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Effect of nonsteroidal anti-inflammatory drugs on pelvic floor muscle regeneration in a preclinical birth injury rat model

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Abstract

BACKGROUND: Pelvic floor muscle injury is a common consequence of vaginal childbirth. Nonsteroidal anti-inflammatory drugs are widely used postpartum analgesics. Multiple studies have reported negative effects of these drugs on limb muscle regeneration, but their impact on pelvic floor muscle recovery following birth injury has not been explored.

OBJECTIVE: Using a validated rat model, we assessed the effects of nonsteroidal anti-inflammatory drug on acute and longer-term pelvic floor muscle recovery following simulated birth injury.

STUDY DESIGN: Three-month old Sprague Dawley rats were randomly assigned to the following groups: (1) controls, (2) simulated birth injury, (3) simulated birth injury+nonsteroidal anti-inflammatory drug, or (4) nonsteroidal anti-inflammatory drug. Simulated birth injury was induced using a well-established vaginal balloon distension protocol. Ibuprofen was administered in drinking water (0.2 mg/mL), which was consumed by the animals ad libitum. Animals were euthanized at 1, 3, 5, 7, 10, and 28 days after birth injury/ibuprofen administration. The pubocaudalis portion of the rat levator ani, which, like the human pubococcygeus, undergoes greater parturition-associated strains, was harvested (N=3–9/time point/group). The cross-sectional areas of regenerating (embryonic myosin heavy chain^{+}) and mature myofibers were assessed at the acute and 28-day time points, respectively. The intramuscular collagen content was assessed

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at the 28-day time point. Myogenesis was evaluated using anti-Pax7 and anti-myogenin antibodies to identify activated and differentiated muscle stem cells, respectively. The overall immune infiltrate was assessed using anti-CD45 antibody. Expression of genes coding for pro- and anti-inflammatory cytokines was assessed by quantitative reverse transcriptase polymerase chain reaction at 3, 5, and 10 days after injury.

RESULTS: The pubocaudalis fiber size was significantly smaller in the simulated birth injury+nonsteroidal anti-inflammatory drug compared with the simulated birth injury group at 28 days after injury ($P<.0001$). The median size of embryonic myosin heavy chain$^+$ fibers was also significantly reduced, with the fiber area distribution enriched with smaller fibers in the simulated birth injury+nonsteroidal anti-inflammatory drug group relative to the simulated birth injury group at 3 days after injury ($P<.0001$), suggesting a delay in the onset of regeneration in the presence of nonsteroidal anti-inflammatory drugs. By 10 days after injury, the median embryonic myosin heavy chain$^+$ fiber size in the simulated birth injury group decreased from 7 days after injury ($P<.0001$) with a tight cross-sectional area distribution, indicating nearing completion of this state of regeneration. However, in the simulated birth injury+nonsteroidal anti-inflammatory drug group, the size of embryonic myosin heavy chain$^+$ fibers continued to increase ($P<.0001$) with expansion of the cross-sectional area distribution, signifying a delay in regeneration in these animals. Nonsteroidal anti-inflammatory drugs decreased the muscle stem cell pool at 7 days after injury ($P<.0001$) and delayed muscle stem cell differentiation, as indicated by persistently elevated number of myogenin$^+$ cells 7 days after injury ($P<.05$). In contrast, a proportion of myogenin$^+$ cells returned to baseline by 5 days after injury in the simulated birth injury group. The analysis of expression of genes coding for pro- and anti-inflammatory cytokines demonstrated only transient elevation of *Tgfb1* in the simulated birth injury+nonsteroidal anti-inflammatory drug group at 5 but not at 10 days after injury. Consistently with previous studies, nonsteroidal anti-inflammatory drug administration following simulated birth injury resulted in increased deposition of intramuscular collagen relative to uninjured animals. There were no significant differences in any outcomes of interest between the nonsteroidal anti-inflammatory drug group and the unperturbed controls.

CONCLUSION: Nonsteroidal anti-inflammatory drugs negatively impacted pelvic floor muscle regeneration in a preclinical simulated birth injury model. This appears to be driven by the negative impact of these drugs on pelvic muscle stem cell function, resulting in delayed temporal progression of pelvic floor muscle regeneration following birth injury. These findings provide impetus to investigate the impact of postpartum nonsteroidal anti-inflammatory drug administration on muscle regeneration in women at high risk for pelvic floor muscle injury.

Keywords

birth injury; muscle stem cells; nonsteroidal anti-inflammatory drug; pelvic floor muscles; rat; regeneration

Introduction

The pelvic floor muscles (PFMs) are skeletal muscles that span the pelvic outlet, providing support to pelvic and abdominal organs and aiding in continence.[1,2] In humans, these muscles include the coccygeus and the levator ani complex, which is comprised

of the puborectalis, pubococcygeus, and iliococcygeus. Computational simulations and radiological studies of the female pelvic floor indicate that, during spontaneous vaginal childbirth of a non-macrosomic infant, PFMs are stretched beyond the physiological limit of skeletal muscles. Mechanical stretch is a key risk factor for PFM injury and the resultant muscle dysfunction—a major risk factor for the development of pelvic floor disorders later in life.[3–6] Interestingly, despite the "supraphysiological" strains necessary to achieve fetal delivery, not all women experience PFM injury during vaginal childbirth. We opine that the above is likely due to the presence of pregnancy-induced adaptations, shown to protect PFMs from stretch injury in the preclinical model,[7,8] and constructive postpartum PFM regeneration following birth injury that is essential for preventing PFM degeneration[9] and the associated morbidities.

The skeletal muscle regeneration starts immediately after injury when muscle stem cells (MuSCs), quiescent under homeostatic conditions, become activated and begin proliferating. Muscle regeneration is tightly regulated by the immune response that is characterized by 2 phases: proinflammatory and anti-inflammatory (proregenerative). The proinflammatory phase, in turn, consists of 2 waves. The first wave begins within 24 hours after injury when the complement system detects the injury, signaling the release of proinflammatory cytokines and the subsequent migration of leukocytes to the damaged site[10] to clear up injured muscle fibers and other cellular debris.[11–14] The increased expression of proinflammatory cytokines such as IL-1β (interleukin-1 beta), IFN-γ (interferon gamma), and TNF-α (tumor necrosis factor alpha) signals the activation of proinflammatory macrophages (M1), initiating the second wave of the proinflammatory response.[10,12,14–16] M1 phenotype macrophages continue to clear up cellular debris while expressing IFN-γ, IL-1β, TNF-α, and IL-6 cytokines that stimulate proliferation of MuSCs.[17] The anti-inflammatory phase of immune response starts approximately 3 days after injury and is characterized by macrophage polarization and a shift from M1 to M2 phenotype. The M2 macrophages release anti-inflammatory cytokines that mediate tissue remodeling and repair[10,16,18–20] while aiding MuSC differentiation and thus promoting muscle regeneration.[21]

In addition to being regulated by various cytokines, the activity of MuSCs is also modulated by prostaglandins (PGs), which are derived from arachidonic acid and released from membrane phospholipids by phospholipase A2 in response to mechanical injury of the skeletal muscle. Arachidonic acid is converted via COX-1 and COX-2 (cyclooxygenases 1 and 2, respectively) into prostaglandin H2 (PGH_2), a precursor to various bioactive PGs that aid in muscle regeneration.[22–24] One example is prostaglandin E2 (PGE_2), which enhances muscle progenitor proliferation and differentiation. PGE_2 also plays a role in mediating both the pro- and anti-inflammatory immune responses by regulating activity of macrophages, T cells, and B cells through binding to PGE_2 membrane receptors 1 to 4 (EP1–EP4) present on the immune cells.[22–24] COX-1 is constitutively expressed, allowing for immediate PG synthesis after release of arachidonic acid,[24,25] whereas COX-2 expression is induced by inflammation and is associated with pain.[26–29] The analgesic properties of nonselective nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen,[30] are due to the inhibition of COX-1 and COX-2 and prevention of PG synthesis.

