The effect of red yeast rice on delayed union fracture in animal model: a molecular study of IL-6, BMP-2, VEGF, BALP, and N-Mid-OC in fracture healing [version 1; peer review: awaiting peer review]

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Abstract

Background
As serious fracture complications, delayed union and non-union are parts of complications from fracture healing. Growth factors such as BMP-2, VEGF, proinflammatory cytokines including IL-6 and bone formation BALP, N-Mid-OC are important regulators of the fracture healing process. Red yeast rice (RYR), produced by fermenting Monascus purpureus rice, monacolin K, which is the main ingredient in RYR, was found to play a major role in the anti-inflammatory process and increasing the proliferation of osteoblast in osteoporosis cases. This study aims to examine the effect of RYR in the fracture healing process in delayed union rats through molecular studies of levels of IL-6, BMP-2, VEGF, BALP, and N-Mid-OC.

Methods
This study was experimental research that used male rats (Rattus norvegicus) which were divided into a control and 3 treatment groups using a random sampling method. Group 1 was given orally 25 mg/kg, Group 2 was 50 mg/kg, Group 3 was 100 mg/kg, and the control group was given a placebo. The rats were then subjected to a delayed union fracture model. Observations were made for two periods on the 14th and 28th days.

Results
There were no significant differences in serology examination between days 0 and 14 between groups. However, there were significant differences between groups on day 28. IL-6, BMP-2, VEGF, BALP, and N-Mid-OC on day 28 between groups (p<0.001). The group
with 100 mg/kg RYR extract was found to be the most influencing serology marker level. RYR 100 mg/kg significantly decreased IL-6, and increased BMP-2, VEGF, BALP, and N-Mid-Osteocalcin, thus enhancing the fracture healing process in the delayed union rats model.

**Conclusion**
A red yeast rice dose of 100 mg/KgBW significantly reduced IL-6, increased BMP-2, VEGF, BALP, N-Mid-OC, and RUST Score so as to improve the fracture healing process in delayed union rats.

**Keywords**
Red Yeast Rice, Delayed Union, ELISA, IL-6, BMP-2, VEGF, Fracture Healing
**Introduction**

Fractures are the type of trauma that most require hospitalization. In 2018 the prevalence of fractures due to accidents ranked third out of all non-natural disasters, 31.4% occurring on roads and the most in the 15–24-year age group at 49.5% (Litbangkes, 2019). Not all fractures can heal completely, some have complications such as delayed union or non-union. Delayed union and non-union account for 5–10% of fracture healing (Kostenuik and Mirza, 2017). Impaired fracture healing significantly influences the quality of life, financial condition, and functional and psychological disorders of patients (Stewart, 2019).

The diamond concept, which includes biological chamber, mechanical stability, osteogenic cells, osteoinductive mediators (growth factors, cytokines), and osteoconductive matrix, needs to be applied in the treatment of fracture healing disorders (Andrzejowski and Giannoudis, 2019). Growth factors such as bone morphogenetic protein (BMP) are important regulators of the fracture healing process. BMP-2 functions for the differentiation of mesenchymal stem cells into osteoblast cells (Wu et al., 2020). Proinflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), help initiate the fracture healing cascade, and may also play a key role in the remodeling phase (Hartono et al., 2022; Ding et al., 2018).

Red yeast rice, produced by fermenting *Monascus purpureus* rice, has been used as a traditional medicine in East Asian countries such as China, Japan, Korea, and Thailand (Patel, 2016; Zhu et al., 2019). Red yeast rice has been reported to have many biological properties with hypolipidemic, anti-atherosclerotic, anti-cancer, neuroprotective, hepatoprotective, anti-osteoporosis, anti-fatigue, anti-diabetic, anti-obesity, immunomodulatory, anti-inflammatory, anti-osteoosteoporotic, and anti-inflammatory, and antibiotic properties (Zhang et al., 2018). Monacolin K, which is the main ingredient in red yeast rice, was found to play a major role in the fracture healing process by increasing the proliferation of osteoblasts (Wu et al., 2020; Song et al., 2019).

The researchers examined the effect of giving red yeast rice which is easily available and widely known to the public in Indonesia on the fracture healing process in delayed union rats through molecular studies of levels of IL-6, BMP-2, and vascular endothelial growth factor (VEGF) as the predictor of the healing process, also N-Mid-Osteocalcin and bone alkali phosphatase (BALP) as the predictor of osteoblast activity.

