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Effect of Interval Tourniquet Use on MDA Levels and Liver Histopathological Damage in the Management of Long Bone Fractures

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ABSTRACT

The use of arterial tourniquets as a tool for maintaining hemostasis in trauma has been widely used. The use of tourniquet serves to allow and accelerate operative procedures in musculoskeletal and vascular cases. In addition, torniquet is generally used to aid hemostasis in trauma cases. Providing torniquet perfusion time interval is known to reduce ischemic injury. However, the effect of reperfusion on the MDA level and histopathological damage of the liver has never been identified, thus requiring further research. This study determines the effect of the reperfusion interval in tourniquet use that causes reperfusion ischemic injury on the MDA level and histopathological damage of the liver in the management of long bone fracture. This study employed the true experimental method involving fractured Wistar rats. The Wistar rats were divided into 3 groups, group without reperfusion (P1), group with reperfusion of 10 minutes (P2), and group with reperfusion of 20 minutes. P1 ware treated with a tourniquet without being given a reperfusion interval for 3 hours. The rats in the P2 group were given a tourniquet with a reperfusion interval of 10 minutes after 2 hours. The rats in the P3 group were treated with a tourniquet with a reperfusion interval of 20 minutes after 2 hours of using the tourniquet, then the tourniquet was re-inflated for one hour. After 14 days, the rats were put down and analyzed for their MDA levels and their liver's histopathological damage. The statistical analysis used one-way ANOVA and Kruskal–Wallis with a significance level of $p < 0.05$. There was a difference in the reperfusion interval between P1, P2, and P3 groups regarding the MDA levels in the liver ($p < 0.05$). The reperfusion intervals of 10 and 20 minutes showed a higher reduction of the MDA level in the liver, compared to the group without reperfusion ($p < 0.05$). The reperfusion interval of 20 minutes showed a higher reduction of the MDA levels in the liver compared to P1 group ($p < 0.05$). There was a difference in the reperfusion interval between the group without reperfusion and the group with reperfusion regarding the liver's histopathological damage ($p < 0.05$). The use of reperfusion intervals of 10 and 20 minutes showed a higher reduction of cell ischemia and hepatic necrosis compared to the P1 group. The reperfusion interval of 20 minutes showed a higher reduction of the hepatic cell necrosis

compared to the reperfusion interval of 10 minutes ($p < 0.05$). The reperfusion interval in tourniquet use has an effect on reducing MDA levels and decreasing the number of ischemic and necrosis cells in the liver. There are differences in the duration of the reperfusion interval in the use of tourniquets in preventing ischemic injury.

1. INTRODUCTION

Bleeding from extremity injuries is the most common cause of preventable death in either the civilian population or on the battlefield [1]. The use of arterial tourniquets as a tool to aid hemostasis has been commonly used clinically in cases of trauma or in other cases [2]. More than 20.000 tourniquet are used per day by medical personnel to obtain a blood-free operating field such as orthopedic surgery, musculoskeletal reconstruction procedures and vascular surgery [3]. However, the use of tourniquets can also cause complications in the form of nerve paralysis, thromboembolism, and compartment syndrome. In addition, at the time of tear deflation, the return of blood circulation, restore tissue oxygenation, can also cause ischemic-reperfusion injury [4], [5]. In orthopedics, tourniquets are often used to reduce bleeding and keep the operating field clear at the time of surgery, for example in the case of surgery due to fractures. Middleton and Varian reported the incidence of complications of tourniquet use in Australia around 1970, which was about one in 5000 applications on the upper limb and one in 13,000 applications on the lower extremities [6- 8]. A fracture is a discontinuity of bone structure caused by trauma or a pathological condition.5 According to the World Health Organization (WHO), there were 13 million cases of fracture worldwide in 2008, with a prevalence rate of 2.7%. Meanwhile, in 2009 there were approximately 18 million people with a prevalence rate of 4.2%. In 2010 it increased to 21 million people with a prevalence rate of 3.5%. A study conducted by [9] at a hospital in Kathmandu, Nepal, from 1337 samples, it was found that the tibia and fibula were the most common bones that had fractures due to motor accidents with a total of 297 people (22%). In a study of patients with cruris fractures who had a traffic accident and were hospitalized at the Sanglah Central General Hospital, Denpasar for the period May 2015-April 2016. Most fractures were open fractures (61.4%) with the most frequent fracture locations in the cruris region. in the right (55.7%) and in the middle of the bone (35.2%). Tibia and fibula were the most fractured bones (62.5%) [9].

