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Research Article
Selenium Supplementation Alters IL-1β and IL-6 Protein Levels in Contusion Model Rats

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Abstract

Background and Objective: Contusion in skeletal muscles were common in athletes. Contusions usually occur when the tissue is exposed to a rapid and strong compressive force, for example, a direct blow, which usually results in the formation of a hematoma within the muscle. Contusion injuries impair the physiological function of the muscle. Supplementation is needed to shorten the healing process. Alternative therapy is antioxidant supplementation. Therefore, we conducted a study on the administration of the antioxidant selenium in contusion rats. **Materials and Methods:** The subject of this study were male Wistar rats. Rats were divided into 3 groups, namely control group, contusion group and selenium group. Each group consisted of 5 rats. Selenium dose was 0.0513 mg kg^{-1} b.wt., dissolved into 2% PGA given once a day, for 3 consecutive days. After treatment periods, CK-MM level, IL-1β and IL-6 level were examined. **Results:** Protein expression of IL-1β and IL-6 were significantly lower in the selenium treatment group compared to the contusion group. These results were confirmed by improved step gait in the selenium group. But there was no significant decrease in serum CK-MM levels expression in the selenium treatment group when compared to the contusion group. **Conclusion:** Selenium supplementation improved gait function after contusion by suppressing IL-1β and IL-6 expression. However, selenium administration did not alter CK-MM levels.

Key words: Antioxidant, CK-MM, contusion, IL-1B, IL-6, gait analysis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Muscle injuries are common in sports and disturb physical activity for a long time. More than 90% of sports injuries are contusions and strains in skeletal muscle[1]. Contusion usually occurs when the tissue is exposed to a rapid and strong compressive force, for example, a direct blow, which usually results in the formation of a hematoma within the muscle[2]. Contusions that occur are caused by blood leaking out of the damaged capillaries into the interstitial tissue[2]. Contusion manifestations are generally pain, tenderness on movement, limited range of motion (ROM), or a combination of these symptoms[3]. Contusions are caused by blunt force trauma that occurs outside the muscle, resulting in tissue and cellular damage and bleeding within the muscle[2-4]. Necrosis and hematoma occur in the tissue causing inflammation[4]. At the time of muscle injury, there is an early detection process that can be seen as increasing levels of muscle creatine kinase. Muscle creatine kinase is a specific enzyme for skeletal muscle that plays a role in the formation of energy in muscle cell activity through anaerobic metabolism. An increase in muscle creatine kinase in serum indicates muscle cell damage[5].

The inflammatory response is seen as increased ROS in mitochondria[6]. Increased ROS in mitochondria triggers the kinase pathway, by altering the redox status of NF-κB which results in NF-κB activation associated with the inflammatory response[7]. NF-κB is then translocated into the nucleus and induces transcription of target genes such as interleukin-1β (IL-1β) and interleukin-6 (IL-6). IL-1β and IL-6 are the main cytokines produced by macrophages/monocytes and play important roles in immunological, inflammatory and hematopoietic reactions[8]. IL-1β induces the synthesis and secretion of prostaglandins by endothelial cells and smooth muscle cells thereby increasing the expression of adhesion molecules, which allows migration of other immune cells to sites of inflammation[9]. IL-1β can directly activate mature immune cells to produce more pro-inflammatory cytokines such as IL-6. IL-1β is a major proinflammatory cytokine that triggers several physiological responses such as fever, lymphocyte activation and initiate an acute-phase protein synthesis[10]. IL-1β increases the activity of T cells that are part of the adaptive immune response and stimulates fibroblast proliferation and collagen production, thereby increasing scarring and chronic inflammatory changes[11]. IL-6 is a pro-inflammatory cytokine and mediator of the acute phase response that is released as the response to inflammation[12]. Physiologically IL-6 causes a decrease in albumin production. The consequences of decreased albumin in the circulatory make extravasation of the blood plasma and accumulated in the injured tissue as oedema[13,14].

