ORIGINAL RESEARCH

REFEREED

Use of a Mycobacterial Cell Wall Extract (MCWE) in Susceptible Mares to Clear Experimentally Induced Endometritis With *Streptococcus zooepidemicus*

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ABSTRACT

The ability of an immunomodulator, mycobacterial cell wall extract (MCWE), to clear uterine infection in susceptible mares after an experimental challenge with *Streptococcus zooepidemicus* was evaluated. Thirty mares susceptible to endometritis, based on the presence of uterine fluid during both diestrus and estrus, were selected from a herd of 896 and inoculated with a live culture of 5×10^6 CFU of *S. zooepidemicus* on day 1 of estrus. Twenty-four hours later, mares were evaluated by ultrasonography, bacteriology, exfoliative cytology, and uterine biopsy to confirm infection. Forty-eight hours after inoculation, and on confirmation of uterine infection, mares were randomly assigned to one of four unbalanced experimental treatments to receive 1500 μg MCWE IU (n = 10) or IV (n = 10), or placebo IU (n = 5) or IV (n = 5). Mares were examined at ovulation and 7 days post-ovulation for uterine fluid via transrectal ultrasonography and for bacteriology, exfoliative cytology, and uterine biopsy. Efficacy was based on the ability of the mare to clear endometritis as determined by negative bacteriology and reduced numbers of polymorphonuclear cells (PMNs) on uterine biopsy. Because no statistical difference was detected between routes of administration on day 7 post-ovulation, the data sets were combined and re-analyzed to evaluate overall efficacy. Endometritis was observed in all placebo-treated mares 7 days post-ovulation, whereas treatment with MCWE resulted in the elimination of endometritis in 35% of the mares by the time of ovulation, and 70% of the mares by 7 days post-ovulation. Treatment with MCWE, compared with the placebo group, resulted in a significant decrease in the number of mares positive for endometritis at ovulation based on exfoliative cytology and bacteriology ($P < .01$) and at 7 days post-ovulation based on biopsy, exfoliative cytology, and bacteriology ($P < .001$). Results indicate that MCWE was an effective treatment for the elimination of endometritis caused by *S. zooepidemicus* in mares.

Keywords: Mycobacterial cell wall extract (MCWE); Immunomodulation; Innate immunity; Endometritis; Mares

INTRODUCTION

Infectious endometritis in mares is an important disease with a high economic impact.[1,2] Bacteria gain entrance into the uterus at the time of foaling or breeding. Mares can be classified as being susceptible or resistant to endometritis based on their ability to clear an experimentally induced *Streptococcus zooepidemicus* infection within 96 hours of inoculation.[3]

Why the immune systems of susceptible mares are not as efficient as those of resistant mares is not clear. The role of the humoral immunological response is not clear, because mares with persistent endometritis have an increased number of immunoglobulin-containing cells when compared with reproductively normal mares. However, antigenic-specific antibodies have not been detected in either susceptible or resistant mares, which suggests that non-humoral immunological responses are critical to the resolution of endometritis in mares.[4]

Innate immunity is very important because other uterine factors may contribute to the impaired function of uterine polymorphonuclear cells (PMNs) in susceptible mares. Troedsson et al.[5] reported inadequate opsonization of bacteria in uterine secretions as the primary cause of uterine dysfunction, as opposed to a primary dysfunction of PMNs. They demonstrated that nonfunctional uterine PMNs from susceptible mares were, in fact, fully functional if provided with the appropriate environment. Additionally, although the phagocytic activity of blood neutrophils in susceptible and resistant mares was similar, the migration of blood neutrophils from susceptible mares was lower. The phagocytic activity of uterine neutrophils was significantly lower in susceptible mares, despite serum from susceptible mares having significantly more chemotactic

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activity than that in resistant mares.[6] Overall, uterine neutrophils from susceptible mares have significantly lower phagocytic activity and reduced migratory responses to chemotactic stimuli than those from resistant mares.[7,8]