Ischemia is a condition in which there is disruption in blood flow which can result in tissue damage and organ dysfunction. Tissues that experience ischemia will experience a period of hypoxia and cause a decrease in ATP in the cells. By restoring blood flow (reperfusion) to ischemic tissue it is hoped that it will reduce tissue damage due to hypoxia, restore ATP production in cells, restore intracellular ion balance and restore tissue function. However, this reperfusion has its own effect which can cause further damage to ischemic tissue and also damage to other organs, through the formation of reactive oxygen species (ROS). This compound is a very potent oxidizing and reducing agent that results in oxidative stress conditions and can cause damage to cell membranes through lipid peroxidase. Oxidative stress is defined as an imbalance in the production of oxidants and the inability of the body to eliminate these oxidants by antioxidants.10 When lipid peroxidation occurs, aldehyde molecules will be formed, namely Malondialdehyde (MDA), 4-Hydroxynonenal (4-HNE) which can be biomarkers of lipid peroxidation, especially MDA [11]. Reperfusion of ischemic tissue is often associated with microvascular injury, particularly due to increased capillary and arteriolar permeability which leads to increased diffusion and the process of filtration of fluid

through the tissue. Activated endothelial cells produce more ROS resulting in an imbalance resulting in a further inflammatory response [12]. Leukocytes, which are carried to the injured area by the bloodstream, will release lysozyme, produce ROS, and release chemotactic agents which will attract more leukocytes. In addition, in the reperfusion phase an antibody complex will appear which will damage the cell membrane. The complement activation that occurs will cause degranulation of gold cells and the release of histamine and other chemical mediators. Ultimately ischemic-reperfusion injury will result in systemic damage [5], [13].

Systemic injury due to ischemic-reperfusion can occur in various organs, such as the lungs, heart, liver, kidneys, intestines, brain, skeletal muscles [14]. Several studies have shown a dysfunction of the liver, renal organs and damage to the lung organs due to bilateral lower leg ischemia accompanied by a reperfusion phase. As blood flow and oxygen supply are rebuilt, reperfusion increases injury caused by the ischemic period, exacerbating the damage caused at the cellular level. This phenomenon, known as ischemia-reperfusion (IR) injury, has a direct impact on the survival of organs, including the liver. During the ischemic period, some functional changes occur at the cellular level leading to cell injury. Decreased oxidative phosphorylation results in ATP depletion and disruption of calcium homeostasis. The adverse effects of modified ATP catabolism are further enhanced by the production of several substances, including reactive oxygen species (ROS), cytokines, adhesion molecules, and vasoactive agents (endothelin and thromboxan-A2). This change is accompanied by a decrease in cytoprotective substances including nitric oxide, prostacyclin, and others. Liver cell death occurs due to necrosis and apoptosis [15]. To reduce the systemic complications of tourniquet use, one of which can be done by providing a perfusion time interval with tourniquet deflation for a certain duration. This can be done by providing a reperfusion interval carried out for 10 minutes or 30 minutes after using a tourniquet for 2 hours [16- 19]. Several studies have shown that tourniquet deflation for a while, will provide reperfusion time and reduce skeletal muscle damage with prolonged use of tourniquets (more than 2 hours) [20]. Newman's research shows that giving a reperfusion duration of 10 minutes every hour of tourniquet use has a metabolic restoration effect and maintains adequate levels of ATP in the distal muscle tissue of the tourniquet [21]. [22] study showed that the presence of a reperfusion interval in the experimental group of rats for 20 minutes after experiencing ischemic for 1.5 hours, showed better blood flow in skeletal muscle compared to the group without reperfusion intervals [16- 19]. Based on this, a study is needed to determine the effect of the interval tourniquet use on the systemic effect of ischemic-reperfusion injury in the liver on the management of long bone fractures by analyzing MDA biomarkers and histological observation of the liver in white rats (Rattus novergicus).

