Haematological and inflammatory responses of intramedullary tibial iron based implant in sheep

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Abstract. Iron-based implants have been widely researched as biodegradable orthopaedic implants although non-degradable metallic materials are still the golden standard as orthopaedic implant to avoid removing implant surgery when bone healing achieved. The aim of this study was to observe haematological and inflammatory responses of iron-based intramedullary biodegradable implants. Eight sheep were divided into two groups according to the material implanted, which were the control group using stainless steel Steinman pin and treatment group using degradable iron-based pin. The pin was inserted into a transverse fracture of sheep tibial bone medullary cavity. Blood sampling was performed on jugular vein on day 0, 1, 3, 7, and 14 after implantation. The examination include the number of erythrocytes, haematocrite values, hemoglobin levels, platelet values, the number of leukocytes, and leukocyte differential cell. Iron-based bone pin implantation did not cause significant changes on erythrocyte profile, leukocyte count and leukocyte differential cell when compared to stainless steel bone pin. The interaction between iron-based implant and bone tissue, acute and chronic inflammatory responses shows a dynamic variation. It can be concluded the iron-based implant is still tolerated sub-chronically in sheep.

1. Introduction
Fracture cases are quite common in Indonesia. According to the regional health research (RISKESDAS) of Indonesia in 2013, the incidence of fracture was ranked 4th with a rate of 5.8% due to injury [1]. Fracture bone could be immobilized using biomaterial implant. Inert biomaterial such as titanium-based is still the golden standard as orthopaedic implant [2]. To avoid implant surgery, iron-based as a biodegradable metallic material is widely developed as orthopaedic bone implants [3, 4]. Iron used as a bone implant is still debated because of its toxicity [5, 6], but iron also had proven has minimal response in mice [7].

Iron is an essential mineral for heme biosynthesis in developing erythroblasts [8] and also known as an element required for cognitive development in human [9]. Implantation procedures in fracture cases also could affect the number of erythrocyte. Because iron is poorly acquired through diet [10], then in most cases iron supply could be obtained from erythrocyte disposal and iron recycling require macrophage in the liver as primary organ supporting [11]. The use of iron-based biomaterial as orthopaedic biodegradable materials will provide the source of iron ion due to it will gradually degrade by the time [12]. Several studies were conducted to explore the potential of iron as a bone
implant, but just a few of them discussed the response of iron as a bone implant to the systemic response of the body. Therefore, the aim of this study was to observe systemic effects of iron-based biodegradable implant using blood examination in sheep.

2. Experimental methods

2.1. Implantation preparation procedures
All of the experimental methods were already approved by animal care and use committee of Bogor Agricultural University number 12-2015 RSHP FKH-IPB. Eight male sheep with 21±4 kg range weight were divided into iron-based and control groups. Control group was implanted with 5 mm of diameter stainless steel based intramedullary implant (Steinmann Pin, sklar®, USA) while an iron-based group was implanted with 5 mm of diameter of iron-based implant with (90.02% of iron, BjPT 6, Tunggal Jaya Steel®, Indonesia). Before implantation, all animal had 2 weeks acclimation and had blood examination at the end of acclimatization period to determine the health condition.

One hour before implantation procedures started, all of the animal was injected with anti-tetanus serum (anti-tetanus serum 1.5®, 1 mL/sheep, intramuscularly) and general antibiotic combination of gentamycin (Bio-genta®, Bioparchemie, Vietnam) and amoxicillin (intramox-150 LA®, Interchemie, Holland). Implantation procedures was performed under general anaesthesia influenced. General anaesthesia we used was xylazin 2% (Xyla®, Interchemie, Holland) and ketamine 10% (Ketamin®, Kepro BV, Netherland) accompanied with Atropin sulfate (Atropine®, PT Indofarma, Indonesia) as premedication.

2.1.1. Implantation and post implantation procedures.
The implantation procedure was performed in right lateral recumbency. The skin incision was applied to the lateral of tibia-fibula. The pin was inserted into a transverse fracture of left-tibial sheep bone intramedullary. The retracted muscle was sutured using polyglactin 910 3/0 (Vicryl®, Ethicon, USA) and wounded skin was sutured using silk 3/0 (PT Inti sumber Hasil Sempurna, Indonesia). The wound was bandaged using antibiotic gauze pad (Sofra-tulle®, Sanafi-aventis, Thailand) and hypafix adhesive (Hypafix®, PT. BSN Medical, Sweeden).

