Original Research Report

EFFECT OF L-CITRULLINE ON CREATINE KINASE MM (CK-MM) ISOENZYMES IN MICE: AN IN VIVO STUDY FOCUSING ON IMMUNOHISTOCHEMISTRY ANALYSIS

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ABSTRACT

Eccentric exercise often induces muscle injuries in athletes, resulting in impaired performance and prolonged recovery time. Creatine kinase MM (CK-MM) is a biomarker for assessing muscle damage, with elevated levels indicating injury. L-citrulline, an amino acid, has shown promise in enhancing performance and reducing recovery time. However, its specific effect on CK-MM remains unclear. This study utilized immunohistochemistry analysis to investigate the effect of L-citrulline supplementation on CK-MM expression post-eccentric exercise in male BALB/c mice. This in vivo study was conducted with a post-test-only design. A total of 25 mice were divided into two control groups (normal/C1 and negative/C2) and three treatment groups (T1, T2, and T3), each containing five mice. The T1, T2, and T3 groups were daily administered 250, 500, and 1,000 mg/kg bw of L-citrulline for seven days, respectively. All mice, except the C1 group, performed a downhill running procedure. The CK-MM expression in skeletal muscle tissue post-eccentric exercise was assessed using immunohistochemistry analysis. The statistical analysis included the Shapiro-Wilk test for data distribution and the Kruskal-Wallis and Mann-Whitney post-hoc tests for significant differences ($p<0.05$). The results showed that CK-MM expression in the C2 group (91.00±2.24%) was significantly higher ($p=0.008$) than that of the C1 group (70.00±10.0%). Subsequently, the T1 (68.00±9.08%, $p=0.008$), T2 (72.00±7.58%, $p=0.008$), and T3 (67.00±9.75%, $p=0.008$) groups exhibited significantly lower expressions than the C2 group. These results were consistent with the role of CK-MM as a marker for muscle damage, and they indicated that L-citrulline might have a protective effect against muscle damage post-eccentric exercise. However, no significant differences were observed among the C1, T1, T2, and T3 groups. In conclusion, L-citrulline supplementation demonstrates promise in attenuating muscle damage following eccentric exercise, as evidenced by reduced CK-MM expression levels. These findings highlight the potential therapeutic role of L-citrulline in enhancing muscle recovery and performance.

Keywords: Creatine kinase MM (CK-MM); exercise; healthy lifestyle; L-citrulline; muscle damage

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Highlights:
1. This study elucidates the effect of L-citrulline supplementation on creatine kinase MM (CK-MM) expression post-eccentric exercise, offering novel insights into its potential role in mitigating muscle damage.
2. The findings demonstrate that L-citrulline administration significantly reduces CK-MM expression levels in skeletal muscle tissue, suggesting its therapeutic potential in enhancing muscle recovery and performance following exercise-induced damage.
3. This study contributes valuable insights into the multifaceted benefits of L-citrulline supplementation for supporting overall muscle health and performance by identifying its protective effects under different mechanisms, including improved blood flow, antioxidant activity, enhanced mitochondrial function, and promotion of muscle protein synthesis.

INTRODUCTION

Muscle injuries, which can vary from contusions to strains or lacerations, are the most prevalent cause of physical disability in sports. This prevalence represents 41% of the total number of injuries (Edouard et al. 2016, SantAnna et al. 2022). Strains, the most common etiology of muscle injury, may arise when muscles fail to withstand the stretching imposed by the forces applied during exercise, particularly while performing eccentric contractions. After the contracting muscle is forcibly lengthened, the myofibril is impaired, resulting in hematoma and myonecrosis (Dueweke et al. 2017, SantAnna et al. 2022). Several biomarkers can be used for measuring muscle viability following an injury. A prior study utilized creatinine kinase (CK) to observe the incidence of myalgia (Adhiatma et al. 2022). It has been found that CK levels increase after the incidence of muscle damage. This indicates a potential association between CK levels and the presence of underlying necrosis or muscle degeneration after an injury (Nagaraju et al. 2017, Schultze et al. 2022).

