Efficacy and clinical safety of pegbovigrastim for preventing naturally occurring clinical mastitis in periparturient primiparous and multiparous cows on US commercial dairies

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ABSTRACT

Periparturient dairy cows experience impaired immune function, exhibited as a transient decrease in neutrophil function. This decrease in immune competence is associated with an increase in susceptibility to bacterial infections, including mastitis and metritis. Bovine granulocyte colony stimulating factor (bG-CSF) is an endogenous protein that enhances neutrophil bactericidal functions and increases the production of neutrophils from bone marrow precursors. Administration of pegbovigrastim (recombinant bG-CSF covalently bound to polyethylene glycol) around the time of calving has been shown to reduce the incidence of new clinical mastitis cases in a natural disease model system. To further explore the application of pegbovigrastim under herd management systems typical of those found in the US dairy industry, we conducted a multicenter field study to evaluate the efficacy and clinical safety of pegbovigrastim administered to multiparous cows and heifers approximately 7 d before calving and within 24 h of calving. Responses of treated cows were compared with those of animals treated with sterile saline. Animals treated with pegbovigrastim exhibited 4- to 5-fold increases in circulating neutrophil numbers within 24 h of treatment initiation, and this increase persisted at least a week beyond the second dose. Pegbovigrastim-treated animals exhibited a 35% decrease in the incidence of clinical mastitis relative to the controls during the first 30 d of lactation. Animals treated with pegbovigrastim also exhibited a 52% reduction in failure to return to visual estrus within 80 d of calving. We observed no differences in somatic cell count or milk composition between treated and control animals. We also found no differences in the duration of pregnancy or proportion of viable calves in treated

cows relative to control animals. These results indicate that administration of pegbovigrastim provides a well-tolerated, novel approach to overcoming periparturient immune suppression, resulting in reduced susceptibility to clinical mastitis during early lactation.

Key words: periparturient immunosuppression, bovine granulocyte colony stimulating factor, clinical mastitis

INTRODUCTION

Despite decades of research and the availability of numerous methods for reducing the incidence of intramammary infections, mastitis remains a major source of economic losses for dairy producers around the world (Ruegg, 2012; Rollin et al., 2015).

Animal genetics and husbandry practices significantly affect mammary gland immunity and susceptibility to intramammary infection (Sordillo, 2005). Protection of the mammary gland from microbial invasion requires a complex and coordinated interaction between the innate and acquired immune responses. Cows experience periods of increased susceptibility to mastitis during the periparturient period, beginning 1 to 2 wk before calving and continuing for several weeks after calving (Oliver and Sordillo, 1988). Neutrophils play a pivotal role in host defense during an IMI and are recruited in large numbers by cytokines secreted by resident macrophages early in the infection of the gland (Paape et al., 2003). Impairment of immune responses during the periparturient period has been well documented (Sordillo et al., 1997). Kehrli et al. (1989) demonstrated that neutrophil functions are impaired in cows during the periparturient period: bovine neutrophils from periparturient cows exhibited decreased oxidative burst and myeloperoxidase-hydrogen peroxide-halide (MPO-H₂O₂-halide) activity, both of which play important roles in neutrophil-mediated antimicrobial activity.

Granulocyte colony stimulating factor (**G-CSF**) is an endogenous hematopoietic growth factor that stimu-

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lates the production and differentiation of neutrophils by progenitor cells in the bone marrow (Nomura et al., 1986; Nagata, 1989). Recombinant bovine G-CSF (**bG-CSF**) induced a pronounced neutrophilia and increased phagocytic and cytotoxic activity by neutrophils (Kehrli et al., 1991; Heidari et al., 2001). Kehrli (1998) evaluated the efficacy of daily injections of human G-CSF (**hG-CSF**) alone or in combination with bovine granulocyte-macrophage colony stimulating factor (**bGM-CSF**) in an *Escherichia coli* infection model in periparturient cows. Cows treated with hG-CSF alone beginning on d 3 of lactation and challenged with *E. coli* by intramammary infusion on d 6 exhibited a 50% reduction in the number of new infections, faster bacterial clearance rates, and reduced clinical severity scores. Animals treated with hG-CSF also exhibited significantly improved milk production and feed consumption relative to the saline-treated controls. In contrast, co-administration of bGM-CSF and hG-CSF resulted in a febrile response and appeared to antagonize the protective effects of hG-CSF monotherapy on coliform mastitis.

The pharmacodynamic response to native sequence bG-CSF as determined by changes in absolute neutrophil counts indicates that daily dosing of the protein is required for therapeutic benefit. Modifying native proteins by covalent binding of water-soluble polymers such as polyethylene glycol extends the duration of activity by increasing the hemodynamic volume of the protein and reducing renal clearance and proteolytic degradation (Molineux, 2003). A single dose of PEGylated hG-CSF (pegfilgrastim) has been shown to provide equivalent efficacy to 11 daily doses of non-PEGylated hG-CSF (Molineux, 2004).

