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Intramammary infusion of *Panax ginseng* extract in bovine mammary gland at cessation of milking induces changes in the expression of toll-like receptors, MyD88 and NF-kB during early involution

Celina Baravalle [a,b,*], Paula Silvestrini [a,b], Mónica C. Cadoche [a,b], Camila Beccaria [a,b], Carolina S. Andreotti [a,b], María S. Renna [a,b], Elizabeth A.L. Pereyra [a,b], Hugo H. Ortega [a,b], Luis F. Calvinho [c], Bibiana E. Dallard [a,b]

[a] Laboratorio de Biología Celular y Molecular Aplicada, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Argentina
[b] Instituto de Ciencias Veterinarias del Litoral (ICIVET-Litoral), Universidad Nacional del Litoral – Consejo Nacional de Investigaciones Científicas y Tecnológicas (UNL-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 purposes of this study were to explore TLR2 and TLR4 participation and MyD88 and NF-κB activation in bovine mammary glands (BMG) treated with *Panax ginseng* (PG) at involution and verify the effect of PG in the cytokine expression. Quarters were infused at the end of lactation with PG solution (3 mg/ml), placebo or kept as uninoculated controls. Cows were slaughtered at 7 d after cessation of milking and mammary tissue samples were taken. A significant increase of TLR2, TLR4, MyD88, NF-κB, IL-1β, IL-6 and TGF-β1 mRNA expression was observed in PG-treated quarters. Immunostaining of TLR2 and TLR4 was significantly higher in PG mammary tissues. The percentages of immunopositive cells for NF-κB-p65 were significantly higher in PG-treated quarters. The BMG responded to PG extract components possibly by TLR2 and TLR4 signaling pathway. These results provide an insight into potential mechanisms by which PG stimulates innate immunity during BMG involution.

1. Introduction

Intramammary infections (IMI) in dairy cows are a major concern for the dairy industry. The bovine mammary gland is highly susceptible to new IMI during physiological transition from lactation to involution (Nickerson, 1989; Oliver and Sordillo, 1988). These infections, which lead to severe milk loss, are potentially fatal and a major cost to dairy farmers (Halasa et al., 2007). The early mammary gland involution is a critical stage, since changes that lead to increased concentration of protective 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.

Development of novel therapeutics, which could nonspecifically increase the innate immune response, represents an ideal strategy for addressing current worldwide concerns regarding how to combat classical and emerging infectious agents. Modulation of cytokine secretion may offer novel approaches in prevention and treatment of a variety of diseases. Certain herbal medicine extracts have been shown to have immunomodulatory activity, modifying the immune function through the dynamic regulation of informational molecules such as cytokines (Spelman et al., 2006).

The root of the plant *Panax ginseng* C.A. Meyer (Asian or Korean ginseng) has a long history as a general tonic to promote health (Pannacci et al., 2006). Ginsenosides, the steroid saponins, are major biologically active compounds of ginseng. They comprise 3–6% of ginseng extracts (Huang, 1999) and are mainly chemically triterpenoid glycosides of the dammarane series. Ginsenosides have a broad range of biological activities, including anti-inflammatory (Surh et al., 2001), antioxidant and anti-tumor effects, as well as adjuvant properties with low hemolytic activity (Hu et al., 2003; Jang and Shin, 2010; Rivera et al., 2003, 2005; Sun et al., 2007). We have previously shown that the intramammary (IM) inoculation of *Panax ginseng* extract (PG) in cows at cessation of milking increased the innate immune responses in the mammary gland revealed by an increase of pro-inflammatory cytokine mRNA transcription in milk and cytokine expression in mammary tissue, together with an increase in somatic cell counts (SCC) in milk (Baravalle et al., 2010, 2011). In addition, we found that the IM inoculation of PG at drying off increased the rate of mammary cell apoptosis without inhibiting cell proliferation (Dallard et al., 2011) and inhibited insulin-like

* Corresponding author. Instituto de Ciencias Veterinarias del Litoral (ICIVET-Litoral). R. P. Kreder 2805, (3080) Esperanza, Santa Fe, Argentina. Tel.: +54 03496 420639; fax: +54 3496 426304.
E-mail address: cbaraval@fcv.unl.edu.ar (C. Baravalle).

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growth factor 1 (IGF1) local production and bioavailability in mammary tissue (Dallard et al., 2013) leading to enhancement of mammary regression rate during early involution. However, there is no information about the effects of PG in the expression levels of molecules that play an important function in the innate immune system during early involution of the bovine mammary gland.

A rapid acting and effective innate immune response is predicated on early recognition of potential pathogens (Akira et al., 2006). Although 'non-specific' in character, innate immunity is initiated when specific pattern recognition receptors (PRR) on the surfaces or within host cells bind particular bacterial molecules termed pathogen-associated molecular patterns (PAMP). Among PRR, Toll-like receptors (TLRs) have been well characterized and are host cell sensors that recognize conserved PAMPs. Interestingly, in addition to microbial components, TLRs also recognize nonpathogenic molecules like plant-derived and stress related proteins such as heat shock proteins and β-defensin (Asea et al., 2002; Biragyn et al., 2002; Nakaya et al., 2004).

The specific response initiated by individual TLRs depends on the recruitment of a single, or a specific combination of, TIR domain-containing adaptor protein (*e.g.*, MyD88, TIRAP, TRIF, or TRAM) (Kawai and Akira, 2010). MyD88 is used by all TLRs (with the exception of TLR3) and members of the IL-1 receptor family, transmitting signals culminating in NF-κB and MAP kinase activation and the induction of inflammatory cytokines. NF-κB is a well-characterized transcription factor that is known to regulate the expression of a wide range of genes, including those of cytokines, chemokines and cytokine receptors (Baeuerle and Baichwal, 1997).

