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Maternal Minocycline as Fetal Therapy in a Rat Model of Myelomeningocele

Juan C. Biancotti, PhD,[1] *Anne M. Sescleifer, MD,*[1] *Shelby R. Sferra, MD, MPH, Annalise B. Penikis, MD, Kyra M. Halbert-Elliott BS, Ciaran R. Bubb, and Shaun M. Kunisaki, MD, MSc*[*]

Division of General Pediatric Surgery, Department of Surgery, Johns Hopkins School of Medicine, Baltimore, Maryland

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ABSTRACT

Introduction: This study aimed to investigate whether the maternal administration of minocycline, a tetracycline antibiotic known to have anti-inflammatory and neuroprotective properties in models of neural injury, reduces inflammation and neural cell death in a fetal rat model of myelomeningocele (MMC).

Methods: E10 pregnant rats were gavaged with olive oil or olive oil + retinoic acid to induce fetal MMC. At E12, the dams were exposed to regular drinking water or water containing minocycline (range, 40-140 mg/kg/day). At E21, fetal lumbosacral spinal cords were isolated for immunohistochemistry and quantitative gene expression studies focused on microglia activity, inflammation, and apoptosis ($P < 0.05$).

Results: There was a trend toward decreased activated Iba1+ microglial cells within the dorsal spinal cord of MMC pups following minocycline exposure when compared to water (H_2O) alone ($P = 0.052$). Prenatal minocycline exposure was correlated with significantly reduced expression of the proinflammatory cytokine, *IL-6* (minocycline: 1.75 *versus* H_2O: 3.52, $P = 0.04$) and apoptosis gene, *Bax* (minocycline: 0.71 *versus* H_2O: 1.04, $P < 0.001$) among MMC pups.

Conclusions: This study found evidence that the maternal administration of minocycline reduces selected markers of inflammation and apoptosis within the exposed dorsal spinal cords of fetal MMC rats. Further study of minocycline as a novel prenatal treatment strategy to mitigate spinal cord damage in MMC is warranted.

[*] *Corresponding author*. Johns Hopkins Children's Center, 1800 Orleans Street, Suite 7353, Baltimore, MD, 21287.
E-mail address: skunisa1@jhmi.edu (S.M. Kunisaki).

[1] These authors contributed equally to this work.

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Introduction

Myelomeningocele (MMC) is a neural tube defect that exposes the developing lumbosacral spinal cord to the intrauterine environment, causing irreversible lower body neural dysfunction, including urinary and bowel incontinence and weak lower extremities at birth. Although the pathogenesis of MMC is not fully understood, mechanical trauma and chemical exposure to amniotic fluid are known to cause progressive damage due to chronic inflammation and neural apoptosis.[1] A "two-hit hypothesis" has been proposed, in which the structurally incomplete neurulation is considered the "first hit" and the subsequent exposure to mechanical injury and amniotic fluid contribute to the "second hit" with progressive loss of neurologic function until term.[2] Prenatal therapies for MMC remain limited to in utero surgical closure of the defect; however, not all fetuses are candidates for the procedure and there are currently no other interventions aimed at addressing the inflammation and neural cell damage that has already occurred.

Minocycline (MIN, 7-dimethylamino-6-dimethyl-6-deoxytetracycline), a second generation, semisynthetic tetracycline with known antibacterial activity via the 30S ribosome pathway, has garnered interest by neuroscience investigators due to its anti-inflammatory and neuroprotective properties.[3] Studies have suggested that minocycline has the potential to mitigate the secondary injury associated with neuroinflammation and to improve neurologic outcomes in a diverse range of pathologies, including traumatic spinal cord injury, stroke, multiple sclerosis, acute spinal cord injury, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis.[3-8] Minocycline has favorable drug distribution properties and has been shown to be well tolerated as a chronic therapy in adults at doses up to 200 mg per day.[9] Unlike most tetracycline medications, minocycline is a highly lipophilic molecule that can pass through the placenta and blood-brain barrier, resulting in drug accumulation in the central nervous system.[10]

Recognizing minocycline's effect on neuroinflammation and neuroprotection, this study aimed to investigate whether the maternal administration of minocycline could attenuate inflammation and neural cell death in a fetal rat model of MMC. Our group hypothesized that minocycline exposure reduces activation of microglia, the resident macrophages responsible for inflammation and phagocytosis, as well as genes associated with inflammation and apoptosis within the MMC spinal cord.