Administration of PEGylated bovine G-CSF (pegbovigrastim) to periparturient dairy cows approximately 7 d before their anticipated calving date and within 24 h of calving enhances the MPO-H₂O₂-halide activity of neutrophils obtained from periparturient cows (Kimura et al., 2014). We have assessed the efficacy of various dose levels of pegbovigrastim administered to periparturient cows and heifers approximately 7 d before calving and on the day of calving against naturally occurring clinical mastitis (Hassfurth et al., 2015). Administration of pegbovigrastim at a dose of 20 µg/kg reduced the incidence of clinical mastitis by up to 72% relative to saline-treated controls.

The objective of the current study was to evaluate the efficacy of pegbovigrastim for preventing clinical mastitis in periparturient multiparous and primiparous cows under husbandry conditions typically found in US commercial dairy herds. The clinical safety of pegbovigrastim was also evaluated in healthy dams and calves exposed to pegbovigrastim in utero.

MATERIALS AND METHODS

Experimental Design

This multicenter study was conducted from January to August 2011 at 4 commercial dairies in Wisconsin, Colorado, California, and Washington. These sites were selected to represent the diversity of management systems in the US commercial dairy herd.

The study was designed as a randomized complete block design, with 2 treatment groups and 4 blocks. Each study site enrolled 160 periparturient cows and heifers. Approximately 30% of the animals enrolled in each treatment group were heifers, and the remaining animals were multiparous cows. To ensure that equal proportions of heifers and multiparous cows were included in each treatment group at each site, we generated separate randomization tables for heifers and multiparous cows in SAS (SAS Institute Inc., Cary, NC) using PROC PLAN. Animals were randomly assigned to treatment groups based on the sequence in the randomization tables on d -7 relative to their individual anticipated calving dates. Anticipated calving dates were determined by farm breeding records and adjusted based on twice-weekly visual evaluations by the herdsman to identify animals that were exhibiting clinical signs associated with impending parturition. Each treatment group contained 80 animals at each facility, yielding 320 animals per treatment across study sites.

Animals

The study protocol was reviewed and approved by the Elanco Animal Health Institutional Animal Care and Use Committee before initiation of the study. This study included both large- and small-frame breeds. The Wisconsin facility was a purebred Jersey dairy. The other sites enrolled Holstein-Friesian and Holstein crossbred cows and heifers. Animals were sourced from the commercial herds at each facility.

Animal Husbandry

The dairies in Wisconsin, Washington, and California were conventional dairies that used dry cow antibiotics, teat sealants, antiseptic teat dips, and *E. coli* J5 vaccines to control intramammary infections. These facilities also used intramammary antibiotics to treat clinical cases of mastitis. The Colorado facility did not use antimicrobial agents in its mastitis control program.

Animals were managed before and after calving in accordance with the standard operating procedures at each dairy facility. Before and after calving, animals

were housed in covered (Wisconsin and Colorado) or open (Washington and California) pens. Pens at the facilities in Wisconsin and Colorado contained straw or cornstalk bedding. Pens at the facilities in Washington and California contained manure or dirt bedding. Animals were given adequate space in accordance with the *Guidelines for Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2010). Calves obtained from animals enrolled in the study were housed in individual hutches (California, Washington, and Colorado) or 3' × 5' individual pens (Wisconsin) with wood shavings or straw bedding. Both bull and heifer calves were retained on the farms through the first 30 d of life to enable health observations.

Animals at all sites were exposed to ambient conditions throughout the study. Cows were milked twice daily in herringbone parlors using manually applied milking units equipped with automatic detachers.

Cows at each site were fed corn/corn silage-based TMR that met or exceeded the NRC nutrient requirements (National Research Council, 2001), in accordance with each facility's standard operating procedures. Animals at the Colorado site were also given access to pasture for grazing from May to the end of the study.

Investigational and Control Products

Sterile saline (0.9% NaCl) was used as a negative control product in this study. The control product was presented in 3-mL prefilled plastic syringes containing 2.7 mL of the control product and was stored under ambient conditions.

Bovine G-CSF was cloned and expressed by recombinant technology in *E. coli*. Following isolation of the expressed protein from inclusion bodies and refolding, the protein was chromatographically purified and covalently bound to 20-kDa activated polyethylene glycol, yielding mono-PEGylated bG-CSF according to the methods of Cho et al. (2011). The protein was formulated in 30 mM citrate buffer containing 250 mM arginine at pH 6.0. The investigational product was presented in 3 mL prefilled plastic syringes containing 2.7 mL of the investigational product and was refrigerated at 2 to 8°C.