Creatinine kinase MM (CK-MM), an isoenzyme of creatinine kinase, is primarily found in the skeletal muscle. An increase in CK-MM levels is a common occurrence following a skeletal injury. This suggests that CK-MM levels play a role in the evaluation of muscle viability (Kumar et al. 2019, Shehata et al. 2022). Muscle injury results in a significant burden on athletes' performance. Athletes who suffer from muscle injury may face an extensive loss of time, primarily due to the prolonged muscle recovery time (Raysmith & Drew 2016). Nowadays, many nutraceuticals have been observed for their potential health benefits. Among these nutraceuticals, L-citrulline has emerged as a promising candidate for enhancing athletes' performance and curtailing the recovery period (Ishak et al. 2017, Gough et al. 2021).

L-citrulline, predominantly found in watermelon, is a non-essential amino acid that plays a role in augmenting L-arginine bioavailability. L-citrulline supplementation has been found to improve muscle repair and performance by elevating blood flow and reducing muscle soreness (Gonzalez & Trexler 2020). Previous research conducted by Rhim et al. (2020) concluded that a single dose (8 g) of citrulline malate supplementation two hours before a workout could significantly reduce the rating of perceived exertion and muscle soreness post-exercise. Subsequently, a six-day consumption of 6 g of L-citrulline significantly improved exercise tolerance and VO_2 levels by upregulating nitric oxide (NO) synthesis (Bailey et al. 2015). A prior study by Valaei et al. (2022) also indicated that 12 g of L-citrulline supplementation pre-exercise significantly improved antioxidant markers after high-intensity exercise. However, trained soccer players demonstrated different results following L-citrulline supplementation. It was shown that administering 6 g of L-citrulline during a short-term pre-exercise did not improve oxidative stress and muscle damage markers (Mirenayat et al. 2024).

Despite the growing body of evidence supporting the ergogenic effects of L-citrulline supplementation, the specific mechanisms underlying its impact on skeletal muscle impairment remain poorly understood, particularly its effect on CK-MM. Therefore, this study aimed to elucidate the effect of L-citrulline supplementation on CK-MM expression levels in BALB/c mice, utilizing immunohistochemistry (IHC) analysis as the primary investigative tool.

MATERIALS AND METHODS

This in vivo study was conducted using a post-test-only design. The experiment was performed in the Anatomical Pathology Laboratory, Universitas Sebelas Maret, Surakarta, Indonesia, from October 2022 to January 2023. The study involved 8-week-old male BALB/c mice of the *Mus musculus* species. Animals with deformity, injury, or inflammation in their forelimbs or hindlimbs were excluded. Twenty-five mice were randomly allocated into two control groups (normal/C1 and negative/C2) and three treatment groups (T1, T2, and T3). Each group consisted of five animals. The randomization process was conducted by a laboratory assistant, and the authors were blinded to the allocation of each group (Lim & In 2019). This study received ethical approval from the Health Research Ethics Committee of the Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia, under protocol No. 01/02/09/2022/117 dated 10/10/2022.

Before treatment, the mice were acclimatized for one week following the same approach used in our prior work (Ghozali et al. 2023). After acclimatization, mice in the C2 group were given tap water. Meanwhile, mice in the T1, T2, and T3 groups were given 250, 500, and 1,000 mg/kg bw of L-citrulline (C7629; Sigma-Aldrich, St. Louis, MO, USA) every day for seven days, respectively. L-citrulline was administered orally by gavage to ensure an accurate dose. All mice except for the C1 group engaged in downhill exercises on the seventh day, four hours after absorption, following the protocol outlined in the study conducted by Purwanto et al. (2016). The mice were given five minutes to adapt to the modified Columbus treadmill (Columbus Instruments, Columbus, OH, USA) before starting downhill running. The running was carried out at an average speed of 30 cm/s for 18

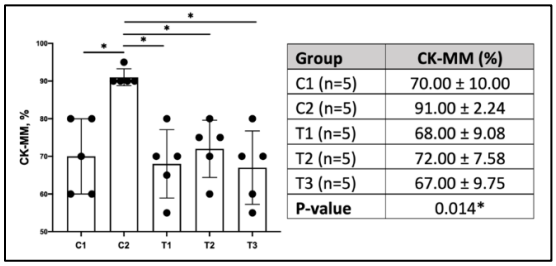
minutes, with a consistent running frequency, on a -15° inclination. At the completion of the experiment, all of the animals were euthanized by inducing carbon dioxyde (CO_2) asphyxiation in accordance with the American Veterinary Medical Association (AVMA) recommendations.

