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Objective: Women in preterm labor are commonly treated with magnesium sulfate (MgSO4) and betamethasone (BMTZ) to reduce complications of prematurity including neurologic injury. MgSO4, however, prevents cerebral palsy in only 1 in 40 women who receive it. Understanding the cellular responses to MgSO4/BMTZ could allow for more effective treatments. Nur77 is a nuclear receptor implicated in apoptosis and immune responses – processes that occur in neuroinflammation. Our objective was to investigate if Nur77 is increased in the setting of inflammation-induced preterm labor and to determine the impact of MgSO4 and BMTZ treatments. Methods: Using an established mouse model, we induced inflammation in pregnant mice using lipopolysaccharide (LPS) then randomized them for treatment with MgSO4 and/or BMTZ or saline. Treatment with MgSO4 and BMTZ in our model significantly reduced Nur77 expression. This finding suggests one mechanism by which these medications prevent neurologic injury in the preterm infant.

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TITLE

Magnesium sulfate and betamethasone reduce *NUR77* expression in a preterm labor mouse model

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ABSTRACT

Objective: Women in preterm labor are commonly treated with magnesium sulfate ($MgSO_4$) and betamethasone (BMTZ) to reduce complications of prematurity including neurologic injury. $MgSO_4$, however, prevents cerebral palsy in only 1 in 40 women who receive it. Understanding the cellular responses to MgSO4/BMTZ could allow for more effective treatments. *Nur77* is a nuclear receptor implicated in apoptosis and immune responses – processes that occur in neuroinflammation. Our objective was to investigate if *Nur77* is increased in the setting of inflammation-induced preterm labor and to determine the impact of $MgSO_4$ and BMTZ treatments. Methods: Using an established mouse model, we induced inflammation in pregnant mice using lipopolysaccharide (LPS) then randomized them for treatment with $MgSO_4$ and/or BMTZ or saline. Fetal brains from the E15 group were harvested six hours after surgery for microarray and confirmatory quantitative-RT-PCR. Brains from the E17 group were collected on post-natal day 15 for histological analysis. Results: Exposure to LPS resulted in a significant increase in inflammatory related genes and *Nur77* expression vs. placebo ($p<0.05$). In contrast, treatment with $MgSO_4$ and/or BMTZ reduced this expression pattern to levels similar to the uninjured controls. Histological analysis of brains from LPS treated pups that were given MgSO4and BMTZ showed reductions in glial cell infiltration and neuronal architecture akin to the uninjured control mice. Conclusion: Treatment with $MgSO_4$ and BMTZ in our model significantly reduced *Nur77* expression. This finding suggests one mechanism by which these medications prevent neurologic injury in the preterm infant. Our next step is to use a model in which the *Nur77* gene is suppressed to further elucidate the role of this receptor in neuroinflammation.

DISCLOSURES/DISCLAIMER

The views expressed in this article are those of the author(s) and do not necessarily reflect the official policy or position of the Department of the Navy, Department of the Army, Department of the Air Force, Department of Defense, or the United States Government. Several authors are military service members. This work was prepared as part of their official duties. Title 17 U.S.C. 105 provides that 'Copyright protection under this title is not available for any work of the United States Government.' Title 17 U.S.C. 101 defines a United States Government work as a work prepared by a military service member or employee of the United States Government as part of that person's official duties. Animals involved in this study were maintained in accordance with the 'Guide for the Care and Use of Laboratory Animals' published by the National Research Council/Institute of Laboratory Animal Research (ILAR).

INTRODUCTION

Preterm labor and delivery remain a common problem worldwide despite considerable medical advances. The underlying mechanism of preterm labor is incompletely understood, however, intrauterine inflammation – whether from overt infection or a more insidious process – leading to cervical shortening and contractions may play a significant role (1). The cost of preterm delivery is dramatic, estimated at $26 billion annually (2), and the long term implications are profound. Cerebral palsy (CP), a non-progressive mainly motor disorder, is one prominent example. Twelve percent of infants born prior to 27 weeks gestation develop CP (3) and the lifetime cost of caring for a single person with this condition was calculated in excess of $900,000 in 2003 (4).

To date, interventions employed in the setting of overt or suspected preterm labor focus on one of two objectives: (1) inhibiting the process and (2) mitigating the complications (5). The former includes tocolysis, progesterone therapy, and cerclage. Historically, the latter has consisted almost exclusively of corticosteroid therapy. Administration of steroids including betamethasone (BMTZ) and dexamethasone to a woman in preterm labor accelerates fetal lung maturation and reduces the incidence of intraventricular hemorrhage (IVH) and necrotizing enterocolitis (NEC) (6). Steroids also have known anti-inflammatory properties.