Currently, NSAIDs are the most prescribed medications for postpartum pain.[31,32] Although NSAIDs are effective at reducing pain and inflammation in various contexts,[31,32] studies examining their effect on injured limb skeletal muscles have shown that NSAIDs delay muscle regeneration by impairing the immune response and myogenesis.[22,33–36] On the basis of the results of studies conducted in limb muscles, we hypothesized that NSAIDs will impair PFM regeneration following mechanical birth injury. However, to date, nothing is known about the effect of NSAIDs on regenerating PFMs after birth injury. Therefore, this study sought to determine the effect of NSAIDs on PFM regeneration after simulated birth injury (SBI) in a previously validated rat model.[7,37–39]

Materials and Methods

Study design

All procedures were conducted with the approval of and in compliance with the University of California San Diego Institutional Animal Care and Use Committee. Three-month-old nulligravid female Sprague Dawley rats (Envigo, Inc., Indianapolis, IN) were randomly assigned to 1 of the following 4 experimental groups: (1) controls—uninjured and not given NSAIDs (N=9); (2) SBI—injured but not given NSAIDs (N=3/time point); (3) SBI+NSAID—injured and given NSAIDs (N=3/time point); and (4) NSAID—uninjured but given NSAIDs (N=3/time point). SBI was performed following a well-established protocol[37,40–44] in which a modified 12-French urinary catheter was placed inside the anesthetized rat vagina, inflated to a 5-mL volume, and left in place for 2 hours with a 130-g weight suspended from the end to simulate circumferential and downward distension associated with fetal crowning. Animals in the SBI+NSAID group were provided with a solution of liquid ibuprofen (0.2 mg/mL) in drinking water immediately after SBI. The animals drank the ibuprofen solution ad libitum until sacrifice at either 1, 3, 5, 7, 10 (acute time points), or 28 (long-term time point) days post injury (dpi). Animals in the NSAID-only group received the same ibuprofen solution administered in the same manner as animals in the SBI+NSAID group, and were sacrificed at 1, 3, 5, 7, 10, or 28 days after NSAID initiation.

Nonsteroidal anti-inflammatory drug administration

The concentration of the NSAID solution used in this study was obtained from the published literature.[45] Throughout the duration of NSAID administration, the volume of ibuprofen solution drunk by the rats and their weights were monitored to ensure analogous ingestion of solution between groups. At the 28-day time point after injury or initiation of NSAID administration, the average amount of ibuprofen solution consumed per day by the animals in the SBI+NSAID and NSAID groups was 60.6±10.0 mL (12.1±2.0 mg).

Tissue collection

Following euthanasia, bilateral pubocaudalis (PCa) muscles were harvested. The PCa portion of the rat levator ani was selected as the focus of these experiments, given analogous stretch ratios observed for this muscle and its human homolog, the pubococcygeus, during SBI and human parturition, respectively.[7,46] Samples designated for histologic analyses were snap-frozen, sectioned into 10 μm-thick slices, and stored at −80°C until staining. For gene

analysis, the whole PCa muscles were placed into RNAlater (Thermo Fisher Scientific, Waltham, MA), and stored at −80°C.

Histology and immunohistochemistry

Muscle morphology—To assess muscle morphology (ie, myofiber packing and shape), PCa cross-sections were stained with hematoxylin and eosin using an established protocol.[9] Muscle cross-sections were then imaged using either the Leica SCN400 slide scanner (Leica Camera, Wetzlar, Germany) or the Keyence BZ-X810 Fluorescence Microscope (Keyence Corporation, Osaka, Japan) at 10× magnification.

Immune infiltrate—Frozen tissue sections were fixed in acetone at −20°C, washed with 1× phosphate-buffered saline (PBS), and blocked with a blocking buffer (1× tris-buffered saline [TBS], 0.05% Tween 20 [Croda International PLC, Snaith, United Kingdom], 1% bovine serum albumin [BSA], 5% goat serum). The sections were incubated with primary antibodies (mouse anti-CD45 and rabbit anti-laminin [Supplemental Table 1]) overnight at 4°C. Slides were then washed in 1× TBS before incubation with secondary antibodies (Supplemental Table 2). Slides were incubated in DAPI (Supplemental Table 2) after washing again, and cover-slipped. Four randomly selected tiles per tissue section were imaged at 20× magnification and $CD45^+$ cells were manually quantified using ImageJ. Bilateral PCa muscles were examined in SBI and SBI+NSAID groups, whereas only 1 side per biological replicate was analyzed in the control and NSAID-only groups. Cell density was calculated by dividing the total number of $CD45^+$ cells by the tissue cross-sectional area (# cells/mm^2).

Myogenic progenitors—PCa cross-sections were fixed in 4% paraformaldehyde (PFA), blocked with blocking buffer (Pax7: 10% goat serum, 0.03% Triton X-100 (Dow Chemical Company, Midland, MI); myogenin: 5% goat serum, 0.3% Triton X-100, 1% BSA), and then incubated overnight with rabbit anti-laminin primary antibody (Supplemental Table 1). Slides were then incubated with filtered secondary antibody (Supplemental Table 2) followed by 4% PFA. Afterward, tissues were incubated in antigen retrieval solution in a heated water bath, cooled, and then incubated in 1× PBS. Slides were blocked in blocking buffer then incubated overnight with either mouse anti-Pax7 antibody to identify quiescent and activated MuSCs or mouse anti-myogenin antibody to identify differentiating MuSCs (Supplemental Table 1). The following day, slides were incubated with filtered secondary antibody (Supplemental Table 2) followed by DAPI, and cover-slipped. Slides were washed with 1× PBS between each step, except after blocking. Stained muscle cross-sections were imaged, and cell density was calculated as described above.