The mice were anesthetized using 5 mg/25 g bw of ketamine via intraperitoneal injection after four hours of exercise. After that, the left gastrocnemius muscle of the mice was dissected by cutting the tendons at both the origin and insertion points using scissors, scalpels, and tweezers. For the immunohistochemical staining, the left gastrocnemius muscle was preserved in 10% buffered formalin (Fisher Chemical, Hampton, NH, USA). The CK-MM expression was assessed through immunohistochemical staining using 120 µL of CKM Polyclonal Antibody (Elabscience Biotechnology Co., USA) in accordance with the manufacturer's guidelines. Subsequently, the tissue sections were mounted and covered with a coverslip for examination. An anatomical pathology expert performed a blinded reading using an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan) at 20 times magnification. The investigation results were classified as positive or negative depending on the staining outcomes of the muscle cells (myocytes). Positive results were indicated by brown staining, while negative results were shown by blue staining. The percentage of cells exhibiting positive staining was calculated by dividing the number of positive cells by the total number of muscle cells (Kim et al. 2016).

Univariate analysis was employed for the statistical analysis to calculate the mean and standard deviation of the continuous variables as well as the frequency and percentage of the dichotomous variables. The Shapiro-Wilk test was used to assess the distribution of the data. The Kruskal-Wallis and Mann-Whitney post-hoc tests were utilized to examine the differences among the study groups. All statistical tests were performed using a two-sided approach, with statistical significance defined as a $p<0.05$ (Ludbrook 2013). The statistical analyses were conducted using IBM SPSS Statistics for Windows, version 27.0 (IBM Corp., Armonk, N.Y., USA).

RESULTS

Twenty-five male BALB/c mice enrolled in this study were assigned to five groups, each consisting of five animals. One of the 25 mice, experienced adverse events that warranted their exclusion from the analysis. Figure 1 shows the creatine kinase MM (CK-MM) expressions and the differences among each group. The data were represented as mean ± standard deviation (SD) and individual values. The lines in the figure indicate significant differences among the groups (*p<0.05).



Group	CK-MM (%)
C1 (n=5)	70.00 ± 10.00
C2 (n=5)	91.00 ± 2.24
T1 (n=5)	68.00 ± 9.08
T2 (n=5)	72.00 ± 7.58
T3 (n=5)	67.00 ± 9.75
P-value	0.014*

Figure 1. Effects of L-citrulline supplementation at doses of 250, 500, and 1,000 mg/kg bw on CK-MM expression levels post-eccentric exercise. The lines indicate significant differences between groups (*p<0.05).

Figure 2 displays the immunochemistry results for CK-MM expressions in the five groups. The immunohistochemistry showed that the CK-MM expressions were 60–80% in the C1 group, 90–95% in the C2 group, 55–80% in the T1 group, 60–80% in the T2 group, and 55–80% in the T3 group. Furthermore, the immunohistochemical staining revealed the presence of positive CK-MM expressions in the cytoplasm of skeletal muscle cells, as indicated by the yellow arrows in the figure. In contrast, the expressions were negative in other areas, as represented by the red arrows.

The post-eccentric exercise examination showed that the C2 group had a significantly higher expression of CK-MM (91.00±2.24%) in the muscle tissues compared to the C1 group (70.00±10.0%), with p=0.008 indicating a statistically significant difference between the two groups.