More recently, magnesium sulfate ($MgSO_4$) for neuroprotection has been added to the clinician's preterm labor armamentarium. While observational studies dating back to the 1990s (7) suggest a possible neuroprotective benefit of $MgSO_4$, widespread clinical use was spurred by meta-analysis of several large randomized controlled trials (8) and a joint statement by the

American College of Obstetricians and Gynecologists and the Society for Maternal Fetal Medicine, most recently in 2013 (9).

The precise mechanism by which $MgSO_4$ exerts a neuroprotective role remains unknown and 46 women in preterm labor must receive the agent to prevent a single case of cerebral palsy (8). Some research (10) suggests potential adverse neonatal effects from $MgSO_4$ administration and maternal toxicity is an established serious complication. Understanding how $MgSO_4$ works may lead to the development of a more targeted therapy with fewer sequelae.

Nur77 (also known as NR4A1) is an example of one such potential target. The protein is an orphan nuclear receptor. Its superfamily has been linked to such diverse conditions as malignancy, diabetes, and atherosclerosis (11) but not perinatal brain injury. *Nur77* in particular appears to play a role in apoptosis by targeting the mitochondria leading to cytochrome C release and cell death (12). Our objective was to investigate if *Nur77* is increased in the setting of inflammation-induced preterm labor and to determine the impact of $MgSO_4$ and BMTZ treatments. We hypothesize that these agents exert a neuroprotective effect in part my inhibiting *Nur77* activation and thereby reducing apoptosis of neuronal cells.

MATERIALS & METHODS

To test our hypothesis, we employed an established mouse model of inflammation-based preterm labor and perinatal brain injury (13). This protocol was approved by our Institutional Animal Care and Use Committee (IACUC) and performed under the auspices of an attending veterinarian. The experiment consisted of two phases. In the first phase, 96 CD1 timed pregnant mice were shipped from Charles River Laboratories (Wilmington, MA) and arrived at our facility for a period of acclimation. On day 15 of a 19-21 day gestation, each animal was anesthetized and a vertical midline laparotomy performed using the technique pioneered by Elovitz et al (13). Mice were randomized to receive an intrauterine injection between the two gestational sacs of either 100 μg lipopolysaccharide (LPS, N=48) derived from *Escherichia coli*, or an equivalent volume of phosphate buffered saline (PBS, N=48). Following injection, the incision was closed and the animal was awakened from anesthesia and recovered in single cage housing.

Thirty minutes after surgery, animals were further randomized to receive subcutaneous $MgSO_4$, BMTZ, a combination of the two, or an equivalent volume of placebo (normal saline). The timing of therapy was selected based on prior research demonstrating an increase in serum cytokines 30 minutes following administration of LPS (14). $MgSO_4$was dosed using the Hallak protocol (15) with administration of a 270 mg/kg loading dose followed by 27 mg/kg every 20 minutes for four hours. This regimen achieves a serum concentration of magnesium sulfate of 3.1 mEq/L which is equivalent to the third quartile (2.6-3.2 mEq/L) of cord blood levels in the largest randomized controlled trial assessing $MgSO_4$ for neuroprotection (16). Animals randomized to receive BMTZ were administered a 0.1 mg subcutaneous dose based on a prior study demonstrating efficacy (17).

Six hours after surgery, dams were euthanized via cervical dislocation under sedation by trained veterinarian staff. We selected this endpoint to ensure collection of tissue prior to delivery and cannibalization of the non-viable offspring. Fetal brains were extracted and flash frozen. The four fetal brains closest to the injection site from 24 litters (four from each group) were processed for gene array using the GeneChip Mouse Gene 2.0 ST Array. *Nur77* gene expression significance was determined by ANOVA between treatment groups, following quality control and analysis of the microarray data. Microarray results were verified using quantitative real time polymerase chain reaction (RT-qPCR). Total RNA from the same samples used for the microarray analysis was converted to cDNA and analyzed for Nur77 by SYBR-green using the following conditions: 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 10 seconds. Relative expression for *Nur77* and inflammatory related genes was normalized to ribosomal *18s* by delta Ct analysis. Primers for *Nur77* were derived from the Harvard Primer Bank (ID# 6754216a1) and *18s* was previously reported by Au CG. et al, 2011 (18). Inflammatory related genes *Plp1*, *Il1b*, *Il6*, *Tlr4*, and *Tnfa* were detected using Roche commercially available probes. Statistical significance between groups in for the RT-qPCR analyses was evaluated by student's *t*-test.

