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14. ABSTRACT In this project(03/01/2017 to 08/31/2019), we performed our research experiments in four groups of mice including sham, tourniquet-induced IR+saline, tourniquet-induced IR+lipo-Dex, and tourniquet-induced IR+Dex groups. These experiments tested the protective effects of lipo-Dex and Dex on morphology and functionof the skeletal muscle and neuromuscular junction in mice with tourniquet-induced IR. Lipo-Dex is liposome-encapsulated Dex. Our study found that lipo-Dex was mainly retained in IR-injured skeletal muscles after 24-hour ischemia/reperfusion (IR), lasted about 1 week, and then disappeared. Unilateral (left) hindlimb ischemia was induced in IR mice by placing an orthodontic rubber band (ORB) at the hip joint through use of a McGivney hemorrhoidal ligator. After 3 hours of ischemia, the ORB tourniquet was released to begin reperfusion for different periods. Treatment with Dex (1mg/kg/day, i.p. injection for 1 wk) or lipo-Dex (7mg/kg, one-time i.v., equivalent to 1 mg/kg/day of Dex for 1 wk) will begin at the beginning of reperfusion on the day of IR induction. Our data demonstrated that treatment of lipo-Dex or Dex suppresses IR-induced inflammation and promotes recovery of skeletal muscle and neuromuscular junction morphology and function from tourniquet-induced IR injury, especially no any side-effects for Lipo-Dex treatment. These experimental results suggest that the therapeutic addition of lipo-Dex supports the current policy of encouraging tourniquet use in combat and civilian medicine.					
15. SUBJECT TERMS Lipo-Dex and Dex promote structural and functional recovery of the skeletal muscle and neuromuscular junction from tourniquet-induced IR injuries in mouse hindlimb					
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TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	2
2. Keywords	2
3. Accomplishments	2-11
4. Impact	11-12
5. Changes/Problems	12-13
6. Products	13
7. Participants & Other Collaborating Organizations	13-15
8. Special Reporting Requirements	15
9. Appendices	15

The text of the report must include all sections addressed in the table of contents to include the following. **DO** include the bolded section headings, but **DO NOT** include the *italicized* descriptions of section contents in your submitted reports.

1. INTRODUCTION:

Tourniquet application is an effective means of arresting life-threatening limb hemorrhage in the military and civilian pre-hospital setting and creating bloodless surgical fields in orthopedic surgery. However, tourniquet-induced ischemia and subsequent reperfusion can cause serious ischemia-reperfusion (IR) injury for the neuromuscular junction and skeletal muscle. Such injury is associated with long-term skeletal muscle contractile dysfunction and permanent neurological deficiency. Although our laboratory and others have uncovered the protective effects of anti-inflammation and anti-oxidation on tourniquet-induced acute IR injury in skeletal muscles, there is currently no effective means to improve long-term functions of the skeletal muscle and neuromuscular junction. Although glucocorticoids such as dexamethasone (Dex) have a potent anti-inflammatory effect, these drugs are also associated with severe side-effects. A novel drug delivery system, liposome encapsulation, can help drugs such as Dex accumulate at the site of inflammation and have high therapeutic efficacy without adverse effects. To address whether the application of liposomal dexamethasone (lipo-Dex) at the early stage of tourniquet-induced IR promotes the structural and functional repair of the neuromuscular junction and skeletal muscle, and restores skeletal muscle contractile function without Dex-related adverse effects, we use tourniquet-induced mouse limb IR model to design *in-vitro* (cells and tissues) and *in-vivo* (conscious and anesthetized mice) studies for comparing lipo-Dex and Dex as to their therapeutic effects on recovery of skeletal muscle contractile function and adverse effects. The application of lipo-Dex in this study will support the development of potential therapeutic strategies for the structural and functional repair of the neuromuscular junction and skeletal muscle after tourniquet-induced limb IR injury. This therapeutic strategy has significant potential to benefit patients with tourniquet-induced IR injury that results from prolonged treatment at combat sites or during civilian pre-hospital treatment, thereby resolving a limitation for tourniquet use and resulting in extensive savings of life, limb use, and financial burden.

2. KEYWORDS:

Civilian, contraction, dexamethasone, glucocorticoid, hemorrhage, inflammation, injury, ischemia-reperfusion, limb, liposome, military, morphology, mouse, neuromuscular junction, side effects, skeletal muscle, tourniquet.

3. ACCOMPLISHMENTS:

o What were the major goals of the project?

The goals of this project is to test the feasibility of liposomal dexamethasone (lipo-Dex) as a novel therapeutic intervention for improving the outcome of post-traumatic skeletal muscle and neuromuscular junction in patients who have been treated with tourniquet.

Milestones	timeline (Total 24 Months)
Milestone 1: Obtain ACURO approval.	1-3
Milestone 2: Obtain lipo-Dex.	1
Milestone 3: Determine the inhibitory effects of lipo-Dex and Dex on inflammatory cytokines during tourniquet-induced IR. Determine lipo-Dex distribution.	1-4
Milestone 4: Obtain lipo-Dex and determine the protective effects of lipo-Dex and Dex on skeletal muscle contraction during tourniquet-induced IR.	5-8
Milestone 5: Obtain lipo-Dex and determine the protective effects of lipo-Dex and Dex on the structure of skeletal muscles during tourniquet-induced IR.	9-12
Milestone 6: Present the above findings at relevant national conferences. Edit and publish peer reviewed paper.	8-12
Milestone 7: Obtain lipo-Dex	13
Milestone 8: Determine the protective effects of lipo-Dex and Dex on function of the neuromuscular junction during	13-17

tourniquet-induced IR.

Milestone 9: Obtain lipo-Dex and determine the protective effects of lipo-Dex and Dex on structure of the neuromuscular junction during tourniquet-induced IR. 18-20

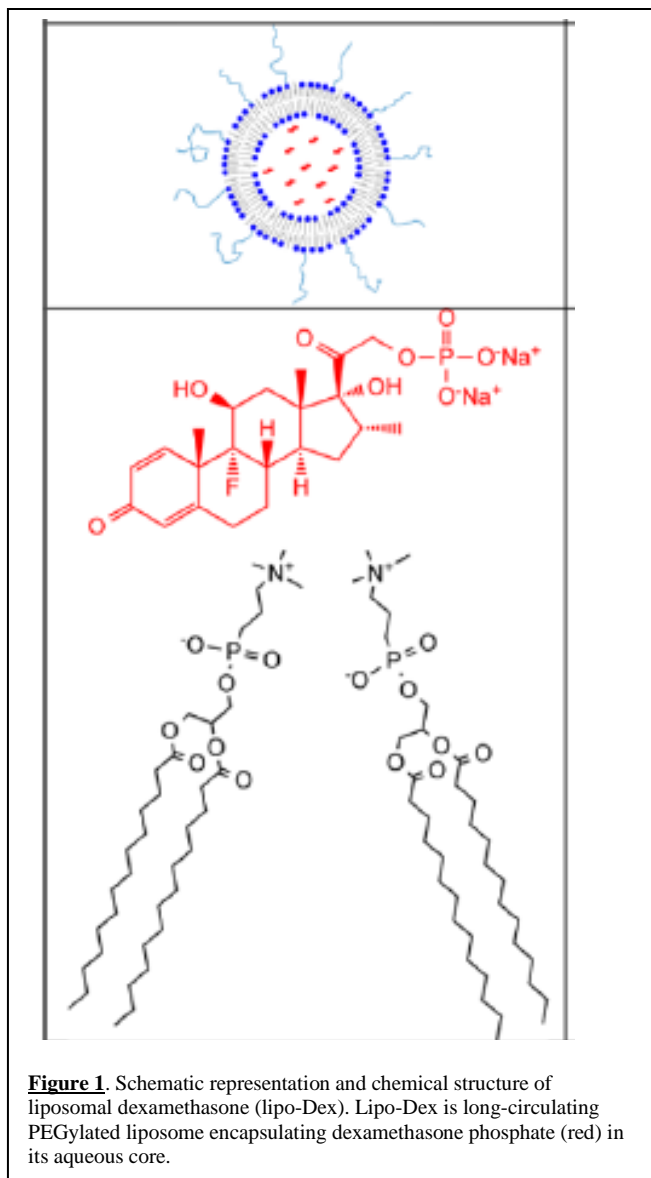
Milestone 10: Obtain lipo-Dex and determine the adverse effects of lipo-Dex and Dex in mice with tourniquet-induced IR. 21-24

Milestone 11: Present the above findings at relevant national conferences. 20-24
Edit and publish peer reviewed papers.

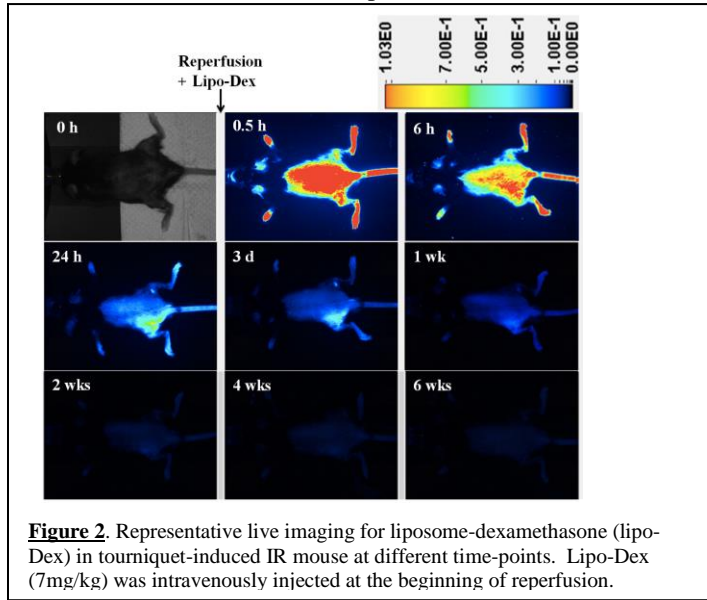
Milestone 12: Edit the video for our experimental demonstration and discuss our results with clinical doctors. Prepare to seek clinical partners in military and/or civilian facilities to conduct a small clinical trial for combining use of pre-hospital tourniquet and lipo-Dex. 22-24

○ **What was accomplished under these goals?**

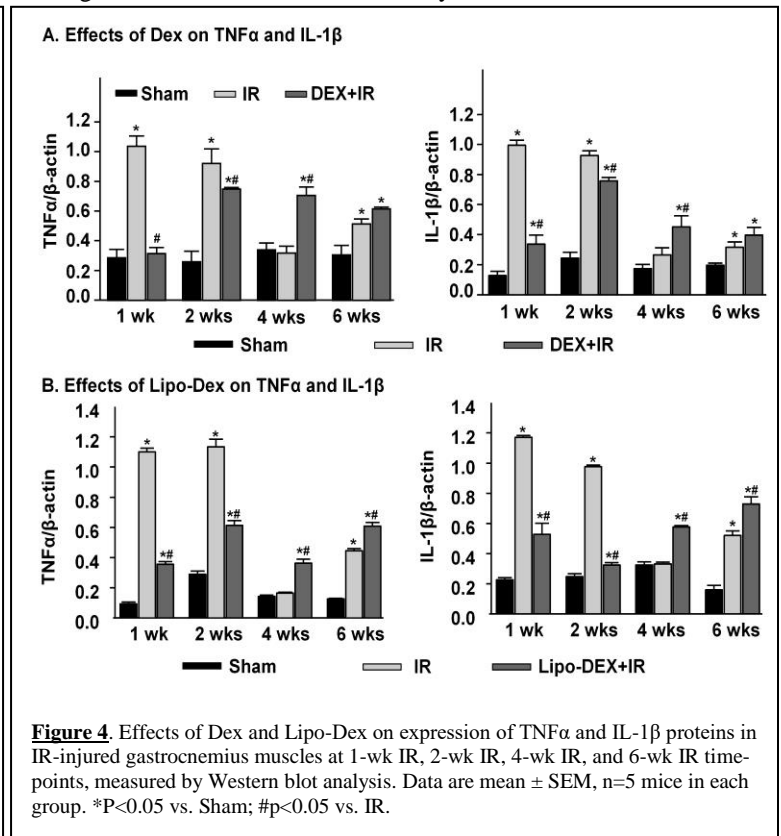
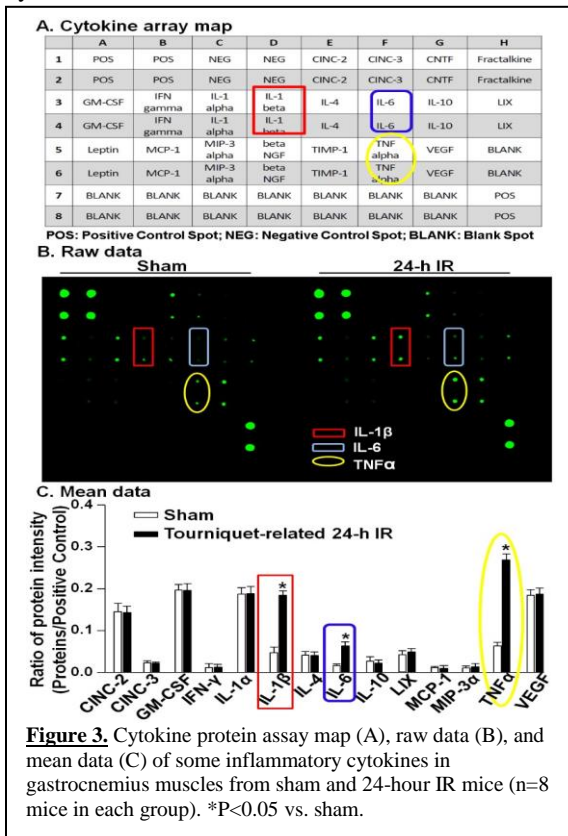
- 1) We submitted IACUC protocol for this project to the University of Nebraska Medical Center (UNMC) IACUC and ACURO and obtained approval from UNMC IACUC on December 21, 2016 and ACURO on February 28, 2017, respectively.
- 2) We prepared lipo-Dex. Lipo-Dex was synthesized by the lipid film-hydration method. Briefly, dipalmitoyl phosphatidylcholine, polyethylene-glycol 2000-distearyl phosphatidylethanolamine, and cholesterol will be dissolved in a 1.85:0.15:1 molar ratio in 5-10 ml ethanol. A lipid film will be formed and hydrated with a 100 mg/ml solution of Dex phosphate disodium (free Dex). Lipo-Dex (about 6.5 mg/ml, **Figure 1**) will be measured, upon extraction of the aqueous phase, by Ultra Performance Liquid Chromatography (UPLC) BEH C18 column.