Contusion injuries impair the physiological function of the muscle[15]. Therefore, the administration of drugs or supplementation is needed to shorten the healing process. As the alternative therapy is the administration of antioxidants. Selenium is an essential mineral and traces element for humans and animals that plays an important role in many physiological processes[16,17]. Selenium exists in 2 forms, namely inorganic and organic[18]. Most of the selenium in plants is in the form of selenomethionine[19]. Selenomethionine is easily oxidized, the antioxidant activity of selenomethionine arises from its ability to reduce ROS[20]. Selenocystein will be converted into hydrogen selenide together with inorganic selenium and with the help of ATP to produce antioxidant selenoproteins[12]. Through these selenoproteins, selenium plays a role as a defensive mechanism for oxidative stress and contributes to regulating cell membrane integrity and reducing the risk of oxidative damage[21].

Until now, no study is conducted to elucidate the effect of selenium to modulate the inflammation process in skeletal muscle contusion. Therefore, we conducted a study on the administration of the antioxidant selenium in contusion rats.

MATERIALS AND METHODS

Study area: The study was carried out at Central Laboratory, Universitas Padjadjaran Bandung Indonesia, from December, 2020-May, 2021.

Sample: The method in this study was an experimental study. The subject of this study were 15 male Wistar rats, weighing between 200-220 g. Rats were divided into 3 groups, namely the control group, the contusion group (rats with contusion treatment) and the selenium group (rats with contusion treatment+selenium supplementation). Each group consisted of 5 rats. The method of this study was approved by the Research Ethics Commission Universitas Padjadjaran Bandung No: 1013/UN6.KEP/EC/2020.

Contusion model: Contusion model rats were developed based on a study conducted by Macedo *et al.*[22] Animals were anaesthetized with a combination of ketamine (95 mg kg^{-1} b.wt.) and xylazine (12 mg kg^{-1} b.wt.) intraperitoneally and kept in the prone position. The right foot was fixed manually in knee extension and 90° dorsiflexion of

the ankle joint. The contusion area was generated in the Right Gastrocnemius Muscle (RGM) using a hollow aluminium tube with a weight of 75% of the rat's body weight. The tube was placed perpendicular to the wooden platform, the tube was dropped from 30 cm high. After the procedure, the absence of tibial fracture was assessed by palpation.

Selenium supplementation: The dose of selenium was 0.0513 mg kg^{-1}, dissolved into 2% PGA by giving 1 × a day, for 3 consecutive days. Selenium administration was carried out in the acute post-contusion phase.

By the end of the treatment period, the rat was euthanized. The target tissue samples such as blood and gastrocnemius muscle samples were separated and frozen in liquid nitrogen to be stored at -80°C until use.

Gait analysis: Gait analysis was carried out to see the changes in muscle function after contusion and the effect of selenium supplementation after contusion[23]. Modification of human's gait analysis has been developed for animal experiment use[24]. All the rats were allowed to walk straight along with the sheet of paper. Experimental rats were visually observed and the footprints made by the diseased leg (contusion) were compared with the healthy foot to assess weight resistance during the movement. Gait analysis was performed using black ink on the ventral surface of the hind legs. Gait analysis was done daily to detect changes in muscle function. At the initial stage, gait analysis was carried out for 5 min for the adaption process. In the next stage, gait analysis was carried out for 2-3 min[25].

Western blot analysis for IL-1β and IL-6 protein expression: The rats were sacrificed on the last day of treatment and a sample of the right gastrocnemius muscle tissue was taken. Muscle tissue samples were taken to examine the protein expression of IL-1β and IL-6 with western blot.

We used the first antibody of IL-1β anti-mouse polyclonal antibody (Cell Signaling, USA) and IL-6 anti-mouse polyclonal antibody (Cell Signaling, USA) with 1:1000 dilution. As an internal control, we used GAPDH anti-rabbit polyclonal antibody (Cell Signaling, USA) with 1:1000 dilution. Secondary antibody we use IR Dye 680 anti-rabbit and antimouse (Li-Cor, USA) with dilution 1:15000. The proteins expression was visualized using Odyssey clx (Li-Cor, USA). Quantification of the western blot band was performed using ImageJ software (NIH).