This finding indicated a consistent pattern that aligned with the recognized role of CK-MM as a marker for muscle damage. The CK-MM expression in the C2 group was also significantly higher than that of the T1 (68.00±9.08%, p=0.008), T2 (72.00±7.58%, p=0.008), and T3 (67.00±9.75%, p=0.008) groups. These expressions provided evidence of the potential protective effect of L-citrulline against muscle damage post-eccentric exercise. Nevertheless, no significant differences were found among the C1, T1, T2, and T3 groups.

DISCUSSION

Effect of eccentric exercise on creatine kinase MM (CK-MM)

Eccentric exercise occurs when the force applied to the muscle exceeds its momentary force, causing the muscles and tendons to lengthen while contracting.

It is a type of muscle contraction that is beneficial for enhancing strength and muscle development. However, eccentric exercise can also carry the risk of muscle injury, particularly if the intensity or volume is excessive (Hody et al. 2019, Harris-Love et al. 2021).

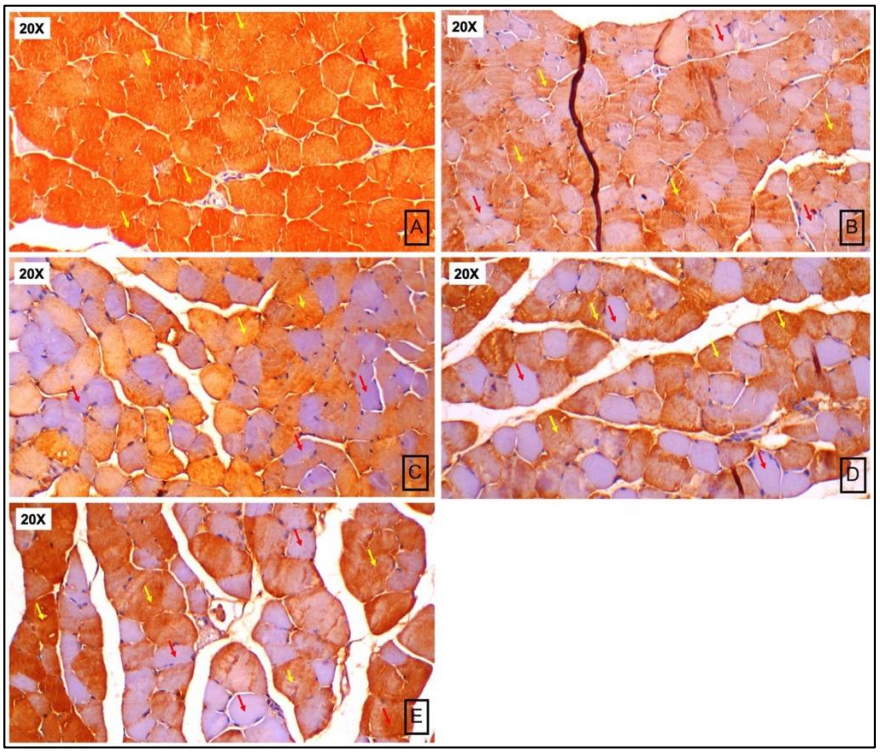


Figure 2. Immunohistochemistry results for CK-MM expressions in the gastrocnemius muscle tissue. Yellow arrows indicate positive CK-MM expressions, while red arrows indicate negative expressions.

Muscle damage following eccentric exercise can result from various mechanisms, primarily involving the disruption of sarcomeres, which are the basic units of muscle contraction. During an eccentric contraction, the muscle fibres lengthen under tension, potentially damaging the actin and myosin filaments within the sarcomeres (Jeon et al. 2022). Such damage may lead to the release of muscle proteins into the bloodstream, including creatine kinase (CK). This enzyme is commonly used as a biomarker to indicate muscle damage.

According to Gao & McNally (2015), myofibrils are attached to the membrane through the dystrophin complex. Excessive stretching of the sarcomere can disrupt the anchoring structures of myofibrils. Subsequently, this has the potential to cause harm to the membrane (Owens et al. 2019). During physical exercise, the cell membrane (sarcolemma) may become disrupted, leading to the leakage of intracellular proteins into the extracellular area. Additionally, eccentric exercise can cause damage to the cytoskeleton, a network of proteins that provide structural support to cells. This damage can lead to increased membrane permeability and the release of intracellular proteins into the bloodstream (Stožer et al. 2020).