Animal Dosing

In a previous study (Hassfurth et al., 2015), animals were dosed with pegbovigrastim in volumes calculated to deliver a specific level (μg/kg) based on each animal's body weight. Because many farms do not have the ability to measure individual animal weights, we altered the dosing in this study to use a consistent volume of saline or pegbovigrastim in prefilled syringes.

Healthy cows and heifers randomized to each treatment group were dosed by subcutaneous injection with the contents of 1 syringe 7 d before their anticipated calving date. Animals were dosed again by subcutaneous injection with the contents of a second syringe within 24 h after calving. Pegbovigrastim syringes contained 15 mg of protein. The dosing regimen used in this study was identical to that specified on the product label. Body weights for individual animals were determined on d -7 relative to anticipated calving to allow us to calculate doses delivered for individual animals. Based on the average body weights of animals enrolled in the study, the average weight-based dose of pegbovigrastim was 22.6 μg/kg for Holsteins and 32.8 μg/kg for Jerseys.

Clinical Observations

Absolute Neutrophil Counts. We determined the effect of pegbovigrastim on absolute neutrophil counts for all cows and heifers on d -7 (before dosing) and d -6 before calving, and on d 7 after calving. Blood samples were collected into Vacutainer tubes containing EDTA by venipuncture of the jugular or coccygeal veins, depending on facility standard practices. Blood samples from all sites were shipped via overnight service to a single clinical pathology laboratory (Physician's Reference Laboratory, Overland Park, KS) for assessment of total and differential leukocyte counts using an Advia 120 hematology analyzer.

Clinical Mastitis Scores. Foremilk samples from each quarter were evaluated before each milking. The clinical status of each quarter was recorded by individuals who were masked to treatment assignments at the morning and evening milking on 3 to 30 DIM to monitor for the development of clinical signs of mastitis. Evaluations were not initiated until d 3 to allow colostrum to clear from the gland and milk to have a normal appearance in healthy quarters. Clinical mastitis evaluations were documented by the assignment of a clinical score using a scale of 1 to 5 as summarized in Table 1.

If a quarter had suspect or abnormal milk, a California Mastitis Test (CMT) was performed on a foremilk sample from the affected quarter. If a quarter appeared abnormal or if the foremilk sample appeared abnormal and exhibited a CMT result ≥ 2 , the cow was assigned a clinical score of 3 or 4 based on the severity of the observed abnormality. The animal's rectal temperature was determined; animals with a rectal temperature $\geq 39^\circ\text{C}$ were considered to be exhibiting signs of systemic inflammation and were assigned a clinical score of 5. Duplicate milk samples were collected for microbiological evaluation according to National Mastitis Council recommended procedures (National Mastitis Council, 2004) before milking.

Following microbiological sample collection and documentation of clinical mastitis status, the affected animal was removed from the study and data collection for that animal was terminated. Immediately following removal from the study, cows were treated according to pre-existing farm management practices and returned to the dairy management system for individual care.

Microbiology. Individual milk samples obtained from affected quarters (clinical score ≥ 3) were stored frozen at -20°C until the conclusion of the study. Frozen samples were forwarded to a single microbiology laboratory for isolation and identification of pathogens by genus and species using aerobic culture on MacConkey and blood agar plates and biochemical tests.

Reproductive Health Observations

We assessed the effect of pegbovigrastim on reproductive health in healthy cows by comparing the incidence of metritis, observed estrus, and first-service conception rates between animals in each treatment group according to criteria used at each facility. Estrus synchronization was performed using prostaglandins, GnRH, or both, at the Washington and Wisconsin dairies, beginning at approximately 45 to 60 DIM. The Colorado and California dairies did not use hormones in their reproductive management program.

Assessments of metritis, visual estrus, and first-service conception rates were performed by the herdsman at each study site according to the site's routine reproductive management protocols. Cows were checked for uterine discharge as part of daily general health observations during the first 2 to 3 wk after calving. Cows were observed for signs of estrus beginning approximately 4 wk after calving through 80 DIM. Cows that did not exhibit visual estrus within 80 DIM were removed from the study, and no additional data were recorded for these animals. First-service conception was

determined at least 35 d after insemination between 85 and 100 DIM, by either rectal palpation or ultrasound.

Clinical Safety Assessment

Daily Health Observations. General health observations were recorded twice per day for 7 d after each treatment administration and once per day on d 8 through 30 of lactation. To avoid the potential alteration of animal's susceptibility to intramammary infections and confounding the mastitis efficacy assessment, animals that exhibited other health problems requiring administration of antibiotics or anti-inflammatory drugs (e.g., corticosteroids) were removed from the study and treated according to the facility protocols for the commercial herd at each location. No additional data were collected from animals after they were removed from the study.

