



# Preventing Ischemic-Reperfusion Injury after Tourniquet Application in Fracture using *Aloe vera* Ethanolic Extract

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## Abstract

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**BACKGROUND:** A tourniquet is a typical equipment used in the orthopedics surgery to decrease blood loss and create a better operating environment. Ischemic-reperfusion damage, which happens when the tourniquet is deflated, is one of the tourniquet's negative effects. Antioxidants can be used to minimize oxidative stress, which is one strategy to mitigate ischemic-reperfusion injury. *Aloe vera*, for example, is widely known for its antioxidant and anti-inflammatory properties.

**AIM:** This study aims to evaluate the antioxidant effect of *Aloe vera* gel to mitigate ischemic-reperfusion injury.

**METHODS:** The tibias of male Wistar strain rats (*Rattus norvegicus*) were fractured in this experiment, and *Aloe vera* gel was given orally as an antioxidant. A total of 18 rats were utilized in this research, which were separated into 6 groups. Varying amounts of *Aloe vera* extract were given to the treatment groups: 40 mg/kgBW, 60 mg/kgBW, and 80 mg/kgBW. The tourniquet would be inflated for two hours then deflated after application of the *Aloe vera* extract. Superoxide dismutase (SOD) and malondialdehyde (MDA) levels were measured in the tibia (MDA).

**RESULTS:** Application of tourniquet increased oxidative stress marker in fracture state, marked by reduction of SOD and increase of MDA. After administration of *Aloe vera* extract, the oxidative stress parameters (SOD and MDA) were reduced significantly.

**CONCLUSION:** The result of this study suggests that tourniquet increased oxidative stress marker in fracture state and *Aloe vera* extract could be used to ameliorate this ischemic-reperfusion injury.

## Introduction

To provide a bloodless operating field, tourniquets are routinely utilized in orthopaedic procedures involving long bone fractures [1]. The use of a tourniquet, on the other hand, has various risks, including local neurovascular complications and systemic complications [2]. According to a study conducted in Norway, tourniquet-related complications in orthopaedic surgery occur roughly 0.032 %, with lower limbs being affected more frequently than upper extremities [3].

Ischemic-reperfusion injury is also one of possible serious complication of tourniquet application [4]. Ischemic reperfusion would affect the limb itself (muscle and joint), and it may affect distant organ [5]. Ischemia-reperfusion injury caused by a tourniquet would enhance superoxide production and reduce endogenous antioxidant activity. Several techniques for reducing ischemia-reperfusion harm after tourniquet application have been proposed, including ischemic preconditioning and postconditioning, reperfusion interval, and the injection of various agents [6], [7], [8]. The inflammatory indicators in the distant organ, in this example the lung, have been

shown to be reduced by reperfusion intervals. The long operating duration is, however, a disadvantage of this method. As a result, the authors propose a preventative intervention that can be implemented prior to surgery.

Exogenous antioxidant administration is one way for reducing oxidative stress that can be done preoperatively. *Aloe vera* gel has been used to counter ischemic-reperfusion injury in the lung, kidney, and liver [9], [10]. The use of this agent in bone tissue is yet to be studied. Thus, the author aims to evaluate the antioxidant effect of *Aloe vera* gel to mitigate ischemic-reperfusion injury.

## Methods

This experimental study was conducted during January 2019–February 2019 at the authors' institution. The acclimatization, maintenance, and intervention of the samples were done in the Animal Model Unit, Laboratory of Pharmacology of the authors' institution. The Malondialdehyde (MDA) and Superoxide dismutase (SOD) measurement was conducted in the Physiology

Laboratory of the authors' institution. The evaluation of bone morphogenetic protein 7 (BMP-7) level was conducted in the Central Biomedical Laboratory of the authors' institution. The callus and osteoblast evaluation were conducted in the Pathology Laboratory of the authors' institution. The Institutional Review Board has approved all animal protocols, and all subsequent experiments were carried out according to the ARRIVE guidelines and regulations.

