UNIVERSITIY OF CALIFORNIA SAN DIEGO

The effect of non-steroidal anti-inflammatory drugs on the female rat pelvic floor muscle regeneration after birth injury

A thesis submitted in partial satisfaction of the requirements
for the degree of Master of Science

in

Biology

by

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Professor Amy Kiger

2020

The Thesis of Emmy Kimchi Do is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-chair

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Chair

University of California San Diego

2020

DEDICATIONS

I dedicate this to my grandfather, Ong Noi, who always believed in me. I hope he is watching me from heaven and is proud of my accomplishments.

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ABBREVIATIONS

PFDs	Pelvic floor disorders
PFMs	Pelvic floor muscles
NSAIDs	Non-steroidal anti-inflammatory drugs
SBI	Simulated birth injury
MuSCs	Muscle stem cells
COX	Cyclooxygenase
PG	Prostaglandin
NOS	Nitric oxide synthase
NO	Nitric oxide
IHC	Immunohistochemistry
eMyHC	Embryonic myosin heavy chain

ACKNOWLEDGMENTS

I would like to acknowledge Dr. Marianna Alperin. Her support as the chair of my committee and guidance throughout my project have been invaluable.

I would also like to acknowledge my other mentor, Ms. Pamela Duran, a doctorate candidate, for her advice and teaching me all the lab skills that I know today.

I would further like to acknowledge the rest of the Alperin lab for all the help, guidance, and encouragement given to me and my project. Immeasurable thanks and appreciation to them all, especially undergraduate volunteer Ms. Alyssa Kobayashi. Without her help, I would not have been able to complete the experimental work presented in my thesis in the timely fashion.

ABSTRACT OF THE THESIS

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Master of Science in Biology

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Professor Marianna Alperin, Chair
Professor Heidi Cook-Andersen, Co-Chair

Following vaginal delivery, the most common treatment is ibuprofen, a non-steroidal anti-inflammatory drug (NSAID) that helps alleviate postpartum pain. In the limb muscles, NSAIDs have been observed to interfere with the immune and myogenic events associated with skeletal muscle regeneration, leading to impaired muscle regeneration. However, the effect of NSAIDs on the pelvic floor muscles (PFMs) injured during parturition has never been evaluated. Thus, we aimed to elucidate: 1) the impact of birth injury on PFM micromorphological properties and 2) the effect of NSAIDs on the cellular events associated with PFM regeneration following birth injury. Utilizing an established rat model of simulated birth injury (SBI), we performed histological analysis of the temporal cellular events in the PFMs along the biologically relevant continuum following SBI. Our data demonstrate a profound negative impact of SBI on the

functionally relevant morphological PFMs' properties. NSAIDs do not impact the overall immune infiltrate following SBI but appear to delay muscle resident stem cells (MuSC) differentiation and decrease MuSC pool. In addition to these alterations in the acute recovery period, our analyses demonstrate a statistically significant reduction in the PFM myofiber size 28 days post-SBI in the group exposed to NSAIDs relative to the animals who did not receive NSAIDs following birth injury. The studies presented here are the first step in the continuum of the novel research evaluating the impact of postpartum NSAIDs on functional PFM properties.

INTRODUCTION

1.1 Impact of Vaginal Delivery on the Female Pelvic Floor

Pelvic floor disorders (PFDs), which include pelvic organ prolapse, and urinary and fecal incontinence, are highly prevalent conditions that adversely impact quality of life of millions of women (Siddiqui et al., 2018; Wu et al., 2014). Pelvic floor muscle (PFM) dysfunction consequent to maternal birth injury is a key risk factor in the pathogenesis of PFDs. PFMs are skeletal muscles that span the pelvic outlet, supporting the pelvic and abdominal organs and aiding in continence. (Bharucha, 2006; Raizada & Mittal, 2008). The human PFMs include coccygeus and the levator ani complex, comprised of puborectalis, pubococcygeus, and iliococcygeus.

One of the main risk factors for the development of symptomatic PFDs is the pelvic floor muscle (PFM) trauma during vaginal delivery. As the fetal head descends, PFMs are stretched up to 300%, past the physiological limits of limb skeletal muscles (Hoyte et al., 2008; Jing et al., 2012). Despite the high prevalence of PFM injury in vaginally parous women, not much is known about the cellular events involved in muscle regeneration after birth injury. In contrast to the sparse studies of the tissue- and cell-level alterations of the PFMs following mechanical injury associated with parturition, multiple studies of the limb muscles have shown that injured muscle recovery is facilitated by temporally regulated interactions between immune response and muscle stem cells (MuSCs) (Dort et al., 2019; Howard et al., 2020; Olguin et al., 2007; Schmidt et al., 2019; Sutcu & Ricchetti, 2018; Tidball, 2017; Tidball et al., 2014; Tidball & Villalta, 2010).

One of the most frequently used postpartum pain management strategies is the administration of ibuprofen, a non-steroidal anti-inflammatory drug (NSAID) (Deussen et al., 2011; Wuytack et al., 2016). Interestingly, the existing literature on recovery of limb muscles after injury demonstrates that NSAIDS interfere with the cellular events associated with skeletal muscle regeneration. These studies observe that NSAIDs impairs immune response and/or myogenesis, leading to possible negative impacts on overall

muscle regeneration (Bondesen et al., 2004, 2006; Bryant et al., 2017; Ho et al., 2017; Järvinen et al., 2013; Liao et al., 2019; Mackey et al., 2007; Mikkelsen et al., 2009). However, to our knowledge the impact of NSAIDs on postpartum recovery of PFMs has never been assessed (Figure 1A). Thus, we sought to investigate the impact of NSAIDS on the regenerative potential of PFMs following birth injury. Given substantial technical and ethical constraints associated with directly probing PFMs, located deep in the pelvis, in women, we utilized a well-established simulated birth injury rat model, previously validate in our lab for the studies of the human PFMs (Alperin et al., 2014).

1.2 Skeletal Muscle Regeneration is Coupled with the Immune Response to Injury

Skeletal muscle regeneration depends on the intact myogenesis, a process during which activation, proliferation, and differentiation of muscle stem cells (MuSCs) lead to the repair of injured myofibers (C. A. Collins et al., 2005; Kassar-duchossoy et al., 2005; Megeney et al., 1996; Pawlikowski et al., 2019; Rantanen et al., 1995; Schmidt et al., 2019; Sutcu & Ricchetti, 2018; Tidball, 2017; Zammit et al., 2006). Upon muscle injury, quiescent MuSCs activate and begin to proliferate to expand the MuSC pool (Tidball, 2017). Upon muscle injury, a population of committed satellite cells undergo differentiation around 4 days post-injury, forming de novo myofibers or fusing with the existing myofibers (Le Moal et al., 2017; Pawlikowski et al., 2019; Tidball, 2017). Another population divides 5 to 7 days after injury, returning to quiescence to replenish the MuSC pool (Pawlikowski et al., 2019).

The different states and progression of MuSCs through the myogenic process can be identified from the expression of specific transcription and myogenic regulatory factors. Pax7 is a transcription factor expressed by quiescent and early activated MuSCs—important for homeostatic and regenerative functions (Kassar-duchossoy et al., 2005; Relaix & Zammit, 2012; Zammit et al., 2006). Early activated MuSCs exhibit a co-expression of Pax7 and MyoD, a transcription factor important in the early commitment of MuSCs (Megeney et al., 1996; Montarras et al., 2005; Olguin et al., 2007; Relaix & Zammit, 2012; Zammit et al., 2006). These early committed MuSCs can proceed to self-renewal or terminal differentiation. The self-renewal phase is characterized by a down regulation of MyoD, while Pax7 is upregulated as MuSC

returns to quiescence (C. A. Collins et al., 2005; Sutcu & Ricchetti, 2018). MuSC differentiation is indicated by the activation of myogenin, a transcription marker for differentiated cells (Le Moal et al., 2017; Tidball, 2017). Upregulation of myogenin along with increased expression embryonic myosin heavy chain (eMyHC), a marker for regenerative myofibers, then corresponds with fusion of differentiated myoblast for formation of de novo fibers (Beylkin et al., 2006; Zanou & Gailly, 2013).

The temporally regulated cellular events during skeletal muscle regeneration are coupled with the two main phases of the immune response: pro-inflammatory and pro-regenerative (Locati et al., 2013; Tidball, 2017; Tidball et al., 2014; Tidball & Villalta, 2010; Varga et al., 2016). Within the first 24 hours following muscle injury, an innate immune response is activated. This activation results in the release of leukocytes and pro-inflammatory cytokines (Tidball et al., 2014). Leukocytes, such as neutrophils and other innate immune cells, infiltrate injured muscles early to clear damaged muscle fibers and cellular debris (Albina et al., 2017; R. A. Collins & Grounds, 2001; Nicholas et al., 2015; Warren et al., 2002). An upregulation in pro-inflammatory cytokines, such as interleukin-1 beta (IL-1ß), interferon-γ (IFN-γ) and tumor necrosis factor alpha (TNF-α), follows the initial immune wave response. These cytokines signal the activation of macrophage response (Cheng et al., 2008; R. A. Collins & Grounds, 2001; Novak, Weinheimer-Haus, & Koh, 2014; Tidball et al., 2014; Warren et al., 2002). The presence of T-helper 1 (Th1) cytokines activates pro-inflammatory M1 macrophages to infiltrate the injury site, where via phagocytosis the cells continue clearing the majority of damaged myofibers while also stimulating the activation and proliferation of MuSCs (Mills et al., 2000; Mills, 2015; Novak et al., 2014; Tidball, 2017; Tidball et al., 2014). Macrophage and T-helper polarization occurs 3 days post injury. Th1 cells transition to Th2 phenotype with the help of T-regulatory cells. The T-cell polarization promotes macrophage polarization, characterized by a shift of the macrophage phenotype from M1 to M2 (Mills, 2015; Mills et al., 2000). Macrophage polarization is further accompanied by an influx of anti-inflammatory and pro-regenerative cytokines that promote constructive tissue remodeling and repair (Mills, 2015; Mills et al., 2000; Novak et al., 2014; Tidball et al., 2014; Tidball & Villalta, 2010). Overall, these temporally regulated cellular events are important for the coordination of the immune and myogenic processes that contribute to

muscle regeneration. Interference in the cascade of these cellular events can negatively impact muscle regeneration (Ho et al., 2017; Mikkelsen et al., 2009; Mo et al., 2019; Soltow et al., 2006).

1.3 NSAIDs Inhibit Cyclooxygenase, Affecting Prostaglandins and Nitric Oxide Synthase

NSAIDs are the most commonly prescribed medications for pain management following vaginal delivery (Deussen et al., 2011; Wuytack et al., 2016). The analgesic properties of NSAIDs are induced through the inhibition of cyclooxygenase (COX), a key enzyme mediator regulating protective homeostatic processes (cardiovascular, gastrointestinal, and central nervous system) and host immune response (Ong et al., 2007). There are two main isoforms of COX: COX-1 and COX-2. COX-1 is constitutively active and expressed in resident immune cells, regulating white blood cell reactivity to inflammatory response and cellular differentiation (McAdam et al., 2000; Morita, 2002; Ricciotti & FitzGerald, 2011; C. J. Smith et al., 1998). COX-2 activation upon injury or inflammatory stimulus mediates the pain and inflammatory responses (Kim, 2011, 2014; Rouzer & Marnett, 2009). In particular, studies have shown that COX-2 along with COX-1 regulate MuSC activity, promoting myogenesis during skeletal muscle regeneration (Bondesen et al., 2004, Bondesen et al., 2006; Ricciotti & FitzGerald, 2011).