Methods

Fetal MMC rat model

This study was approved by the Johns Hopkins University animal welfare committee under protocol RA22M192, in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. To induce fetal MMC, timed-pregnant Sprague–Dawley dams (Charles River) at E10 (gestational age 10 d) were gavage-fed olive oil + 50 mg/kg all-trans retinoic acid (Sigma–Aldrich) based on protocols described elsewhere.[11] Control animals were gavaged olive oil alone. At E12 (gestational age 12 d), control and retinoic acid–exposed dams were randomized to either regular drinking water (H_2O) or water containing minocycline (Sigma–Aldrich) ad libitum at concentrations of 40, 100, or 140 mg/kg/day until euthanasia. Minocycline doses were based on studies described elsewhere.[12-14] Minocycline water was replaced every other day to ensure antibiotic stability.

Tissue collection and processing

On E21 (gestational age 21 d, where term is 22 d), the dams were euthanized, and fetuses (approximately 10 per dam) were harvested by a midline laparotomy. Fetuses were euthanized by decapitation, and the whole fetus was fixed in 4% paraformaldehyde, dehydrated, and paraffin-embedded for immunohistochemistry. Eight micron sections of the spinal cord were cut and mounted on glass slides and dried overnight under air circulation before initiating immunostaining. For RNA isolation, lumbar spinal cords were sterilely collected using a dissecting microscope and fast-frozen in liquid nitrogen. In MMC cases, sections at the thoracic level (MMC covered) were also harvested for comparison with lumbar sections (MMC exposed) to determine the effect of exposure to the amniotic cavity on the spinal neural tissue.

Immunostaining

To determine the degree of inflammation and apoptosis within each spinal cord, transverse sections from controls (n = 4-6 per group) and retinoic acid–exposed fetuses with confirmed lumbosacral MMC defects (n = 4-16 per group) were stained for microglia using an antibody against the ionized calcium binding adaptor molecule 1 (Iba1, 1:2000; Cat. No. Ab178846; Abcam) and for apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (DeadEnd Fluorometric TUNEL System, Promega). Goat anti-rabbit Alexa Fluor 488 (1:200; Jackson ImmunoResearch) was used as secondary antibody. Tissue sections were imaged using a Leica wide-field (DMi8) fluorescent microscope and analyzed in a blinded fashion for total microglia, activated (ameboid) microglia, and TUNEL using Fiji ImageJ software (v2.3.0/1.53p; https://imagej.net/software/fiji/). Activated microglia were defined based on their round morphology with smaller cell area, perimeter, and convex hull area with fewer skeletal branches and end points.[15]

Quantitative gene expression

Relative expression of proinflammatory markers (*IL-1β*, *TNFα*, *IL-1α*, and *IL6*) and apoptotic genes (*Bad* and *Bax*) within the spinal cord (n = 4-10 per group) were assessed by quantitative reverse transcription polymerase chain reaction (PCR). Total RNA was isolated using RNeasy Mini Kits (Qiagen). RNA quantity and quality were determined spectrophotometrically using NanoDrop 2000 spectrophotometer (Thermo Fisher). Reverse transcription was conducted using the SuperScript Verso cDNA Synthesis kit (Thermo Fisher) according to the

manufacturer's protocol. Finally, quantitative PCR was performed using Power Up SYBR Green Master Mix (Applied Biosystems) and an AB-Quant Studio 3 real-time PCR machine (Thermo Fisher). PCR primers were designed with Primer3 plus, and *Gapdh* was used as a reference gene for the normalization of target gene expression using the ΔΔCt method.

Statistical analysis

Data were tested for normality using Shapiro–Wilk normality test. Normally distributed data were presented as mean ± standard error of the mean, and non-normally distributed data were presented as median (Q1, Q3). Statistical analyses of normally distributed data were performed for multiple comparison groups by one-way analysis of variance and two-way analysis of variance with Tukey posthoc analysis, and non-normally distributed data were analyzed with the Wilcoxon Mann-Whitney test using GraphPad Prism (v10.0.3, GraphPad Software). Statistical significance was set at $P < 0.05$.

