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# Influence of ischemic injury on post-tetanic depression of contractional force of rat gastrocnemius muscle

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*Ischemia-reperfusion injury of skeletal muscles is a significant clinical problem caused by trauma, peripheral artery disease, or prolonged immobility, such as during surgical procedures. The present study aimed to examine the effect of 3 h of ischemia on post-tetanic modulation of gastrocnemius muscle contraction force in rats. Ischemia was induced by surgical clamping of the femoral artery. Muscle contractile activity was evaluated in situ using a sciatic nerve stimulation protocol that included single twitches before and after a 5-second tetanic stimulation (40 Hz). A linear mixed model (LMM) was employed to analyze changes in the ratio of the second twitch amplitude to the first (P2/P1) across 10 measurements. The LMM analysis revealed a statistically significant difference in the slope of change of the P2/P1 ratio between the groups. The estimated slope for the experimental group was -0.11 rel. un. per measurement, demonstrating a significantly faster decrease in the indicator than in the control group (-0.042). Our findings suggest that three hours of ischemia cause a substantial enhancement of post-tetanic depression and a concurrent suppression of post-tetanic potentiation mechanisms in the rat gastrocnemius muscle. These results indicate a notable impairment of excitation-contraction mechanisms and an elevated susceptibility of ischemic muscle to fatigue.*

*Key words: ischemia; skeletal muscle; calf muscle; fatigue; post-tetanic potentiation; post-tetanic depression; contractile force; rat; linear mixed model.*

## INTRODUCTION

Ischemic-reperfusion (I/R) injury of skeletal muscles constitutes a significant clinical problem arising from traumatic injuries, reconstructive vascular surgery, tissue transplantation, and pathologies such as peripheral artery disease [1]. Accordingly, the development of new therapeutic strategies to minimize the consequences of such injuries remains a key research priority, including both regenerative medicine, such as stem cell therapy [2], and the use of new pharmacological agents, particularly water-soluble $C_{60}$ fullerenes.

The pathophysiology of I/R injury is biphasic. The initial ischemic phase is marked by restricted blood flow, leading to hypoxia and depletion of energy substrates. Because mitochondria play a central role in muscle bioenergetics, they are especially susceptible to ischemic insults, resulting in metabolic dysfunction that underlies much of the ensuing cellular damage [3]. This dysfunction disrupts mitochondrial metabolism and disturbs ionic balance. The subsequent reperfusion phase, while essential for restoring blood supply, paradoxically exacerbates damage due to the rapid generation of reactive oxygen species (ROS), activation of inflammatory cascades, and further damage to cellular structures [4].

The functional state of skeletal muscle during repeated activity is determined by the dynamic balance between fatigue processes and short-term plasticity phenomena, particularly post-tetanic depression (PTD) and post-tetanic potentiation (PTP) [5, 6]. Fatigue is defined as a temporary, activity-induced reduction in the ability of a muscle to generate force [7]. In contrast, PTP refers to a transient enhancement of the contractile response following brief, high-frequency stimulation. The primary

mechanism underlying PTP is thought to involve phosphorylation of the myosin regulatory light chains by $Ca^{2+}$/calmodulin-dependent kinase (MLCK), which increases the $Ca^{2+}$ sensitivity of myofibrils. PTD can be regarded as a manifestation of fatigue that occurs during tetanic contraction. Accordingly, the ratio of the amplitudes of single contractions before (P1) and after (P2) tetanus (P2/P1) serves as a sensitive, integrative indicator reflecting the combined effect of these two opposing processes [6].

Ischemia has been shown to create an unfavorable intracellular environment that simultaneously suppresses the mechanisms underlying PTP and enhances factors contributing to fatigue. Understanding these processes is essential for the development and testing of new therapeutic strategies. Reliable electrophysiological methods, such as those employed in the present study, provide an objective, instrumental means of assessing tissue damage and complement other approaches, including the analysis of evoked potentials used to investigate nociception [8]. Disruption of fundamental muscle properties, such as PTP, has profound consequences for motor control, impairing the stability and efficiency of movements that depend on complex phenomena, such as the coactivation of antagonist muscles [9].

This study assessed the effect of three-hour acute ischemia on the balance between post-tetanic potentiation and depression in the rat gastrocnemius muscle using a repeated stimulation protocol.

## METHODS

All experimental procedures involving rats were conducted in accordance with international ethical standards, in particular the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986), and Article 26 of the Law of Ukraine No. 3447-IV on the protection of animals from cruel treatment (February 21, 2006). The study was conducted in accordance with the bioethical and biological safety standards implemented by the Bogomoletz Institute of Physiology of the NAS of Ukraine (according to Protocol No. 4/18 dated December 27, 2018). The Biomedical Ethics Committee authorized the execution of these experiments on February 29, 2024 (Protocol No. 1/24).