### **Animal model**

The animal models in this study were 18 male rats (*Rattus norvegicus*), divided into 6 groups with 3 rats in each group. Male rats were used because the use of male rats is already established in fracture experimental study [11]. Furthermore, the authors used only male rats to reduce variability because the previous study found that female rat fracture model exhibits different mechanical properties [12].

The inclusion criteria were healthy 3-month-old male *Rattus norvegicus* weighing 180–200 g. The exclusion criteria were abnormal extremity, infection, and death. The animals were acclimatized for a week in a controlled environment with a 12-h light/dark cycle and a temperature of 23.6° C.; they were fed a regular chow diet with unrestricted water.

The rats were divided into 6 groups; they were sacrificed directly after fracturing the tibia. The details of the groups and their following intervention are as follows Table 1:

### **Tibia fracture model**

The right tibia of the rat was fractured using the closed bone cutting technique [13]. Before the operation, the animals were fasted for 3 h. Ketamine HCl, 40 mg/kgBW, was used to anesthetize the animals. The fracture was started by using two forceps to grip the rat's leg on the proximal and distal sides until false movement was observed. If the rats showed signs of pain, such as lethargy, trouble feeding, or shivering, they were given analgesic medication in the form of paracetamol 10 mg/kg.

### **Tourniquet**

A 4.5 ounce orthodontic rubber band with a 1/8 inch diameter was used as the tourniquet. The rat's proximal thigh leg was fitted with an orthodontic rubber band. In a previous study, the application of orthodontic rubber bands in the murine model of ischemic-reperfusion injury was demonstrated. Because it enables appropriate blockage of the femoral artery and its multiple collateral branches, tourniquets are frequently utilized in the ischemic-reperfusion rat model. The rubber band used in orthodontics is superior because it produces less soft tissue injury [14].

**Table 1: The details of treatment in each animal model group**

Group	Intervention
Group 1	Normal rats, without fracture
Group 2	Fractured tibia, without tourniquet
Group 3	Fractured tibia, 2 h tourniquet
Group 4	Fractured tibia, 2 h tourniquet, 40 mg/kgBW <i>Aloe vera</i> gel administration
Group 5	Fractured tibia, 2 h tourniquet, 60 mg/kgBW <i>Aloe vera</i> gel administration
Group 6	Fractured tibia, 2 h tourniquet, 80 mg/kgBW <i>Aloe vera</i> gel administration

### **Aloe vera Dosage**

The Aloe vera gel was created with Soxhlet method, then diluted with dimethylsulfoxide (DMSO). The lowest dose of 40 mg/kgBW was determined using a conversion table by Laurence and Bacharach [15]. The second dose of 60 mg/kgBW was determined as the half of the lethal dose of *Aloe vera* in rats (120.65 mg/kgBW/day) [16]. The period between the first and second doses was used to determine the third dose of 80 mg/kg BW. The Aloe vera gel was given three hours before the tourniquet was applied, and it was given with a probe.

### **Superoxide dismutase Measurement**

SOD is an enzyme acting as an endogenous antioxidant. In this study, the authors measured the SOD level using SOD enzyme-linked immunoassay kit. The measurement was conducted to the serum level and the tissue (bone) level.

### **Malondialdehyde measurement**

MDA is the byproduct of lipid peroxidation, a process occurring in oxidative stress [17]. The authors measured MDA level in the serum and bone tissue using TBARS (Thiobarbituric acid reactive substances) assay.

### **Statistical analysis**

The normality test was used to assess the distribution of the data obtained. The data's homogeneity was also examined. After the data matched the required assumptions of normality and homogeneity, the one-way ANOVA test was applied. Furthermore, the Tukey test was used to examine all pair-wise comparisons. If the assumptions were not met, the Kruskal-Wallis test and post-hoc Dunn test were used to compare the results. SPSS version 25 software, NY Armonk, USA, was used to analyze the data.

## **Results**

There were 18 Wistar strain rats used in this study. The measurement results of each variable in group 2 to 6 are depicted in Table 2.