The translocation of circulating neutrophils into the inflamed tissues, or extravasation, is initiated by chemokines such as interleukin 8 (IL-8), macrophage inflammatory protein (MIP-1β), and other bacterial components including *Streptococcal* sp. culture filtrate.[9] Translocation of circulating neutrophils is followed by the attachment of neutrophils to endothelial cells, which is critical for the phagocytic process and is mediated through leukocyte-specific cell adhesion molecules (CAMs), which stabilize the adhesion between the neutrophils and the endothelial cells in large numbers. Therefore, potent chemokines such as IL-8 and bacterial cell components, along with CAMs, play a pivotal role in clearing bacteria-induced endometritis.

Zerbe et al.[10] have assessed in mares the endometrial PMN response after experimental challenge with *S. zooepidemicus* and the effect of subsequent treatment with recombinant human IL-8. Mares demonstrated a profound response to recombinant IL-8 treatment, and PMNs were attracted and translocated into the uterus within 6 hours of administration. These intrauterine PMNs were determined to have decreased expression of major histocompatibility complex (MHC-1) receptor, but a significantly increased expression of the intercellular adhesion molecule, CD11a, in addition to an increased ability to generate reactive oxygen species (ROS).

Traditionally, endometritis in mares has been treated using antimicrobials or oxytocin, which has been effective in clearing the bacterial infection in most cases of post-breeding and post-parturition endometritis.[11,12] However, recent data[13-16] indicate that nonspecific immunomodulation with mycobacterial cell wall extract (MCWE) can restore homeostasis of local inflammatory mechanisms and consequently assist in the resolution of this condition in mares.[17,18]

Fumuso et al.[14] evaluated endometrial mRNA transcription patterns of three pro-inflammatory cytokines (IL-1β, IL-6, and tumor necrosis factor alpha [TNFα]) in mares resistant or susceptible to persistent post-breeding endometritis (PPBE) over three consecutive estrous cycles after treatment with the immunomodulator MCWE (Settle™, Bioniche Animal Health, Bogart, GA). Susceptible mares had significantly higher mRNA expression levels for all cytokines at estrus and significantly higher IL-1β and TNFα levels during diestrus compared with resistant mares. Treatment of susceptible mares with MCWE served to downregulate IL-1β and TNFα levels at estrus and IL-6 and TNFα levels during diestrus, resulting in cytokine levels similar to those of resistant mares.

In another study, Fumuso et al.[13] evaluated the ability of MCWE to improve the conception rate of foal-heat mares. Within 1 week after foaling, 85% of the mares receiving MCWE treatment had achieved bacterial clearance and demonstrated a significant decrease in exfoliative cytology scores as compared with the control group, which was statistically unchanged. Ultimately, almost three times more MCWE-treated mares conceived during foal heat than mares in the control group.

In a third study, Fumuso et al.[16] evaluated the potential for MCWE to ameliorate the breeding response of barren mares with a history of failing to conceive in the previous 2 years. Treatment with MCWE resulted in 86% of mares achieving bacterial clearance, with 39% of the infertile mares becoming pregnant.

The current study is the first designed to investigate MCWE (Settle™) within a controlled challenge model for its ability to clear an experimentally induced infection of *S. zooepidemicus* in mares susceptible to endometritis as a stand-alone therapy.

MATERIALS AND METHODS

Animals

Thirty mares were selected from a PMSG production herd of 896 mares. Mares were cross-bred, non-pregnant, had been barren for two breeding seasons, and had an average age of 7.5 years. All mares were determined to be susceptible to endometritis as indicated by the presence of uterine fluid (>2 cm) during both diestrus and estrus.[19] All mares were treated with antibiotics until all existing endometritis was resolved (negative bacterial culture and exfoliative cytology, and absence of uterine fluid). On resolution, mares were treated with an intramuscular injection of 250 μg racemic cloprostenol sodic (Estroplan prostaglandin; Parnell Laboratories, Australia) to minimize the variation of the cycles among the group.