Regenerating myofibers—Cross-sectional area of embryonic myosin heavy chain (eMyHC) positive fibers, a marker of regenerating myofibers,[47] was analyzed at 3, 7, and 10 dpi. Frozen tissue sections were fixed in acetone at −20°C and then incubated in PBS. Slides were then blocked in blocking buffer (10% goat serum and 0.03% Triton X-100) before primary antibody solution (mouse anti-eMyHC and rabbit anti-laminin [Supplemental Table 1]) was added for incubation overnight. Afterward, slides were incubated with filtered secondary antibody (Supplemental Table 2) followed by DAPI, and cover-slipped. Slides

were washed between each step, except after blocking. Entire cross-sections of bilateral PCa muscles were imaged using the Leica AF6000 Modular System for fluorescent microscopy (Leica Camera) at 10× magnification. eMyHC^{+} fiber area was quantified using a custom fiber area macro on ImageJ.

Mature fiber size—Fiber cross-sectional area was assessed 28 dpi or 28 days after the start of NSAID administration. Data were compared between SBI and SBI+NSAID groups. PCa sections were stained with rabbit anti-laminin antibody. The entire tissue section was imaged at 20× magnification. Fiber area was quantified using a custom fiber area macro on ImageJ.

Fibrotic degeneration—Fibrosis, defined as excessive deposition and replacement of the contractile myofibers by collagen, was assessed 28 dpi or 28 days after the start of NSAID administration using anti-collagen I antibody because type I collagen is the main constituent of the intramuscular extracellular matrix.[48] Slides were fixed in 4% PFA, washed, incubated with blocking buffer (10% goat serum, 1% BSA, 0.3% Triton X-100), and then incubated with anti-collagen I primary antibody (Supplemental Table 1) overnight. The next day, slides were washed and incubated with filtered secondary antibody (Supplemental Table 2) followed by DAPI, and cover-slipped. Stained PCa cross-sections were imaged using the Keyence BZ-X810 Fluorescence Microscope (Keyence Corporation) at 4× magnification with the threshold for positive collagen staining adjusted to a minimum of 7.69 and maximum of 1e30. Collagen area fraction was quantified in ImageJ.

Gene expression analysis

Quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) was used to assess the expression of immunogenic genes relevant to skeletal muscle recovery from injury. RNA was isolated from control PCa samples and from samples at 3, 5, and 10 dpi using the miRNeasy Micro Kit (QIAGEN, Hilden, Germany) following the manufacturer protocol. QIAxpert (QIAGEN) and Qubit (Thermo Fisher Scientific) were used for RNA quantification. Complementary DNA (cDNA) was obtained with SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific, 18091050). The *Power* SYBR Green PCR Master Mix (Thermo Fisher Scientific, 4367659) was combined with 1-ng cDNA and 10-μM concentration primers (Supplemental Table 3) for qRT-PCR analysis on the CFX96 Touch Real-Time PCR Detection System (1855195; Bio-Rad Laboratories, Hercules, CA). *Rplp0* (acidic ribosomal phosphoprotein P0) was used as a housekeeping gene. Because the left and right PCa muscles from an individual animal do not always undergo the same level of injury during SBI, *Myh3* expression levels (given by ΔCT values) of technical replicates for each muscle sample were averaged and compared with average expression levels of all control muscles to confirm the activation of injury–recovery pathways. Only samples with significantly increased *Myh3* expression relative to controls were included in the analysis of immunogenic genes because a lack of such difference indicated that that particular muscle did not undergo significant injury. Consequently, 1 muscle sample/group at 5 dpi and 1 muscle sample/group at 10 dpi were eliminated from the SBI and SBI+NSAID groups.

Statistical analysis

Normally distributed data were analyzed using a 2-way (immune cell density, quiescent/activated and differentiating MuSC reservoir) or a 1-way (collagen area fraction, gene expression) analysis of variance, followed by pairwise comparisons with Tukey or Dunnett tests. Nonparametric data, which included cross-sectional areas of $eMyHC^+$ and mature myofibers, were analyzed with a Kruskal–Wallis (post-hoc Dunn) test and Mann–Whitney test, respectively. Significance for all analyses was set at $\alpha < 0.05$. All results are presented as mean ± SEM or as median (range). Given the novelty of these investigations and lack of previous data, a priori power analysis was not feasible. We chose the sample size of n=3/group/time point, commonly used in other investigations of pelvic muscles and in similar studies of appendicular skeletal muscles.[9,22,49,50]

Results

Effect of simulated birth injury with and without nonsteroidal anti-inflammatory drugs on pelvic floor muscle regeneration

Representative images of PCa at 1, 3, and 7 dpi for all 4 experimental groups are presented in Figure 1. As expected, muscles from the control group had tightly packed myofibers, whereas PCa from the SBI group demonstrated myofiber death at 1 dpi, followed by separation of myofibers and influx of immune cellular infiltrate at 3 dpi. By 7 dpi, myofibers appear to become more tightly packed compared with 3 dpi. Similarly, in the SBI+NSAID group, marked myofiber death was observed at 1 dpi followed by the influx of cellular infiltrate at 3 dpi. However, the morphologic PCa appearance was divergent from that of the SBI-alone group at 7 dpi as myofibers continued to appear separate. Myofibers remained tightly packed, similar to uninjured controls, for all time points in the NSAID-only group.

Given that NSAID administration appeared to affect PCa recovery following SBI, we sought to delve further into assessing the effect of NSAIDs on PFM regeneration along a biologically relevant time course. Comparison of $eMyHC^+$ fiber cross-sectional area revealed significantly reduced median size with fiber area distribution enriched with smaller fibers at 3 dpi in the SBI+NSAID (86.47 μm^2, 14.85–285.6 μm^2) relative to the SBI group (210.97 μm^2, 17.96–741.1 μm^2) ($P<.0001$), suggesting a potential delay in the onset of regeneration in the SBI+NSAID group (Figure 2, A–C). By 7 dpi, $eMyHC^+$ median fiber size and distribution were similar in both injured groups (SBI+NSAID: 444.30 μm^2, 24.86–1355 μm^2; SBI: 426.10 μm^2, 25.90–1483 μm^2; $P>.999$) (Figure 2, B and C). The median regenerating fiber size in the SBI group decreased at 10 dpi compared with 7 dpi (248.63 μm^2, 32.58–895.9 μm^2) ($P<.0001$), with a tight cross-sectional area distribution. In contrast, the size of $eMyHC^+$ fibers continued to increase at 10 dpi compared with 7 dpi ($P<.0001$), with a substantial expansion of the cross-sectional area distribution in the SBI+NSAID group (717.59 μm^2, 56.77–2039 μm^2). The above further indicates a delay in regeneration in the SBI+NSAID relative to the SBI group, as $eMyHC^+$ fibers in the SBI+NSAID group appear to still be in various stages of regeneration, whereas those in the SBI group indicate that this phase of regeneration is reaching completion. Overall, these data indicate that NSAIDs impact the temporal progression of PFM regeneration across acute time points.

To determine whether the acute effects of NSAID administration on muscle regeneration, described above, impact PFMs long-term, we analyzed cross-sectional area of mature fibers at 28 dpi (Figure 2, D). Importantly, PCa fiber size was significantly smaller in the SBI+NSAID (1216.03 μm^2, 121.8–4169 μm^2) compared with the SBI group (1329.49 μm^2, 59.72–4146 μm^2) ($P<.0001$) at this longer time point (Figure 2, E and F).

