



Reperfusion interval effect in prevention of ischemic-reperfusion injury due to tourniquet use on fracture healing

Thomas Erwin Christian Junus HUWAE, Fiki NURANDANI, Retty RATNAWATI

From the Saiful Anwar General Hospital, Malang, East Java, Indonesia

Tourniquet is widely used in orthopedic surgery but its application may induce ischemic-reperfusion injury. It may lead to the disruption of fracture healing by altering the balance between osteoblast and osteoclast. One way to reduce the negative effects is by reducing the ischemic period. The aim of this study is to evaluate the effect of reperfusion interval on ischemic-reperfusion injury and fracture healing. This study involved 24 Wistar strain male rats, divided into four groups. Their tibias were fractured, and tourniquet was applied on their proximal thighs for three hours. Group one is the control group (C) with no reperfusion interval, group two, three and four were given the reperfusion interval of 5 (R1), 10 (R2), and 15 (R3) minutes after 2 hours tourniquet use. On the 14th day, the tibia was harvested to measure the level of malondialdehyde (MDA), diameter of the callus, and osteoblast cell count. The statistical comparison was conducted using One-way ANOVA, followed by Tukey test.

There was a significant difference of the MDA level, callus diameter, and osteoblast cell count between control group and all reperfusion groups. However, there were no significant differences in regards of the MDA level and callus diameter in all pairwise comparisons between all reperfusion groups. In regards of osteoblast cell count, the highest was observed in group R2. The MDA level was inversely correlated with both the callus diameter and the osteoblast count.

Reperfusion interval can reduce ischemic reperfusion injury in rat's fracture healing process.

Keywords : fracture healing ; ischemic-reperfusion injury ; malondialdehyde ; reperfusion interval.

INTRODUCTION

The patients who undergo orthopedic operations usually lose a large amount of blood. Pneumatic tourniquet is routinely used to achieve bloodless operation field (1). However, the use of this device poses risk of several complications. One of the most common complications is ischemic-reperfusion injury resulting from the direct pressure of the tourniquet (2). Reperfusion injury

- Thomas Erwin Christian Junus Huwae^{1,2},
- Fiki Nurandani³,
- Retty Ratnawati⁴.

¹Orthopaedic and Traumatology Department, Saiful Anwar General Hospital Malang, East Java, Indonesia.

²Doctoral Program of Medical Science, Brawijaya University, Malang, East Java, Indonesia.

³Resident of Orthopaedic and Traumatology Department, Saiful Anwar General Hospital

⁴Physiology Department, Brawijaya University, Malang, East Java, Indonesia.

Correspondence : Thomas Erwin Christian Junus Huwae, Jl. Jaks Agung Suprpto No.2, Malang, East Java, Indonesia 65111. Phone : +62818300802.

E-mail : huwaethomas@ub.ac.id

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which occurs due to formation of reactive oxygen species (ROS) following the ischemic period may also occur after deflation of the device (3). There are several biomarkers of oxidative stress which can be measured by either immunologic or spectrophotometric method. One of the markers of ROS formation is malondialdehyde (MDA) which forms after lipid peroxidation reaction (4).

Various strategies are proposed to reduce the risk of ischemia-reperfusion injury. Some of the options available are the application of antioxidants, the use of specific type of anesthetics, and others (5). One of the simplest way to achieve it is by applying a short reperfusion period in order to decrease the ischemic period of the limb (6).

The effect of ischemic-reperfusion injury on skeletal muscle of murine model has been demonstrated in several studies (7,8). However, there is limited study regarding the effect of this injury to bone healing process in fractures (9). Lu et al. found that ischemia would increase the risk of delayed and non-union fractures (10). On the contrary, Liu et al. demonstrated that repetitive brief ischemia could lead to bone healing improvement by promoting the synthesis of BMP-2, VEGF, TGF- β , and ALP (11). Wauquier et al. stated that ROS formation inhibits osteoblast formation but promotes osteoclast differentiation by increasing RANKL synthesis (12). Even so, there is currently no study evaluating the effect of a single reperfusion period on bone healing process.

To the authors' knowledge, this is the first study evaluating the effect of reperfusion interval on bone healing process in murine model. The aim of this study is to evaluate the effect of different single reperfusion interval on the prevention of ischemic-reperfusion injury, evaluated by the MDA level, callus diameter, and osteoblast cell count.