Experimental Design

All mares were inoculated with a live culture of 5×10^6 CFU *S. zooepidemicus* the day after estrus detection (as confirmed by rectal palpation and ultrasonography) (schedule shown in Fig. 1). Twenty-four hours after inoculation, infection was confirmed by the presence of uterine fluid via transrectal ultrasonography, and positive sampling for bacteriology and exfoliative cytology. Forty-eight hours after inoculation, mares with confirmed *S. zooepidemicus* infections were randomly assigned to one of four unbalanced experimental treatments: intrauterine administration (IU) of 1.5 mg MCWE (Settle™) (n = 10); intravenous administration (IV) of 1.5 mg MCWE (n = 10); placebo administration IU (n = 5); placebo administration IV (n = 5). The placebo consisted of the same diluents and carriers as the commercial formulation (mineral oil emulsion containing Tween 80 in phosphate-buffered saline [PBS]) but lacked MCWE as an active ingredient. Ovulation occurred 3 to 6 days after infection. Mares were evaluated 1 day post-ovulation and again 7 days post-ovulation for uterine fluid accumulation via transrectal ultrasonography, and were sampled for bacteriology, exfoliative cytology, and uterine biopsy to determine the degree of clearance of the previously established *S. zooepidemicus* infection. Efficacy was based on the ability of the mare to

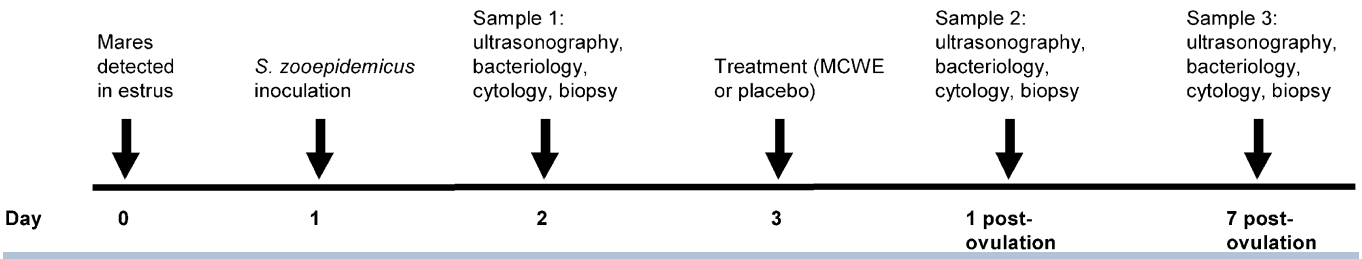

Figure 1. Experimental schedule, showing mare inoculation and sampling points.

clear endometritis as determined by negative bacteriology and reduced numbers of PMNs on uterine biopsy.

Ultrasonography

Uterine fluid was visualized by transrectal ultrasonography and classified based on uterine echogenicity and fluid volume according to Losinno and Aguilar.[20] An anechoic uterus with <50 ml and no change in uterine size was considered negative. A hypoechoic, semi-echogenic, hyperechogenic, or strongly echogenic uterus with >50 ml was considered positive for endometritis.

Infection With *Streptococcus zooepidemicus*

Endometritis was induced by inoculating each mare with a live culture of *Streptococcus zooepidemicus* as described by Cadario et al.[21] with minor modifications. Briefly, a virulent genital strain of *S. zooepidemicus* was grown overnight in Brain Heart Infusion Broth (Merck, Argentina) at 37°C, washed twice in PBS, diluted to 1×10^9 bacterial cells/ml, and stored at −70°C until inoculation. Immediately before inoculation, the culture was thawed and diluted in PBS to obtain a suspension of 1×10^6 cells/ml, of which 5 ml (5×10^6 cells) was mixed with 20 ml PBS as a carrier and introduced into the uterus through a plastic pipette.