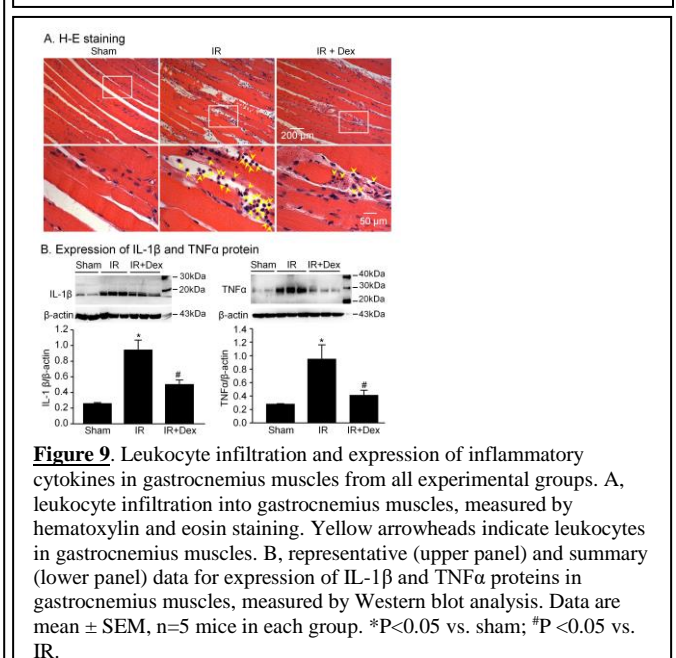
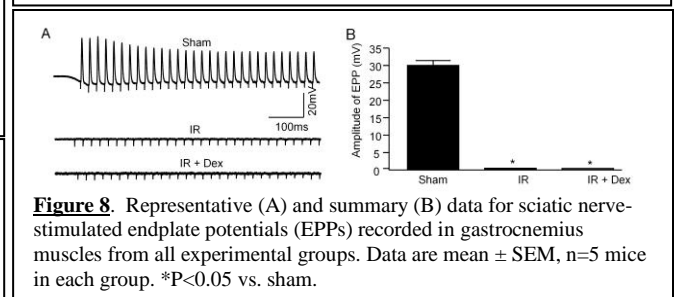
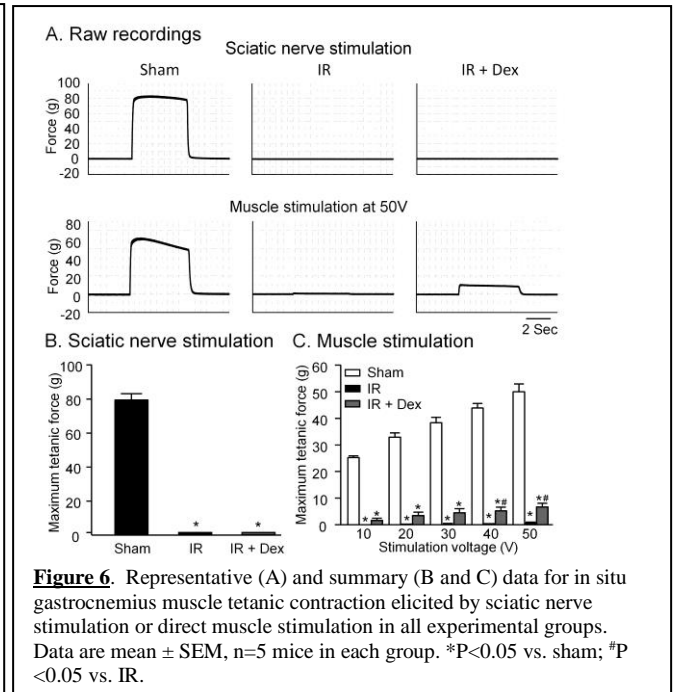
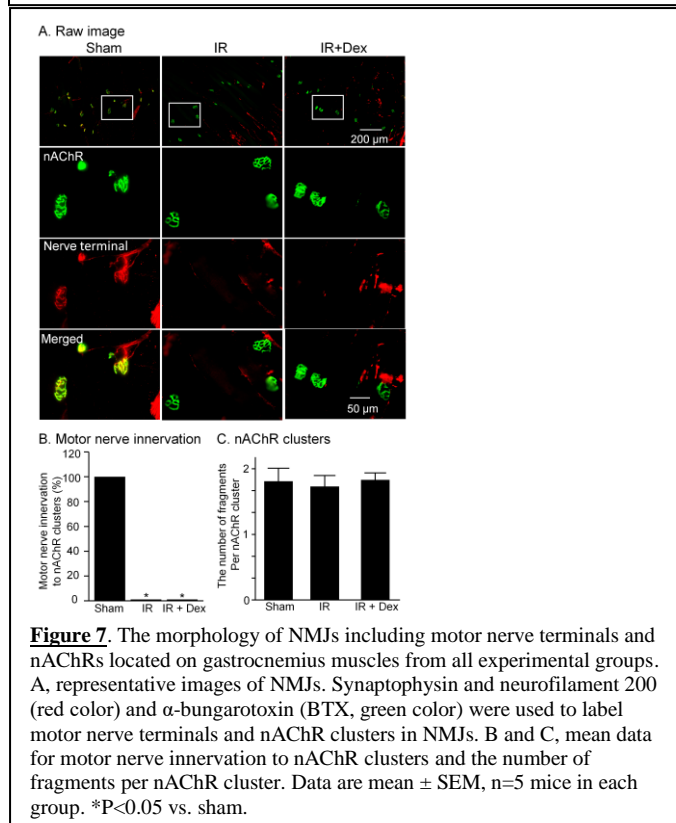
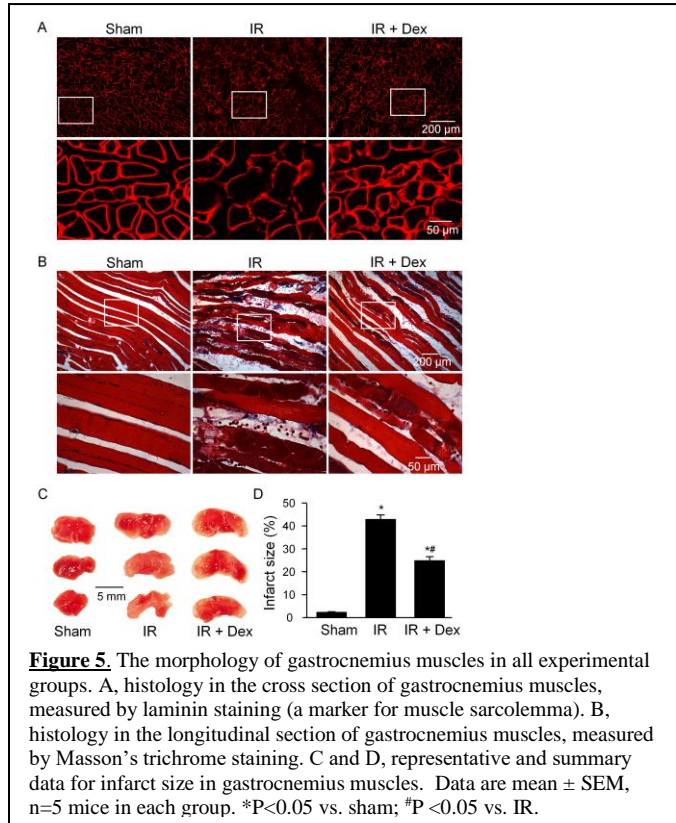
- 3) We measured lipo-Dex distribution using near-infrared imaging analysis. At the beginning of reperfusion after 3-hours of ischemia, mice received lipo-Dex (7 mg/kg) by tail-vein injection under anesthesia. They were imaged at IR 0.5 hour, 6 hours, 1 day, 3 days, 1 wk, 2 wks, 4 wks, and 6 wks. We found that lipo-Dex was mainly retained in IR-injured skeletal muscles after 24-hour ischemia/reperfusion (IR), lasted about 1 week, and then disappeared (**Figure 2**).



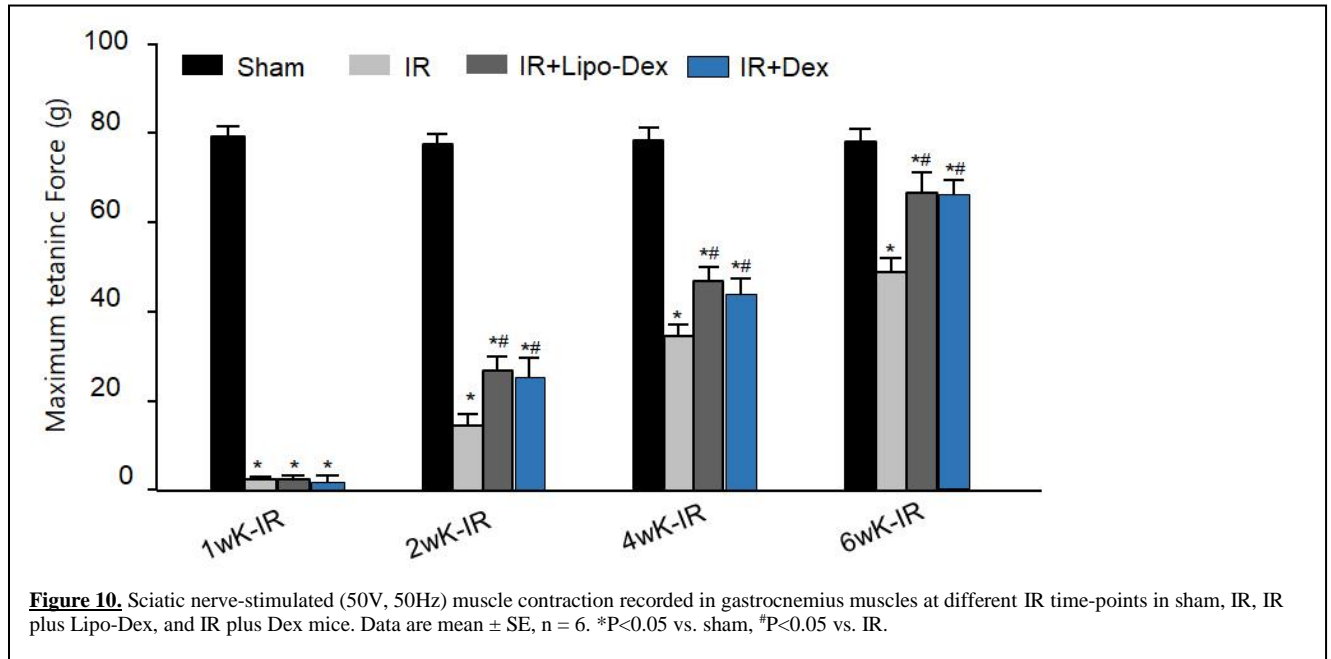
- 4) Using harvested gastrocnemius muscles, we measured inflammatory cytokines. A cytokine protein assay was used to measure 19 cytokines at 24-hour IR (**Figure 3**). The data showed that 14 cytokines were detectable, in which only 3 cytokines (TNF α , IL-1 β , and IL-6) were increased in gastrocnemius muscles after 24-hour IR, compared to sham (**Figure 3**). Therefore, we used Western blot analysis to measure the levels of TNF α and IL-1 β at 1-wk IR, 2-wk IR, 4-wk IR, and 6-wk IR time-points. We found that the levels of TNF α and IL-1 β were elevated in gastrocnemius muscles at 1-wk and 2-wks IR, returned back to the normal level at 4-wk IR, and then increased again at 6-wk IR (Figure 4). Lipo-Dex and Dex significantly inhibited the elevation of cytokines in gastrocnemius muscles at 1-wk and 2-wks IR, whereas they increased the levels of TNF α and IL-1 β in gastrocnemius muscles at 4-wk and 6-wk IR (Figure 4). These results suggest that inflammation in IR-injured muscles at the early stage of tourniquet-induced IR might link to the tissue damage and inflammation in the late stage of tourniquet-induced IR might be involved in tissue regeneration. Some research papers reported that inflammatory cytokines are also involved in nerve and muscle structural regeneration and functional recovery.



- 5) We tested protective effect of dexamethasone against tourniquet-induced acute IR injury in mouse hindlimb. Three hours of tourniquet and 24-hour of release (24-hour IR) resulted in leukocyte infiltration and elevation of inflammatory cytokines, caused gastrocnemius muscle injuries and contractile dysfunction including rupture of the muscle sarcolemma, muscle necrosis, and no muscle contraction, induced disappearance of motor nerve terminals and endplate potentials. One-time treatment with Dex at the beginning of reperfusion (1 mg/kg) reduced IR-induced skeletal muscle damage and direct muscle stimulation-triggered muscle contraction in 24-hour IR mice (**Figure 5-9**). However, this anti-inflammatory drug did not improve neuromuscular junction (NMJ) morphology and function, and sciatic nerve-stimulated muscle contraction in 24-hour IR mice (**Figure 5-9**). The results suggest that one-time treatment with Dex at the beginning of reperfusion only reduced structural and functional impairments of the skeletal muscle but not the NMJ through inhibiting inflammatory cytokines.

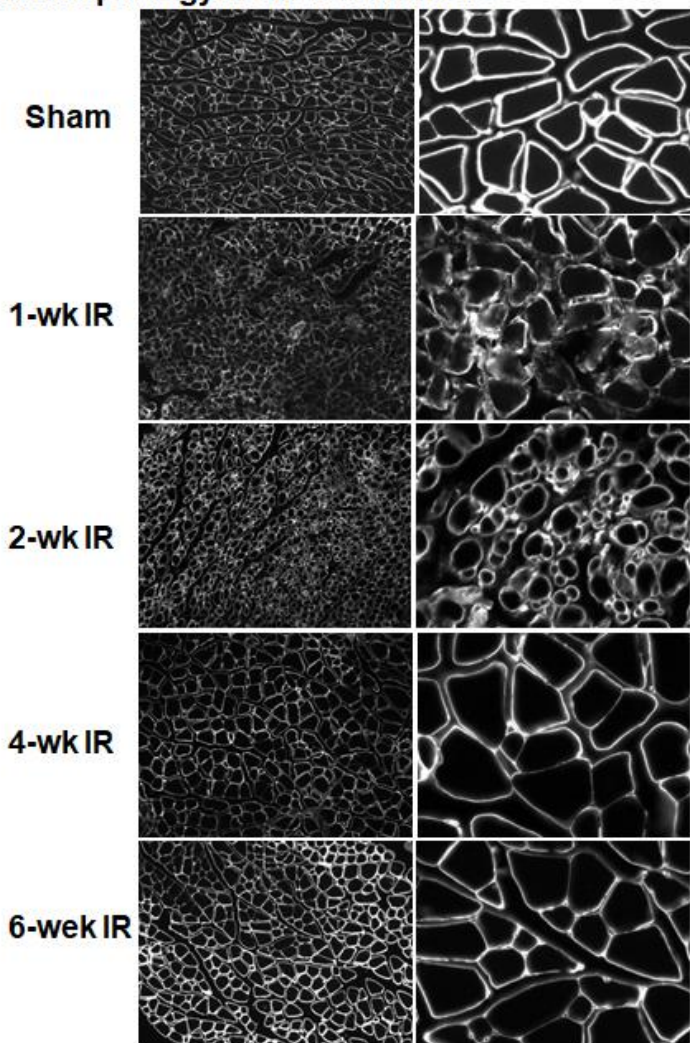


- 6) Using general electrophysiological technique, we measured skeletal muscle contraction in sham, tourniquet-induced IR+saline, tourniquet-induced IR+lipo-Dex, and tourniquet-induced IR+Dex. Unilateral (left) hindlimb ischemia was induced in IR mice by placing an orthodontic rubber band (ORB) at the hip joint through use of a McGivney hemorrhoidal ligator. After 3 hours of ischemia, the ORB tourniquet was released to begin reperfusion for different periods. Treatment with Dex (1mg/kg/day, i.p. injection for 1 wk) or lipo-Dex (7mg/kg, one-time i.v., equivalent to 1 mg/kg/day of Dex for 1 wk) will begin at the beginning of reperfusion on the day of IR induction. At 1-wk IR, 2-wk IR, 4-wk IR, and 6-wk IR, the mice in all groups were anesthetized. Then sciatic nerve-stimulated gastrocnemius muscle contraction was measured. We found that tourniquet-induced IR significantly decreased sciatic nerve-stimulated gastrocnemius muscle contraction at 1-wk IR. Following prolongation of reperfusion, gastrocnemius muscle contraction gradually strengthened at 2-wk IR, 4-wk IR, and 6-wk IR. However, gastrocnemius muscle contraction did not recover to the normal level, even at 6-wk IR (**Figure 10**). Lipo-Dex and Dex markedly improved gastrocnemius muscle contraction at 2-wk IR, 4-wk IR, and 6-wk IR (**Figure 10**).



- 7) We also measured the structure of skeletal muscles at 1-wk IR, 2-wk IR, 4-wk IR, and 6-wk IR time-points. At 1-wk IR, the morphology of gastrocnemius muscles still showed rupture of the muscle sarcolemma and muscle necrosis. At 2-wk IR, the muscle regeneration started and many immature new muscle fibers (small-diameter muscle fibers) appeared in IR-injured gastrocnemius muscles (**Figure 11**). IR-injured gastrocnemius muscles recovered to the normal muscle morphology at 4-wk and 6-wk IR (**Figure 11**). In particular, there are no difference in direct muscle stimulation-induced gastrocnemius muscle contraction between sham and 6-wk IR mice (**Figure 11**). These results suggest that the skeletal muscle itself could not be involved in skeletal muscle contractile dysfunction after 6-wk IR. Lipo-Dex and Dex reduces rupture of the muscle sarcolemma and infarct size at 1-wk IR time-point. However, there is no significant difference in the structure of skeletal muscles among all groups of mice at 2-wk IR, 4-wk IR, and 6-wk IR time-points.

