Original Research

THE EFFECT OF DICLOFENAC SODIUM ON CALLUS FORMATION IN WHITE MALE RAT (*Rattus norvegicus*) CRURIS FRACTURE HEALING

Herry Wibowo[1], Prihartini Widiyanti[2]
[1]Physiology Laboratory, Department of Biomedicine, Faculty of Medicine, Universitas Surabaya, Surabaya, Indonesia
[2]Biomedical Engineering Study Program, Department of Physics, Faculty of Science and Technology, Universitas Airlangga/Institute of Tropical Disease, Universitas Airlangga, Indonesia

ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs), such as diclofenac sodium, are standard treatments to relieve pain associated with bone fractures. The bone healing process consists of four stages: inflammation, soft callus formation, complex callus formation, and bone remodeling. Previous studies mentioned that intake of NSAIDs (sodium diclofenac) could inhibit the bone healing process. This study examined the effect of diclofenac sodium intake on callus formation in fracture healing. In this study, thirty-six rats (Rattus Norvegicus) with fractures were used and divided into two groups, namely 18 rats for the control and 18 rats for the treatment group. In the treatment group, each rat was given 1.8 mg sodium diclofenac/150 grams of body weight per day. In the control group, each rat was given CMC-Na 0.5% with equal volume as diclofenac sodium in the treatment group. After 28 days, all the rats were stunned until dead, and the diameter and strength of their calluses were measured. In the treatment group with diclofenac sodium1.8 mg/ 150 grams BW/ 28 days after the tibia bone callus was pressed using the Shimadzu tool, the lowest callus strength was found to be 56.500 N, and the highest callus strength was 59.000 N. The lowest callus diameter in the treatment group was 4 mm, the highest was 5 mm. In the control group, the lowest callus strength was 76 N, and the highest callus strength was 77 N. The lowest callus diameter in the control group was 6 mm, and the highest was 8 mm. The strongest callus in the treatment group was found in the ^{sixth} observation, with a value of 59 N and a diameter of 4 mm. In the control group, the highest callus strength was 77 N, with a diameter of 7-8 mm. These measurements were found on the 5^{th}, 7^{th}, 8^{th}, 9^{th}, 16^{th}, and 17^{th} observations. Diclofenac sodium with a dose of 1.8 mg/150 grams of body weight could decrease the callus quality parameters, such as callus strength and diameter on fracture healing.

***Keywords**: Bone fracture; sodium diclofenac; Rattus norvegicus; callus strength; callus diameter*

ABSTRAK

Non-steroidal anti-inflammatory drugs (NSAID), seperti sodium diklofenak merupakan pengobatan yang umum dilakukan untuk menghilangkan rasa sakit pada patah tulang. Penelitian sebelumnya menyatakan bahwa NSAID (natrium diklofenak) dapat menghambat proses penyembuhan tulang. Proses penyembuhan tulang sendiri terdiri atas empat tahap, yaitu inflamasi, pembentukan kalus lunak, pembentukan kalus keras, dan remodeling tulang. Penelitian ini bertujuan untuk mengetahui pengaruh natrium diklofenak terhadap pembentukan kalus pada penyembuhan patah tulang. Pada penelitian ini digunakan 36 ekor tikus (Rattus norvegicus) yang mengalami patah tulang, dimana objek penelitian dibagi menjadi 2 kelompok yaitu kelompok kontrol 18 ekor dan kelompok perlakuan 18 ekor. Setiap tikus pada kelompok perlakuan diberi natrium diklofenak 1,8 mg/150-gram BB per hari, sedangkan pada kelompok kontrol masing-masing tikus diberi CMC-Na 0,5% dengan volume yang sama dengan natrium diklofenak pada kelompok perlakuan. Setelah 28 hari, semua tikus distunning sampai mati. Kemudian diameter dan kekuatan kalusnya diukur. Pada kelompok perlakuan dengan sodium diklofenak 1,8 mg/150-gram BB/28 hari setelah kalus tulang tibia ditekan menggunakan alat Shimadzu, kekuatan kalus terendah 56.500 N dan kekuatan kalus tertinggi. adalah 59.000 N. Diameter kalus terendah pada kelompok perlakuan adalah 4 mm, tertinggi adalah 5 mm. Pada kelompok kontrol kekuatan kalus terendah 76 N dan kekuatan kalus tertinggi 77 N. Diameter kalus terendah pada kelompok kontrol 6 mm dan tertinggi 8 mm. Kalus terkuat pada kelompok perlakuan terdapat pada kelompok kontrol. Pengamatan ke-6, dengan nilai 59 N dan diameter 4 mm. Pada kelompok kontrol, kekuatan kalus tertinggi adalah 77 N dengan diameter 7-8 mm. Penelitian ini menemukan bahwa pengamatan pengukuran ke-5, 7, 8, 9, 16, dan 17 natrium diklofenak dengan dosis 1,8 mg/150-gram berat badan dapat menurunkan parameter kualitas kalus seperti kekuatan kalus dan diameter kalus pada penyembuhan fraktur.