Experiments were performed on adult male Wistar rats (Rattus norvegicus) weighing 250-270 g. The rats were divided into two groups of 11 each: a control group and an experimental group. Prior to the experiment, all animals were subjected to 24 h of food deprivation and 4 h of water deprivation.

Anesthesia was induced via intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The depth of anesthesia was carefully monitored by assessing heart rate and pupillary response to light. After achieving surgical anesthesia, each rat was positioned in a stereotaxic frame. Animals in the experimental group underwent surgical induction of ischemia in the right hind limb by applying a non-stretchable ligature to the femoral artery proximal to its bifurcation (Fig. 1A) for a duration of 3 h. Control animals underwent the same anesthetic and positioning procedures without surgical intervention.

To assess the contractile function of the gastrocnemius muscle, the sciatic nerve (*nervus ischiadicus*) was electrically stimulated. The surgical procedure involved isolating the nerve, clearing adjacent tissues, and establishing contact with a servo-controlled electrical stimulator (Fig. 1B). To prevent nerve dehydration, the stimulation site was wrapped in surgical absorbent material and continuously moistened with mineral oil. The distal tendon of the muscle was surgically excised and then connected to a force sensor to record isometric contractions (Fig. 1C), while the limb was immobilized to prevent movement.

The sciatic nerve stimulation sequence was generated using Spike2 software on a personal computer. The signal was transmitted through a

A B C

Fig. 1. Key steps in preparing a rat for tensometric examination: A - a ligature applied to the femoral artery; B - isolation of the gastrocnemius muscle and sciatic nerve; C - attachment to the tensometric recording system

digital-to-analog converter to a Master-8 AMPI pulse isolator (Israel) and then to a DS2A stimulator ("Digimeter Ltd", USA), with electrodes in direct contact with the prepared nerve. The programmable protocol delivered signals in a predefined sequence: an initial single pulse (P1, 2 ms), a 500-ms delay, a tetanic train of 2-ms pulses at 40 Hz for 3000 ms, followed by a 500-ms delay and a final single pulse (P2, 2 ms). The stimulation current was set at 1.5 times the threshold for muscle contraction. This sequence was repeated ten times at 10-second intervals.

The mechanograms obtained (Fig. 2) were subjected to quantitative analysis. The P1 and P2 amplitudes were defined as the maximum signal values within their respective time windows relative to the baseline. Within each series of 10 mechanograms, raw force data were normalized to the series' global maximum and minimum. To standardize the data and assess the dynamics of post-tetanic modulation, P2/P1 was calculated for each mechanogram and used as the dependent variable in subsequent statistical analysis.

Statistical analysis was conducted using R (R Core Team, 2024) for advanced modeling and OriginPro 2021 for data handling and visualization. Due to repeated measurements (10 mechanograms per animal), a Linear Mixed Model (LMM) was used for analysis [10]. LMMs are well-suited for hierarchical data, as they simultaneously account for fixed effects (experimental conditions) and random

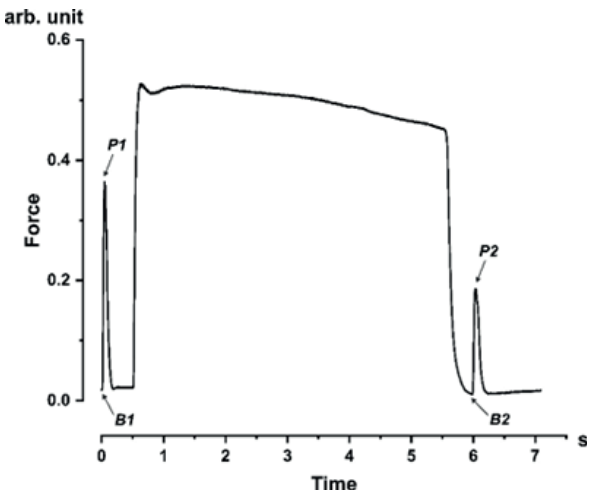


Fig. 2. Representative mechanogram of the gastrocnemius muscle contractile response to sciatic nerve stimulation. P1 indicates the single twitch before tetanic stimulation (40 Hz), with B1 as the corresponding baseline. P2 indicates the single twitch following tetanic stimulation, with B2 as the post-stimulation baseline

effects (individual differences), providing more accurate estimates than traditional methods [11]. In our LMM model, P2/P1 was set as the dependent variable. Fixed effects included Group ("Control" vs. "Ischemia") and Mechanogram number (0-9, representing the measurement sequence), with their interaction term ("Group * Mechanogram number") used to assess whether the rate of change in P2/P1 differed between groups. Sample ID was included as a random intercept to account for individual baseline differences. Statistical significance was set as $P < 0.05$.

