

Investigating the role of teat seal in mastitis control: In addition to its effectiveness in preventing mastitis, why is teat seal effective in curing existing cases of mastitis?

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Abstract

The presence of mastitis in bred dairy heifers can adversely affect the development of milk-producing tissues, leading to less than maximal milk production and increased somatic cell counts (SCC) during the first lactation. Use of nonlactating cow therapy has been beneficial in curing existing intramammary infections (IMI), and teat sealants have been beneficial in preventing new IMI from developing. When used together, the combination of the two products may be more effective than either alone in controlling mastitis in these young dairy animals. Recent studies to examine this combination demonstrated that cure rates after the infusion of nonlactating cow therapy + teat seal, although 96.1% effective, were similar to nonlactating cow therapy alone (100% cure) and teat seal alone (85.7% cure), all of which were significantly ($P < 0.001$) elevated over untreated controls (55.2% cure); prevention rates ranged between 92.2 and 97.9% for all 4 treatments. Thus, although all 3 infusion treatments were effective in curing existing IMI and preventing new ones, the combination of nonlactating cow therapy + teat seal was no more effective than either treatment alone in controlling mastitis as initially hypothesized. What was unexpected was the 85.7% cure rate ($P < 0.001$) in quarters infused with teat seal alone. The purpose of the proposed research was to determine why teat seal was effective in curing existing IMI, the results of which may be instrumental in supporting claims that teat seal is a product that not only prevents new IMI as the product label states, but in addition, cures existing IMI. To accomplish this, mammary secretions were collected 24, 48, and 72 h after treatment from quarters of heifers randomly assigned to: 1) untreated control, 2) nonlactating cow therapy, 3) teat seal, or 4) the combination of the two products. The SCC, differential leukocyte count, and cytokine activity of secretions were compared among treatments to determine if quarters infused with a treatment that included teat seal, e.g., teat seal alone or teat seal + antibiotic, elicited an SCC, differential leukocyte, or cytokine response that could be the basis for the elevated cure rate in teat seal-infused quarters that was previously observed. Results demonstrated that SCC were not different among treatments; however, significant changes in differential leukocyte counts and cytokine activity were observed. The percentage of neutrophils increased significantly after all 3 infused treatments and were elevated over controls. Concomitantly, percentages of lymphocytes and macrophages decreased after all 3 infused treatments, and were lower than controls. Eosinophil counts were significantly elevated after infusion with nonlactating cow therapy and teat seal treatments. Concentrations of tumor necrosis factor alpha (TNF- α) and the ratio of TNF- α to interleukin (IL)-8 were elevated in quarters treated with teat seal compared with other treatments. Results suggest that recruitment of neutrophils into quarters infused with teat seal, as well as the high level of TNF- α in mammary secretions may have provided antibacterial activity that resulted in the high cure rate after treatment with this infusion product. As observed in previous trials, infusion with nonlactating cow therapy (100% cure), teat seal (87.5% cure), or the combination of the two products (100% cure) were effective in curing existing IMI, and prevention rates ranged between 95 and 100%.

Introduction

Because of the importance of bred heifers to the future milk production of any dairy operation, it is critical that udder health be maximized to ensure that animals freshen free of IMI and with low SCC. During a heifer's first gestation, the presence of mastitis can compromise the development of milk-producing tissues, and in the case of *Staphylococcus aureus*, milk yield may be reduced up to 10% over the first lactation (Nickerson, 2009; Owens et al., 1991). Milk quality is also reduced due to an increase in SCC for the duration of the lactation (Paradis et al., 2010). In some of the worst cases, mammary tissue is replaced with scar tissue, causing heifers to calve with blind (nonfunctional) quarters.

Greater than 90% of breeding age and bred heifers may have IMI caused by the coagulase-negative staphylococci (CNS) and *Staph. aureus*, and up to 30% is caused by *Staph. aureus* alone (Nickerson, 2009). Such IMI induce a chronic inflammation, which is associated with elevated SCC ($>10 \times 10^6/\text{ml}$) and damage to the developing milk-producing tissues (Trinidad et al., 1990). Thus, an udder health program should be in place to eliminate existing IMI and to prevent new ones in bred heifers so that they freshen free of mastitis with low SCC and the potential for maximum milk yield.

Use of nonlactating (dry cow) antibiotic infusion products in dairy heifers has been successful in curing existing IMI that develop during pregnancy and in preventing new cases that occur late gestation. For example, Owens et al. (2001) evaluated the efficacy of 5 different nonlactating cow antimicrobial products administered 8 to 12 weeks prepartum and found that cure rates for *Staph. aureus* IMI ranged from 67 to 100%, and were higher than the spontaneous cure rate (25%) observed in untreated control quarters. In another study (Owens et al., 1994), the infusion of nonlactating cow therapy into uninfected quarters 8 to 12 weeks prepartum reduced the development of new environmental streptococcal IMI by 93%. Thus, use of nonlactating cow therapy was effective in both curing existing IMI and preventing new cases of mastitis.

Other studies have tested the efficacy of internal teat sealant barriers (bismuth subnitrate) in preventing the development of new IMI by physically impeding bacterial entry to the teat canal and distal teat cistern. Parker et al. (2008) found that the placement of a teat seal approximately 1 month prior to calving in heifers reduced the risk of new IMI by 74% and prevalence of post-calving IMI by 65%.