A. Morphology of skeletal muscle



B. Direct muscle stimulation-induced muscle contraction

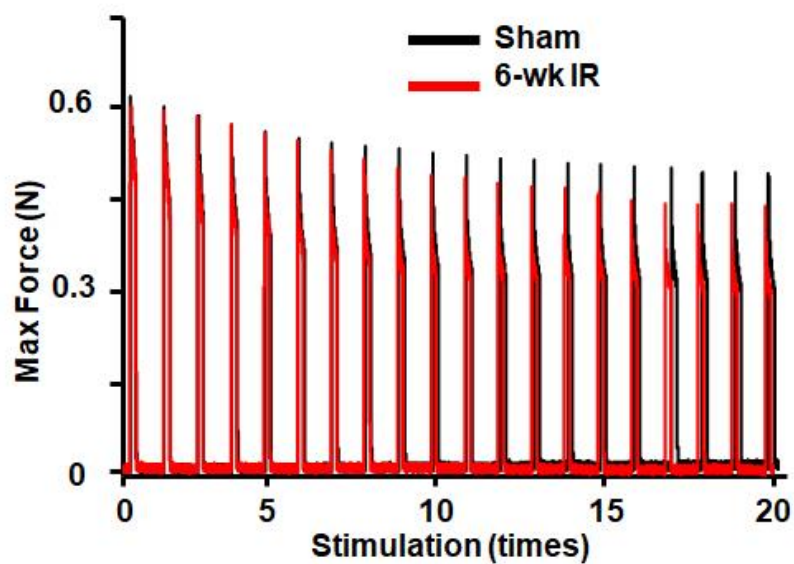
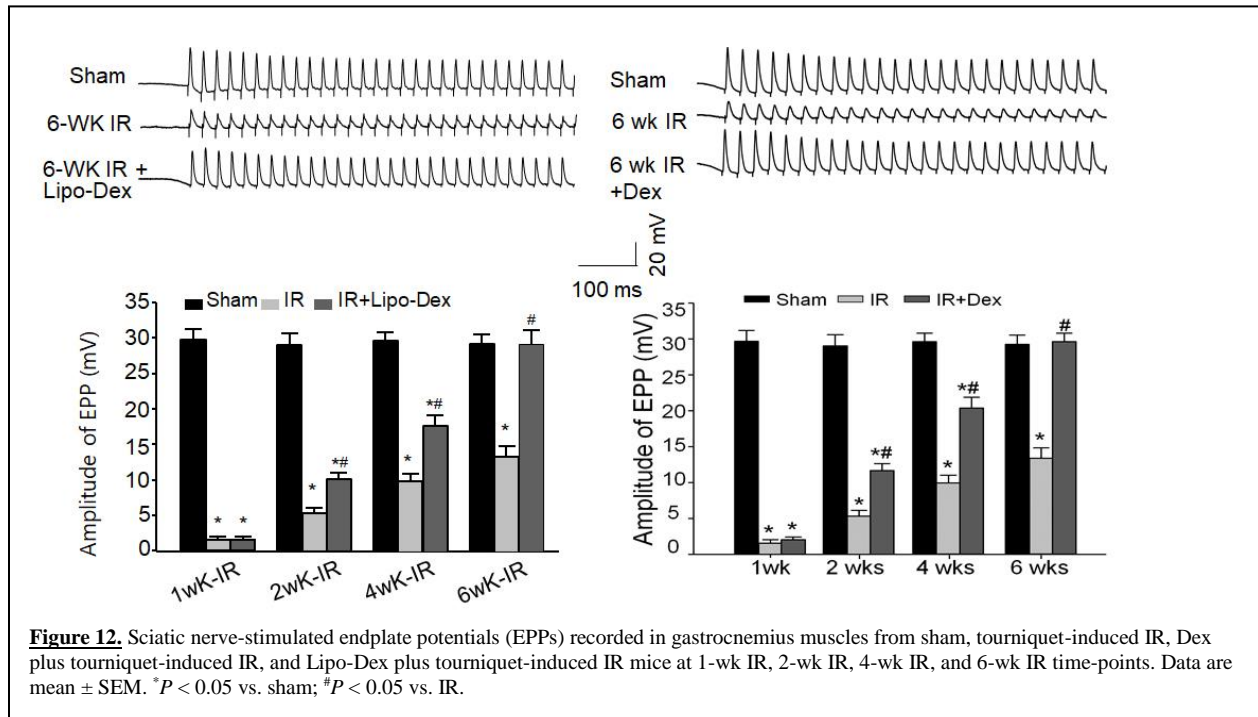
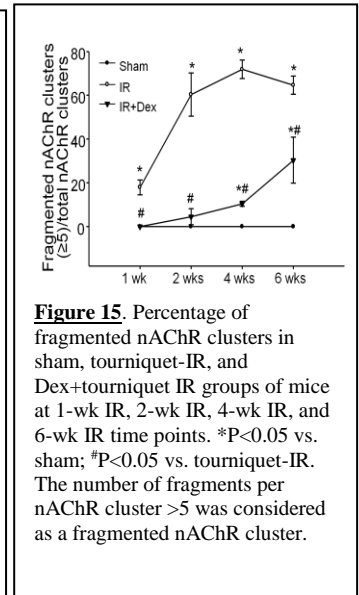
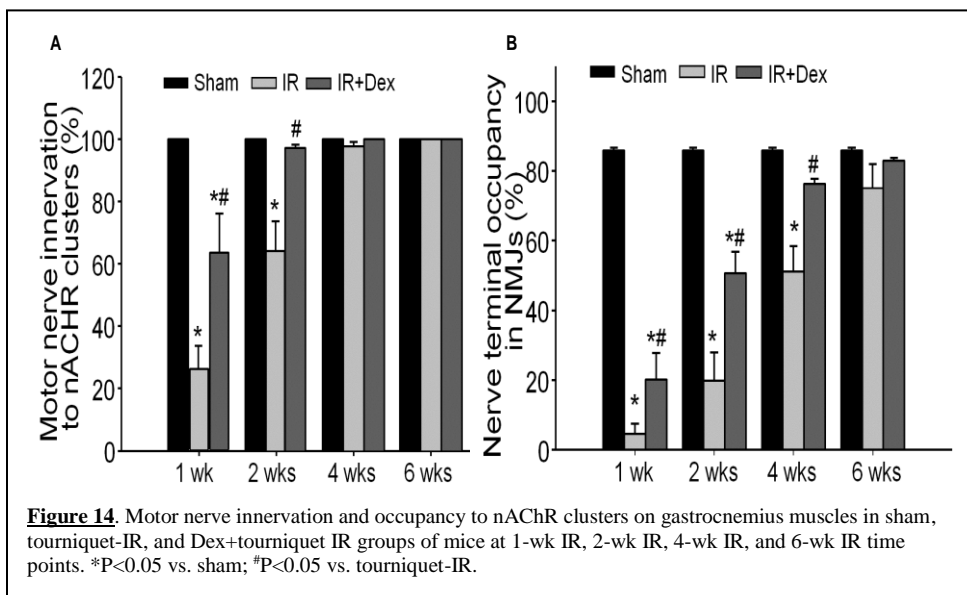
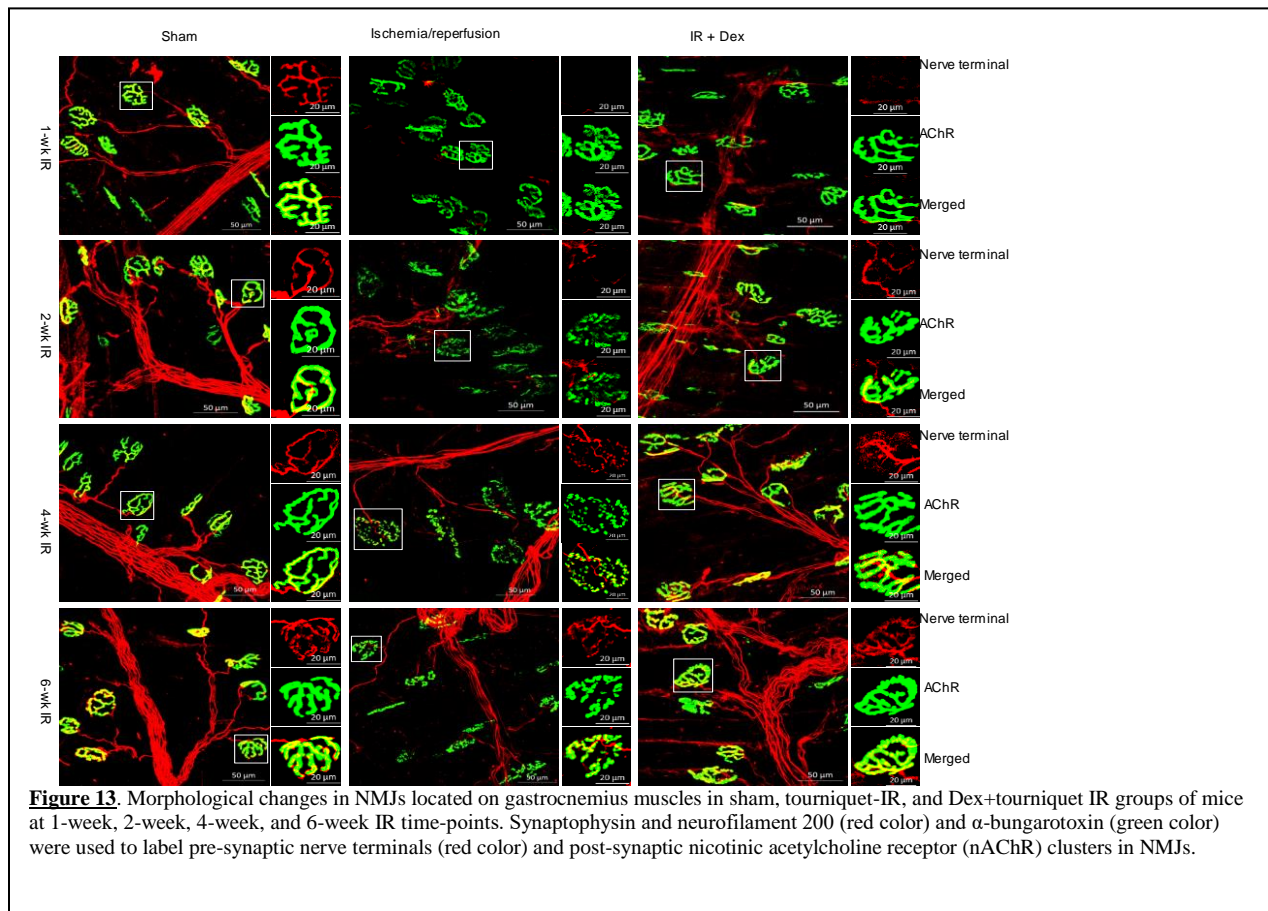


Figure 11. Muscle morphology (A) stained by laminin (a marker for muscle sarcolemma) and direct muscle stimulation-induced gastrocnemius muscle contraction (B) in sham and IR mice. Each muscle contraction lasted for 5-sec (10 V, 50 Hz, 0.1 ms pulse) with 25-sec interval.

- 8) We completed the recording of endplate potentials (EPPs) in gastrocnemius muscles in 4 groups of mice including sham, tourniquet-induced IR+saline, tourniquet-induced IR+lipo-Dex, and tourniquet-induced IR+Dex. In sham animals, the amplitude of sciatic nerve-stimulated EPPs is about 30 mV (29.4 ± 0.6 mV). In 1-week tourniquet-induced IR, the amplitude of sciatic nerve-stimulated EPPs is about 1.5 mV (1.5 ± 0.5 mV) and then the amplitude of sciatic nerve-stimulated EPPs gradually increases with longer IR time (5.8 ± 0.7 mV, 9.5 ± 0.9 mV, 13.9 ± 1.1 mV for 2-week IR, 4-week IR, and 6-week IR, respectively). The results indicate that the amplitude of sciatic nerve-stimulated EPPs does not recover to the normal level, even after 6-week IR (**Figure 12**). After treatment of lipo-Dex and Dex, the amplitude of sciatic nerve-stimulated EPPs is significantly improved in 1-week IR, 2-week IR, 4-week IR, and 6-week IR, compared to tourniquet-induced IR alone. In particular, the amplitude of sciatic nerve-stimulated EPPs in 6-week IR recovers to the normal level after treatment of lipo-Dex or Dex (**Figure 12**). These results suggest that treatment with lipo-Dex or Dex promotes recovery of neuromuscular junction function from tourniquet-induced IR injury.



- 9) We used immunofluorescent staining to measure morphological changes of the neuromuscular junction (NMJ) in gastrocnemius muscles during 1-week IR and 2-week IR time-points in 3 groups of mice including sham, tourniquet-induced IR+saline, tourniquet-induced IR+ dexamethasone (Dex) (Figs. 13-15). Motor nerve terminals and post-synaptic nicotinic acetylcholine receptor (nAChR) clusters in NMJs were labeled by neurofilament/synaptophysin (red color) and α -bungarotoxin (BTX, green color), respectively. In sham mice, pretzel-like nAChR clusters remained an intact structure, and motor nerve terminals innervated nAChR clusters to form functional synapses in 1, 2, 4, and 6 weeks of sham treatment. Quantitative data showed that 100% of nAChR clusters were innervated by motor nerve terminals, about 86% of the nerve terminal occupancy was observed in NMJs, and there were no fragmented nAChR clusters in 1, 2, 4, and 6 weeks of sham treatment (Figs. 13-5). In tourniquet-induced IR mice, a few innervated motor nerve terminals were observed (about 26% of the motor nerve innervation and 5% of the nerve terminal occupancy, Figs. 13 and 14), and there are some fragmented nAChR clusters in 1-week IR (Figs. 13 and 15; 17.9% fragmented nAChR clusters). In 2-week IR, the motor nerve terminals were regenerated, and the motor nerve innervation and occupancy were increased (Figs. 13 and 14, 63.8% and 19.8%); whereas the number of fragmented nAChR clusters was increased (60.3%, Fig. 15), compared to 1-week IR. In tourniquet-induced 4-week IR, motor nerve terminals almost sprouted into the endplates and formed new NMJs (97.8 ± 1.3 of the motor nerve innervation and 51.1 ± 7.3 of the nerve terminal occupancy in NMJs, Figs. 13 and 14), and many nAChR clusters remained to be fragmented (71.9 ± 4.2 fragmented nAChR clusters, Figs. 13 and 15). In 6-week IR, the motor nerve innervation and occupancy were increased close to the level seen in sham mice (100% of the motor nerve innervation and 71.6 ± 6.6 of the nerve terminal occupancy, $P > 0.05$ vs. sham group, Figs. 13-14); whereas the number of fragmented nAChR clusters still kept at the high level (64.6 ± 4.1 , $P < 0.05$ vs. sham group, Fig. 15). When Dex (1 mg/kg) was treated at the beginning of reperfusion on the day of IR induction and lasted for 1 week, the motor nerve innervation and occupancy were improved (62.8% and 20.2% in 1-wk IR+Dex, and 96.7% and 50.6% in 2-wk IR+Dex) and fragmented nAChR clusters are reduced in 1-week and 2-week IR (0% in 1-wk IR+Dex and 4.5% in 2-wk IR+Dex) (Figs. 13-15). Then the motor nerve innervation and occupancy were further improved (100% and 76.3 ± 1.4 in 4-wk IR+Dex, and 100% and 82.8 ± 0.9 in 6-wk IR+Dex) and fragmented nAChR clusters were further reduced in 4-week and 6-week IR (10.4 ± 2.0 in 4-wk IR+Dex and 30.4 ± 10.5 in 6-wk IR+Dex) (Figs. 13-15). From these data, we conclude that Dex stimulates recovery of the NMJ morphology from tourniquet-induced IR injury.