***Kata Kunci:** Patah tulang; sodium diklofenak; Rattus norvegicus; kekuatan kalus; diameter kalus*

Correspondence: Herry Wibowo. Laboratory of Physiology, Department of Biomedicine, Faculty of Medicine, Universitas Surabaya, Surabaya, Indonesia. Email: drherrywibowo@staff.ubaya.ac.id

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INTRODUCTION

In daily practice, non-steroidal anti-inflammatory drugs (NSAIDs) are widely used to treat pain due to fractures (Akman et al. 2002). In America, more than 35 million NSAIDs are prescribed each year, and more than 1% of the population in America uses NSAIDs. In Australia, more than 20% use NSAID drugs as anti-pain and anti-inflammatory drugs.

Bone fracture cases usually happen with pain. Thus, NSAIDs, such as diclofenac sodium, relieve pain, heat, and swelling through prostaglandin synthesis inhibition (Maroon et al. 2010). The oral application of diclofenac sodium significantly extends the fracture healing period (Bissinger et al. 2016).

Prostaglandins are formed mainly in the fracture site in the inflammation and soft callus formation stage. They are formed in the healing process, stimulate osteoclast accumulation, and increase activity (Lisowska et al. 2018). Cyclooxygenase (COX) is a rate-limiting enzyme that converts arachidonic acid to prostaglandin H2 as the precursor of several molecules, including prostaglandins, prostacyclin, and thromboxanes (Moro et al. 2017). Diclofenac sodium intake inhibits the cyclooxygenase enzyme. In a previous study, NSAID intake interfered with fracture healing (Suhana 2002). NSAID was caused by the disruption of osteoclast and osteoblast activities which decreased callus quality and fracture healing. Mefenamic acid intake for handling pain associated with fracture inhibited prostaglandin synthesis (Kress et al. 2016).

Tissue damage and hematoma are present in the fracture site. Prostaglandins are formed in the inflammation stage (Phase II). They are also secreted in the soft callus stage (Phase III). The prostaglandins increase osteoclast activity and stimulate new osteoclast accumulation. Dead bone tissue is cleaned from the fracture site and followed by new blood vessel formation, osteoblast placement, active substance release, and new bone matrix formation. Osteoblast activity is preceded by osteoclast activity, and if there is a disturbance in osteoclast activity, osteoblast activity is disturbed, too (Shapiro et al. 2014).

Diclofenac sodium intake inhibits osteoclast activity. It can disturb the osteoblast placement. This study observed the effect of diclofenac sodium intake on callus formation in fracture healing and proved that diclofenac sodium intake could decrease callus quality (diameter and strength) of cruris fractures in rats. The significance of this study was related to the scope of the role of diclofenac sodium in bone healing. Therefore, this study focused on diclofenac sodium's physiological and pharmacologic role in bone healing.