Different NSAIDs have varying selectivity for COX-1, COX-2, or both. The most commonly used and prescribed NSAID for postpartum pain management is ibuprofen (Deussen et al., 2011; Wuytack et al., 2016). Ibuprofen is a non-selective COX-inhibitor, wherein both COX-1 and COX-2 (COX-1/2) are inhibited (Bushra & Aslam, 2010). Non-selective COX-inhibitor NSAIDs are clinically preferred because they have lower gastrointestinal and cardiovascular risks compared to selective COX inhibitors (Deussen et al., 2011; Ong et al., 2007; Rao & Knaus, 2008; Wuytack et al., 2016). However, published literature on limb muscles have suggested that inhibition of COX-1 and COX-2 may negative impact muscle regeneration (Ho et al., 2017; Horsley & Pavlath, 2003; Jansen & Pavlath, 2008; Sheppe et al., 2018). This is because COX-1/2 enzymes play a key role modulating inflammatory immune response and myogenesis via the regulating of prostaglandin synthesis (Ong et al., 2007; Ricciotti & FitzGerald, 2011). Inhibition of

COX-1/2 decreases prostaglandin synthesis, potentially contributing to the impairment of muscle regeneration (Ho et al., 2017; Horsley & Pavlath, 2003; Jansen & Pavlath, 2008; Sheppe et al., 2018).

1.3.1 Effect of NSAIDs on Prostaglandin Synthesis

Prostaglandin (PG) synthesis is essential for muscle regeneration as prostaglandins help modulate inflammatory immune response and myogenesis (Ho et al., 2017; Horsley & Pavlath, 2003; Jansen & Pavlath, 2008; Ricciotti & FitzGerald, 2011; Sheppe et al., 2018). Upon an inflammatory stimulus, such as muscle injury, phospholipase A_2 enzymes activates to mediate the release of arachidonic acids from the membrane phospholipids. COX-1 and COX-2 act as lipid mediators, converting arachidonic acids to PGH_2 (Figure 2A). Both COX enzymes, along with various synthases, metabolize PGH_2 and help trigger downstream production of biosynthesized prostaglandin proteins (Ricciotti & FitzGerald, 2011). For muscle regeneration, PGE_2 and $PGF_{2\alpha}$ are the most relevant as they promote key inflammatory immune functions and myogenic processes (Ho et al., 2017; Horsley & Pavlath, 2003; Jansen & Pavlath, 2008; Ricciotti & FitzGerald, 2011; Sheppe et al., 2018) (Figure 2A-B). PGE_2 is key for enhancing myoblast proliferation and differentiation for myofiber repair (Ho et al., 2017; Sheppe et al., 2018). PGE_2 is also an immunomodulator, helping regulate macrophage polarization (Ho et al., 2017; Sheppe et al., 2018). This was confirmed in a study of injured mouse hindlimb muscles. The knockdown or COX1/2 NSAID inhibition of PGE_2 result in decreased MuSC expansion and overall impaired muscle regeneration (Ho et al., 2017; Mo et al., 2019). In conjunction, $PGF_{2\alpha}$ promotes MuSC survival and fusion (Horsley & Pavlath, 2003; Jansen & Pavlath, 2008). Inhibition of $PGF_{2\alpha}$ in mouse hindlimb muscles post injury was observed to impede muscle regeneration by increasing MuSC apoptosis and reducing the MuSC pool needed for myogenesis and constructive muscle remodeling (Jansen & Pavlath, 2008). Hence, COX1/2 inhibition of prostaglandin synthesis reduces production of PGE_2 and $PGF_{2\alpha}$—mediators of skeletal myogenic processes essential for proper muscle regeneration.

1.3.2 Effect of NSAIDs on Nitric Oxide Synthase

Nitric oxide (NO) plays a regulatory role for inflammatory immune cells and MuSC maintenance, essential for the activation, growth, and death of various immune and stem cells (Rigamonti et al., 2013). These regulatory functions occur because of the crosstalk interaction between nitric oxide synthase (NOS) and COX (Salvemini et al., 2013; Salvemini et al., 1993). NOS and COX are often co-expressed and share similar physiological functions. Constitutively expressed NOS (cNOS) is paired with COX-1 expression, producing NOs and PGs important for regulating the reactivity of white blood cells (Salvemini et al., 1993, 2013). Inducible NOS (iNOS) is associated with COX-2 expression. Both iNOS and COX-2 are activated by inflammation, producing pro-inflammatory NOs and PGs that mediate inflammation and pain, immune defense, and regulation of other cellular responses associated with cytotoxicity and tissue damage (Salvemini et al., 2013, Salvemini et al., 1993). The mechanism by which the NOS-COX crosstalk regulates PG synthesis remains not well defined. Studies proposed that NOS oxidatively modifies COX, activating COX to mediate the conversion of arachidonic acids into prostaglandins (Kim, 2014; Landino et al., 1996; Salvemini et al., 2013; Upmacis, Deeb, & Hajjar, 1999) (Figure 2B). Furthermore, NO-derived iNOS is known to be an important immunoregulator, regulating not only homeostatic processes but myogenic and immune responses. Specifically, iNOS induces T-helper cell polarization (Tripathi et al., 2007) and promotes activation, differentiation, and fusion of MuSCs (Filippin et al., 2009, 2011; Palma & Clementi, 2012; Rigamonti et al., 2013; Tatsumi et al., 2006). Hence, it has been suggested that NSAIDs may also impact NOS to a certain extent, given the reciprocal interaction between COX and NOS (Salvemini et al., 2013). However, there is conflicting evidence regarding the positive vs negative effect of NSAIDs on NOS and its related immunomodulatory activities in the context of skeletal muscle injury (Cheung & Tidball, 2003; Pizza et al., 1998).

1.4 NSAIDs Effect on Skeletal Muscle Regeneration

Considering significant role that COX and NOS play in driving prostaglandin synthesis, studies have surmised the probability that NSAIDs negatively interfere with muscle regeneration (Mackey et al.,

2007; Mendias, Tatsumi, & Allen, 2004; Mikkelsen et al., 2009; Mo et al., 2019). However, rigorous support for this notion is lacking, especially in the context of the mechanistic impact of NSAIDs on skeletal muscle regeneration (Deussen et al., 2011; Wuytack et al., 2016). Limb muscle studies generally suggest that COX inhibition using non-NSAID COX inhibitors or NSAIDs inhibits COX-dependent muscle immune and myogenic response to injury, leading to delayed muscle regeneration (Ho et al., 2017; Mackey et al., 2012; Mackey et al., 2007; Mikkelsen et al., 2009; Mo et al., 2019; Rigamonti et al., 2013). Most of the existing studies, which focus on the use of COX-2 selective NSAIDs, show that use of COX-2 selective NSAIDs significantly decrease MuSC activity, causing impaired muscle repair (Bondesen et al., 2004, 2006; Na et al., 2013; Nakanishi et al., 2011; Novak et al., 2014; Shen et al., 2005). This is because COX-2 primarily facilitates pain and inflammatory response and, thus, believed to be more important in muscle regeneration (Bondesen et al., 2004, 2006; Ong et al., 2007; Ricciotti & FitzGerald, 2011). Although COX-2 does indeed play a more prevalent role in muscle regeneration, it has been shown that both COX-1 and COX-2 help facilitate muscle regeneration (Mendias, Tatsumi, & Allen, 2004; Mo et al., 2019; Morita, 2002; Ong et al., 2007; Rao & Knaus, 2008; Ricciotti & FitzGerald, 2011). The studies investigating the role of COX enzymes in the hindlimb myogenesis in the rodent models have demonstrated that COX-1/2 inhibition via NSAIDs significantly decreased differentiation and fusion of the myoblasts compared to the inhibition of either COX-1 or COX-2 alone (Mendias et al., 2004; Mo et al., 2019). Taken together, the existing data indicate that COX-1 and COX-2 enzymes are required for proper myogenesis as inhibition of both enzymes impairs the proper function of MuSCs and myoblasts (Mendias, Tatsumi, & Allen, 2004; Mo et al., 2019).

The few human studies that assess the effect of NSAIDs on muscle regeneration demonstrate that non-selective NSAIDs negatively impact myogenesis following eccentric exercise. Similar to muscle injury, eccentric exercise is known to induce myogenesis (Gomez-Cabrera et al., 2016; Schoenfeld, 2012). During eccentric exercise, myofiber trauma occurs. MuSCs then activate in order to repair the damaged myofibers and promote skeletal muscle hypertrophy—the enlargement and growth of muscle (Gomez-Cabrera et al., 2016; Schoenfeld, 2012). NSAIDs are often used after eccentric exercise to help mediate

pain and inflammation during recovery, but some studies have shown that NSAIDs had a negative impact on myogenesis. Two studies have evaluated in human leg muscles that 8 days following eccentric exercise, both non-continuous and continuous use of a non-selective NSAID resulted in significantly decreased MuSC activity (Mackey et al., 2007; Mikkelsen et al., 2009). However, despite evidence that NSAIDs impaired protein synthesis, both studies did not observe significant impacts to immune response or myofiber regeneration. The lack of observed differences to muscle regeneration can be likely attributed to limited timepoint of assessment (only 8 days after injury) and small sample size.

Overall, although existing limb muscle literature on NSAIDs focuses on assessing the effects of myogenesis, with few investigating effects NSAIDs have on inflammatory immune cell response, the studies provide some insight that suggest COX1/2 inhibition delays skeletal muscle regenerative cellular events by impacting MuSC activity. ***Thus, we posit that the use of NSAIDs, intended to aid women's recovery after vaginal delivery, impair postpartum PFM recovery by inhibiting normal cellular events associated with muscle regeneration.***

1.5 The Rat Model of the Pelvic Floor Muscle Birth Injury

To investigated the above, we utilized a widely-used stimulated birth injury (SBI) rat model (Alperin et al., 2010; Alperin, et al., 2010; Cannon et al., 2002; Damaser et al., 2003; Lin et al., 1998; Resplande et al., 2002; Sievert et al., 2001). It is not realistically feasible to obtain human pelvic floor muscles for study because PFMs are located deep within the pelvis and would require invasive surgical methods to access. Studies have previously validated the rat model for the studies of human PFMs (Alperin et al., 2014; Stewart et al., 2017). The rat PFM architectural properties that predict muscle force-generating and excursional capacity is most similar to human PFM architecture (Alperin et al., 2014). Moreover, the Alperin lab has previously shown that the rat PFMs experience substantial mechanical injury in response to strains induced by SBI, with pubocaudalis demonstrating stretch ratios analogous to those experienced by the human pubococcygeus in the computational models of human parturition (Catanzarite et al., 2018).

Therefore, the simulated birth injury rat model allows for the investigation of cellular events during PFM regeneration that are likely to be very relevant to the human PFMs.

1.6 Research Objective

Our primary aim was to elucidate the impact that non-selective NSAIDs have on the rat PFM regeneration across a biologically relevant time course following simulated birth injury. We hypothesized that NSAIDs will impair PFM regeneration by altering immune and myogenic cellular events following birth injury.

RESULTS

Our study first established the impact simulated birth injury (SBI) has on PFMs. Hematoxylin and eosin (H&E) staining was performed at early, middle, and late timepoints (1, 3, and 7 days) on PFM cross-sections after SBI to evaluate changes in muscle morphology. Uninjured control showed tightly packed myofibers, demonstrating the unperturbed morphology of PFMs (Figure 3A). Following birth injury, we observed that at 1-day post-SBI PFMs exhibited profound myofiber death (Figure 3B). PFM myofibers then appeared separated along with the influx of cellular infiltrate 3 days post-SBI (Figure 3C) followed by the presence of centralized nuclei, indicating regenerating myofibers at 7 days post-SBI (Figure 3D). Together, the H&E illustrates the striking impact birth injury alone has on PCa muscle, causing pathological alterations to PFM muscle.

