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Magnesium Sulfate Differentially Modulates Fetal Membrane Inflammation in a Time-Dependent Manner

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Abstract

Problem—Chorioamnionitis and infection-associated inflammation are major causes of preterm birth. Magnesium sulfate (MgSO₄) is widely used in obstetrics as a tocolytic, however, its mechanism of action is unclear. This study sought to investigate how MgSO₄ modulates infection-associated inflammation in fetal membranes (FMs), and whether the response was time dependent.

Method of Study—Human FM explants were treated with or without bacterial lipopolysaccharide (LPS); with or without MgSO₄ added either: 1 hr before LPS; at the same time as LPS; 1 hr post-LPS; or 2 hrs post-LPS. Explants were also treated with or without viral dsRNA and LPS, alone or in combination; and MgSO₄ added 1 hr post-LPS. After 24hrs, supernatants were measured for cytokines/chemokines; and tissue lysates measured for caspase-1 activity.

Results—LPS induced FM inflammation by upregulating the secretion of a number of inflammatory cytokines/chemokines. MgSO₄ administered 1 hr post-LPS inhibited FM secretion of IL-1 β , IL-6, G-CSF, RANTES and TNF α . MgSO₄ administered 2 hrs post-LPS augmented FM secretion of these factors as well as IL-8, IFN γ , VEGF, GRO α , and IP-10. MgSO₄ delivered 1 hr post-LPS inhibited LPS-induced caspase-1 activity, and inhibited the augmented IL-1 β response triggered by combination viral dsRNA and LPS.

Conclusions—MgSO₄ differentially modulates LPS-induced FM inflammation in a time-dependent manner, in part through its modulation of caspase-1 activity. Thus, the timing of MgSO₄ administration may be critical in optimizing its anti-inflammatory effects in the clinical setting. MgSO₄ might also be useful at preventing FM inflammation triggered by a polymicrobial viral-bacterial infection.

Keywords

Fetal membranes; Infection; Inflammation; Magnesium sulfate

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Introduction

Preterm birth affects 9.6% of US pregnancies¹ and is the major cause of neonatal morbidity and mortality. Currently there is no way to prevent preterm birth in the general population. However, MgSO₄ is used in routine obstetric practice as a tocolytic agent to suppress preterm labor by preventing preterm contractions. It is also used to prevent seizures in women with preeclampsia; and to prevent neurologic injury in fetuses threatening to deliver <32 weeks²⁻⁶. Despite its wide clinical use in high-risk pregnancies, our understanding of its mechanism of action are limited.

Bacterial infection-associated inflammation at the maternal-fetal interface is a major cause of preterm birth⁷. More recently, a role for viral infection in the predisposition of bacterial-induced preterm birth has been examined using experimental models⁸⁻¹⁰. Infection and/or inflammation of the fetal membranes (chorioamnionitis) is an important risk factor¹¹, complicating 40-70% of spontaneous premature births¹². A number of pro-inflammatory cytokines have been associated with chorioamnionitis and preterm birth, such as IL-1 β , IL-6, and TNF α ¹³. IL-1 β , in particular, is an important mediator^{11, 14-16}, and elevated inflammasome activity has been detected in fetal membranes from patients with preterm birth and acute histologic chorioamnionitis¹⁷.

Studies suggest that MgSO₄ has anti-inflammatory properties. In pregnant rats, MgSO₄ prevented bacterial lipopolysaccharide (LPS)-induced inflammation in the placenta, amniotic fluid, fetal brain, and maternal and fetal circulation^{18, 19}. In human placental perfusion studies, MgSO₄ reduced IL-1 β , TNF α and IL-6 production^{20, 21}. *In vitro*, MgSO₄ inhibited human term placental, decidual and amniotic cell cytokine production in response to LPS^{19, 22}. However, nothing is known about the effects on MgSO₄ on intact human chorioamniotic tissues. Therefore, using an established *in vitro* human fetal membrane (FM) explant system^{23,24-26}, we sought to: 1) characterize the effect of MgSO₄ on the FM response to bacterial infection using LPS as our model; and 2) determine whether altering the time of MgSO₄ administration with respect to LPS exposure altered the response. Based on our recent study showing that viral infection augments LPS-induced FM inflammation²⁶, we also examined the effect of MgSO₄ on FM responses to a polymicrobial infection using combination viral dsRNA (Poly(I:C)) and LPS as our model.