10) We also used immunofluorescent staining to measure morphological changes of the NMJ in gastrocnemius muscles during 1-week IR, 2-week IR, 4-week IR, and 6-week IR time-points in 3 groups of mice including sham, tourniquet-induced IR+saline, tourniquet-induced IR+lipo-Dex (Figs. 16-18). Synaptophysin and neurofilament 200 (red color) and α -bungarotoxin (green color) were used to label pre-synaptic nerve terminals (red color) and post-synaptic nicotinic acetylcholine receptor (nAChR) clusters in NMJs. Similarly to experimental 9) (above experiments), in sham animals, the structure of pretzel-like nAChR clusters normally remained intact, and motor nerve terminals innervated nAChR clusters to form synapses for signal transmission. In 1-week IR, only a few motor nerve fibers appeared and some motor nerve terminals innervated nAChR clusters (Figs.16-17; 4.7%), whereas there are no significant alterations in nAChR clusters (Figs, 16 and 18; 19.3% fragmented nAChR clusters). In 2-week IR, although many motor nerve fibers regenerated within muscle nerve fascicles and approached NMJs, these motor nerve fibers did not enter endplates or only a part of motor nerve terminals formed synapses with nAChR clusters (19.8%). Many nAChR clusters were fragmented in 2-week IR (60.3%). In 4-week IR,

motor nerve terminals began to sprout into endplates and form new NMJs (51.1 % innervation), and many nAChR clusters remained to be fragmented (Fig. 16 and 18; 72.8% fragmented nAChR clusters). In 6-week IR, many motor nerve terminals were overlapped with nAChR clusters (71.6% innervation), whereas a part of nAChR clusters were still fragmented (64% fragmented nAChR clusters). After treatment of lipo-Dex, motor nerve innervation is improved (15.3% in 1-wk IR+lipo-Dex and 33.5% in 2-wk IR+lipo-Dex) and fragmented nAChR clusters are reduced (0.1% in 1-wk IR+lipo-Dex and 4.5% in 2-wk IR+lipo-Dex) (Figs. 16-18). Then motor nerve innervation is further improved (62.5% in 4-wk IR+lipo-Dex and 84.5% in 6-wk IR+lipo-Dex) and fragmented nAChR clusters are significantly reduced (19.7 % in 4-wk IR+lipo-Dex and 24.6% in 6-wk IR+lipo-Dex) (Figs. 16-18). Like Dex, lipo-Dex promotes recovery of the NMJ morphology from tourniquet-induced IR injury.

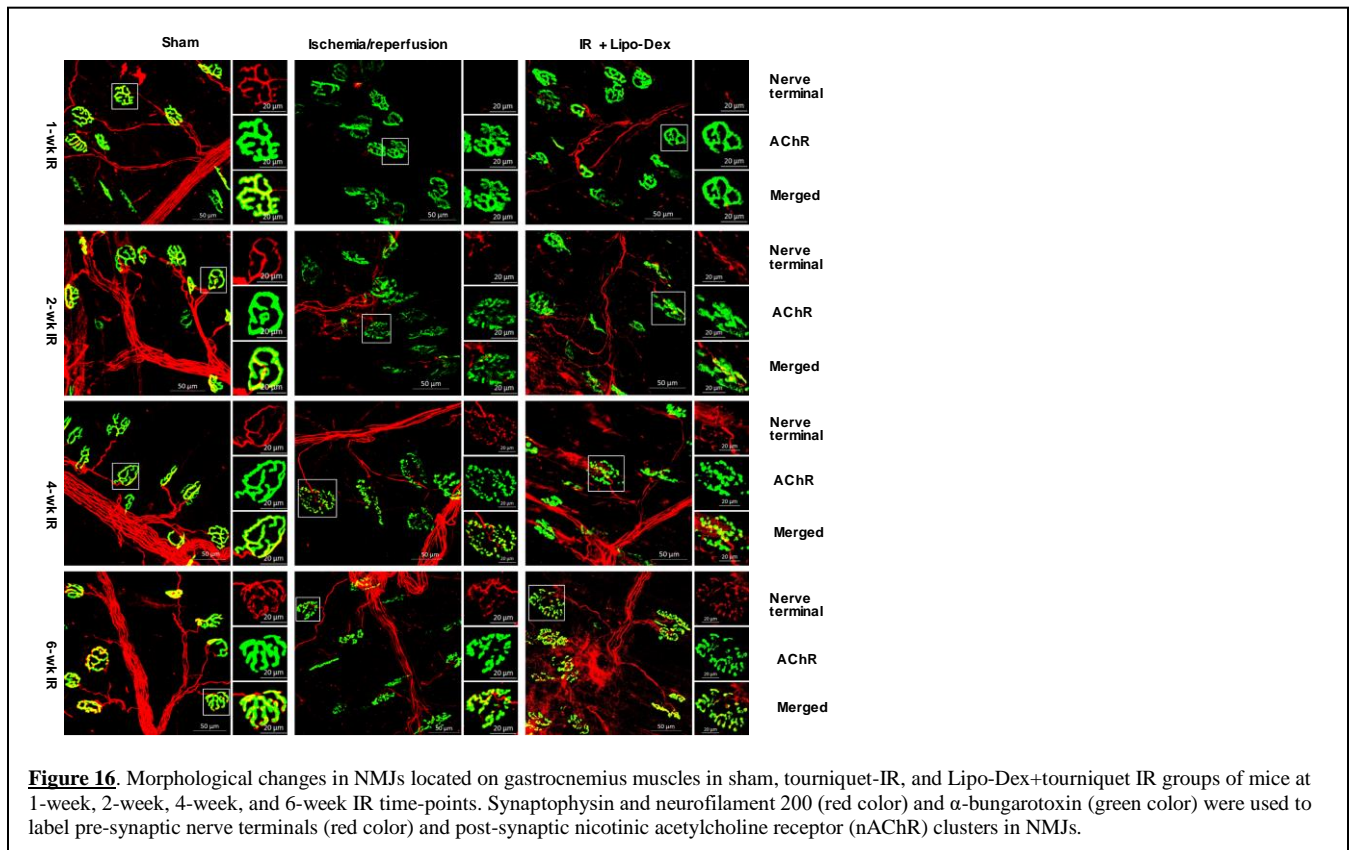


Figure 16. Morphological changes in NMJs located on gastrocnemius muscles in sham, tourniquet-IR, and Lipo-Dex+tourniquet IR groups of mice at 1-week, 2-week, 4-week, and 6-week IR time-points. Synaptophysin and neurofilament 200 (red color) and α -bungarotoxin (green color) were used to label pre-synaptic nerve terminals (red color) and post-synaptic nicotinic acetylcholine receptor (nAChR) clusters in NMJs.

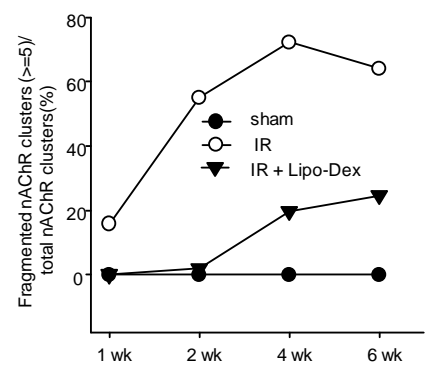
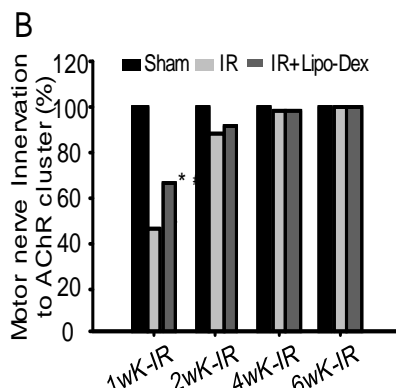
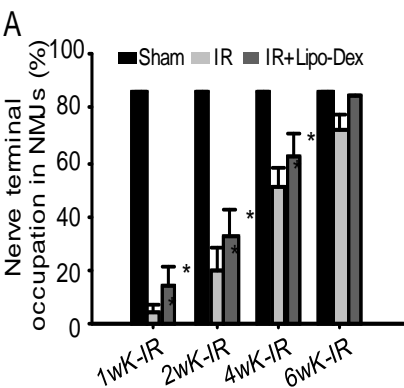
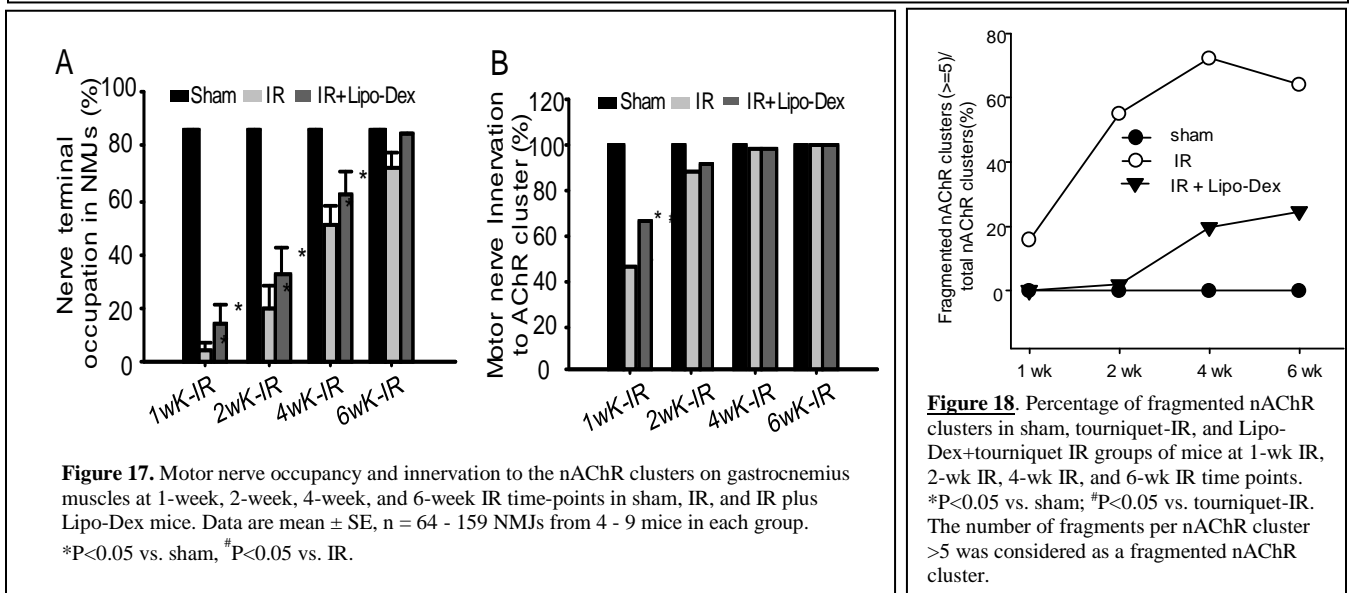


Figure 17. Motor nerve occupancy and innervation to the nAChR clusters on gastrocnemius muscles at 1-week, 2-week, 4-week, and 6-week IR time-points in sham, IR, and IR plus Lipo-Dex mice. Data are mean \pm SE, n = 64 - 159 NMJs from 4 - 9 mice in each group. *P<0.05 vs. sham, #P<0.05 vs. IR.

Figure 18. Percentage of fragmented nAChR clusters in sham, tourniquet-IR, and Lipo-Dex+tourniquet IR groups of mice at 1-wk IR, 2-wk IR, 4-wk IR, and 6-wk IR time points. *P<0.05 vs. sham; #P<0.05 vs. tourniquet-IR. The number of fragments per nAChR cluster >5 was considered as a fragmented nAChR cluster.

11) We measured blood pressure and glucose in all groups including sham, tourniquet-induced IR+saline, tourniquet-induced IR+lipo-Dex, and tourniquet-induced IR+Dex. There was no significant difference in blood pressure among all experimental groups. Hyperglycemia was observed in 33% of mice with tourniquet-induced IR+Dex but not in the mice with sham treatment, tourniquet-induced IR+saline, or tourniquet-induced IR+lipo-Dex. As mentioned in our Quarterly-Technical-Progress-Reports, we could not complete this project on time due to the hard work for the measurement of NMJ morphology.

Although we got the approval for a 6-month no-cost extension until Aug 31, 2019, we could not conduct the measurement of bone density due to the limitation of the time and budget.

- 12) We presented our research data at 2017 Experimental Biology conference (April, 2017 in Chicago, IL). The title is “Morphological regeneration and functional recovery of neuromuscular junctions after tourniquet-induced injuries in mouse hindlimb”.
- 13) We presented our research results at 2017 Military Health System Research Symposium. The title is “protective effect of dexamethasone against tourniquet-induced acute ischemia-reperfusion injury in mouse hindlimb”.
- 14) We presented our research data at 2018 Military Health System Research Symposium. The title is “Liposomal dexamethasone improves outcomes of post-traumatic skeletal muscle and neuromuscular junction”.
- 15) One research paper has been published in *Frontiers in Physiology*. Corrick RM, Tu, H, Zhang D, Muelleman RL, Wadman MC, Li YL. Dexamethasone protect against tourniquet-induced acute ischemia-reperfusion injury in mouse hindlimb. *Front Physiol* 2018; 9: 244.
- 16) We presented our research data at 2019 Military Health System Research Symposium. The title is “Dexamethasone improves neuromuscular junction recovery from ischemia-reperfusion injury induced by tourniquet application in mouse hindlimb”.

Summary: In this project (03/01/2017 to 08/31/2019), we used tourniquet-induced mouse IR model to investigate the protective effects of Dex and lipo-Dex against tourniquet-induced IR injuries for NMJs and skeletal muscles. First, we found that one-time intravenous administration with lipo-Dex at the beginning of reperfusion is mainly retained in IR-injured skeletal muscles after 24-hour IR, lasted about 1 week, and then disappeared. Second, we investigated the protective effect of Dex against tourniquet-induced acute IR injury in mouse hindlimb. The aforementioned results suggest that one-time treatment with Dex at the beginning of reperfusion only reduces structural and functional impairments of the skeletal muscle but not the NMJ through inhibiting inflammatory cytokines in 24-hour tourniquet/IR mice. Third, we investigated the effects of Dex and lipo-Dex on the recovery process of NMJs and skeletal muscle and skeletal muscles after tourniquet-induced IR injuries in mouse hindlimb. Our results mentioned above indicate that treatment with Dex or lipo-Dex for one week at the early stage of reperfusion significantly promotes the recovery of the NMJ morphology and function as well as the skeletal muscle contraction from tourniquet-induced chronic IR injuries in 6-week IR mice. These protective effects of Dex and lipo-Dex are associated with their anti-inflammatory effects at the early stage of reperfusion. Therefore, considering the advantages of lipo-Dex including being not refrigerated and daily administrated without side-effects, treatment with lipo-Dex at an optimal time-window might be a potential clinical therapeutic intervention for tourniquet-induced IR injuries and help improve the outcome and quality of life for patients with these injuries.

- **What opportunities for training and professional development has the project provided?**

Nothing to Report.

- **How were the results disseminated to communities of interest?**

Nothing to Report.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**

This project would provide an effective therapeutic intervention to improve the prognosis of patients with extremity injury by mitigating tourniquet-induced limb ischemia-reperfusion (IR) injury. Severe hemorrhage from extremity injuries is a significant cause of battlefield deaths and an important cause of preventable trauma fatalities in civilian medicine. As an effective means of mitigating life-threatening limb hemorrhage, tourniquet use is common and has improved survival. According to two clinical reports from Operation Iraqi Freedom, 57% of possible deaths were prevented by earlier tourniquet use (Beekley, et al. *J trauma* 2008,64:S28-S37), and the survival rate was higher at 87% with pre-hospital tourniquet use than without tourniquet use reported at 0% (Kragh, et al. *Ann Surg* 2009,249:1-7). Recent retrospective studies demonstrate that tourniquets could be used in the management of civilian trauma patients with severe limb injuries (Schroll, et al. *J Trauma Acute Care Surg* 2015,79:10-14; Inaba, et al. *J Trauma Acute Care Surg* 2015,79:232-237; Ode, et al. *J Trauma Acute Care Surg* 2015,79:586-591). Worldwide, over 20,000 tourniquet applications are performed daily to produce bloodless operating fields in orthopedic, musculoskeletal reconstructive procedures, and in vascular surgeries. Although tourniquets are effective at controlling hemorrhage, tourniquet-induced IR injury is a significant concern. Prolonged ischemia results in damage to both nerves and skeletal muscles; subsequent inflammatory response accompanying reperfusion promotes further destruction of nerves and skeletal muscles. So far, there are no established, effective means to improve long-term recovery of skeletal muscle function from tourniquet-induced IR injury. Our project focused

on the protective effect of anti-inflammation on long-term structural and functional recoveries of the neuromuscular junction and skeletal muscle from tourniquet-induced IR injury. The information from this project could be beneficial for further understanding the relationship between inflammation and neural-skeletal muscle IR injury and repair. From the results of this project, we see a significant potential therapeutic strategy for long-term recovery of tourniquet-injured neuromuscular junction and skeletal muscle, and for optimizing limb salvage in clinical tourniquet applications.