The following immunohistochemistry (IHC) analyses were performed to assess changes in immune response and myogenic cells during regeneration with and without ibuprofen. Data for each outcome of interest were compared between the groups and time-points using a two-way ANOVA, followed by post-hoc pairwise comparisons with Tukey's range test when indicated. The independent variables are the timepoints (1, 3, 5, 7, and 10 days) and groups (control, SBI, SBI+NSAID, and NSAID). The dependent variables include immune and myogenic cellular events, identified using antibodies against 1) CD45 for leukocytes (Figure 4A-E), 2) myogenin for differentiated MuSCs (Figure 5A-E), and 3) Pax7 for quiescent and activated MuSCs (Figure 6A-E). Note, NSAID implies the use of ibuprofen.

Pan-leukocyte immune response was assessed using anti-CD45 antibody. In the SBI group, leukocytes peaked 1-day post-birth injury (53.55 ± 4.87 cells/mm^2) ($p<0.0001$) before returning to control levels (14.41 ± 3.04 cells/mm^2) by 7 days post-SBI (21.57 ± 3.38 cells/mm^2) ($p=0.7$) (Figure 4A-C). NSAID alone group exhibited no significant differences in CD45$^+$ cell density compared to controls ($p>0.05$; Table 1 & 2) (Figure 2A-B & E). SBI+NSAID group did not show significant differences in leukocyte infiltration compared to the SBI group at any of the timepoints ($p>0.05$; Table 1 & 2) (Figure 4A & C- D). Thus, the data indicated leukocyte activity peak 1-day post SBI and that NSAIDs do not impact leukocyte response.

Changes in myogenic cells present during regeneration with and without the ibuprofen were assessed through the expression of myogenin, a marker of differentiated cells, and Pax7, a marker of MuSCs. Myogenin$^+$ cell density following SBI peaked at 3 days (16.85 ± 4.76 cells/mm^2) compared to controls (0.94 ± 0.24 cells/mm^2) ($p>0.0001$) and quickly returned to the control levels by 5 days (4.15 ± 1.99 cells/mm^2) ($p=0.43$) (Figure 5A-C). NSAID group was not statistically different from controls, consistent with previous observations of the NSAID group ($p>005$, Table 3 & 4) (Figure 5A-B & E). Myogenin$^+$ cell density was not statistically different in the SBI+NSAID group compared to the SBI group at any timepoint ($p>0.05$; Table 3 & 4) (Figure 5A). However, SBI+NSAID myogenin$^+$ cell density 5 days post-SBI maintained a significantly higher myogenin$^+$ cell density (9.48 ± 14.14 cells/mm^2) compared to controls (0.94 ± 0.24 cells/mm^2) ($p = 0.008$). Myogenin$^+$ cell density in SBI+NSAID group was nearly double the SBI group 5 days post birth injury (4.15 ± 1.99 cells/mm^2) ($p=0.17$) (Figure 5A-D). The above data demonstrate that MuSC differentiation peaks 3 days following SBI and that NSAID treatment appears to delay MuSC differentiation—an alteration that may impact recovery long-term.

In evaluating the impact SBI had on the MuSC pool, we observed that compared to controls (15.40± 2.02 cells/mm^2) Pax7$^+$ MuSC density post-SBI increased as early as 5 days after injury (27.44 ± 7.58 cells/mm^2) ($p=0.0012$). Pax7$^+$ MuSC density peaked 7 days post-SBI (80.42 ± 4.04 cells/mm^2) ($p<0.0001$) before returning to baseline levels by day 10 (22.00 ± 1.22; $p=0.32$) (Figure 6A-C). NSAID group did not show any significant difference from the controls ($p>0.05$; Table 5 & 6) (Figure 6A-B & E). The SBI+NSAID groups showed a similar trend to SBI group with respect to Pax7$^+$ cell density levels across all time points, however, Pax7$^+$ cell density for SBI+NSAID groups was consistently lower than SBI groups (Table 5). Particularly, SBI+NSAID Pax7$^+$ cell density 7 days after birth injury was decreased nearly 2-fold compared to the SBI alone group (44.32 ± 2.36 v. 80.42 ± 4.04 cells/mm^2) ($p<0.0001$) (Figure 6A & C-D). These data show that the MuSC pool peaks 7 days after birth injury, while NSAID treatment reduces the expansion of the MuSC pool. Reduction in the MuSC pool may contribute to delayed muscle regeneration, as well as impaired muscle recovery in a setting of repeat birth injury.

To compare the extent of muscle regeneration between the SBI and SBI+NSAID groups, an antibody against embryonic myosin heavy chain (eMyHC) was used to identify regenerating myofibers. Cross-sectional area of $eMyHC^+$ myofibers was quantified to measure regeneration progression. The fiber size distribution was graphed employing violin plots, with median (min-max) calculated given non-normal distribution. In both SBI and SBI+NSAID groups, fiber area was significantly increased at 7 days compared to 3- and 10-day time points ($p>0.0001$; Table 7 & 8) (Figure 7A-C). This is consistent with the limb muscle studies, where regenerating myofibers reach their maximum size 7 days after injury (Mishra, Friden, Schmitz, & Lieber, 1995; Pawlikowski et al., 2019; Schmidt et al., 2019; Tidball, 2017). Surprisingly, $eMyHC^+$ fibers size of SBI group at 3, 7, and 10 days was significantly smaller than that of SBI+NSAID ($p>0.0001$; Table 7) (Figure 7A-D). Although these differences may be attributed to the large variation in fiber size in the SBI+NSAID group, the fiber size distribution was within the known physiological limits of skeletal muscle myofiber size, including regenerating myofibers (50-5000 μm^2). Rather, the differences observed likely represents a potential compensatory hypertrophy of the $eMyHC^+$ myofibers still present in the NSAID groups at the acute timepoints after birth injury (Schoenfeld, 2012; Trappe et al., 2011; Zanou & Gailly, 2013).

Taking together, our results demonstrate that NSAIDs administration after simulated birth injury delays muscle cells differentiation and decreases the expansion of the MuSCs pool. Although this did not result in a delay in the early regenerative process, as indicated by the non-inferior size of the regenerating myofibers, NSAID-induced alterations in the myogenic events may impact muscle recovery long-term.

To determine whether long-term NSAID administration further impacts muscle regeneration, we assessed mature fiber cross-sectional area 4 weeks after birth injury. We observed a reduction in myofiber cross sectional area in the SBI group (1363 μm^2 (62-4146 μm^2)) compared to the controls (1851 μm^2 (81-4724 μm^2) ($p<0.0001$), indicating long-term muscle atrophy (Figure 8A-C). SBI+NSAID group showed a further statistically significant reduction in fiber size (1304 μm^2 (90-4990 μm^2) compared to SBI alone ($p<0.0001$) (Figure 8A, C-D). Surprisingly, prolonged ibuprofen administration to the uninjured animals also resulted in the reduction of the PFM fiber size relative to the controls (1526 μm^2 (65-4981 μm^2) v.

(1851 μm^2 (81-4724 μm^2)) ($p<0.0001$) (Figure 8A-B, E). This suggests that prolonged NSAID use negatively impacts PFMs, possibly impacting muscle homeostasis. It is important to note that the magnitude of the differences in fiber size distribution and median size in SBI vs SBI+NSAID group is small. Therefore, despite statistical significance, the identified differences may not be clinically significant. Comparison of the PFM functional properties between the experimental groups is needed to definitively answer this question.

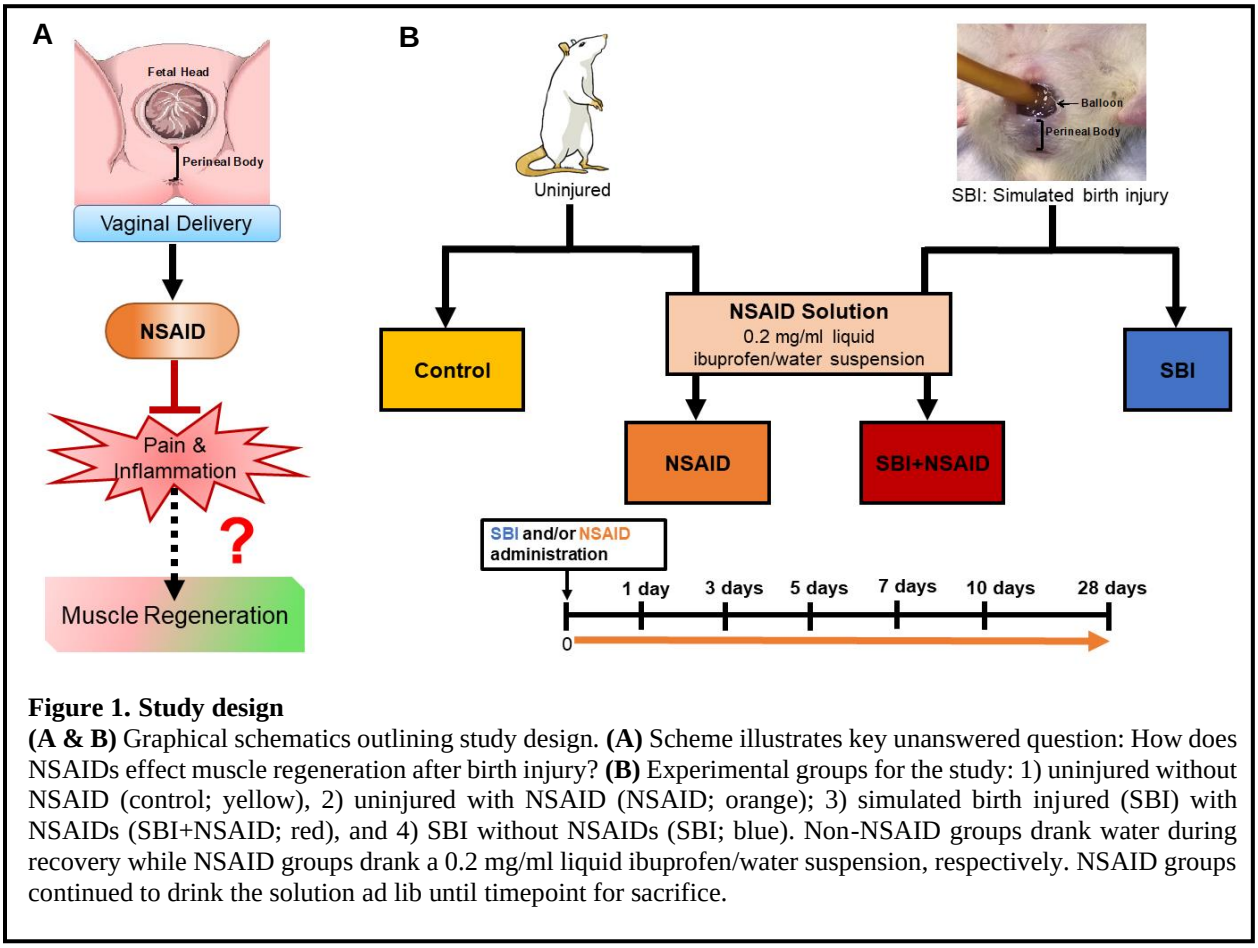

Figure 1. Study design
(A & B) Graphical schematics outlining study design. **(A)** Scheme illustrates key unanswered question: How does NSAIDs effect muscle regeneration after birth injury? **(B)** Experimental groups for the study: 1) uninjured without NSAID (control; yellow), 2) uninjured with NSAID (NSAID; orange); 3) simulated birth injured (SBI) with NSAIDs (SBI+NSAID; red), and 4) SBI without NSAIDs (SBI; blue). Non-NSAID groups drank water during recovery while NSAID groups drank a 0.2 mg/ml liquid ibuprofen/water suspension, respectively. NSAID groups continued to drink the solution ad lib until timepoint for sacrifice.