The question becomes, from a heifer management standpoint, which tool is most beneficial for mastitis control: 1) infusion of nonlactating cow therapy, 2) placement of teat seals, or 3) the combination of the two products? When used together, the combination of the two products may be more effective than either alone in controlling mastitis in these young dairy animals. Recent studies (Nickerson et al., 2016) demonstrated that cure rates after the infusion of nonlactating cow therapy (100% cure), teat seal (85.7% cure), or the combination of the two products (96.1% cure) were significantly ($P < 0.001$) higher than those observed in untreated controls (55.2% cure); prevention rates ranged between 92.2 and 97.9% for all 4 treatments. Thus, although all 3 infusion treatments were effective in curing existing IMI and preventing new ones, the combination of nonlactating cow therapy plus teat seal was not more effective than either alone in controlling mastitis. What was unexpected was the 85.7% cure rate ($P < 0.001$) in quarters infused with teat seal alone.

The purpose of the proposed research was to determine why teat seal was effective in curing existing IMI, the results of which may be instrumental in supporting the additional product label claim that teat seal is a product that not only prevents new IMI, but also cures existing IMI. To accomplish this, mammary secretions were collected at certain times after treatment from quarters of heifers randomly assigned to: 1) untreated control, 2) nonlactating cow therapy, 3) teat seal, or 4) the combination of the two products. The SCC, differential leukocyte count, and cytokine activity were compared among treatments to determine if quarters infused with a treatment that included teat seal, e.g., teat seal alone or teat seal + antibiotic, elicited an elevated SCC, differential leukocyte count, or cytokine response that could be the basis for the elevated cure rate observed in teat seal-infused quarters.

Materials and Methods

Animals:

Thirty pregnant Holstein heifers were enrolled in this trial and housed in a far-off pasture at the UGA Teaching Dairy. Animals were fed a total mixed ration (TMR) once daily based on wheat or sorghum silage and 2.3 kg/head/day of dry cow grain mix. Between 30 and 60 days prior to the expected calving date, mammary secretion samples were collected aseptically from each quarter of each heifer and processed for bacteriological analysis, SCC, differential leukocyte counts, and cytokine activity as described below under *Sample collections*.

At approximately 2 to 3 weeks prepartum, heifers were relocated to a close-up pasture, and the TMR was top-dressed with approximately 0.8 kg/head/day of dietary cation anion diet (DCAD) mix, 2.7 kg/head/day of dry cow grain mix, and 0.11 kg/head/day of limestone. All husbandry procedures were carried out according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999).

Heifers calved in maternity paddocks, and within 24 hours of parturition, all animals began twice daily milking in a double-6 herringbone parlor using a DeLaval system equipped with automatic milking unit takeoffs, milk volume meters, and electronic cow identification. Quarter milk samples were collected on days 3 and 10 postpartum, analyzed bacteriologically, and processed to determine SCC. A third milk sample was collected if culture results on days 3 and 10 did not agree. A composite 3-day milk sample from each heifer was tested for the presence of antibiotic residues before milk was added to the bulk tank (DSM, 2011).

Intramammary treatments:

After the mammary secretion sample collection was performed 30 to 60 days prepartum, 4 treatments were administered as follows: 1) untreated control; 2) nonlactating cow antibiotic (Spectramast[®] DC, ceftiofur hydrochloride, Zoetis, Florham Park, NJ); 3) teat sealant (Orbeseal[®], bismuth subnitrate, Zoetis); and 4) nonlactating cow antibiotic + teat sealant. Spectramast[®] DC sterile suspension is a dry cow intramammary antimicrobial indicated for the treatment of subclinical mastitis in dairy cattle at the time of dry-off associated with *Staph. aureus*, *Streptococcus dysgalactiae*, and *Streptococcus uberis*. Orbeseal[®] is a sterile, non-antibiotic intramammary infusion in the form of a viscous paste indicated for the prevention of new IMI during the dry period. Infusion of Orbeseal[®] into the teat canal acts as an inert physical barrier against mastitis-causing bacteria for the entire dry period, and by mimicking the function of the cow's innate anatomical defense system (formation of a keratin plug). Under microscopic

examination, teat seal appeared as glass-like shards that presumably adhered to the teat cisternal lining to form the physical barrier (Figure 1). These shards appeared similar to the lath-shaped particles, which form a precipitate exhibited by bismuth subnitrate upon hydrolysis with sodium carbonate (Troy, 2006). The use of teat sealant as an adjunct to a nonlactating cow antibiotic has been shown to reduce the risk of acquiring new IMI, reducing the prevalence of IMI, and reducing the risk of experiencing a clinical mastitis event during the dry period (Parker et al., 2008).

Treatments were randomly distributed in such a way that a different pattern of quarters was allotted the 4 treatments for each heifer to account for any dependence among quarters with respect to the incidence of mastitis. After treatment was administered, teats were sprayed using a postmilking teat germicide to eliminate any bacterial contaminants from inadvertently being placed on the teat end via the sampling and treating processes.



Figure 1. Microscopic examination (x1000) of Orbeseal® prior to infusion.

Sample collections:

Mammary secretion samples from each quarter were taken from all heifers 30 to 60 days prepartum immediately prior to treatment. After treatments were administered, quarters were sampled at 24 h (10 heifers), 48 h (10 heifers), and 72 h (10 heifers), and processed for SCC, differential leukocyte counts, and cytokine activity to examine differences among treatments and differences in posttreatment collection times. Samples were also scored at these 3 times for visual characteristics including: viscosity, color, and presence of a precipitate (Figure 2).