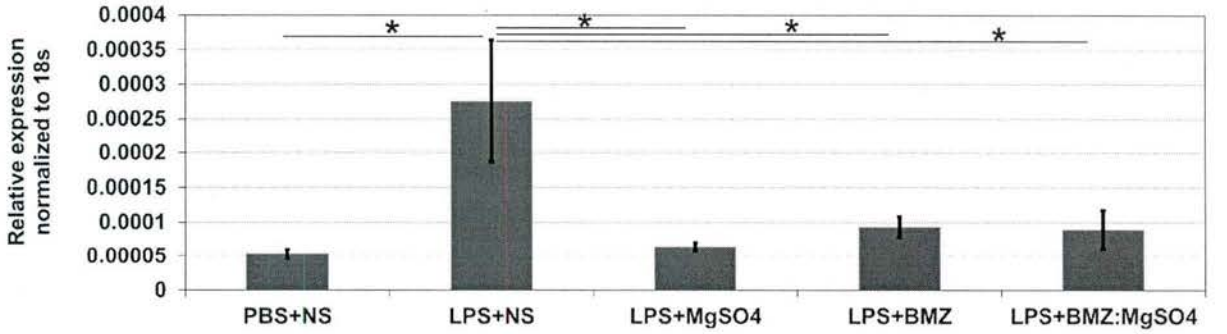
In the second phase of this experiment, we investigated histological differences between treatment groups. A separate cohort of timed pregnant mice (n=4 per condition) was shipped to our facility and randomized to undergo surgery on day of gestation 17 with intrauterine injection of a lower dose of LPS (50 μg) or PBS as outlined above followed by $MgSO_4$, BMTZ,

combination treatment, or placebo. Surgery at this more advanced gestational age with a lower dose of LPS ensured survival of some offspring and preserved the intracranial architecture for histological analysis. Mice were closely monitored until delivery which occurred within 24h of surgery for all groups. Surviving offspring were reared until post-natal day 15 then euthanized using Fatal-Plus. Brains from these animals were harvested and processed for histology. Briefly, brains were fixed in 4% formaldehyde for 2 hours, run through a sucrose gradient beginning with 10%, 20% then left overnight in 30% at 4°C. Brains were then embedded and frozen in isopentane cooled OCT and transverse sections were subsequently cut 8μm thick for immunostaining. Sections were first blocked with 10% donkey serum in 1%BSA for 30 minutes prior to the addition of antibodies. Glial were identified by staining with polyclonal chicken anti-GFAP at a dilution of 1:4000, and neurons with polyclonal rabbit anti-NeuN diluted1:1000; both from Abcam and applied overnight at 4°C. Donkey secondary antibodies against chicken conjugated to Alexa Fluor 488 and rabbit conjugated to Alexa Fluor 555 (both from Jackson Immunoresearch), were diluted 1:1000 and applied for 1 hour before counterstaining with DAPI.

RESULTS

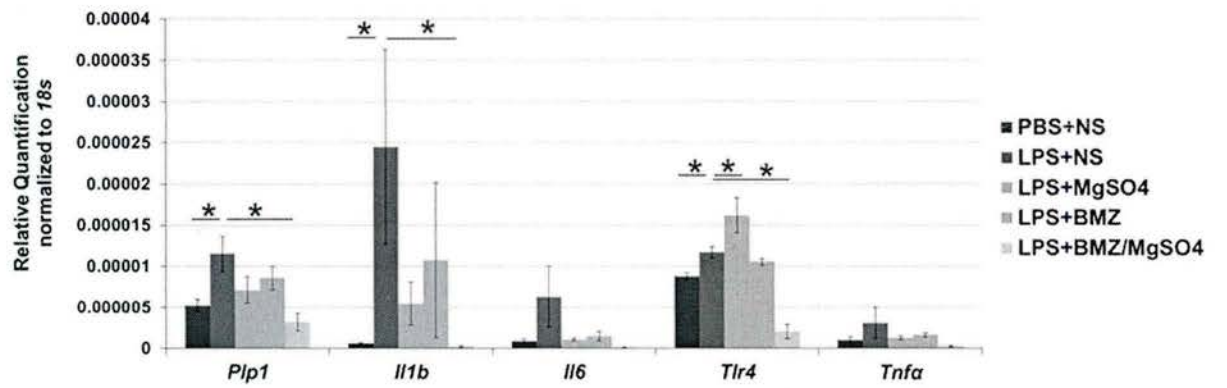
By microarray analysis, brains from pups exposed to LPS and harvested on day of gestation 15 demonstrated a 4.6 increase in *Nur77*expression over PBS controls. Pups of dams who received LPS and $MgSO_4$ had a 4-fold decrease in *Nur77* expression over LPS treated brains that received normal saline. Pups of dams who received BMTZ also demonstrated a reduction in *Nur77* expression, 2.6 fold lower vs. LPS treated brains that received normal saline. Likewise MgSO4 in combination with BMTZ resulted in a 3 fold reduction in *Nur77*. This observed pattern of elevated *Nur77* mRNA levels and decline with the addition of $MgSO_4$/BMTZ was significantly different ($p<0.05$) in the background of LPS injury. Subsequent validation by RT-qPCR the substantiated that *Nur77* is not different between $MgSO_4$/BMTZ treatments and the PBS control, but significantly elevated with LPS injury alone (Figure 1.* denote $p<0.05$).