## RESULTS

Analysis of the dynamics of P2/P1 using a Linear Mixed Model. The dynamics of P2/P1 were analyzed using a LMM to quantitatively assess the effects of three-hour acute ischemia and to identify differences between the control and ischemic groups over time. Key findings from the fixed effects analysis are presented in Table.

The LMM analysis revealed a statistically significant ($p < 0.001$) negative effect of "Mechanogram number", indicating a progressive decline in P2/P1 over time in both groups. At the start of the experiment, there was no significant difference ($P = 0.236$) between the experimental ("Group: Ischemia") and control groups, confirming the groups comparability. The model's key finding was a significant negative interaction between "Mechanogram number" and "Group: Ischemia" (estimate = -0.067, $P < 0.001$), demonstrating that the rate of decline in P2/P1 differed markedly between groups. The trend slope for the control group was -0.042, while it was significantly steeper in the ischemia group at -0.11 (sum of -0.042 and -0.067). These results provide strong evidence that ischemia amplifies the development of post-tetanic depression. Fig. 3 illustrates the distinct trajectories of P2/P1 in both groups over time.

## DISCUSSION

The primary outcome of this study is the quantitative demonstration that 3 h of acute ischemia markedly increases skeletal muscle susceptibility to fatigue. This is reflected by

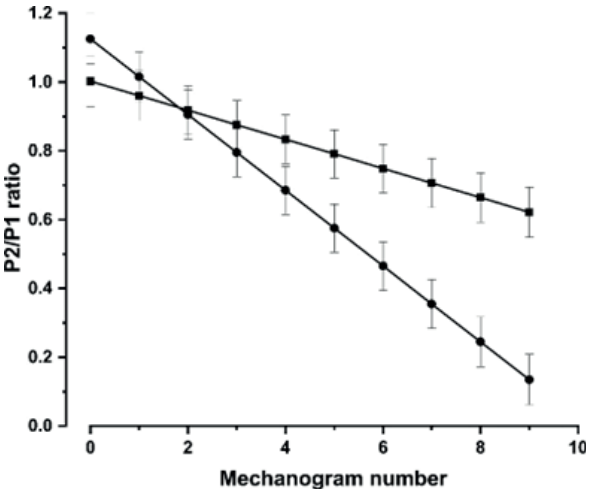


Fig. 3. Dynamics of the P2/P1 ratio in control and experimental groups. The estimated marginal means from the Linear Mixed Model over 10 consecutive measurements are shown. Black squares represent the control group, and black circles represent the experimental (ischemia) group. Error bars indicate the standard error of the mean. The steeper decline in the experimental group reflects enhanced post-tetanic depression and greater susceptibility to fatigue

**Estimation of fixed effects of the Linear Mixed Model for P2/P1**

| Effect | Estimate | Standard error | t-value | P-value | 95% CI Lower | 95% CI Upper |
|---|---|---|---|---|---|---|
| Intercept | 1.002 | 0.07321 | 13.695 | < 0.001 | 0.858 | 1.146 |
| Mechanogram number | -0.042 | 0.00448 | -9.457 | < 0.001 | -0.051 | -0.033 |
| Group: Ischemia | 0.122 | 0.10353 | 1.186 | 0.236 | -0.081 | 0.327 |
| Mechanogram number * Group: Ischemia | -0.067 | 0.00633 | -10.704 | < 0.001 | -0.08 | -0.055 |

a pronounced decline in the relative strength of individual contractions following tetanic stimulation. The LMM analysis showed that the rate of P2/P1 decline in ischemic muscle was more than twice that of the control group, indicating a substantial imbalance between post-tetanic potentiation and fatigue, with potentiation being less pronounced.

Transient changes in muscle contractility following exercise arise from a complex interplay between two opposing processes. High-frequency activation triggers post-tetanic potentiation (PTP), historically, a subject of extensive research, with its complex underlying mechanisms gradually uncovered [12]. Conversely, the same level of intense activity can induce fatigue processes [7]. In the context of acute ischemia, a rapid depletion of ATP reserves occurs [13], which limits MLCK activity and disrupts a key step in PTP, as demonstrated in mechanokinetic analyses of post-ischemic muscles [14]. The phosphorylation state of myosin regulatory light chains, essential for PTP, is dynamically regulated by the balance between MLCK and phosphatase activities [15]. Previous studies have also documented complex alterations in contractile dynamics in ischemic rat soleus muscle [16]. Furthermore, ischemia profoundly disrupts $Ca^{2+}$ homeostasis, including reduced calcium transient amplitude due to sarcoplasmic reticulum (SR) dysfunction, which is associated with impaired DHP receptor activity [6, 17].