Results

Inflammation and apoptosis within exposed MMC spinal cords

Iba1 immunostaining of normal control, MMC-covered thoracic, and MMC-exposed lumbosacral spinal cord sections from E21 pups did not significantly differ in the total number of microglial cells (Fig. 1A). However, when focused exclusively on microglial morphology consistent with the ameboid activated phenotype, blinded quantitative analyses revealed a statistically significant increase in cell density within MMC-exposed spinal cords when compared to MMC-covered (overall: 23.80 [15.75, 43.85] cells/mm^2 *versus* 3.20 [0.00, 11.12] cells/mm^2, $P = 0.005$; dorsal: 33.70 [23.38, 64.40] cells/mm^2 *versus* 1.85 [0.00, 19.98] cells/mm^2, $P = 0.007$; ventral: 5.07 [6.28, 22.45] cells/mm^2 *versus* 0.00 [0.00, 6.83] cells/mm^2, $P = 0.39$; Fig 1B). MMC-exposed spinal cords showed significantly higher levels of expression of proinflammatory cytokine genes, including *IL-1α* (5.7 [4.60, 7.97] *versus* 1.30 [0.71, 1.47], $P = 0.026$), *IL6* (4.36 [2.42, 5.50] *versus* 1.11 [0.82, 1.27], $P = 0.026$), *IL-1β* (0.43 [0.26, 0.49]] *versus* 0.13 [0.12, 0.14], $P = 0.004$), and *TNF-α* (0.70 [0.49, 1.35] *versus* 0.14 [0.07, 0.18], $P = 0.004$), compared to MMC-covered spinal cords (Fig. 2).

Baseline apoptotic activity within the MMC spinal cord is shown in Figure 3. There was a significant increase in TUNEL + cells in MMC-exposed spinal cords when compared to the MMC-covered spinal cords (140.00 [101.00, 147.00] cells/mm^2 *versus* 68.00 [62.00, 84.00] cells/mm^2, respectively; $P = 0.036$), which was localized to the dorsal half (exposed: 217.00 [120.00, 245.00] cells/mm^2 *versus* covered: 81.00 [73.25, 94.75] cells/mm^2; $P = 0.016$). The TUNEL + cell density within the ventral MMC spinal cord was not significantly different (exposed: 74.00 [43.00, 80.00] cells/mm^2 *versus* covered: 54.50 [50.00, 58.25] cells/mm^2; $P = 0.776$).

MMC spinal cord inflammation after minocycline delivery

After oral maternal minocycline administration, inflammation within the dorsal half of E21 MMC-exposed spinal cords was evaluated by activated Iba1+ microglial cell density. Escalating dose of minocycline were associated with a dose-response trend toward reduced activated Iba1+ cell density when compared to MMC-exposed pups that did not received any minocycline (Fig. 4). However, the association between minocycline exposure and activated microglial density narrowly missed statistical significance ($P = 0.052$).

Subsequent experiments performed with maternal minocycline exposure at 100 mg/kg/day showed significantly reduced relative expression of *IL6* (MIN: 1.61 [1.56, 2.25] *versus* H_2O: 2.89 [261, 3.92], $P = 0.04$) within affected MMC spinal cords (Fig. 5A), whereas *IL6* levels remained uniformly low upon minocycline delivery in control pups (MIN: 0.50 [0.47,

