Histomorphometric Assessment of Fracture Healing in Contaminated Segmental Fracture after Sterilization using Microwave Irradiation: An experimental study on Wistar rats

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Abstract. Open fractures with large bone fragment is major problems. Preservation of bone fragment is essential for fracture healing however infection due to contaminated fragment is a challenging threat. Restoration of contaminated bone fragments requires prior sterilization to prevent infection. A 7-minute microwave irradiation has been proven sterilized a contaminated bone fragment however the effect of irradiation toward incorporation to normal bone has not been established. This study investigates the effect of microwave irradiation toward fracture healing in 16 Wistar rats with segmentally contaminated fracture. The rats randomly assigned into two groups. In control group the segmental fragment was directly reimplanted and stabilized using intramedullary K wire while in treatment group the bone fragment was inoculated with Staphylococcus Aureus ATCC 25923 and exposed to 7 minutes microwave irradiation prior to reimplantation and K wire stabilization. At 2nd and 4th week, healing process was assessed using histomorphometric analysis (total fibrous tissue area, cartilage formation and woven bone area). At week 2, there is significant differences in formation of cartilage (p=0.010) and woven bone (p=0.004) between control and treatment group with treatment group has smaller size. At week 4, there is no statistically significant difference found between the treatment and control groups. Interval between week 2 and week 4 is the ideal time to compare normal process of fracture healing to fracture healing under treatment in Wistar rat. Microwave irradiation serve as a means to eliminate the infection yet preserve the nonorganic environment that eventually delivers conducive environment for fracture healing.

1. Introduction

Open fracture can cause complications particularly the risk of infection due to the contaminated free bone fragment [1-3]. While management of an open fracture by doing adequate debridement, administering antibiotics and stabilization is widely accepted, the management of contaminated large bone fragments is still controversial. Preservation of the free bone fragment is essential for the process of healing since bone defect in an open fracture will deliver even more complicated problem, however on the other hand contamination of the free bone fragment may lead to infection which also delivers a
challenging problem. Thus, sterilization of the free bone fragment prior restoration is essential to prevent infection and eventually to ensure fracture healing [4-6].

Several studies have claimed microwaves as a sterilization method [7,8]. Oppusunggu et al [9] reported a successful use of 7 and 10 minute of irradiation using a domestic microwave for sterilising contaminated free bone fragments in an animal model of open fracture. Oppusunggu et al. [9] also stated that exposure more than 7 min can lead to bone cell death and matrix destruction. Until recently, there is no study assessed the osteogenic viability and potency of fracture healing after domestic microwave irradiation on contaminated bone fragments. This study was performed to comprehend the impact of microwave irradiation as a mean of sterilization on a large bone fragments of an animal model with contaminated bone fragment. Specifically, this study aimed to describe the effect of 7 minute of microwave irradiation on a contaminated large fragment toward its ability to incorporate into healthy bone using histomorphometry.

2. Methods
An experimental study was performed using 16 male Wistar rats weighed 250-300 gram randomly assigned into two groups, that is 8 Wistar rats for each control and treatment group. The protocol of the study had been approved by the Health Research Ethics Committee, Faculty of Medicine Universitas Indonesia-Cipto Mangunkusumo Hospital. The body weight distribution was p > 0.05, which showed a normal data distribution and did not impact the measurement. A segmental femoral fracture with 1 cm free bone fragment was created in all 16 Wistar rats. In the control group, the segmental free bone fragment was reimplanted directly into the host bone and stabilized using intramedullary K wire. In the treatment group, the segmental free bone fragments were inoculated with Staphylococcus Aureus ATCC 25923 bacteria and then wrapped within sterile gauze before being exposed to 7 minute of microwave irradiation. A domestic 2,450 MHz Samsung ME86V-BBH microwave with 800-watt power is used. Afterwards, the free bone fragment is reimplanted into the host bone and stabilized using intramedullary K wire. During surgery each rat received anaesthesia with an intraperitoneal injection of ketamine (Ketalar® 40–80 mg/kg) and xylazine (5–10 mg/kg). Both groups were kept in the Animal Laboratorium of Research and Development, Indonesian Ministry of Health.