**Methods**

**Research design**

This is experimental research with a completely randomized pre- and post-test control group. The experimental animals/male rats that met the inclusion and exclusion criteria were taken in as many as eight animals/group (three treatment groups and one control group) using a random sampling method. The Sprague Dawley rat model was chosen because the model rat has been widely used in previous studies so genetic data is easy to obtain and it can provide research results with a high level of validity. In addition, the stages of fracture healing in rats also resemble humans even though they occur twice as fast, genetic similarity, lamellar bone architects also resemble humans, and have a relatively short life with a fast bone turnover rate, making it easier to carry out multigenerational research. Care and maintenance are relatively inexpensive, and have the ability to adapt to a laboratory environment. Rats are also considered more suitable than other four-legged animal species to study the morphology of the femur bone (Gutierreza et al., 2006). The sample in each group was randomly chosen by giving each trial animal a tag number. Following that, the researcher randomly chose the tag numbers. The animal experiment and examination were carried out at the experimental animal husbandry in the Inter-University Center Building, Universitas Gadjah Mada (PAU-UGM) Yogyakarta. Identification and analysis of IL-6, BMP-2, VEGF, BALP and N-Mid-Osteocalcin were carried out at the Biomedical Laboratory, Faculty of Medicine, Gadjah Mada University. This study had been approved by The Ethic Committee No.513/IV/HREC/2021.

The population in this study were male rats (*Rattus norvegicus*) Sprague Dawley strain aged 12 weeks with a body weight of 150–200 grams which were developed and maintained at PAU-UGM totaling 8 individuals each group with 3 treatment groups and 1 control group so that a total of 32 animals. male rats (*Rattus norvegicus*) were calculated using the Steel & Torry (1980) formula:

\[ (n - 1)(k - 1) > 15 \]

- \( n \) = total samples
- \( k \) = number of groups
This study used four groups so,

\[(n - 1)(4 - 1) > 15\]

Then, the animals should also meet the inclusion criteria (healthy, active, good appetite, 12 weeks old, and weighs 150–200 grams) and exclusion criteria (dead during the study, unwilling to eat, and infection in the operating area). All experimental procedures involving animals were carried out in keeping with guidelines from the National Institutes of Health Guide for the Care and Use of Laboratory Animals to ameliorate any suffering of animals (Tan, 2004). Expected and unexpected adverse events were recorded to identify deficiencies in procedures or study design.

**Procedures**

Before the intervention, the experimental animals were kept for 1 week for acclimatization. The animal models were acclimatized for a week at a temperature of 21–23°C with controlled humidity (50±5%) in a 12-hour artificial light cycle (08:00 h to 20:00 h) to help them to adapt to the same conditions as their various origins. All rates were located individually in polycarbonate cages (0.90×0.60×0.60 m). Every animal model was fed with a standard pellet and water was provided *ad libitum* with the husk replaced every three days. All animal models were routinely inspected and observed regarding their food consumption and fecal characteristics. After being anesthetized by administering Ketamine (Dexa Medica, Tangerang) 35 mg/kg body weight (BW) and xylazine (Inter Chemie, Holland) 5 mg/kg BW intramuscularly, the animals were then subjected to a delayed union fracture model by antiseptics of the right lower leg. Perform a 2-cm long incision on the posterolateral side of the femur, the vastus lateral muscle is separated from the biceps femoris, then the vastus lateral and biceps femorius muscles are elevated while maintaining the peristeum intact along the surface of the femur bone, performed osteotomy/fracture in the diaphysis of the femur with a 1-mm manual saw to eliminate the effects of heat when using a chainsaw, the delayed union rat model in this study refers to the research of Kasman and Kurniawan (2018). It is in the form of stripping the periosteum/damaging the periosteum in a circular manner with a surgical blade as far as 5 mm from the fracture line towards the proximal and distal according to the Kokabu et al. (2003) and Utvåg et al. (1996) method. After the fracture and periosteal stripping procedure, we performed intramedullary reaming using a 23G needle followed by fixation. Internally, using intramedullary k-wire measuring 1.2-1.4 mm retrograde, the surgical wound was closed using catgut 3.0 and the skin with silk 3.0. Blood samples were taken through puncture of the orbital vein and analyzed for cellular, IL-6, BMP 2, VEGF, N-Mid-Osteocalcin, and BALP by using the ELISA method. Randomization was performed with 1 control group and 3 treatment groups, each consisting of 8 experimental animals.