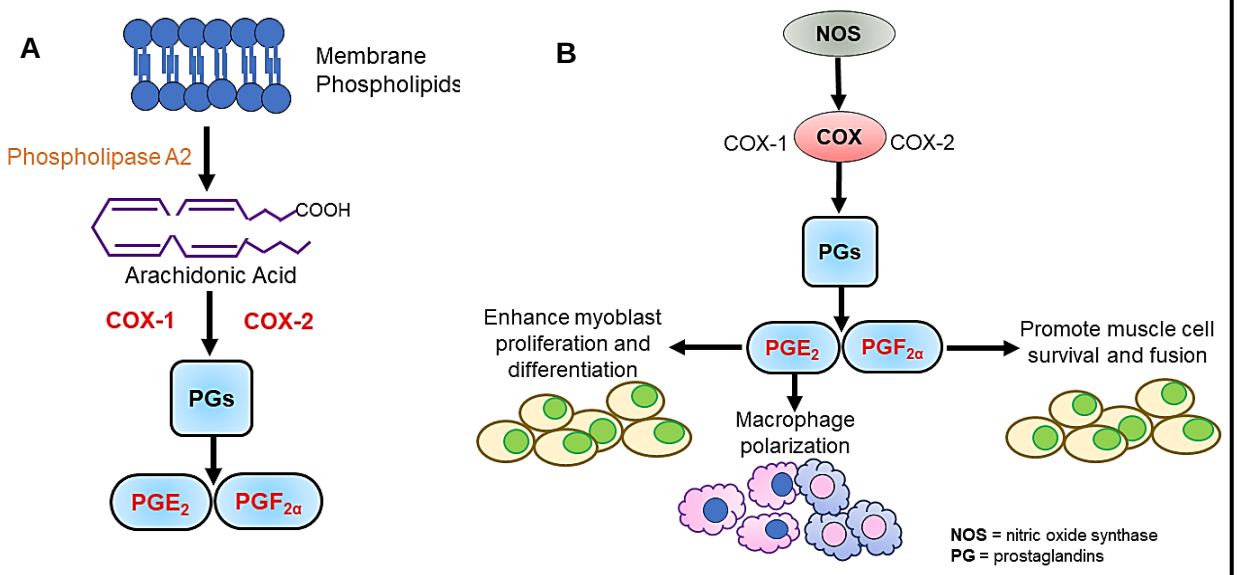

Figure 2. Prostaglandin synthesis and nitric oxide synthase-COX interaction with PGs
(A) Graphical schematic of prostaglandin (PG) synthesis. **(B)** Simplified outline of nitric oxide synthase (NOS)-COX interaction with PGs and overview of PGs most prevalent during muscle regeneration, PGE_2 and $PGF_{2\alpha}$.

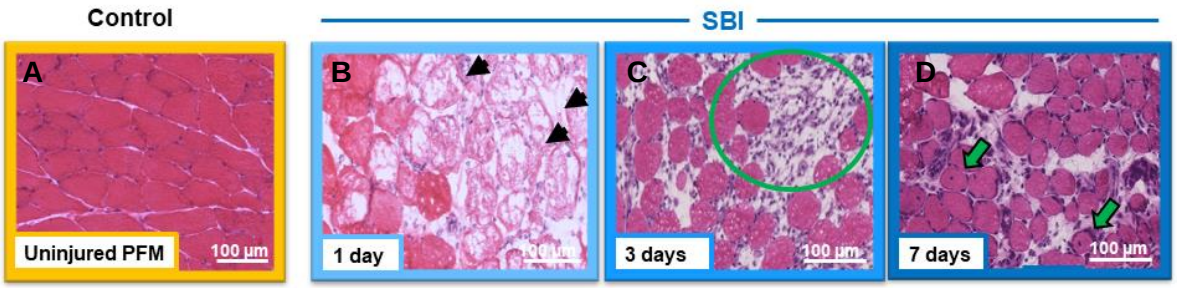

Figure 3. Simulated birth injury (SBI) causes pathological alterations to PFMs
(A-E) Hematoxylin and eosin (H&E) staining of control (yellow) and 1, 3, and 7 days post-SBI (blue) PFM cross-sections. **(A)** Uninjured control. **(B)** 1-day post-SBI, where the black arrows indicate myofiber death. **(C)** 3 days post-SBI, where the green circle indicates myofiber separation and cellular infiltration. **(D)** 7 days post-SBI, where green arrows point to regenerative myofibers as indicated by centralized nuclei.

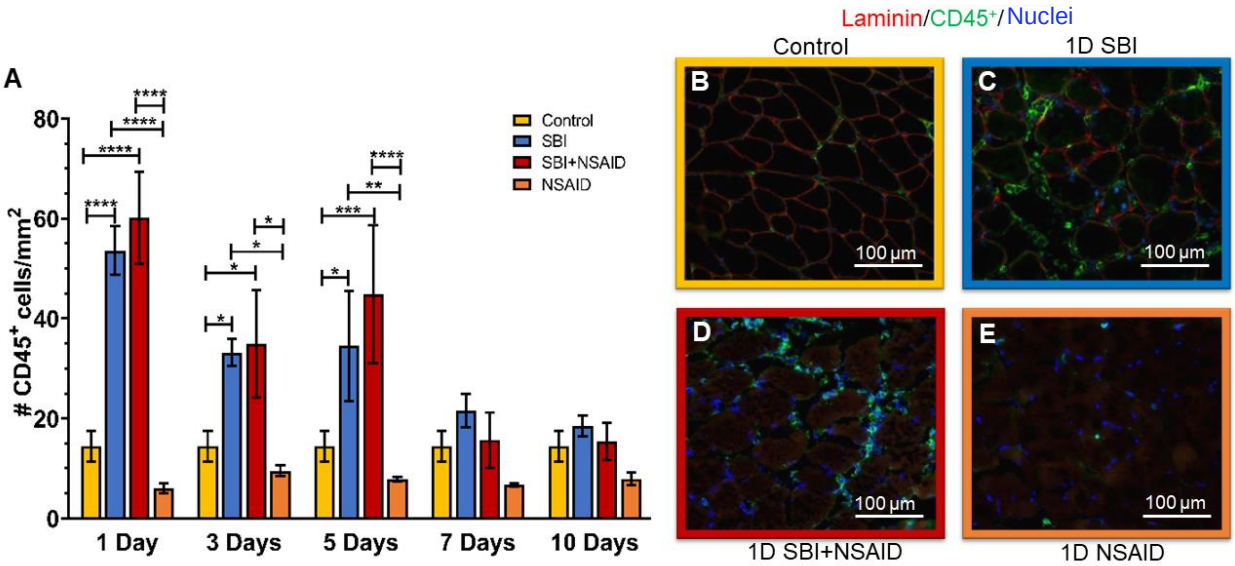

Figure 4. NSAIDs do not affect leukocyte infiltration
(A) Bar graphs CD45$^+$ cell density quantification for control (yellow), SBI (blue), SBI+NSAID (red), and NSAID (orange) groups across acute timepoints (1, 3, 5, 7, and 10 Days). Quantification is reported as mean ± SEM and statistically analyzed using two-way ANOVA (post-hoc Tukey's Test). *p<0.5, **p<0.01, ***p<0.001, ****p<0.001. N=9 for controls while N=3 for all other groups. CD45$^+$ cell density illustrates that NSAIDs did not affect leukocyte infiltrate for birth injured groups across timepoints (p>0.05). **(B-E)** Representative images of CD45$^+$ control and 1-day SBI, SBI+NSAID, and NSAID only stains.

Table 1. CD45 mean cell density values
Mean CD45 cell density values (#CD45$^+$ cells/mm^2) and standard error of the mean (SEM) to quantify leukocyte infiltration across a time course after birth injury. The same control (yellow) was used to compare at each timepoint (N=9). SBI (blue), SBI+NSAID, (red), and NSAID (orange) was N=3/timepoint/group.

	Control		SBI		SBI+NSAID		NSAID	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
1 Day	14.41	3.04	53.55	4.87	60.13	3.20	5.97	0.99
3 Days	14.41	3.04	33.21	2.66	34.37	10.78	9.49	1.07
5 Days	14.41	3.04	34.51	11.03	44.85	13.75	7.86	0.45
7 Days	14.41	3.04	21.57	3.38	15.67	5.53	6.71	0.33
10 Day	14.41	3.04	18.54	2.11	15.34	3.71	7.91	1.27

Table 2. CD45 p-values
Table of CD45 p-values/timepoint/groups as determined from Two-way ANOVA (post-hoc Tukey Test), where n.s. = no significant differences, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001

	Group 1 v. Group 2		Significance	p-value
1 Day	Control	SBI	****	< 0.0001
	Control	SBI+NSAID	****	< 0.0001
	Control	NSAID	n.s.	0.58
	SBI	SBI+NSAID	n.s.	0.85
	SBI	NSAID	****	< 0.0001
	SBI+NSAID	NSAID	****	< 0.0001
3 Days	Control	SBI	*	0.029
	Control	SBI+NSAID	*	0.014
	Control	NSAID	n.s.	0.87
	SBI	SBI+NSAID	n.s.	0.99
	SBI	NSAID	*	0.023
	SBI+NSAID	NSAID	*	0.013
5 Days	Control	SBI	*	0.017
	Control	SBI+NSAID	***	0.001
	Control	NSAID	n.s.	0.69
	SBI	SBI+NSAID	n.s.	0.58
	SBI	NSAID	**	0.0041
	SBI+NSAID	NSAID	****	< 0.0001
7 Days	Control	SBI	n.s.	0.70
	Control	SBI+NSAID	n.s.	1.0
	Control	NSAID	n.s.	0.65
	SBI	SBI+NSAID	n.s.	0.89
	SBI	NSAID	n.s.	0.27
	SBI+NSAID	NSAID	n.s.	0.69
10 Day	Control	SBI	n.s.	0.92
	Control	SBI+NSAID	n.s.	1.0
	Control	NSAID	n.s.	0.76
	SBI	SBI+NSAID	n.s.	0.98
	SBI	NSAID	n.s.	0.56
	SBI+NSAID	NSAID	n.s.	0.80