Exfoliative Cytology

Samples were collected from the uterine body using guarded protective Culturvet swabs (Culturvet, Argentina), rolled on glass slides, dried at room temperature, and stained with Giemsa stain (15" Biopur, Argentina).[22,23] Samples were viewed under 40×, and PMN cells were counted and averaged in 10 fields of view. Those samples with an average greater than 4 PMN per field were classified as positive for endometritis.[24]

Bacteriology

Endometrial samples were collected using guarded Culturvet swabs (Culturvet, Argentina) and immediately placed in Amie's transport medium (Oxoid, United Kingdom). Samples were cultured according to Ricketts[22,23] to determine the presence (positive) or absence (negative) of *S. zooepidemicus.*

Uterine Biopsies

Uterine biopsy samples were collected using an alligator jaw biopsy punch (Pilling, PA) and analyzed as described by Miragaya et al.[25] Results were obtained by counting and averaging the number of PMN cells in each of 20 fields of view under 40× magnification and classified as either negative or positive. Samples with an average of 0 to 17 PMN cells were classified as negative and samples with an average of ≥18 PMN cells were positive for endometritis.

Statistical Analysis

For comparisons between the MCWE-treated and placebo groups, statistical analysis was performed using Fisher's exact test (SAS System, FREQ Procedure). For comparisons between the two routes of administration and between treatments on different sampling days, statistical analysis was conducted using INSTAT (HALLoGRAM Publishing, Aurora, CO). The Mann-Whitney test was used to evaluate uterine fluid and uterine biopsy classifications, whereas the categorical data of bacteriology and exfoliative cytology was analyzed using contingency tables and Fisher's exact test with a 95% confidence interval. Because no statistical difference was detected between the two routes of administration, the data sets were combined and re-analyzed to evaluate overall efficacy.

RESULTS

All mares developed uterine infection within 1 day after inoculation with *Streptococcus zooepidemicus* as confirmed by uterine fluid, bacteriology, exfoliative cytology, and uterine biopsy (Figs. 2–5). This infection persisted through 7 days post-ovulation in mares treated with the placebo (both IV and IU), whereas a significant decrease was seen in the number of MCWE-treated mares diagnosed with endometritis.

No observable difference was seen in the presence of uterine fluid by day 7 post-ovulation for either MCWE IU or IV treatment groups as compared with the respective placebo groups (Fig. 2). However, by day 7 post-ovulation, 80% of IU treated mares ($P < .05$) and 70% of the IV treated mares ($P < .05$) had negative *S. zooepidemicus* cultures (Fig. 3), and all mares in the placebo groups remained positive at day 7. Additionally, 80% of IU-treated mares ($P < .05$) and 60% of IV-treated mares ($P < .05$) were negative for exfoliative cytology by day 7 post-ovulation (Fig. 4), in contrast to the placebo groups, all of which remained positive. Finally, 70% of IU treated mares ($P < .05$) and 90% of IV treated mares ($P < .05$) were negative

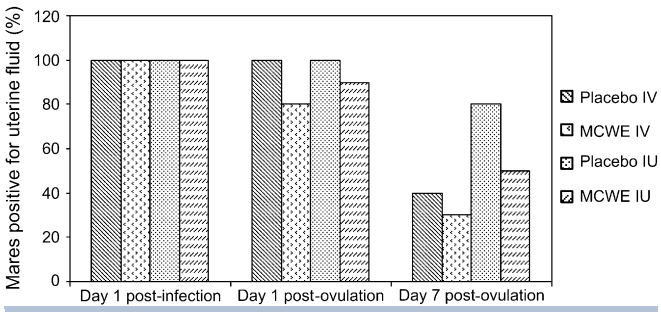

Figure 2. Percentage of mares positive for endometritis as determined by the ultrasonographic detection of >2 cm uterine fluid volume. Testing was performed 1 day after experimental infection, 1 day post-ovulation, and 7 days post-ovulation.