Figure 2. Visual characteristics of secretion samples taken after treatment. 1) Control: Thin, watery, clear, yellowish or milky fluid. 2) Nonlactating cow antibiotic: Thin, clear, yellowish or milky fluid with slimy yellowish precipitate; ropey in consistency. 3) Teat seal: Clear, thin, milky or yellowish fluid with white precipitate. 4) Nonlactating cow antibiotic + teat seal: Thin, brownish fluid with a brownish-tan precipitate with white flecks indicating teat seal, with or without a slimy yellowish precipitate.

The white precipitate in samples from quarters treated with teat seal and the combination of nonlactating cow therapy + teat seal was analyzed microscopically. This substance was believed to be the teat seal (bismuth subnitrate) that was inevitably removed from the teat canal into the sample tube during sample collection.

After calving, samples were taken from all heifers ($n = 29$) at 3 days and at 10 days post parturition and processed for bacteriology, SCC, and antibiotic residues (day 3 only). Of the 30 heifers sampled prepartum, one animal was subsequently found to be open (not bred); thus, only 29 heifers calved in this study.

In order to ensure that antibiotic residues were not present in milk from treated heifers post parturition, 3-day postcalving samples were tested using a Delvotest broad spectrum antibiotic residual testing kit (DSM, 2011). All heifers freshened with no antibiotic residues detected in milk samples.

Sample processing for bacteriology, SCC, and differential leukocyte counts:

Mammary secretions collected prepartum and milk samples collected postpartum were mixed by vortexing and plated on trypticase soy agar with 5% sheep blood plates using sterile, flamed 10- μ L loops. Plates were incubated for 48 h at 37°C and visually inspected for presence of microbial colonial growth and hemolysis. Presumptive identification of microbial growth was performed following procedures outlined by the National Mastitis Council (2004). After presumptive identification, bacteria were further identified as follows: Staphylococci were differentiated from streptococci by means of the catalase test, and staphylococci were differentiated as coagulase positive or negative by conducting the coagulase test. Final identification of *Staphylococcus* spp. was performed using the API Staph test (bioMerieux, Inc., Marcy l'Etoile, France).

Identification of *Streptococcus* spp. was verified by means of the Slidex® Strepto Plus (bioMerieux, Inc.). The culture of bacteria in 2 out of 3 postcalving milk samples, when necessary, qualified as an infection. The vast majority of mastitis was caused by *Staph. aureus*, CNS, and *Strep. dysgalactiae*.

The SCC of mammary secretions (collected 30-60 days prepartum) and milk samples (collected 3 and 10 days postpartum) were determined using a Direct Cell Counter (DeLaval, Tumba, Sweden). Secretion samples were diluted 1:1 using a physiological saline solution in order to overcome the high viscosity inherent in mammary secretions of heifers. Differential leukocyte counts of secretions were determined as follows: For the preparation of the differential smear, 50 µL of 7.5% bovine serum albumin (BSA) and 25 µL of secretion sample were added to a cytopsin well. After being secured in a metal holder with a clean microscope slide, the prepared secretion sample was placed in a Cytospin 2 Centrifuge (Shandon, Pittsburgh PA) and operated for 2 min at 1200 rpm. After the slide was removed and air-dried, the smear was stained using the Wright stain method (Wright, 1902). After staining and drying, smears were examined at 1000x under an oil immersion lens, and percentages of lymphocytes, macrophages, neutrophils, and eosinophils were recorded. A total of 100 cells/slide was counted to determine the population distribution.

Cytokine activity

Sample preparation for ELISA assessment of IL-8 and TNF- α : Mammary secretion samples were diluted with an equal volume of phosphate-buffered saline (PBS) containing 1.0% F-127 detergent (Molecular Probes, Eugene, OR). Diluted samples were vortexed vigorously for 10 cycles on maximum power, then centrifuged at 7500 rpm for 2 minutes in a microcentrifuge to remove the teat seal material from the liquid. The liquid portions were transferred to new tubes and stored at -80°C until used in the assays. When possible, 2-mL aliquots of secretion were processed.

To measure IL-8, a bovine IL-8 DIY antibody pair (capture plus detection antibody) and recombinant cytokine standard from Kingfisher Biotech (Minneapolis, MN) were used. Plates were coated with 100 µl of IL-8 coating antibody at 1.5 µg/ml in PBS (pH 7.4) in Flat-Bottom Maxisorb 96-well ELISA plates (Thermo Scientific, Waltham, MA). Plates were sealed and incubated overnight at room temperature (20-25°C), then washed 3 times with PBS and 0.05% tween before use. Bovine IL-8 standards were prepared over a range of 2,000 to 16 pg/mL in reagent diluent (4% BSA in PBS + 0.05% Tween 20 detergent) to generate a standard curve. Standards and mammary secretion samples (diluted 1:2 and 1:4) were added to each ELISA test plate in duplicate. Plates were sealed and incubated for 1 h at room temperature and washed 3 times. IL-8 detection antibody conjugated to biotin was diluted to 0.075 µg/ml in reagent diluent, and 100 µl was added to each well and incubated for 1 h; plates were washed 3 times. Streptavidin-HRP (Pharmingen, Haryana, India), diluted 1:6000 in reagent diluent, was added to each well. Plates were incubated for 30 minutes and washed 3 times. Finally, 100 µl of tetramethylbenzidine (TMB) substrate solution was added to each well and incubated in the dark for 10-30 minutes (based on color development of maximum standard). The reaction was terminated by adding 100 µl of 0.05 M sulfuric acid stop solution to each well. Plates were read using a plate reader (BioTek, Burlington, VT) at 450 nm.

