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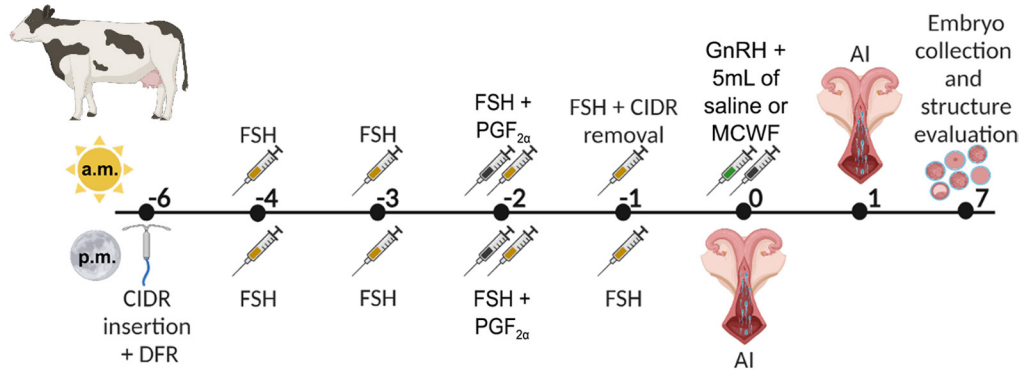
Short Communication
Physiology

Effects of administration of mycobacterium cell wall fraction during the periovulatory period on embryo development following superovulation in virgin dairy heifers

W. Brown,[1] M. Oliveira,[2] R. Reis Silva,[3] D. Demetrio,[2] and J. Block[1]*

Graphical Abstract



Variable	Treatment	
	Control	MCWF
Donor animals (n)	16	18
Total structures (n)	11.64 ± 1.55	9.28 ± 1.57
Unfertilized oocytes (n)	2.52 ± 0.90	1.79 ± 0.91
Degenerate embryos (n)	1.79 ± 0.76	1.68 ± 0.77
Transferable embryos (n)	6.02 ± 0.86	5.54 ± 0.87
Blastocysts (n)	0.28 ± 0.18	0.68 ± 0.19

Data are presented as least squares means ± SEM.

Variable	Treatment	
	Control	MCWF
Donor animals (n)	16	18
Unfertilized oocytes (%)	21.18 ± 5.55	13.26 ± 5.61
Degenerate embryos (%)	11.68 ± 4.56	18.93 ± 4.61
Transferable embryos (%)	55.59 ± 6.24	63.31 ± 6.31
Blastocysts (%)†	4.30 ± 1.90	8.63 ± 1.93

Data are presented as least squares means ± SEM.
†$P < 0.10$

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Summary

The objective of this study was to evaluate the effects of stimulating proinflammatory cytokines during the periovulatory period on embryo development using superovulation as a model. Animals submitted to a superovulation protocol received either a control treatment (sterile saline) or mycobacterium cell wall fraction (MCWF, Amplimune) on the day of induced estrus. Seven days after artificial insemination, embryos were recovered through nonsurgical recovery, and the number and development of each collected structure were evaluated. There were no statistical differences in any of the measures evaluated except for a trend in animals receiving MCWF having a greater proportion of blastocysts recovered ($P < 0.10$).

Highlights

- Embryo development was evaluated after using a nonspecific immune stimulant.
- Stimulation of proinflammatory cytokines did not affect embryo development.
- The use of MCWF is not detrimental to the number of structures recovered.
- The proportion of blastocysts recovered tended to increase when animals were treated with MCWF.