Post-implantation treatment was using the combination of gentamycin and amoxicillin general and tramadol. Combination of gentamycin and amoxicillin general antibiotic was administered intramuscularly to prevent secondary infection. Tramadol (Tramadol®, PT Indofarma, Indonesia) also administered intravenously as an analgesic. The bandage was replaced every two days until day 14 using antibiotic gauze pad.

2.1.2. Blood examination.
Blood sampling was performed on jugularis vein at day 0, 1, 3, 7, and 14 after implantation. The whole blood sample was examined to have the number of erythrocytes, hematocrite values, hemoglobin levels, platelet values, the number of leukocytes and leukocyte differential cell.

2.1.3. Data analytics.
Data were conducted using SPSS for Windows with a P value of P ≤ 0.05 as statistically significant and expressed as mean ± standard deviation. Data were analyzed using analysis of variance (ANOVA) and then post-hoc DUNCAN.

3. Result and discussion

3.1. Erythrocyte profile
Erythrocytes, also known as red blood cells, are the most common type of blood cell that plays the main role to deliver oxygen to the soft tissue. The importance of the role of erythrocyte then the observation of erythrocyte profile is the major concern to know the health condition. The blood examinations of
male sheep include erythrocyte counts, hematocrit counts, hemoglobin level, and thrombocyte counts (Table 1, Figure 1).

**Table 1.** Erythrocyte profile of control and iron-based implant in tibial bone of male sheep on different observation day.

| Group             | Day 0          | Day 1          | Day 3          | Day 7          | Day 14         |
|-------------------|----------------|----------------|----------------|----------------|----------------|
| **Erythrocyte count (10⁶/µL)** |                |                |                |                |                |
| Control           | 3.43±0.10<sup>x,a</sup> | 3.33±0.10<sup>x,a</sup> | 3.18±0.26<sup>x,a</sup> | 3.10±0.20<sup>x,a</sup> | 3.18±0.30<sup>x,a</sup> |
| Iron-based        | 3.48±0.21<sup>x,a</sup> | 3.53±0.29<sup>x,a</sup> | 3.10±0.22<sup>x,a</sup> | 3.20±0.36<sup>x,a</sup> | 3.10±0.42<sup>x,a</sup> |
| **Haematocrit (%)** |                |                |                |                |                |
| Control           | 30.50±1.73<sup>x,a</sup> | 31.00±2.45<sup>x,a</sup> | 28.25±2.75<sup>x,a</sup> | 28.00±2.94<sup>x,a</sup> | 29.75±1.50<sup>x,a</sup> |
| Iron-based        | 30.75±2.63<sup>x,a</sup> | 31.25±2.87<sup>x,a</sup> | 28.50±2.08<sup>x,a</sup> | 28.50±3.42<sup>x,a</sup> | 28.25±4.03<sup>x,a</sup> |
| **Haemoglobin (g/dL)** |                |                |                |                |                |
| Control           | 10.10±0.52<sup>x,b</sup> | 10.20±0.83<sup>x,b</sup> | 9.40±0.89<sup>x,ab</sup> | 8.83±0.36<sup>x,a</sup> | 9.73±0.67<sup>x,ab</sup> |
| Iron-based        | 10.43±1.03<sup>x,a</sup> | 10.38±0.95<sup>x,a</sup> | 9.38±0.67<sup>x,a</sup> | 9.28±1.05<sup>x,a</sup> | 9.30±1.32<sup>x,a</sup> |
| **Thrombocyte (10⁶/µL)** |                |                |                |                |                |
| Control           | 0.27±0.11<sup>x,a</sup> | 0.22±0.14<sup>x,a</sup> | 0.23±0.13<sup>x,a</sup> | 0.312±0.14<sup>x,a</sup> | 0.37±0.14<sup>x,a</sup> |
| Iron-based        | 0.12±0.03<sup>x,a</sup> | 0.19±0.12<sup>x,a</sup> | 0.20±0.10<sup>x,a</sup> | 0.15±0.04<sup>x,a</sup> | 0.15±0.09<sup>x,a</sup> |

Description: data presented in the average ± standard deviation. The same superscript letters (x,y) in different columns showed no significant differences (P>0.05). The same superscript letters (a,b,c) in different row showed no significant differences (P>0.05).