To measure TNF- α activity, a bovine TNF- α DIY antibody pair and recombinant cytokine standard from Kingfisher Biotech were used. Plates were coated with 100 µl of TNF- α coating antibody at 2.0 µg/ml in PBS (pH 7.4) in Flat-Bottom Maxisorb 96-well ELISA plates (Thermo Scientific).

Plates were sealed, incubated overnight at room temperature, then washed 3 times with wash buffer (PBS and 0.05% tween) before use. Bovine TNF- α standards were prepared over a range of 5,000 to 78 pg/mL in reagent diluent (4% BSA in PBS + 0.05% Tween 20 detergent) to generate a standard curve. Standards and mammary secretion samples (diluted 1:2 and 1:4) were added to each ELISA test plate in duplicate. Plates were sealed and incubated for 1 h at room temperature, then washed 3 times. TNF- α detection antibody conjugated to biotin was diluted to 0.5 μ g/ml in reagent diluent, and 100 μ l was added to each well and incubated for 1 h. Plates were washed 3 times. Streptavidin-HRP (Pharmingen) diluted 1:6000 in reagent diluent was added to each well; plates were incubated for 30 minutes then washed 3 times. Finally, 100 μ l of tetramethylbenzidine (TMB) substrate solution was added to each well and incubated in the dark for 10-30 minutes (based on color development of maximum standard). The reaction was terminated by adding 100 μ l of 0.05 M sulfuric acid stop solution to each well. The plates were read using a plate reader (BioTek) at 450 nm.

Bovine IL-17A VetSet ELISA Development Kit (Kingfisher Biotech) was used for this assay. Bovine IL-17A standards were prepared over a concentration range of 1200 to 18 pg/mL in reagent diluent (4% BSA in PBS) to generate a standard curve. On each plate, duplicate wells of standards and samples (diluted 1:2 and 1:4) were applied to the bovine IL-17A capture antibody coated and blocked plates. Plates were sealed and incubated for 1 h at room temperature, then washed 3 times. This was followed by addition of 100 μ l of IL-17A detection antibody, diluted 1:23 in reagent diluent, followed by incubation for 2 h at room temperature and washing 3 times. Streptavidin-HRP, diluted to 1:200 in reagent diluent, was added to each well, and plates were incubated for 30 minutes at room temperature followed by washing 4 times. The TMB substrate was added to each well of all plates followed by incubation in the dark for 15 minutes. The reaction was stopped by adding 100 μ l of 0.5 M sulfuric acid. The plates were read using a plate reader (BioTek) at 450 nm.

Statistical analyses:

After calving, infection data collected on days 3 and 10 were compared with infection data collected prepartum, and results used to determine 1) the percentage cure of existing IMI at time of treatment and 2) the percentage of new IMI prevented across all 4 treatments. The SCC means among treatments were calculated for secretions collected at time of treatment, on the 3 times (24 h, 48 h, and 72 h) posttreatment, and for days 3 and 10 postpartum. Mean percentages of differential leukocyte populations (lymphocytes, macrophages, neutrophils, and eosinophils) among treatments and sample times were also determined. Means, expressed on a per treatment basis, were separated using SAS 9.3 Proc Glim for Windows (SAS, 2013). Analysis of SCC and differential counts collected 24, 48, and 72 h after treatment showed no differences among times, so data were combined as one post-treatment sample (24 to 72 h). A random subset of secretions ($n = 28$) from all 4 treatments was analyzed for cytokine activity (control $n = 12$, nonlactating cow therapy $n = 7$, teat seal $n = 4$, nonlactating cow therapy + teat seal $n = 5$). The differences in SCC and differential counts before and after treatment were determined using SAS as above.

Results

SCC and differential leukocyte counts

For secretion samples collected prior to treatment and the combined posttreatment sample (24 to 72 h), an examination of SCC revealed no differences among treatments (Figure 3). Across treatments, SCC ranged from 2,494,000 to 3,361,000/ml for pretreatment samples (SCC Before) and from 2,565,000 to 3,999,000/ml for posttreatment samples (SCC After). However significant changes in differential leukocyte counts and cytokine activity were observed. Results showed that, although neutrophil percentages were similar across treatments prior to infusion, treatment with nonlactating cow therapy, teat seal, and the combination of the two products resulted in significant increases in the percentages of neutrophils relative controls ($P < 0.001$) (Figure 4). In addition, percentages of neutrophils were significantly elevated after treatment compared with before treatment.

Lymphocyte percentages were similar across treatments prior to infusion, but treatment with nonlactating cow therapy, teat seal, and the combination of the two products resulted in significant decreases in the percentages of lymphocytes relative controls ($P < 0.001$), and percentages were lower after treatment with teat seal and teat seal + nonlactating cow therapy compared with values before treatment ($P < 0.001$); percentages were numerically lower in quarters infused with nonlactating cow therapy (Figure 5).

Likewise, macrophage percentages were similar across treatments prior to infusion (Figure 6). However, treatment with nonlactating cow therapy and the combination of the two products resulted in significant decreases in the percentages of macrophages relative controls; percentages were numerically lower in quarters infused with teat seal. In quarters treated with nonlactating cow therapy and the combination of the two products, there was a significant decrease in percentages of macrophages after treatment.