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Abstract: Proinflammatory cytokines are involved in regulating several reproductive processes that occur during the periovulatory period, including ovulation, corpus luteum formation, and preimplantation embryo development. The objective of this study was to determine whether stimulation of proinflammatory cytokines through administration of mycobacterium cell wall fraction (MCWF; Amplimune, NovaVive) could improve embryo development following superovulation in dairy heifers. A total of 34 independent embryo recovery procedures were performed using nulliparous Holstein heifers (n = 20; age 12–18 mo) as donors. For superovulation, dominant follicle removal was performed and an intravaginal progesterone device was inserted on d −6. Thirty-six hours later, on d −4, FSH (420 IU total) was administered in a decreasing dose regimen consisting of 8 injections given twice daily at 12-h intervals. Prostaglandin $F_{2\alpha}$ was administered in conjunction with the fifth and sixth injections of FSH on d −2 and the intravaginal progesterone device was removed on the morning of d −1. Twenty-four hours later, on d 0, donors received their randomly assigned treatment (sterile saline or MCWF, 5 mL, i.m.) and gonadotropin-releasing hormone was administered to induce ovulation. Donors were artificially inseminated with frozen-thawed semen at 12 and 24 h after induced ovulation. Nonsurgical embryo recovery procedures were performed on d 7. Recovered structures were evaluated using a stereomicroscope to assess embryo development. There was no effect of MCWF treatment on the numbers of total structures, unfertilized oocytes, degenerate embryos, transferable embryos, or blastocysts. However, there was a trend for donors treated with MCWF to have a greater proportion of blastocysts out of total structures recovered. Overall, the efficacy of superovulation in virgin dairy heifer donors was not improved by administration of MCWF during the peri-ovulatory period, but results indicate that MCWF treatment may enhance embryo developmental kinetics.

Early embryonic mortality accounts for a significant proportion of pregnancy failure that occurs in cattle (Wiltbank et al., 2016). Moreover, failure of pregnancy establishment and maintenance during the first month postconception contributes to significant economic losses in the cattle industry $300–$600 per animal (De Vries, 2006; Cabrera, 2012). Successful preimplantation embryo development and survival is regulated in part by a complex cross-talk between the developing embryo and the maternal reproductive tract (Mamo et al., 2012; Ding et al., 2021). Communication between the oviduct or uterus and the preimplantation embryo involves a multitude of molecules, including hormones, growth factors, cytokines, and various other regulatory molecules (Hugentobler et al., 2007; Lonergan et al., 2016). Among these molecules, several proinflammatory cytokines are expressed by the bovine oviduct and uterus and their respective receptors are correspondingly expressed by the developing embryo (Austgulen et al., 1995; Talukder et al., 2020), indicating a putative role in regulating early embryo development.

Previous research in mice and pigs has demonstrated a role for proinflammatory cytokines in the early postmating period. In particular, exposure of the maternal reproductive tract to seminal plasma following mating induces a proinflammatory response that results in improved pregnancy rates (Rozeboom et al., 2000; Bromfield et al., 2014). The maternal proinflammatory response that occurs after postmating exposure to seminal plasma involves greater expression of the proinflammatory cytokines IL-6, IL-1β, and colony stimulating factor-2 (CSF-2; Tremellen et al., 1998; Sharkey et al., 2007; Chan et al., 2021). In cattle, IL-6, IL-1β, and CSF-2 act as embryokines and promote embryo development in vitro (Paula-Lopes et al., 1998; Loureiro et al., 2009; Wooldridge and Ealy, 2019). Moreover, bovine embryos produced following culture with CSF-2 and IL-6 have a greater capacity for establishment of pregnancy following embryo transfer. While studies evaluating the effects of administration of seminal plasma on pregnancy establishment in cattle have been equivocal (Odhiambo et al., 2009; Ibrahim et al., 2019; Mateo-Otero et al., 2020), other options for stimulating proinflammatory cytokines in the postmating period could be more effective.