Treatment of experimental animals on the same day by giving red yeast rice (Monacolin K/Cholestimax®, Jakarta) one capsule of Cholestimax containing 600 mg of red yeast rice dissolved in 150 mL of distilled water so that every 1 mL of solution contains 4 mg of red yeast rice. The solution was diluted with distilled water according to the required dose for each experimental animal (25, 50, and 100 mg/kg), assuming a 50 mg dose was the optimum dose for delayed union cases and for a 25 mg dose which is half of the optimum dose assessed whether it is still effective. In accelerating the healing of delayed union cases, while the dose of 100 mg is the maximum dose (twice the optimum dose) assessed whether it is still safe/lethal and does not cause side effects. Then the solution was probed into the mouth of the experimental animal using a 1 ml syringe. It is given at the same time as meals, to avoid side effects of digestive system disorders. General observations for signs of pain or suffering in the animal were conducted daily as needed. The moribund condition was used as a humane endpoint (Tan, 2004).

**Evaluation**

All groups were observed on the 14th and 28th days for blood sampling through a puncture in the orbital vein and cellular analysis including, IL-6, BMP 2, VEGF, BALP, and N-Mid-Osteocalcin with the ELISA method. In this study, observations were made for 2 periods, namely on the 14th and 28th days, to be able to assess any changes that occurred during the fracture healing process.

5 mL of blood were collected through a puncture in the orbital vein under anaesthesia, using a serum separator tube (SST). Samples were centrifuged at 3,500 rpm (~1,000 × g) for 20 min. Blood serum is separated into a sterile 1.5-mL microcentrifuge tube, immediately tested, or stored in a deep freezer at −80°C until the analysis is carried out. A commercially available research ELISA kit was used to measure serum concentrations of IL-6, BMP 2, VEGF, BALP, and N-Mid-Osteocalcin (FineTest, Wuhan).

**Statistical analysis**

To determine whether there is a difference in the results of the examination between the four groups of treatment preparations in this study, an unpaired difference test was carried out using the ANOVA test. The test results are considered significant if the p-value <0.05. This research uses the SPSS for Windows Release program (IBM).
Results

Result in each treatment groups

Changes in serological levels of each observation in each treatment were found from day 0 to day 14 and day 14 to day 28 (Table 1).

Based on Table 1 and Figure 1 above, it can be seen that in treatment group P1 (red yeast rice 25 mg/kg) the IL-6 level experienced a significant increase (p≤0.001) between day 0–14, then there was a statistically significant decrease (p≤0.001) on days 14–28. Treatment group P1 (red yeast rice 25 mg/kg) VEGF level experienced a significant increase (p≤0.001) between days 0–14, then a statistically significant decrease (p=0.001) on days 14–28. Treatment group P1 (red yeast rice 25 mg/kg) BALP level experienced a significant increase (p≤0.001) between days 0–14, then there was a statistically significant decrease (p≤0.001) on days 14–28. Treatment group P1 (red yeast rice 25 mg/kg) N-mid-OC level experienced a significant increase (p≤0.001) between days 0–14, then a statistically significant decrease (p≤0.001) on days 14–28. Treatment group P1 (red yeast rice 25 mg/kg) BMP2 level experienced a significant decrease (p≤0.001) between days 0–14, then there was a statistically significant increase (p≤0.001) on days 14–28.

Based on the Table 2 and Figure 2 below, it can be seen that the treatment group P2 (red yeast rice 50 mg/kg) The IL-6 level experienced a significant increase (p≤0.001) between days 0–14, then there was a statistically significant decrease (p≤0.001) on day 14–28. Treatment group P2 (red yeast rice 50 mg/kg) VEGF level experienced a significant increase (p≤0.001) between days 0–14, then a statistically significant decrease (p≤0.001) on days 14–28. Treatment group P2 (red yeast rice 50 mg/kg) BALP level experienced a significant increase (p≤0.001) between days 0–14, then there was a statistically significant decrease (p≤0.001) on days 14–28. The P2 group (red yeast rice 50 mg/kg) N-mid-OC level

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| Table 1. Changes in serological levels in treatment group P1 (red yeast rice 25 mg/kg). |
|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| Treatment group P1 (read yeast rice 25 mg/kg) (mean) | IL-6 | VEGF | BALP | N mid OC | BMP2 |
|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| Day 0 | 27.05 | 22.06 | 0.87 | 6.07 | 12.66 |
| Day 14 | 79.97 | 35.17 | 14.21 | 13.02 | 5.94 |
| Day 28 | 60.85 | 30.84 | 5.11 | 10.75 | 8.46 |

| p-value |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Day 0–Day 14   | <0.001*         | <0.001*         | <0.001*         | <0.001*         |
| Day 14–Day 28  | <0.001*         | <0.001*         | <0.001*         | <0.001*         | <0.001*         |

*Significant p<0.05.
experienced a significant increase (p=0.012) between days 0–14, then a statistically significant decrease (p=0.018) on days 14–28. The P2 group (red yeast rice 50 mg/kg) BMP2 level experienced a significant decrease (p ≤ 0.001) between days 0–14, then there was a statistically significant increase (p ≤ 0.001) on days 14–28.