Eosinophil percentages were similar across treatments prior to infusion, but treatment with nonlactating cow therapy resulted in significant increases in the percentages of eosinophils relative to controls ($P < 0.001$); percentages were numerically higher in quarters infused with teat seal and nonlactating cow therapy + teat seal (Figure 7). Eosinophil percentages were higher after treatment with nonlactating cow therapy and teat seal compared to before treatment ($P < 0.001$); percentages were numerically higher in quarters infused with nonlactating cow therapy + teat seal.

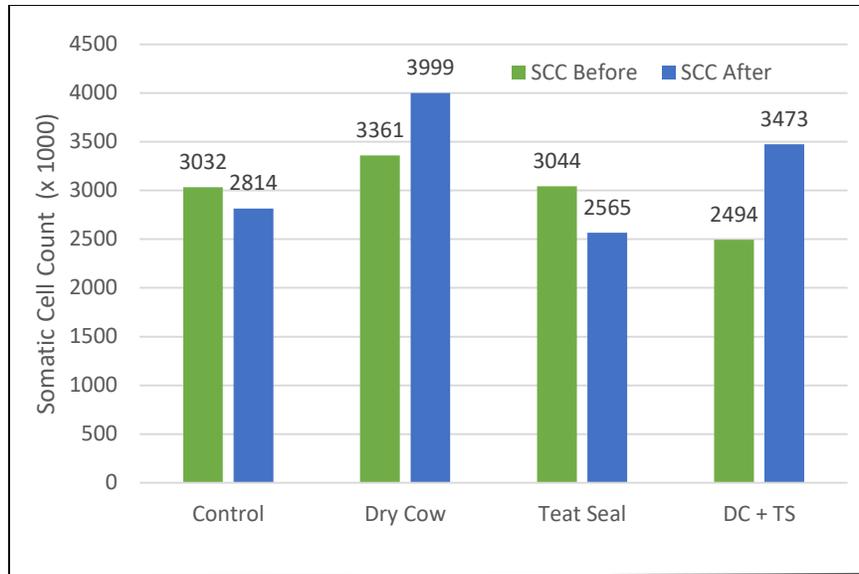


Figure 3. Total SCC values ($\times 10^3$) at the initial sampling (SCC Before) and after treatment (SCC After) by treatment. Dry Cow = Nonlactating cow therapy; DC+TS = Nonlactating cow therapy + Teat Seal.

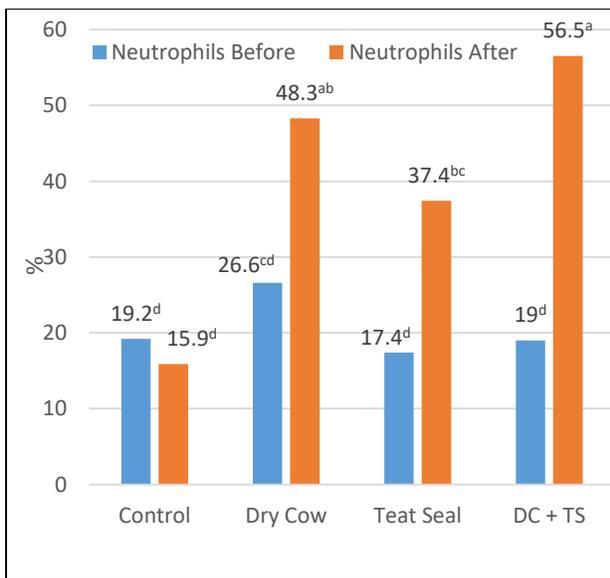


Figure 4. Neutrophil counts (%) before and after treatment by treatment. Dry Cow = Nonlactating cow therapy; DC+TS = Nonlactating cow therapy + Teat Seal. a, b, c, d. Values with different superscripts are different ($P < 0.001$).

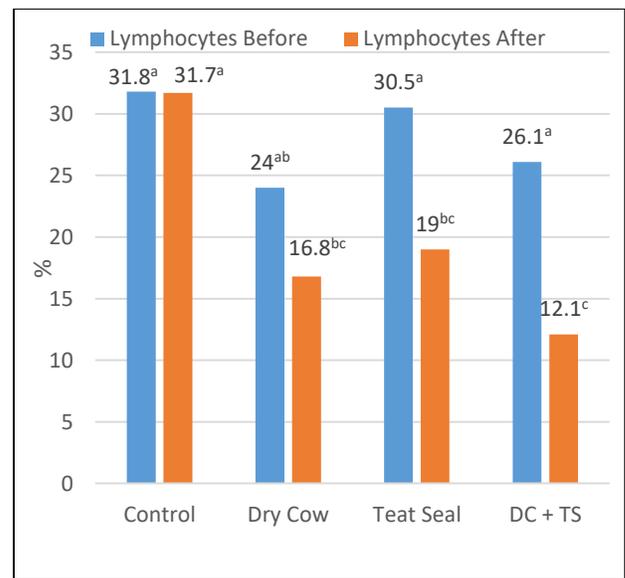


Figure 5. Lymphocyte counts (%) before and after treatment by treatment. Dry Cow = Nonlactating cow therapy; DC+TS = Nonlactating cow therapy + Teat Seal. a, b, c, d. Values with different superscripts are different ($P < 0.001$).