Milk Quality and Composition. To determine the effect of pegbovigrastim on milk composition, composite milk samples were collected from all animals at 1 milking on d 7, 14, 21, and 28 of lactation. Milk samples were collected from milk meters to obtain samples representative of the entire milking. Milk samples were analyzed at local milk quality laboratories for milk solids, fat, protein, lactose, and SCC.

Milk Production. We determined the effect of pegbovigrastim on daily milk production by recording milk production for each animal at each milking on d 1 to 30.

Gestation Length and Calf Viability. To assess the effect of precalving exposure to pegbovigrastim on the duration of gestation, we compared the mean number of days between the d -7 dose and calving for each treatment group. To assess the influence of prepartum exposure to pegbovigrastim on in utero survival, we recorded the viability of calves at birth to allow calculation of the percentage of live births.

Table 1. Clinical mastitis scoring system. Cows exhibiting either an abnormal quarter or abnormal milk in combination with a California Mastitis Test (CMT) ≥ 2 were diagnosed

Appearance of quarter	Appearance of milk	CMT results	Clinical score
Normal	Normal	No test required	1
Normal	Suspect (few transient flakes or clots)	Negative, trace, or 1	2
Normal	Abnormal (flakes, clots, or discoloration)	≥ 2	3
Abnormal (slight inflammation/swelling, warm to touch, or both)	Normal		
Abnormal (moderate inflammation/swelling, hot to touch, or both)	Abnormal (obvious flake, clots, or discoloration)	≥ 2	4
Abnormal (severe inflammation/swelling, hot to touch, or both)	Abnormal or agalactic (numerous flakes, clots, or severe discoloration)	≥ 2	5 (fever)

Calf Health Observations. To evaluate the effect of in utero exposure to pegbovigrastim on calf health, daily health observations were recorded for all calves for the first 30 d of life according to the programs used to assess calf health at each facility.

Statistical Analyses

Statistical analyses were conducted on the combined data set from all 4 study locations using SAS software (version 9.2; SAS Institute Inc.). The experimental unit was the individual animal. Statistical analyses were performed on the pooled data from the study sites.

Individual study sites were used as a blocking factor for analyses. We did not include parity in the statistical models, because we did not have sufficient numbers of animals to perform meaningful comparisons between treatments within each parity group. We used 2-sided tests to compare pegbovigrastim group mean to the control mean within an analysis. The level of significance was set to at $\alpha = 0.05$ for clinical observations and $\alpha = 0.1$ for clinical safety variables. The less stringent *P*-value was used for the clinical safety observations to ensure an abundance of caution in identifying potential safety concerns associated with the use of the product.

Clinical Observations

Absolute Neutrophil Counts. A generalized mixed model was used to analyze absolute neutrophil counts, where a normal distribution was assumed. If distribution was not normal, an appropriate transformation was examined (such as square root and log). If a suitable transformation could not be discovered, we used an appropriate non-parametric method to carry out the analyses and compare the treatment means (or medians) to the control means (or medians) using aligned data. Data within a site was aligned by subtracting the site median from each of the observations.

Clinical Mastitis Incidence. A generalized mixed model for binomial data was used to compare clinical mastitis incidence between treatment groups. Treatment was considered to be a fixed effect.

The Poisson distribution or negative binomial distribution was used to describe the number of morbidity events. We chose the distribution that fit the data best to carry out the analysis of the treatment effects as described for the morbidity data.

Clinical Safety Endpoints

Milk Composition. A repeated-measures ANOVA was used where treatment and day were fixed effects, and cows nested within sites and treatment was consid-

ered as a random effect. Time points were not equally spaced, so the possible covariance structures evaluated were (1) compound symmetry, (2) unequal time variances, (3) anti-dependent, (4) spatial covariance with the power as a function of time, and (5) unstructured. We used the minimum Akaike information criterion of each covariance structure for which Proc MIXED converged to select the covariance structure. Least squares means were used to compare treatment groups. When compound symmetry and unstructured were used as covariance structures, the term ID (site treatment) was eliminated from the model specification.

Milk Production. A repeated-measures ANOVA was used to analyze total daily milk production. The possible covariance structures evaluated were (1) compound symmetry, (2) compound symmetry with unequal time variances, (3) autoregressive, (4) autoregressive with unequal variances at each time, and (5) unstructured. The minimum Akaike information criterion of each covariance structure for which Proc MIXED converges, was used to select the covariance structure. Least squares means were used to compare the effects of pegbovigrastim to the controls. When compound symmetry, compound symmetry with unequal time variances, and unstructured were used as covariance structures, the term ID (site treatment) was eliminated from the model specification.