Table 2. Changes in serological levels in the treatment group P2 (red yeast rice 50 mg/Kg).

| Treatment group P2 (red yeast rice 50 mg/kg) (mean) |
|-----------------------------------------------------|
| IL-6      | VEGF | BALP | N mid | OC | BMP2 |
| Day 0     | 27.94 | 22.35 | 0.94  | 6.15| 12.70|
| Day 14    | 80.14 | 35.28 | 15.08 | 13.07| 5.95 |
| Day 28    | 49.18 | 29.85 | 2.92  | 7.91| 10.01|
| p-value   |       |       |       |     |      |
| Day 0–Day 14 | <0.001* | <0.001* | <0.001* | 0.012* | <0.001* |
| Day 14–Day 28 | <0.001* | <0.001* | <0.001* | 0.018* | <0.001* |

*Significant p<0.05.

Figure 2. Histogram of changes in serological levels in treatment group P2 (red yeast rice 50 mg/kg).

Table 3. Changes in serological levels in the treatment group P3 (red yeast rice 100 mg/kg).

| Treatment group P3 (red yeast rice 100mg/kg) (mean) |
|-----------------------------------------------------|
| Interleukin 6 | VEGF | BALP | N mid | OC | BMP2 |
| Day 0         | 28.36 | 22.06 | 0.92  | 6.11| 12.67|
| Day 14        | 80.04 | 35.25 | 14.74 | 13.14| 5.92 |
| Day 28        | 32.75 | 25.47 | 1.57  | 6.82| 11.35|
| p-value       |       |       |       |     |      |
| Day 0–Day14   | <0.001* | <0.001* | <0.001* | <0.001* | <0.001* |
| Day 14–Day 28 | <0.001* | <0.001* | <0.001* | 0.001* | <0.001* |

*Significant p<0.05.

experienced a significant increase (p=0.012) between days 0–14, then a statistically significant decrease (p=0.018) on days 14–28. The P2 group (red yeast rice 50 mg/kg) BMP2 level experienced a significant decrease (p ≤ 0.001) between days 0–14, then there was a statistically significant increase (p ≤ 0.001) on days 14–28.

Based on the Table 3 and Figure 3 above, it can be seen that the treatment group P3 (red yeast rice 100 mg/kg) The IL-6 level experienced a significant increase (p≤0.001) between days 0–14, then there was a statistically significant decrease...
Treatment Group P3 (red yeast rice 100 mg/kg) VEGF level experienced a significant increase \( (p \leq 0.001) \) between days 0–14, then a statistically significant decrease \( (p \leq 0.001) \) on days 14–28. Treatment group P3 (red yeast rice 100 mg/kg) BALP level experienced a significant increase \( (p \leq 0.001) \) between days 0–14, then there was a statistically significant decrease \( (p \leq 0.001) \) on days 14–28. Treatment group P3 (red yeast rice 100 mg/kg) N-mid-OcC level experienced a significant increase \( (p \leq 0.001) \) between days 0–14, then there was a statistically significant decrease \( (p=0.001) \) on days 14–28. Treatment group P3 (red yeast rice 100 mg/kg) BMP2 level experienced a significant decrease \( (p \leq 0.001) \) between days 0–14, then there was a statistically significant increase \( (p \leq 0.001) \) on days 14–28. The treatment group P3 (red yeast rice 100 mg/kg).

**Discussion**

From the experiment of the animal population sample rats that as many as 8 animals/group (3 treatment groups and 1 control group) using a random sampling method. All groups were periosteal damaged which made the model of delayed union. The Sprague Dawley rat as an experimental animal was used because they have been used in several studies so that the required data is easy to obtain, and standard strains with uniform genetic backgrounds are available, that this type of research can produce data with high validity and the treatment can be regulated by researchers (Sastroasmoro and Ismael, 2015). In addition, the stages of fracture healing in rats also resemble humans even though they occur twice as fast, have genetic similarities to humans, have similar lamellar bone architecture, relatively short life cycle with a fast bone turnover rate so that multigenerational research can be carried out, care and maintenance is relatively inexpensive, can adapt in a laboratory environment. Rats are also considered more suitable than four-legged animal species to study femur morphology (Gutierrez et al., 2006). The selection of experimental animals was male rats with the reason to minimize the biased influence of the hormone estrogen on the process of bone remodeling.