Glucocorticoids such as dexamethasone (Dex) are used clinically to treat inflammation and autoimmune phenomena. Their pharmacokinetics of these drugs require daily administration and can have serious adverse effects such as increased blood glucose and osteoporosis. In this project, we employed a liposomal nanoparticle-delivered Dex (lipo-Dex). Our proposed study is the first to test the protective effects of lipo-Dex on long-term structural and functional recoveries of both skeletal muscle and neuromuscular junction from tourniquet-induced IR injury. As a liposomal drug product, lipo-Dex translocates into the injured area and slowly releases the drug within target sites, prolonging the effect of the drug and limiting potential systemic toxicity. Lipo-Dex does not need refrigeration and can be administered at the beginning of limb reperfusion during definitive surgical intervention. The ease of dosing and storage ensures convenient and rapid application of lipo-Dex especially for the military continuum of care. Dex is widely used clinically as a potent anti-inflammatory drug, and the liposomal nanoparticle has been shown in numerous clinical trials to be a safe and efficient drug delivery system for the treatment of other diseases. Therefore, the FDA approval process for this therapeutic intervention should face fewer impediments and permit more rapid transition to clinical trials and applications. Therefore, lipo-Dex could soon become a novel therapeutic intervention for tourniquet-induced limb IR injury in the Role 3 medical care settings. Following this project, we will identify a clinical partner and advance this intervention to clinical trials. Our current project validates that pre-hospital tourniquet application to control fatal extremity hemorrhage with the addition of lipo-Dex at reperfusion could optimize limb performance outcomes and provide a reduction of complete limb paralysis and secondary amputation. The widespread application of the tourniquet in military and civilian pre-hospital settings makes the anticipated use of lipo-Dex a tangible possibility. Pre-hospital tourniquet use with the addition of lipo-Dex could soon be recommended to Advanced Trauma Life Support (ATLS) as the ATLS principle to manage the patients with traumatic extremity bleeding.

- **What was the impact on other disciplines?**

Nothing to Report.

- **What was the impact on technology transfer?**

Nothing to Report.

- **What was the impact on society beyond science and technology?**

Nothing to Report.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**

Nothing to Report.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to Report.

- **Changes that had a significant impact on expenditures**

Nothing to Report.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to Report.

- **Significant changes in use or care of human subjects**

N/A

- **Significant changes in use or care of vertebrate animals.**

Nothing to Report.

- **Significant changes in use of biohazards and/or select agents**

Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations.**

- **Journal publications.**

Corrick RM, Tu, H, Zhang D, Muelleman RL, Wadman MC, Li YL. Dexamethasone protect against tourniquet-induced acute ischemia-reperfusion injury in mouse hindlimb. *Front Physiol* 9: 2018; 244; (published); acknowledgement of federal support (yes).

- **Books or other non-periodical, one-time publications.**

Nothing to Report.

- **Other publications, conference papers, and presentations.**

1. Tu H, Zhang D, Corrick RM, Muelleman RL, Wadman MC, **Li YL**. Morphological regeneration and functional recovery of neuromuscular junctions after tourniquet-induced injuries in mouse hindlimb. *FASEB J* 2017; 31: 1022.4 (2017 Experimental Biology conference.)
2. **Li YL**, Corrick RM, Tu H, Zhang D, Muelleman RL, Wadman MC. Protective effect of dexamethasone against tourniquet-induced acute ischemia-reperfusion injury in mouse hindlimb. *MHSRS symposium*, August, 2017.
3. Zhang D, Tu H, Zhao, G, Wang D, Wadman MC, **Li YL**. Liposomal dexamethasone improves outcomes of post-traumatic skeletal muscle and neuromuscular junction. *MHSRS symposium*, August, 2018.
4. Tu H, Zhang D, Wadman MC, **Li YL**. Dexamethasone improves neuromuscular junction recovery from ischemia-reperfusion injury induced by tourniquet application in mouse hindlimb. *MHSRS symposium*, August, 2019.

- **Website(s) or other Internet site(s)**

DMRDP research highlight for this project

https://cdmrp.army.mil/dmrdp/research_highlights/19yulong_li_highlight

- **Technologies or techniques**

Nothing to Report.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name: Yulong Li

Project Role: Principal Investigator

Research Identifier: 0000-0001-5407-2761 (ORCID ID)

Nearest person month worked: 6

Contribution to Project: To design the experimental protocols and monitor all data to assure its quality.

Name: Dong Wang

Project Role: Co-Investigator

Research Identifier: 0000-0002-8340-2763 (ORCID ID)

Nearest person month worked: 6

Contribution to Project: To assist and supervise Dr. Wang's graduate student and Dr. Li's postdoctoral fellow to synthesize lipo-Dex and trace lipo-Dex distribution.

Name: Dongze Zhang

Project Role: Postdoctoral fellow

Research Identifier: none

Nearest person month worked: 24

Contribution to Project: to collaborate with Dr. Wang's graduate student to trace lipo-Dex distribution. He also performs in-vivo and in-vitro animal experiments.

Name: Gang Zhao

Project Role: Graduate Student

Research Identifier: none

Nearest person month worked: 6

Contribution to Project: To synthesize lipo-Dex and trace lipo-Dex distribution.

Name: Huiyin Tu

Project Role: Senior research associate

Research Identifier: none

Nearest person month worked: 6

Contribution to Project: To measure morphology of skeletal muscles.

Funding Support: NIH R01 grant.

Name: Ryan Corrick

Project Role: Clinical resident in Emergency Medicine

Research Identifier: none

Nearest person month worked: 2

Contribution to Project: To measure inflammation including cytokine.

Funding Support: Departmental residence training program.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to Report.

- **What other organizations were involved as partners?**

Nothing to Report.

- **Organization Name:**
- **Location of Organization:**
- **Partner's contribution to the project**
 - **Financial support;**
 - **In-kind support**
 - **Facilities**
 - **Collaboration**
 - **Personnel exchanges**

- **Other.**

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** N/A
- **QUAD CHARTS:** Attached.

9. APPENDICES:

1. QUAD CHARTS
2. One copy of published research paper.
3. Abstracts for research conferences

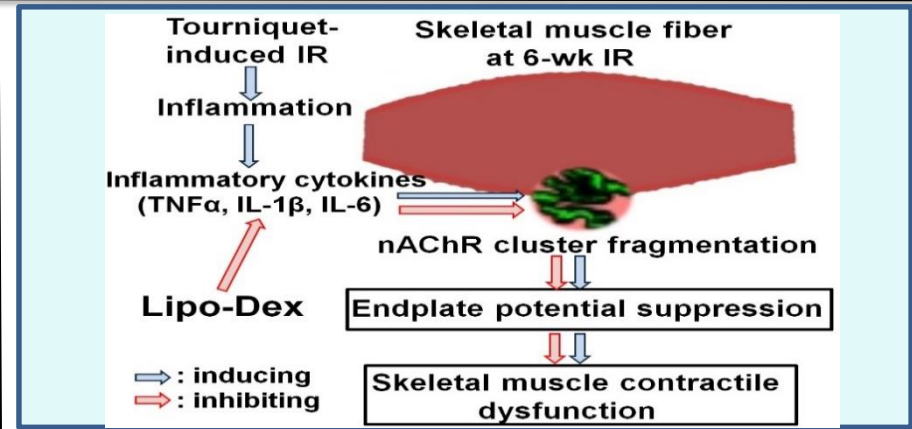


Study/Product Aim(s)

- To test the effects of lipo-Dex and Dex on inflammatory cytokines, structure of skeletal muscles, and skeletal muscle contraction during tourniquet-induced IR.
- To test the effects of lipo-Dex and Dex on the structure and function of the neuromuscular junction during tourniquet-induced IR.
- To test adverse effects of lipo-Dex and Dex in mice with tourniquet-induced IR.

Approach

- Tourniquet-induced limb IR injury will be induced by placing and releasing an orthodontic rubber band at the hip joint in mice.
- We will synthesize lipo-Dex by ourselves.
- We will design *in-vitro* (cells and tissues) and *in-vivo* (conscious and anesthetized mice) studies.



Accomplishment: 1. Lipo-Dex and Dex inhibit tourniquet-IR-induced inflammation including elevation of cytokines, and promote recovery of skeletal muscle and neuromuscular junction structure and function from tourniquet-induced IR injury. 2. The data have been presented in conferences and published in *Frontiers in Physiology*.

Timeline and Cost

Activities	CY	17	18	19	20
To test effect of lipo-Dex on skeletal muscle function		█			
To test effect of lipo-Dex on function of neuromuscular junction			█		
To test adverse effect of lipo-Dex				█	
Estimated Budget (\$K)		\$150	\$150	\$60	\$0

Goals/Milestones

CY17 Goal – To test effect of lipo-Dex on skeletal muscle function

- To obtain IACUC and ACURO approval.
- To test effect of lipo-Dex on morphology and function of the skeletal muscle during tourniquet-induced IR.

CY18 Goals – To test effect of lipo-Dex on morphology and function of neuromuscular junction

- To test effect of lipo-Dex on morphology and function of neuromuscular junction during tourniquet-induced IR.

CY19 Goal – To test adverse effect of lipo-Dex

- To test adverse effect of lipo-Dex in mice with tourniquet-induced IR.
- To present the finding at relevant national conferences and publish peer reviewed papers.
- To prepare seek clinical partners to conduct a small clinical trial.

Budget Expenditure to Date

Projected Expenditure: \$360,645.00

Actual Expenditure: \$360,645.00 (Mar. 1, 2017 to Aug. 31, 2019)



Dexamethasone Protects Against Tourniquet-Induced Acute Ischemia-Reperfusion Injury in Mouse Hindlimb

Ryan M. Corrick [†], Huiyin Tu [†], Dongze Zhang [†], Aaron N. Barksdale, Robert L. Muelleman, Michael C. Wadman and Yu-Long Li ^{*}

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Extremity injuries with hemorrhage have been a significant cause of death in civilian medicine and on the battlefield. The use of a tourniquet as an intervention is necessary for treatment to an injured limb; however, the tourniquet and subsequent release results in serious acute ischemia-reperfusion (IR) injury in the skeletal muscle and neuromuscular junction (NMJ). Much evidence demonstrates that inflammation is an important factor to cause acute IR injury. To find effective therapeutic interventions for tourniquet-induced acute IR injuries, our current study investigated effect of dexamethasone, an anti-inflammatory drug, on tourniquet-induced acute IR injury in mouse hindlimb. In C57/BL6 mice, a tourniquet was placed on unilateral hindlimb (left hindlimb) at the hip joint for 3 h, and then released for 24 h to induce IR. Three hours of tourniquet and 24 h of release (24-h IR) caused gastrocnemius muscle injuries including rupture of the muscle sarcolemma and necrosis ($42.8 \pm 2.3\%$ for infarct size of the gastrocnemius muscle). In the NMJ, motor nerve terminals disappeared, and endplate potentials were undetectable in 24-h IR mice. There was no gastrocnemius muscle contraction in 24-h IR mice. Western blot data showed that inflammatory cytokines (TNF α and IL-1 β) were increased in the gastrocnemius muscle after 24-h IR. Treatment with dexamethasone at the beginning of reperfusion (1 mg/kg, i.p.) significantly inhibited expression of TNF α and IL-1 β , reduced rupture of the muscle sarcolemma and infarct size ($24.8 \pm 2.0\%$), and improved direct muscle stimulation-induced gastrocnemius muscle contraction in 24-h IR mice. However, this anti-inflammatory drug did not improve NMJ morphology and function, and sciatic nerve-stimulated skeletal muscle contraction in 24-h IR mice. The data suggest that one-time treatment with dexamethasone at the beginning of reperfusion only reduced structural and functional impairments of the skeletal muscle but not the NMJ through inhibiting inflammatory cytokines.

Keywords: tourniquet, acute ischemia-reperfusion injury, skeletal muscle, neuromuscular junction, cytokine, dexamethasone

INTRODUCTION

Based on the data from the Center for Disease Control and Prevention, over 14 million persons with extremity injuries visit emergency rooms, with an estimated cost of \$80 billion each year (Center for Disease Control and Prevention, 2017). Exsanguinating injury of the extremities is a key factor of trauma fatalities in civilian medicine and on the battlefield (Mabry, 2006; Inaba et al., 2015; Ode et al., 2015). Tourniquet use is a primary tool in first-line treatment of severe limb hemorrhage (Mabry, 2006; Beekley et al., 2008; Kragh et al., 2012; Welling et al., 2012). The tourniquet is also used to create an optimal operating fields with bloodless in vascular surgeries and orthopedic, musculoskeletal reconstructive procedures (an estimated 15,000 procedures daily in the US and elsewhere) (Tai et al., 2011; Halladin et al., 2014). However, acute ischemia-reperfusion (IR) injuries induced by a tourniquet use with subsequent reperfusion usually occur, which include tissue apoptosis and necrosis in the skeletal muscle, and severe structural and functional damage in the neuromuscular junction (NMJ) (Aho et al., 1983; Mohler et al., 1999; Schoen et al., 2007; Tran et al., 2011, 2012; Gillani et al., 2012). These acute injuries also affect long-term recovery of NMJ function and skeletal muscle contraction from IR injuries, even resulting in complete limb paralysis as well as the need for secondary amputation (Kam et al., 2001; Noordin et al., 2009; Vignaud et al., 2010). These complications have led to the demerit and limitations of tourniquet use (Clasper et al., 2009; Kue et al., 2015).

Until now the cellular and molecular mechanisms responsible limb acute IR injuries are unclear. Some studies demonstrated that overproduction of inflammatory cytokines and reactive oxygen species in injured skeletal muscles during the acute IR causes neuronal damage and deficit of skeletal muscle function (Seekamp et al., 1993; Clementsen and Reikeras, 2008; Chen and Nunez, 2010; Eltzschig and Eckle, 2011). Based on this fact, inhibition of production and release of inflammatory cytokines is likely considered as one of the strategies for limb acute IR injury. Dexamethasone (Dex), a synthetic glucocorticoid and potent anti-inflammatory drug, is used clinically to treat many inflammatory and autoimmune diseases, including asthma, rheumatoid arthritis, lupus nephritis, inflammatory shock, etc. (Barnes, 2011). Therefore, we hypothesized that Dex could protect the limb from acute IR injury through inhibiting inflammatory cytokines because glucocorticoids reduce production and release of inflammatory cytokines in whole blood (Schwiebert et al., 1996; Smits et al., 1998; Schuld et al., 2001).

In general, both innervated motor nerve-muscle transmission (i.e., NMJ) and skeletal muscle are involved in skeletal muscle contractile function (Chervu et al., 1989; Fish et al., 1989; Eastlack et al., 2004). In this study, therefore, three purposes were performed: (1) to assess the morphological and functional changes of the skeletal muscle and NMJ; (2) to test whether Dex could attenuate the structural and functional impairment of the skeletal muscle and NMJ; (3) to measure the inhibitory effect of Dex on inflammatory cytokines in a murine model of tourniquet-induced 3-h ischemia and subsequent 24-h reperfusion.