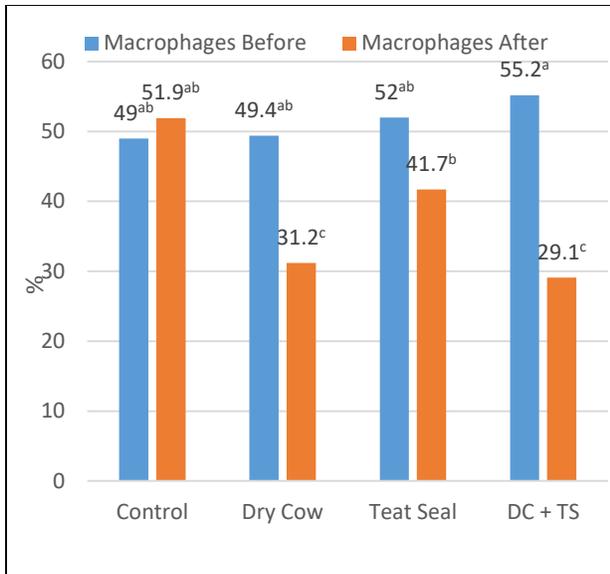


Figure 6. Macrophage counts (%) before and after treatment by treatment. Dry Cow = Nonlactating cow therapy; DC+TS = Nonlactating cow therapy + Teat Seal. a, b, c, d. Values with different superscripts are different ($P < 0.001$).

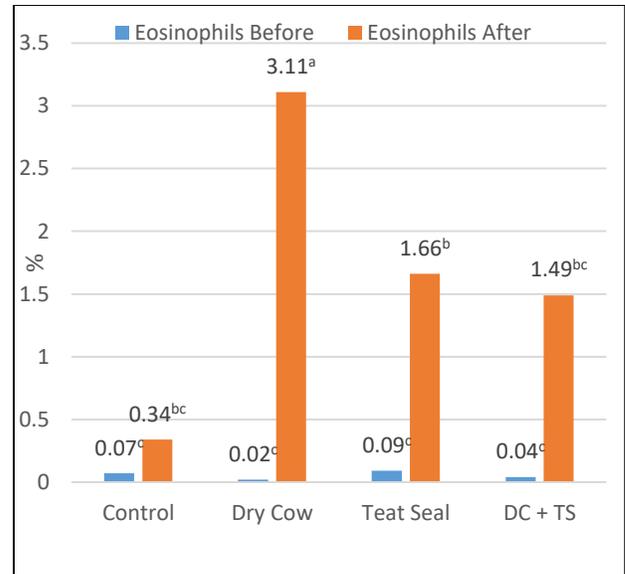


Figure 7. Eosinophil counts (%) before and after treatment by treatment. Dry Cow = Nonlactating cow therapy; DC+TS = Nonlactating cow therapy + Teat Seal. a, b, c, d. Values with different superscripts are different ($P < 0.001$).

Cytokine activity

Analysis of cell signaling proteins (cytokines) found in mammary quarters postinfusion with teat seal indicated a nonsignificant, yet notable increase in TNF- α activity compared with the other treatments (Figure 8). Likewise, when analyzing the ratio of TNF- α :IL-8, it was found that there was a marked increase in activity in the teat seal treatment group ($P < 0.001$) compared to both nonlactating cow therapy, and the combination of nonlactating cow therapy + teat sealant, and numerically higher than control (Figure 9).

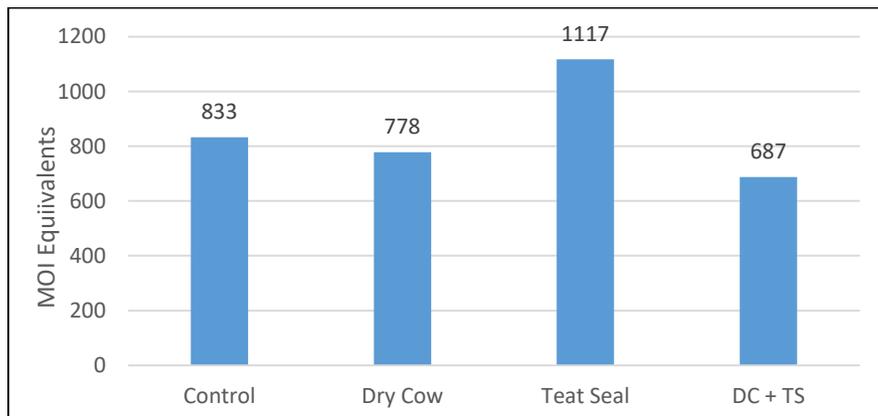


Figure 8. TNF- α activity in mammary secretions by treatment ($P > 0.05$) Dry Cow = Nonlactating cow therapy; DC+TS = Nonlactating cow therapy + Teat Seal.

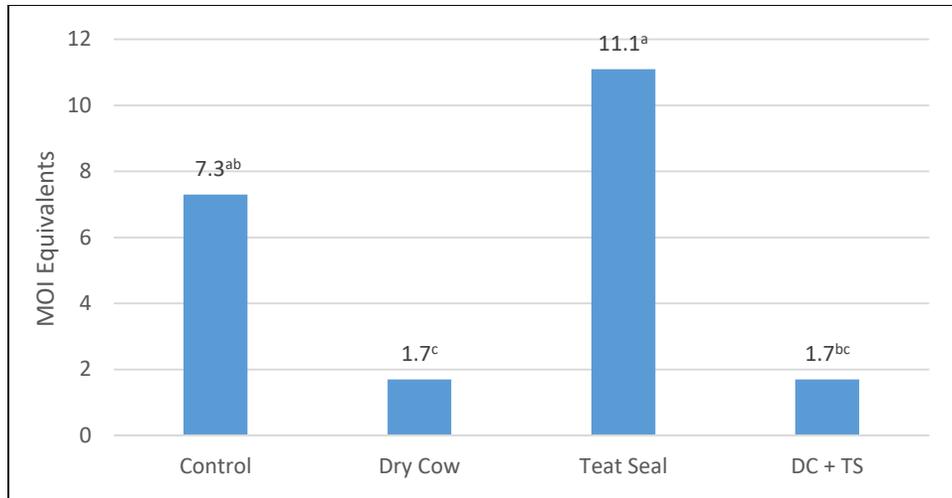


Figure 9. TNF- α :IL-8 ratio in mammary secretions by treatment. Dry Cow = Nonlactating cow therapy; DC+TS = Nonlactating cow therapy + Teat Seal.