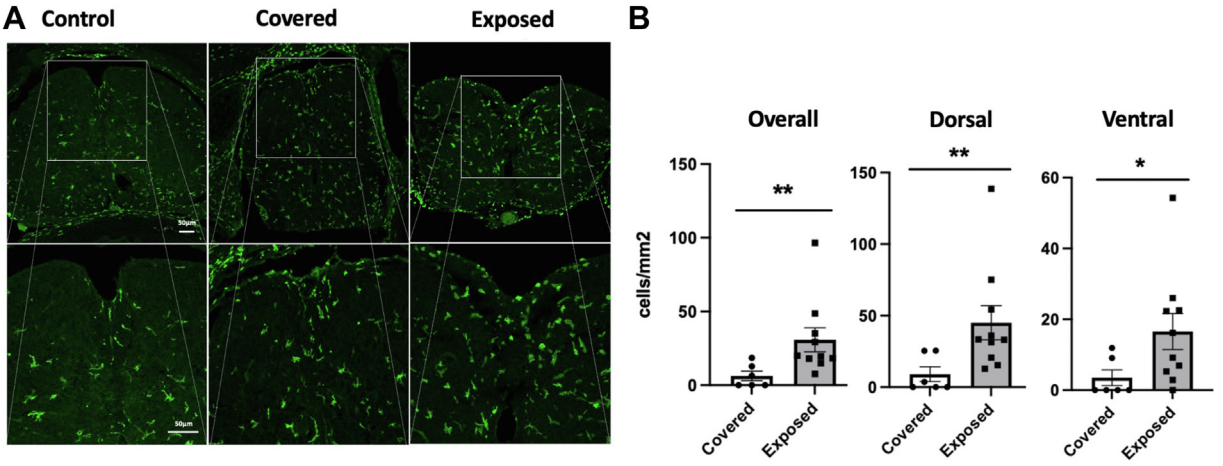

Fig. 1 – Baseline microglia activity within the fetal spinal cord (E21) of control and myelomeningocele (MMC) pups. (A) Representative Iba1 immunostaining of transverse sections of control lumbosacral, MMC-covered thoracic, and MMC-exposed lumbosacral spinal cords at 100x and 200x magnification. (B) Quantification of activated Iba1+ cell density within the fetal spinal cord of MMC-covered pups and MMC-exposed pups, overall and by dorsal or ventral regions, $^{**}P < 0.01$.

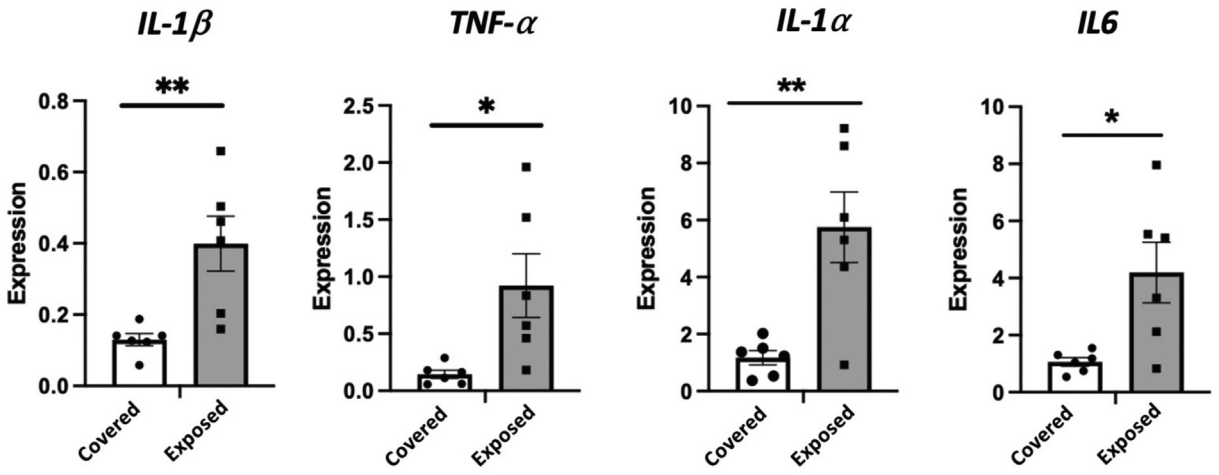

Fig. 2 – Scatter plot bar graphs showing the relative expression of proinflammatory cytokine (IL-1β, TNFα, IL-1α, and IL6) genes in covered (thoracic) and exposed (lumbar) regions of the myelomeningocele (MMC) fetal spinal cord. Data were normalized relative to housekeeping gene (*Gapdh*) and presented as the mean ± standard error of the mean, * denotes $P < 0.05$, **$P < 0.01$.

0.52] *versus* H_2O: 0.95 [0.77, 1.18], $P = 0.91$). There was a nonsignificant trend toward reduced expression of *IL-1α* (MIN: 9.36 [8.09, 10.92] *versus* H_2O: 24.9 [14.97, 27.50], $P = 0.09$) and *TNF-α* (MIN: 441.20 [101.00, 594.06] *versus* H_2O: 828.93 [473.67, 1362.23], $P = 0.21$) after minocycline delivery to MMC-exposed spinal cords. Inducible nitric oxide synthase, a downstream marker of inflammation, was not different between groups (MIN: 1.98 [1.18, 2.23] *versus* H_2O: 1.14 [0.89, 2.08], $P = 0.995$).