2. Methods

This research is a True Experimental research on experimental male white rats (Rattus novergicus). The aim was to determine the effect of reperfusion interval tourniquet use on the liver organs of white rats (Strain Wistar Rattus Novergicus) that had long bone fractures, by looking at organ histology and levels of MDA.

Determination of research replication was determined by random sampling. The amount of replication is determined based on the calculation of the formula according to Supranto J (2000), as follows: At least 5 individuals/ group; Additional 20%/ minimum amount.23 Total number of rats used:

$$(T-1)\ (n-1) \geq 15 \text{ rats}$$

T, Treatment; n, amount of replication

The amount of replication in this study is a minimum of 7 rats for each group. So that a minimum of 21 rats

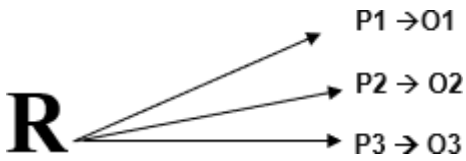
is needed for the three groups.

This research was conducted in several places, including: RSSA Surgery Section for proposal preparation and data processing; Laboratory of Parasitology, Faculty of Medicine, University of Brawijaya Malang for the maintenance of experimental animals, treatment, sacrifice of experimental animals; Physiological Laboratory of the Faculty of Medicine, University of Brawijaya Malang for measuring MDA levels; Laboratory of Pathology Anatomy, Faculty of Medicine, University of Brawijaya Malang for organ histology assessment. This research was conducted from December 2020 - April 2021.

The study inclusion criteria included:
1. White rat (Rattus norvegicus Wistar strain)
2. Male
3. Age 3-4 months
4. Weight 180-200 gr
5. Healthy, characterized by active movement and no limb defects

The study exclusion criteria included: Defects in the extremities; infection; Mice died before the study; Cast is broken / damaged during sampling.

2.1 Research Protocol



R = Randomization of all samples.
P1 = Control. Treatment with a tourniquet without reperfusion intervals for three hours.
P2 = Treatment with tourniquets with reperfusion intervals of 10 minutes after two hours of tourniquet use, then tourniquet inflation again for one hour.
P3 = Tourniquet treatment with a reperfusion interval of 20 minutes after two hours of tourniquet use, then tourniquet inflation again for one hour.
O1 = Observation of tourniquet groups without reperfusion intervals
O2 = Observation of the tourniquet group with a reperfusion interval of 10 minutes after two hours of tourniquet use, then the tourniquet inflation returns for one hour.
O3 = Observation of the tourniquet group with a reperfusion interval of 20 minutes after two hours of tourniquet use, then the tourniquet inflation returns for one hour

Experimental steps include: acclimatization; fracture action; tourniquet preparation; fixation with plaster of Paris; collecting of liver organ specimens; specimen examination.

2.2 Acclimatization

Acclimatization of experimental animals for 7 days in laboratory conditions. Conducted in a cage of 30 X 20 X 15 cm each 1 experimental animal with BR-1 feeding without any other additions. If there is food leftovers, the rest is discarded and then replaced with new ones. Rats were also given distilled water as needed and the husks in the cages were changed every day so that cleanliness was maintained.

2.3 Fracture action

1. The experimental animals were fasted for 3 hours before surgery, initiated by anesthesia with

ketamine hydroxychloride (ketalar) 40 mg / kg followed by prophylactic antibiotic cefazolin 5 mg / kg IM.
2. The operating field is cleaned by shaving and cleaned with a solution of savlon, 70% alcohol, and povidone iodine, covered with sterile linen.
3. After being sedated (indicated by the mouse's eyes starting to close, the movement becomes slow) the rats will undergo surgery, the operator wears sterile gloves and a gown as an asepsis procedure.
4. Anterior incision in the cruris shaft region of the mouse, deepen layer by layer until the bone
5. Fracture of the middle third of the tibia was performed with osteotomy using bone cutting forceps.
6. Wash the wound with sterile NS, and suture the wound with prolen 5.0 thread and cover it with a wound dressing.