Statistical analysis: Statistical analysis using the GraphPad Prism Version 8.1.3 program, the data were presented in mean and Standard Error of the Mean (SEM). Differences between all groups were analyzed with one-way ANOVA followed by the Least Significant Differences test (LSD).

RESULT

Selenium supplementation improves gait analysis performance after contusion: To see the contusion effect on the affected limb, we examined the gait length of rats. The gait length was measured at 1 and 3 days after contusion. Then the difference between long and short steps was calculated. We found that there was a change in stride length in contusion legs.

In the control group, the difference between long and short steps was 1 cm. After contusion, the step difference in the contusion and selenium group was greater than in the control group. We observed the difference between long steps and short steps was around 2 cm in 1 day after contusion (Fig. 1a). In contrast, the administration of selenium improved the muscle healing process, as shown in Fig. 1b. Selenium group's delta step length returns to the normal level (compared to the control group). The quantification of the steps gait test was presented in Fig. 1c.

Selenium supplementation on CK-MM levels after contusion: In this study, serum CK-MM was taken after contusion treatment and contusion+selenium treatment for the selenium group. Serum CK-MM examination in the study was done 24-hr after treatment.

Contusion increase CK-MM serum levels in all treatment group. Based on the graph, the mean serum CK-MM concentration was higher in the contusion group (without selenium supplementation) and tend to be lower in the selenium group (given contusion injury+selenium supplementation) but not statistically significant (Fig. 2).

Selenium supplementation on changes expression of IL-1β and IL-6 after contusion: Structural muscle damage after contusion injury in this study can recruit cytokines such as IL-1β and IL-6. To examine the selenium effect on inflammation cytokines cascade, we measured the IL-1β and IL-6 protein expression with western blot. The representative figure of protein blotting were presented in Fig. 3a. Western blot results showed protein expression levels of IL-1β and IL-6 were higher in the contusion group and lower in the selenium group. The IL-1β level was 1/3 fold lower in the selenium group than the contusion group ($p<0.05$, Fig. 3b). A similar result was observed in IL-6 expression, the IL-6 protein expression in affected muscle was decreased 1/4 fold in the selenium group ($p<0.05$, Fig. 3c).