The active NF-κB transcription factor consists of functional homo or heterodimers of its five monomeric family member subunits p50 (NF-κB1), p52 (NF-κB2) p65 (Rel A), c-Rel and Rel B. Ordinarily, NF-κB is sequestered in the cytoplasm through a physical association with inhibitory proteins of the IκB family (p100, p105, Bcl-3, IκB-a, IκB-b, IκB-e), which maintains the transcription factor in an inactive state (Siebenlist et al., 1994; Whiteside et al., 1997). A wide range of stimuli, including pro-inflammatory cytokines, induce intracellular signaling cascades, which result in the dissociation of the inhibitory protein from NF-κB (Chen et al., 2001).

Given the background on the role of TLRs in the recognition of microbial components and nonpathogenic molecules and in the initiation of the immune response, and given that little is known about the mechanisms of production of pro-inflammatory cytokines in response to PG extract in bovine mammary gland we considered that these receptors (TLR2 and TLR4) could represent a target for some components of ginseng extract. Moreover, previous reports suggest that PG components possess a putative TLR ligand in different study models (Nakaya et al., 2004; Pannacci et al., 2006). Aims of this study were to explore TLR2 and TLR4 participation, and MyD88 and NF-κB activation in bovine mammary glands treated with PG at cessation of milking. In addition, to verify the effect of PG in bovine mammary gland we detected the mRNA expression of pro and anti-inflammatory cytokines not previously evaluated in mammary tissue.

2. Materials and methods

2.1. *Preparation of* Panax ginseng *inoculum*

The PG extract was provided by Indena Company (Indena® SpA, Milan, Italy). The spectrophotometric content of saponins expressed as ginsenoside Rg1 with reference to the dried substance was 27%. High performance liquid chromatography analysis on the composition of protopanaxatriol ginsenosides Rg1, Rf, Re, calculated as Rg1 and of protopanaxadiol ginsenosides Rc, Rd, Rb2, Rb1, calculated as Rb1, with reference to the dried substance was 23.9%. The PG solution was prepared as previously described (Dallard et al., 2013). The endotoxin level in the purified PG solution was examined by Pyrotell *Limulus* amebocyte lysate assay kit (Associates of Cape Cod, East Falmouth, MA, USA) according to the manufacturer's instructions. The PG dose used (3 mg/ml) was selected according to previous studies carried out in four Holstein nonpregnant cows in late lactation (Baravalle et al., 2010).

2.2. *Selection of cows and experimental protocol*

All of the experiments were performed according to the Guide for the Care and Use of Agricultural Animals in Research and Teaching (2011), and approved by the Committee of Animal Ethics and Security of the Facultad de Ciencias Veterinarias, UNL. Six healthy Holstein non-pregnant dairy cows in late lactation (weeks 31–36), in their third to fifth lactation periods with average milk yield of 10 kg per cow per day at the end of lactation, were selected from the Rafaela Experiment Station of Instituto Nacional de Tecnología Agropecuaria (INTA). The animals were kept under grazing conditions, received concentrates in the milking parlor and were milked twice daily before initiation of the study. Cows were included in the study based on previous bacteriological studies and SCC. All the quarters used in this study were free of infection.

The experimental protocol followed has been described in detail previously (Baravalle et al., 2010). The mammary quarter was the experimental unit. Cows were divided in three equal groups and mammary quarters were randomly assigned to each of the treatment groups, verifying that all treatments were administered within each udder. Briefly, eight quarters were intramammarily infused with 10 ml of ginseng solution (3 mg/ml) (group I), six quarters were treated with 10 ml of placebo (saline solution) (group II) and six quarters were kept as uninoculated controls (group III). Two quarters from each of groups II and III were not considered for the treatment owing to high SCC at the time of inoculation. In all cases, cows were no longer milked after intrammamary infusion.

2.3. *Sample collection*

Cows were slaughtered at a local abattoir 7 days after inoculation and mammary gland tissue sections from the animals included in the three groups were collected (Dallard et al., 2011). Tissue samples were obtained from three zones of mammary quarters as previously described (Dallard et al., 2013): upper limit of the gland cistern, approximately midway between the upper limit of the gland cistern and the dorsal boundary of the mammary gland at a depth of 4 cm (lobulo-alveolar zone), and then were immediately frozen at –80 °C in a freezing vial until further use for gene expression and western blotting assays. Additional sections of mammary tissue from the three zones 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 in an ascending series of ethanol, cleared in xylene and embedded in paraffin. Four-micrometer thick sections were mounted on slides previously treated with 3-aminopropyltriethoxysilane (Sigma–Aldrich, St. Louis, MO, USA) for use in immunohistochemistry (IHC) procedures (Dallard et al., 2013).

2.4. *RNA extraction and quantitative real time reverse transcription-PCR*

Total RNA was isolated from tissue aliquots from different zones of the mammary quarters after treatment with Trizol LS reagent (Invitrogen, Life technology, CA, USA) according to the manufacturer's instructions but with slight modifications (Dallard et al., 2013). Quantity and quality of RNA for all obtained samples from the different treatment groups were determined using a fluoroscopic method (Qubit, Invitrogen, CA, USA) and by visualization on a denaturating agarose gel. Two micrograms of RNA samples was treated with DNAse I (Invitrogen) in accordance with the manufacturer's instructions. The

Table 1
Quantitative real-time PCR primers. Probe sequences (5′–3′).