An alternative to seminal plasma for the stimulation of proinflammatory cytokines during the periovulatory period could be the administration of an immunostimulant, such as mycobacterium cell wall fraction (**MCWF**). Components of MCWF, including lipid mycolic acid, trehalose dimycolate, and muramyl dipeptides, are potent inducers of proinflammatory cytokines (Korf et al.,

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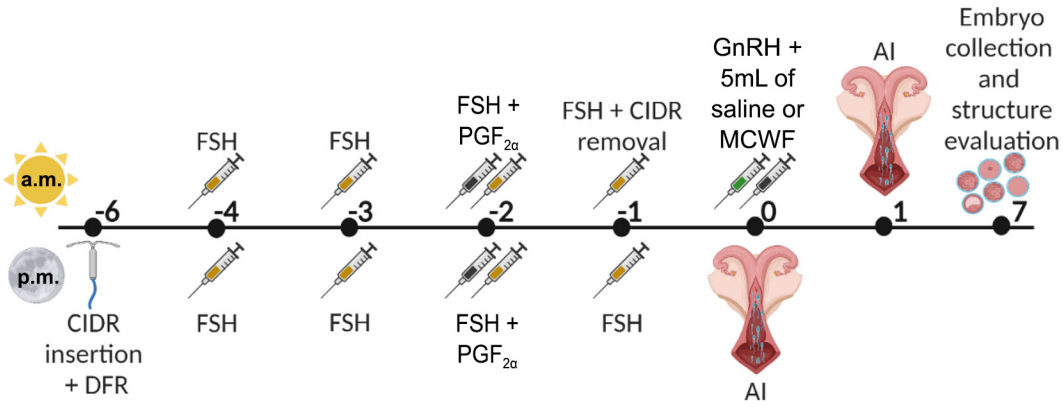

Figure 1. Schematic depiction of the experimental schedule, including superovulation, treatment, and embryo recovery. Controlled internal drug-releasing device (CIDR), mycobacterium cell wall fraction (MCWF), dominant follicle removal (DFR). Created with BioRender.com.

2005; Medellin-Peña, 2017). Administration of MCWF at the time of estrus increased the proportion of dairy heifer recipients that had a corpus luteum (**CL**) ≥22 mm in diameter and resulted in a greater number of pregnancies per embryo transfer (Palomino et al., 2021). Moreover, treatment of dairy heifer donors with MCWF at the initiation of a follicle superstimulation regimen tended to increase the number of transferable embryos produced following in vitro fertilization (Brown et al., 2024).

The objective of the present study was to determine whether administration of MCWF during the periovulatory period could increase the number and quality of embryos recovered following superovulation in dairy heifer donors.

Unless specified otherwise, all materials were purchased from Millipore-Sigma (Burlington, MA). Animal use and experimental procedures were approved by the University of Wyoming Institutional Animal Care and Use Committee. This experiment was conducted at a commercial dairy in central California (RuAnn Dairy, Riverdale, CA, 36.4727778°N, −119.9263889°W) from February to May and November to December of 2022. Nulliparous Holstein heifers (n = 20), 12 to 18 mo of age, were used as embryo donors. Heifers were housed in dry lots and fed a TMR formulated to meet the nutrient requirements of yearling dairy heifers.

Animals enrolled in the study were randomly assigned to 1 of 2 treatments, saline (n = 16) or MCWF (n = 18) derived from *Mycobacterium phlei* (Amplimune, NovaVive Inc., Napanee, ON, Canada). Animals assigned to treatment were administered 1 mg of MCWF i.m. in a volume of 5 mL. Heifers assigned to the control treatment were administered 5 mL of sterile saline i.m. The dose and route of administration of MCWF in the present study were based on a prior report in which administration of 5 mL of MCWF to dairy heifer recipients increased pregnancy per embryo transfer (Palomino et al., 2021). The experiment included 34 independent superovulation, treatment, and embryo recovery procedures. Of the 20 heifers used on the study, some were submitted to either 2 (n = 12) or 3 (n = 1) separate rounds of superovulation, treatment, and embryo recovery. Before the first round of embryo recovery, animals were randomly assigned to treatment groups. For animals used on more than one occasion, the treatment administered was the opposite of the previous round. The washout period between treatments for animals that were used for more than one recovery procedure was 6.8 ± 0.80 (6–8) wk. The study included 13 independent replicates.