MATERIALS AND METHODS

This experimental study was conducted in the authors' institution from February-March 2019. The animal samples were handled in the Parasitology Laboratory of the authors' institution. The measurement of MDA level was conducted in the

Physiology Laboratory of the authors' institution. The quantitative osteoblast counting was conducted in the Anatomical Pathology Laboratory of the authors' institution.

Twenty-four adult male Wistar rats were used in this study. The inclusion criteria were rats aged 12 weeks, weighted 180-200 g, healthy, and ambulatory. The exclusion criteria were sick animals and deformity of the extremity. The dropout criteria were infection, death before the end of the experiment, and broken cast during tissue collection. The animals were acclimatized in a controlled condition of 12-h light/dark cycle at the temperature of 23.6°C ; they were fed a standard chow diet with free water intake. The acclimatization period was continued for one week.

The animals were divided into four groups of six rats : one control group (C) which didn't receive any reperfusion period, one group receiving five minutes reperfusion (R1), one group receiving ten minutes reperfusion (R2), and one group receiving 15 minutes reperfusion (R3). These reperfusion intervals were chosen to reflect possible future application in operating room settings. At the end of the experiment (day 14), the rats were euthanized using cervical dislocation technique. The 14-day period was chosen because previous study showed that the peak callus mass was formed after that time period (13).

The right tibia of the rat was fractured using open bone cutting technique. Following disinfection and aseptic technique, longitudinal incision on the anterior tibia was made, both cortices was fractured in the mid-diaphysis using bone cutting forceps after intraperitoneal anesthesia with ketamine (40 mg/kgBW). The wound was then stitched with non-absorbable suture. The fracture was immobilized with long leg cast with plaster of Paris (POP).

The tourniquet used was a 4.5 oz orthodontic rubber band with a diameter of 1/8 inch. Orthodontic rubber band was applied to one leg of the rat on the proximal thigh. Orthodontic rubber band use in the ischemic-reperfusion injury in murine model has been established in the previous study (14). The rubber was installed for 3 hours in the control group, while the treatment group was given a reperfusion

interval of 5, 10, and 15 minutes after 2 hours of tourniquet use. The tourniquet was then reapplied for another hour and only applied on the first day.

For evaluation of fracture healing, the right tibial bone was harvested after euthanasia. The fracture callus diameter was measured using manual micrometer. After measurement, 1 cm of bone tissue from the fracture site and fracture callus was harvested. The tissue was fixed in 10% neutral buffered formalin. The bone tissue was decalcified with 15% aqueous formic acid, then embedded in paraffin. All the histologic sections were stained with hematoxylin and eosin (HE) staining for light microscopic examination. Observation of osteoblast cells was done by quantitative counting per 10 large viewing fields using the Olympus BX-51 dot slide microscope with Olympus XC10 camera, 400x magnification.

MDA represents the end product of lipid peroxidation, one of the markers of oxidative stress. The MDA levels in the bone was evaluated using thiobarbituric acid reactive substances (TBARS) method. The right tibia was ground in a porcelain mortar to homogenize the bone sample. An amount of 50 mg bone tissue was weighed and mixed with 1 ml of buffer phosphate, 1 ml of 100% trichloroacetic acid (TCA), 1 ml of 1 N HCl, and 1 ml of 1% Na Thiobarbiturates in a petri dish. The solution was then heated in the water bath with the temperature of 100° C for 25 minutes. It was then centrifuged at 2000-3000 rpm for 15 minutes. The supernatant was taken and diluted with aquabides to 3 ml. The absorbance of each sample was measured at 532 nm using spectrophotometry. The results were expressed as nanomol of MDA per miligram of tissue (nmol/mg).

Data normality was analyzed using Kolmogorov-Smirnoff test, and homogeneity was tested using Levene's test. One-way ANOVA was used to compare the difference of means among all groups. The post-hoc test was conducted using Tukey test to conduct all possible pair-wise comparisons between the groups. Pearson correlation test was used to further evaluate the correlation between the MDA level and the callus diameter and between MDA level and the osteoblast cell count. The p-value of <0.05 was considered as statistically significant.

All statistical analysis was conducted using SPSS version 20.0, Armonk NY, USA.

All procedures involving the animal samples were performed in accordance with the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) and approved by the Institutional Review Board.

RESULTS

The animal samples included in this study were 24 healthy male Wistar strain rats, aged 12 weeks, and weighted 180-200 g. There were no dropouts in this study.