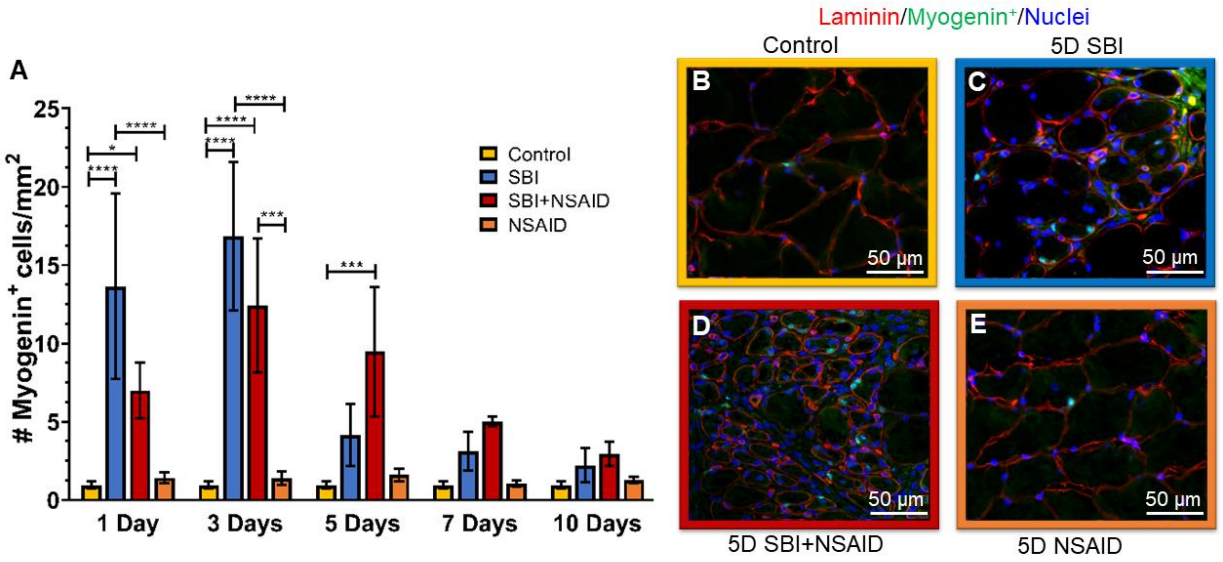

Figure 5. NSAIDs potentially delayed MuSC differentiation
(A) Bar graphs myogenin$^+$ cell density quantification for control (yellow), SBI (blue), SBI+NSAID (red), and NSAID (orange) groups across acute timepoints (1, 3, 5, 7, and 10 Days). Quantification is reported as mean ± SEM and statistically analyzed using two-way ANOVA (post-hoc Tukey's Test). *p<0.5, **p<0.01, ***p<0.001, ****p<0.001. N=8-9 for controls while N=3 for all other groups. Myogenin$^+$ cell density illustrates that statistically NSAIDs did not impact expression of differentiated MuSCs between birth injured groups across all timepoints (SBI v. SBI+NSAIDs; p>0.05). However, as suggested by the 5D timepoint, NSAIDs may possibly be prolonging the MuSC differentiation phase and contributing to a delay in overall muscle regeneration. **(B-E)** Representative images of myogenin$^+$ control and 5 days SBI, SBI+NSAID, and NSAID stains.

Table 3. Myogenin mean cell density values
Mean myogenin cell density values (#myogenin$^+$ cells/mm^2) and standard error of the mean (SEM) to quantify differentiated MuSCs across a time course after birth injury. The same control (yellow) was used to compare at each timepoint (N=9). SBI (blue), SBI+NSAID, (red), and NSAID (orange) was N=3/timepoint/group.

	Control		SBI		SBI+NSAID		NSAID	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
1 Day	0.94	0.24	13.63	5.92	6.99	1.78	1.42	0.33
3 Days	0.94	0.24	16.85	4.76	12.40	4.27	1.39	0.44
5 Days	0.94	0.24	4.15	1.99	9.48	4.14	1.60	0.41
7 Days	0.94	0.24	3.13	1.23	5.01	0.30	1.05	0.21
10 Day	0.94	0.24	2.22	1.08	2.94	0.78	1.28	0.21

Table 4. Myogenin p-values
Table of myogenin p-values/timepoint/groups as determined from Two-way ANOVA (post-hoc Tukey Test), where n.s. = no significant differences, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001

	Group 1 v. Group 2		Significance	p-value
1 Day	Control	SBI	****	< 0.0001
	Control	SBI+NSAID	*	0.028
	Control	NSAID	n.s.	1.00
	SBI	SBI+NSAID	n.s.	0.053
	SBI	NSAID	****	< 0.0001
	SBI+NSAID	NSAID	n.s.	0.14
3 Days	Control	SBI	****	< 0.0001
	Control	SBI+NSAID	****	< 0.0001
	Control	NSAID	n.s.	1.00
	SBI	SBI+NSAID	n.s.	0.31
	SBI	NSAID	****	< 0.0001
	SBI+NSAID	NSAID	***	< 0.0003
5 Days	Control	SBI	n.s.	0.43
	Control	SBI+NSAID	***	0.008
	Control	NSAID	n.s.	0.99
	SBI	SBI+NSAID	n.s.	0.17
	SBI	NSAID	n.s.	0.71
	SBI+NSAID	NSAID	**	0.0081
7 Days	Control	SBI	n.s.	0.73
	Control	SBI+NSAID	n.s.	0.23
	Control	NSAID	n.s.	>1.00
	SBI	SBI+NSAID	n.s.	0.88
	SBI	NSAID	n.s.	0.85
	SBI+NSAID	NSAID	n.s.	0.41
10 Day	Control	SBI	n.s.	0.93
	Control	SBI+NSAID	n.s.	0.78
	Control	NSAID	n.s.	1.00
	SBI	SBI+NSAID	n.s.	0.99
	SBI	NSAID	n.s.	0.98
	SBI+NSAID	NSAID	n.s.	0.91

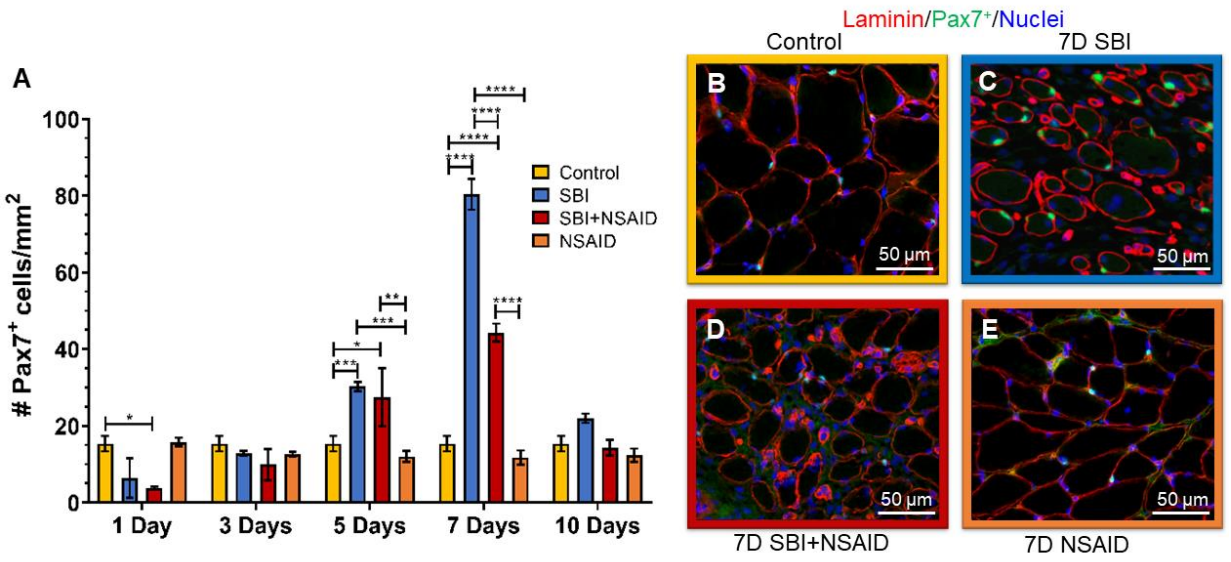

Figure 6. NSAIDs decreased activity of the MuSC pool
(A) Bar graphs Pax7$^+$ cell density quantification for control (yellow), SBI (blue), SBI+NSAID (red), and NSAID (orange) groups across acute timepoints (1, 3, 5, 7, and 10 Days). Quantification is reported as mean ± SEM and statistically analyzed using two-way ANOVA (post-hoc Tukey's Test). *p<0.5, **p<0.01, ***p<0.001, ****p<0.001. N=8-9 for controls while N=3 for all other groups. Pax7$^+$ cell density illustrates that significantly decreased activity of the MuSC pool 7 days post-birth injury (SBI v. SBI+NSAIDs; p<0.0001). **(B-E)** Representative images of myogenin$^+$ control and 5 days SBI, SBI+NSAID, and NSAID stains.

Table 5. Pax7 mean cell density values
Mean Pax7 cell density values (#Pax7$^+$ cells/mm^2) and standard error of the mean (SEM) to quantify activity of MuSC pool across a time course after birth injury. The same control (yellow) was used to compare at each timepoint (N=9). SBI (blue), SBI+NSAID, (red), and NSAID (orange) was N=3/timepoint/group.

	Control		SBI		SBI+NSAID		NSAID	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
1 Day	15.40	2.02	6.48	5.14	3.83	0.40	15.80	1.15
3 Days	15.40	2.02	12.88	0.65	9.95	4.06	12.69	0.64
5 Days	15.40	2.02	30.28	1.21	27.44	7.58	12.03	1.40
7 Days	15.40	2.02	80.42	4.04	44.32	2.36	11.77	1.84
10 Day	15.40	2.02	22.00	1.22	14.36	2.03	12.38	1.72

Table 6. Pax7 p-values
Table of Pax7 p-values/timepoint/groups as determined from Two-way ANOVA (post-hoc Tukey Test), where n.s. = no significant differences, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001

	Group 1 v. Group 2		Significance	p-value
1 Day	Control	SBI	n.s.	0.10
	Control	SBI+NSAID	*	0.017
	Control	NSAID	n.s.	1.00
	SBI	SBI+NSAID	n.s.	0.94
	SBI	NSAID	n.s.	0.20
	SBI+NSAID	NSAID	n.s.	0.059
3 Days	Control	SBI	n.s.	0.91
	Control	SBI+NSAID	n.s.	0.49
	Control	NSAID	n.s.	0.89
	SBI	SBI+NSAID	n.s.	0.92
	SBI	NSAID	n.s.	>1.00
	SBI+NSAID	NSAID	n.s.	0.94
5 Days	Control	SBI	**	0.0012
	Control	SBI+NSAID	*	0.012
	Control	NSAID	n.s.	0.76
	SBI	SBI+NSAID	n.s.	0.93
	SBI	NSAID	***	0.0005
	SBI+NSAID	NSAID	**	0.0040
7 Days	Control	SBI	****	< 0.0001
	Control	SBI+NSAID	****	< 0.0001
	Control	NSAID	n.s.	0.78
	SBI	SBI+NSAID	****	< 0.0001
	SBI	NSAID	****	< 0.0001
	SBI+NSAID	NSAID	****	< 0.0001
10 Day	Control	SBI	n.s.	0.32
	Control	SBI+NSAID	n.s.	1.00
	Control	NSAID	n.s.	0.86
	SBI	SBI+NSAID	n.s.	0.37
	SBI	NSAID	n.s.	0.18
	SBI+NSAID	NSAID	n.s.	0.97

