Original Research Article

The Effect of Vitamin C, E, and B12 on the reduction of Ischemic Skeletal Muscle Damage on Rat (Rattus norvegicus) due to Reperfusion Injury

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ABSTRACT

Introduction: Tourniquet is usually used in limb surgery, both orthopaedic surgery and plastic surgery.[1] The use of pneumatic tourniquets has become a standard for creating bloodless fields in the upper and the lower limb surgery. However, the use of tourniquets is dangerous because it causes complications. Complications of tourniquet use occur due to mechanical suppression of the underlying tissue, ischemia, and reperfusion effects. This causes local and systemic complications.

Objectives: This study aims to determine the effect of vitamin C, vitamin E and B12 in reducing rat skeletal muscle damage due to tourniquets.

Methods: This research is an experimental laboratory with a post-test-controlled group design. The sample consisted of 20 female Wistar rats divided into 4 groups, where each group consisted of controlled group, vitamin C group, vitamin E group, and vitamin B12 group. All the rats were anesthetized and then a tourniquet (orthodontic rubber) was placed on the thigh of each rat for 2 hours and continued reperfusion was performed for 1 hour after the tourniquet was released. Histological picture of the muscle cells (myocytes) was observed and the number of the damaged cells and the healthy ones were counted. Data were analyzed using the One-Way ANOVA test ($\alpha = 0.05$) and continued with the Post Hoc Multiple Comparisons (LSD) test ($\alpha = 0.05$).

Results: The result of One-Way ANOVA test showed significant differences between the four treatment groups. The result of Post Hoc test showed significant differences between the control group and the groups P2, P3 and P4. Whereas the P3 group showed significant difference with the P2 and P4 groups.

Conclusions: Oral administration of vitamins C, E and B12 for 5 days reduces histological damage of rat myocytes in ischemic reperfusion injuries due to tourniquet. Vitamin E has the best effectiveness compared to vitamins C and B12.

Keywords: antioxidants, myocyte damage, reperfusion injury
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INTRODUCTION

Tourniquet is usually used in limb surgery, both orthopaedic surgery and plastic surgery.[1] The use of pneumatic tourniquets has become a standard for creating bloodless fields in the upper and the lower limb surgery.[2] However, the use of tourniquets is dangerous because it causes complications. Complications of tourniquet use occur due to mechanical suppression of the underlying tissue, ischemia, and reperfusion effects. This causes local and systemic complications.[3]

Skeletal muscles in the extremities are very sensitive to ischaemic changes.[4] Ischaemic-reperfusion (IR) injuries due to tourniquet use increase with greater inflationary pressure, as well as an increase in the duration of ischemia.[3] The molecular mechanisms underlying IR injury have been extensively investigated over the past few decades. It has been shown that reactive oxygen species (ROS), polymorphonuclear neutrophils (PMN) and nitric oxide (NO) play an important role in IR injury.[5,6]

The ROS formed can be reduced using antioxidants.[7] Researches on antioxidants and IR injuries have been carried out, but there are no recommendations regarding the type of antioxidant and the dosage needed to prevent IR injury to skeletal muscles. This study aims to determine the effect of enteral antioxidants (vitamin C, vitamin E, and vitamin B12) in the prevention of skeletal muscle ischaemic-reperfusion injuries due to the use of tourniquets.

MATERIAL AND METHODS

This study was a true experimental study, post-test controlled-group design. This study was conducted in Integrated Research and Testing Laboratory, Gadjah Mada University, Indonesia, for maintenance, experiment, and sacrifice of experimental animals, and in Pathology Anatomy Laboratory, Faculty of Medicine, Sebelas Maret University, Indonesia, for myocyte count, in February 2020. Ethical permission for undertaking this study was granted by Gadjah Mada University, with certificate number: 00072/04/LPPT/2020.