Molecule	Primer sequence (5′–3′)	Reference sequence
TLR2*	For[a] CAGGCTTCTTCTCTGTCTTGT	NM174197
	Rev[b] CTGTTGCCGACATAGGTGATA	
TLR4	For AGCTTCAACCGTATCATGGCCTCT	AY634630
	Rev ACTAAGCACTGGCATGTCCTCCAT	
IL-1β	For TGGGAGATGGAAACATCCAG	M37211
	Rev TTTATTGACTGCACGGGTGC	
IL-6	For GATGCAGTCTTCAAACGAGTGGGT	X57317
	Rev AGGTTTCTGACCAGAGGAGGGAAT	
TGF-β1	For CCTGAGCCAGAGGCGGACTAC	M36271
	Rev GCTCGGACGTGTTGAAGAAC	
NF-κB	For GCTGAGTTGAGAGAGAGTAACC	NM001076409.1
	Rev CTTTCTGTTGTCACTGCTGC	
MyD88	For CGCGGCTTACTTGTCCT	NM002468
	Rev CTGCCAGTCGTCCAGGAG	
GAPDH	For CACCCTCAAGATTGTCAGCA	AY538775
	Rev GGTCATAAGTCCCTCCACGA	

* References: TLR2: toll-like receptor 2; TLR4: toll-like receptor 4; IL-1β: interleukin 1 beta; IL-6: interleukin 6; TGF-β1: Transforming growth factor beta; NF-κB: nuclear factor κB; MyD88: myeloid differentiation factor 88; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.
[a] Forward (For).
[b] Reverse (Rev).

first strand cDNA was synthesized using a master mix (Moloney Murine Leukemia Virus (MMLV) buffer, dithiothreitol (DTT), RNAout, MMLV reverse transcriptase, deoxyribonucleotide triphosphate (dNTP) and random hexamer primers (Invitrogen)). The reverse transcription conditions were performed as described previously (Baravalle et al., 2010). To validate the absence of genomic DNA, non-transcriptional (without the reverse transcriptase and with RNA as sample) controls were run for each sample and gene.

Transcript levels for TLRs, NF-κB, MyD88 and cytokines were measured by relative quantitative real time PCR using a StepOne Real Time PCR System (Applied Biosystems, Life Technology, CA, USA). An optimized protocol was used: initial denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s and annealing 62 °C (TLR2, NF-κB and TGFβ1), 58 °C (TLR4, IL-1β and glyceraldehyde 3-phosphate dehydrogenase, GAPDH) and 56 °C (IL-6) for 30 s, extension at 72 °C for 30 s and fluorescence reading at 74 °C for 10 s. All measurements for each sample were performed in duplicate. The efficiency of PCRs and relative quantities were determined from a six-point standard curve. Standard curves were constructed from a dilution series of pooled cDNAs, and PCR efficiency was calculated using the StepOne software v2.2 (Applied Biosystems).

For amplification, each PCR was performed in a total volume of 20 μl containing: 4 μl of cDNA (500 ng/ml) previously quantified by the Qubit method (Invitrogen), 4 μl of 5X Phire reaction buffer, 0.5 μl of each 10 μM forward/reverse primers, 0.2 mM dNTPs, 1 μl SYBR Green I (Invitrogen, Life Technology, CA, USA), 0.05 μl Phire Taq polymerase (Thermo Fisher Scientific Company, Finland) and 14 μl of sterilized DEPC treated water.

The primer sequences used for PCR assays were purchased from Invitrogen and are described in Table 1. All primers were designed to ensure cDNA specific amplification using Primer-Quest software (http://www.idtdna.com/Primerquest/Home/Index). Primers and amplification products were verified *in silico* using Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST) to confirm gene specificity. The forward and reverse primers for all genes were designed when possible to produce amplification products that spanned at least two exons. The GAPDH gene sequence was included as the housekeeping gene. The mRNA expression levels were recorded as cycle threshold (Ct) values that corresponded to the number of cycles at which the fluorescence signal can be detected above a threshold value. The Ct was calculated using StepOne v2.2. Negative DNA template controls were included in all the assays, and yielded no consistent amplification. Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. Fold change was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.5. Immunohistochemistry (IHC)

Protein expression of TLR2, TLR4 and NF-κB-p65 was detected using polyclonal antibodies (Table 2). The homology between the target peptide of each antibody and the correspondent bovine protein was tested with the Basic Local Alignment Search Tool (BLAST software; http://www.ncbi.nlm.nih.gov/BLAST) to determine the peptide location and to confirm antigen specificity. Briefly, after deparaffinization and hydration, antigens were unmasked in citrate buffer (pH 6.0) using a microwave oven. Endogenous peroxidase activity was quenched with hydrogen peroxide in methanol for 20 min. After rinsing in PBS, non-specific binding sites were blocked by goat serum for 15 min. The sections were then incubated overnight at 4 °C with primary antibodies diluted in PBS with bovine serum albumin. The samples were washed with PBS and then incubated with a biotinylated goat anti-rabbit secondary antibody (Table 2). The extravidin biotin immunoperoxidase method used has been previously described by Dallard et al. (2011). For antigen visualization 3.3-diaminobenzidine (DAB) was used as the chromogen (Table 2). Negative control sections in which the primary antibody was replaced by a non-immune rabbit serum were included (Baravalle et al., 2011). Some sections were incubated only with DAB reagent to exclude the possibility that endogenous peroxidase activity had been unsuccessfully blocked.

2.6. Western blotting

To test the specificity of the primary antibodies used in the present study, a pool of mammary gland tissue sections from the different treatment groups were homogenized in a radio-immunoprecipitation 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.),

Table 2
Antibodies, conditions and commercially purchased reagents used.