Animals were subjected to superovulation and embryo recovery as outlined in Figure 1. To synchronize follicle wave emergence, donor animals were submitted to dominant follicle removal (**DFR**) on the afternoon d −6. For DFR, donors were restrained and then animals were administered caudal epidural anesthesia via administration of 4 mL of 2% (wt/vol) lidocaine hydrochloride (Aspen Veterinary Resources, Liberty, MO) in the first coccygeal intervertebral space. The DFR procedure was performed using an ultrasound (Mindray DP50, Mindray, Shenzhen, China) equipped with a 6.5-MHz convex ultrasound probe encased within a plastic transvaginal handle (WTA, College Station, TX). All follicles ≥5 mm were ablated transvaginally using a 20-gauge, 8.5-cm needle (WTA) that was attached to the end of a stainless-steel needle guide. Following DFR, an intravaginal controlled internal drug-releasing device impregnated with 1.38 g of progesterone (Eazi-Breed CIDR, Zoetis, Parsippany-Troy Hills, NJ) was inserted. Thirty-six hours later, follicle superstimulation was induced with 420 IU of FSH (Folltropin, Vetoquinol, Fort Worth, TX) administered i.m. every 12 h in a decreasing dose regimen consisting of 8 doses (70, 70, 70, 70, 35, 35, 35, and 35 IU of FSH, respectively). Animals were administered $PGF_{2\alpha}$ in conjunction with the fifth (Estrumate, 750 µg, i.m., Merck Animal Health, Madison, NJ) and sixth (500 µg, i.m.) injections of FSH in the a.m. and p.m. of d −2. The CIDR device was removed in the a.m. of d −1. On d 0 in the a.m., ovulation was induced with GnRH (129 µg, i.m., Fertagyl, Merck Animal Health) and animals received their assigned treatment (saline or MCWF). Donor animals were artificially inseminated 12 and 24 h later with either 1 (conventional semen) or 2 (sex-sorted semen) units of semen per AI. Semen from 11 different bulls was used in the study, including conventional semen from one bull and X-sorted semen from 10 bulls. Of the 34 superovulation and embryo recovery procedures performed, 5 included donors inseminated with conventional semen (n = 3 control and 2 MCWF, respectively) and 29 with X-sorted semen (n = 13 control and 16 MCWF, respectively).

On d 7, donor animals were submitted to nonsurgical embryo recovery procedures as described previously (Elsden et al., 1976; Lonergan and Boland, 2011). Briefly, donors were restrained

Table 1. Effect of administration of mycobacterium cell wall fraction (MCWF) during the periovulatory period on the number of various structures recovered following superovulation[1]

	Treatment	
Variable	Control	MCWF
Donor animals (n)	16	18
Total structures (n)	11.64 ± 1.55	9.28 ± 1.57
Unfertilized oocytes (n)	2.52 ± 0.90	1.79 ± 0.91
Degenerate embryos (n)	1.79 ± 0.76	1.68 ± 0.77
Transferable embryos (n)	6.02 ± 0.86	5.54 ± 0.87
Blastocysts (n)	0.28 ± 0.18	0.68 ± 0.19

[1]Data are presented as LSM ± SEM.

Table 2. Effect of administration of mycobacterium cell wall fraction (MCWF) during the periovulatory period on the proportion of various structures recovered following superovulation[1]

	Treatment	
Variable	Control	MCWF
Donor animals (n)	16	18
Unfertilized oocytes (%)	21.18 ± 5.55	13.26 ± 5.61
Degenerate embryos (%)	11.68 ± 4.56	18.93 ± 4.61
Transferable embryos (%)	55.59 ± 6.24	63.31 ± 6.31
Blastocysts† (%)	4.30 ± 1.90	8.63 ± 1.93

[1]Data are presented as LSM ± SEM.