Length of Gestation. A generalized mixed model was used to analyze the length of gestation data where a normal distribution was assumed. If the distribution of the residuals was not normal, an appropriate transformation was examined (such as square root and log). If a suitable transformation could not be discovered, we used an appropriate nonparametric method to carry out the analyses and compare the treatment means (or medians) to the control means (or medians) using aligned data. Data within a site were aligned by subtracting the site median from each of the observations.

Percentage Live Births. The model we used for clinical mastitis incidence rates was used to analyze the percentage live birth data, where the binomial distribution was assumed.

RESULTS

Summary of Removals

Each site enrolled 80 animals per treatment group. Animals were removed from the study when they developed clinical mastitis or when they developed another health disorder that required antibiotic or anti-inflammatory therapy. Animals that had not exhibited visual estrus by 80 DIM were also removed from the study. Removals per site are summarized in Table 2. Data

Table 2. Number of animals removed from each study location due to concomitant disease requiring antibiotics or anti-inflammatories or failure to exhibit estrus by 80 DIM

Treatment	Wisconsin	Washington	Colorado	California
Saline				
Antibiotic/anti-inflammatory	4	7	5	4
Failure to return to estrus	4	2	21	0
Pegbovigrastim				
Antibiotic/anti-inflammatory	3	11	8	3
Failure to return to estrus	2	0	10	1

generated for each animal before removal was included in data analyses. Animals treated with antibiotics were removed at a mean of 15 DIM for both treatment groups.

Absolute Neutrophil Counts

The effect of pegbovigrastim on absolute neutrophil counts is summarized in Figure 1. We observed a significant ($P < 0.01$) increase in circulating neutrophil numbers relative to saline-treated controls within 24 h of administering the first dose in animals treated with pegbovigrastim. Circulating neutrophil numbers remained significantly ($P < 0.01$) elevated relative to the saline-treated controls 7 d after the second dose of pegbovigrastim, administered within 24 h of calving.

Clinical Mastitis Incidence Rates

A cow was identified as a treatment failure if 1 of more quarters met the clinical mastitis criteria (clinical score ≥ 3) at any milking during d 3 to 30 of lactation. The incidence of clinical mastitis in each treatment group is summarized in Table 3.

Administration of pegbovigrastim numerically reduced the incidence of clinical mastitis at each study site relative to the saline-treated controls. The overall incidence of clinical mastitis across study sites was significantly ($P < 0.01$) reduced by 35% relative to controls. The distribution of clinical mastitis events between primiparous and multiparous cows was similar for both treatment groups: approximately 60% of the clinical mastitis cases occurred in multiparous cows in both groups.

Microbiological Evaluation

Microbiological evaluation of milk samples collected from individual quarters at the time a cow met the clinical mastitis criteria indicated that the intramammary infections we observed in this study were associated with a diverse range of gram-positive and gram-negative pathogens typical of those found at commercial dairies (Wilson et al., 1997; Bradley, 2002). The frequency of the most common isolates are summarized in Table 4.

Escherichia coli, environmental streptococci (*Streptococcus uberis* and *Streptococcus dysgalactiae*) and coagulase-negative staphylococci (*Staphylococcus chromogenes*) were the most commonly isolated pathogens from morbid animals. The distribution of pathogens was similar for both treatment groups. Pegbovigrastim-treated cows had a reduced incidence of clinical mastitis associated with both gram-positive and gram-negative pathogens.

Cow Mortalities

We monitored cow mortalities at each study site during the first 30 d of lactation. The causes were typical of those observed in transition cows, including toxic mastitis, metritis, hepatic lipidosis, dystocia, and pneumonia (Kelton et al., 1998; Bobe et al., 2004; Mee, 2008). The incidence of cow mortalities from all causes is summarized in Table 5.

The overall incidence of cow mortalities was numerically lower for pegbovigrastim-treated animals than for the saline-treated controls, but this difference was not statistically significant ($P > 0.1$).

Table 3. Effect of pegbovigrastim on the incidence of clinical mastitis (number of cows) in periparturient cows and heifers at 4 United States dairies

Treatment	Wisconsin	Washington	Colorado	California	Overall
Saline	26	14	14	20	74 ^a
Pegbovigrastim	20	7	7	14	48 ^b
Calculated % reduction	23	50	50	30	35

^{a,b}Means within a column with different superscripts differ ($P < 0.01$).

Table 4. Frequency of common mastitis pathogens isolated from milk samples collected from animals with clinical mastitis

Bacterial pathogen	Frequency of isolation ¹	
	Saline	Pegbovigrastim
<i>Escherichia coli</i>	12/86	10/85
<i>Streptococcus uberis</i>	31/86	25/85
<i>Streptococcus dysgalactiae</i>	4/86	10/85
<i>Staphylococcus chromogenes</i>	9/86	6/85

¹Number of isolates/total number of isolates per treatment.