2.4 Tourniquet preparation

Orthodontic rubber was tied around one leg of the mouse at the groin. Rubber was applied for 3 hours in the group without reperfusion interval treatment, while the treatment group was given reperfusion intervals for 10 and 20 minutes after 2 hours of use, then re-applied for 1 hour.

2.5 Fixation with plaster of Paris

1. Plaster of Paris of the tibia (lower leg) was placed using the long leg cast method (from 1/3 of the middle of the thigh to the ankle) in a straight position (full extend)
2. The experimental animals are put into their cages, with each day given food according to their habits. Rats received pain medication in the form of paracetamol 100 mg / kg if there were signs of pain in the form of lethargy, difficulty eating, and shivering.

2.6 Collecting of liver organ specimens

1. On the 14th day after treatment, the rats would be sacrificed using the cervical dislocation technique.
2. Then take the liver as a sample for examination of MDA tissue and histological preparations of anatomical pathology.
3. Mice that have been harvested the organs to be examined are confirmed to be dead.
4. Then the death rats were placed in a basin container. Experimental rats are buried in the ground with a minimum depth of 50 cm and a hole area of 0.25 m^2. Each hole is only used to bury 10 rats together, this is to prevent the rat carcasses from being dug up by other animals such as cats. The hole is closed again with soil and then the hole is compacted so that it doesn't smell.

Specimen examination

Malondialdehyde (MDA)

1. After taking the liver, the levels of Malondialdehyde (MDA) were measured by:
2. The sample of the hepatic organ is mashed, weighed 50-100 mg
3. Put in the Petri tube, add 1 cc phosphate buffer
4. Added TCA 100% of 1 cc
5. Added 1 cc of HCL of 1 cc
6. Added 1 cc of Na-Thio-Barbiturate as much as 1 cc
7. Heat with a water batch temperature of 100 degrees Celsius for 25 minutes
8. Conducted a centrifuge at a speed of 2000-3000 rpm for 15 minute
9. Take supernatant, diluted with aquades to 3cc
10. Read by spectrophotometry with a wavelength of 532 nm

(6.2) Histological Examination

1. The taken tissue is immersed in a medium containing 10% formaldehyde.
2. Dehydration process using alcohol: 70% alcohol for 1 hour; 80% alcohol for 1 hour; 90% alcohol for 1 hour; 95% alcohol for 1 hour; 99% alcohol for 1 hour; 100% alcohol for 1 hour
3. The clearing process is carried out by inserting the dehydrated material into the xylol solution for 2 x 30 minutes
4. The process of making blocks (Embedding) is carried out
5. Installing the block on the rotary microtome, then making thin cuts in a longitudinal direction with a thickness of 3 - 5 μm.
6. The results of the incision are then transferred into a water bath so that it can expand properly. After that it is transferred to the glass object that has been labeled.
7. Performed painting with Hematoxylin & Eosin (HE) covered with a glass cover.
8. Histological observation of the liver was carried out by quantitative counting per 10 small fields of view using an Olympus BX-51 dot Slide microscope with a 400x magnification Olympus XC10 camera. Then the calculation results are analyzed.

The data obtained were analyzed and displayed in tables and figures. The data obtained were tested for normality and homogeneity test. The differences in MDA levels between the three treatment groups were analyzed using the one-way ANOVA test which was then followed by the post-hoc test. The differences in the number of ischemia and necrosis cells in the three groups were analyzed using the Kruskal-wallis test and followed by the Mann Whitney test.