The highest MDA concentration was observed in group C and the lowest was in group R2. There was a significant difference between mean MDA level of all groups ($p < 0.05$). Post-hoc test also showed significant difference between group C and all reperfusion groups. Nevertheless, there was no difference between each reperfusion groups. The details of the MDA level measurements are depicted in table 1.

Table 1. — Measurements of MDA level of control and reperfusion groups

Group	MDA level (ng/ml)	p-value ^e
C	551.15±39.01 ^{a#}	<0.001*
R1	325.22±39.70 ^{b#}	
R2	310.04±34.14 ^{b#}	
R3	362.63±31.81 ^{b#}	

* $p < 0.05$ was considered statistically significant, ^ep-value from One-way ANOVA test, [#]Post hoc comparison using Tukey test. Values in a row with different superscript are significantly different.

Significant difference in callus diameter was observed between the control and treatment groups. There was an increment of size proportionate to the reperfusion interval. The one-way ANOVA test showed there was a significant difference of callus diameter between all groups. The post-hoc test, using Tukey test, showed there was a significant difference between group C and group R1, R2, and R3. However, there was no significant difference between all reperfusion groups. The callus diameter of all groups is depicted in table 2.

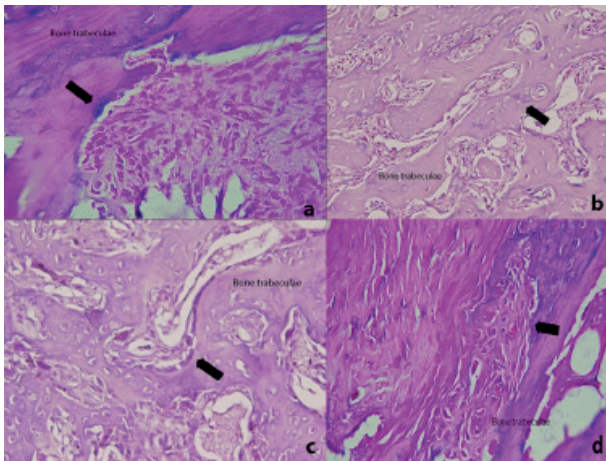


Figure 1. — Histopathologic examination of the callus tissue stained with hematoxylin eosin (HE) under 400x magnification. a) control (no reperfusion), b) group R1 (5 minutes reperfusion), c) R2 (10 minutes reperfusion), and d) R3 (15 minutes reperfusion); the black arrow points to osteoblast.

Table 2. — Callus diameter of control and reperfusion groups

Group	Callus Diameter (cm)	p-value [†]
C	0.33±0.013 ^{a#}	<0.001*
R1	0.42±0.04 ^{ba}	
R2	0.48±0.09 ^{ba}	
R3	0.48±0.05 ^{ba}	

*p<0.05 was considered statistically significant, [†]p-value from One-way ANOVA test, [#]Post hoc comparison using Tukey test. Values in a row with different superscript are significantly different.

Table 3. — Osteoblast cell count of control and reperfusion groups

Group	Osteoblast Count	p-value [†]
C	1.23±0.32 ^{a#}	<0.001*
R1	2.85±0.73 ^{ba}	
R2	5.47±1.20 ^{cb}	
R3	3.72±0.83 ^{ba}	

*p<0.05 was considered statistically significant, [†]p-value from One-way ANOVA test, [#]Post hoc comparison using Tukey test. Values in a row with different superscript are significantly different.

The lowest osteoblast cell count was observed in group C and the highest one was in group R2. The result of one-way ANOVA test also showed significant difference between all groups. The Tukey

test showed significant difference between group R2 and all other reperfusion groups. In contrast, there was no significant difference between group R1 and R3. The histopathologic sample results of each group are depicted in figure 1. The osteoblast cell count is presented in table 3.

Further Pearson correlation test showed that there was a strong inverse correlation between the MDA levels and the callus diameter ($r = -0.610$, $p < 0.05$). There was also an inverse correlation between the MDA level and the osteoblast cell count ($r = -0.734$, $p < 0.05$).

DISCUSSION

The concept of reperfusion interval used in this study is different from ischemic preconditioning applied in previous studies; ischemic preconditioning in those studies refers to a bout of intermittent ischemic and reperfusion periods before the definitive tourniquet-induced ischemia and the following reperfusion (15,16). In this study, the authors used only a brief reperfusion period in the middle of two tourniquet-induced ischemic periods, aimed to reflect a possible implementation intraoperatively.