MMC spinal cord apoptosis after minocycline delivery

Gene expression of the proapoptotic marker, *Bax,* was significantly reduced (MIN: 0.71 [0.70, 0.73] *versus* H_2O: 1.05 [0.96, 1.11], $P = 0.0006$) in E21 MMC-exposed spinal cords after minocycline exposure at 100 mg/kg/day (Fig. 5B). Expression of the proapoptotic gene, *Bad*, was lower after minocycline exposure (MIN: 0.72 [0.68, 0.82] *versus* H_2O: 0.91 [0.81, 1.01], $P = 0.50$), with a similar trend toward reduced expression of *Bcl2*, an antiapoptotic marker that blocks programmed cell death (MIN: 0.54 [0.43, 0.64] *versus* H_2O: 0.76 [0.67, 0.82], $P = 0.16$); but these associations failed to reach statistical significance. TUNEL + cell density was not significantly different in any region of MMC-exposed spinal cords following minocycline administration (Fig. 6).

Discussion

In this pilot study of experimental MMC in fetal rats, we used gene expression and immunohistochemistry data to show that prenatal exposure to minocycline drinking water correlated with decreased microglia, inflammation, and apoptosis

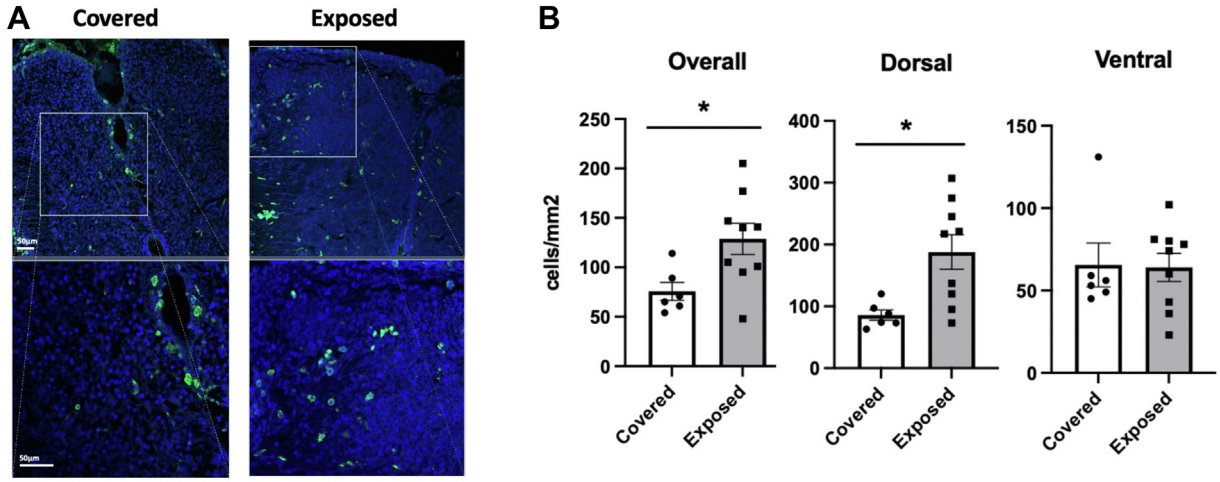

Fig. 3 – Apoptotic activity within the fetal spinal cord (E21) of myelomeningocele (MMC) pups. (A) Representative TUNEL staining of transverse sections of MMC-covered control and MMC-exposed spinal cords at 200× and 400× magnification. (B) Quantification of TUNEL + cell density staining within the fetal spinal cord of MMC-covered (control) and MMC-exposed pups, overall and by dorsal or ventral regions, *$P < 0.05$.

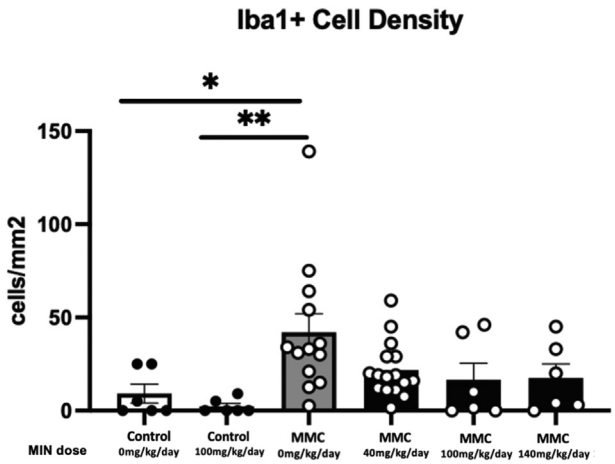

Fig. 4 – Scatter plot of activated Iba1+ cell density within the dorsal aspect of the fetal lumbosacral spinal cord in control and myelomeningocele (MMC) pups after maternal minocycline (MIN) administration (dose range, 0-140 mg/kg/day).