Effect of nonsteroidal anti-inflammatory drugs on myogenesis following simulated birth injury

Given that NSAIDs negatively impacted PFM regeneration after SBI, we sought to elucidate the putative mechanisms governing the above. We first examined the impact of NSAIDs on transcription factors that control MuSC activation and differentiation following muscle injury, termed myogenic factors. Pax7 was used to evaluate the MuSC pool at 1, 3, 5, 7, 10, and 28 dpi (Figure 3, A). Unsurprisingly, quantification of quiescent and early activated MuSCs showed substantially increased $Pax7^+$ cell density in injured groups relative to controls, peaking at 7 dpi before returning to baseline by day 10. Importantly, although both groups followed this expected trend, the $Pax7^+$ cell density was significantly lower in the SBI+NSAID compared with the SBI group across all time points, with an almost 2-fold reduction at day 7 (SBI+NSAID: 44.32±2.362 cells/mm^2; SBI: 80.42±4.038 cells/mm^2; $P<.0001$) (Figure 3, B and C). The NSAID group did not differ from controls at any time point, indicating that NSAIDs alone do not impact the MuSC pool. Next, we evaluated the effect of NSAIDs on MuSC differentiation (Figure 3, D). $Myogenin^+$ cells peaked at 3 dpi in both the SBI and SBI+NSAID groups. Although the amount of differentiating muscle progenitors rapidly returned to baseline by 5 dpi in the SBI group, the increased $myogenin^+$ cell density persisted until 7 dpi in the SBI+NSAID group (Figure 3, E and F). Taken together, these data demonstrate that NSAID treatment following birth injury decreases PFM stem cell proliferation and prolongs MuSC differentiation, consistent with our findings of delayed growth of $eMyHC^+$ regenerating myofibers in the SBI+NSAID relative to the SBI group.

Effect of nonsteroidal anti-inflammatory drugs on pelvic floor muscle immune response to simulated birth injury

We next investigated the impact of NSAIDs on the PFM immune response using CD45—a panleukocyte marker (Figure 4, A). The SBI (53.55±4.868 cells/mm^2) and SBI+NSAID (60.13±9.200 cells/mm^2) groups had similarly elevated $CD45^+$ cell densities compared with uninjured controls (8.456±0.7424 cells/mm^2) at 1 dpi ($P<.0001$) (Figure 4, B and C). No significant differences were observed in the NSAID-alone group relative to controls at any time point examined. In the absence of quantitative differences in the immune infiltrate as a result of NSAID administration, we proceeded to compare the expression of pro- and anti-inflammatory genes between the groups relative to controls because temporal regulation of the immune response phases is essential for MuSC activation and differentiation (Figure 4, D and E). A significant increase in expression of *Stat1* was observed in the SBI+NSAID group (3.79-fold; $P<.01$) at 3 dpi. In the SBI group, expression of *Irf5* (35.0-fold; $P<.05$) and *Tgfb1* (3.42-fold; $P<.01$) was significantly elevated at 3 dpi, whereas in the SBI+NSAID group, *Tgfb1* expression was not significantly different until 5 dpi (2.46-fold; $P<.01$). Expression of the anti-inflammatory gene *Il4* (0.19-fold; $P<.001$) was

significantly decreased, whereas that of *Stat3* was significantly increased in both the SBI and SBI+NSAID groups (SBI: 6.30-fold; $P<.05$; SBI+NSAID: 7.50-fold; $P<.01$) at 3 dpi. Overall, these data suggest that although NSAIDs do not affect leukocyte accumulation, they do impact other facets of the immune response.

Effect of nonsteroidal anti-inflammatory drugs on fibrotic degeneration of pelvic floor muscles following simulated birth injury

Given that muscle atrophy (Figure 2, E and F) is usually accompanied by fibrosis, we also assessed intramuscular collagen content at 28 dpi (Figure 4, F). Collagen content in the SBI group was more than double that of uninjured controls (SBI: 14.61%±0.8%; control: 6.86%±1.6% collagen; $P=.054$), consistent with our previous findings.[9,51] Collagen content was similarly increased in the SBI+NSAID group, reaching 18.91%±2.87% ($P=.01$ relative to controls; $P=.3$ relative to the SBI group) (Figure 4, G). These data indicate that birth injury leads to fibrotic degeneration of PFMs irrespective of NSAIDs.

Comment

Principal findings

The main finding of the current study is that NSAID administration following SBI negatively impacts the rat PFM regeneration, as evidenced by the significantly smaller fiber size observed 4 weeks after NSAID administration following SBI compared with birth injury alone. Our initial investigation of the potential mechanisms accountable for these findings implicates NSAID-induced impairment of myogenesis following SBI. We observed that following SBI, NSAID treatment decreased MuSC proliferation and delayed differentiation relative to SBI alone. Although a differential impact of SBI vs SBI+NSAID was not observed on the overall immune infiltrate, NSAIDs did affect the expression of *Irf5*, *Tgfb1*, and *Stat1* genes. Consistent with previous studies, SBI resulted in elevated collagen deposition, an indicator of fibrosis. The fibrotic degeneration of PFMs, observed 4 weeks after SBI, was similar between injured animals who did and did not receive NSAIDs.

Results in the context of what is known

Although there are conflicting findings in the published literature regarding the long-term effect of NSAID administration, the results of our investigation in the context of mechanical PFM injury are in line with multiple studies that have shown that NSAIDs hinder limb muscle regeneration.[22,33,49,50] Furthermore, the study conducted by Rooney et al[52] demonstrates a decrease in the fiber cross-sectional area of the exercised rat supraspinatus muscles after 2 weeks of ibuprofen administration.

In regard to the myogenic response, our finding of decreased pelvic MuSC proliferation is consistent with the existing murine and human limb muscle studies that have demonstrated that NSAIDs impair MuSC activation and proliferation.[33,36,53] Moreover, our finding that NSAIDs delayed MuSC differentiation is also consistent with previous in vitro studies that have demonstrated that NSAID use or COX-1/COX-2 knockdown negatively impact murine MuSC differentiation.[50,54]

Our findings in regard to the immune response are in contrast to a previous study that found that the use of COX-2 selective inhibitors in freeze-damaged murine tibialis anterior muscle resulted in reduced proinflammatory response.[33] Another in vitro study, in which polymorphonuclear leukocytes or lymphocytes isolated from human blood were incubated in varying concentrations of NSAIDs before being seeded onto a collagen matrix, showed that NSAIDs inhibited migration of immune cells in a dose-dependent manner.[55] The current differential findings are likely due to the variability in the concentrations of NSAIDs used[55] or differences in NSAID type (ie, selective vs nonselective inhibitors). In fact, previous studies found that nonselective NSAIDs, such as the ibuprofen used in this study, may not inhibit COX-2 to the same extent as COX-2 inhibitors and COX-2 selective NSAIDs.[24,56] Other studies have found that the use of nonselective COX inhibitors does not change either leukocyte infiltration or activity in murine hind limb muscle injured via repeated electrical stimulation[57] or rat hind limb muscle injured with prolonged muscle loading,[58] consistent with our results.