**Table 2: Comparison and post hoc test of Group 2–6**

Parameter	Group	n	Mean ± SD	p
Plasma SOD (IU/mL)	2	3	49.72 ± 1.56 <sup>a,c</sup>	0.012 <sup>*,†</sup>
	3	3	36.94 ± 2.66 <sup>a</sup>	
	4	3	43.77 ± 1.68 <sup>a,c</sup>	
	5	3	49.30 ± 0.88 <sup>a,c</sup>	
	6	3	59.65 ± 4.95 <sup>b,c</sup>	
Bone SOD (IU/mL)	2	3	5.31 ± 0.10 <sup>a</sup>	<0.0001 <sup>*,†</sup>
	3	3	2.42 ± 0.11 <sup>b</sup>	
	4	3	4.61 ± 1.00 <sup>a,b,c</sup>	
	5	3	6.76 ± 0.12 <sup>b,c,d</sup>	
	6	3	7.50 ± 0.19 <sup>b,c,d,e</sup>	
Plasma MDA (µmol/L)	2	3	118.79 ± 1.76 <sup>a</sup>	<0.0001 <sup>*,†</sup>
	3	3	358.00 ± 36.43 <sup>b</sup>	
	4	3	313.56 ± 8.68 <sup>b,c</sup>	
	5	3	253.92 ± 24.98 <sup>d</sup>	
	6	3	220.96 ± 4.50 <sup>d,e</sup>	
Bone MDA (ng/g)	2	3	173.55 ± 7.78 <sup>a</sup>	<0.0001 <sup>*,†</sup>
	3	3	273.56 ± 6.76 <sup>b</sup>	
	4	3	250.67 ± 4.42 <sup>c</sup>	
	5	3	215.04 ± 4.21 <sup>d</sup>	
	6	3	203.18 ± 2.32 <sup>d,e</sup>	

<sup>†</sup>p < 0.05 is considered as significant, <sup>†</sup>p-value from one-way ANOVA test, <sup>†</sup>p-value from Kruskal–Wallis test. Values in a row with different superscript letters (when paired) indicate significant pair-wise comparisons. In contrast, values in a row with same superscript letters (when paired) indicate nonsignificant pair-wise comparisons. SD: Standard deviation, SOD: Superoxide dismutase, MDA: Malondialdehyde.

Values in a row with different superscript letters (when paired) indicate significant pair-wise comparisons. In contrast, values in a row with same superscript letters (when paired) indicate non-significant pair-wise comparisons.

- <sup>†</sup> p-value from one-way ANOVA test
- <sup>\*</sup> p-value from Kruskal-Wallis test
- \*p-value <0.05 is considered as significant.

We observed a decrease of both plasma and SOD level in group 3 in which tourniquet was applied for two hours. There was a decrease of plasma SOD level of about 25%, and the decrease of bone SOD level reached more than 50%. After administration of *Aloe vera* gel, there was a dose-dependent increment of plasma and bone SOD level.

The contrary was observed in regards of plasma and bone MDA level. In group 3, plasma MDA level tripled compared to Group 2, and the bone MDA level doubled. With administration of *Aloe vera* gel, we observed dose-dependent decrease of MDA level both in plasma and bone tissue.

Variables that are normally distributed can be tested using parametric statistics, namely ANOVA, while variables that are not normally distributed will be tested using non-parametric statistics, namely the Kruskal-Wallis test. The results of the ANOVA test showed that in all variables there were significant differences between all groups. The Kruskal-Wallis test was used to analyze the differences between groups regarding the plasma SOD variable, because this variable was not normally distributed. The analysis showed that there were significant differences in plasma SOD of all groups.

The authors also analyzed the comparison across group 1-3. In comparison of the parameters between group 1-3, there was no significant difference of SOD plasma level (p=0.066). Other parameters showed significant difference (p < 0.05), but not all pairwise comparisons showed significant difference among group 1-3.

## Discussion

In the case of long bone fractures, the use of a tourniquet during surgery intends to minimize bleeding and maintain the operating field clear. However, one of the drawbacks of employing a tourniquet is that it might lead to issues such as ischemic-reperfusion injury when the tourniquet is deflated. Our findings revealed that using a tourniquet elevated oxidative stress markers in the fractured state, as seen by a decrease in SOD and an increase in MDA. This study also showed that *Aloe vera* extract could be used to ameliorate the ischemic-reperfusion injury caused by tourniquet, marked by reduced oxidative stress parameters (SOD and MDA).