Materials and Methods

Human FM collection and preparation

Human FMs were collected from planned uncomplicated term (37-41 weeks) cesarean deliveries without labor or known infection/inflammation, as previously described^{24, 25}. The study, including tissue collection, was approved by the University's Human Research Protection Program. After washing the FM with sterile PBS supplemented with penicillin (100U/ml) and streptomycin (100 μ g/ml) (Gibco, Grand Island, NY), blood clots were removed and explants where both the chorion and amnion were intact were obtained using a 6mm biopsy punch. The FM explants were placed in 0.4 μ m cell culture inserts (BD Falcon, Franklin Lakes, NJ), with 500 μ l Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), and these were

placed in a 24-well plate containing 500µl of the same DMEM media for 24 hrs, as previously described^{23–25}. The next day the media was removed and replaced with serum-free OptiMEM (Gibco). After 3 hrs, treatments were initiated in serum-free OptiMEM.

Human FM treatments

FM explants were treated with or without LPS isolated from *Escherichia coli* 0111:B4 (Sigma-Aldrich, St Louis, MO) at 1ng/ml. Magnesium sulfate (MgSO₄; 5mM) from Sigma Aldrich (St Louis, MO) was added to the explants either: 1 hr before LPS; at the same time as LPS; 1 hr post-LPS; or 2 hrs post-LPS. Explants were incubated for 24 hrs after which supernatants and tissues were collected, snap frozen, and stored at –80°C. For some experiments, FM explants treated with or without the viral dsRNA mimic, Poly(I:C) (20µg/ml) for 24 hrs. LPS (1ng/ml) was then added or not, and MgSO₄ (5mM) was added 1 hr post-LPS. The FMs were then incubated for another 24 hrs after which, supernatants and FM tissues were collected, snap frozen, and stored at –80°C.

Cytokine/chemokine analysis

Supernatants were measured for IL-1β by ELISA (R&D Systems, Minneapolis, MN). The following cytokines/chemokines were measured by multiplex analysis (BioRad, Hercules, CA): IL-1β, IL-6, IL-8, IL-10, IL-12, IL-17, granulocyte-colony stimulating factor (G-CSF), interferon gamma (IFN-γ), monocyte chemotactic protein-1 (MCP-1/CCL2), macrophage inflammatory protein-1 beta (MIP-1β/CCL4), regulated on activation, normal T cell expressed and secreted (RANTES/CCL5), tumor necrosis factor alpha (TNFα) vascular endothelial growth factor (VEGF), growth regulated oncogene-alpha (GROα) and interferon gamma-induced protein 10 (IP-10/CXCL10)^{24, 25}.

Caspase-1 activity assay

Caspase-1 activity was determined using the Caspase-Glo 1 Inflammasome assay (Promega, Madison, WI). Briefly, 10µg of the FM tissue lysates were incubated at room temperature in the dark for 1 hr with the caspase-1 substrate. Luminescence was measured using a TD-20/20 illuminometer (Turner Designs). The amount of luminescence detected as relative light units (RLU) was proportional to caspase-1 activity. All samples were assayed in triplicate.

Statistical analysis

Each FM treatment experiment was performed in triplicate. All data are reported as mean ± standard error of the mean (SEM) of pooled experiments. Statistical significance was set at $p < 0.05$ and determined using Prism Software (Graphpad, Inc; La Jolla, CA). For normally distributed data, significance was determined using ANOVA or a paired t-test. For data not normally distributed, significance was determined using a non-parametric test or the Wilcoxon matched-pairs signed rank test.

Results

MgSO₄ differentially modulates LPS-induced fetal membrane IL-1 β secretion in a time-dependent manner

As previously reported^{24, 26}, bacterial LPS significantly increased FM IL-1 β secretion compared to the no treatment (NT) control (Figure 1). FM secretion of IL-1 β under NT control conditions was either unaffected by MgSO₄ or significantly inhibited by 65.8 $\pm$ 13.4% (when given at the same time as LPS) or 38.3 $\pm$ 20.0% (when given 1 hr post-LPS) (Figure 1). Treatment of FMs with MgSO₄ had differential effects on the LPS-induced IL-1 β response, and this was dependent upon the timing of MgSO₄ delivery. As shown in Figure 1, when MgSO₄ was delivered either 1 hr before or at the same time as LPS, IL-1 β levels were not significantly different when compared to LPS alone. MgSO₄ given 1 hr post-LPS significantly inhibited FM IL-1 β secretion by 31.1 $\pm$ 9.4% when compared to LPS alone. Treatment with MgSO₄ 2 hrs post-LPS augmented FM IL-1 β secretion by 1.4 $\pm$ 0.3 fold, although this did not reach significance (Figure 1).