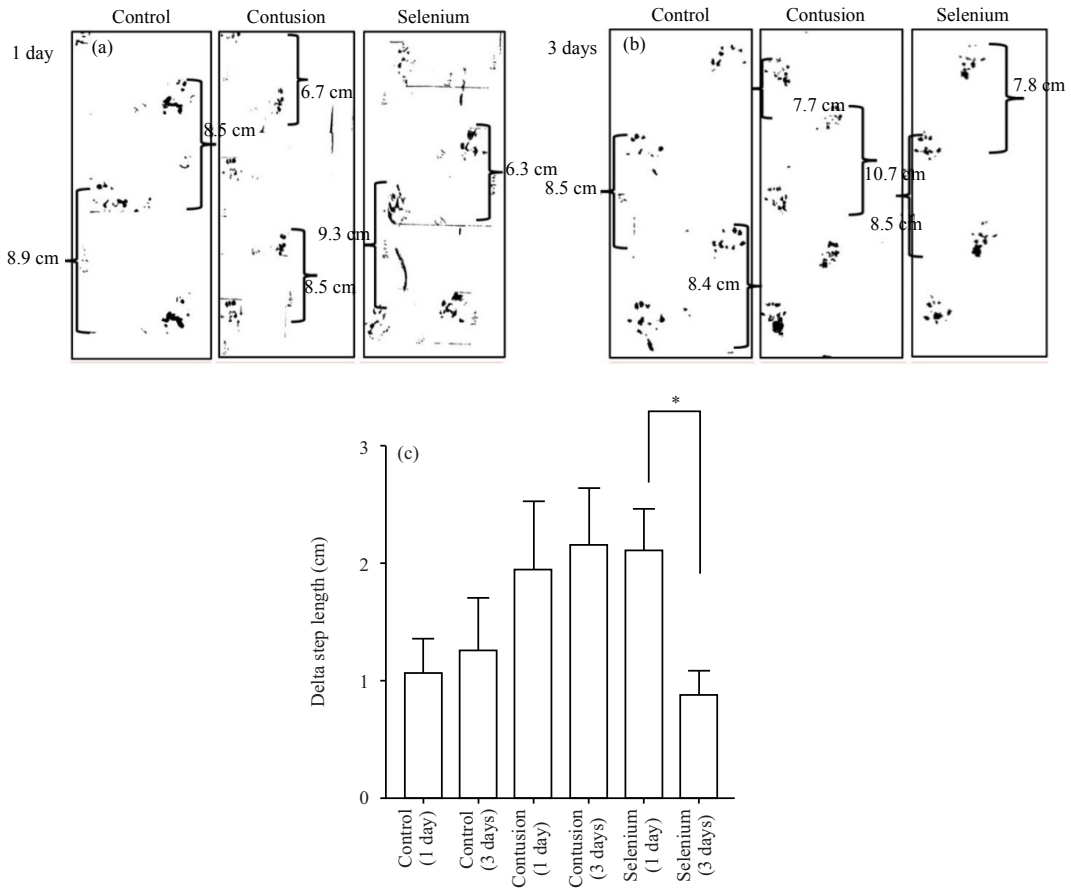

Fig. 1(a-c): Gait length of animal model, (a) Footprints of rats on 1day, (b) 3 days after contusion and (c) Average of subtraction between 2 limbs 1 and 3 days after contusion
Mean±SEM and *p<0.05

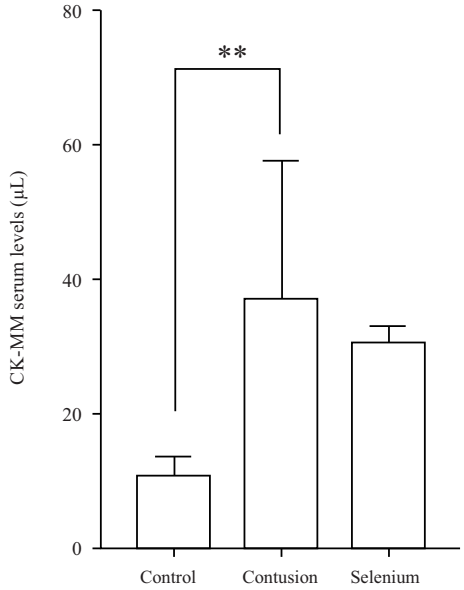

Fig. 2: CK-MM serum levels 3 days after contussion
Average concentration of CK-MM serum levels after contusion, Mean±SEM *p<0.05 and asterisks *p<0.05