Health Disorders

Health disorders that required veterinary intervention resulted in the removal of cows from the study. Health disorders were limited to those that frequently occur in periparturient cows (Kelton et al., 1998; Santos et al., 2015). The relative incidence of abnormal health observations is summarized in Table 6.

Health disorders other than clinical mastitis occurred with similar frequencies between treatment groups. We observed no significant differences ($P > 0.1$) in the incidence of health disorders between groups. We also observed no differences in the incidence of abnormalities between primiparous and multiparous cows.

Reproductive Health Observations

The incidence of reproductive health problems, including metritis, failure to return to estrus, and failure of first-service conception are summarized in Table 7. The incidence of metritis was not significantly different ($P > 0.1$) between treatment groups. We observed a significant difference ($P < 0.03$) between groups in failure to return to estrus by 80 DIM. Animals treated with pegbovigrastim exhibited a 52% reduction in failure to return to estrus relative to the saline-treated controls. We observed no significant difference ($P > 0.1$) in first-service conception rates between treatment groups. The percentages of cows pregnant as a proportion of the at-risk population was 17.5% for the saline-treated controls and 31.6% for the animals treated with pegbovigrastim. This difference between treatments was not significantly different ($P > 0.1$).

Figure 1. Effect of pegbovigrastim on circulating absolute neutrophil counts in periparturient cows and heifers (means $\pm$ SEM).

Milk Quality and Composition

We evaluated the effect of pegbovigrastim on SCC and milk composition weekly for the first 4 weeks of lactation. The results of the SCC are summarized in Figure 2. We observed no significant ($P > 0.5$) differences in SCC between pegbovigrastim-treated cows and the saline-treated controls, indicating that the increased numbers of circulating neutrophils observed was not associated with increased SCC.

The effect of pegbovigrastim on milk fat, lactose, protein, and milk solids is summarized in Figure 3. The administration of pegbovigrastim had no significant effect on milk fat ($P = 0.5778$), milk protein ($P = 0.8507$), lactose ($P = 0.8624$), or milk solids ($P = 0.8722$) during the first 4 wk of lactation.

Milk Production

We determined the effect of pegbovigrastim on daily milk production for the first 30 DIM. Daily milk production for both large-frame (Holsteins) and small-frame (Jersey) cattle are summarized in Figure 4. Milk production was similar for animals in both treatment

Table 5. Incidence of cow mortalities (number of cows) up through 100 DIM

Treatment	Wisconsin	Washington	Colorado	California	Overall
Saline	5	2	3	4	14 ^a
Pegbovigrastim	1	2	4	3	10 ^a
Calculated % reduction	80	0	25	25	29

^aMeans within a column with identical superscripts were not significantly different ($P > 0.1$).

Table 6. Incidence of health disorders (number of cows) leading to early removal of cows or heifers from the study¹

Study site	Dystocia		Lameness		Low milk production		Ketosis, left displaced abomasum, peritonitis		Pneumonia	
	Saline	PEG	Saline	PEG	Saline	PEG	Saline	PEG	Saline	PEG
Wisconsin	0	0	1	0	2	2	2	3	1	0
Washington	1	2	0	0	0	0	1	2	0	1
Colorado	0	0	0	1	0	0	0	1	1	0
California	2	0	0	1	3	2	2	1	0	1
Combined	3	2 ^a	1	2 ^a	5	4 ^a	5	7 ^a	2	2 ^a

^aIncidence of each disorder not significantly different between treatments ($P > 0.1$).

¹Treatments: Sal = saline (N = 80/site); PEG = pegbovigrastim (N = 80/site).

groups, regardless of frame size. We observed no significant differences ($P = 0.8480$) between treatments.

Length of Gestation and Calf Viability

Because administration of pegbovigrastim was initiated before calving, we evaluated the length of gestation after dosing and the viability of calves to identify any negative effects of in utero exposure to pegbovigrastim on the ability of cows to maintain pregnancy or the health of the calves. The effects of pegbovigrastim on the length of gestation and proportion of live births are summarized in Table 8.

We observed no significant difference ($P = 0.2889$) between treatment groups in length of gestation after treatment initiation. In addition, these data confirmed that the first dose of pegbovigrastim was delivered approximately 7 d before calving, as intended.

We observed no significant difference ($P = 0.5485$) in the proportion of live births, suggesting that administration of pegbovigrastim before calving had no adverse effect on calf viability in utero.

Calf Health

Abnormal health conditions observed in calves during the first 30 d of life included abnormalities frequently

observed in neonatal calves in commercial production environments.