At the end of second week, 4 Wistar rats from control group and 4 Wistar rats from treatment group were euthanized and sacrificed. The femur previously fixed with K wire was harvested and processed into histopathological slides in the Pathology Anatomy Department, Faculty of Medicine, Universitas Indonesia. Haematoxylin Eosin (HE) stain is used for assessing total fibrous area woven bone area while Safranin O/fast green stain is used for cartilage area. The slides are digitally photographed and assessed using Image J software for histomorphometry analysis. Histomorphometry data were presented in millimeters square and evaluated using an independent t-test. At the end of 4th week, the same procedures were performed to evaluate the process of fracture healing using histomorphometry analysis.

Figure 1. (1A) Slide using HE stain for analysis of total fibrous area and woven bone are, (1B) Slide using Safranin O stain for analysis of cartilage area
3. Results
The analysis of histomorphometry at the second week shows significant differences in cartilage and woven area between the control and treatment group with the size of cartilage and woven area of the treatment group were smaller than the controls. The difference in the size of fibrous tissue area between control and treatment group was not significant, still with the size of the treatment group smaller than the control. (Table 1)

Table 1. Histomorphometry evaluation at week 2

| Parameter          | Comparison            | Mean ± SD (mm) | Statistic Test | p value |
|--------------------|-----------------------|----------------|----------------|---------|
| Fibrous tissue area| Control group week 2  | 1.76 ± 1.05    | Independent t-test | 0.733   |
|                    | Treatment group week 2| 1.52 ± 1.58    |                |         |
| Cartilage area     | Control group week 2  | 0.81 ± 0.48    | Independent t-test | 0.004   |
|                    | Treatment group week 2| 0.18 ± 0.20    |                |         |
| Woven bone area    | Control group week 2  | 0.64 ± 0.28    | Independent t-test | 0.010   |
|                    | Treatment group week 2| 0.28 ± 0.20    |                |         |

The analysis of histomorphometry at week 4 doesn’t show any significant differences in the fibrous tissue, cartilage and woven bone areas (p > 0.05). The size of fibrous tissue and cartilage areas in the treatment group were higher compared to those in the control group while the size of woven bone in the treatment group was lower compared to the size of woven bone in the control group. (Table 2)

Table 2. Histomorphometry evaluation at week 4

| Parameter          | Comparison            | Mean ± SD (mm) | Statistic Test | p value |
|--------------------|-----------------------|----------------|----------------|---------|
| Fibrous tissue area| Control group week 4  | 1.50 ± 1.00    | Independent t-test | 0.060   |
|                    | Treatment group week 4| 2.62 ± 1.23    |                |         |
| Cartilage area     | Control group week 4  | 0.05 ± 0.05    | Independent t-test | 0.069   |
|                    | Treatment group week 4| 0.38 ± 0.46    |                |         |
| Woven bone area    | Control group week 4  | 1.53 ± 1.00    | Independent t-test | 0.947   |
|                    | Treatment group week 4| 1.50 ± 0.93    |                |         |

The histomorphometric data of control group on Table 3 shows significant differences in the size of cartilage and woven tissue area between week 2 and week 4. There was no significant difference in the size of fibrous tissue between week 2 and week 4.

Table 3. Histomorphometry evaluation of the control group

| Parameter | Comparison          | Mean ± SD(mm) | Statistic test | P value |
|-----------|---------------------|----------------|----------------|---------|
| Fibrous   | Control group week 2| 1.76 ± 1.05    | Independent t-test | 0.623   |
|           | Control group week 4| 1.50 ± 1.00    |                |         |
| Cartilage | Control group week 2| 0.81 ± 0.48    | Independent t-test | 0.001   |
|           | Control group week 4| 0.05 ± 0.05    |                |         |
| Woven     | Control group week 2| 0.64 ± 0.28    | Independent t-test | 0.001   |
The histomorphometric data of the treatment group on Table 4 shows no significant differences in the size of fibrous and cartilage area between week 2 and week 4. The size of woven bone in treatment group is significantly different between week 2 compared to week 4.