MATERIALS AND METHODS

Animals

Totally 63 male C57/BL6 mice (21 sham, 21 tourniquet-induced IR, and 21 tourniquet-induced IR + Dex mice) weighing 22–25 g (Charles River Laboratory, Wilmington, MA) were used in this study. The mice were housed under controlled temperature and humidity and a 12:12-h dark-light cycle, and were provided water and mouse chow *ad libitum*. All experimental procedures were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and were carried out in accordance with the National Institutes of Health (NIH Publication No. 85–23, revised 1996) and the American Physiological Society's "Guides for the Care and Use of Laboratory Animals."

A Mouse Model of Tourniquet-Induced Hindlimb IR and Drug Treatment

Tourniquet-induced mouse hindlimb IR is a mature animal model and has been used in our previous studies (Tran et al., 2011, 2012; Tu et al., 2017; Zhang et al., 2017). Briefly, under anesthesia with a cocktail consisting of 100 mg/kg ketamine and 10 mg/kg xylazine, given as an intraperitoneal injection (10 ml/kg body weight), the tourniquet in unilateral hindlimb (left hindlimb) was done for 3 h by setting an orthodontic rubber band at the hip joint, using a McGivney hemorrhoidal ligatorer. Then the orthodontic rubber band was released to begin reperfusion for 24 h. During the period of this procedure, the level of anesthesia was continuously monitored by testing the respiratory patterns and toe pinch reflex. A heating pad was used to maintain body temperature at 37°C until the animals woke up. Our previous study has verified skeletal muscle IR in this animal model through testing blood flow in IR gastrocnemius muscles (Tran et al., 2011). Before waking up, mice were kept well hydrated with an intraperitoneal injection of 0.2 ml normal saline every 2 h.

Usually the contralateral skeletal muscle is used as an uninjured control muscle in a unilateral tourniquet-induced IR model (Woitaska and McCarter, 1998; Walters et al., 2008; Lotfi et al., 2013). However, it is possible that metabolites from injured skeletal muscles and drug treatments are also distributed into contralateral NMJs and skeletal muscles, affecting their functions. A sham group of mice was designed as the control group in the present study to avoid this possibility. The same procedure except for the application of the orthodontic rubber band was performed in sham-operated animals.

Dex (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.9% NaCl. IR mice were randomly divided into two groups for administration of normal saline or Dex (1 mg/kg), respectively. Normal saline solution or Dex (0.2 ml) was intraperitoneally administered at the beginning of reperfusion. All terminal experiments were performed on mice after 24 h of reperfusion.

In-Situ Detection of the Gastrocnemius Muscle Contractile Force

The gastrocnemius muscle contractile force was measured in five sham, five tourniquet-induced IR, and five tourniquet-induced

IR + Dex mice as described previously (Tu et al., 2017; Zhang et al., 2017). Briefly, under anesthesia (800 mg kg⁻¹ urethane and 40 mg kg⁻¹ chloralose, a mixing solution of 20% urethane and 1% chloralose, 4 ml kg⁻¹, i.p.), the mouse was kept in prone position and maintained at 37°C. The isolated middle and distal ends of the left gastrocnemius muscle were moistened by warmed saline. The distal end of the gastrocnemius muscle was sutured and connected to a wide range force transducer (AD Instruments, Colorado Springs, CO). The left sciatic nerve was exposed through splitting the left biceps femoris muscle along muscle fibers. Maximal electrical tetanus stimulation (10 V, 50 Hz, 0.1-ms pulse, 5-s duration) was produced by a bipolar platinum electrode placed on the distal cut end of the sciatic nerve to induce tetanic contractile force of the gastrocnemius muscle. A similar procedure was performed for direct gastrocnemius muscle stimulation (10–50 V, 50 Hz, 0.1-ms pulse, 5-s duration). Considering spatial variability for direct gastrocnemius muscle stimulation, we selected 4 areas of gastrocnemius muscles for this measurement and collected these results as the data for one measured muscle. PowerLab 8/30 Data Acquisition Systems with LabChart 7 (AD Instruments, Colorado Springs, CO, USA) were used to record and analyze the muscle contractile force.

Measurement of Infarct Size in the Gastrocnemius Muscle (Tran et al., 2011)

Infarct size of the gastrocnemius muscle induced by tourniquet-related IR was tested by triphenyl tetrazolium chloride assay. After measurement of the gastrocnemius muscle contractile force, mouse gastrocnemius muscle was quickly isolated and cut into 1.5 mm transverse slices. The cut slices were cleaned by cold saline solution, and subsequently incubated with 1% triphenyl tetrazolium chloride solution (Sigma-Aldrich, St. Louis, MO) at room temperature for 1 h. Slice images were digitalized by a Canon camera. In the obtained muscle images, the muscle with a dark purple red color stain was defined as the viable muscle, and the muscle with a pale brown color stain was defined as the infarcted muscle. After the viable muscle and infarcted muscle were quantified by Adobe Photoshop CS5, the ratio (% wet weight/wet weight) of the infarcted muscle to total gastrocnemius muscle (viable plus infarcted muscles) was calculated as the infarct size.

Histological Evaluation of Skeletal Muscle Damage and Inflammatory Infiltration

In the first experiment, gastrocnemius muscles from three sham, three tourniquet-induced IR, and three tourniquet-induced IR + Dex mice were successively fixed in Methacarn solution (300 ml methanol, 150 ml chloroform, and 50 ml acetic acid) for 48 h, and 60% ethanol for 72 h. The fixed muscle was embedded into paraffin wax after routine processing, and then cut into 4- μ m-thick longitudinal sections. After deparaffinization, sections were stained with hematoxylin and eosin (H&E, Sigma-Aldrich, St. Louis, MO) for inflammatory infiltration and with Masson's trichrome (Sigma-Aldrich, St. Louis, MO) for skeletal muscle damage. Stained sections were captured by a bright-field

microscope (Leica Microsystems Inc., Buffalo Grove, IL) with digital camera (Qimaging MicroPublisher 3.3RTV).

In the second experiment, gastrocnemius muscles from three sham, three tourniquet-induced IR, and three tourniquet-induced IR + Dex mice were postfixed with 4% paraformaldehyde for 12 h, and then soaked in 30% sucrose for 12 h at 4°C for cryostat procedure. The muscle was cut into 10- μ m-thick cross-sections in a freezing cryostat at -20°C. Tissue sections were processed with standard immunocytochemical staining procedure. Briefly, tissue sections were permeabilized with 0.3% Triton X-100 (Thermo Fisher Scientific, Waltham, MA) in PBS at room temperature for 20 min. After tissue sections were successively incubated with 10% normal goat serum (Jackson ImmunoResearch Labs Inc.), rabbit anti-laminin antibody (Sigma-Aldrich, St. Louis, MO), and goat anti-rabbit IgG labeled with Alexa Fluor 594 (Thermo Fisher Scientific, Waltham, MA), tissue images were obtained using a Leica fluorescent microscope with digital camera (Qimaging Retiga Exi Fast 1394).

Electrophysiological Recording of the EPP In-situ

The EPP recording was performed in five sham, five tourniquet-induced IR, and five tourniquet-induced IR + Dex mice as described previously (Tu et al., 2017; Zhang et al., 2017). Under anesthesia (800 mg/kg urethane and 40 mg/kg chloralose, i.p.), the isolated middle and distal end of the left gastrocnemius muscle was moistened by warmed saline solution. To avoid the influence of muscle contraction on the EPP recording, a specific muscle Na⁺ channel blocker, μ -conotoxin GIIIB (4 μ M, 200 μ L) was locally provided into the gastrocnemius muscle to inhibit muscle contraction. A glass microelectrode filled with 3 M KCl (5–15 MQ pipette resistance) was slowly inserted into the gastrocnemius muscle fiber and connected with an intracellular preamplifier (IX1; Dagan Corporation, Minneapolis, MN, USA) for the EPP recording. When miniature endplate potentials (mEPPs) could be recorded to determine an endplate close to the electrode, the EPPs were recorded by intracellular recording technique under the electrical stimulation of exposed sciatic nerve (10 V, 50 Hz, 0.1 ms). Finally sciatic nerve stimulation-evoked EPPs was digitized by PowerLab 8/30 Data Acquisition System with LabChart 7 (AD Instruments), and stored on computer for analyzing the amplitude of EPPs. In the gastrocnemius muscle from each mouse, 6–8 sites were selected for EPPs recording.

Immunohistochemistry of the NMJs (Tu et al., 2017)

After recording of the EPP in-situ, mouse gastrocnemius muscle was quickly isolated for postfixing with 4% paraformaldehyde for 15 min, and subsequently incubating with 0.1 M glycine for 15 min. To facilitate probe penetrations into NMJs, The gastrocnemius muscle was divided into 8–10 small longitudinal segments, and then permeabilized in -20°C methanol for 10 min. After blocking with PBS containing 0.5% Triton and 1% BSA for 1 h, small longitudinal segments of the gastrocnemius

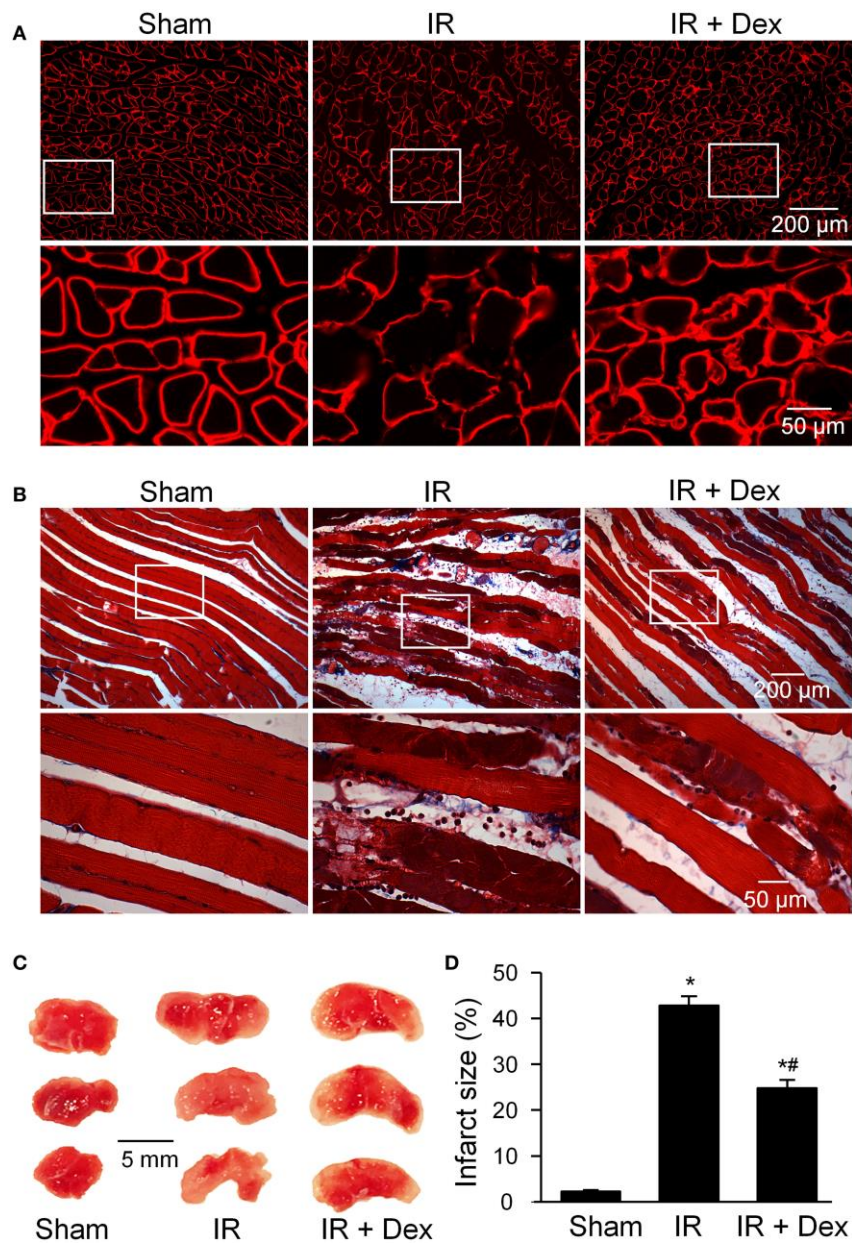
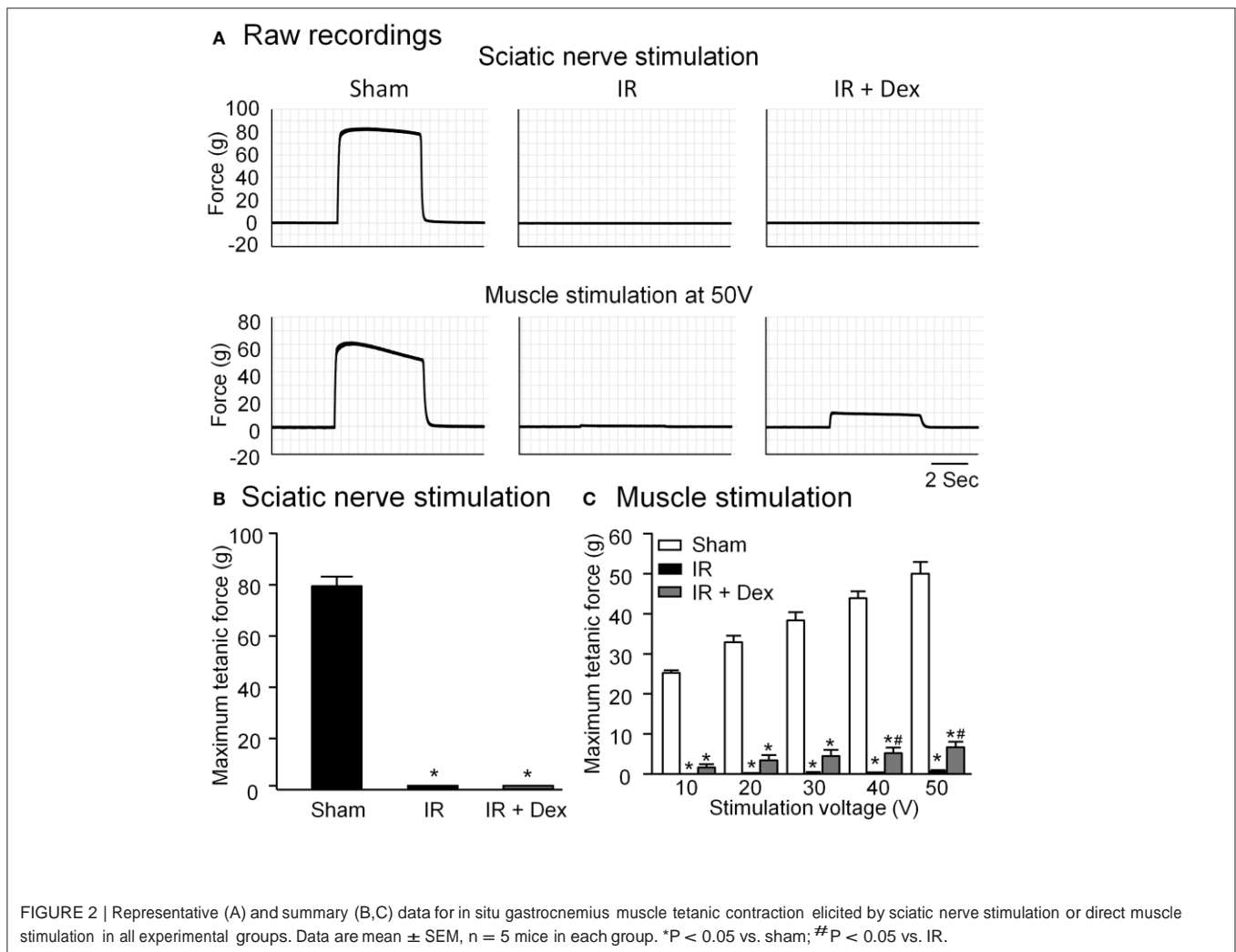


FIGURE 1 | The morphology of gastrocnemius muscles in all experimental groups. (A), histology in the cross section of gastrocnemius muscles, measured by laminin staining (a marker for muscle sarcolemma). (B), histology in the longitudinal section of gastrocnemius muscles, measured by Masson's trichrome staining. (C,D), representative and summary data for infarct size in gastrocnemius muscles. Data are mean \pm SEM, $n = 5$ mice in each group. * $P < 0.05$ vs. sham, # $P < 0.05$ vs. IR.

muscle were incubated overnight at 4°C in a cocktail of primary antibodies, including mouse anti-neurofilament 200 (Sigma-Aldrich, St. Louis, MO) and rabbit anti-synaptophysin (Thermo Fisher Scientific, Waltham, MA) antibodies for axon and nerve terminal labeling. Then muscle segments were incubated overnight at 4°C with Alexa Fluor® 594 labeled donkey anti-mouse (Thermo Fisher Scientific, Waltham, MA) and anti-rabbit (Thermo Fisher Scientific, Waltham, MA) IgGs, and Alexa

Fluor® 488 labeled α -Bungarotoxin (α -BTX, Thermo Fisher Scientific, Waltham, MA). Finally, images of muscle segments mounted on glass slides were captured using a Leica fluorescent microscope (Leica DMR, Leica Microsystems Inc., Buffalo Grove, IL) to analyze immunohistochemically labeled NMJs including motor nerve terminals and nAChR clusters.