We did not observe any significant differences ($P > 0.1$) in the incidence of health disorders between treatment groups (Table 9), indicating that administration of pegbovigrastim had no adverse effect on the health of calves.

DISCUSSION

The primary objective of this study was to assess the efficacy of pegbovigrastim for reducing the incidence of clinical mastitis in periparturient primiparous and multiparous cows. Monitoring of cows was discontinued after they were diagnosed with their first case of clinical mastitis. Cows that developed health conditions requiring administration of antibiotics or anti-inflammatories were removed from the study because of the potential effect of these products on susceptibility to IMI. We collected no further data on animals that were removed from the study. Removing animals during the first 30 d of the experiment potentially limited the utility of the study results to generate conclusions about other potential health consequences of pegbovigrastim-induced enhancement of neutrophil activity. Animals that did not exhibit visual estrus by 80 DIM were also removed from the study. Data collected before removal of these animals were included in the analyses.

Table 7. Incidence of reproductive health problems (number of cows) in periparturient cows and heifers treated with pegbovigrastim (PEG) or sterile saline

Study site	Metritis				Failure to return to estrus				Failed to conceive after first			
	N	Saline	N	PEG	N	Saline	N	PEG	N	Saline	N	PEG
Wisconsin	76	0	77	2	71	4	76	2	67	40	74	47
Washington	73	5	69	6	71	2	67	0	69	39	67	35
Colorado	75	4	72	6	72	21	68	10	51	26	58	31
California	76	0	77	0	72	0	74	1	72	55	73	43
Combined	300	9	295	14 ^a	286	27	285	13 ^b	259	160	272	156 ^a

^aIncidence between treatments not significantly different ($P > 0.1$).

^bIncidence between treatments differed ($P < 0.03$).

Figure 2. Effect of pegbovigrastim on SCC (means $\pm$ SEM).

Pegbovigrastim is supplied in a prefilled syringe. Because the entire contents of the syringe were administered at each dose, we considered it relevant, particularly for the safety assessments, to demonstrate utility and safety across the range of body weights the product would likely be used for. Given that pegbovigrastim alters the immune response of treated cows, we also wanted to identify any clinically apparent negative health consequence in treated periparturient cows and calves exposed to pegbovigrastim in utero.

Administration of pegbovigrastim to periparturient cows and heifers induced a 4- to 5-fold increase in circulating neutrophils within 24 h of treatment initiation. These results were consistent with those of previous studies (Kehrli et al., 1991; Cullor et al., 1992) evaluating the hemogram changes associated with daily injections of bovine G-CSF. In the present study, pegbovigrastim-treated animals maintained el-

Figure 3. Effect of pegbovigrastim on (A) milk fat, (B) milk protein, (C) milk solids, and (D) lactose (means $\pm$ SEM). Saline, black squares; pegbovigrastim, white squares.

Figure 4. Effect of pegbovigrastim on daily milk production (means $\pm$ SEM) in Jersey and Holstein cows.

evated neutrophil counts relative to the saline-treated controls for more than 7 d after the second dose. These results were similar to those observed with PEGylated human G-CSF (Molineux, 2004), which demonstrated the increased duration of activity associated with the PEGylated protein. The prolonged duration of activity of pegbovigrastim enables a reduction in the number of doses required for a pharmacologically beneficial response compared with non-PEGylated bG-CSF (Cullor et al., 1992).

Administration of pegbovigrastim resulted in an overall reduction in the incidence of clinical mastitis infections by 35% relative to the saline-treated controls. The decrease in disease incidence varied from 23 to 50% across sites, and reductions were observed across all herd management systems represented in the study. The proportions of clinical mastitis events between treatment groups for primiparous and multiparous cows were similar, suggesting that pegbovigrastim was equivalently efficacious in primiparous and multiparous animals.

Clinical mastitis in the present study was associated with bacterial pathogens typically observed in US dairy operations (Wilson et al., 1997; Bradley, 2002).

The causative agents were similar in both treatment groups, indicating that the animals were exposed to similar pathogens across farms and locations. Pathogen distribution in the clinical mastitis events in the pegbovigrastim-treated cows suggested that no gap existed in the protection associated with a particular genus or species. This finding was consistent with the role of neutrophils as a key effector cell in the innate immune system, with broad-spectrum microbicidal activity (Mócsai, 2013).

Administration of pegbovigrastim resulted in a numerical decrease in incidence mortality relative to saline-treated controls across the 4 study sites. However, the observed decrease was not statistically significant ($P > 0.1$), and the reduction was primarily limited to 1 study site. It is possible that the observed decrease in mortality could have been influenced by other management practices in addition to the activity of pegbovigrastim.