Animals

Twenty healthy, actively moved, and were not disabled in the extremities, female white rats (*Rattus norvegicus Wistar strain*), 2-3 months old (weighing about 180-200 g) were purchased from The Pre-clinical Research Service Unit, Integrated Testing Research Laboratory, Gadjah Mada University. These rats were selected randomly and divided into control, Vitamin C, Vitamin E, and Vitamin B12 groups. The rats had infection or died before this study completed were excluded. The animals were quarantined and acclimatized for one week before the experiment. The animals were kept in the animal room where the temperature was automatically maintained at temperature 25 ± 2°C, relative humidity 70-90%, and a dark/light cycle 12 hours per day. We maintained the rats with food and water *ad libitum*. Rats were kept in a cage with a size of 50 x 40 x 20 cm^3. The cage was placed in a quiet condition and the outside noise was minimized.

Procedure

Administration of Enteral Antioxidants

All groups were given a standard diet for 5 days. In addition to the standard diet, the experiment group 2 (P2), group 3 (P3) and group 4 (P4) were given additional oral vitamin C, vitamin E, and vitamin B12, respectively, for 5 days. The reference dose of vitamin C used is 2 g per kg for 5 days.[8] The reference dose of vitamin E is 1 x 200 mg,[9] and the dose of vitamin B12 is 6 mcg.[10] After the dose conversion from human to mice,[11] we got doses of vitamin C, vitamin E, and vitamin B12 per 200 g of rat weight, 36 mg, 3.6 mg, and 108 mcg, respectively. We gave the vitamins in 2 cc volume via enteral using syringe.

Torniqueting and Specimen Collection

In day 5, all animals were fasted for 3 hours before the surgery with ketamine hydroxychloride (ketalar) as anesthesia, 100 mg/kg, then the surgical preparation was performed. Once all the rats were anesthetized, the procedure were performed asepsis, a tourniquet (orthodontic rubber 4.0-4.5 oz) was then placed on the thigh of each rat for 2 hours and continued reperfusion was performed for 1 hour after the tourniquet was released.

Samples were collected under the tourniquet location with a lateral incision of the proximal femur up to the muscle, then the muscle tissues were taken 0.5-0.5 cm. The preparations were fixed with 4% paraformaldehyde and stored overnight at 4°C. The preparations were subsequently cut transversely into 4 μm thick with a

microtome and stained with hematoxylin-eosin (HE). The wound was cleaned, sutured and closed with a wound dressing. The rats that had been given the action were sacrificed by giving a triple dose of ketamine. The carcasses of the rats were then put in an incinerator to burn to ashes.

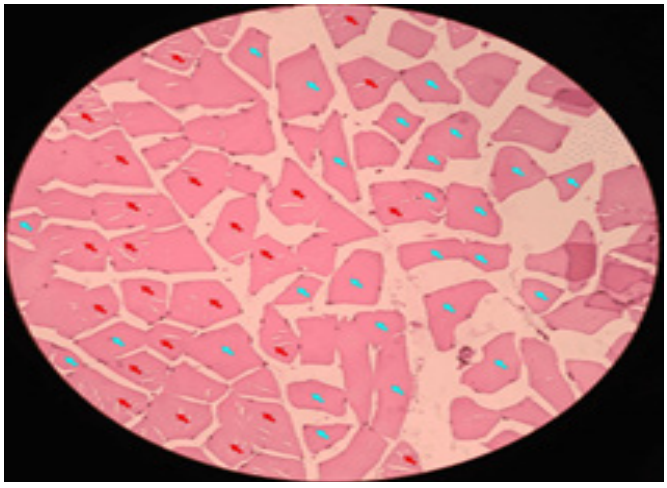

Figure 1. Histology of skeletal muscle tissue with ischemic reperfusion injury (control group). Red arrow: damaged myocyte (72%); blue arrow: normal myocyte (28%).