†$P < 0.10$.

and then animals were administered caudal epidural anesthesia as described above. The perineal region was cleaned thoroughly and then a 20-French Foley catheter (Bard, Covington, GA) was inserted transcervically into the uterus. The cuff of the catheter was placed at the base of one of the uterine horns and then inflated with 5 to 10 mL of air. Gravity flow was then used to fill the uterine horn with embryo collection medium (Vetivex Lactated Ringer's, Dechra Veterinary Products, Overland Park, KS) and subsequently recover the medium in an embryo collection filter (EZ-Way Filter, SPI, Charlotte, NC). This process was repeated 4 to 5 times with up to 500 mL of embryo collection medium and then the process was repeated for the second uterine horn. After both horns were flushed, recovered structures were identified and evaluated using an Olympus SZ10 stereomicroscope (Olympus Corporation, Shinjuku City, Tokyo, Japan). All recovered structures were classified based on stage of development and quality according to the criteria of the International Embryo Technology Society (Barfield and Demetrio, 2022; Demetrio and Barfield, 2022).

Statistical analyses were performed using the Statistical Analysis Software package (version 9.4, SAS Institute Inc. Cary, NC). Data for the number of total structures, number of unfertilized oocytes, number of degenerate embryos, number of transferable embryos, and number of blastocysts were analyzed by ANOVA using the PROC GLM procedure. Transferable embryos were classified as embryos that were grade 1 or 2 and had reached at least the morula stage of development, whereas blastocysts were classified as embryos that were grade 1 or 2 and had reached at least the blastocyst stage of development. Data are presented as least squares means ± standard error of the means. The proportions of unfertilized oocytes, degenerate embryos, transferable embryos, and blastocysts out of total structures were also analyzed by ANOVA using PROC GLM. Statistical analysis of proportional data was performed following arcsine transformation. For proportional data, the reported least squares means ± standard error of the means are based on untransformed data, whereas the associated *P*-values are based on the arcsine-transformed data. The statistical models used for both analyses included the fixed effects of replicate, bull, treatment, and the bull × treatment interaction or replicate, semen type, treatment, and the semen type × treatment interaction.

Data for the effects of MCWF treatment on the numbers of total structures recovered, unfertilized oocytes, degenerate embryos, transferable embryos, and blastocysts are summarized in Table 1. The total number of structures recovered following superovulation did not differ between treatments. There was also no difference between the control and MCWF treatment groups for the number of unfertilized oocytes, degenerate embryos, transferable embryos, or blastocysts. Data for the proportion of total structures that were unfertilized oocytes, degenerate embryos, transferable embryos, and blastocysts can be found in Table 2. Administration of MCWF during the periovulatory period had no effect on the proportion of total structures that were unfertilized oocytes, degenerate embryos, or transferable embryos. However, there was a trend ($P < 0.10$) for the proportion of blastocysts recovered to be greater in animals treated with MCWF. The number and proportion of blastocysts recovered following superovulation was affected by bull ($P < 0.03$) and there was also a tendency ($P < 0.06$) for bull to affect the number of structures recovered, but there was no interaction between bull and treatment for any of the variables analyzed. Additionally, there was no effect of semen type or an interaction between semen type and treatment for any of the variables analyzed.

It is estimated that 60 to 80% of pregnancy loss in cattle occurs within the first month following conception (Diskin and Morris, 2008; Wiltbank et al., 2016) and these early pregnancy failures can contribute to significant economic losses in the cattle industry (De Vries, 2006; Cabrera, 2012). One strategy to improve early embryo survival is to manipulate the maternal reproductive tract environment to better support preimplantation development. Among the maternal factors that help to regulate preimplantation embryo development and survival are proinflammatory cytokines (Loureiro et al., 2009; Silva et al., 2020). It was hypothesized that stimulation of proinflammatory cytokines during the periovulatory period through administration of the immunostimulant MCWF could improve preimplantation embryo development. However, the results of the present study indicate that treatment of dairy heifer donors with MCWF had minimal impact on preimplantation embryo development following superovulation.