	Source	Type of antibody	Specificity of antibody	Dilution IHC	DilutionWB
Anti-TLR2[a,b]	GenWay Biotech, Inc	Polyclonal	Human, bovine, mouse and rat	1:200	1:400
Anti-TLR4[a,b]	GenWay Biotech, Inc	Polyclonal	Human, bovine, mouse and rat	1:200	1:400
Anti-NF-κB-p65[a,b]	Santa Cruz Biotechnology	Polyclonal	Human, equine, canine, bovine and porcine	1:500	1:1000
Biotinylated anti-rabbit IgG[a]	Zymed	Polyclonal		1:100	
Streptavidin-peroxidase solution[a]	BioGenex				
diaminobenzidine[a]	Dako Corp				
Anti-rabbit IgG peroxidase[b]	Amershan	Polyclonal			1:200

[a] Used for immunohistochemical staining.
[b] Used for Western blot study.

0.1 M PBS, and a protease inhibitor cocktail (Complete Mini Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany). The homogenate was then centrifuged at 12,000 × *g* at 4 °C for 30 min and the supernatant was frozen at –80 °C. Protein concentration in the supernatants was estimated using fluorescence methods (Qubit™, Invitrogen). Proteins (40 μg), along with pre-stained molecular weight markers (Bio-Rad, Hercules, CA, USA), were separated in SDS–polyacrylamide gels (10% resolving gel for TLR2 and TLR4, and 15% resolving gel for NF-κB-p65) and transferred electrophoretically to a nitrocellulose membrane (Hybond ECL Nitrocellulose Membrane, GE Healthcare, Buckinghamshire, UK). After blotting, the membranes were blocked with Tris-buffered saline (TBS) containing 0.05% (v/v) Tween20 (Sigma–Aldrich Corp.) and 5% (w/v) non-fat milk, and then incubated overnight at 4 °C with specific primary antibodies (Table 2). Bound antibody was detected using anti-rabbit IgG peroxidase antibody (Amersham). Immunoblots were visualized with an enhanced chemiluminescence system (GE Healthcare).

2.7. Image analysis

Images were digitized via a color video camera (Motic 2,000, Motic China Group, China) mounted on top of a conventional light microscope (Olympus BH-2, Olympus Co., Japan), using an objective magnification of 40× as described and validated previously (Baravalle et al., 2011; Dallard et al., 2011). The immunohistochemical stained areas (IHCSA) for antibodies TLR2 and TLR4 reactions were calculated as a percentage of the total area evaluated through color segmentation analysis. Briefly, the average density (% of positive area) of the TLR antibody reaction was calculated from at least 50 images of each area (parenchymal and stromal cells) in each section as a percentage of the total area evaluated through color segmentation analysis, which extracts objects by locating all objects of a specific color (brown stain) (Baravalle et al., 2011; Dallard et al., 2013).

Positive cells for NF-κB-p65 were quantified in the epithelium (parenchyma) and stroma of mammary gland as described previously (Dallard et al., 2008). Cells were classified as epithelial, stromal, labeled epithelial or labeled stromal cells. Labeled brown nuclei were readily visible; a cell was classified as labeled when the nuclear staining was at least twice as intense as the background. A minimum of 1000 cells in at least 20 microscopic fields at 40× magnification were counted in each slide. Epithelial and stromal cells showing an intense nuclear staining were quantified and results were expressed as a percentage of immunopositive cells.

For the western ligand blot, the exposed films were scanned at 1200 dpi (scanner HP Officejet J5, 780).

2.8. Statistical analysis

All statistical analyses were performed using a software package (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL). Medians from each treatment were analyzed by Kruskal–Wallis non-parametric test and those showing statistically significant differences ($P < 0.05$) were further analyzed by a 2-tailed Mann–Whitney test. Results were expressed as mean ± SEM (standard error of the mean).

3. Results

3.1. Analysis of endotoxin levels of PG extract

The endotoxin levels in PG at 10 mg/ml were lower than the detection limit of the test (<0.05 ng/ml), indicating that the biological effects of PG were not due to endotoxin contamination.