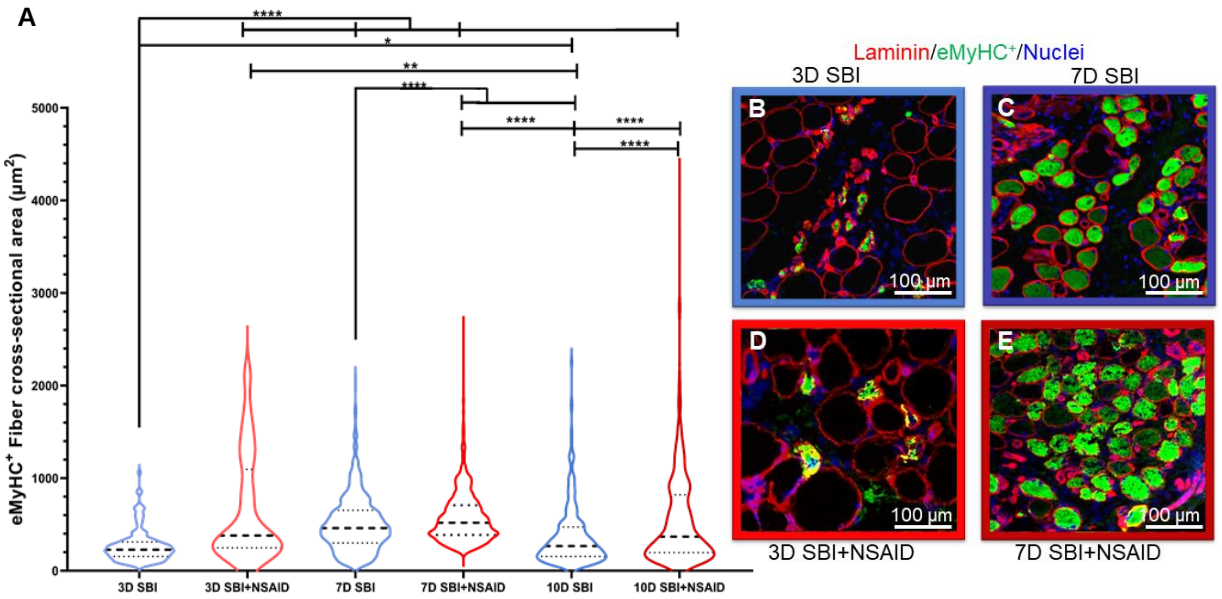

Figure 7. NSAIDs led to transient hypertrophy during regeneration
(A) Violin plots of embryonic myosin heavy chain positive (eMyHC$^+$) cross-sectional fiber area (µm^2) after simulated birth injury (SBI) (blue) or SBI + NSAIDs (red). Data demonstrates that NSAID groups had significantly increased fiber size across all compared timepoints compared to the SBI groups. (**B-E**) Representative images of eMyHC$^+$ sections for SBI and SBI+NSAID groups 3- and 7-days post-birth injury. Not normally distributed data was analyzed by Kruskal-Wallis test (post-hoc Dunn's test). ****p<0.0001.

Table 7. eMyHC$^+$ cross-sectional fiber area distribution values
Table of eMyHC$^+$ fiber area distribution values (median, minimum, and maximum cross-sectional fiber area (µm^2)) for 3, 7, and 10 days SBI (blue) and SBI+NSAID.

		Median (µm^2)	Minimum (µm^2)	Maximum (µm^2)
SBI	3 Days	228	57	1062
	7 Days	461	55	2076
	10 Days	268	33	2258
SBI+NSAID	3 Days	381	90	2141
	7 Days	519	159	2636
	10 Days	370	57	4197

Table 8. eMyHC$^+$cross-sectional fiber area p-values
Table of eMyHC$^+$ fiber area p-values for 3, 7, and 10 days SBI and SBI+NSAID as determined by Kruskal-Wallis (post-host Dunn's Test), where n.s. = no significant differences, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001

Group 1 v Group 2		Significance	p-value
3D SBI	3D SBI+NSAID	****	< 0.0001
3D SBI	7D SBI	****	< 0.0001
3D SBI	7D SBI+NSAID	****	< 0.0001
3D SBI	10D SBI	*	0.021
3D SBI	10D SBI+NSAID	****	< 0.0001
3D SBI+NSAID	7D SBI	n.s.	> 1.00
3D SBI+NSAID	7D SBI+NSAID	n.s.	0.70
3D SBI+NSAID	10D SBI	**	0.0034
3D SBI+NSAID	10D SBI+NSAID	n.s.	> 1.00
7D SBI	7D SBI+NSAID	****	< 0.0001
7D SBI	10D SBI	****	< 0.0001
7D SBI	10D SBI+NSAID	n.s.	0.24
7D SBI+NSAID	10D SBI	****	< 0.0001
7D SBI+NSAID	10D SBI+NSAID	****	< 0.0001
10D SBI	10D SBI+NSAID	****	< 0.0001

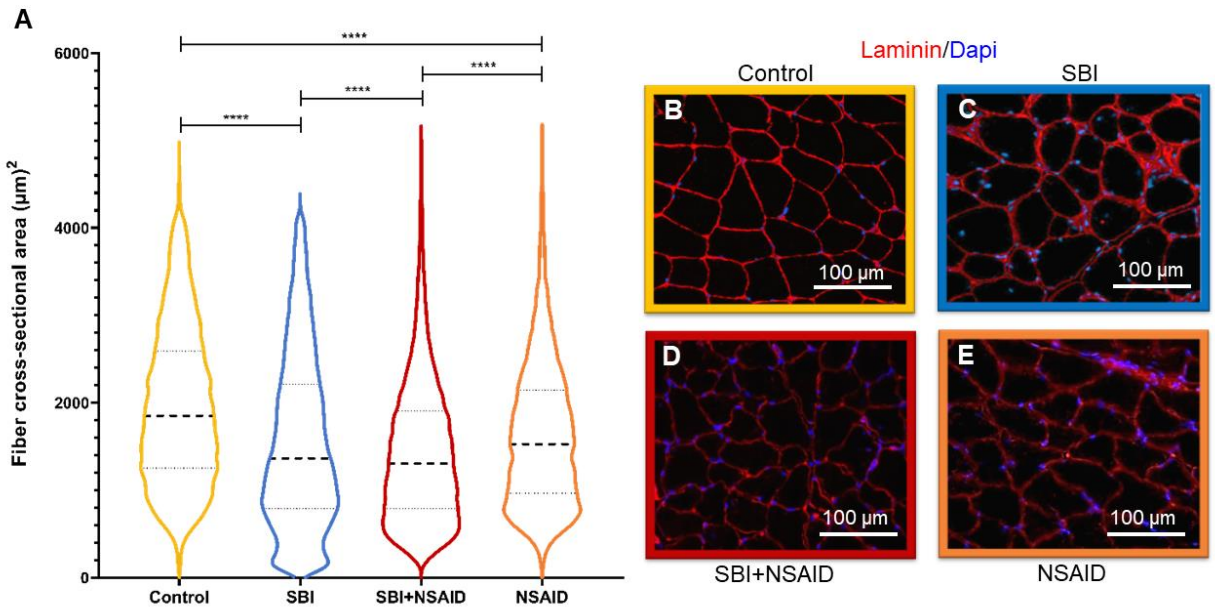

Figure 8. NSAIDs decreased fiber size 28 days after birth injury

(A) Violin plot of fiber cross-sectional area (μm^2) 28 days birth injured and uninjured groups. The plot illustrates that NSAIDs decreased fiber size for both injured (SBI+NSAIDs) and uninjured (NSAID; orange) conditions. (B-E) Representative images of myofiber size differences between groups: **(B)** control, **(C)** SBI alone (blue), **(D)** SBI+NSAID (red), and **(E)** NSAIDs alone (orange). Laminin stains for myofibers while Dapi stains for cell nuclei. Not normally distributed data was analyzed by Kruskal-Wallis (post-host Dunn's test). ****$p<0.0001$. All distribution values are listed in the results section.

DISCUSSION

Ibuprofen, the most commonly prescribed NSAID during postpartum recovery (Deussen et al., 2011; Wuytack et al., 2016), is a non-selective COX inhibitor that blocks COX-1 and COX-2 (COX-1/2) enzymes and reduces pain and inflammation (Ricciotti & FitzGerald, 2011). Limb skeletal muscle studies have shown that COX-1/2 are essential enzyme regulators of the immune and myogenic cells relevant to muscle homeostasis and recovery from injury (Bondesen et al., 2004, 2006; Mo et al., 2019; Morita, 2002; Ong et al., 2007; Ricciotti & FitzGerald, 2011; Salvemini et al., 2013; C. J. Smith et al., 1998). Studies of mouse and rat hindlimb muscles further confirmed the importance of COX to the aforementioned muscle regenerative cellular events by showing that knockdown or NSAID-induced inhibition of COX-1/2 lead to decreased immune cell response (leukocytes and macrophages) and impaired myogenesis, resulting in delayed muscle regeneration (Bryant et al., 2017; Ho et al., 2017; Mackey et al., 2012; Mikkelsen et al., 2009; Mo et al., 2019).

Using previously validated rat simulated birth injury (SBI) model, the current study investigated the effect of non-selective NSAID, ibuprofen, on the recovery of the pelvic floor muscles (PFMs) following birth injury. To achieve the above, we first established the expected time course in which muscle regenerative immune cell response and myogenesis would occur post-SBI in PFMs. We observed that following SBIs, leukocytes ($CD45^+$ cells) peaked at 1 day (Figure 4A). This is consistent with previously published studies in the limb muscle that detail leukocytes infiltrating within 24 hours after muscle injury to clear damaged myofibers (Albina et al., 2017; R. A. Collins & Grounds, 2001; Nicholas et al., 2015; Warren et al., 2002). In examining the changes to myogenesis post-SBI, differentiated MuSCs ($myogenin^+$ cells) peaked 3 days (Figure 5A) followed by an increase in the MuSC pool ($Pax7^+$ cells) at 7 days (Figure 6A). These observed myogenic cell events is consistent with the myogenic regeneration timeline previously described in the introduction (Pawlikowski et al., 2019; Rantanen et al., 1995; Schmidt et al., 2019; Tidball, 2017; Yang & Hu, 2018). In turn, the cellular events associated with PFM regeneration after SBI followed the expected temporally-regulated events established in published limb muscle literature.

NSAID alone groups (NSAID) were included to control for potential NSAID effects on unperturbed muscles. We observed no statistical differences between control and NSAID groups for neither leukocyte response, MuSC differentiation, or overall MuSC activity (Figure 4A, 5A, 6A). This outcome is as we expected. As previously described in the introduction, ibuprofen is a non-selective NSAID that inhibits both COX-1 and COX-2 (COX-1/2) (Mitchell et al., 1993; Ong et al., 2007; Ricciotti & FitzGerald, 2011). COX-2, in particular, plays a more predominate role in mediating immune response and myogenesis associated with muscle regeneration (Bondesen et al., 2004; Bondesen et al., 2006; Morita, 2002). However, COX-2 requires an inflammatory stimulus, such as muscle injury, in order to activate and upregulate. In unperturbed conditions, COX-2 activity remains extremely low and barely detectable (Gilroy et al., 1999; Kang et al., 1996). Therefore, NSAIDs should not have an effect on uninjured muscles as little to no COX-2 activity for the NSAID to inhibit.

Birth injured groups with NSAIDs (SBI+NSAID) served as the main experimental group in order to assess the impact NSAIDs have on the muscle regenerative cellular events following birth injury. NSAID use following SBI did not affect the overall leukocyte infiltration at the acute time points examined (Figure 4A-E). Our observations contrast expectations based on published NSAID literature. Extensive studies investigating the importance of the COX-2 pathway on muscle regeneration have demonstrated that inhibition of COX-2 through the use of COX-2 inhibitors or COX-2 selective NSAIDs significantly reduced pro-inflammatory immune response (leukocytes and pro-inflammatory macrophages). This interference in pro-inflammatory immune response coupled with decreased myogenesis then contributes to increased muscle atrophy and impair muscle regeneration (Bondesen et al., 2004, 2006; Paskauskas et al., 2011; Shen et al., 2005). Note, non-selective NSAIDs do not inhibit COX-2 to the same extent of COX-2 inhibitors or COX-2 selective NSAIDs (Ong et al., 2007; Ricciotti & FitzGerald, 2011), but some non-selective NSAID studies have also reported observing slightly reduced leukocyte infiltration or activity (Bryant et al., 2017; Cheung & Tidball, 2003; Giannakis et al., 2019). However, the majority of these and other non-selective NSAID studies conclude the observed differences from NSAID treatment were not significant compared to non-NSAID controls (Bryant et al., 2017; Cheung & Tidball, 2003; Giannakis et al., 2019; Mikkelsen et

al., 2009). Thus, our results are in line with this second body of literature, suggesting that the COX-inhibitory effects of non-selective NSAIDs is not strong enough to significantly inhibit leukocyte response.