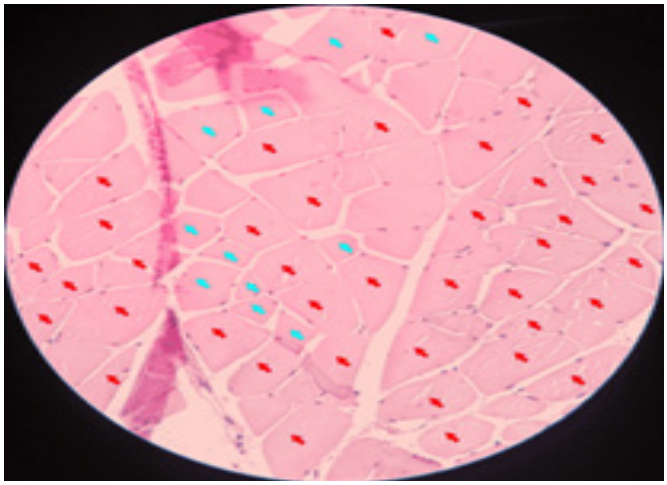

Figure 2. Histology of skeletal muscle tissuewith ischemic reperfusion injury (Vitamin C group). Red arrow: damaged myocyte (45%); blue arrow: normal myocyte (55%).

Histological Examination

Histological examination was carried out on specimens obtained at the Laboratory of Pathology Anatomy, Sebelas Maret University. The preparations were observed under a microscope with a magnification of 400x. The tissue damage assessment was measured in percent, the percentile of the number of defect myocytes in the total number of myocytes examined. Myocytes were categorized into injured and uninjured based on the morphological assessment of each individual myocyte. Uninjured myocytes had firm edges, consistent texture, and uniformity in the myocyte unity. Pericellular or satellite cells can sometimes be seen in healthy myocytes. Injured myocytes were described with uneven edges, inconsistent textures and colors (not artifacts), and/or lost nuclei.[12] Each experimental animal was made into one preparation, therefore there were five preparations in each group. The number of damaged (injured) and healthy (uninjured) cells were counted in a total of 100 myocytes counted from the entire field of view.

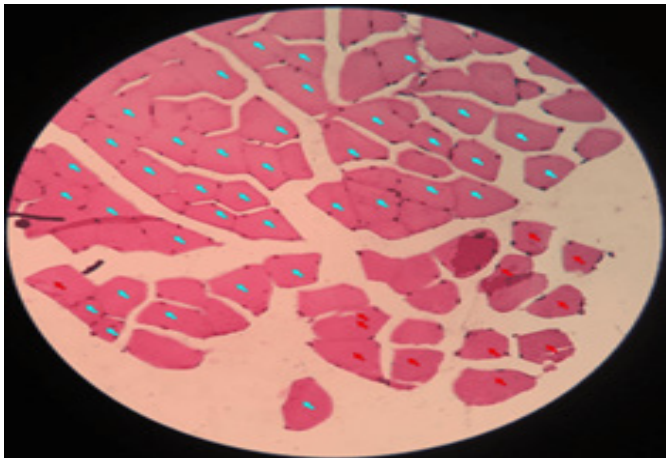

Figure 3. Histology of skeletal muscle tissue with ischemic reperfusion injury (Vitamin E group). Red arrow: damaged myocyte (22%); blue arrow: normal myocyte (78%).

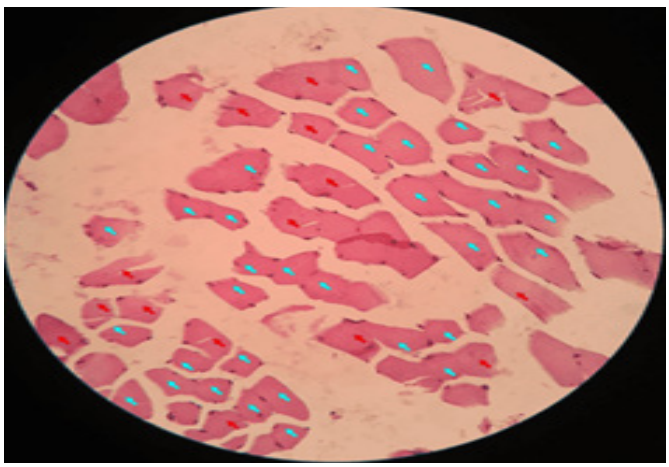

Figure 4. Histology of skeletal muscle tissue with ischemic reperfusion injury (Vitamin B12 group). Red arrow: damaged myocyte (33%); blue arrow: normal myocyte (67%).