When an ischemic condition occurs, energy production to meet cell needs is inhibited. In the initial phase, energy needs are met from the process of glycolysis (breakdown of glucose). Along with the glycolysis process, there is also a buildup of lactate (↓pH) and other toxic products. This triggers the activation of chemical mediators and enzymes including phospholipase A2 and lysozyme. After deflation, the return of circulation can lead to ischemic-reperfusion injury that produces reactive oxygen species (ROS). The increase in ROS will cause oxidative stress that can damage the lipid components of cell membranes [2].

The administration of 60 mg/kgBW *Aloe vera* gel could increase SOD level approximately close to control group. Administration with higher doses (80 mg/kg) further increased SOD levels. This increase was observed both in the plasma and bone tissue SOD level. When the authors compared the oxidative stress parameters of Group 1 (no fracture) and 2 (fractured tibia without tourniquet), there was no significant difference of SOD levels, these findings indicate that fracture conditions do not cause oxidative stress. So that oxidative stress conditions occur due to the use of tourniquets. This finding is in line with the results of a study by Prasad *et al.* In their study, the oxidative stress parameters did not increase significantly in the first week after fracture, but they were increased during the second and third week during callus formation in which there is a reperfusion of the fracture site [18].

Both the level of plasma and bone tissue MDA increased significantly after application of tourniquet. This signified that the application of tourniquet and the subsequent reperfusion during its deflation caused a marked oxidative stress in local tissue and systematically. The same result was observed in the preliminary study by the authors [4]. Previous studies recommended the safe time of tourniquet use of 1-3 h [19]. The results of this study, however, suggests that even the duration of application is still within that limit, oxidative stress is still occurring. The administration of *Aloe vera* ethanolic extract was proven to reduce both plasma MDA and

bone MDA levels, and there was a negative correlation between MDA levels and Aloe vera dose. There was no significant difference in MDA levels in groups 1 and 2, both in plasma and bone as with SOD levels.

After administration of *Aloe vera* gel, there was a serial increase of SOD level and a serial decline of MDA level with increasing dose of the extract administered. *Aloe vera* is well known for its antioxidant property; the phenolic compound found in *Aloe vera* is a strong reductant and hydrogen donor [19]. Other studies have demonstrated the benefit of *Aloe vera* in ameliorating ischemic-reperfusion injury in other organs [20], [21]. The results of our study suggest that *Aloe vera* gel may be used preoperatively to reduce the deleterious effect of ischemic-reperfusion injury after tourniquet application in bone fracture cases.

There were significant differences between groups in the variables of plasma and bone SOD, plasma & bone MDA, plasma BMP 7. The significant difference was mainly found between groups without a tourniquet and with a tourniquet fitted, as well as between groups without Aloe vera and groups given Aloe vera (especially in the group given Aloe vera at a dose of 80 mg/kgBW). Correlation analysis showed a directly proportional relationship between aloe vera and plasma and bone SOD variables, plasma BMP 7, and an inverse relationship between plasma and bone MDA and bone BMP-7 variables.

There are still some limitations to this research. To begin with, the authors unable to identify the specific active polyphenol or ethanolic component responsible for Aloe vera extract's antioxidant action. In addition, the authors did not quantify the concentration of active component in Aloe vera extract. Furthermore, the weight of the animal models varied in this investigation, which could have resulted in a difference in the metabolic rate of Aloe vera extract and its active component due to body weight differences. Future research could include more time points for measurements to better understand the metabolic changes carried on by ischemic-reperfusion injury and antioxidant treatment. Nonetheless, this study could serve as a starting point for future research on the utilization of antioxidants in the treatment of ischemic-reperfusion injury caused by tourniquet use.

## Conclusion

The result of this study suggests that tourniquet increased oxidative stress marker in fracture state and Aloe vera extract could be used to ameliorate this ischemic-reperfusion injury marked by reduced oxidative stress parameters (SOD and MDA).

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