3. Results

3.1 Reperfusion interval analysis of tourniquet use on MDA levels

In this study, it was found that there were significant differences in liver MDA levels in the three groups of the One-way ANOVA test. The results of the post-hoc test for MDA levels were significant in each group. The MDA level in the P1 group was higher than the MDA level in the group with reperfusion intervals. The lowest MDA levels were found in the group with a reperfusion interval of 20 minutes (P3). (Table 1)

Table 1. Differences in liver MDA levels based on treatment groups

Variable	Organ	Group	Mean ± SD	*p-value*
MDA	Liver	P1	36.03 ± 8.98^{a}	<0.001
		P2	16.61 ± 3.85^{b}	
		P3	4.22 ± 1.56^{c}	

Different notations indicate significant differences (a, b, c); P1: treatment without reperfusion; P2: treat with reperfusion for 10 minutes; P3: treat with reperfusion for 20 minutes

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3.2 Reperfusion interval analysis of tourniquet use on liver histopathology

In this study, there were significant differences between the three groups in the types of ischemia and necrosis cells using the Kruskal Wallis test. Therefore, it was continued using the Mann Whitney test. The percentage of the number of ischemic cells in the P1 group was higher than the reperfusion interval treatment group. There was no significant difference in the number of ischemic cells between the P2 and P3

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groups. The percentage of the number of necrosis cells in the three treatment groups also showed a significant difference. The percentage of necrosis cells in the P1 group was higher than the reperfusion interval group. The percentage of the number of necrosis cells in the P3 group was lower than the P2 and P1 groups. (Table 2)

Table 2. Difference in the number of ischemia and necrosis cells in the liver based on the treatment group

Liver Histopathology	Groups (n=7)	Median (min-max)	*p-value*
Ischemia	P1	57.5 (29-67)[a]	<0,001
	P2	15 (5-25)[b]	
	P3	11 (6-23)[b]	
Necrosis	P1	43.5 (19-72)[a]	<0,001
	P2	5.5(1-13)[b]	
	P3	1.5 (0-5)[c]	

Different notations indicate significant differences (a, b, c); P1: treatment without reperfusion; P2: treat with reperfusion for 10 minutes; P3: treat with reperfusion for 20 minutes