Table 4. Histomorphometry evaluation of the treatment group

| Parameter | Comparison              | Mean±SD (mm) | Statistic test | P value |
|-----------|-------------------------|--------------|----------------|---------|
| Fibrous   | Treatment group week 2  | 1.52 ± 1.58  | Independent t-test | 0.144   |
|           | Treatment group week 4  | 2.62 ± 1.23  |                |         |
| Cartilage | Treatment group week 2  | 0.81 ± 0.48  | Independent t-test | 0.288   |
|           | Treatment group week 4  | 0.38 ± 0.46  |                |         |
| Woven     | Treatment group week 2  | 0.28 ± 0.20  | Independent t-test | 0.003   |
|           | Treatment group week 4  | 1.50 ± 0.93  |                |         |

4. Discussion

Preservation and restoration of a free bone fragment in a fracture plays an essential role in the process of fracture healing. The prevalence of fracture nonunion is around 2.5% among long bone fracture. However, in the setting of segmental bone loss, this rate approaches 100% secondary to the limited ability of the skeletal system to repair and fill defects [10-12]. In the setting of a contaminated open fracture with segmental bone fragment, preservation of the free bone fragment is controversial because in on hand the restoration is essential for fracture healing however on the other hand it carries a significant risk of infection which may hinder the process of fracture healing itself [2,13]. Therefore, preservation and restoration of contaminated free bone fragment with prior sterilization plays important roles in this setting [3-9].

Microwave irradiation has been proven as one of procedures to sterilize infected anatomical structure because it delivers heat that result in protein precipitation and cell death [7-9]. However, microwave irradiation may also harmful for any osteogenic cells involve in the process of fracture healing as well as damaging any osteoinductive growth factors and osteoconductive components of fracture healing. Giannoudis [14] proposed “diamond concept” to summarize important factors which play significant roles in the process of fracture healing which consist of osteoconductive scaffold, osteogenic cells, mechanical stability, osteoinductive growth factors, vascularisation and the host. Although microwave irradiation damages all osteogenic cells and osteoinductive protein in the free bone fragment, the process of fracture healing is expected to occur from the intact mechanical integrity and various host supportive factors. Our hypothesis is that exposure to microwave irradiation will eliminate the inoculated bacteria rendering the free bone fragment sterile for incorporation into the intact host bone with the support of local osteogenic cells and osteoinductive growth factors. To our knowledge till date, there is no studies have evaluated the process of bone healing after domestic, sterilizing microwave irradiation.

For our study, we performed a histomorphometric quantitative analysis and the final calculation was made using Image J software from the National Institutes of Health [15]. We measured the total area of cartilage, fibrous tissue and woven bone. The samples were stained using haematoxylin and eosin, safranin O and fast green. Haematoxylin and eosin staining was sufficient to assess the fibrous tissue and bone tissue, whereas the cartilage was observed using safranin O staining. The measurement of selected areas was semi-automatic and calculated using Image J. The disadvantage of using semi-automatic assessment was subjectivity and error in the human researcher; however, the results were not significantly different from those using automatic assessment [16].
Comparison of histomorphometry between control and treatment group at the second week (Table 1) shows the size of cartilage and woven area of the treatment group were significantly smaller than the controls. This is in accordance with the process of endochondral ossification in fracture healing, in which the process of fracture healing within 2 weeks period in a Wistar rat has reached the phase of cartilage formation and even some of them have already reached the formation of a woven bone. The process of fracture healing in the treatment group is lagging behind the control group because the electromagnetic energy delivered by microwave irradiation will transformed into heat which eliminate osteogenic cells and osteoinductive growth factors [9,17].

Comparison of histomorphometry between control and treatment group at the fourth week (Table 2) shows there is no statistically significant difference found between the treatment and control groups. The treatment groups shows a larger size of cartilage area whereas the control group has larger size of woven bone even though, once again, not statistically significant. This data shows that the control group has a tendency to attain a more advanced stage of fracture healing and that the treatment group has already catch up and overcoming its shortage in osteogenic cells and osteoinductive growth factors. Ye et al [18] reported microwave as a method for improving fracture repair in New Zealand rabbits with a 3.0 mm bone defect internally fixed with titanium alloy systems, which showed radiographic, histologic and histomorphometric evaluation revealed significant improvement in the healing bone in the middle of femur. Study reported by Ye et al. may partly explain why the treatment group in our study regain its healing process of fracture. However, because the methodology is different, Ye et al. expose the living New Zealand rabbits to microwave irradiation while our study exposes free bone fragment to microwave irradiation, we prefer to presume that the surrounding tissue provides the osteogenic cells and osteoinductive growth factors to restart the cascade process of fracture healing. Microwave irradiation serve as a means to eliminate the infection yet preserve the nonorganic environment that eventually microwave irradiation delivers conducive environment for fracture healing.

5. Conclusion
The interval between week 2 and week 4 of fracture healing in Wistar rat is the ideal time to assess the normal process of fracture healing and ideal time to compare normal process of fracture healing with process of fracture healing under treatment.

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