We assessed the clinical safety of pegbovigrastim for dams and calves from the initiation of treatment through confirmation of pregnancy. Cows treated with pegbovigrastim exhibited a 52% reduction in failure to return to estrus by 80 DIM compared with saline-treated

Table 8. Length of treatment to calving interval and proportion of live births in cows and heifers treated with pegbovigrastim or sterile saline

Treatment	Treatment to calving interval, d (mean $\pm$ SEM)	Live births (%)
Saline	7.4 $\pm$ 0.3 ^a	96.1 ^a
Pegbovigrastim	7.0 $\pm$ 0.3 ^a	95.1 ^a

^aMeans within a column with identical superscripts were not significantly different ($P > 0.1$).

Table 9. Incidence of health disorders in calves during the first 30 d after calving (no. of animals with condition)

Abnormal health observation	Saline	Pegbovigrastim
Depression	15	11
Diarrhea	180	169
Ear infection	4	5
Fever	1	2
Respiratory disease	86	79
Refusing water	4	1

ed controls. This reduction was most evident at the Colorado dairy, which did not use an estrus synchronization protocol. These results were consistent with a recent study conducted at dairy farms in Mexico, which demonstrated that cows treated with pegbovigrastim had a 5.8% greater chance of being inseminated within the first 100 DIM than control cows (Ruiz et al., 2017). Further studies will be required to understand the underlying mechanism.

We did not observe a difference in first-service conception rates in the present study, based on either the number of open cows per treatment or the percentage of the at-risk population that were confirmed pregnant. The lack of a difference in first-service conception rates in this study may have been influenced by the removal of animals with concomitant diseases, which could have altered the ability of the cows to be rebred successfully. Additional studies should be pursued to better understand the effect of pegbovigrastim on reproductive health in periparturient cows.

Because pegbovigrastim induces pronounced neutrophilia, we were interested in determining the effect of the protein on milk SCC in healthy quarters. The results of the SCC evaluations indicated that healthy cows treated with pegbovigrastim had milk SCC similar to control cows. These results indicate that the increase in circulating neutrophil numbers after administration of pegbovigrastim was dissociated from the numbers of neutrophils in healthy glands. These results are significant given the important role of milk SCC in assessing milk quality.

It was beyond the scope of the present study to assess the effect of pegbovigrastim on milk SCC in cows that developed clinical mastitis. Additional studies are needed to confirm whether the enhanced neutrophil activity from cows treated with pegbovigrastim reported by Kimura et al. (2014) would result in more rapid elimination of the pathogen in infected quarters, reducing the need for a sustained influx of inflammatory cells and the persistent increase in milk SCC.

Animals treated with pegbovigrastim also exhibited similar milk composition to the saline-treated controls, indicating that the protein was unlikely to affect milk processing.

The results of the daily milk production assessments indicated that healthy treated cows exhibited similar milk production to healthy control animals, and that administration of pegbovigrastim had no negative effect on milk production in healthy animals. Because cows that developed clinical mastitis were removed from this study, we were unable to collect data on their milk production. Additional studies evaluating the effect of pegbovigrastim on milk production and milk discarded would help increase our understanding of the

economic benefits of preventing clinical mastitis during the periparturient period.

Periparturient cows experience impaired neutrophil function around the time of calving (Kehrli et al., 1989). This immune suppression may help the cow maintain her pregnancy (Hansen, 2013). We were interested in determining whether increasing neutrophil numbers and activity in dams would have any negative effect on their ability to maintain pregnancy. The length of gestation after treatment was similar between groups, indicating that administration of pegbovigrastim had no adverse effect on the ability of the cows to maintain pregnancy. These findings also indicate that the use of breeding records and herdsmen observations twice per week for signs of impending parturition enabled the prediction of calving dates with reasonable accuracy for the intended dosing regimen.

Calf viability at parturition was similar for both treatment groups, indicating that enhancing neutrophil numbers and activity before parturition had no negative effect on calf viability. We also found no significant differences in the incidence of health disorders in calves during the first 30 d postpartum, suggesting that exposure to pegbovigrastim in utero and potentially in colostrum had neither a positive nor a negative effect on calf health.

CONCLUSIONS

Traditional approaches to controlling clinical mastitis have focused on reducing exposure to pathogens (teat sealants and antiseptic teat dips) or eliminating pathogens through antimicrobial therapy. Administration of pegbovigrastim enhances neutrophil function in periparturient cows, and this study demonstrated that pegbovigrastim reduced the incidence of clinical mastitis in primiparous and multiparous cows during the periparturient period. We observed these benefits under diverse herd management systems typical of those found in the United States. Pegbovigrastim was well tolerated by cows, and was not associated with changes in milk production or milk composition. We conclude that pegbovigrastim represents a novel approach to reducing the incidence of clinical mastitis by addressing the periparturient immune suppression of the dam.

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