In general, the efficacy of superovulation as measured by the total number of structures and transferable stage embryos recovered was not affected by treatment with MCWF. No prior studies have evaluated the effects of administration of MCWF during the periovulatory period on in vivo embryo development and survival during the first week postfertilization. However, administration of MCWF at the initiation of follicle superstimulation in dairy heifers submitted to ovum pick-up tended to increase the number of transferable embryos produced following in vitro fertilization (Brown et al., 2024). There was a tendency for MCWF to increase the proportion of recovered structures that developed to the blastocyst stage, suggesting that alterations in the oviductal or uterine environment post-MCWF treatment could have enhanced embryo developmental kinetics.

One potential explanation for the lack of an effect of MCWF on most of the parameters of preimplantation embryo development evaluated in the present study is that there is no benefit to stimulation of proinflammatory cytokines when females are exposed to seminal plasma or sperm. Although the effects of seminal plasma on pregnancy establishment in cattle are inconsistent (Odhiambo et al., 2009; Ortiz et al., 2019), seminal plasma alters endometrial gene expression both in vitro (Ibrahim et al., 2019; Nongbua et al., 2020; Donnellan et al., 2021) and in vivo (Forde and Lonergan, 2012; Recuero et al., 2020). Moreover, exposure of bovine endometrial tissue to sperm induces a proinflammatory response through activation of the toll-like receptor 2 signaling pathway (Ezz et al., 2019; Akthar et al., 2021; Mansouri et al., 2023).

Levels of proinflammatory cytokines in blood or in uterine fluid following administration of MCWF were not evaluated, but it is possible that either MCWF treatment did not induce a proinflammatory response or the proinflammatory response induced by MCWF was insufficient to have an impact on the reproductive tract. Intraperitoneal administration of MCWF to mice during the periovulatory period induced an inflammatory response that resulted in increased spleen weights (Brown et al., 2023). However, 2 separate studies in cattle have reported limited effects of MCWF treatment on serum levels of specific proinflammatory cytokines. In one study in dairy heifers, i.m. administration of MCWF during the periovulatory period had no effect on serum levels of IL-1β, IL-6, TNF-α, and IFN-γ at 6 and 24 h posttreatment (Brown et al., 2024). In another study, treatment of beef heifers and steers with MCWF increased blood levels of TNF-α when administered s.c. but there was no effect on the levels of IL-1β, IL-6, and IL-12, and when administered i.m. there was no effect of MCWF on any of the proinflammatory cytokines evaluated (Alexander et al., 2022).

Studies evaluating the effects of MCWF on parameters of embryo development and survival in cattle are limited and the studies that have been conducted have yielded variable results (Palomino et al., 2021; Brown et al., 2024; Vidlund et al., 2023). While there are many potential reasons for the varying results observed with MCWF, one possibility is that the optimal dose of MCWF has yet to be identified. None of the studies cited above have tested a dose of MCWF other than 5 mL. It is also possible that the effectiveness of MCWF depends on the timing of administration, the particular reproductive outcome being evaluated, or both. At present, the half-life of MCWF has not been reported and this information could be useful in determining the optimal timing of MCWF administration. Moreover, the capacity of MCWF to induce a proinflammatory response may be dependent on the route of administration (Alexander et al., 2022; Brown, 2023). In studies in cattle evaluating the effects of MCWF on different reproductive outcomes, only i.m. administration of MCWF has been tested.

Overall, the results of the present study indicate that administration of MCWF during the periovulatory period has limited effects on the number of viable embryos recovered following superovulation when administered i.m. as a 5-mL dose. Prior studies in cattle have not directly evaluated the effects of MCWF treatment on in vivo embryo development during the first week following fertilization, and specific reasons for the lack of an effect of MCWF on embryo development following superovulation in the current experiment are unclear. Future research aimed at testing varying doses, as well as timing and routes of administration, may provide more clarity on the effectiveness of MCWF for improving bovine preimplantation embryo development.

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Nonstandard abbreviations used: CIDR = controlled internal drug-releasing device; CL = corpus luteum; DFR = dominant follicle removal; MCWF = mycobacterium cell wall fraction.