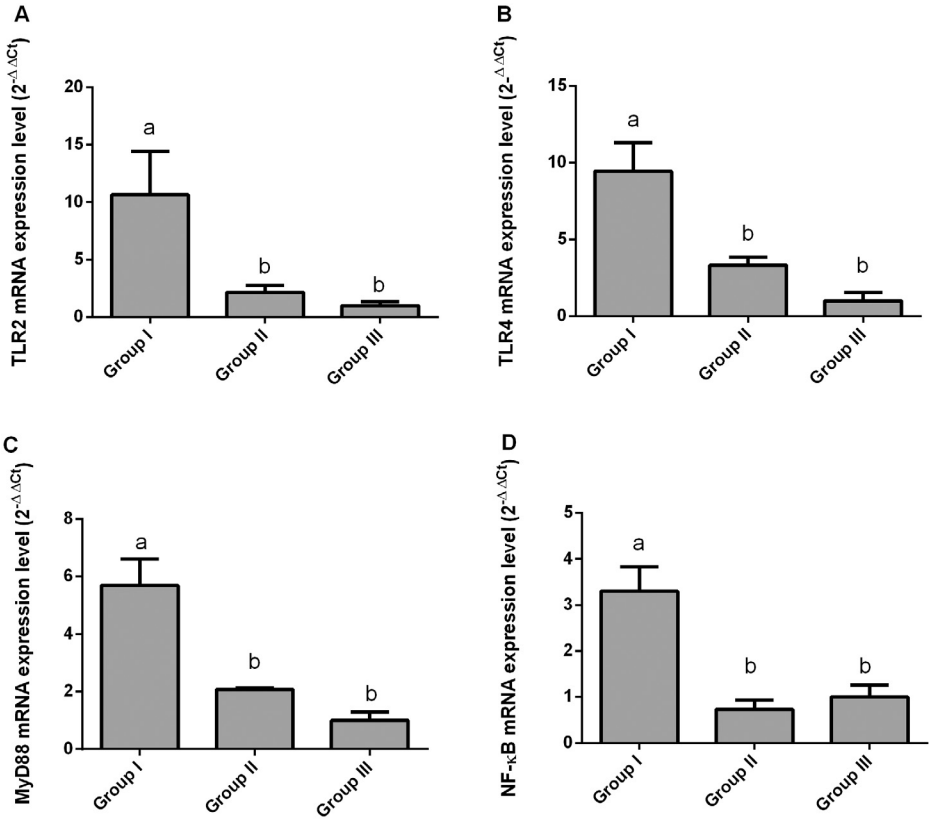

Fig. 1. Relative expression of TLR2 (A), TLR4 (B), MyD88 (C), NF-κB (D) in mammary quarters treated with *Panax ginseng* (group I), with placebo (group II) and untreated controls (group III) at 7 d of drying off. Values represent the means ± standard error of the mean (SEM). Bars with different superscript letters denote significant differences ($P < 0.05$).

3.2. Clinical signs and post inoculation observations

A preliminary experiment was designed to evaluate the doses of PG that triggered an immunomodulatory response when infused in the mammary gland through the teat canal (Baravalle et al., 2010). With the selected dose (3 mg/ml) none of the cows showed clinical signs or any visible local inflammatory reaction in mammary quarters. Only macroscopic changes (small clots) in mammary secretions and moderate swelling were observed in mammary quarters of group I at 24 h.

3.3. Gene expression in mammary tissue

3.3.1. Toll-like receptors, protein adaptor and transcription factor expression

Transcripts for TLRs, protein adaptor (MyD88) and transcription factor NF-κB were detected in every treatment group. Fig. 1 shows the effects of PG treatment on transcriptional activity of TLR2, TLR4, MyD88 and NF-κB genes at 7 d of post-lactational involution.

In mammary quarters treated with PG (group I) TLR2 ($P < 0.01$) and TLR4 ($P < 0.01$) gene expression was higher than for groups II and III. No differences were detected in the transcript levels for TLR2 and TLR4 between groups II and III ($P = 0.289$ and $P = 0.275$, respectively; Fig. 1).

In group I the transcript levels for MyD88 ($P < 0.01$) and NF-κB ($P < 0.01$) genes were higher than for groups II and III. No significant differences were found between the groups II and III for both genes ($P = 0.052$ and $P = 0.289$ respectively; Fig. 1).

3.3.2. Cytokine expression

The effect of PG on IL-1β, IL-6 and TGF-β1 mRNA expression levels was detected in bovine mammary tissue. At 7 d post inoculation, IL-1β, IL-6 and TGF-β1 were detected in all the evaluated groups. Transcript levels for IL-1β, IL-6 and TGF-β1 were higher in group I compared with groups II and III ($P = 0.029$; $P = 0.002$ and $P = 0.004$). No differences in the levels of the three evaluated cytokines between control groups (II and III) ($P = 0.376$; $P = 0.513$ and $P = 0.275$; Fig. 2) were observed.

3.4. Antibody specificity for immunohistochemistry

Fig. 3 shows the results from western blot analysis of mammary gland tissue homogenates. TLR2 and TLR4 antibodies recognized bands of 89.8 and 95.7 kDa respectively, both with appropriate sizes for each protein studied. On the other hand, western blot allowed to confirm the presence of one band of approximately 65 kDa for anti-NF-κB-p65 (Fig. 3).

3.5. Immunohistochemistry of mammary tissue for TLR2, TLR4 and NF-κB-p65 detection

To obtain quantitative data for immunohistochemical labeling of TLR receptors and information about the localization in the bovine mammary gland, brown cytoplasmic staining was detected and then evaluated by the IHCSA. TLR2 staining was present in all tissue sections at all evaluated groups and was primarily associated with the apical domain of alveolar epithelial cells and ducts of the mammary parenchyma (Fig. 3). TLR4 staining was present in all tissue sections at all evaluated groups and was associated with parenchymal and stromal mammary components. 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 plasma cells showed intense staining for TLR4 in their cytoplasm (Fig. 3).