In each muscle segment, five different regions were selected to obtain Z-stack images of the NMJ. All analyses were done by



ImageJ software (NIH Image) on en-face NMJs. In the NMJ, the percentage of motor nerve innervation to nAChR clusters was quantified by measurements of nerve terminal sizes labeled with neurofilament and synaptophysin. The endplate with or without labeling for neurofilament and synaptophysin was defined as an innervated or denervated endplate, respectively. The nAChR areas in NMJs labeled with α -BTX were used to calculate the area per fragment, the nAChR area per nAChR cluster, and the number of discrete fragments per nAChR cluster. A fragmented nAChR cluster was defined when the number of discrete fragments per nAChR cluster ≥ 5 .

Protein Expression of IL-1 β and TNF α in Gastrocnemius Muscles

Gastrocnemius muscles from five sham, five tourniquet-induced IR, and five tourniquet-induced IR + Dex mice were rapidly removed and stored at -80°C until analyzed. A lysing buffer (10 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% SDS, 1 mM PMSF; pH 7.4) plus protease inhibitor cocktail (100 $\mu\text{l/ml}$, P2714, Sigma-Aldrich, St. Louis, MO) was added into the muscle tissue for homogenization. Homogenized muscle tissue was centrifuged

at 12,000 g for 20 min at 4°C . Total protein concentration in the supernatant was measured by a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA). Same volume of the loading buffer was added into protein samples to mix and heat for 5 min at 100°C . Protein samples (40 $\mu\text{g/well}$) loaded in the stacking gel were separated on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide running gel. Proteins of these samples were electrophoretically transferred at 200 mA for 3 h onto PVDF membrane (EMD Millipore, Billerica, MA). The membrane was blocked with 5% non-fat milk for 1 h. Then the membrane was probed with rabbit anti TNF- α antibody (Cell Signaling Technology, Beverly, MA), mouse anti IL-1 β antibody (Cell Signaling Technology, Beverly, MA), or mouse anti β -actin antibody (Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C . After washing by PBS, the membrane was incubated with peroxidase-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA), or goat anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA) for 1 h. The signal was developed by enhanced chemiluminescence substrate (Thermo Fisher Scientific, Waltham, MA), and captured by UVP bioimaging system (UVP, Upland, CA).

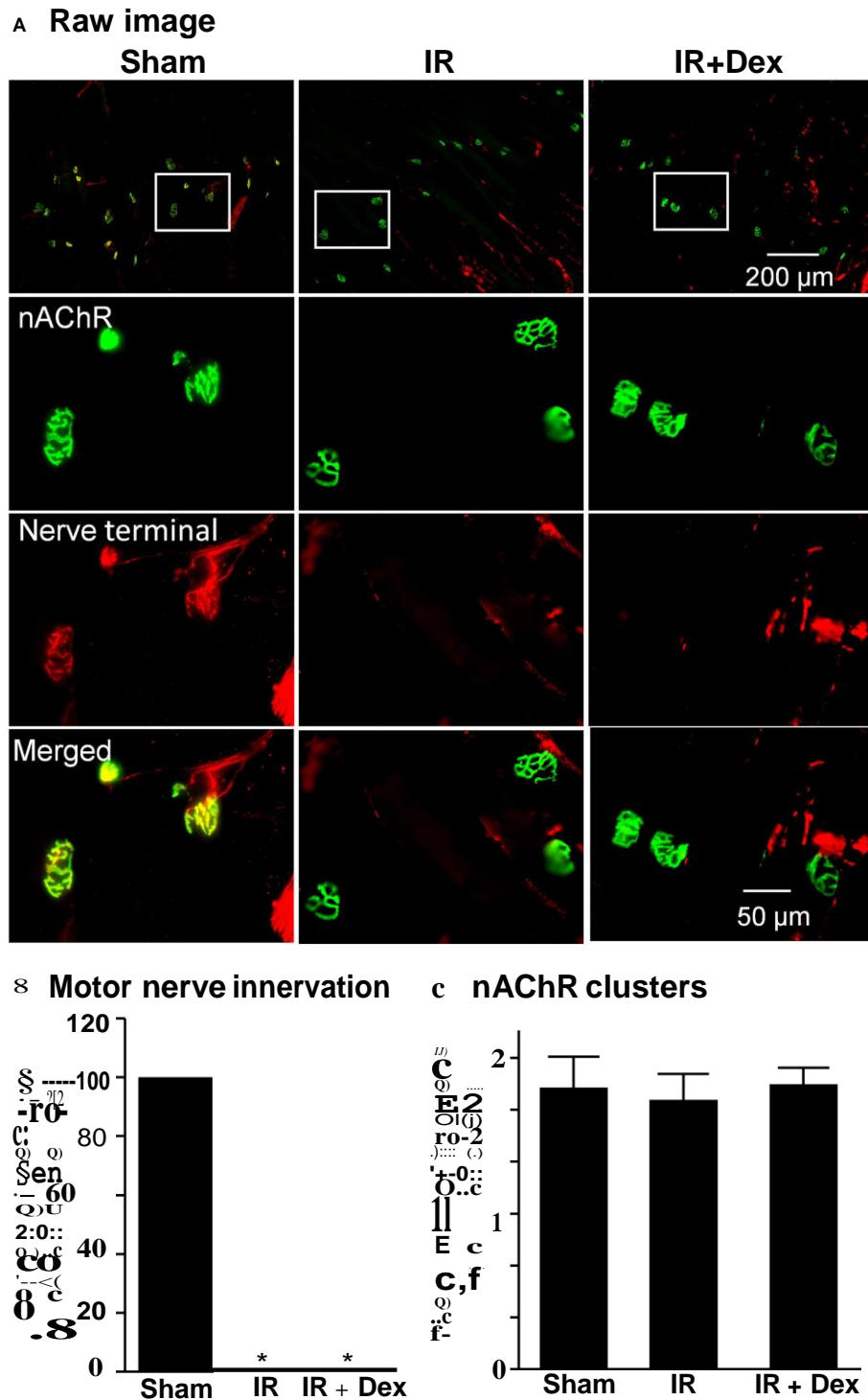


FIGURE 3 | The morphology of NMJs including motor nerve terminals and nAChRs located on gastrocnemius muscles from all experimental groups. **(A)**, representative images of NMJs. Synaptophysin and neurofilament 200 (red color) and u-bungarotoxin (B1X, green color) were used to label motor nerve terminals and nAChR clusters in NMJs. **(B,C)**, mean data for motor nerve innervation to nAChR clusters and the number of fragments per nAChR cluster. Data are mean \pm SEM, $n = 5$ mice in each group. * $p < 0.05$ vs. sham.

Statistical Analysis

All data are presented as means \pm SE. SigmaPlot 12 was used for data analysis. A one-way ANOVA with *post hoc* Bonferroni test was used to determine statistical significance for multi group comparison. A Chi-Square test was performed for percentage of nerve innervation in the NMJ. Normal distribution of data was confirmed with Kolmogorov–Smirnov test and equal variance with Levene's test. Statistical significance was accepted when $p < 0.05$.

RESULTS

Effects of Dex on Acute IR-Induced Morphological and Functional Changes in Gastrocnemius Muscles

Acute IR-induced gastrocnemius muscle injuries were shown in **Figure 1**. An obvious breakage of muscle fiber was observed in tourniquet-induced IR group of mice, revealed by laminin staining of gastrocnemius muscles in cross-section (**Figure 1A**). Tourniquet-induced IR caused undulating muscle cell borders, irregular spacing of muscle striations, and disruption of muscle fibers, evidenced by Massion's trichrome staining of gastrocnemius muscles in longitudinal-section (**Figure 1B**). Additionally, infarct size of the skeletal muscle was measured by triphenyl tetrazolium chloride assay. Tourniquet-induced IR also markedly resulted in infarction in gastrocnemius muscles ($42.8 \pm 2.4\%$), compared to that in sham group of mice (0% , $p < 0.05$, **Figures 1C,D**). Dex treatment (1 mg/kg, i.p.) at the beginning of reperfusion significantly attenuated, but did not abolish, acute IR-induced gastrocnemius muscle injuries including broken muscle sarcolemmata, disrupted muscle fibers, and muscle infarct size, measured by above three methods (**Figure 1**).

Gastrocnemius muscle contraction was measured by sciatic nerve stimulation or direct muscle stimulation, which can determine if IR-induced skeletal muscle contractile dysfunction is caused by muscle injury alone and/or by nerve-muscle transmission failure (Chervu et al., 1989; Eastlack et al., 2004). In tourniquet-induced IR group, both sciatic nerve stimulation and direct muscle stimulation did not evoke gastrocnemius muscle contraction (**Figure 2**). Dex treatment improved direct muscle stimulation-induced muscle contraction in IR-injured gastrocnemius muscles ($P < 0.05$ vs. IR group, **Figure 2C**), whereas it did not affect sciatic nerve stimulation-induced gastrocnemius muscle contraction (**Figure 2B**).

Effects of Dex on Structural and Functional Alterations in NMJs

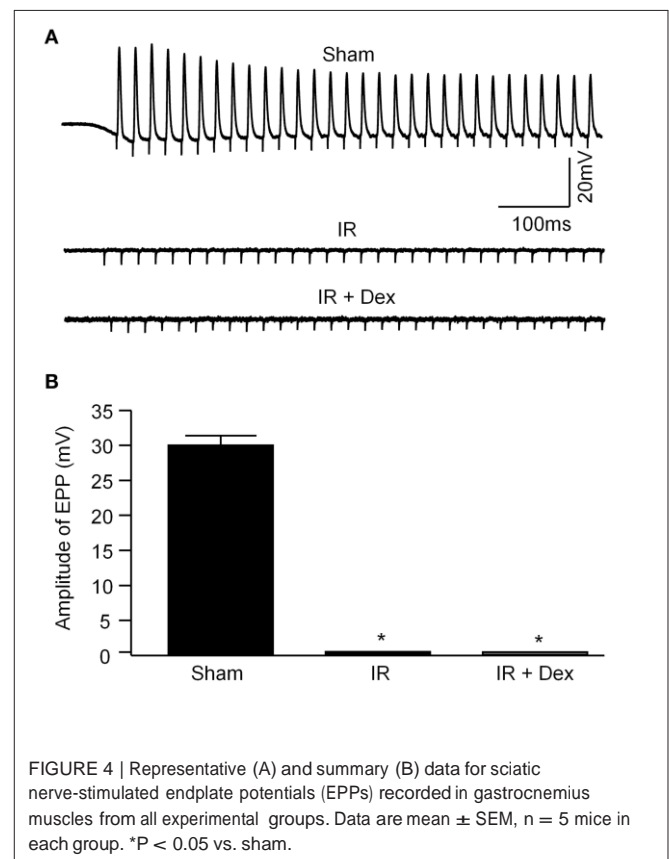
In **Figure 3**, motor nerve terminals labeled by neurofilament and synaptophysin and nAChR clusters labeled by BTX were investigated. As a major feature of NMJs, a specific synapse is normally formed by the presynaptic membrane (motor nerve terminals) and postsynaptic membrane (nAChR clusters), which can transmit the signal from motor neurons to skeletal muscles (**Figure 3A**). In IR group, the gastrocnemius muscle was totally denervated by motor nerve terminals in all NMJs (0% motor

nerve innervation to nAChR clusters, $p < 0.05$ vs. sham). Compared to sham mice, the structure of nAChR clusters did not show significant alterations, and the number of fragments per nAChR cluster had no markedly changes (1.65 ± 0.13 vs. sham 1.80 ± 0.21 , **Figure 3C**). Treatment with Dex (1 mg/kg) did not blunt IR-induced disappearance of motor nerve terminals in NMJs from the gastrocnemius muscle (**Figure 3**).

As noted above, electrical signals are usually transmitted from motor neurons to skeletal muscles through the NMJ (including motor nerve terminals and nAChR clusters), which can trigger skeletal muscle contraction. Recording of sciatic nerve-stimulated EPPs was used to NMJ function in the gastrocnemius muscle (**Figure 4**). In sham animals, sciatic nerve stimulation evokes regular, steady EPPs and the amplitude of EPPs was 29.9 ± 1.4 mV. In the IR group, sciatic nerve-stimulated EPPs were not detectable in all NMJs (**Figure 4**). When Dex (1 mg/kg) was administered at the beginning of reperfusion, IR-induced disappearance of sciatic nerve-stimulated EPPs was not improved (**Figure 4**).

Effects of Dex on Inflammatory Cytokines in IR Gastrocnemius Muscles

In the sham group, there was no obvious leukocyte infiltration and expression of IL-1 β and TNF α (2 proinflammatory cytokines) kept at a low level in the gastrocnemius muscle (**Figure 5**). However, after 3h of tourniquet and 24h of

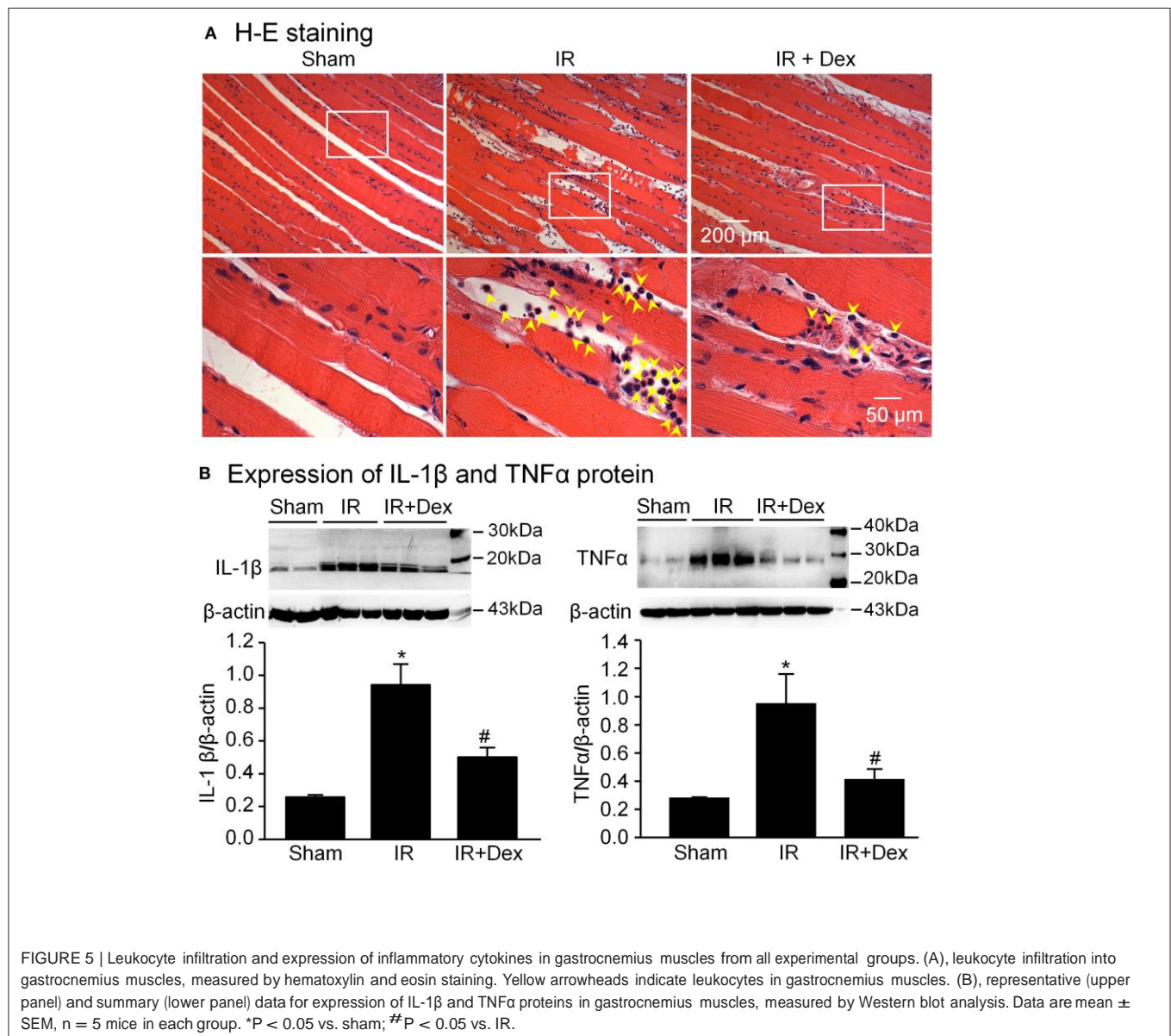


reperfusion, a large number of leukocytes, including neutrophils, lymphocytes, and monocytes were infiltrated into the IR-injured gastrocnemius muscle. Likewise, expression of IL-1 β and TNF α significantly increased in the IR-injured gastrocnemius muscle. When Dex (1 mg/kg) was administered at the beginning of reperfusion, IR-induced leukocyte infiltration and overexpression of IL-1 β and TNF α were markedly decreased ($p < 0.05$ vs. IR group, **Figure 5**).