Figure 1. Erythrocyte profile of stainless steel and iron-based implant in tibial bone of male sheep on different observation day. (a) Erythrocyte (10⁶/µL), (b) Haematocrit (%), (c) Haemoglobin (g/dL), (d) Thrombocyte (10⁶/µL).

The number of erythrocytes fluctuates insignificantly different at each observation time (Table 1, Figure 1a). In the first two weeks after bone pin implantation, the erythropoiesis response has not been stable. The decrease in erythrocyte number can be due to the cytotoxicity of the material. The erythrocyte membrane ruptured and the hemoglobin released into the blood plasma that induced by the implant design and the mechanical properties of the implanted surface [7].

Haematocrit is the percentage of erythrocyte in the blood volume so that the capacity of oxygen that is carried in the blood can be calculated [13]. Based on observations of acute hematocrit values (Table 1, Figure 1b), there was an increase in hematocrit value on day 1 and decrease on day 3, 7, and 14 when compared with day 0 before implantation. Increased hematocrit values on the first day after implantation correspond to an increased erythrocyte number. Low hematocrit values can be caused by anemia or blood loss, hemorrhage, increased erythrolysis, anemia, myeloma, lymphoproliferative hepatocellular carcinoma, and excessive hydration. High hematocrit values can be caused by spleen contractions, hypoproteinemia, erythrocytosis, and dehydration [14].
Hemoglobin is a protein formed from the symmetrical pair of polypeptide chains to carry oxygen from the lungs which play an important role in the biological cycle [15]. Observation of acute hemoglobin levels after implantation of the iron-based implant are listed in Table 1. Based on observation, hemoglobin values insignificantly decreased on day 1, 3, and 7 in the iron-based implant group. The hemoglobin increment may be due to blood loss during implantation and the new hemoglobin has not formed. Hemoglobin accumulates in 3-4 days along with the formation of reticulocytes. Otherwise, on day 14 the value of hemoglobin has insignificantly increased. Iron is also known as a major component of hemoglobin [14], therefore iron-based implantation could accelerate the formation of hemoglobin.

Thrombocyte or platelet value is insignificantly increasing in the first two weeks due to the process of wound healing after implantation (Table 1, Figure 1d). The main role of platelets is to repair the vascular damage and proven hemorrhage by forming a hemostatic plug [13]. The correlations between iron and platelet count were negligible [16].

3.2. Leucocyte profile
Leucocyte and its differentials have the primary role to defend the body through foreign body phagocytic process and antibodies proceed, transportation, and distribution in immune response [17]. The leucocyte count and differentiation are presented in Table 2 and Figure 2.

Table 2. Leucocyte profile of control and iron-based implant in tibial bone of male sheep on different observation.