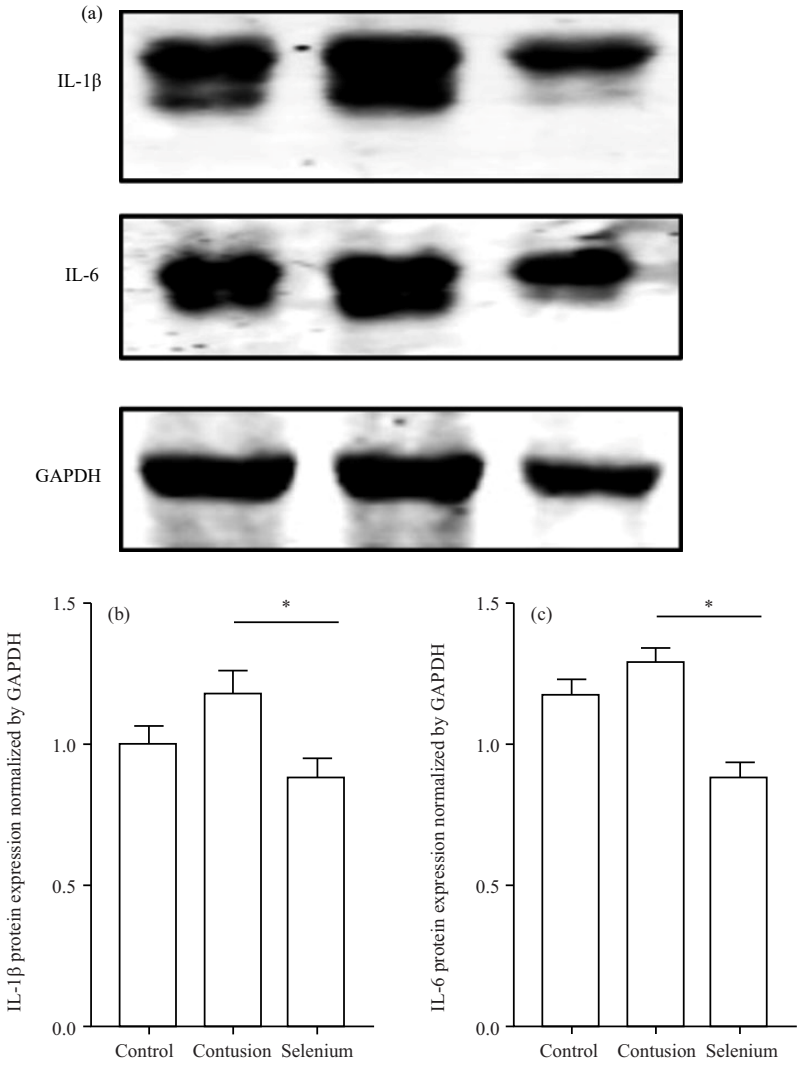

Fig. 3(a-c): Protein expression of IL-1β and IL-6, (a) Representative blot of IL-1β and IL-6, (b) Quantification of IL-1β and (c) IL-6 protein expression after normalized by GAPDH
Mean±SEM and asterisk *p<0.05

DISCUSSION

In this study, we examined the effect of selenium supplementation on gait step test, CK-MM level and IL-1β and IL-6 protein expression level after contusion in the animal model. These results are in line with several related studies. Gait analysis is a functional parameter that can be evaluated in animals, as information in determining muscle response to injury[25]. CK-MM levels measured in serum samples are known to be a marker of skeletal muscle injury[26].

We found that administration of selenium after contusion injury decreased levels of IL-1β and IL-6 proteins expression in the injured muscle. In addition, selenium supplementation after contusion injury resulted in significant changes in gait analysis stride length in the selenium group when compared to the contusion group. Structural muscle damage can lead to activation of NF-κB which plays an important role in the inflammatory response[6]. NF-κB activation can recruit cytokines such as IL-1β and IL-6 that can cause tissue damage[27].

Our study showed that selenium supplementation effectively reduced the inflammation cytokines after injury and enhanced the healing process. Selenium is known as a trace element that acts as an antioxidant through selenoproteins that act in preventing the formation of ROS and free radicals that cause cell damage and behave as scavengers to regenerate or repair oxidative damage[28-32]. Based on research by Gholami *et al.*[33] selenium supplementation can reduce oedema, mast cell infiltration, TNF- expression and

inactivated NF-κB in ischemia-reperfusion injury in the right gastrocnemius muscle[34]. In this study, muscle injury in the form of contusions in male rats aged 8 weeks was carried out with selenium supplementation for 3 days, then an analysis of the muscle tissue was carried out to see the healing process that occurred. In this study, the authors also analyzed the effect of selenium supplementation on CK-MM levels, IL-1β, IL-6 and gait analysis after contusion injury.

After contusion, we observed potentially physical changes in the impact area. This result is in agreement with the previous study by Dantas *et al.*[34], who found that the gastrocnemius muscle after contusion injury with an impact mass of 324 g, at a height of 30 cm showed a mild inflammatory response such as oedema, slight bruising and muscle lesions[34]. This shows that physical trauma given in the form of contusions lead to muscle injury characterized by tearing and subsequent necrosis of myofibrils, hematoma formation in the spaces created in the torn muscle and proliferation of inflammatory cells[35].

Selenium supplementation in this study at a dose of 0.0513 mg kg^{-1} b.wt., in rats, for 3 days. Administration of selenium with such a dose taking into account the effects of toxicity in rats and the half-life of the carrier protein selenoprotein is 57 hrs. Selenium treatment prolonged the half-life of selenoprotein 2-fold[36]. Administration of selenium for 3 days was seen from the acute phase of inflammation that lasted for 3 days, so it is expected that administration of selenium for 3 days can suppress the acute phase of inflammation and accelerate muscle healing response after contusion[32,33].