The MDA level of the control group was significantly higher compared to the reperfusion groups. Cetinus et al. found that transient ischemic period would increase the MDA level in similar rat tibia fracture model (17). The animal model used in their study was quite similar to this study but the authors also observed the effect of different reperfusion interval to the MDA level. The damage caused by ischemia is proportionate to the duration of the ischemic period (18). In orthopedics surgery, longer duration of tourniquet application is proven to produce more oxidative damage (19).

Ischemia would induce increased production of ROS in skeletal muscle xanthine oxidase pathway, and the following reperfusion would lead to more ROS production (20). The implementation of ischemic pre-conditioning (IPC) is proven to lower the stress response caused by ischemic-reperfusion injury (16). The effect of IPC in orthopedic surgery has been explored in total knee arthroplasty (TKA) procedure, in which it may reduce the oxidative

damage to skeletal muscle (5). Zhang et al. concluded that the ischemic-reperfusion injury in the bone tissue is mediated by the plasma cascade system, like the process observed in various organs (9). However, the effect of ischemia and the following reperfusion on bone fracture healing is still undiscovered.

The reduction of MDA level in this study suggests that brief reperfusion interval may also attenuate oxidative insults not unlike ischemic preconditioning. Ischemic precondition provides protection to oxidative insult via activation of cell protective pathways and apoptosis abatement by activation of mitogen-activated protein kinase (MAPK) pathway and decrease of mitochondrial permeability transition pores (mPTPs) (21). Future studies are still needed to explore whether the phenomenon caused by a single brief reperfusion period is triggered by the same physiologic changes observed in ischemic preconditioning. The reperfusion period of 15 minutes also yielded higher MDA level compared to 5 and 10 minutes although their differences were not statistically significant.

Greater callus formation was observed in the reperfusion groups compared to the control group. Oxidative stress is known to increase osteoclast differentiation and induce apoptosis of osteocyte (22). As the reperfusion groups exhibit both lower MDA level and greater callus diameter, this result suggests that the reperfusion interval exerts its beneficial effect to callus formation by reducing oxidative stress and in turn reducing osteoclast activation and osteoblast suppression.

The osteoblast cell count in the reperfusion group was also comparatively higher than the control group. This supports the finding of greater callus formation in the reperfusion group, as osteoblast and the osteoinductive substances it produces are both determinants of callus formation (23). Interestingly, the highest cell count and the lowest MDA level was observed in group R2, which received a 10-minute reperfusion period and it was significantly different from other groups. Further investigation should examine the effect of longer reperfusion duration to oxidative stress.

There were no significant differences of the MDA level and average callus diameter between each

reperfusion groups, suggesting the interval of 5, 10, or 15 minutes are equally effective in preventing ischemic-reperfusion injury in animal models.

However, this conclusion is not supported by the osteoblast cell count, in which group R2 had significantly higher count. This phenomenon suggested that the interval of 5 minutes in the group R1 was not enough to mitigate the ischemic-reperfusion injury. On the other hand, the paradoxical lower osteoblast count after 15 min reperfusion might be attributed to the induction of cellular inflammation and ROS production during that 15 min lapse. The period of 10 minutes was more optimal to preserve the osteoblast in our study. Overall, the effect of the reperfusion interval in this study could possibly be explained by the fact that shorter ischemic-reperfusion duration would promote cell programming to control ROS formation and mitigate the cell damage (24). This study didn't include longer duration to reflect efficacy under the possibility of it being applied during operation, but future studies may explore the effect of longer reperfusion duration to better understand the effect of reperfusion interval.

This suggests that the lower the MDA level, the higher the number of osteoblasts could be found and the greater the callus diameter formed. This result is in accordance to a study by Domazetovic et al. in which ROS promotes osteoclastogenesis. Conversely, antioxidants may induce a beneficial effect on bone remodeling and even promoting the apoptosis of osteoclast (22).

Although this study showed that reperfusion interval had good effect in fracture healing, we still can't draw a clear conclusion whether it is truly caused by reperfusion interval or ischemic condition. Ideally, a negative control of unfractured rats could be used to measure the levels of MDA and cell counts against the positive control group to ascertain the ischemic effects of tourniquet. Other than that, subsequent research should evaluate the implementation of antioxidant agents to prevent or attenuate the deleterious effect of tourniquet use.

In conclusion, reperfusion interval can possibly reduce ischemic reperfusion injury in rat's fracture healing process as shown by the decrease in MDA

level, increase in the callus diameter and increase in osteoblast count of reperfusion interval groups.

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