| Groups                  | Day 0      | Day 1      | Day 3      | Day 7      | Day 14     |
|-------------------------|------------|------------|------------|------------|------------|
| **Leucocyte Count (10^3/µL)** |            |            |            |            |            |
| Control                 | 16.67±3.39 x,y,a | 18.37±2.08 x,y,a | 19.32±5.45 x,y,a | 16.47±2.54 x,y,a | 15.67±1.08 x,y,a |
| Iron-based              | 20.4±2.30x,y,a | 27.72±8.82 x,y,a | 18.90±7.16 x,y,a | 15.52±5.92 x,y,a | 17.32±4.29 x,y,a |
| **Eosinophil (%)**      |            |            |            |            |            |
| Control                 | 0.25±0.5 x,a | 0.25±0.5 x,a | 1.25±0.95 x,a | 0.5±0.57 x,a | 0.25±0.5 x,a |
| Iron-based              | 0.75±0.95 x,a | 1.25±0.95 x,a | 0.25±0.5 x,a | 1.5±0.57 x,a | 1±0.81 x,a |
| **Band Neutrophil (%)** |            |            |            |            |            |
| Control                 | 0.25±0.5 x,y,a | 0.25±0.5 x,y,a | 1.25±0.95 x,y,a | 0.5±0.57 x,y,a | 0.25±0.5 x,a |
| Iron-based              | 0.75±0.95 x,a | 1.25±0.95 x,a | 0.25±0.5 x,a | 1.5±0.57 x,a | 1±0.81 x,a |
| **Segmented Neutrophil (%)** |            |            |            |            |            |
| Control                 | 22.5±6.75 x,y,a | 27.75±14.17 x,y,a | 30±7.11 x,y,a | 27.5±8.34 x,y,a | 21.75±1.26 x,y,a |
| Iron-based              | 22.25±10.04 x,y,a | 36±29.4 x,y,a | 27.5±6.45 x,y,a | 25.25±4.92 x,y,a | 28.75±10.11 x,y,a |
| **Lymphocyte (%)**      |            |            |            |            |            |
| Control                 | 70.25±8.18 x,a | 67.75±12.92 x,a | 63.75±9.7 x,a | 69.5±7.59 x,a | 72.75±2.21 x,a |
| Iron-based              | 73±10.13 x,a | 59.25±33.5 x,a | 67.5±7.93 x,a | 68.5±4.65 x,a | 64.75±9.56 x,a |
| **Monocyte (%)**        |            |            |            |            |            |
| Control                 | 4±1.82 x,a | 1.5±1.73 x,a | 4±1.41 x,a | 3.25±1.25 x,a | 3.75±1.5 x,a |
| Iron-based              | 2.75±0.95 x,a | 3.25±1.25 x,a | 3.25±1.89 x,a | 4±1.41 x,a | 3.75±1.7 x,a |
| **Basophil (%)**        |            |            |            |            |            |
| Control                 | 0±0       | 0±0       | 0±0       | 0±0       | 0±0       |
| Iron-based              | 0±0       | 0±0       | 0±0       | 0±0       | 0±0       |

Description: data presented in the average ± standard deviation. The same superscript letters (x,y) in different columns showed no significant differences (P>0.05). The same superscript letters (a,b,c) in different row showed no significant differences (P>0.05).

Leucocyte count increment in both groups (Table 2, Figure 2a) indicates the presence of a post-implantation response caused by the procedure or the implanted implant. Leukocytosis occurs as a normal protective response to the physiologic stressor [18]. The highest total number of leucocyte in
control and the iron-based group were on day 3 and day 1 post-implantation, respectively. After the bone injury, leucocyte rapidly infiltrates the fracture site, which marks the inflammatory phase of the fracture healing. Insignificantly changes in this study indicated the inflammatory response was still tolerable to the sheep.

Eosinophils count data showed insignificantly fluctuated results and still in normal range (Table 2, Figure 2b). Eosinophils are a variety of white blood cells and one of the immune systems responsible for combating multicellular parasites and associated with the allergic disorder [18]. The iron administration is known have no exacerbate response in allergic experimental [19]. It indicated both implants did not trigger an allergic reaction.

Figure 2. Leucocyte profile of stainless steel and iron-based implant in tibial bone of male sheep on different observation day. (a) Leucocyte (10³/µL), (b) Eosinophil (%), (c) Band neutrophil (%), (d) Segmented neutrophil (%), (e) Lymphocyte (%), (f) Monocyte (%), (g) Basophil (%).

The inflammation process could activate neutrophils and macrophages and lead to phagocytosis of foreign materials, which may occur in the early phase of bone healing. Table 2 shows the highest neutrophil number in control and iron-based implant are day 3 and 1, respectively. The highest monocyte number in control and iron-based implant are occurred on day 3 and 7, respectively. The results of this study in accordance with previous research that neutrophils and then followed by macrophages appeared in the early stages of inflammation [20]. These insignificant neutrophils and monocytes changes between both groups indicated there was no excessive inflammatory response at various observation times.

Basophils counts were zero in both groups (Table 2, Figure 2g). An increase in the circulating number of Basophils is a rare and generally, is a response to inflammation and immediate hypersensitivity reactions [15]. Basophils play a role in inflammatory reactions during an immune response, as well as in the formation of acute and chronic allergic disease. Based on the basophils count, both implanted implant groups have no hypersensitive reaction.

4. Conclusion

Generally, iron-based implant did not cause significant changes in systemic effects of an iron-based biodegradable implant through erythrocyte and leucocyte profile observation. It can be concluded the iron-based implant is still tolerated sub-chronically in sheep.
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