a, b, c, d. Values with different superscripts are different ($P < 0.001$).

Postpartum cure rates and SCC

The infusion of nonlactating cow therapy (100% cure), teat seal (87.5% cure), or the combination of the two products (100% cure) resulted in mastitis cure rates above that observed in untreated control quarters (62.5% cure) (Table 1); prevention rates ranged between 95 and 100%.

Table 1. Cure rate of existing IMI and prevention rate against new IMI across treatments.

Variable	Control	Dry Cow (DC)	Teat Seal (TS)	DC+TS	SE	<i>P</i> value
Cure rate (%)	62.6 ^b	100 ^a	87.5 ^{ab}	100 ^a	10.53	0.046
Prevention rate (%)	100	100	95.5	95	3.38	0.614

a, b Values in row with different superscripts are different ($P < 0.001$).

The SCC on days 3 and 10 postpartum based on quarter infection status at time of treatment and status at calving are shown in Table 2. For the quarter status uninfected at time of treatment that acquired a new IMI at calving, there were only 2 quarters that fit the criteria: one in teat seal treated and one in nonlactating + teat seal treated quarters. Both quarters exhibited SCC $> 3 \times 10^6$ on day 3 but decreased markedly by day 10.

In quarters uninfected at the time of treatment that remained uninfected at calving, SCC were numerically higher in all 3 treatment-infused quarters relative to controls at 3 d, but by day 10 postcalving, SCC had decreased for all 4 treatments.

For the quarter status infected at time of treatment that remained infected at calving, there were only 4 that fit the criteria: 3 in controls and one in teat seal treated quarters. Controls exhibited SCC $> 2 \times 10^3$ on day 3 and day 10, but SCC in teat seal treated quarters had lowered to 339×10^3 by day 10.

Quarters with IMI at the time of treatment that were uninfected at calving, exhibited elevated SCC on day 3 (with the exception of nonlactating cow treatment); however, by day 10, SCC were numerically lower in all 3 infused treatments compared with controls.

Table 2. SCC x 10³/ml on days 3 and 10 postpartum based on quarter infection status at time of treatment and status at calving.

Treatment	Status at treatment	Status at calving	n	SCC 3 day	SCC 10 day
Control	Uninfected	New IMI	0	- ¹	-
Dry cow	Uninfected	New IMI	0	-	-
Teat seal	Uninfected	New IMI	1	3,704	194
DC + TS	Uninfected	New IMI	1	3,845	405
			SE	--- ²	---
			<i>P</i>	---	---
Control	Uninfected	Uninfected	20	321	204
Dry cow	Uninfected	Uninfected	18	552	333
Teat seal	Uninfected	Uninfected	21	637	247
DC + TS	Uninfected	Uninfected	19	458	214
			SE	157	51
			<i>P</i>	0.546	0.339
Control	Infected	Infected	3	2,735	2,053
Dry cow	Infected	Infected	0	-	-
Teat seal	Infected	Infected	1	2,069	339
DC + TS	Infected	Infected	0	-	-
			SE	---	---
			<i>P</i>	---	---
Control	Infected	Uninfected	5	528	326
Dry cow	Infected	Uninfected	10	227	124
Teat seal	Infected	Uninfected	7	1,481	253
DC + TS	Infected	Uninfected	9	525	181
			SE	427	72
			<i>P</i>	0.174	0.255

¹No data for that status category.

²Insufficient n for valid analysis.

Dry Cow = Nonlactating cow therapy; DC+TS = Nonlactating cow therapy + Teat Seal.

Discussion

The infusion of nonlactating cow therapy (100% cure), teat seal (87.5% cure), and the combination of the two products (100% cure) 30 to 60 days prepartum resulted in effective cure rates relative to untreated controls (62.5% cure); prevention rates among the 4 treatments ranged between 95% and 100%. As observed in previous trials (Booth et al., 2016; Harding et al., 2015; Nickerson et al., 2016), infusion with teat seal was not only effective in preventing new cases of IMI, but also curing existing IMI. Thus, for the present study, the SCC, leukocyte differential count, and

cytokine activity in mammary secretions were monitored in attempts to explain this unexpected cure rate attributed to teat seal.

Somatic cell counts taken before and after infusion showed no significant differences across treatments; however, examination of differential leukocyte concentrations demonstrated significant changes in leukocyte populations in response to treatment. Neutrophil concentrations were elevated in all quarters after infusion with any of the 3 products relative to control quarters, whereas lymphocyte and macrophage concentrations were decreased in all quarters infused with any of the 3 products relative to controls. Interestingly, eosinophil concentrations were elevated in quarters infused with nonlactating cow therapy and with teat sealant. Thus, after treatment, there was a shift toward increasing neutrophil and eosinophil concentrations as lymphocytes and macrophages decreased. Neutrophils are the major leukocyte type involved in engulfing and killing mastitis-causing bacteria (Paape et al., 2000), and the increases in neutrophil concentrations observed in teat seal infused quarters may have been responsible, in part, for the elevated cure rate observed.