In examining the effects of NSAIDs on myogenesis, we first evaluated the effects NSAIDs had on differentiation of MuSCs. While there were no statistical differences between the SBI+NSAID compared to the SBI group, the mean cell density for SBI+NSAID group was doubled at 5 days post-SBI compared to the SBI alone group returned to control levels 5 days post-SBI (Figure 5A-E). This suggest NSAIDs altered the normal time course for MuSC differentiation. Limb studies have demonstrated that NSAID use or COX-1/2 knockdown significantly decreased myoblast differentiation (Mendias et al., 2004b; Mo et al., 2019). In particular, Mo et al., revealed that decrease in myogenic differentiation led to morphological changes to the formation of myotubes as fusion of myoblast was significantly reduced (Mo et al., 2019). Although we did not observe a decrease in differentiated MuSCs from NSAIDs as shown in published literature, the delay in normal myogenic response could still point towards alterations in muscle regeneration.

As a secondary assessment of the effects NSAIDs have on myogenesis post-SBI, we evaluated the impact NSAIDs had on the overall activity of the MuSC pool. We observed that NSAIDs significantly decreased the MuSC pool 7 days post-SBI (Figure 6A-E). Our results are consistent with existing rodent and human limb muscle studies that have demonstrated that the use of COX-2 selective and non-selective NSAIDs impeded MuSC activity (Bondesen et al., 2004; Bondesen et al., 2006; Ho et al., 2017; Mackey et al., 2007; Mikkelsen et al., 2009). This is because COX activity is key for MuSC activation. COX activation is responsible for mediating the production of prostaglandins, which are important for regulating and mediating myogenic response to injury (Ricciotti & FitzGerald, 2011). For instance, PGE_2 is associated with the enhancement of myoblast proliferation and differentiation (Giagoudakis & Markantonis, 2005; Ho et al., 2017; Sheppe et al., 2018; Trappe & Liu, 2013). A recent study on mice injured hindlimbs illustrated that non-selective NSAID treatment decreased PGE_2 signaling as well as $Pax7^+$ MuSC pool, resulting in impaired muscle regeneration and reduced muscle strength. The observed phenotypes were then rescued by intramuscular delivery of PGE_2, directly connecting the necessity for PGE_2 in muscle regeneration and

MuSCs function (Ho et al., 2017). Thus, while we did not directly assess whether PGE_2 is contributing to reduction of $Pax7^+$ MuSCs, we can posit that the decrease in MuSCs number is potentially due to NSAID-mediated COX inhibition of muscle regenerative prostaglandins.

The observed NSAID-induced alterations in myogenic response—delayed MuSC differentiation and decreased expansion of the MuSC pool—during acute timepoints of injury likely contributed to the assessed long-term negative impacts NSAIDs had on PFM fiber size. Firstly, 28 days following SBI alone, we observed a significantly decreased fiber size compared to controls (Figure 8A-C). This indicates evidence of increased muscle atrophy due to birth injury, consistent with limb studies that show even after 28 days or more following severe muscle trauma the muscle does not fully recover (Järvinen et al., 2013; Winkler et al., 2011). The additional of NSAID treatment for both uninjured (NSAID) and birth injured groups (SBI+NSAID) resulted in a significant reduction in fiber size compare to control and SBI alone (Figure 8A-E). The reduced fiber size for the uninjured NSAID group was most surprising. As previously described, we did not anticipate NSAIDs to have an effect on unperturbed muscle (Ricciotti & FitzGerald, 2011). We hypothesize that the decreased fiber size in the NSAID group could be due to the animals becoming hyperglycemic from the high sugar content (30 % of sucrose) in the NSAID used for the study. Various published literature on limb muscles have described how high glucose intake can cause glucose toxicity or hyperglycemia, conditions in which have been linked to causing both inhibition of myogenesis and muscle regeneration (Kawahito et al., 2009; Luo et al., 2019; Riedinger et al., 2018; Röhling et al., 2016; Saki et al., 2013). For the SBI+NSAID group, the reduced fiber size was consistent with limb studies that suggest long-term NSAID use contributes to increased long-term muscle atrophy after injury (Bondesen et al., 2006; Mackey et al., 2012; Morelli et al., 2018; Shen et al., 2006; Urso, 2013). As previously explained, the COX-2 pathway is important in regulating muscle growth and regeneration after injury. Many studies have demonstrated in mouse models that knock out of COX-2 or continuous use of a COX-2 selective inhibitor for up to 14 days after hindlimb muscle injury that myofibers were significantly smaller, indicating increased signs of atrophy compared to wild-type or uninhibited controls (Bondesen et al., 2006; Shen et al., 2005; Shen et al., 2006). One non-selective NSAID study observed in rabbit hindlimbs

a similar negative impacts to myofiber regeneration after 28 days of continuous flurbiprofen use 28 days after injury (Mishra et al., 1995), but unfortunately, the majority of non-selective NSAID studies do not evaluate the impacts of NSAIDs on muscle regeneration beyond acute timepoints after injury (max 8-10 days) (Mackey et al., 2012; Morelli et al., 2018). Our study still presents the possibly that prolonged NSAID use after birth injury has a negative impact towards PFM regeneration.

Overall, our study is unique in that it is the first known study to elucidate the immune and myogenic cellular events associated with PFM regeneration following SBI. The majority of existing muscle regeneration studies focus on evaluating limb muscles. There are no known publications that assessed the pathological events that occur during PFM regeneration, especially in the context of birth injuries. Furthermore, we are the first to investigate the impact NSAID have on cellular events associated with PFM regeneration. Despite the established clinical practice to prescribe ibuprofen to women for postpartum recovery, no research has been done to assess the immediate or long-term effects continuous use of NSAIDs may have on PFM regeneration An additional strength of our study that we assessed the cellular events across multiple acute timepoints after injury. A major flaw of currently publish literature on limb muscles is that only 2-3 key acute timepoints are chosen based on the expected timepoint immune or myogenic events are expected to peak. However, the large gap between timepoints is not sufficient to capture slight delays or shifts in the cellular events which could lead to major impacts in overall regeneration. For instance, the delay we observed in differentiation MuSCs due to NSAIDs would not have been captured if we had not assessed all the possible acute timepoint and would likely have concluded that NSAIDs had no effect MuSC differentiation. Hence, our novel approach at assessing multiple timepoints allowed us to capture the slight but significant changes in MuSC differentiation due to NSAID, which likely contributed to the overall impairment in myofiber size observed at the long-term timepoint.

Nevertheless, there are limitations to our study. The use of a rat model, although previously validated for the study of PFMs and birth injuries, is not completely representative to human PFM. Pelvic muscles from nonhuman primates are considered a more ideal model. It has been shown that in terms of muscle composition and size as well as functionally of levator ani muscles, non-human primate pelvic

muscle are a more analogous comparison to human PFMs (Couri et al., 2012; Otto et al., 2002). Research utilizing nonhuman primates, however, is severely limited due to strict regulatory challenges and extremely expensive procurement and housing cost. Therefore, for given the amount of timepoints and group of our NSAID study, it is more cost effective to use a rat model. In addition, the large variability evident in our leukocyte and myogenin data is most likely due to our small sample size (N=3/timepoint/group). The sample size selected for this study was determined using a power analysis (priori analysis) performed on G*power of pre-existing SBI data (α=0.05, power set at 80). We then re-analyzed our sample size for leukocytes and myogenin based on our current SBI+NSAID data. Using G*power, the power to detect the standardized mean difference of 0.52 for the leukocyte data with α=0.05, when N=3/group is 13% (post-hoc analysis). Based on sample size calculations (priori analysis), a size of 48/group is required for leukocyte data to achieve an 80% power with α=0.05. For the myogenin data, the power to detect the standardized mean difference of 0.95 with α=0.05, when N=3/group is 25% (post-hoc analysis). Based on sample size calculations (priori analysis), a size of 14/group is required for myogenin data to achieve an 80% power with α=0.05. In other words, both leukocyte and myogenin studies were underpowered and more animals would be needed to account for the variation and achieve an 80% power.

Conclusions and Future Directions

In summary, we demonstrated not only the profound negative impact SBIs have on the morphological properties of PFMs but that NSAIDs further attenuate the muscle regenerative cellular events following birth injury. SBIs have a striking morphological effect on PFM and that severe muscle trauma from SBI causes pathological alteration to the temporally-regulated cellular events, contributing to impaired muscle regeneration long-term. Upon treatment with NSAID during acute recovery period, NSAIDs did not appear to impact leukocyte infiltration, but did delay MuSC differentiation and decreased the MuSC pool. The alteration to the myogenic events likely contributed to the long-term negative impacts NSAIDs treatment had for both birth-injured and uninjured groups as demonstrated by the significant reduction in PFM myofiber size compared animals that did not receive NSAIDS. Therefore, our study suggest that NSAIDs may negatively impact PFM regeneration.

For future study, we seek to elucidate the immune mechanisms by which NSAIDs impact PFM regeneration. Previous work in the lab had highlighted a sustained pro-inflammatory response after birth injury. Specifically, pro-inflammatory cell surface markers and cytokines related to immune cells polarization were significantly upregulated up to one month after injury whereas anti-inflammatory markers and cytokines quickly returned to baseline levels. Hence, the study indicated the prevalence of pro-inflammatory response during muscle regeneration after birth injury. To evaluate the impact of NSAIDs on immune cells polarization, qRT-PCR will be utilized. A panel of target genes, including immune cell makers, chemokines, cytokines, and transcription factors will be designed to achieve this goal. In particular, we focused on evaluating changes in macrophage immune response and polarization because, as discuss in the introduction, it is tightly coupled with myogenesis and thus a key modulator of muscle regeneration (Dort et al., 2019; Locati et al., 2013; Martinez & Gordon, 2014; Mills, 2015; Mills et al., 2000; Novak et al., 2014; Pillon et al., 2013; Tidball, 2017; Tidball & Villalta, 2010; Wynn & Vannella, 2017). The experimental groups will be the same as those used in the current study: control, SBI, SBI+NSAID, and NSAID. However, only two timepoints, 5 and 10 days, will be assessed for gene expression analysis. Based on limb studies 5 and 10 days encompasses the macrophage polarization process, and thus should allow us to capture the shift from pro-inflammatory to anti-inflammatory response (C. Smith et al., 2008; Tidball, 2017; Tidball et al., 2014). Based on literature, we expect NSAIDs to decrease pro-inflammatory expression—as observed in previous studies (Giannakis et al., 2019; Mikkelsen et al., 2011; Paccani et al., 2002). We also expect a reduction in COX-1/2 expression in response to NSAID treatment (Mikkelsen et al., 2011; Mitchell et al., 1993).

For instance, employing $CD4^+$ and $CD8^+$ as makers for the identification of T-cells, an *in vitro* study demonstrated that NSAIDs suppresses general T-cell activation (Paccani et al., 2002). Another study on local non-selective NSAID effect after eccentric exercise in human leg muscle reported that pro-inflammatory IL-1b and TNF-α were significantly downregulated compared to injury alone (Mikkelsen et al., 2011). Regarding macrophages polarization, it has been shown that as consequence of 4 days daily administration of ibuprofen on cardiotoxin injured mice, number of M1 macrophages was low, while that

of M2 macrophages was high compared to injury alone controls. This suggests that ibuprofen can alter macrophage polarization kinetics (Giannakis et al., 2019). We also expect a reduction in COX-1/2 expression in response to NSAIDs treatments. An early *in vitro* study reported that ibuprofen was more potent at inhibiting COX-1/2 activity, particularly COX-2, compared to some other non-selective NSAIDs (Mitchell et al., 1993). Unfortunately, there are few recent studies assessing the effects of NSAIDs on COX activity or gene expression after muscle injury. The few studies that do examine COX gene expression do not observe significant inhibition of COX-1/2 expression from NSAIDs, but large variations in data (Dale et al., 2020) and non-continuous administration of NSAIDs (Mikkelsen et al., 2009) may contribute to the lack of significant differences.