MATERIALS AND METHODS

Data from experimental research on the effect of diclofenac sodium on callus quality on fracture healing of male white rat cruris were the result of measuring the strength and weight of tibia bone callus in experimental animals were measured using Shimadzu and Spencer's Dissecting microscope. The data were described and processed using the SPSS 10.0 program. Group 1 received treatment with diclofenac sodium 1.8 mg/200-grams BW/ day for 28 days. Group 2 was the control group.

Table 1. Descriptive data of callus strength (N) and callus diameter (mm) from the treatment group and control group

	Treatment group	Control group
Lowest callus strength	56,500 N	76,000 N
Highest callus strength	59,000 N	77,000 N
Mean of callus strength	57,556	76,556
Standard deviation of callus strength	0,684	0,379
Lowest callus diameter	4,000 mm	6,000 mm
Highest callus diameter	5,000 mm	8,000 mm
Mean of callus diameter	4,556	7,333
Standard deviation of callus diameter	0,511	0,594

Table 1 explains that the lowest callus strength measured in observations made in the treatment group was recorded at 56.5 N, while in the control group was recorded at 76 N. In the observations to measure the highest callus strength recorded in the treatment group, it was 59 N. In contrast, in the control group, the highest strength was recorded at 77 N. The data for the lowest callus diameter in the treatment group was recorded at 4 mm and the control group was 6 mm. In contrast, the highest callus diameter observed in this study obtained 5 mm in the treatment group and 88 mm in the control group. In this study, diclofenac sodium concentration was the free variable. Callus diameter and strength were the bound variables. The control variables were the animal type, gender, physical condition, and animal care. The body weight of the animals was the moderate variable.

The treatment and care of each rat were carried out in a 30x20x15 cm cage. The cage was made from plastic and closed by woven wires. Each cage had husk bedding. Every day, the husks were replaced to keep the cages clean. Thirty-six rats were randomly allocated into two groups: group 1 as the treatment group and group 2 as the control group, with 18 rats in each group.

The intake of 1.8 mg sodium diclofenac/150-grams of body weight per day (Reynolds 1993) was performed

in the treatment group, while the control group was treated with CMC-Na 0.5%. Diclofenac sodium was given by sonde by using a size 8 nasogastric tube. The sonde was inserted through the rat's mouth to the stomach. The drug solution volume given to each animal was 2 ml (Donatus & Nurlaila 1986).

All thirty-six rats were stunned with an ether solution in a hood. Anesthesia was performed with a titration method. The anesthesia began 2 minutes after the rats closed their eyes and slowed their movements. Then, the factorization and immobilization of one side of its lower limbs were performed.

Figure 1. Fracture making process of the tibial bone of the rat

After factorization, group 1 was given 1.8 mg sodium diclofenac/150-grams daily by sonde. The duration of the intake was 28 days. Group 2 was treated with a placebo solution of CMC-Na 0.5% using sonde with the same volume as the diclofenac sodium in group 1. On day 28, all rats were sacrificed with stunning (inner anesthesia) until the rats died. Furthermore, the diameter of each callus was measured in millimeters with a dissecting microscope Spencer® type 501909 manufactured by American Company Instrument Division Buffalo, New York, USA. The strength of each callus was measured with Shimadzu Autograph in newtons (N). The Ethical Committee had approved this protocol of the Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia.

RESULTS

The callus strength was calculated using a three-point bending test to find the perpendicular load. The lowest callus strength in the treatment group was 56.5 N, while the control group was 76 N. The highest callus strength in the treatment group was 59 N, while the control group was 77 N. The average callus strength of the treatment group and the control group were 57.556 N and 76.556 N, respectively (Figure 2). The standard deviations of callus strength in the treatment and control groups were 0.684 and 0.379.