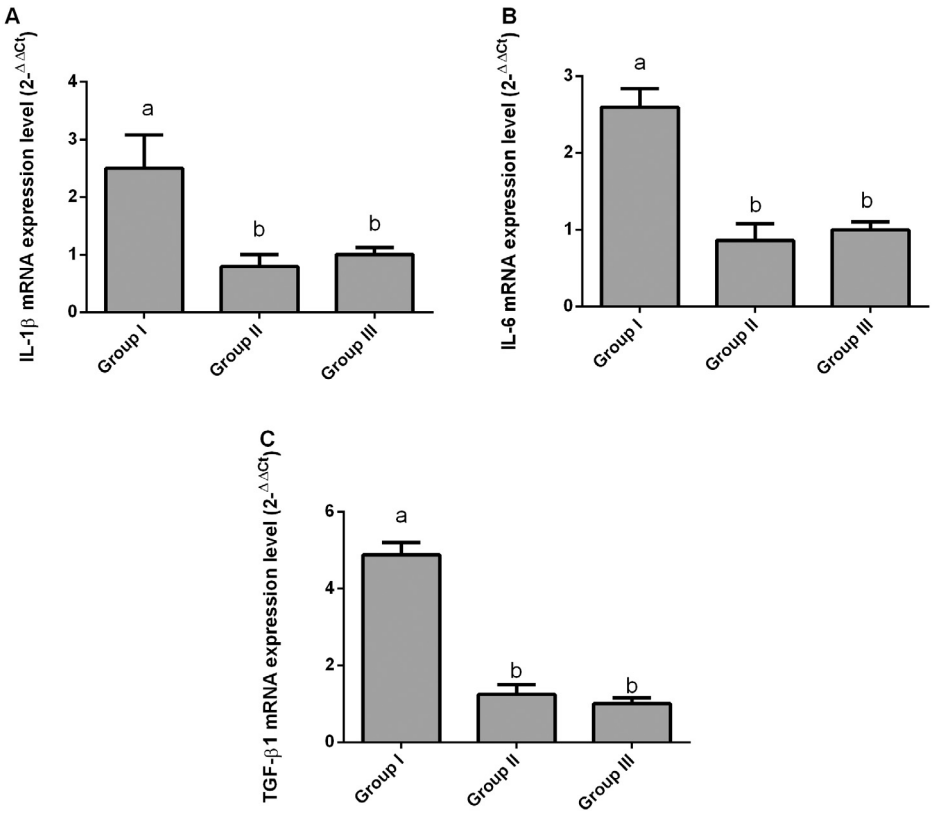

Fig. 2. Relative expression of IL-1β (A), IL-6 (B) and TGF-β1 (C) in mammary quarters treated with *Panax ginseng* (group I), with placebo (group II) and untreated controls (group III) at 7 d of drying off. Values represent the means ± standard error of the mean (SEM). Bars with different superscript letters denote significant differences ($P < 0.05$).

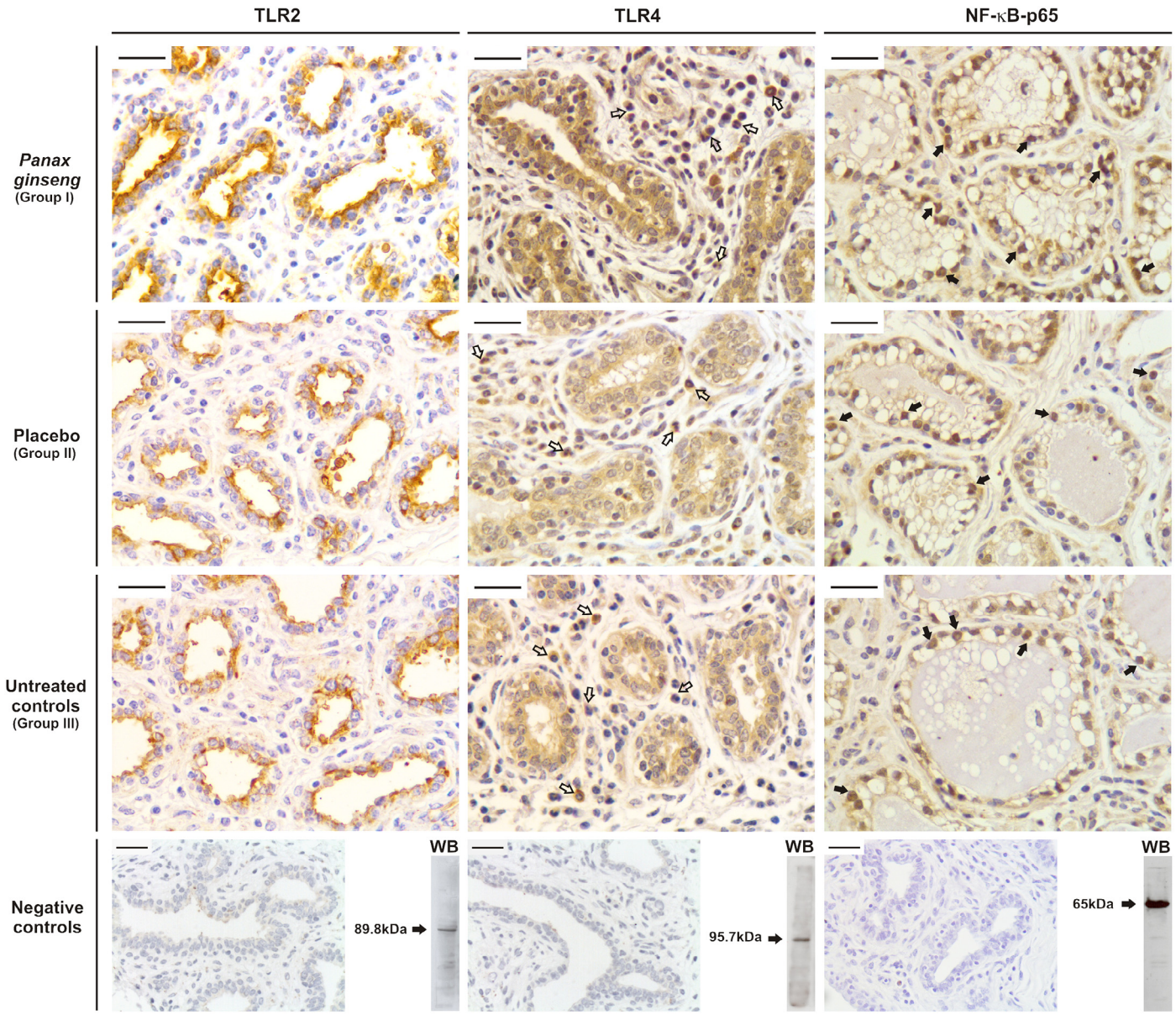

Fig. 3. Representative images of TLR2, TLR4 and NF-κB-p65 immunostaining in mammary quarters treated with *Panax ginseng* (group I), with placebo (group II) and untreated controls (group III). Verification of antibody specificity by western blot (WB) analysis of mammary gland homogenates (10% resolving gel for TLR2 and TLR4, and 15% resolving gel for NF-κB-p65) and negative controls for immunostaining are shown in the bottom panel. Immunoreactivity of immune cells to antibody against TLR4 is indicated with transparent arrows. Nuclear immunoreactivity of mammary epithelial and stromal cells to antibody against NF-κB-p65 is indicated with black arrows. Magnification is the same for all panels (400×) and is indicated by 25-μm bars.