Although previous studies examining the effect of NSAIDs on the expression of various immune genes in skeletal muscles are sparse, our finding of increased anti-inflammatory *Irf5* expression in the SBI but not in the SBI+NSAID group relative to controls suggests that NSAIDs may decrease the presence of M1 macrophages after birth injury given that IRF5, present in monocytes and macrophages, is critical for the commitment of monocytes to an M1 phenotype.[59–61] Because M1 macrophages release cytokines that stimulate the proliferation of MuSCs,[17] our finding that NSAIDs reduce the presence of M1 macrophages is consistent with our finding of a decreased MuSC pool in the SBI+NSAID group compared with the SBI group. Moreover, although *Tgfb1* expression increased relative to controls in the SBI group by 3 dpi, it did not increase in the SBI+NSAID group until 5 dpi, suggesting that NSAIDs delayed *Tgfb1* expression. Previous studies found that *Tgfb1* expression to be significantly elevated relative to controls by 48 hours after injury induced by repeated eccentric contractions in mice transverse abdominal muscles and rat plantar flexor muscles.[62,63] As an anti-inflammatory cytokine, TGFB1 promotes MuSC differentiation,[21] and thus a delay in *Tgfb1* expression in the SBI+NSAID group led to a delay in MuSC differentiation. This is consistent with our findings of a persistently elevated presence of myogenin$^+$ cells in the SBI+NSAID group relative to the SBI group. Finally, although *Stat1* expression increased only in the SBI+NSAID group, *Stat3* expression was elevated in both the SBI and SBI+NSAID groups. Conventionally, STAT1 is known to promote proinflammatory activities mediated by IFN-γ, and STAT3 is known to promote anti-inflammatory activities mediated by IL-10. However, some studies have indicated that there is cross talk between these signaling pathways[64,65] that complicates the designation of these STAT proteins as strictly pro- or anti-inflammatory. Therefore, the functional implications of the differential expression of *Stat1* between SBI and SBI+NSAID groups are uncertain in the context of our study and in the skeletal muscle regeneration field overall.

Clinical implications

The results of this study are significant because they suggest that the use of NSAIDs in the acute postpartum period might be detrimental for women at high risk of PFM injury. Thus, this study provides the impetus for the effect of NSAIDs to be studied in women

postpartum. Moreover, the results of this study suggest that individuals who sustain external anal sphincter injuries postpartum would also benefit from the use of an alternative analgesic to NSAIDs to manage their pain.

Research implications

In the future, it is important to explore whether NSAID use is detrimental to PFM regeneration after vaginal childbirth in women, and to further delve into the mechanisms through which NSAIDs impair myogenesis of PFMs. Moreover, we seek to explore the impact of other nonopioid pain-relieving drugs, such as acetaminophen, on PFM regeneration to identify potential alternatives to NSAIDs for postpartum pain treatment in women at high risk for PFM birth injury.

Strengths and limitations

The strengths of this study include the use of multiple time points along a biologically relevant continuum that capture the progress of PFM regeneration following birth injury in the presence and absence of NSAIDs. Moreover, this study used multiple controls, including uninjured controls, the SBI-only group, and the NSAID group, allowing it to capture the interactive effect of NSAIDs and birth injury on PFM regeneration. The use of a validated rat model with specific focus on the most translationally relevant portion of the rat levator ani, which undergoes similar strains to those of the pubococcygeus portion of the human levator ani complex, further strengthens the relevance of the current investigation to women. Similarly, the dosage of ibuprofen consumed daily per rat weighing an average of ~300 g was ~40.4 mg/kg, which was comparable to the average dosage of 31.0 mg/kg that women consume per day after childbirth (given a daily intake of 2400 mg of ibuprofen and an average weight of US women aged >20 years of 77 kg according to the Centers for Disease Control and Prevention). However, this study also has limitations inherent to the use of animal models that do not fully capture the human condition. Secondly, in this initial study, we only assessed the expression of specific genes, precluding potential discovery of novel mechanisms governing the impact of NSAIDs on PFM regeneration. Unbiased multiomics investigation is a fruitful avenue for future research.

Conclusions

In summary, our data indicate that nonselective NSAID treatment negatively impacts PFM regeneration following SBI in the validated preclinical model, leading to a more significant decrease of myofiber size compared with SBI alone. This effect is mediated by the negative impact of NSAID administration on pelvic MuSC proliferation and differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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AJOG at a Glance

Why was this study conducted?

This study was conducted to determine the effect of nonsteroidal anti-inflammatory drugs (NSAIDs) on pelvic floor muscle regeneration following simulated birth injury.

Key findings

This study demonstrated that NSAIDs negatively impact pelvic floor muscle regeneration in a simulated birth injury preclinical model. NSAID administration following birth injury decreased the muscle stem cell pool and delayed muscle stem cell differentiation, leading to smaller myofiber size of the pelvic floor muscles.

What does this add to what is known?

The effect of NSAIDs on skeletal muscle regeneration after injury has been widely studied in limb muscles. However, the effect of these drugs—commonly prescribed in the postpartum period—on pelvic floor muscle regeneration following birth injury has not been previously elucidated. The findings of this translationally relevant study provide impetus to assess the impact of NSAIDs on pelvic floor muscle recovery in women, and to consider avoiding NSAIDs in the acute postpartum period in women at high risk for pelvic floor muscle injury.

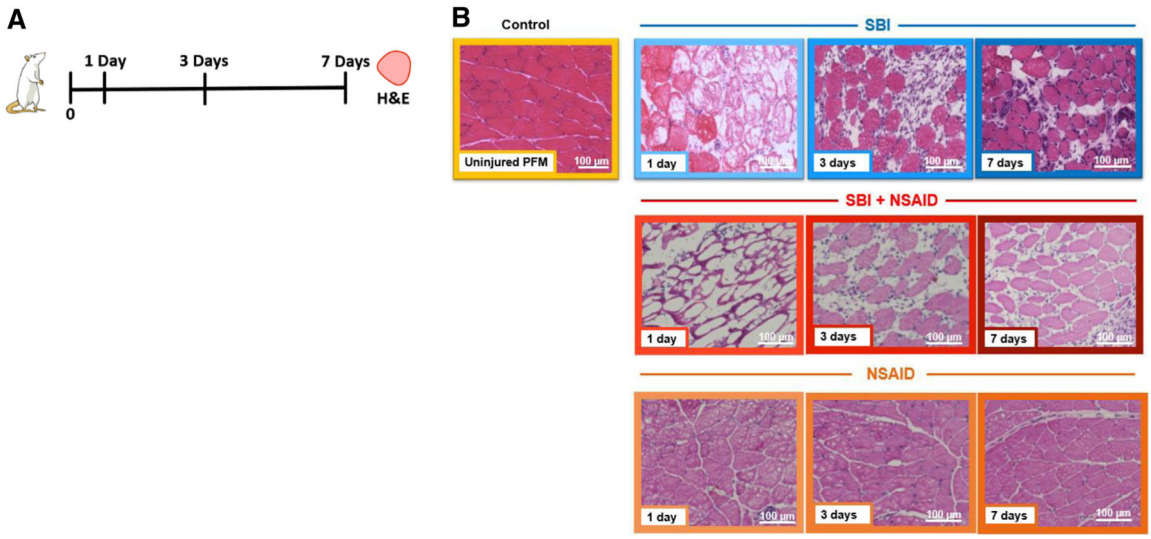


FIGURE 1. NSAIDs affect PFM regeneration following SBI

A, Schematic representation of the experimental approach in panel B. **B**, Qualitative assessment of H&E staining of pubocaudalis cross-sections procured from control, SBI, SBI+NSAID, and NSAID experimental groups showing extensive myofiber death, separation, and regeneration at 1, 3, and 7 days after injury, respectively, in the SBI group, with delayed regeneration in the SBI+NSAID group.