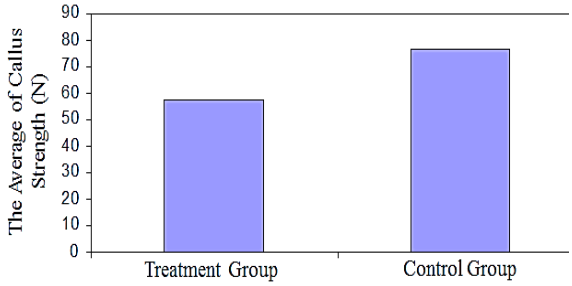

Figure 2. The results of callus strength

The callus diameter was obtained by measuring the distance between two calli through their center.

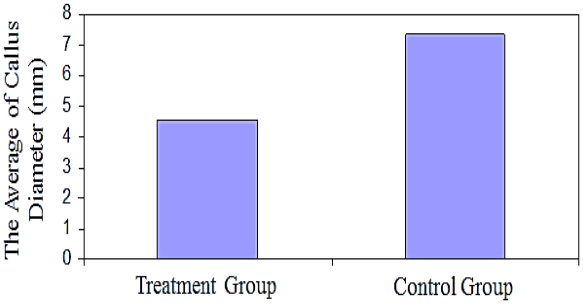

Figure 3. The average callus diameter

The lowest callus diameter in the treatment group was 4 mm and the control group was 6 mm. The highest callus diameter in the treatment group was 5 mm, and the control group was 8 mm. The average callus diameter of the treatment group was 4.556 mm, while the control group was 7.333 mm. The standard deviations of callus diameter in the treatment and control groups were 0.511 and 0.594, respectively.

A homogeneity test was performed to test whether the variance of the sample was different. This test was carried out by using Levene's test, and the result was shown that the body weight of the rats in the treatment and the control groups was homogeneous ($p>0.05$). A normality test was performed to observe whether the data were normally distributed. A normality test on callus strength and diameter was performed using the Kolmogorov-Smirnov test. The Kolmogorov-Smirnov test showed that the callus strength data were normally distributed because the probability value was more than 0.01, while the callus diameter data were not normally distributed because the probability value was less than 0.01.

A t-test was performed to determine whether the sample's average was different. There was no difference between the body weight of the rats in the treatment group (diclofenac sodium) and that in the control group (no diclofenac sodium). That could be

seen from the significance level t-test of 0.411, which was more significant than the tolerance for error (α=0.05). The callus strength data was normally distributed so that the t-test could be performed. According to the t-test result, the significance level was less than 0.01, which concluded a significant difference between the average callus strength of the treatment group and the average callus strength of the control group. According to the test, the Z calculation was more significant than the Z table, with a 1% error. It was concluded that there was a significant difference in the average callus diameter between the treatment and control groups due to the intake of sodium diclofenac.

DISCUSSION

In a previous study, the intake of NSAIDs interfered with the fracture healing process through disturbance in osteoclast and osteoblast activities (Suhana 2002). This method impacts this research on the mechanism of NSAIDs interference to fracture healing. NSAIDs act by inhibiting the production of PGs. PGs participate in inflammatory responses and increase osteoclast and osteoblast activity, bone resorption, and new bone formation (Harder & An 2003). Another study was performed using Wistar-strain rats with transverse osteotomy on the left proximal tibial bone (Beck et al. 2003). The rats were divided into four groups, with ten rats in each group. Group 1 was the control group, group 2 was treated with tramadol (20 mg/kg BW/day), and group 3 was treated with diclofenac sodium(5 mg/kg BW/day) for seven days and continued for 14 days without any drug intake. Group 4 was treated with diclofenac sodium(5 mg/kg BW/day) for more than 21 days. On day 21, the rats were sacrificed, and each leg was examined under an X-ray. Their tibial bones were examined under a CT scan, the three-point bending method, and histology.

The previous study showed that the rats in group 3 experienced inhibition of fracture healing compared to the rats in group 2 and the rats in the control group. Fracture healing was evaluated through bone density and bone strength parameters. The highest bone strength was obtained from group 1 and the control group. The rats that received diclofenac sodium therapy (group 3 and group 4) had lower bone density levels, bone strength, and stiffness than those that received tramadol therapy (group 2). The study concluded that diclofenac sodium significantly inhibited fracture healing in rats.