Several muscle proteins, such as myoglobin, lactate dehydrogenase, myosin heavy chain, skeletal troponin I, α-actin, fatty acid-binding protein, aspartate aminotransferase, carbonic anhydrase II isoenzymes, and CK, may be elevated in the bloodstream following exercise-induced muscle damage (Brentano & Martins Kruel 2011). While these markers indicate muscle damage, their expression levels depend on leakage from injured muscles and clearance rates. CK has been frequently used as a biomarker due to its significant rise in concentration following exercise-induced muscle injury and its lower laboratory cost than other biomarkers.

Our study found significantly higher expression levels of CK-MM in mice from the negative control group (C2) compared to those in the normal control group (C1). This finding aligns with the established theory that eccentric exercises, such as downhill running, often lead to muscle soreness and upsurge circulating CK-MM levels (Frimpong et al. 2019).

CK-MM represents a substance found primarily in the muscle tissue and released into the bloodstream when muscle tissue becomes damaged. The CK-MM gene generates CK's cytosolic muscular isoform, a protein crucial for immediate adenosine triphosphate (ATP) replenishment. It functions by catalyzing the reversible phosphorylation of creatine to phosphocreatine and adenosine diphosphate (ADP) to ATP during robust skeletal muscle contractions (Bekkelund 2020).

Downhill running is an exercise involving an eccentrically oriented movement where muscles contract while being stretched. This exercise has been associated with skeletal muscle injury and increased CK levels (Kindermann 2016). Mechanical stress on the muscle filaments during eccentric contractions may result in microtears in the muscle tissue. Diminished levels of the CK enzyme may be accountable for muscular fatigue, potentially resulting from a spike in intracellular inorganic phosphate quantities. Additionally, eccentric contractions may increase calcium levels within resting muscle fibres, which can contribute to muscle damage and the release of CK into the bloodstream (Giorgi et al. 2018, Touron et al. 2021). Thus, this enzyme could serve as a reliable biomarker for exercise-induced skeletal muscle damage.

Effect of L-citrulline on CK-MM post-eccentric exercise

L-citrulline is a non-essential amino acid found in watermelon and naturally synthesized within the body. It serves as a precursor to L-arginine, a key component in protein synthesis (Shatanawi et al. 2020). Despite not directly contributing to protein formation, L-citrulline has garnered attention for its potential benefits in various health conditions. Currently, research exploring the antioxidant and anti-inflammatory properties of L-citrulline in the context of skeletal muscle damage following eccentric exercises is limited. Therefore, this study investigated the effect of varying doses of L-citrulline on skeletal muscle damage, utilizing CK-MM expression as a marker for muscular injury.

Our findings revealed that CK-MM expression levels in mice subjected to acute eccentric exercise and administered L-citrulline at doses of 250, 500, and 1,000 mg/kg bw were lower than those without L-citrulline administration. This suggests that L-citrulline might exert a protective effect against muscle damage following acute eccentric exercise, potentially enhancing the body's molecular response to oxidative stress. L-citrulline serves as a precursor to L-arginine, facilitating the production of nitric oxide (NO) via nitric oxide synthase (NOS) enzymes (Shatanawi et al. 2020). According to Allerton et al. (2018), NO is known for its vasodilatory properties, as it can regulate blood flow by dilating blood vessels. It has been highlighted that L-citrulline improves blood flow and increases NO bioavailability. This aids in the removal of metabolic byproducts, such as H+ ions and free radicals, and enhances antioxidant production within muscle tissue (Theodorou et al. 2021, Douglass et al. 2021). Improved blood flow may also enhance the delivery of oxygen and essential nutrients required for muscle repair and regeneration following exercise-induced damage. While NO does not directly influence CK-MM levels, the improved muscle recovery associated with enhanced blood flow may mitigate muscle damage and subsequently lower CK-MM release.