Figure1. quantitative reverse transcription-PCR for *Nur77* in embryonic brains collected at E15 post in utero post injury and treatments



Further analysis indicated that *Nur77* expression, coincided with the upregulation of inflammatory related genes expected to rise with LPS injury (19) (Figure 2. * denote $p<0.05$). Although all genes examined showed increases, only *Plp1*, *Il1b*, and *Tlr4* were significantly elevated with LPS and normal saline in our analysis. Interestingly, alterations in the expression of these same genes was treatment dependent. Specifically, *Tlr4* was elevated with $MgSO_4$ but declined with the addition of BMTZ. In contrast, *PlP1* and *Il1b* were only significantly different when both MgSO4 and BMTZ were administered. Such results suggest that these treatments have individual and synergistic effects on neuroinflammation that may involve the ablation of *Nur77* signaling.

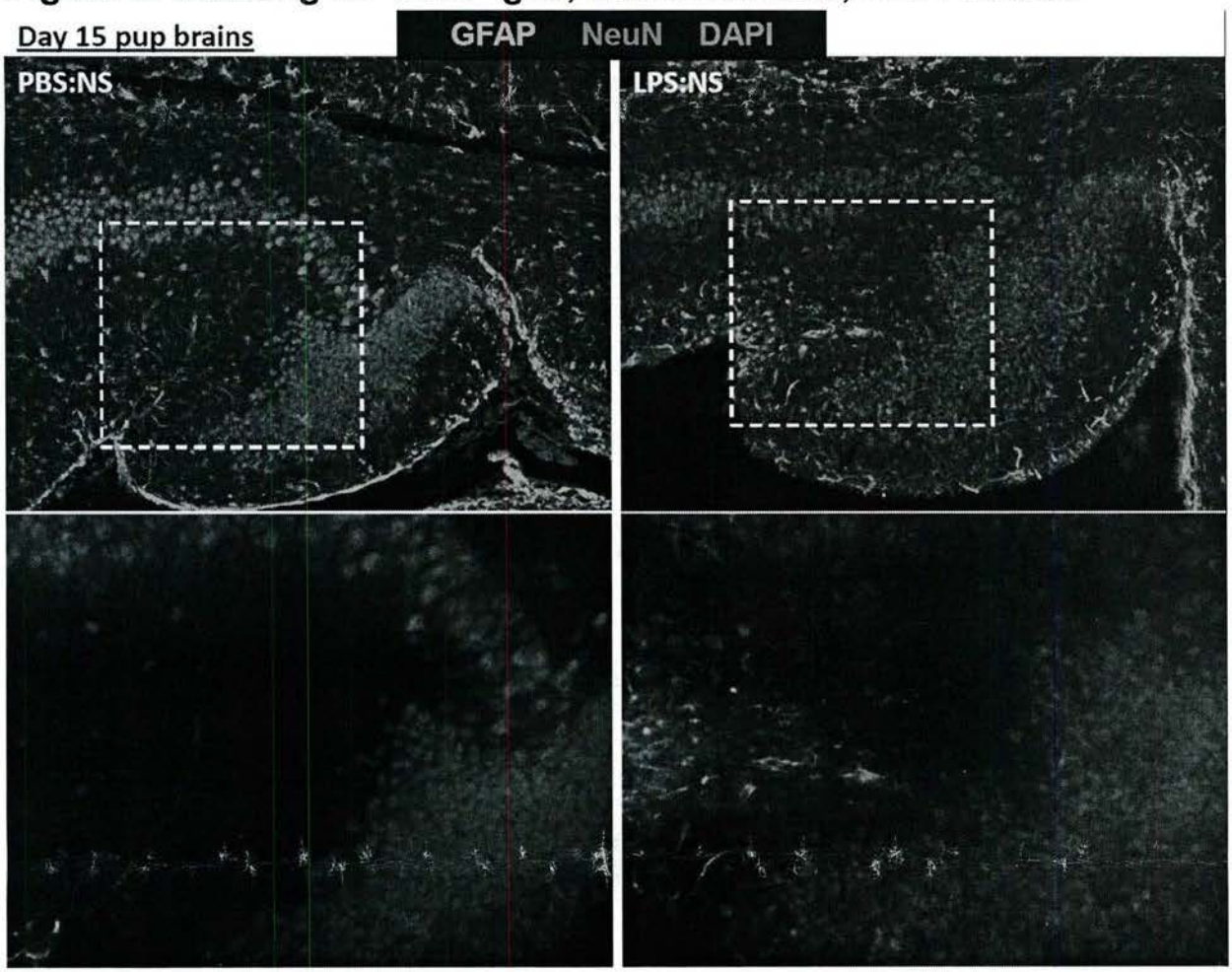
Figure2. quantitative reverse transcription-PCR for inflammatory genes in embryonic brains collected at E15 post in utero post injury and