Fig. 4 shows the effects of PG treatment on IHCSA for TLR2 and TLR4 and the percentages of immunopositive cells for NF-κB-p65 at 7 d of post-lactational involution. The percentages of IHCSA for TLR2 and TLR4 were higher in group I than in groups II and III ($P < 0.001$; Fig. 4). No differences were observed for TLR2 and TLR4 percentages of IHCSA between groups II and III ($P = 0.819$ and $P = 0.456$ respectively; Fig. 4). No differences in percentages of IHCSA between mammary zones sampled for the two evaluated receptors were detected ($P = 0.976$ for TLR2 and $P = 0.893$ for TLR4) across the three experimental groups.

To confirm the effect of PG treatment at drying off on activation of NF-κB, we performed immunohistochemistry on paraffin-embedded sections of mammary glands using an antibody to NF-κB-p65. Activated NF-κB was detected by the nuclear localization of p65 in mammary epithelial and stromal cells of the three experimental groups, whereas was poorly visualized in the cytoplasm of all mammary gland cells studied (Fig. 3). Both in stroma and parenchyma mammary cells from group I, the immunoexpression of NF-κB-p65 was higher than in cells from groups II and III ($P < 0.001$; Fig. 4). No differences were observed in the immunolabeling for NF-κB-p65 in stroma and parenchyma cells between groups II and III ($P = 0.289$ and $P = 0.724$, respectively; Fig. 4). No differences in percentages of positive cells for NF-κB-p65 between mammary zones sampled were detected in the three evaluated groups in stroma and parenchyma mammary cells ($P = 0.345$ and $P = 0.623$ respectively) across the three experimental groups.

4. Discussion

The increased susceptibility to IMI during early mammary gland involution and growing concerns about the use of blanket dry cow therapy due to potential development of antibiotic-resistant bacteria (Oliver et al., 2011) requires continued development of novel therapeutics to combat microbial pathogens. A novel strategy is to

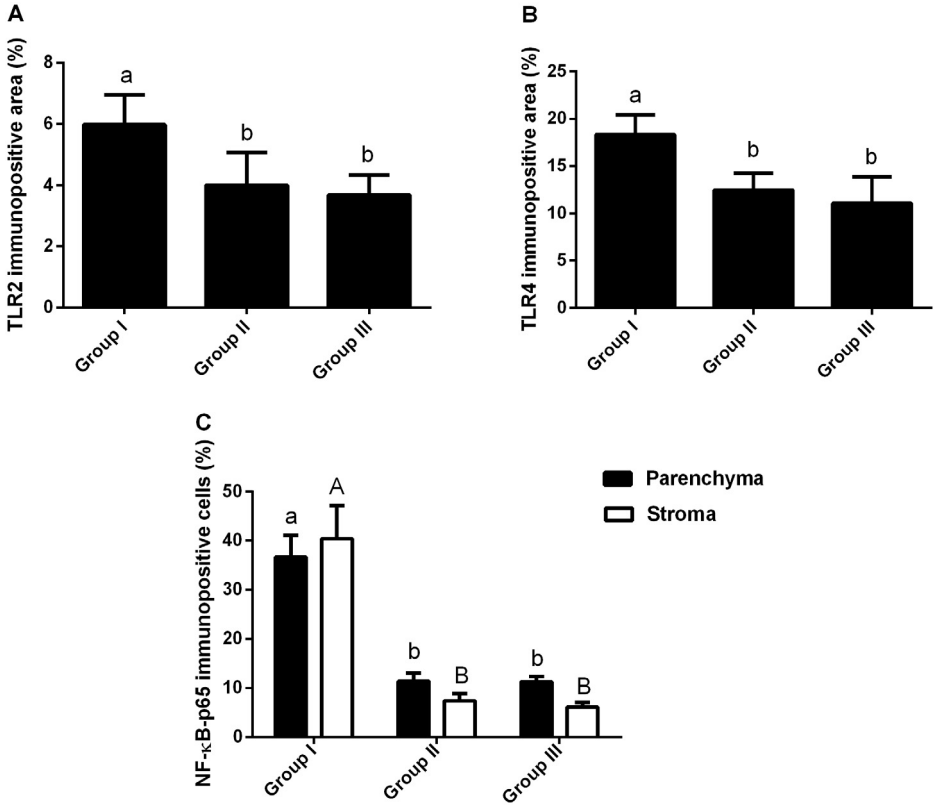

Fig. 4. Relative expression (measured as % of immunopositive area) of (A) TLR2 and (B) TLR4, and relative expression (measured as % of immunopositive cells) of (C) NF-κB-p65 in the epithelium (parenchyma) and stroma of mammary quarters treated with *Panax ginseng* (group I), placebo (group II) and untreated controls (group III) at 7 d of drying off. Values represent the mean ± standard error of the mean (SEM). Means (a, b; A, B) without a common letter differ ($P < 0.05$).

manipulate host immune responses to combat or prevent infection. Unlike antibiotics, immunomodulation would enhance a preexisting system that is designed to have broad spectrum antimicrobial activity (Nicholls et al., 2010) and can be conducive to improved animal health and economic performance of dairy farms (Trevisi et al., 2014).