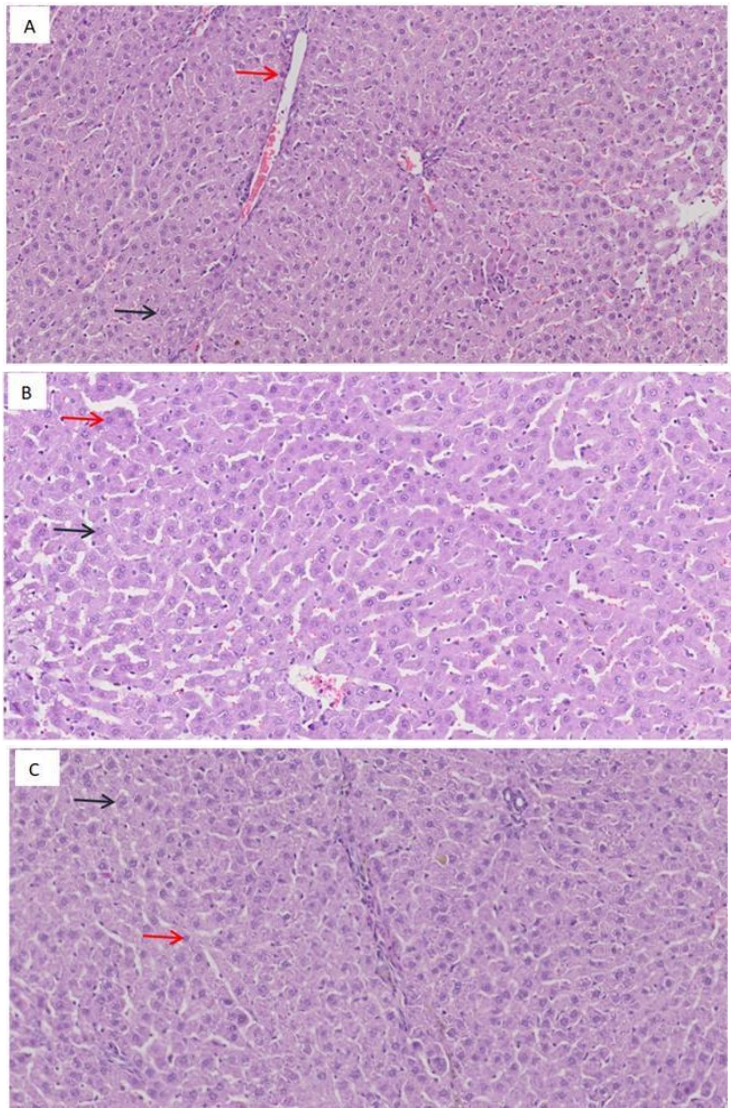

Figure 1. Histopathology of the liver

(A) P1; (B), P2; (C), P3; blue arrows indicate ischemic cells; red arrows indicate cell necrosis.

This study showed that there was an increase in the percentage of necrotic cells in the P1 group compared to the P2 and P3 groups. In addition, there was an increase in the percentage of ischemia from the P1 group compared to the P2 and P3 groups.

4. Discussion

4.1 Effect of reperfusion interval of tourniquet use on MDA levels

Based on the results of the data analysis test, it was found that the liver MDA levels in both the P2 and P3 treatment samples proved to be significantly lower than the P1 group. The P3 group was also significantly lower than the P2 group. This suggests that the administration of reperfusion intervals at the tourniquet insertion has been shown to reduce the ischemic injury of hepatocyte cells in the liver in white wistar rats with tibia fractures. In a fracture, there is damage to the surrounding tissue, including damage to muscle tissue and blood vessels, including arteries, veins and capillaries. Excessive blood loss can lead to hypovolemic shock which is fatal, such as death of surrounding tissue cells which can lead to amputation, organ damage or even death. The use of a tourniquet is intended to stop or control bleeding [24]. However, the use of a tourniquet that lasts more than 2 hours has been shown to interfere with the process of oxygenation chain formation and ATP formation in the mitochondria.4 If it lasts long, the surrounding tissue will induce ischemia. In addition, disturbances in mitochondria will trigger the production of ROS (reactive oxigene species). Reperfusion after use of the tourniquet can also cause injury that can lead to the formation of ROS. ROS will cause excessive inflammatory reactions and further damage to cells and organs further. ROS can also affect the process of the electron transport chain and damage the tissue through lipid peroxidase which is characterized by an increase in MDA [4], [24]. Ischemic injury/ hepatic reperfusion occurs in two phases, namely the initial phase and the final phase. In the early phase, ischemia occurs due to reduced blood flow to the liver during the use of the tourniquet so that nutrient intake and oxygen levels of hepatic tissue cells are also reduced. In the early phase, which is relatively fast, the tissue hypoxia can still be tolerated by the liver. During this phase, liver cells will produce intracellular xanthine oxidase and NADPH. Shortly after reperfusion, kupffer cells are activated by complement, high mobility group box 1 (HMGB1), and IL-23. Activation of Kupffer cells will produce (Reactive oxygen species) ROS such as superoxide anion and hydrogen peroxide. ROS will induce oxidative stress to the liver and other distant organs and produce cytokines that cause injury to the parenchyma and blood vessels. In addition, reduced NO levels will reduce endogenous antioxidants such as superoxide dismutase (SOD) in mitochondria and increase superoxide.Error! Reference source not found. The imbalance between the production of endothelin- 1 and nitric oxide (NO) from NO synthase (NOS) will also cause sinusoid vasoconstriction.

The excessive inflammatory response through the production of local cytokines (IL-1b, IL-17 and TNF-α) is a precursor to the final phase of reperfusion injury. This phase is a cellular phase characterized by the activation and migration of neutrophils, CD4+ T lymphocytes, and platelets into the liver. Cell surface adhesion molecules such as intracellular cell adhesion molecules (ICAM) and vascular cell adhesion molecules (VCAM) will also be expressed by endothelial cells and hepatocytes in the inflammatory process. These pro-inflammatory molecules will become trapped and attached to the sinusoids. This situation causes an excessive inflammatory reaction to constriction of the sinusoids. In addition, sinusoid constriction also causes platelets and neutrophils to become trapped and adhere to endothelial cells, causing hepatocyte injury and microvascular failure. CD4+ T lymphocytes produce granulocyte colony stimulating factors for macrophages, interferon gamma, and TNFα which enhance the activation of Kupffer cells and release of cytokines. Microcirculation failure worsens and prolongs ischemia because hepatocyte cells