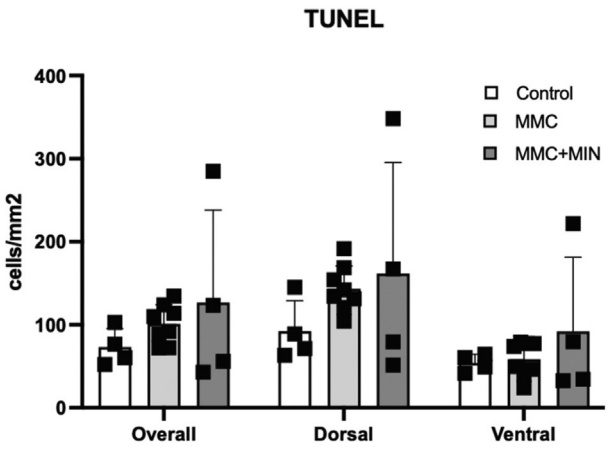

Fig. 6 – Scatter plot bar graphs showing TUNEL + cell density within fetal lumbosacral spinal cord sections in control and myelomeningocele (MMC) pups, with or without maternal minocycline (MIN) at doses of 100 mg/kg/day. Data presented as the mean ± standard error of the mean.

activity within the dorsal spinal cord. Specifically, we found that activated microglial activity, as measured by ameboid Iba1+ cell density, was reduced at all doses of minocycline exposure when compared to water alone, although these differences narrowly missed statistical significance. Moreover, prenatal minocycline exposure was associated with a