Data Analysis

Statistical analysis was performed using One Way ANOVA to see the difference between the number of myocytes in each group and the significance level was

set at p<0.05. If the difference obtained was significant, the analysis proceeded with The Post Hoc Multiple Comparison test

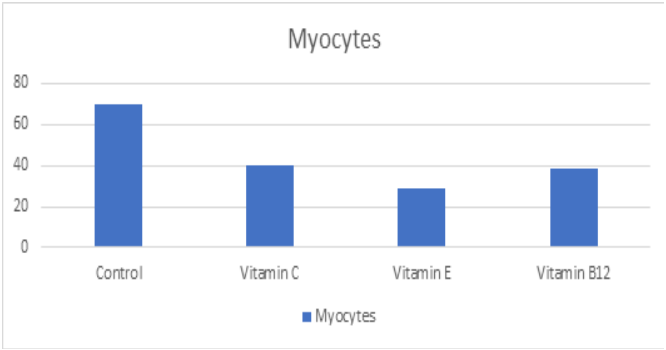

Figure 5. Diagram of Myocyte Damage on Various Treatment Preparations

RESULTS

In this study, a histological examination of 100 myocytes was performed under a microscope magnification of 400x. The myocytes were categorized into damaged and healthy based on the morphology of the myocytes.

Table 1. Myocyte Damage on Various Treatment Preparations

Group	Mean (myocytes)
Control	70,00 + 4,64
Vitamin C	40,40 +6,11
Vitamin E	29,20 +4,21
Vitamin B12	38,60 +6,80
p-value	<0.001*

Table 2. Myocytes Damage Comparrison on Various Treatment Preparations

Myocytes	p-value		
	Vitamin C	Vitamin C	Vitamin C
Control	<0,001*	<0,001*	<0,001*
Vitamin C		0,006*	0,615
Vitamin E			0,016*

Based on Table 1, it is showed that the rats taken Vitamin E had the least damage of all with an average damage of 29.20+4.21. While the most damage occurred in the control group with an average damage of 70.00+4.64. ANOVA test showed a value of p = <0.001 (p <0.05) which indicated a significant difference in myocytes damage between the four groups.

Based on the post hoc multiple comparisons test, there was a significant difference in the group of rats given vitamin C (p = <0.001), vitamin E (p = <0.001), and vitamin B12 (p = <0.001) (Table 2). This study shows that treatment with antioxidants vitamin C, Vitamin E, and Vitamin B12 can prevent an increase in myocyte damage. The post hoc test also demonstrated that the Vitamin E, vitamin C (p = 0.006), and vitamin B12 (p = 0.016) groups showed significant difference. From this study, it was found that compared to the other three groups, Vitamin E, as an antioxidant, showed the best effectiveness in preventing myocyte damage.

DISCUSSION

The results of statistical test using one way ANOVA (α = 0.05) showed significant differences between the four treatment groups. The control group was the group with the most damage, while the vitamin E group had the lowest number of myocyte damage, followed by vitamin B12 and vitamin C groups, respectively. The results of the Post Hoc test with LSD test showed a significant difference (p> 0.05) in the P1-P2, P1-P3, P1-P4 groups. This showed that giving antioxidants, *i.e.* vitamin C, vitamin E, and vitamin B12, can reduce myocyte damage. The results of the post hoc test also showed significant difference between P2-P3 and P3-P4. However, the P2-P4 groups did not show any significant difference. This study is in accordance with the theory that the administration of antioxidants can reduce the explosion of ROS that occurs due to ischemic-reperfusion injuries in the use of tourniquets on skeletal muscles and, as the consequences, will reduce the damage to the muscle cells.

The effect of oral vitamin C on prevention of IR injury in rat cremaster muscle was studied[8] by using cross-clamping for 2 hours and reperfusion was performed for 1 hour, and the muscle function was assessed electrophysiologically by electric field stimulation, infiltration by neutrophils was determined by tissue myeloperoxidase (MPO) activity, and tissue edema was determined with wet-dry ratio. In this study, pre-treatment with vitamin C maintained muscle function and reduced tissue edema and neutrophil infiltration. Neutrophil infiltration blast activity was reduced in the vitamin C-treated group compared to the control group. It can be concluded from this study that pre-treatment with oral vitamin C protects muscles against acute IR injury by attenuating neutrophil infiltration activity.