MgSO₄ differentially modulates LPS-induced fetal membrane cytokine/chemokine secretion in a time-dependent manner

Having observed a differential effect of MgSO₄ on FM LPS-induced FM IL-1 β secretion when given either 1 or 2 hrs post-LPS, supernatants were measured for the secretion of IL-1 β and a wider panel of cytokines and chemokines using multiplex analysis. Compared to the NT control, LPS significantly increased FM secretion of IL-1 β , IL-6, G-CSF, RANTES, TNF α , IL-10, IL-8, IFN γ , VEGF, GRO- α , IP-10, IL-12, IL-17, MIP-1 β and MCP-1 (Figure 2). The only significant effect of MgSO₄ on basal FM cytokine/chemokine secretion was a slight reduction in IL-17 and MCP-1 levels (Figure 2). As detected by ELISA (Figure 1), MgSO₄ given 1 hr post-LPS significantly inhibited FM IL-1 β secretion by 23.6 $\pm$ 0.9% when compared to LPS alone, while treatment with MgSO₄ 2 hrs post-LPS significantly augmented FM IL-1 β secretion by 1.3 $\pm$ 0.0 fold (Figure 2). A similar differential response was observed for IL-6, G-CSF, RANTES and TNF α . When compared to LPS alone, MgSO₄ given 1 hr post-LPS significantly inhibited FM secretion of IL-6 by 17.7 $\pm$ 0.2%; G-CSF by 28.2 $\pm$ 1.3%; RANTES by 30.0 $\pm$ 1.9%; and TNF α by 23.0 $\pm$ 3.0% (Figure 2). Compared to LPS alone, treatment with MgSO₄ 2 hrs post-LPS significantly augmented FM secretion of IL-6 by 1.9 $\pm$ 0.0 fold; G-CSF by 1.3 $\pm$ 0.0 fold; RANTES by 2.4 $\pm$ 0.3 fold; and TNF α by 2.2 $\pm$ 0.0 fold (Figure 2). MgSO₄ given 1 hr post-LPS significantly inhibited FM IL-10 secretion by 22.5 $\pm$ 3.5% when compared to LPS alone, while treatment with MgSO₄ 2 hrs post-LPS had no effect on IL-10 (Figure 2). FM secretion of IL-8, IFN γ , VEGF, GRO α , and IP-10 in response to LPS were unaffected by MgSO₄ given 1 hr post-LPS. However, when compared to LPS alone, treatment with MgSO₄ 2 hrs post-LPS significantly augmented FM secretion of IL-8 by 2.3 $\pm$ 0.1 fold; IFN γ by 1.3 $\pm$ 0.0 fold; VEGF by 1.3 $\pm$ 0.0 fold; GRO α by 1.9 $\pm$ 0.1 fold; and IP-10 by 1.3 $\pm$ 0.0 fold (Figure 2).

MgSO₄ inhibits LPS-induced FM caspase-1 activity

Given the importance of IL-1 β in FM responses to infection or infectious products^{24, 26, 27–29}, and in infection-associated preterm birth^{11, 14–16}, the effect of MgSO₄ on the mechanisms governing IL-1 β production was examined. FM IL-1 β secretion in

response to infectious stimuli is mediated by the inflammasome^{24, 26}, where caspase-1 is a common marker of inflammasome activity³⁰. As shown in Figure 3, LPS treatment significantly increased FM caspase-1 activity by 2.7 ± 0.6 fold, and this was significantly inhibited $36.5 \pm 10.9\%$ by MgSO_4 given 1 hr post-LPS (Figure 3). While MgSO_4 given 2 hrs post-LPS also inhibited LPS-induced FM caspase-1 activity levels were significantly 2.4 ± 0.3 fold higher than MgSO_4 given 1 hr post-LPS (Figure 3). In the absence of LPS, MgSO_4 given at the 2 hr time point significantly increased FM caspase-1 activity by 4.3 ± 1.2 fold (Figure 3).