Based on a study by Einhorn and Gerstenfeld in 2015, the fracture healing period on day 14 is the peak of cellular proliferation in the intramembranous fracture healing process, as well as bone formation from periosteal osteoprogenitor cells and an increase in cartilage tissue. On day 28, there was a mineralization process, the formation of woven bone, and the change of callus into the lamellar bone by osteoclasts so that there was a combination of calcified cartilage and woven bone and lamellar bone. Due to the calcification process, the cartilage area becomes smaller, and also due to the remodeling process in the callus, the total callus area begins to decrease.

Analysis of changes in serological levels in the treatment group was evaluated. In all treatment groups P1, P2, and P3 all serological levels: IL-6, VEGF, BALP, and N-Mid-Osteocalcin, experienced a significant increase on day 0 to day 14. This is consistent with the previous discussion that the early phase or endochondral ossification of bone grafting requires the role of IL-6 as an inflammatory response, VEGF as growth factor for angiogenesis and osteogenesis, while BALP and N-Mid-Osteocalcin as markers of metabolism and osteoblast activity (Dong et al., 2014; Einhorn and Gerstenfeld, 2015; Cunningham et al., 2017). The administration of red yeast rice in this experimental animal model was
able to significantly increase the serological levels. The BMP-2 which decreased significantly on day 0 to day 14 is in accordance with in vivo studies conducted on dogs showing the same decrease in the initial week which is the initial phase of bone grafting (Rady et al., 2020). Furthermore, there was an increase in BMP-2 levels the following week. This happens because the inflammatory process is in accordance with research conducted on previous experimental animals that increased inflammation will reduce BMP-2 levels (Huang et al., 2014). Although theoretically, BMP-2 is indispensable in the early phase of fracture healing (Street et al., 2002), increased levels of TNF-α and IL-1β show a suppressive effect on BMP-2 levels (Huang et al., 2014). Thus, in the early or inflammatory phase, BMP-2 levels will decrease first and the process of osteogenesis in this phase is induced by VEGF (Rady et al., 2020). This indicates that the serological increase is significant because it is evidenced by an increase in callus formation and fracture union.

On day 14 to day 28, there was a significant decrease in levels of IL-6, VEGF, BALP, and N-Mid-Osteocalcin. As explained in the previous discussion, this decrease indicates that the union process has been completed (Dong et al., 2014; Einhorn and Gerstenfeld, 2015; Cunningham et al., 2017). Meanwhile, BMP-2 levels should always increase until the final phase of fracture healing because it is needed in the process of ossification and remodeling (Halloran et al., 2020).

Some limitations of this study is that we did not conduct the histological examination to see directly the union histologically or use immunohistochemistry directly to see the expression of IL-6, BMP-2, VEGF, BALP, and N-Mid-Osteocalcin on the fracture site. However, the union process was not clearly seen by using laboratory markers because the evaluation interval period was longer than the union process provided by the administration of red yeast rice unpredictably faster. Hopefully, this finding can provide the information that red yeast rice is a promising, safe, and effective therapeutic option for delayed union. However, further research on the effect on humans should be conducted in translational studies or further clinical trials.

**Conclusion**

Red yeast rice can decrease the IL-6 and increase BMP-2, VEGF, BALP, N-Mid-Osteocalcin, and enhance fracture healing in the delayed union Sprague Dawley rat model. However further studies to see the histopathology using immunohistochemistry should be conducted to make sure the expression of IL-6, BMP-2, VEGF, BALP, and N-Mid-Osteocalcin, directly on the fracture site. Moreover, the red yeast rice can be a promising and safe option for treatment option for delayed union fracture cases and can proceed to the next translational study or clinical trial to see the effect on patients.

**Author contributions**

U.H.N.: research concept, literature search, data analysis, manuscript preparation, drafting the manuscript, reviewing and editing the manuscript; H.: research concept, data analysis, manuscript preparation, reviewing and editing the manuscript; D.I.: literature search, data analysis, manuscript preparation, reviewing and editing the manuscript; A.K.: literature search, data analysis, manuscript preparation, reviewing and editing the manuscript.

**Data availability**

**Underlying data**

- Dataset available at: Nefihancoro, Udi Heru, Hartono, Indarto, Dono, & Kurniawan, Aryadi. (2022). Statistic Dataset (SPSS) [Data set]. Zenodo. https://doi.org/10.5281/zenodo.7042279 (Nefihancoro et al., 2022).

**Reporting guidelines**

- ARRIVE author checklist: Nefihancoro, Udi Heru. (2022). ARRIVE author checklist. https://doi.org/10.5281/zenodo.7042336.

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