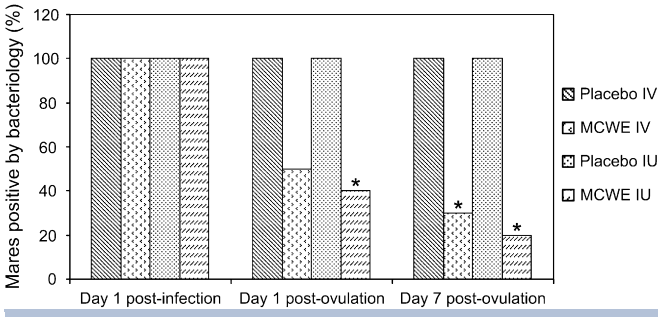

Figure 3. Percentage of mares positive for endometritis as determined by the presence of bacterial culture for *Streptococcus zooepidemicus*. Testing was performed 1 day after experimental infection, 1 day post-ovulation, and 7 days post-ovulation. *$P < .05$.

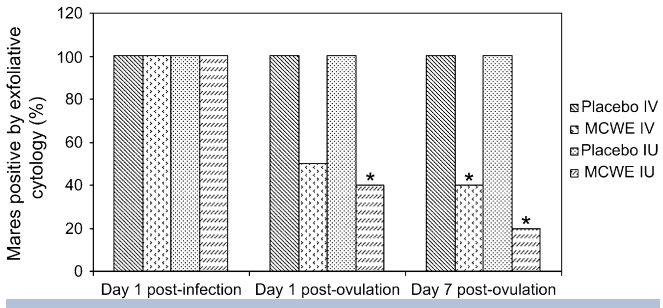

Figure 4. Percentage of mares positive for endometritis (an average > 4 PMN per field at 400× magnification) as determined by endometrial exfoliative cytology. Testing was performed 1 day after experimental infection, 1 day post-ovulation, and 7 days post-ovulation. *$P < .05$.

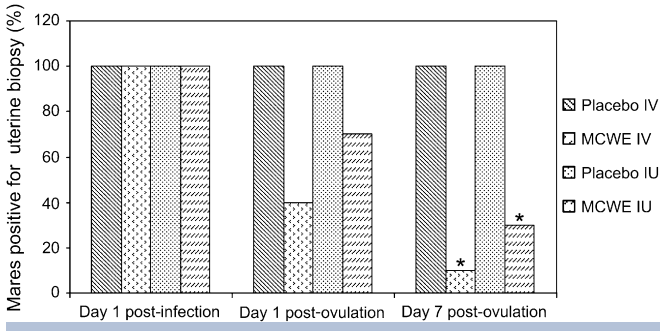

Figure 5. Percentage of mares positive for endometritis (an average ≥18 PMN per field at 400× magnification) as determined by endometrial biopsies. Testing was performed 1 day after experimental infection, 1 day post-ovulation, and 7 days post-ovulation. *$P < .05$.

on uterine biopsy (Fig. 5) as compared with the placebo groups, all of which remained positive.

Because no statistical difference was found between MCWE IV and MCWE IU in the number of mares positive by exfoliative cytology ($P = .63$), bacteriology ($P = 1.00$), uterine biopsies ($P = .86$), or for the presence of uterine fluid ($P = .68$) on day 7 post-ovulation, the two groups were combined and analyzed together to assess the overall efficacy of the MCWE treatment and ability to clear the endometrial *S. zooepidemicus* infection. By the time MCWE-treated mares ovulated, a 55% reduction in the number of mares positive by bacteriology ($P < .05$) and a 55% reduction in the number of mares positive by exfoliative cytology ($P < .05$) had occurred. By 7 days post-ovulation, a 75% reduction in the number of mares positive by bacteriology ($P < .05$), a 70% reduction in the number of mares positive by exfoliative cytology ($P < .05$), and an 80% reduction in the number of mares positive on uterine biopsy ($P < .05$) occurred. Based on the number of mares negative on bacteriology and uterine biopsy, the overall efficacy of the ability of treatment with MCWE to eliminate *S. zooepidemicus* infection compared with placebo was 35% by day 1 post-ovulation and 70% by day 7 post-ovulation, irrespective of the route of administration.