However, the safety of administering vitamin C has also

been questioned because of its contradictory role in IR injury. In a model of IR injury-induced liver damage, ascorbic acid was shown to have anti- and pro-oxidant properties. After IR injury, decreased glutathione oxidation ratio is reduced and increased lipid peroxidation levels and mitochondrial swelling can be prevented by exposure to ascorbic acid at a dose of 0.5 mM. On the contrary, a dose of 2.0 mM ascorbic acid increases tissue injury.[5]

Several studies on the protection of vitamin E in IR injury to various organs have been conducted. Kohler, in his study, stated that enteral pre-treatment of vitamin E reduced ischemic reperfusion injury in the rat jejenum, which was indicated by less loss of villi height and weakened neutrophil infiltration.[5] Meanwhile, the group given with vitamin C did not show any significant results.

Research conducted by Medling showed that vitamin E infusion had a protective effect in preventing IR injury in mouse muscle as measured by increased muscle viability and reperfused blood flow compared to control.[13] Vitamin E also had the effect of reducing edema, although the results were not significant. Furthermore, Medling *et al*. revealed that, apart from the release of ROS, the tissue damage that occurred during reperfusion was potentiated by the infiltration of inflammatory cells and the release of humoral mediators.[13] In addition to necrotic mortality, apoptosis appears to be induced by activation of the signal transduction cascade promoted by the changing environment observed during reperfusion. The transduction cascade is a delayed process that can potentially be reversed if the pathway can be interrupted. The initiation and spread of IR injury depends on the activation of transcription factors which, in turn, are responsible for the induction of inflammatory genes required for the rapid production of proteins (cytokines, adhesion molecules, complement factors, and induced nitric oxide synthase). Vitamin E has been shown to function not only as an antioxidant but also as a regulator of this signal transduction.

Vitamin B12 or cobalamin protects against oxidative stress induced by hydrogen peroxide. The antioxidant effect of cobalamin is likely to result from a combination of direct and indirect effects: stimulation of methionine synthetase (MS) activity, direct reaction with reactive oxygen and nitrogen species, glutathione sparing effects, and modification of molecular signals.[14] Vitamin B12 is a cofactor of the MS enzyme. MS plays a key role in homocysteine metabolism (L-Hcy, Hcy). Impaired MS activity due to cobalamin deficiency leads to increased Hcy or hyperhomocysteinemia (HHcy / HHCY).[15] HHcy is known to produce oxidative stress, which interferes with different signaling pathways and also inhibits methylation reactions. Research by Moreira suggested that a combination supplement with folic acid, vitamin B6, and vitamin B12 is a very effective treatment for HHcy patients. From the study, it can be concluded that intervention with B12 combined with other B vitamins could be a treatment for skeletal muscle dysfunction in HHcy condition. But how ROS affects skeletal muscle during HHcy is not clear. Further research must be done to confirm this association.

Antioxidant, together with the results of previous studies, support the results of this study, where in this study all groups that were pre-treated with antioxidant showed significant results in protection against histological damage to rat skeletal muscle cells (myocytes) due to IR injury caused by the use of tourniquets. In addition, in this study, the group treated with vitamin E gave the most effective results compared to vitamins C and B12. Because of the effect of vitamin E, vitamin C, and vitamin B12 in preventing reperfusion ischemic injury, their readily available preparations, and low cost, it is hoped that this study can become a reference in developing new therapies for the prevention of complications of tourniquets in muscles.

The results of this study are consistent with the theory that the administration of antioxidants can reduce the explosion of ROS that occurs due to ischemic-reperfusion injuries in the use of tourniquets on skeletal muscles so that it can reduce muscle cell damage.

CONCLUSION

The administration of vitamin C, vitamin E and vitamin B12 can significantly reduce the histological damage to rat myocytes due to the use of tourniquets, and vitamin E shows the best effectiveness compared to vitamins C and B12. Additionally, giving vitamin C and vitamin B12 has the same effectiveness in preventing the increase in rat myocyte damage.

Acknowledgments

Nil

Disclosure

The author reports no conflicts of interest in this work.

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