H&E, hematoxylin and eosin; *NSAID*, nonsteroidal anti-inflammatory drugs; *PFM*, pelvic floor muscle; *SBI*, simulated birth injury.

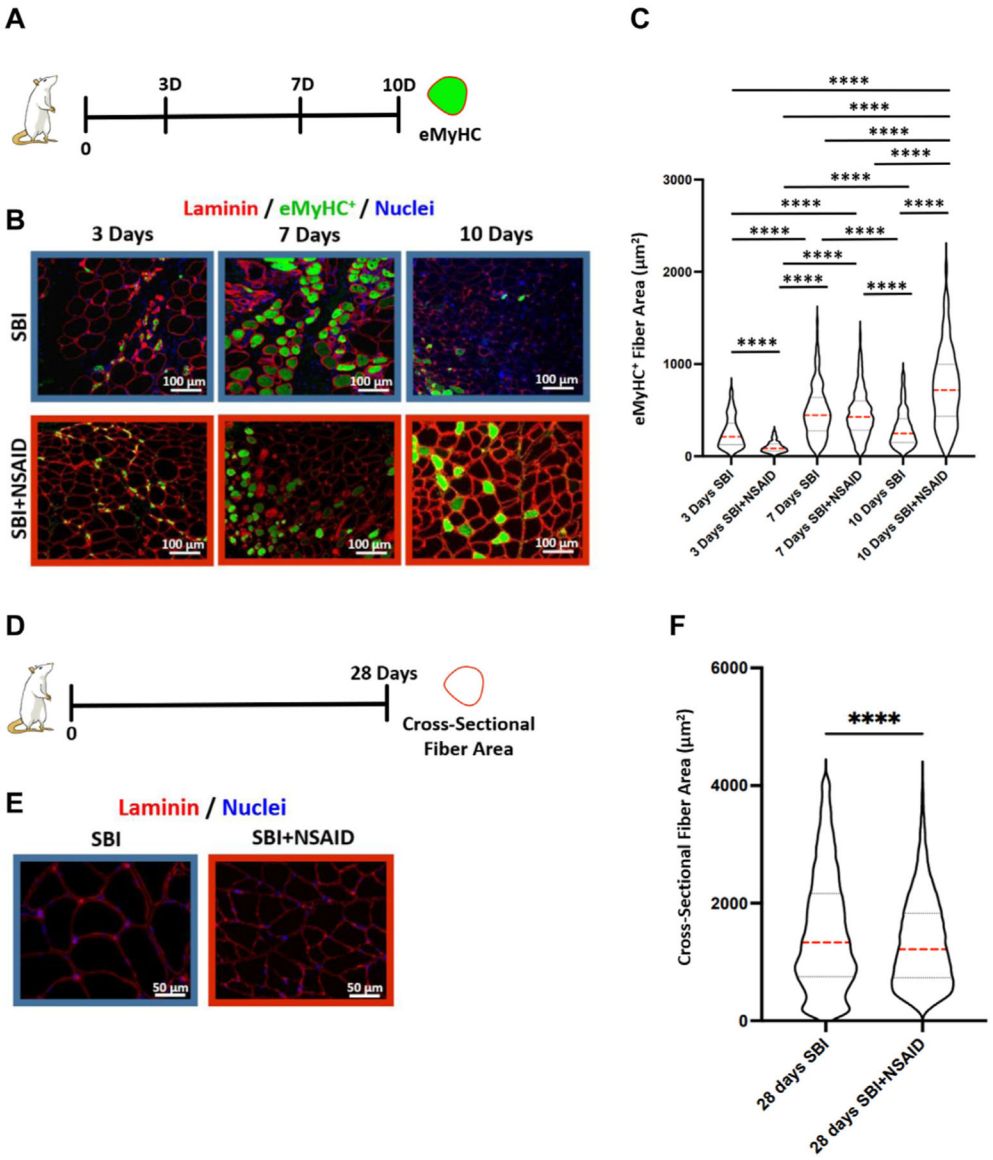


FIGURE 2. Manifestation of SBI differs between SBI and SBIDNSAID groups

A, Schematic representation of the experimental approach in panels B and C. **B**, Representative images of eMyHC staining for SBI and SBI+NSAID groups at 3, 7, and 10 days post injury (dpi). **C**, Violin plot comparing eMyHC^{+} fiber areas of SBI and SBI+NSAID groups at different time points shows that although the range and median of regenerative fiber areas in the NSAID group are initially significantly reduced at 3 days relative to the SBI group, they become significantly increased at 10 days. *Four asterisks* denote $P<.0001$. **D**, Schematic representation of the experimental approach in panels E and F. **E**, Representative images of myofiber areas for SBI and SBI+NSAID groups at 28 dpi. **F**, Violin plot comparing cross-sectional myofiber areas at 28 dpi for SBI and SBI+NSAID groups indicates that fiber areas are significantly reduced in the NSAID group. *eMyHC*, embryonic myosin heavy chain; *NSAID*, nonsteroidal anti-inflammatory drugs; *SBI*, simulated birth injury.