Several *in vitro* and *in vivo* studies have shown that PG extract modulates different parameters of the immune response (Hu et al., 1995, 2001; Scaglione et al., 1996). In previous studies we have demonstrated that the intramammary inoculation of PG at cessation of milking increased the proinflammatory cytokine expression in milk and mammary tissue, associated with an increase of SCC in milk (Baravalle et al., 2010, 2011). Nevertheless, the mechanism underlying production of these cytokines in response to PG has not been defined. We propose that the immunomodulatory action of PG could be regulated by components of PG extract, like ginsenosides, that could exert their activity through the TLR2 and TLR4 pathways.

In the present study, TLR2 and TLR4 at the mRNA levels were detected in all experimental groups; however, in mammary quarters treated with PG the transcript levels for both receptors were significantly higher than in placebo-treated quarters and untreated controls. Bovine mammary tissue was shown to express mRNA for TLR2, 4 and 9. Among these receptors, expression of TLR2 and 4 substantially increased in quarters suffering from subclinical, moderate or severe infections, which correlated with severity of infection (Goldammer et al., 2004). TLR2 and TLR4 gene expression by mammary epithelial cells, monocytes-macrophages and neutrophyls has been reported (Gilbert et al., 2013; Strandberg et al., 2005; Wright et al., 1990); however, expression at the protein level remains to be documented. We performed IHC studies with antibodies to TLR2 and TLR4 to gain information on the localization of these receptors in bovine mammary tissue. Protein expression of TLR2 was abundantly expressed and localized in the apical domain of ductal and alveolar epithelium and was significantly higher in PG-treated quarters than in placebo-treated quarters and untreated controls. Protein expression of TLR4 was observed within the parenchymal and stromal mammary tissues and was significantly higher in PG-treated quarters than in placebo-treated quarters and untreated controls. Moreover, we observed positive reaction in the cytoplasm of immune cells like monocytes-macrophages, lymphocytes, neutrophyls and plasma cells.

Immunohistochemical staining using antibodies for TLR2 and TLR4 has confirmed the results of our gene expression studies regarding the presence of TLR2 and TLR4 molecules in mammary tissue. These results demonstrate that intramammary inoculation of PG at drying-off was able to significantly increase the expression of TLR2 and TLR4 at mRNA level and protein level in mammary tissue.

Previous studies have demonstrated that PG extract is able to increase the production of pro-inflammatory cytokines like TNF-α and IL-1β in mice under physical stress through an increased expression of TLR4 in macrophages (Pannacci et al., 2006). In addition, Nakaya et al. (2004), demonstrated that IFN-γ and TNF-α were produced by GRE-stimulated (Ginseng radix extract) spleen cells and peritoneal macrophages in C3H/HeN mice but not in C3H/HeJ (TLR4-deficient) mice. In accord with these results, in the present study, we demonstrated that the intramammary inoculation of PG increased significantly the expression of proinflammatory cytokines like IL-1β and IL-6 in mammary tissue at 7 d of cessation of milking. Moreover, we demonstrated a significant TGF-β1 gene expression increase in quarters treated with PG compared with control quarters. Transforming growth factor-β has been reported to regulate ductal growth and patterning, as well as alveolar functional differentiation. The ability of TGF-β to regulate these events is largely attributed to its growth inhibitory effects on epithelial cells and stimulatory effects on fibroblasts and other

stromal cells (Bannerman, 2009). In addition to its role in mammary gland development, TGF-β has been shown to suppress immune and inflammatory responses, although some pro-inflammatory properties have been ascribed to this cytokine (Andreotti et al., 2014; Flanders and Wakefield, 2009). The higher expression of TGF-β1 observed in mammary quarters treated with PG could be an essential response for limiting the scope of inflammation and potential injury to the host. These results, as well as those from a previous report from our laboratory (Dallard et al., 2011), provide further evidence that PG contributes to accelerate early mammary involution.

It has been determined that expression of pro-inflammatory mediators is modulated by NF-κB pathways (Kawai and Akira, 2010). Once activated, NF-κB unit p65 dissociates from its inhibitory protein I kBa and translocates from the cytoplasm to the nucleus where they may trigger transcription of specific target genes such as TNF-α, IL-1β and IL-6 (Fu et al., 2013). In the present study, we demonstrated that intramammary inoculation of PG at cessation of milking stimulated the activation of NF-κB in mammary tissue. This activation was confirmed by immunohistochemical nuclear staining of the NF-κB-p65 subunit in epithelial and stromal cells.

Our results indicate that bovine mammary epithelial and stromal cells respond to PG extract components affecting the TLR2 and TLR4 genes and protein expression. We propose that activation of MyD88-dependent pathway leads to a release of transcription factors, like NF-κB that translocates into the nucleus and regulates the expression of pro- and anti-inflammatory cytokines like TGF-β1 playing a crucial role in immune response regulation and mammary gland remodeling. However, to further evaluate the role of intracellular signaling pathways in the response to PG, inhibitors targeting TLRs should be tested using small interfering RNA (siRNA) targeting TLR2 or TLR4. This could provide further evidence to verify if the mammary epithelial, stromal and immune cells mount a pro-inflammatory response to PG by TLR2 and TLR4 pathways. In conclusion, the increased expression of pro-inflammatory cytokines in response to PG extract was associated to an increased expression of TLR2 and TLR4 in bovine mammary cells. Moreover, activation of the transcription factor NF-κB was verified in bovine mammary glands inoculated with PG. These results provide an insight into potential mechanisms by which PG stimulates innate immunity during bovine mammary gland involution.

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