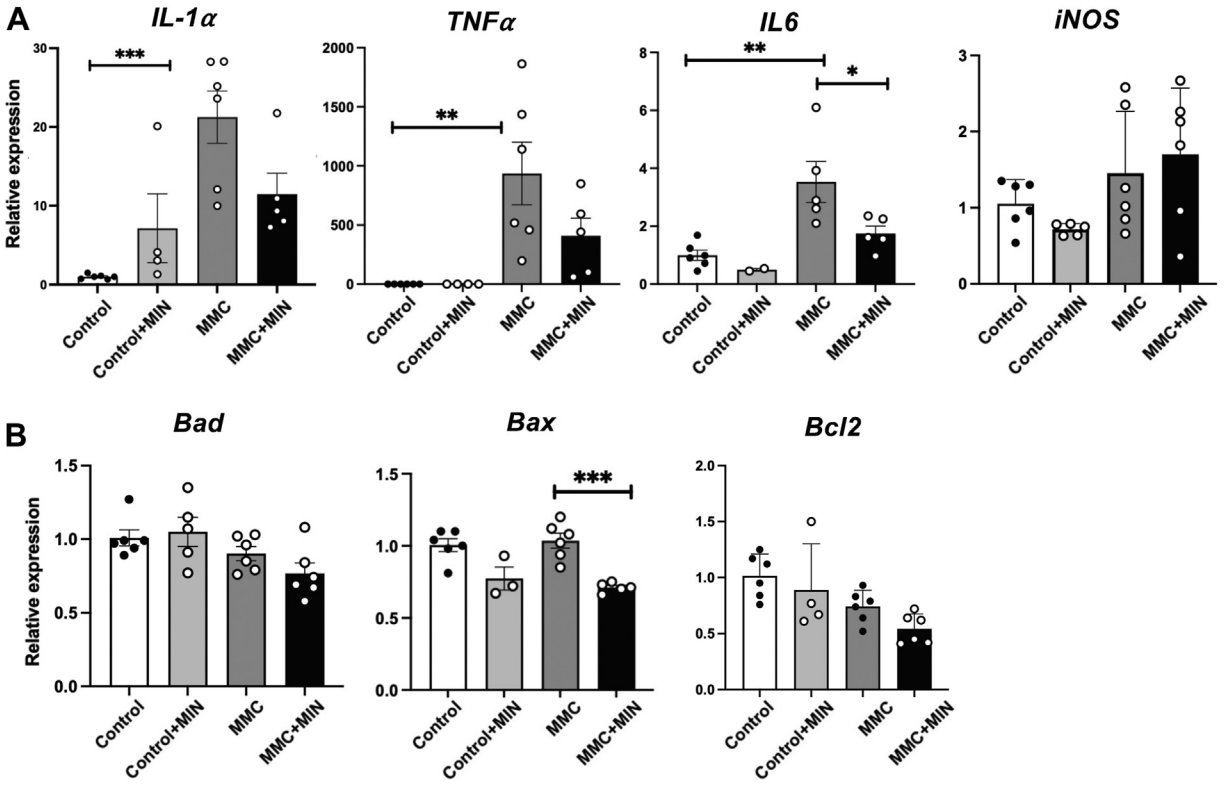

Fig. 5 – Inflammatory and apoptotic activity in control and myelomeningocele (MMC) fetal lumbosacral spinal cord, with and without maternal minocycline (MIN, 100 mg/kg/day) exposure. (A) Scatter plot bar graphs showing the relative expression of proinflammatory cytokines IL-1α, IL6, and TNFα, alongside a plot of inducible nitric oxide synthase (iNOS), a downstream mediator of inflammation. (B) Scatter plot bar graphs showing the relative expression of proapoptotic genes *Bax* and *Bad*, as well as antiapoptotic gene *Bcl2*. Data were normalized relative to housekeeping gene (*Gapdh*) and presented as the mean ± standard error of the mean, * denotes $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

significant decrease in the relative gene expression of proinflammatory marker, *IL6*, as well as apoptotic marker, *Bax*. A trend toward reduced inflammation, including *TNFα* and *IL-1α*, was also observed after prenatal exposure to minocycline, but these did not meet statistical significance. Taken together, these results suggest a mild trend toward neuroprotection in a fetal model of chronic spinal cord damage. Other studies have shown the impact of minocycline on reducing spinal damage and inflammation in cases of acute spinal cord injury, but to our knowledge, our study is the first of its kind to explore the application of minocycline as a strategy to attenuate the inflammatory milieu in fetal MMC.[16]

Identifying the specific molecular mechanism through which minocycline provides neuroprotection falls outside the immediate scope of this study. However, the data suggest that minocycline may reduce local inflammation and may act antagonistically on the Bcl-2 proapoptotic pathway, as evidenced by the reduced expression of *Bax* and *Bad* in drug-exposed spinal cords. Iba1+ and TUNEL staining further supports the notion that minocycline may be acting directly on the microglial cells to attenuate apoptosis within the MMC spinal cord.

Despite our modest results following minocycline administration, prenatal minocycline delivery warrants further investigation as a potential fetal therapy in spina bifida. Mitigation of the deleterious effects of secondary spinal cord injury from potential chemical components of the amniotic fluid remains a high priority given that there continues to be a paucity of prenatal treatments available for MMC except for in utero surgical closure.[17] Minocycline has been shown in postnatal experimental models to attenuate microglial activation, astrocyte reactivity, and mitochondrial cell death, all key inciting events in the pathophysiology of secondary spinal cord injury.[5,7,8,18,19] Previous work has also demonstrated that the number of microglial cells within fetal MMC spinal cords increases during gestation.[20] Under pathologic conditions, ramified (resting) microglia transform into an ameboid (activated) form, which has been shown to exacerbate neuronal death.[21]

While the broad-spectrum antibiotic properties of minocycline have been known since the late 1940s, the anti-inflammatory and neuroprotective properties of the drug have only recently been appreciated in experimental models of neurologic and spinal cord pathology.[19,22] Minocycline has been found to target many downstream mechanisms associated with inflammation, such as free radicals, oxidative stress, glutamate excitotoxicity, calcium influx, mitochondrial dysfunction, ischemia, hemorrhage, and edema.[23-25] Minocycline has also been shown to have positive effects on endogenous neural stem cell survival.[26] Accordingly, minocycline has been explored in a broad spectrum of other neurologic disease models, including in the attenuation of inflammation in West Nile virus–associated spinal cord infection, acute ischemic spinal cord injury, stroke, Huntington's disease, and fetal alcohol syndrome.[27-31] One study has suggested benefit from minocycline as a prevention strategy for imminent spinal cord ischemia in a rat model of aortic occlusion.[32] Another group has reported on the beneficial effects of minocycline on cerebral edema in a neonatal rat model of hydrocephalus.[33]