Concurrently with the suppression of PTP, ischemia has been shown to significantly enhance key factors contributing to peripheral fatigue. One of the primary mediators of fatigue is the accumulation of inorganic phosphate (Pi), formed during the hydrolysis of ATP and phosphocreatine [18]. Elevated Pi concentrations (up to 30 mmol/l) directly inhibit the force-generating states of actomyosin cross-bridges and impair $Ca^{2+}$ release from the SR [19]. The cyclical interaction of these cross-bridges is the fundamental molecular basis of muscle force generation [20]. Another important contributor is intracellular acidosis, which reduces the sensitivity of myofilaments to $Ca^{2+}$ [6]. Finally, ATP depletion disrupts the functioning of ion pumps, leading to membrane depolarization and a vicious cycle of excitation-contraction dysfunction [4].

The reperfusion phase was not examined in this study; however, it is well established that a three-hour period of ischemia is sufficient to initiate oxidative stress-related cell damage [7, 21]. This provides a logical link to studies demonstrating the protective effects of antioxidants [22]. In particular, $C_{60}$ fullerene nanoparticles, potent scavengers of reactive oxygen species, have been shown to attenuate the reduction in muscle contractile force under inflammatory conditions, supporting the hypothesis that oxidative stress is a key mediator of functional decline [23]. Thus, the model we propose can serve as a sensitive functional platform for testing the efficacy of new myoprotective compounds.

The findings are also relevant to the broader field of motor control physiology. Disruption of fundamental muscle properties, such as PTP, has been shown to impair the central nervous system's ability to precisely regulate muscle force and movement stability, particularly through coactivation mechanisms [9]. Functionally, PTP enhances neuromuscular efficiency, a factor that is significantly compromised under ischemic conditions [24]. The pronounced functional deficits observed in this study highlight the complexity of muscle recovery following ischemic injury and emphasize the need for novel approaches, such as stem cell therapy [2]. The application of objective quantitative methods provides a powerful tool for assessing the extent of damage and the efficacy of therapeutic interventions [8]. Furthermore, the selection of appropriate experimental models and evaluation methods is a crucial aspect in preclinical studies of traumatic muscle injuries [25].

*The authors of this study confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of co-authors of the article.*

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## ВПЛИВ ІШЕМІЧНОЇ ТРАВМИ НА ПОСТТЕТАНІЧНУ ДЕПРЕСІЮ СИЛИ СКОРОЧЕННЯ ЛИТКОВОГО М'ЯЗА ЩУРА

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Ішемічно-реперфузійне пошкодження скелетних м'язів виникає внаслідок травм, захворювань периферичних артерій або тривалої нерухомості, зокрема під час хірургічних втручань. Метою нашого дослідження було вивчення впливу тригодинної ішемії на динаміку посттетанічної модуляції сили скорочення литкового м'яза задньої кінцівки щурів. Ішемію викликали хірургічним перетисканням стегнової артерії. Скоротливу активність м›язів оцінювали *in situ*, використовуючи протокол стимуляції сідничного нерва, який включав поодинокі скорочення до і після трисекундної тетанічної стимуляції (40 Гц). Для цього застосовували лінійну змішану модель (LMM) для аналізу змін відношення амплітуди другого поодинокого скорочення до першого (P2/P1) протягом 10 вимірювань. LMM-аналіз виявив статистично значущу взаємодію дослідної групи з часом стимуляції, що вказує на різну швидкість зміни співвідношення P2/P1 між групами. Розрахунковий нахил тренду для дослідної групи становив -0,11 ум. од. на вимірювання, демонструючи значно швидше зниження показника, ніж у контрольній групі (-0,042). Таким чином, тригодинна ішемія призводить до суттєвого посилення посттетанічної депресії та одночасного пригнічення механізмів посттетанічної потенціації у литковому м›язі щура. Ці зміни вказують на значне порушення механізмів збудження-скорочення та підвищену чутливість ішемізованого м'яза до втоми.

Ключові слова: ішемія; скелетний м›яз; литковий м›яз; втома; посттетанічна потенціація; посттетанічна депресія; сила скорочення; щур; лінійна змішана модель.

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