Histological analysis of pup brains obtained on post-natal day 15 supported the above findings. Animals exposed *in utero* to LPS demonstrated increased glial cell infiltration indicated by greater GFAP labeling in the hippocampus as compared to PBS controls (Figure 3. Dotted box outlines higher magnification photo shown in second row of the selected regions).

This was accompanied by alterations in neural architecture visualized by staining for NeuN. In contrast, treatment with $MgSO_4$ and BMTZ appeared to mitigate the expansion of glia and loss of neurons in the hippocampus. Such results provide confirmation of our injury model and suggest that the protective effects of these treatments may directly or indirectly be a result of preserving the cellular architecture.

Figure 3: Staining for GFAP glia, NeuN neurons, DAPI nuclei.



DISCUSSION & CONCLUSION

Our study demonstrates that exposure to intrauterine inflammation in a preterm labor mouse model leads to increased *NUR77* expression at the gene and messenger RNA levels. Research in other fields suggests that this orphan nuclear receptor plays a key role in apoptosis and the immune responses; however, it has not previously been linked to perinatal brain injury. Treatment with either $MgSO_4$, BMTZ, or a combination of the two – therapies commonly used to

mitigate complications of preterm delivery – reduced expression of *Nur77*. The decrease in *Nur77* correlated with reduced evidence of cerebral injury on histologic analysis.

The mechanism by which $MgSO_4$ exerts a neuroprotective effect remains unknown though it may work in part by antioxidant and/or anti-inflammatory effects. Ongoing research suggests that *Nur77* plays a complex role in other inflammatory states such as sepsis and has pro and anti-inflammatory effects downstream (20). Understanding this nuclear receptor's role may provide greater insight into the pathophysiology of inflammation-induced perinatal brain injury and serve as a better pharmacologic target than current available therapies.

Our study has several limitations. The animal model we used is designed to replicate neuroinflammation observed in humans. Because pups exposed to this dose of LPS at 15 days gestation are not viable, specific neonatal sequelae including cerebral palsy cannot be assessed. Additionally, the fetal mouse brain is significantly less developed than its human counterpart and substantial maturation, including development of a more robust blood brain barrier, occurs in the post-natal period. Generalization of this study's findings to the human fetus should be approached with caution and prior research demonstrating the impact of *Nur77* on LPS induced sepsis does not rule out the possibility that this finding is unique to LPS exposure and may not persist in other forms of preterm labor.

Our study has several strengths. We used an established animal model that induces preterm labor through inflammation rather than hormone withdrawal or direct infection which more accurately represents our understanding of preterm labor in humans. While the heterogeneity of this outbred animal strain can impact the effect in a small sample size, it also reflects the variation seen in human populations. Additionally, we took care to base the timing and dosing of interventions on previously published data. Finally, our analysis of open source microarray data (NCBI GEO) derived from an analogous study published by Oskvig DB et al. 2012, indicates that *Nur77* expression is also elevated in rat neonatal brains isolated from dams treated with LPS in utero (21). Therefore, it is less likely that our results are an artifact of the mouse model and merit further research in order to establish a translational link to human neuroinflammation.

Treatment with $MgSO_4$and BMTZ in this animal model significantly reduced *NUR77* expression. This finding suggests a synergistic mechanism by which these medications prevent neurologic injury in the preterm infant. Our next step is to use a model in which the *NUR77* gene is suppressed to further elucidate the role of this receptor in neuroinflammation and to study the precise interaction between these agents using cell cultures.

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