Unfortunately, there are no known studies that have investigated the impact NSAID have on anti-inflammatory immune response, but we posit that NSAIDs will not impact anti-inflammatory response to injury. However, based on previously observed anti-inflammatory response after birth injury alone and alteration to myogenic events that led to impair PFM regeneration long-term, we posit that NSAIDs will have little to no significant changes to the expression of the majority of anti-inflammatory response to injury.

MATERIALS AND METHODS

4.1 Study Design

All procedures performed were approved and in compliance with The University of California, San Diego Institutional Animal Care Committee. Female 3-months old Sprague-Dawley rats (Envigo) were used in this study. Animals were divided into four experimental groups: 1) uninjured untreated controls (Control; N = 9), 2) uninjured, NSAID exposure (NSAID), 3) simulated birth injured without NSAID (SBI), and 4) SBI with NSAID (SBI+NSAID) (N = 3/timepoint/group). Simulated birth injury (SBI) was performed using vaginal balloon distention using previously published protocol (Alperin et al., 2010; Alperin, et al., 2010; Cannon et al., 2002; Damaser et al., 2003; Lin et al., 1998; Resplande et al., 2002; Sievert et al., 2001). Animals assigned to SBI+NSAID group were given an ibuprofen solution (0.2 mg/ml liquid ibuprofen mixed with drinking water) immediately after SBI and were allowed to drink the solution ad lib as they recovered for either 1, 3, 5, 7, 10 (acute timepoints), or 28 days (long-term timepoint) post birth injury before sacrifice. The timepoints were chosen corresponds with peak infiltration of immune cells and/or MuSCs after skeletal muscle injury (Tidball, 2017). The same solution was added to the drinking water of uninjured NSAID animals who were housed for the same duration as injured animals. Uninjured rats and animals subjected to SBI without NSAID administration were given drinking water only. All animals were euthanized via CO2 inhalation followed by thoracotomy.

4.2 Simulated Birth Injury

Rats were anesthetized under 2.5% isoflurane in oxygen and placed supine on an edge of a table. A 12-French transurethral catheter was trimmed flushed to the end of balloon when inflated before insertion into the vagina. The balloon was inflated to a 5 mL volume and left free hanging with a 130g weight off the edge of the table for 2 hours to replicate circumferential and downward strains associated with parturition. Afterwards with the balloon still inflated, the catheter is removed.

4.3 NSAID Administration

The NSAID concentration was determined by referencing an established ad lib NSAID protocol from the Ohio State University Laboratory Animal Resources Vet Guidelines, the ibuprofen solution protocol described in the Soltow et al. paper (Soltow et al., 2008). Various other rodent pain studies also reference that same ad lib ibuprofen solution protocol for the management of pain management (Ezell et al., 2012; Salama et al., 2016). To ensure the rats were ingesting at least 30 ml of the solution per day to receive a 20 mg/kg dose of the NSAID solution, the average volume of the solution between 3 rats/cage was monitored and recorded frequently. The weight of the rats were also monitored and recorded concurrently to further confirm that the rats were drinking the solution and to monitor their overall health and well-being.

4.4 Tissue Histology and Immunohistochemistry

Following sacrifice, the public synthesis was disarticulated. Vagina and anus sphincter was dissected away for access to the PFMs. Pubocaudalis was dissected and imbedded in mold filled with Optimal Cutting Temperature media (Sakura Finetek Inc). The muscle was then pinned at the outermost edge of the muscle (origin and insertion) before frozen in liquid nitrogen-cooled isopentane. Samples were cut into 10 µm-thick sections with a cryostat. Tissue section slides were stored at -80°C until use for histology and immunohistochemistry. The overall muscle morphology for control and post-SBI groups (1, 3, and 7 days) was evaluated using hematoxylin and eosin. Sections were stained with antibodies against CD45 (leukocytes), myogenin (differentiated MuSCs), Pax7 (quiescent and activated MuSCs), and embryonic myosin heavy chain (eMyHC) (regenerated fibers) were used to assess the cellular events associated with PFM regeneration during acute timepoints post-birth injury (1, 3, 5, 7, and 10 days). Note, eMyHC was only evaluated for 3, 7, and 10 days post-birth injury with and without NSAID administration as those are the known timepoints in which myofiber regeneration occurs (Beylkin et al., 2006; Pawlikowski et al., 2019; Tidball, 2017; Zammit et al., 2006). Long-term fiber area analysis at 28 days timepoints for all groups was assessed using an antibody against laminin to stain for the muscle fibers.

4.4.1 Hematoxylin and Eoin

Frozen tissue section slides were placed in a dipping rack and rinsed in tap water for 30 secs. Racks were then incubated in Harris' hematoxylin for 3-5 mins. Slides were then rinsed in tap water 3 times before a quick dip in 0.5% acidic alcohol and rinses in tap water again. Slides were then dipped 30 times in a saturated lithium carbonate solution and rinsed in tap water. A quick drip in 1% alcoholic eosin follows, blotting away excess on a paper towel. Slides are then dipped 30 times in 95% ethanol alcohol, blotting away excess from slides, and repeated in a separate 95% ethanol alcohol container. Next, slides are dipped in three sets of 99% isopropanol, 30 dips each and blotting slides between each set. Dips in three sets of Citri-Solv follows. For the first Citri-Solv dip, slides are dipped clear while the next two Citri-Solv sets are dipped 30 times each. Slides are left in the last Citri-Solv dip as slides were individually mounted with vectamount permanent mounting medium. Slides were placed on a slide rack and left to dry in the dark before imaging.

4.4.2 Leukocytes

Frozen tissue sections were fixed using -20°C acetone for 10 min. The tissue sections were washed using 1xPBS agitating on a shaker and blocked with a blocking buffer (1xTBS, 0.05% tween-20, 1% BSA, 5% goat serum) for 2 hours at room temperature. The slides were incubated in primary antibodies mouse CD45 (1:100; Bio-Rad) and rabbit laminin (1:100, Sigma-Aldrich – L9393) overnight at 4°C. The slides are then washed in1xTBS agitating on a shaker before 1-h secondary antibody incubation (Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 546 goat anti-mouse IgG; 1:500; Thermo Fisher Scientific). Washes in 1xTBS agitating on a shaker followed before incubation in DAPI (1:10,000, Thermo Fisher Scientific - 62248) for 10 mins, agitated washes in 1xTBS, slide mounting, and storage at 4°C.

4.4.3 MuSCs (Pax7, Myogenin, and eMyHC)

Frozen sections were fixed using 4% paraformaldehyde (PFA) for 15 min at room temperature. The tissues sections were washed using 1xPBS and blocked with a blocking buffer (Pax7: 10% goat serum, 0.03% triton-100x; Myogenin: 5% goat serum, 0.3% triton 100-x, 1% bovine serum albumin) for 1-h at

room temperature. Primary antibody incubation follows using laminin (1:100, Sigma-Aldrich – L9393), incubating overnight at room temperature. PBS washes are done before 1-h filtered secondary antibody incubation (Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 546 goat anti-mouse IgG; 1:500; Thermo Fisher Scientific). The tissue section are washed using PBS before fixing in 4% PFA for 15 min at room temperature once more. Heat activated antigen retrieval follows, incubating the tissues in a citrate buffer in a heated water bath for 15 min. The slides are left to cool for 5 min in the dark before at least 5 min incubation in PBS. Tissue section are dipped in a 0.1% triton-100x PBS solution before more PBS washes in preparation for 1-h blocking in blocking buffer at room temperature. Primary antibody Pax7 (1:100; Developmental Studies Hybridoma Bank (DHSB)) or myogenin (1:200; BD Pharmingen) was added, incubating overnight at room temperature. PBS washes follows. The tissue section are incubated in filtered secondary antibodies for 1-h before PBS washes and incubation in DAPI (1:10,000; Thermo Fisher Scientific - 62248) for 10 mins. Slides are then mounted and stored at 4°C.

For eMyHC, frozen tissue sections were fixed in -20°C acetone for incubated for 15 mins. The tissue sections are then moved into PBS before incubating for 30 mins in blocking buffer (10% goat serum and 0.03% triton-100x). Primary antibody mouse eMyHC (1:100; DSHB – F1.652) and rabbit laminin (1:200; Sigma-Aldrich – L9393) is added and incubates overnight at room temperature. PBS washes follow before incubating tissues sections in filtered secondary antibodies (Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 546 goat anti-mouse; 1:250; Thermo Fisher Scientific), for 1-h. Sections are then washed with PBS for 10 mins. A PBS DAPI solution (1:10,000; Thermo Fisher Scientific - 62248) is placed over the slides before mounting and storage at 4°C.

4.5 Imaging Analysis

4.5.1 Hematoxylin and Eosin

Hematoxylin and Eosin staining was imaged on a Lecia SCN400 slide scanner. Whole tissue sections were imaged per group per timepoint (9-10 sections per muscle per animal) before morphology was visually assessed on ImageJ.

4.5.2 Leukocytes and MuSCs

Leukocytes and MuSC tissue section stains for SBI only were imaged using Lecia Ariol slide scanner while NSAID groups were imaged using Lecia AF6000 Modular System fluorescent microscopy. Pax7 and leukocyte stains were imaged 4 tiles per tissue section while myogenin stains were imaged as whole tissue section images (all at 20X magnification). The images were manually quantified, counting positively stained cells on Image scope for controls and SBI alone groups and on ImageJ for controls and all NSAID groups. Cell counts were normalized with the tissue section area in the image for cell density (#cells/mm^2 tissue section area).

4.5.3 Fiber cross-sectional area

Fiber cross-sectional area was quantified automatically from whole tissue section images of control and 28D SBI, SBI+NSAID, and NSAID groups (9-10 sections per muscle; 20X magnification) using a fiber area macro program on ImageJ. The quantified fiber cross sectional areas (μm^2) for each group was then collated together and sorted into a violin plot to assess overall distribution density of fiber size between the experimental groups.

4.5.4 Embryonic myosin heavy chain

Embryonic myosin heavy chain stains were also imaged using Lecia AF6000 Modular System fluorescent microscopy. Whole tissue section images were taken of 3 and 7 days SBI groups and 3, 5, 7, and 10 days SBI+NSAID (10X magnification). The tissue section with the most $eMyHC^+$ stained fibers per animal was visually assessed and imaged for analysis. On ImageJ, fibers without $eMyHC^+$ were manually deleted before running the modified image through a fiber area macro program. The quantified fiber cross sectional areas (μm^2) were then collated together per group per timepoint and sorted into a violin plot to observed distribution density of the fiber size between the experimental groups and timepoints to comparatively evaluate differences in regenerative fiber size.

4.6 Statistical Analysis

IHC data was statistically analyzed using Two-way ANOVA (post-hoc Tukey Test for pairwise comparison), while non-parametric eMyHC and 28 days fiber area data was statistically analyzed using Kruskal-Wallis (post-hoc Dunn Test for pairwise comparison). Significance for both analyses were set at $\alpha<0.05$. All statistical analyses were performed using Prism 8.

Power analysis for sample size (post-hoc analysis and Priori analysis) was performed using G*power. See discussion for more details.

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