DISCUSSION

In the present study, we mimicked the clinical tourniquet use to reproduce the critical limb syndrome in a mouse model subjected to 3 h of tourniquet and 24 h of reperfusion. In this mouse model, tourniquet-induced IR caused not

only the morphological and functional damage of skeletal muscles and NMJs, but also the leukocyte infiltration and over-production of inflammatory cytokines in skeletal muscles. Treatment of Dex at the beginning of reperfusion significantly inhibited IR-induced leukocyte infiltration and overexpression of IL-1 β and TNF α in gastrocnemius muscles. This anti-inflammatory drug also blunted acute IR-induced gastrocnemius muscle injuries and ameliorated direct muscle stimulation-evoked muscle contraction in IR-injured gastrocnemius muscles. However, Dex did not avert IR-induced disappearance of motor nerve terminals in NMJs from gastrocnemius muscles, and did not improve sciatic nerve-stimulated EPPs and muscle contraction in IR-injured gastrocnemius muscles. These results suggest that treatment of Dex at the beginning of reperfusion, through inhibiting inflammatory cytokines, alleviate acute IR-induced structural



and functional impairments of gastrocnemius muscles but not NMJs.

There is a long history for tourniquet use in first-line treatment of severe limb hemorrhage. Although limited clinical evidence exists about complications associated with the use of this device due to the impossibility of distinguishing between tourniquet-induced and original hemorrhage-induced complications, concerns about tourniquet-induced nerve palsy, skeletal muscle injury, and amputation have led to the limitation of tourniquet use, suggesting as a last option after other hemorrhage control interventions fail (Clasper et al., 2009; Kue et al., 2015). Therefore, finding effective therapy for tourniquet-induced IR injuries can resolve the limitations of tourniquet use, and significantly improve outcomes and quality of life for this large group of patients.

Glucocorticoids such as Dex are widely used in clinical patients as potent anti-inflammatory drugs (Urbanska et al., 2014; Rodriguez et al., 2017). A previous study reported that the intramuscular administration of Dex 3 h prior to tourniquet successfully attenuated IR-induced muscle contractile dysfunction seen in rat extensor digitorum longus muscles following tourniquet-induced 3 h of ischemia and 24 h of reperfusion (Chen et al., 1996). As everyone knows, however, it is unrealistic to expect clinical pretreatment of drugs for tourniquet-induced IR injuries because extremity hemorrhage and tourniquet use are not predicted. To determine the clinical relevance of drugs, therefore, treatment of drugs at the beginning of reperfusion is an optimal timing for investigation of drugs' effects on tourniquet-induced IR injuries. In the present study, treatment of Dex at the beginning of reperfusion attenuated acute IR-induced gastrocnemius muscle injuries and improved direct muscle stimulation-evoked muscle contraction in IR-injured gastrocnemius muscles. It has been shown that Dex binding to the glucocorticoid receptor inhibits inflammatory cytokines through inhibition of NF- κ B in some inflammatory conditions (Uddin et al., 2011). Our current study found that Dex significantly inhibited IR-induced overexpression of IL-1 β and TNF α in gastrocnemius muscles. Analyzing above results, we thought that treatment of Dex at the beginning of reperfusion could improve the structure and function of skeletal muscles experienced 3 h of tourniquet and 24 h of reperfusion, through inhibition of inflammatory cytokine production. Nevertheless, treatment of Dex did not affect the structure and function of NMJs in IR-injured gastrocnemius muscles. It is possible that other cellular and molecular mechanisms (such as overproduction of reactive oxygen species and dysfunction of the endogenous antioxidative system) are responsible for acute IR-induced morphological and functional

impairments of NMJs in the skeletal muscle, because G93A mutation of superoxide dismutase significantly increases the denervation of motor nerve terminals during skeletal muscle tourniquet-induced IR (David et al., 2007).

Inflammatory cytokines are mainly produced by activated leukocytes, including neutrophils, monocytes, and lymphocytes (Arango and Descoteaux, 2014). In the current study, tourniquet-induced IR caused the leukocyte infiltration into IR-injured gastrocnemius muscles. Treatment of Dex obviously blunted IR-induced leukocyte infiltration, which suggests that Dex decreased expression of IL-1 β and TNF α in IR-injured gastrocnemius muscles, through inhibiting leukocyte activation and infiltration. It is possible that the skeletal muscle itself is also involved in IR-induced overexpression of IL-1 β and TNF α , because the skeletal muscle is thought to be an endocrine organ for muscle-derived cytokine production (Pedersen and Febbraio, 2008; Borge et al., 2009; Arango and Descoteaux, 2014).

Our current study did not identify the contribution of each cytokine (IL-1 β and TNF α) to tourniquet-induced IR injuries. Additionally, clinical use of Dex is hampered by its severe systemic side effects (Curtis et al., 2006; Fardet et al., 2007; Hanaoka et al., 2012). Therefore, the effects of IL-1 receptor antagonist (IL-1RA) and TNF α inhibitors (etanercept, infliximab, adalimumab, etc.) on tourniquet-induced IR injuries will be investigated in future study to discover the optimum clinical therapeutics.

In conclusion, tourniquet-induced IR results in morphological and functional impairments in mouse skeletal muscles and NMJs. Treatment of Dex at the beginning of reperfusion, through inhibiting inflammatory cytokines, protects the skeletal muscle from acute IR injuries, whereas it does not ameliorate the structure and function of NMJs in IR-injured skeletal muscles.

AUTHOR CONTRIBUTIONS

RC, HT, DZ, and Y-LL designed and performed experiments, analyzed data, and prepared the manuscript. RC, AB, RM, MW, and Y-LL wrote, edited, and revised the manuscript. All authors approved the version to be published.

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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Abstract for 2017 Military Health System Research Symposium

Protective effect of dexamethasone against tourniquet-induced acute ischemia-reperfusion injury in mouse hindlimb

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Background: Extremity injuries with hemorrhage have been a significant cause of death in civilian medicine and on the battlefield. The use of a tourniquet as an intervention is necessary for treatment to an injured limb; however, the tourniquet and subsequent release cause serious acute ischemia-reperfusion (IR) injury in the neuromuscular junction (NMJ) and skeletal muscle. Much evidence demonstrates that inflammation is an important factor to cause acute IR injury. Our current study investigated effect of dexamethasone, a synthetic glucocorticoid and anti-inflammatory drug, on tourniquet-induced acute IR injury in mouse hindlimb.

Methods: Unilateral hindlimb of C57/BL6 mice was subjected to 3 hours of ischemia following 24 hours of reperfusion (24-h IR) via placement and release of a tourniquet at the hip joint. The endplate potential and skeletal muscle contractile force were recorded by general electrophysiological technique. The morphology of the NMJ and skeletal muscle in isolated gastrocnemius muscles was measured using immunofluorescent staining. Cytokines were measured by western blot analysis.

Results: Three hours of tourniquet and 24 hours of release caused gastrocnemius muscle injuries including rupture of the muscle sarcolemma and necrosis ($42.8 \pm 2.3\%$ for infarct size of the gastrocnemius muscle). In the NMJ, motor nerve terminals disappeared, and endplate potentials were undetectable in 24-h IR mice. There was no contraction of the gastrocnemius muscle triggered by sciatic nerve stimulation or direct muscle stimulation in 24-h IR mice. Western blot data showed that TNF α and IL-1 β were increased in the gastrocnemius muscle after 24-h IR. Treatment with dexamethasone at the beginning of reperfusion (1 mg/kg/day, i.p.) significantly inhibited expression of TNF α and IL-1 β , reduced rupture of the muscle sarcolemma and infarct size ($24.8 \pm 2.0\%$), and improved direct muscle stimulation-induced gastrocnemius muscle contraction in 24-h IR mice. However, this anti-inflammatory drug did not improve the morphology and function of the NMJ and sciatic nerve-stimulated gastrocnemius muscle contraction in 24-h IR mice.

Conclusion: Tourniquet-related IR induced serious acute IR injuries in the NMJ and skeletal muscle. Dexamethasone only reduced the structural and functional impairment of the skeletal muscle but not the NMJ through inhibiting proinflammatory cytokines.

Abstract for 2018 Military Health System Research Symposium

Liposomal dexamethasone improves outcomes of post-traumatic skeletal muscle and neuromuscular junction

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Background: Tourniquet-induced ischemia and subsequent release cause serious ischemia-reperfusion (IR) injury in the skeletal muscle although the tourniquet is an effective tool of arresting life-threatening limb hemorrhage in pre-hospital setting. To find effective therapeutic interventions for tourniquet-induced IR injuries, we observed the effect of liposomal dexamethasone (lipo-Dex) in tourniquet-Induced IR injuries in mouse hindlimb.

Methods and Results: Unilateral hindlimb of C57/BL6 mice was subjected to 3 hours of ischemia by placing a rubber band and followed different periods of reperfusion (1 week, 2 weeks, 4 weeks, and 6 weeks). Before tourniquet was released, lipo-Dex (7 mg/kg, equivalent to 1 mg/kg/day of Dex for 1 week) was administered via tail vein injection. After 3 hours of ischemia and different periods of reperfusion, gastrocnemius muscle showed significantly morphological and functional damages. TNF α and IL-1 β were overexpressed in the gastrocnemius muscle after 1, 2, 4, and 6 weeks of tourniquet-induced IR. Treatment with Lipo-Dex inhibited the elevation of TNF α and IL-1 β , improved structure of the muscle sarcolemma, reduced fragmentation of nicotinic acetylcholine receptor clusters, and promoted recovery of gastrocnemius muscle contraction.

Conclusion: The data suggest that tourniquet-related IR induces serious skeletal muscle inflammation and degradation. Treatment with lipo-Dex suppresses IR-induced inflammation and promotes recovery of skeletal muscle morphology and function from tourniquet-induced IR injury.

Abstract for 2019 Military Health System Research Symposium

Dexamethasone improves neuromuscular junction recovery from ischemia-reperfusion injury induced by tourniquet application in mouse hindlimb

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Abstract

Background: Tourniquet-induced ischemia-reperfusion (IR) injuries impede long-term recovery of skeletal muscle contraction. The neuromuscular junction (NMJ) is an important factor to influence skeletal muscle contractile function. Here, we observed the effect of dexamethasone (Dex) on morphological and functional recovery of the NMJ from tourniquet-induced IR injuries in mouse hindlimb.

Methods and Results: Unilateral hindlimb of C57/BL6 mice were subjected to 3 hours of ischemia by placing a rubber band and followed different periods of reperfusion (1 week, 2 weeks, 4 weeks, and 6 weeks). Dex treatment (1 mg/kg/day, i.p.) began on the day of IR induction and lasted for 7 days. After 3 hours of ischemia and different periods of reperfusion, NMJs in the gastrocnemius muscle showed significantly the morphological and functional damage. In the gastrocnemius muscle, TNF α and IL-1 β (two inflammatory cytokines) were increased after 1, 2 and 6 weeks of tourniquet-induced IR. Treatment with Dex inhibited the elevation of TNF α and IL-1 β , mitigated fragmentation of nicotinic acetylcholine receptor (nAChR) clusters, promoted re-innervation of nAChR clusters, and increased nerve-stimulated end plate potentials (EPPs).

Conclusion: The data suggested that tourniquet-related IR induced serious skeletal muscle inflammation for a long period. Dex treatment for 1 week inhibits IR-caused inflammation in the early period of IR, and improves the morphological and functional recovery of the NMJ from tourniquet-induced IR injuries.

Abstract for 2017 Experimental Biology conference

Morphological regeneration and functional recovery of neuromuscular junction after ischemic/reperfusion injury induced by tourniquet in mouse hind limb

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Background: Tourniquet-induced ischemia and subsequent reperfusion cause serious acute ischemia-reperfusion (IR) injury in the neuromuscular junction (NMJ) and skeletal muscle. These injuries are associated with permanent neurological deficiency, and long-term skeletal muscle contractile dysfunction, even complete limb paralysis. Monitoring structural and functional repair of the NMJ and skeletal muscle after tourniquet-induced IR is beneficial to exploring the mechanisms responsible for tourniquet-induced IR injury and finding effective therapeutic interventions. Here, we observed long-term morphological and functional alterations of the NMJ in tourniquet-induced hindlimb IR.

Method: Unilateral hindlimb of C57/BL6 mice was subjected to 3 hours of ischemia by placing an orthodontic rubber band and followed different periods of reperfusion (1 day, 3 days, 1 weeks, 2 weeks, 4 weeks, and 6 weeks). At each time point of reperfusion, the gastrocnemius muscle was isolated for measurement of the NMJ morphology and function. Motor nerve terminals and nicotinic acetylcholine receptors (nAChRs) in the NMJ were measured by immunofluorescence staining. The end plate potential (EPP) was recorded by the intracellular recording technique.

Results: In the NMJ of sham mice, the structure of the nAChR clusters remained intact pretzel-like shape and motor nerve terminals innervated the nAChR clusters to form synapses for signal transmission. The number of fragments in one nAChR cluster was 1.83 ± 0.07 and all nAChR clusters were innervated by motor nerve terminals (100%). The amplitude of EPPs was 29.84 ± 1.02 mV. At 1 day and 3 days of reperfusion, the motor nerve terminals disappeared, the AChR clusters kept normal shape, and no EPPs were recorded. From 1 week to 6 weeks of reperfusion, the motor nerve terminals gradually reinnervated (46.88%, 88.05%, 99.2%, and 100% for 1, 2, 4, and 6 weeks of reperfusion, respectively). However, the AChR clusters were gradually fragmented and the number of fragments in one nAChR cluster increased with longer period of reperfusion (2.92 ± 0.14 , 5.50 ± 0.30 , 8.94 ± 0.67 , and 6.88 ± 0.50 for 1, 2, 4, and 6 weeks of reperfusion, respectively; $p < 0.05$ vs. sham). The amplitude of EPPs also increased gradually with longer period of reperfusion, but the amplitude of EPPs at 6 weeks of reperfusion didn't restore towards the level seen in sham mice (13.92 ± 1.11 mV, $p < 0.05$ vs. sham).

Conclusion: Tourniquet-induced ischemia and subsequent reperfusion impaired the NMJ. The morphological change of motor nerve terminals was faster than that of the nAChR clusters. The slow recovery of fragmented nAChR clusters could attenuate neuromuscular transmission.