While it is possible that the lack of statistical significance for many of our outcome measures may be secondary to small sample size, we believe that several other important factors also limited significant findings. First, because MMC is a chronic inflammatory process, it is possible that the sustained inflammatory insult is not well captured by the acute inflammatory cytokines and apoptotic gene markers examined in this study. This stands in contrast to traumatic spinal cord injury in children and adults.[34] The inflammatory response toward spinal cord injury in MMC may also be less robust due to the relatively immature fetal immune system.[35] Second, despite a trend toward reduced activated microglia activity after minocycline delivery, we were unable to show a significant dose-response effect in our model due, at least in part to reduced pup sample size at higher minocycline concentrations. We found that dams exposed to high doses of minocycline had reduced water intake and became dehydrated and sickly in appearance by E21, likely resulting in subtherapeutic dosing of minocycline. The exact reason for this observation is unclear, but minocycline at higher doses in drinking water may have become unpalatable or may have induced gastrointestinal and/or systemic side effects. Regardless, since the poor state of these dams toward the end of pregnancy raised animal welfare concerns, plans for additional high-dose minocycline experiments were discontinued.

In addition to concerns regarding the maternal side effect profile, clinical translation of maternal minocycline therapy for MMC would be hampered by other known toxicities associated with tetracyclines, which include fetal teeth discoloration, impairment of long bone growth, and spontaneous abortion.[36-38] As a Food and Drug Administration category D medication in pregnant women, the use of minocycline may still be warranted if potential benefits can be demonstrated despite potential risks. For these reasons, ongoing work in our laboratory has recently focused on local drug delivery approaches, such as ultrasound-guided, intra-amniotic minocycline injection on the exposed spinal cord. We have also begun preliminary work on controlled release minocycline strategies that could be applied directly onto the spinal cord defect at the time of prenatal MMC repair. Both of these approaches may be viable approaches for clinical translation, thereby allowing therapeutic levels to the spinal cord while minimizing teratogenic and systemic toxic effects. Local delivery systems with minocycline have shown benefit in adult mouse models of spinal cord injury.[39-42] Adult clinical trials have already demonstrated that successful delivery of minocycline directly to injured neural cells in high concentrations can attenuate microglial activation.[43]

This study has several other limitations. First, our dosing strategy was based on the known transplacental passage of minocycline as well as experimental animal work described elsewhere.[12-14] These papers described the potent neuroprotective effects of minocycline at high concentrations (15-75 μg/mL), but noted that systemic administration of minocycline at a dose of 50 mg/kg only results in a concentration of 0.5 μg/mL in the cerebral spinal fluid.[14] Based on these reports, the levels of minocycline in the fetal rat spinal cord were likely subtherapeutic for inducing the wide-array neuroprotective effects attributed from this drug. We did not collect any pharmacokinetic data on the plasma concentrations of

minocycline in pregnant dams or fetal pups. Such studies would ultimately be required to optimize the risk-benefit profile of minocycline therapy. Second, we did not characterize the effect of minocycline on other neural cell phenotypes, including mature neurons and macroglia, within the dorsal aspect of the MMC spinal cord after minocycline administration.[44] Third, we did not explored specific mechanistic pathways involved in reduced inflammation and apoptosis as reported by others.[28] Finally, since MMC pups in the retinoic acid model do not survive beyond the first few minutes of life, we were unable to perform reliable neurologic testing after minocycline exposure. Such analysis would require adopting a larger fetal animal model to understand the functional impact of MMC therapy.[45]

Conclusions

In conclusion, this novel study found that maternal administration of minocycline reduced selected markers of inflammation and apoptosis within the exposed dorsal spinal cords of fetal MMC rats. Given the potential of minocycline for reducing neurologic morbidity in fetal MMC, further studies on minocycline as fetal therapy are warranted.

Disclosure

None declared.

Funding

This study was funded in part by the Barnes Fund through the Johns Hopkins University Department of Surgery. The funder had no role in design, data collection, data analysis, or reporting of this study.

Availability of Data

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Statements

This study discusses an off-label use of minocycline in an animal model of myelomeningocele. This is not a Food and Drug Administration–approved indication for this medication at this time.

Ethics Approval

This study protocol was reviewed and approved by the Johns Hopkins University Institutional Animal Care and Use Committee, approval number RA22M192.

CRediT authorship contribution statement

Juan C. Biancotti: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Anne M. Sescleifer:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Shelby R. Sferra:** Writing – review & editing, Data curation. **Annalise B. Penikis:** Writing – review & editing, Data curation. **Kyra M. Halbert-Elliot:** Writing – review & editing, Data curation. **Ciaran R. Bubb:** Writing – review & editing, Data curation. **Shaun M. Kunisaki:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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