MgSO_4 inhibits FM IL-1 β secretion in response to combination viral dsRNA and bacterial LPS

A viral infection may sensitize pregnancies to a subsequent bacterial challenge, leading to preterm birth^{8–10}. Recent studies have highlighted a role for viral infections in modulating FM and placental responses to bacterial LPS^{8, 9, 26}. Using this model, we sought to test the effect of MgSO_4 when given 1 hr post-LPS. As previously reported²⁶, treatment of FMs with combination Poly(I:C) [a viral dsRNA mimic] and bacterial LPS, significantly and synergistically augmented FM IL-1 β secretion by 5.0 ± 2.7 fold when compared to LPS alone (Figure 4). MgSO_4 significantly inhibited the LPS, and the combination Poly(I:C) and LPS, induced FM IL-1 β responses by $41.4 \pm 22.7\%$ and $47.7 \pm 13.5\%$, respectively (Figure 4).

Discussion

Magnesium sulfate is used in obstetrics for several indications: as a short-term tocolytic; to prevent maternal seizure; and to protect the preterm neonatal brain^{2–6}. Magnesium deficiency is associated with increased levels of inflammatory cytokines such as IL-6, IL-1 β and TNF α ^{31, 32}. However, despite MgSO_4 being studied as an inflammatory modulator for decades^{33, 34}, its mechanism of action in obstetrical use is still unclear. Since chorioamnionitis is a major risk factor for preterm birth, the objective of this study was to determine whether MgSO_4 could modulate FM inflammation in response to infectious products, and whether timing of MgSO_4 exposure was important using an *in vitro* human FM explant system.

In seeking to investigate if MgSO_4 modulation of FM inflammation was time dependent we chose to deliver MgSO_4 post-LPS exposure in order to model the clinical scenario. However, we also examined other time points. We found no effect on LPS-induced FM IL-1 β production when MgSO_4 was delivered prior to, or at the same time as, LPS. However, MgSO_4 did modulate FM responses when given 1 hr or 2hrs post-LPS. MgSO_4 given 1 hr post-LPS inhibited inflammatory cytokine secretion by FM, while MgSO_4 given 2 hrs post-LPS augmented the FM inflammatory response to bacterial LPS. Specifically, LPS-induced FM secretion of the preterm-associated cytokines, IL-1 β , IL-6 and TNF α ¹³, were differentially modulated in this way, as well as the chemokines G-CSF and RANTES, and the anti-inflammatory cytokine, IL-10. In addition, the inflammatory cytokines and chemokines: IL-8, IFN γ , VEGF, GRO α , and IP-10 were only augmented by MgSO_4 when given 2 hrs post-LPS.

The inhibition of LPS-induced FM inflammation by MgSO₄ is in keeping with a number of other *in vitro* studies. In human placental perfusion studies, MgSO₄ reduced IL-1 β , TNF α and IL-6 production^{20, 21}. MgSO₄ inhibited LPS-induced TNF α mRNA expression in the BeWo trophoblast cell line³⁵. In human term placental cells, MgSO₄ inhibited IL-6 and TNF α secretion in response to LPS¹⁹. While this study also observed a decreased in MCP-1 production, in our studies FM MCP-1 was not modulated by MgSO₄. Similarly while, MgSO₄ inhibited human term placental decidual and amniotic cell IL-8 secretion in response to LPS²², in our studies FM IL-8 production was only augmented by MgSO₄. These differences are likely a reflection of the specific cell types studied (placental, decidual amniotic epithelial)^{19, 22}, compared with our system of intact chorioamniotic explants.

The differential effects of MgSO₄ depending upon the timing of administration has also been examined by other groups. In a pregnant rat model of LPS-induced inflammation, MgSO₄ inhibited maternal serum and amniotic fluid levels of TNF α , IL-6, MCP-1 and GRO/KC when given both before and after LPS; and inhibited maternal serum TNF α and amniotic fluid GRO/KC when only given prior to LPS exposure. When MgSO₄ was given after LPS treatment there was no effect on the LPS-induced inflammation¹⁸. In a similar model, placental IL-6, TNF α and MCP-1 levels were inhibited when MgSO₄ was given both prior to and after LPS¹⁹. In another study using isolated umbilical cord blood monocytes stimulated with LPS, supplementation of MgSO₄ 15 minutes post-LPS exposure reduced TNF α and IL-6 levels, while pre-exposure to MgSO₄ had no effect³⁶. This *in vitro* study closely correlates with our inhibitory findings. Furthermore, this study found that the time-dependency of MgSO₄'s anti-inflammatory properties was directly associated with the rapid elevation in intracellular magnesium levels following supplementation, and inhibition of the NF κ B pathway^{36, 37}. This suggests that in human FM explants, when MgSO₄ is given 1 hr post-LPS there may be a similar rapid uptake and increase in intracellular magnesium content, leading to the observed inhibitory effects, and that based on our pre-treatment data this would not be sufficiently sustained over a 1 hr period in order to block the ability of LPS to initiate the inflammatory cascade.