The effect of diclofenac sodium on the union of tibial fractures in rats was also performed (Akman et al. 2002b). In the study, there were three groups: a control group, a group with an intake of 1 mg/kg BW/day, and a group with an intake of 2 mg/kg BW/day. Closed diaphyseal fractures were performed on the right tibial bone of rats. Then, clinical, radiological, and histological tests were conducted after the second week, the fourth week, and the sixth week. At the end of the second week, the clinical test showed a difference between the treatment and control groups. The control group produced a more stable callus.

Diclofenac sodium triggers changes in bone metabolism and fracture healing because diclofenac sodium affects the healing inflammation phase (Zhou et al. 2018). By inhibiting cyclooxygenase, diclofenac sodium decreases the first synthesis of the inflammation mediator (Pratiwi & Kertia 2019), including prostaglandin, which is responsible for chemotaxis in the first phase of fracture healing. It also decreases the cell number in the fracture site, absorbs the tissue again, and allows the modification of the number of cells for callus formation (Santos et al. 2017). As the cause of prostaglandin inhibition and thromboxane synthesis, NSAIDs could affect neoangiogenesis, resulting in lower oxygen allocated to the mesenchymal cells. In the healing process, there is a tendency to differ between chondroblast and fibroblast, which are responsible for extracellular matrix synthesis. Thus, the immature and less mineralized bone callus will be produced (Painter et al. 2006).

Systemic and non-systemic factors that affected bone remodeling were explained in the literature (Painter et al. 2006). One of the most critical factors in bone healing is several pharmacological agents. Steroids, chemotherapy drugs, and some antibiotics have been reported to affect bone healing negatively. Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most commonly prescribed drugs for pain relief and inflammation. However, they have also been found to have the potential to delay and inhibit fracture healing (Pountos et al. 2008). NSAIDs affect the osteoblastic cell cycle and cell death. A study showed that osteoblastic density was significantly decreased in groups exposed to diclofenac sodium compared to the control group (Hadjicharalambous et al. 2021). The osteoclastic densities were found to be statistically significantly higher in a group exposed to diclofenac sodium than in the control group ($p< 0.05$). The osteoblastic densities showed a statistically significant decrease in groups with exposure to diclofenac sodium compared to the control group ($p< 0.05$).

In this study, the intake of diclofenac sodium diminished the callus quality. These results were observed by examining callus strength and diameter in male Wistar rats. Based on the result of the t-test, the Z calculation was more significant than the Z table, with a 1% error level. There was a significant difference in the average callus diameters between the treatment and

control groups due to diclofenac sodium intake. The callus diameter and callus strength decreased in line with the theory that the intake of NSAIDs could delay bone regeneration by inhibiting the prostaglandin at an early stage of healing as relevant to the findings of the delay of callus maturation (Krischak et al. 2007). One of the shreds of evidence was confirmed by the study result that diclofenac sodium with an intake of 1.8 mg/150- grams could decrease the quality (diameter and strength) of fracture healing callus. This finding could strengthen the theory about the effect of diclofenac sodium on the bone mechanism to guide the usage of medicament wisely and gain the best healing action.

CONCLUSION

Diclofenac sodium at a dose of 1.8 mg/200g could reduce the callus strength and diameter as indicators of callus quality of fracture healing. Further research is needed to perform by involving biochemical measurement parameters and the osteocalcin levels. Osteocalcin has a role in the body's metabolic regulation to enhance osteoblasts' activity during bone healing.

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2. Chapter in a book

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***3. Homepage/Web site* [Edited 12 May 2009]**

Cancer-Pain.org (2002). New York: Association of Cancer Online Resources, Inc.; c2000-01. [updated 2002 May 16]. Available from http://www.cancer-pain.org/. Accessed July 9, 2002

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