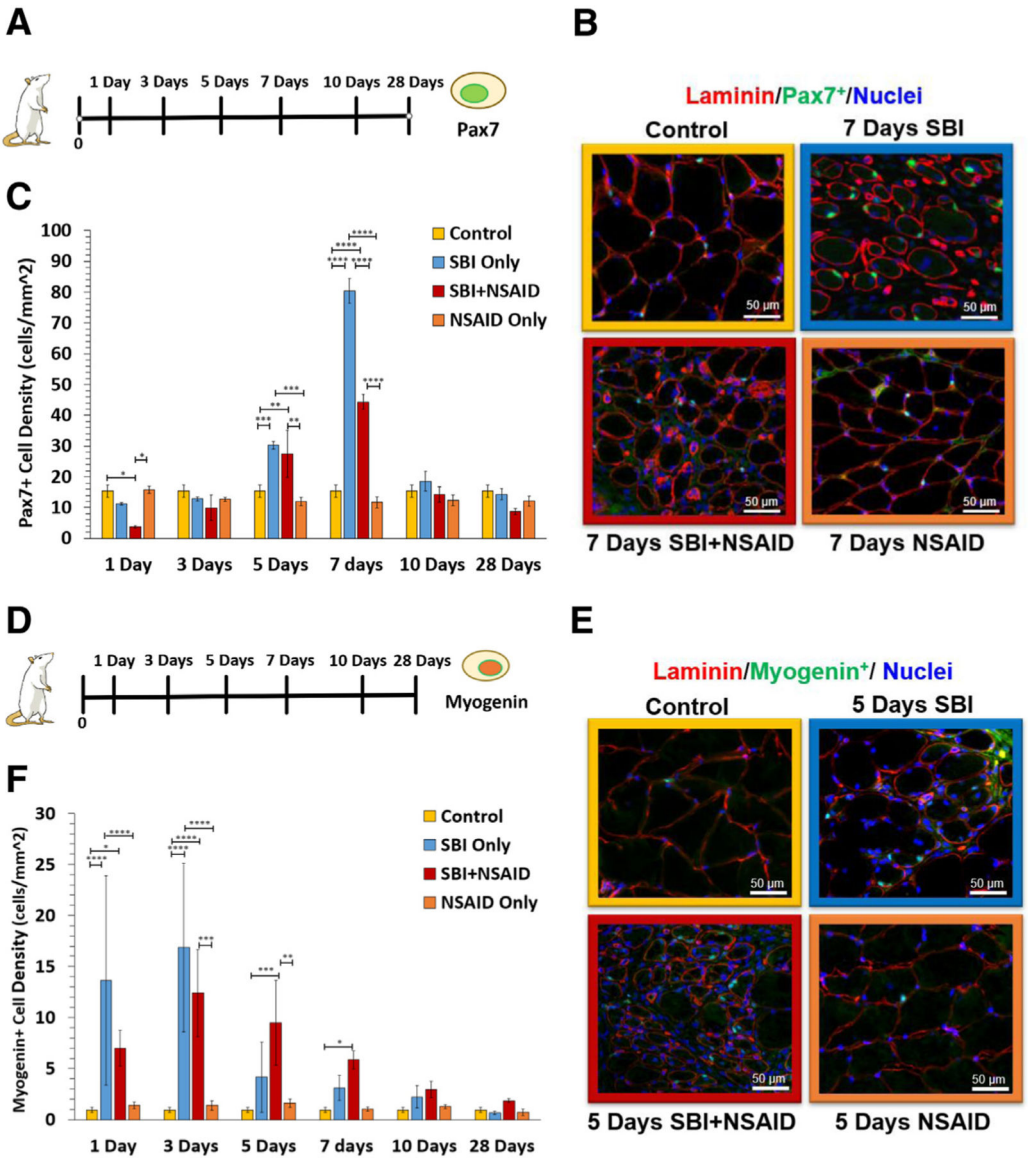
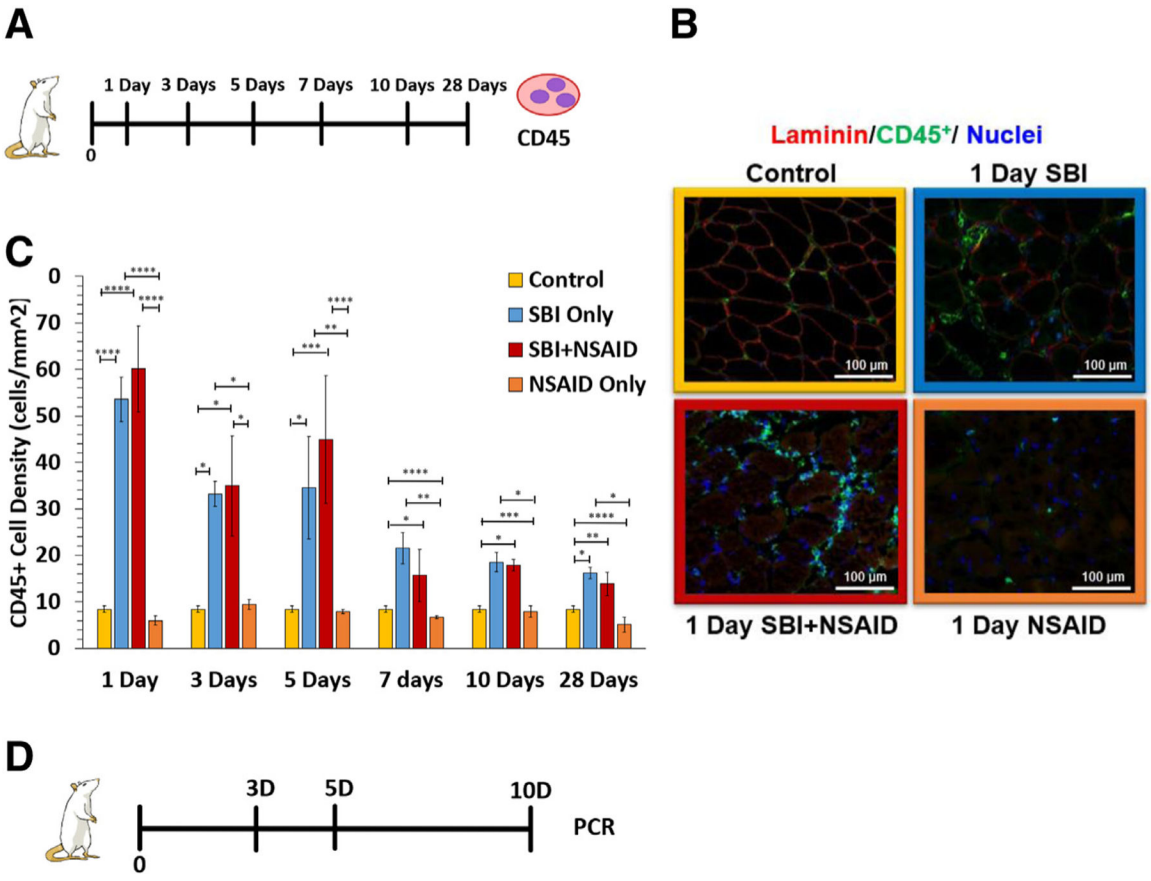
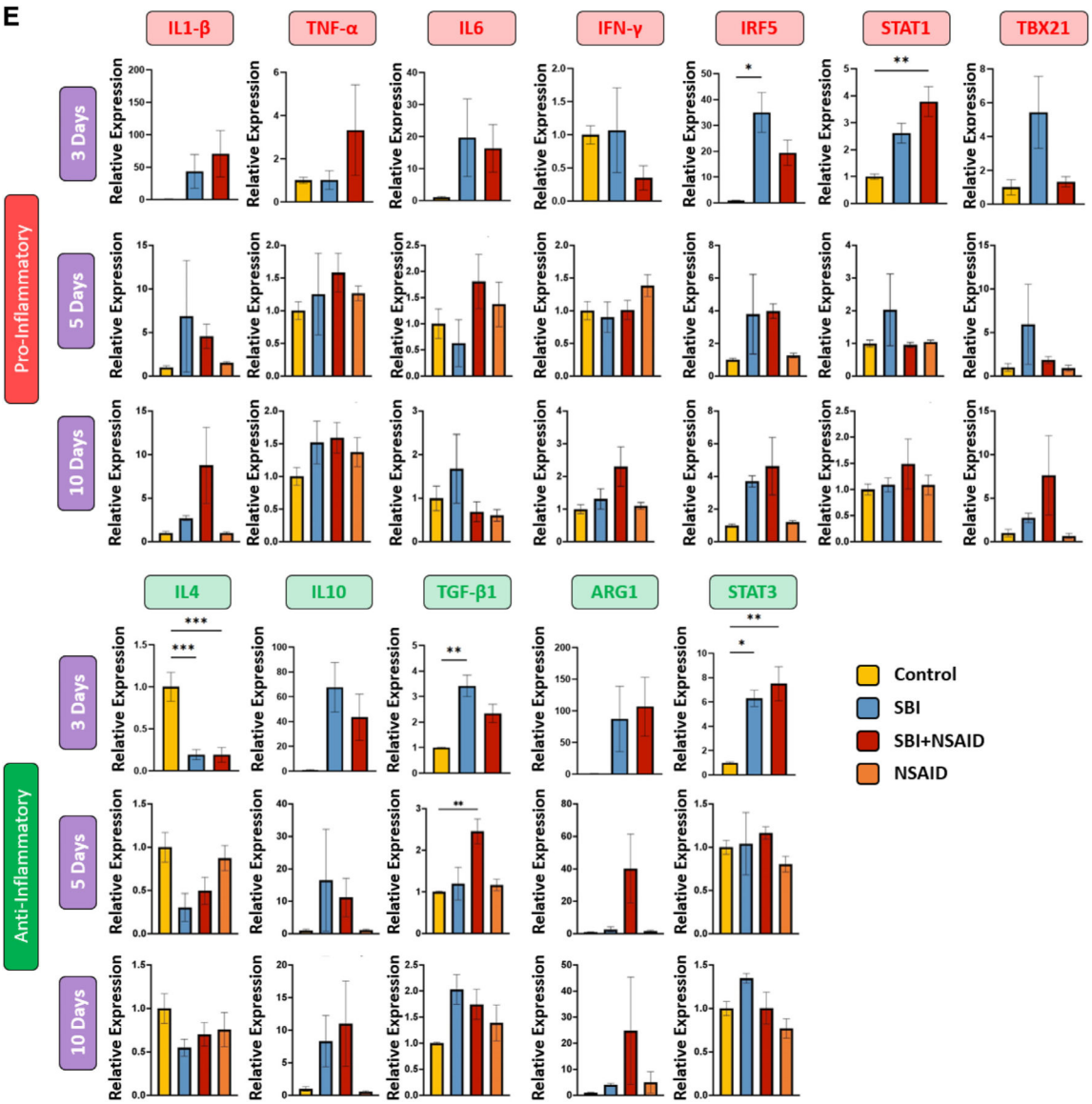