Since IL-1 β is a potent pro-inflammatory cytokine and mediator of preterm birth^{11, 14–16}, we examined the mechanism by which MgSO₄ modulated this response. Caspase-1 is a common mediator of IL-1 β processing and subsequent secretion, typically activated by an inflammasome³⁰. We found that MgSO₄ delivered 1 hr post-LPS treatment inhibited LPS-induced caspase-1 activity, suggesting this was one mechanism by which MgSO₄ reduced IL-1 β production. When MgSO₄ was given 2 hrs post-LPS treatment, caspase-1 levels were significantly higher both alone and with LPS compared to the 1 hr post-LPS levels. MgSO₄ modulation of caspase-1 activity might explain, in part, how the IL-1 β response was augmented at this later time point. How MgSO₄ delivered 2 hrs post-LPS is able to have a stimulatory effect on FM secretion of other cytokines/chemokine is still not fully understood.

In keeping with our observation that MgSO₄ can inhibit FM caspase-1 activation in response to LPS, a study using THP-1 cells reported that MgSO₄ can inhibit the NLRP3 inflammasome³⁸. We previously reported that a viral infection of the FM can augment LPS-induced IL-1 β production through activation of the NLRP3 inflammasome²⁶. This combined

with an increasing awareness of the potential impact viral infections may have on the maternal-fetal interface, and on pregnancy outcomes^{10, 39, 40}, prompted us to test the effects of MgSO₄ using this model. We report herein that MgSO₄ given one hr post-LPS inhibited the augmented FM inflammation induced by combination viral dsRNA and bacterial LPS.

In summary, MgSO₄ differentially modulates bacterial LPS-induced human fetal membrane inflammation in a time-dependent manner, in part through modulation of caspase-1 activity. Thus, the timing of MgSO₄ administration may be critical in optimizing its anti-inflammatory effects in the clinical setting. MgSO₄ might also be useful at preventing FM inflammation triggered by a polymicrobial viral-bacterial infection. Further studies are warranted to elucidate the time-dependent molecular mechanisms by which MgSO₄ modulates FM inflammation.

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Figure 1. MgSO₄ differentially modulates LPS-induced fetal membrane IL-1 β secretion in a time-dependent manner

Human FM explants were treated with no treatment (NT) or LPS (1ng/ml). MgSO₄ (5mM) was added either: 1 hr pre-LPS treatment; at the same time as LPS treatment; 1 hr post-LPS; or 2 hrs post-LPS. After 24 hrs, supernatants were collected and measured for IL-1 β by ELISA. * p <0.05 relative to the NT control unless otherwise indicated. Data are pooled from 4-10 independent experiments.

Figure 2. MgSO₄ differentially modulates LPS-induced fetal membrane cytokine/chemokine secretion in a time-dependent manner

Human FM explants were treated with no treatment (NT) or LPS (1ng/ml). MgSO₄ (5mM) was added either 1 hr post-LPS or 2 hrs post-LPS. After 24 hrs, supernatants were collected and measured for a panel of cytokines/chemokines by multiplex analysis. **p* < 0.05 relative to the NT control unless otherwise indicated. Data are pooled from 4 independent experiments.

Figure 3. MgSO₄ modulates LPS fetal membrane caspase-1 activity

Human FM explants were treated with no treatment (NT) or LPS in the presence of media; MgSO₄ added 1 hr post-LPS or MgSO₄ 2 hrs added post-LPS. After 24 hrs, tissue lysates were collected, homogenized for protein and caspase-1 activity measured. * $p < 0.05$ relative to the NT/media control unless otherwise indicated. Data are pooled from 10 independent experiments.

Figure 4. MgSO₄ inhibits FM IL-1 β secretion in response to combination viral dsRNA and bacterial LPS

Human FM explants were treated with NT, LPS (1ng/ml), Poly(I:C) (20 μ g/ml) the the presence of media or MgSO₄ (5mM). MgSO₄ was delivered 1 hr post-LPS. Supernatants were measured for IL-1 β by ELISA. * $p < 0.05$ relative to the NT control unless otherwise indicated. Data are pooled from 6 independent experiments.