To examine the contusion effect on muscle, we analyzed CK-MM levels from blood serum. We found that serum CK-MM level in the contusion group was significantly increased compared to the control group. The elevated level of CK-MM in serum indicated the muscle damage process after contusion. Moreover, selenium supplementation for 3 days tended to decrease serum CK-MM levels although not statistically significant (Fig. 2). Possibly, that selenium has potential properties to reduce CK-MM levels. Selenium effect on suppressing CK-MM expression is not as potent as chlorella vulgaris[37,38].

Structural muscle damage can lead to an inflammatory response[32]. Activation of NF-κB plays an important role in the inflammatory response[7,40]. Activation of NF-κB can recruit cytokines and chemokines, such as TNF-α, IL-1β and IL-6, which cause tissue damage[33]. IL-1β and IL-6 are important pro-inflammatory cytokines that regulate the inflammatory response during injury[39]. The overreaction of pro-inflammatory cytokines can lead to an exaggerated inflammatory response in the tissues[40]. Increased expression of IL-1β and IL-6 indicates a strong inflammatory response[41]. Therefore, the expression and increase of these pro-inflammatory cytokines should be considered[43,44]. Our results showed that the expression of IL-1β and IL-6 increased in the contusion group and a significant decrease in the selenium treatment group when compared to the contusion group. The results of this study are following previous studies, which found that selenium supplementation inhibited inflammation caused by infection/wounds, with a significant decrease in the expression of IL-1β and IL-6[44]. Another study found that short-term administration of high doses of selenium for 7 days significantly reduced inflammatory cytokines such as TNF-α, IL-1β and CXCL1 in mice with arthritis model[42].

The excessive inflammatory response causes incomplete muscle healing which results in decreased muscle function[2]. Contusion injuries cause oedema, bruising and tenderness, which disrupts gait compensation in rats[3,43]. Injured animals tend to protect the affected limb from movement[44,45]. Gait analysis is a functional parameter that can be evaluated in animals, as information in determining conclusions from muscle response to injury[45].

In this study, gait analysis after being given contusions and selenium supplementation showed that there was a change in long strides (healthy legs) and short strides (sick feet). Gait analysis results showed a significant change in stride length in the selenium group when compared to the contusion group. There is an improvement in the inflammatory response, such as improvement of oedema and also selenium-modulated pain sensation, which causes a decrease in IL-1β and IL-6 expression[41,42]. The results of this study are consistent with previous studies which found that selenium supplementation increased skeletal muscle performance in rats muscles due to downregulation of myostatin and related cytokines[46,47]. This suggests that selenium as an antioxidant trace element plays an important role in repairing muscle function[45-47].

Based on the results, it suggests that selenium supplementation during the first 3 days of the injury was beneficial to suppress the inflammation process and shorten the duration of the muscle healing process. In the future, selenium has the potential capacity for supplementation therapy in sports injuries. However, in this study, we only used one dose of selenium supplementation. Thus, further study to determine the optimal selenium dose for contusion injury is needed.

CONCLUSION

In conclusion, selenium supplementation in the early phase of contusion is necessary to reduce the inflammation

process. Selenium supplementation reduces the IL-1β and IL-6 protein expression in the injured muscle. Selenium also improved the gait step of the impacted muscle. In the future, selenium could be used as alternative supplementation for contusion injury in sport.

SIGNIFICANCE STATEMENT

This study discovers that selenium supplementation after contusion injury can decrease the expression of IL-1β, IL-6 protein and increase the activity of muscle function which can be beneficial for professional athletes as part of the treatment after sports-induced contusion muscle injury in the early phase so that it can help in the healing process after injury. This study found the possible antioxidant effects of selenium could be beneficial for athletes with contusion injuries. This study will help researchers to uncover critical areas of the gastrocnemius muscle after contusion and supplementation of selenium that many researchers were not able to explore. Thus, a new theory on trace elements like selenium or other trace elements may be arrived at.

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