FIGURE 3. Myogenic effects of NSAIDs after SBI

A, Schematic representation of the experimental approach in panels B and C. **B**, Representative images of Pax7$^+$ staining at 7 days post injury (dpi) for all experimental groups. **C**, Quantification of Pax7$^+$ cell density across all time points for all experimental groups indicating a decrease in muscle stem cell (MuSC) pool at 7 dpi as a result of NSAID administration. *Asterisk* denotes $P<.05$; *double asterisks* denote $P<.01$; *triple asterisks* denote $P<.001$; *four asterisks* denote $P<.0001$. **D**, Schematic representation of the experimental approach in panels E and F. E, Representative images of myogenin$^+$ staining at 5 dpi. **F**, Quantification of myogenin$^+$ cell density across all time points for all experimental groups indicating a delay in MuSC differentiation at 5 dpi as a result of NSAID administration.

NSAID, nonsteroidal anti-inflammatory drugs; *SBI*, simulated birth injury.





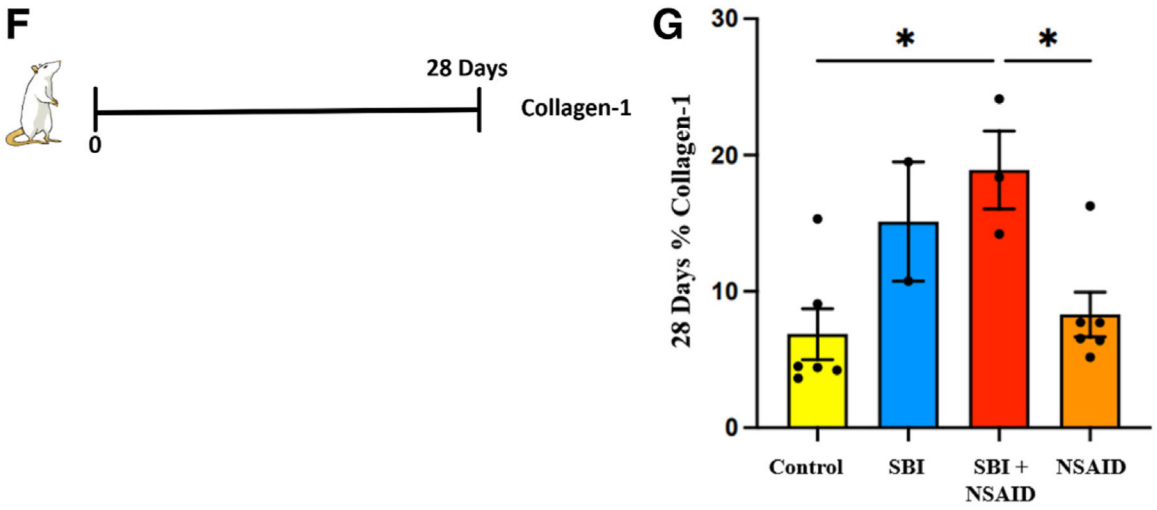


FIGURE 4. Effect of NSAIDs on inflammatory immune response after SBI

A, Schematic representation of the experimental approach in panels B and C. **B**, Representative images of $CD45^+$ staining 1 day after SBI for all experimental groups. *Asterisk* denotes $P<.05$; *double asterisks* denote $P<.01$; *triple asterisks* denote $P<.001$; *four asterisks* denote $P<.0001$. **C**, Quantification of $CD45^+$ cell density across all time points for all experimental groups indicating no difference in early immune response upon NSAID administration. **D**, Schematic representation of the experimental approach in panel E. **E**, Messenger RNA levels relative to controls of various genes involved in the proinflammatory and anti-inflammatory response, indicating an effect of NSAID administration on *Irf5*, *Stat1*, and *Tgfb1*. **F**, Schematic representation of the experimental approach in panel G. **G**, Percentages of collagen levels at 28 days for all experimental groups depicting increased collagen I levels in SBI and SBI+NSAID groups relative to controls.

NSAID, nonsteroidal anti-inflammatory drugs; *PCR*, polymerase chain reaction; *SBI*, simulated birth injury.