The inflammatory response of the cow's immune system in response to presence of bacteria is well understood. In normally functioning cellular immune systems, the presence of bacteria signals leukocytes (macrophages) already present in mammary tissue to engulf and kill the pathogens, and to release cell signaling proteins (cytokines) to recruit more leukocytes (neutrophils) into the infected quarter (Nickerson and Sordillo, 2016). In this study, it was found that differential leukocyte counts changed significantly in response to all treatments infused into the mammary gland, mainly an increase in neutrophils and eosinophils at the expense of macrophages and lymphocytes.

Cytokines are a group of glycoproteins secreted by immune and nonimmune cells that regulate all aspects of inflammation and immunity including the rapidity of leukocyte migratory response to the site of infection. Mammary epithelial cells and macrophages present in the gland are among the first to become activated by factors associated with bacterial colonization, and will quickly begin to produce a variety of cytokines including TNF- α , IL-1, IL-6, and IL-8, which will in turn regulate the magnitude and duration of leukocyte (neutrophil) infiltration into the infected tissue.

TNF- α activity as well as the ratio of TNF- α to IL-8 were found to be elevated in quarters infused with teat seal, indicating that TNF- α may play a role in the immune response in teat seal infused quarters. TNF- α is a potent chemoattractant for neutrophils, and helps to enhance the expression of adhesion molecules on endothelial cells, allowing neutrophils to migrate to specific tissues. High levels TNF- α in quarters infused with teat seal in conjunction with an elevated concentration of neutrophils in the present study indicates that this cytokine may have played a role in neutrophil recruitment. This neutrophil recruitment may have provided the mechanistic antibacterial activity that resulted in the high cure rate (87.5%) observed with this infusion product, e.g., effectively eliminating (curing) existing mastitis pathogens in infected quarters.

Eosinophils, commonly found in tissues experiencing an allergic response to medications or food (Rothenberg and Hogan, 2006), were found to be elevated in tissues infused with nonlactating cow therapy and with teat seal in the present study. Cephalosporins (the class of drug incorporated in the nonlactating cow therapy used in this study) have been found to be associated with drug-induced eosinophilia in human patients (Maidment and Williams, 2000). Literature on drug-induced eosinophilia is sparse and mainly consists of individual case reports; however, incidence

of eosinophilic reactions in patients taking cephalosporins is quoted as approximately 8%. The question becomes could this cephalosporin hypersensitivity reaction found in humans be similar to what was occurring in cow mammary quarters infused with nonlactating cow therapy?

In quarters infused with the combination of nonlactating cow therapy + teat seal, samples consisted of a brown viscous fluid with precipitated solids indicative of teat seal. This reaction was also consistent among quarters infused with combination therapy and was not found to occur in any other treatment group. Under microscopic examination, erythrocytes were not found to be a major component of secretions. The cause of this reaction is unknown; however, it is believed that infusion of both nonlactating cow therapy + teat seal may have altered the osmolarity of the secretion, thereby causing erythrocytes to hemolyze, releasing hemoglobin evidenced by a medium brown pigment. At the time of calving, quarters samples from all treated quarters were normal in appearance.

Although teat seal is an inert product, the internal epithelial tissues of the teat cistern and teat canal may react to teat seal as a foreign substance and as a mild irritant. Under microscopic examination of teat seal, the “shards of glass” appearance supports the notion that this product may irritate the cisternal lining, release subsequent signaling factors, and recruit neutrophils into the area. In mammary quarters treated with teat sealant, it was found that neutrophils were recruited into the quarter similar to that found in both nonlactating cow therapy group and combination treatment.

Similarly, a histological response of the bovine mammary gland to the presence of three intramammary device (IMD) models (abraded, star, or grooved) was studied in 12 lactating cows (Nickerson et al., 1997). Uninfected quarters fitted with devices that served as mild irritants exhibited greater leukocyte infiltration into teat and gland cistern linings as well as into mammary parenchyma adjacent to the gland cistern compared with unfitted control quarters. Infiltrating neutrophils were observed in the teat cistern epithelium in various stages of migration across the epithelial layer. In addition to neutrophils, others have observed eosinophilic infiltration into the subepithelial tissues of the gland cistern in quarters fitted with abraded IMD (Collins et al., 1989).

In another study, neutrophil infiltration into the cisternal linings of IMD quarters was identical to that observed in intracisternal bead (ICB)-fitted quarters (Nickerson et al., 1987). The ICB devices provided a mild irritant and were successful in stimulating a localized leukocytosis into teat cisternal tissues. The irritation provided by the teat seal may have acted similarly to the IMD and ICB, which may serve to amplify the leukocyte response to mastitis-causing organisms once they penetrate the teat duct.

Conclusions

The elevated neutrophil concentrations and TNF- α activity in infected mammary quarters infused with teat seal may be responsible for the high cure rates observed after treatment with this product. In addition, the physical properties of teat seal (crystalline morphology/lath-shaped particles) may have led to an irritation of the teat cisternal lining, which initiated a neutrophil influx into this area of the gland, leading to bacterial elimination and enhancing the cure rate. Results support the additional claim that teat seal is a product that not only prevents new IMI, but also cures existing IMI.

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