM and placebo at 0, 24, 48 and 72 h pt. Values represent the mean ± SEM. Means without a common letters (a) and (b) differ ($P < 0.05$).

groups. In contrast IL-2, IL-4, IL-6 and INF-γ mRNA were not found in any milk sample, despite the presence of β-Actin RT-PCR products in every sample evaluated (Fig. 1).

A significant increase of IL-1α ($P = 0.0031$), IL-1β ($P = 0.0135$) and TNF-α ($P = 0.0377$) mRNA expression was observed in BRM-treated compared with placebo-treated quarters at 48 h pt (Fig. 2), while no differences were detected at 72 h pt. At 72 h pt, a non-significant decrease in mRNA expression for IL-1β in BRM-treated quarters was observed; meanwhile, the expression of IL-1α and TNF-α mRNA showed a significant decrease compared with 48 h pt ($P = 0.0031$, $P = 0.025$; respectively). A previous study found a significant production of IL-1 following stimulation of mice peritoneal macrophages with 25 μg/mL of ginsenoside Rg1 *in vitro*, (Kenarova et al., 1990). The sharp increase and decrease in the transcriptional level of IL-1α and IL-1β at 48 h pt could be the result of concomitant SCC increment in treated quarters at this time point.

Bovine IL-8 mRNA has been detected both in healthy and infected mammary glands (Alluwaimi, 2004). Although, the dose applied in the present study stimulated the expression of IL-8 mRNA in milk extracted cells at 24 and 48 h pt, no significant differences were observed among BRM-treated and controls at any experimental time point ($P = 0.9184$). Interleukine-8 is a chemokine produced by many cell types, including monocytes/macrophages, fibroblasts, epithelial cells, endothelial cells, and neutrophils (Baggiolini et al., 1989). Its production is presumed to be a sensitive indicator of macrophage activation and useful for the evaluation of biological response modifiers in various diseases. Sonoda et al. (1998) found that ginsenan S-IIA, a component of acidic polysaccharide of GS, is a potent inducer of IL-8 in human monocytes and THP-1 cells. The differences found in this cytokine expression between studies may not only rely in the experimental model used, but also in the different chemical nature of the extract used.

3.3. Presence of TNF-α in milk by Western blot

TNF-α is among the earliest secreted cytokines from immuno-competent cells in response to microbial stimuli. This cytokine has been detected both in healthy and infected bovine mammary glands (Alluwaimi, 2004).

In this study, production of TNF-α in mammary secretions collected from BRM and placebo-treated quarters at drying off was examined by density of immunoreactive bands on electrophoresis gel. No immunoreaction was detected in any placebo samples. However, in reduced condition, two bands of 17 and a 26 kDa, respectively, were observed at 24 and 48 pt in BRM-treated quarters (Fig. 3). TNF-α, the prototype member of the TNF superfamily, forms trimers and is expressed both as a membrane-bound 26-kDa and a soluble 17-kDa form, both of which are biologically active (Rahman et al., 2006). Release of 17 kDa TNF-α from membrane-anchored 26 kDa pro TNF-α is an early and rapid response to environmental or endogenous stimuli (Rahman et al., 2006). In the current study, a significant increase ($P < 0.05$) of soluble 17 kDa TNF-α level in

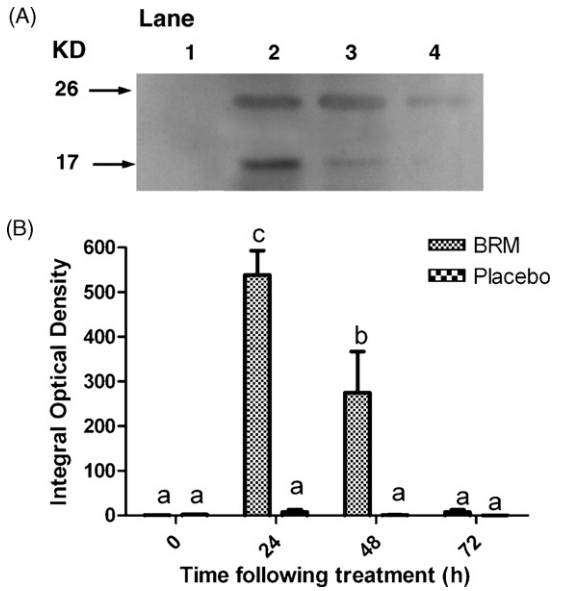

Fig. 3. (A) Western blotting image of TNF-α in milk from bovine mammary quarters treated with a BRM. References: lane 1, 0 h pt; lane 2, 24 h pt; lane 3, 48 h pt; lane 4, 72 h pt. The 17 and 26 kDa bands were also observed at 24 and 48 h pt. (B) Representative Western blot analysis of TNF-α (17 kDa band) in milk from bovine mammary quarters treated with a BRM. Values represent the mean ± SEM. Means without a common letters (a, b, c) differ ($P < 0.05$).

mammary gland secretion was observed in BRM-treated quarters at 24 h pt, while it was not detected in placebo-treated quarters (Fig. 3). This result suggests an active secretion of this cytokine from resident mammary cells as macrophages and epithelial cells. TNF-α has a profound effect on tissue remodelling, repair, and inflammation by coordinating the activities of many other cells, including endothelial cells, granulocytes, fibroblasts, and lymphoid cells (Larrick and Kunkel, 1988). The TNF-α transcript in cells extracted from milk from BRM-treated quarters showed a peak at 48 h pt to decrease significantly at 72 h pt ($P < 0.05$), indicating the activation of leukocytes in udder secretions and the important role of TNF-α in somatic cells recruitment (Persson et al., 1996).

In summary, cytokine RT-PCR products for IL-1α, IL-1β and TNF-α were found in all samples from BRM-treated glands, indicating that GS inoculation at drying off was associated with the recruitment of leukocytes in milk. This compound may play a role in mastitis control at drying off enhancing intramammary defenses, either alone or in conjunction with dry cow antibiotic therapy. Further studies are required to establish the exact mechanism of its action and to evaluate the modulatory activity of the other components of *P. ginseng* on the immune system.

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