Prior research conducted by Allerton et al. (2018) has demonstrated that L-citrulline has a protective effect against reactive oxygen species (ROS) and oxidative stress induced by exercise. The protective effect may be attributable to the antioxidant capacity of L-citrulline. During eccentric exercise, ROS are generated as byproducts, resulting in oxidative damage to cellular components such as proteins, lipids, and deoxyribonucleic acid (DNA) (He et al. 2016, Powers et al. 2020). This contributes to muscle damage and inflammation, which can impact CK-MM levels. By scavenging free radicals and reducing oxidative damage, the antioxidative capacity of L-citrulline may preserve muscle integrity and function. This can potentially lead to diminished CK-MM release following exercise-induced stress.

In their study, Yu et al. (2023) have implicated that L-citrulline has the potential to enhance mitochondrial function. Mitochondria are the cellular organelles responsible for energy production. These organelles play a crucial role in muscle metabolism, particularly during exercise (Giorgi et al. 2018, Touron et al. 2021). Through this mechanism, L-citrulline may boost energy production and reduce the likelihood of muscle fatigue and damage during exercise. Enhanced mitochondrial function can lead to more efficient energy utilization by muscle cells, potentially reducing the stress on the muscle fibres and decreasing the likelihood of extensive damage. As a result, it may decrease the release of CK-MM.

L-citrulline has also been associated with increased muscle protein synthesis rates, which is essential for muscle repair and growth. L-citrulline has consistently demonstrated its role as an activator of muscle protein synthesis (MPS) via the phospho-inositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) signalling pathway. On top of that, L-citrulline can enhance the expression of skeletal muscle myofibrillar

constituents (Jourdan et al. 2015, Le Plénier et al. 2017). However, the underlying mechanism remains unknown. By promoting the synthesis of new muscle proteins, L-citrulline may aid in repairing damaged muscle fibres and regenerating healthy tissue. Enhanced muscle protein synthesis has the potential to accelerate recovery from exercise-induced muscle damage, thereby reducing the duration and severity of CK-MM release associated with muscle repair processes.

Strength and limitations

This study provides valuable insights into the dose-response effect of L-citrulline on CK-MM expression levels in skeletal muscle tissue. Additionally, this study offers a comprehensive analysis of the potential effects of multiple doses of L-citrulline on muscle damage. By employing immunohistochemistry, this study provides detailed insights into the cellular localization of CK-MM expression in response to L-citrulline administration. However, this study has several limitations. Since this study was conducted in vivo, there might be limitations in extrapolating findings from animal models to humans. Differences in metabolism, physiology, and response to interventions between mice and humans could affect the potential to apply the findings to human physiology. Moreover, the focus of this study was on acute responses to L-citrulline administration restricted understanding of its long-term effects on muscle health and performance. Future studies with more extended intervention periods could elucidate the sustained effect of L-citrulline on CK-MM expression levels and muscle function over time.

CONCLUSION

This study presents evidence suggesting that mice exhibit increased CK-MM expression after eccentric exercise. Therefore, CK-MM may function as a marker of exercise induced muscle damage in mice. The CK-MM expression can subsequently decrease after administering L-citrulline at various doses. This indicates that L-citrulline may provide a protective effect against post-eccentric exercise muscle damage. This study also highlights that L-citrulline administration has the potential to attenuate skeletal muscle damage following eccentric exercise through various mechanisms, including improved blood flow, antioxidant activity, enhanced mitochondrial function, and the promotion of muscle protein synthesis. Further research is warranted to gain an enhanced understanding of the precise mechanisms underlying this effect and optimize the L-citrulline administration protocols for the purpose of maximizing muscle recovery and performance.

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Conflict of interest

None.

Ethical consideration

The Health Research Ethics Committee of the Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia, issued the ethical approval for this study, with protocol No. 01/02/09/2022/117 dated 10/10/2022.

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None.

Author contribution

DAG contributed to the conception and design, collection and assembly of data, and critical revision of the article for important intellectual content. MFI analyzed and interpreted the data. MFI, AAR and SNH drafted the article. All authors provided their final approval of the article.

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