remain hypoxic and in a state of oxidative stress [26]. ROS will react with cell membranes through fat peroxidation and produce MDA as a sign of tissue damage, especially in endothelial cells so that it affects platelets and leukocytes. A significant increase in MDA levels will appear 5 minutes after reperfusion. Another study showed a significant increase in MDA levels occurred on days 7 and 14 after tibial fracture of experimental animals. They concluded that oxidative stress occurred at weeks 2 and 3 after fracture [27], [28]. This situation occurred in the control group where the liver tissue damage in wistar rats tibial fracture model due to ischemia-reperfusion injury was shown with very high MDA results. In P2 and P3, MDA levels decreased after the tourniquet interval was applied. The MDA level of normal rats was 0.2202 0.03731. The research of Proved that the installation of a tourniquet for 3 hours and an interval reperfusion for 5-15 minutes after the first 2 hours was shown to reduce MDA levels in the lung organs [24]. In the study of Also described rats with tibial fractures that were treated with a tourniquet for 1 hour and subjected to a tourniquet inflation/ deflation for 10 minutes which was shown to accelerate bone healing and reduce tissue damage by increasing cytokine production and increasing tissue tolerance to hypoxia [29]. The initiation of tourniquet deflation should be undertaken. This supports the findings of this study that MDA levels in the liver IR injury were reduced in the presence of the tourniquet reperfusion interval.

In general, the damage to the liver's ability to tolerate tissue injury is better than other organs. However, if there has been damage to the liver tissue, it is certain that there has been damage to other tissues. The use of a tourniquet to induce ischemia should not be more than 1 hour, because it will cause the death of more tissue cells [30]. The results of this study are supported by previous research on human and experimental animal research subjects. Research by [24] shows that giving a reperfusion interval can prevent an increase in MDA in Wistar rats. This study used 4 groups, namely control, 5 minutes, 10 minutes, and 15 minutes of repertusion intervals in fracture models. The results in this study indicated that there was a decrease in liver MDA levels compared to controls along with the increase in the reperfusion interval [24]. Similar results were obtained for human research subjects. Research by showed that giving reperfusion intervals to patients with lower limb injuries can reduce serum MDA levels [31]. This suggests that the provision of reperfusion intervals plays an important role in liver MDA. Patients with lower limb fractures are thought to have liver damage if the reperfusion interval is not given. In clinical conditions, liver damage can be measured using AST and ALT levels. Reperfusion interval is thought to prevent the increase in AST and ALT in fracture patients.

4.2 Effect of reperfusion interval on tourniquet use on liver histopathology

Based on the results of the study, it appears that the liver histopathological features of the P1 group were significantly different from those of the P2 and P3 groups. Both the percentage of ischemic cells and necrotic cells in the P2 and P3 treatment groups appeared to be less than the P1 group. Meanwhile, the observation of ischemic cells in the P2 and P3 groups did not show a significant difference. In the observation of the histopathological results of the necrosis of the cells in the treatment group, it looked significantly different with a decrease in the percentage of necrosis reaching 73.6% in the P3 group compared to the P2 group. The percentage of ischemic and necrotic cells observed in the P3 group was less than the P2 group. This is directly proportional to the decrease in MDA levels that are less and less in the P3 group. All rats in all groups experienced liver ischemia and necrosis except for the P3 group which experienced necrosis in 5 rats. In this study, the percentage of necrosis cells was obtained from the comparison between cells that did not have a nucleus compared to all cells seen in the field of view. The percentage of necrotic cells is obtained from the ratio of cells that have large vacuoles compared to all cells seen on the preparations. Hepatocyte cell damage begins with a degeneration process either due to ischemic or reperfusion. Based on research (Himawan, 2003), cell damage in rat liver includes hydropic