DISCUSSION

Mycobacterium species are known to have immunomodulatory activity and immunotherapeutic effect in mammals[26] when administered as either a live organism, inactivated whole mycobacteria, cell wall skeletal fraction,[27] or as individual cell wall components such as muramyl dipeptide or trehalose mycolate.[28,29] Because of its ability to enhance the body's immunological response to immunizing antigens, this effect also has been exploited for use as a vaccine adjuvant.

MCWE has been shown to stimulate pro-inflammatory cytokines, activate macrophages and neutrophils, and generally upregulate the host's immunologic system. Mycolic acid, which is a component of MCWE, has been shown to cause a rapid and widespread influx of PMNs, which would primarily be responsible for the clearance of endometrial infection, while stimulating TNFα, interferon gamma (IFN-γ), IL-6, IL-2, and myeloperoxidase production and suppressing IL-10 production.[30] Trehalose 6,6′-dimicolate (TDM or cord factor), which is also a component of MCWE, also has been identified as an inducer of the pro-inflammatory cytokines IL-1β, IL-6, and TNFα[31]; whereas muramyl dipeptide, the smallest peptidoglycan

component of MCWE, upregulates the production of IL-2, IL-4, IL-10, IL-12, and IFNγ.[32] Finally, the MCWE component glycolipid lipoarabinomannan (LAM) has been identified as a trigger for the production of TNFα, granulocyte-macrophage colony-stimulating factor, transforming growth factor beta (TGFβ), IL-1α, IL-β, IL-6, IL-8, IL-10, and IL-12, as well as being a chemoattractant for monocytes and neutrophils.[33] Many distinct mycobacterial cell wall components may interact with different members of the toll-like receptor family, which are involved in innate and adaptive immunological responses; for example, LAM is recognized through TLR-4 whereas phosphatidylinositol mannoside serves as a ligand for TLR-2.[34,35] Thus, MCWE is easily recognized by multiple immunological mechanisms, resulting in an enhanced host response.

Systemic (intravenous) administration of MCWE elicits mononuclear cells that trigger the release of cytokines, resulting in the activation of intrauterine PMNs.[13] However, intrauterine administration of *Mycobacterial sp.* and tuberculin elicits PMN activation through cell-mediated immune reaction[36] but also may directly activate PMNs, as has been demonstrated with inactivated *Streptococcus* cells,[37] gram-negative cell wall components such as LPS,[38] and the 19-kd lipoprotein from mycobacterium.[39]

CONCLUSIONS

Although antibiotic treatment is widely recognized as an effective stand-alone therapy for endometritis, there is a growing desire to minimize its use because of concerns related to microbial resistance.[40] Recently, an antibiotic-free approach for the treatment of endometritis in mares has become possible with the use of MCWE. The ability of MCWE to stimulate the induction of cytokines and chemokines, activate neutrophils, and enhance the overall host immunological response has been well documented.[13-18] Several constitutive components of MCWE are known to play crucial roles in the upregulation of the immune system and act as chemoattractants after administration, including mycolic acid, lipoarabinomannan, cord factor trehalose 6,6′-dimicolate, and muramyl dipeptide. The activation of neutrophils is critical for the successful clearance of endometrial infection, and we present evidence that suggests that MCWE not only activates neutrophils, but also increases their phagocytic activity, which contributes to clearance of bacteria from the uterus.

Notwithstanding a nonsignificant trend that suggested that intrauterine administration of MCWE may be more efficacious than intravenous administration, the data generated in this study confirm that both routes of administration are effective in clearing endometrial infection. Based on uterine biopsy and bacterial culture results, the overall efficacy of MCWE when administered early in estrus was 35% at ovulation and 70% by 7 days post-ovulation.

This study indicates that MCWE immunotherapy is a safe, effective, and convenient treatment for endometritis in mares. Furthermore, one may reasonably hypothesize that MCWE immunotherapy combined with antibiotic treatment could be a highly effective and complementary approach.

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