degeneration, fat degeneration, amyloid degeneration, cloudy swollen degeneration, glycogen degeneration, and necrosis. Ischemic cells observed were hepatocyte cells that looked pale accompanied by vascular congestion (the presence of dilated blood vessels in the liver) [32]. In this situation the cells are deprived of nutrients and oxygen due to the ischemic injury reperfusion of the liver from using a tourniquet for 2-3 hours. Reperfusion conditions will trigger oxidative stress by producing free radicals in the form of ROS and excess inflammatory processes. ROS will change the nature and cell membrane and cytoplasmic elements of cells such as mitochondria and lysozyme through lipid peroxidase. Free radicals can react with DNA thymine in the cell nucleus to damage DNA and trigger p53 activation. This condition will activate the execution caspase and then activate endonuclease and latent cytoplasmic protease so that it degrades cytoskeletal and nuclear proteins. Subsequently a cascade of intracellular degradation occurs, including breakdown of the cytoskeleton and endonuclease-mediated fragmentation of nuclear chromatin. The end result is the formation of apoptotic bodies containing various intracellular organelles and other cytosolic contents. The apoptotic bodies express new ligands that mediate the binding and uptake of phagocytic cells [33], [34].

The abnormal cell structure will end in necrosis. Cells that experience necrosis will secrete various inflammatory mediators and attract inflammatory cells. Hepatic cells that are hypoxic in the ischemic phase will continue to become necrotic if microvascular failure lasts longer. The morphology of necrotic cells changes by increased eosinophilia, vacuoles in the cytoplasm after the digestive system cytoplasmic organelles, and nuclear including pyknosis, karyorrhexis, and karyolysis.33 In pycnosis, the cell nucleus shrinks and a "dark cloud" appears due to the solidified chromatin. In karyorrhexis, the cell nucleus is destroyed, leaving the largest fraction in the nucleus. karyolysis occurs when the cell nucleus is lost or lysis so that it appears as an empty cell [35].

In addition, the number of ischemic cells and necrosis in the liver tissue are thought to be related to the tissue repair process based on time. Improvement of the degree of liver tissue damage was generally obtained on day 14 after discontinuation of exposure. This improvement is thought to be due to the liver regeneration process originating from mature hepatocytes and liver progenitor cells (oval cells). Regeneration by mature hepatocytes takes place much faster than oval cell regeneration. Mature hepatocyte cells have been shown to play a role in liver regeneration after partial hepatectomy. All classes of hepatocytes, including diploid, tetraploid, and octaploid cells participate in this regeneration, either by mononuclear cell mitosis or through binuclear or tetranuclear hepatocyte cytokinesis, after DNA synthesis in all nuclei. Oval cells will play a role in replacing mature hepatocytes when the proliferation of mature hepatocytes is inhibited by certain toxic substances or physical injuries [34]. In this study, we did not use rats without fracturization so that it could not compare the histopathological effect with normal controls. It is necessary to carry out further research on the measurement of superoxide dissmutase levels for oxidative stress, which is a major factor in hepatic reperfusion ischemic injury; The use of negative controls without fractureization was used to compare MDA levels and normal histopathology; and measuring the levels of the SOD enzyme so that it can be ascertained that oxidative stress is a major factor in ischemic reperfusion hepatic injury.

5. Conclusions

According to this research result, it could be concluced that:

1. The reperfusion interval in the tourniquet use has an effect in reducing liver MDA levels.
2. The reperfusion interval in the tourniquet use decreases the number of hepatic ischemia cells.
3. The reperfusion interval for tourniquet use reduces the number of hepatic necrosis.
4. There is a difference in the duration of the reperfusion interval in the use of tourniquets in

preventing ischemic injury.

Ethical Clearance
The Ethical Clearance from Health Research Ethics Commission of Faculty of Medicine, Brawijaya University Malang number: 63/EC/KEPK/03/2020 on March 10th, 2020.

Conflict of Interest
There is no conflict of interest.

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Author Contribution
All authors are contributed to conceived and design the analysis, cellected the data; contributed data and analysis tools; performed analysis and wrote the paper.

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