International Invention of Scientific Journal
Available Online at http://www.iisj.in
eISSN: 2457-0958
Volume 03 | Issue 04 | April, 2019 |

Original Article

OPEN ACCESS

Received: February 16, 2019
Accepted: April 12, 2019
Published: April 29, 2019

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Human Recombinant Erythropoietin in Rat Caput Femur with Steroid Exposed Increase Number of Osteocytes, Osteoblasts, BMP-2, VEGF, and Reduce Adipose Cells

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Abstract

Osteonecrosis is a process of death bone which causes disturbance of the bone blood vessel. It has been considered that erythropoietin (EPO) may affect cell proliferation by stimulating angiogenesis. It also has been proved that cell proliferation during osteogenesis depends on the formation of new blood vessels. Function of EPO is to stimulate angiogenesis, indirectly, it has role in bone repair. This research is an experimental study designed using Randomized post-test control. Our sample is using Winstar rats, which were divided into 2 groups, Group P0 received intervention of dexametasone injection for 5 weeks without rHuEpo, P1 group received intervention of dexametasone injection and rHuEpo for 5 weeks. On the last day of week 5th, rat femoral heads were taken for VEGF, BMP 2, osteocyte, osteoblast and adipocyte levels testing. From immunohistochemical and histopathologic examination, results were analyzed using SPSS. Our results conclude that number of osteocyte cells in rat femoral heads which injected with dexamethasone and rHuEpo were higher than those injected with dexamethasone only, significant values ($p < 0.05$), also number of osteoblast cells values ($p < 0.05$). As well as expression of BMP-2 and VEGF were higher in rats with dexamethasone injected and rHuEPO had significant value ($p < 0.05$). Meanwhile, number of adipocyte cells in rat femoral heads which dexamethasone injected and given rHuEpo was lower than those were injected with dexamethasone only ($p < 0.05$). Administration of rHuEPO in rats which injected with dexamethasone was shown to increase the number of osteoblasts, osteocyte, BMP-2 expression, VEGF and adipocyte cells were lower than in rats which injected with dexamethasone only.

Key Words: Osteonecrosis, eritropoetin, osteocyte, osteoblast, BMP-2, VEGF, adipocyte

Introduction

Osteonecrosis (ON) is the process of death bone which causes disturbance of the bone blood vessel. Besides from idiopathic causes, main cause of osteonecrosis may include trauma (most frequent causes) and non-traumatic causes (systemic conditions), such as alcoholism, steroid therapy, hematological diseases, and Systemic Lupus Erythematosus (SLE).1 There are two categories of Femoral Head Osteonecrosis (ONFH), traumatic and non-traumatic. ONFH leads to a pathology and clinical manifestations caused by malfunction or interruption of the blood supply of the femoral head which causes the bone marrow and osteocytes cells become necrosis.2

The relationship between excessive corticosteroid use and the incidence of ON has been understood since the first case reports on patients with rheumatoid arthritis in 1957. The incidence of ON parallel with increasing of systemic corticosteroid therapy as well as organ transplants. Glucocorticoids are the most common cause of nontraumatic osteonecrosis. Dose-effect of corticosteroid therapy in osteonecrosis had not yet known, recent research suggests that corticosteroid dose above 25-40 mg/day may causes a significant risk of nontraumatic ON on a kidney transplant and patients with SLE.3 Steroids were not only inhibiting function of osteoblasts and osteoclasts but also induces apoptosis of osteoblasts and osteoclasts. Clinical research showed that nitric oxide metabolism changes in bone cells of the development of osteonecrosis in rat femoral heads and accompanied by extensive apoptosis of osteoblasts and osteocytes, which shows the nitric oxide-mediated apoptosis is a potential mechanism of Steroid-Associated Osteonecrosis (SAON).5 Studies in rat and humans had showed that dexamethasone were given in certain doses, had triggered the differentiation of stem cells that obtained from Bone Marrow Cells (BMCS) into adipocytes which then inhibit osteogenesis. Dexamethasone has been shown to inhibit the expression of collagen type-I and osteocalcin, and then reduce the differentiation of BMCS.3 Dexamethasone has been shown to increase the expression of mRNA Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ) and reduce mRNA expression of Cbfa1. Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ) and factor Core Binding Factor Alpha 1(Cbfa1) is an important transcription factor in the differentiation of pluripotent cells into adipogenic and osteogenic cells. These findings support the idea that dexamethasone functions were to inhibit adipogenesis and osteogenesis. In addition, recent study was also found that dexamethasone can be interfered (with angiogenesis by suppressing the production of Vascular Endothelial Growth Factor (VEGF)).3 Some of erythropoietin (EPO) studies, also found that EPO structure, production, and how hematopoietic has successfully been described in detail, while the disposal and degradation of EPO could not understand thoroughly. In 1985, the discovery of EPO nucleotides strands enables the production of EPO human recombinant (rHuEPO) for clinical use. Erythropoietin worked as a primary modulator of erythropoiesis with resistance effect, proliferation, also differentiation of erythroid progenitor cells and regulate erythrocytes which circulate in peripheral blood.6 Erythropoietin (EPO) is a glycoprotein hormone that regulates the production of red blood cells, produced mainly by kidney in adults and liver during fetal life. The use of recombinant human EPO (rHuEPO) has been approved by the Food and Drug Administration (FDA) and is widely used for the treatment of anemia associated with kidney failure, cancer, prematurity, chronic inflammatory diseases and human immunodeficiency virus infection.8 Some studies have shown that EPO can improve bone healing, yet the mechanisms which regulate the process is still unclear. One study says that EPO has role in the new bone regeneration by stimulating the JAK-STAT signaling pathway in HSCs through Epo-R. This process stimulates the production of BMPs, especially BMP2 and BMP6. The results from the production of BMPs and HSCs can induce osteogenic progenitor cells to differentiate into osteoblasts and stimulates the production of cartilage through interaction with the BMP receptors cell surface (BMPRs).9 It is envisaged that the EPO can affect cell proliferation by stimulating angiogenesis. It has been demonstrated that the proliferation of cells during osteogenesis highly dependent on new blood vessel formation. VEGF is a potent angiogenic and osteogenic growth factors in bone repair process. Interestingly, EPO has a similarity genetic and functional to VEGF, so it has a similar role in bone repair. EPO has been reported to stimulate tissue regeneration after skin injury and myocardial infarction through the VEGF pathway. Giving Epo help to upregulates the expression of VEGF during the initial phase of healing. 1

Materials and Methods

The study was conducted from February until March 2018 at Pathology Veterinary laboratorium in Fakultas Kedokteran Hewan Universitas Udayana Bali. The aim of this study is to strengthen the theory of osteonecrosis using rHuEPO through role VEGF and BMP-2. This study is an experimental study was designed using randomized post-test control only. This study sample was using male Winstar Rat who has been conditioned in certain environment and food. The use of experimental animals will be required a certificate Eligible Research Ethics. Rat will be studied with any adjustment to the place and the food. Food was given diverted from eating vegetables to extract rat food comprising 20-25% protein, 5% fat, 40-50% starch, 5% crude fiber. Every day of every mouse was fed a diet of 12-20 grams. For drinking water will be provided 80-100 cc/kg per day and will remain available drinking water ad libitum. Each rat will occupy at rat cage

made of wood or bamboo and will be kept clean, sheltered from the wind, rain and direct sunlight, an ambient temperature of about 15-20 ° C (Smith and Mangkoewidjojo, 1988). Both groups of rats were housed in the Veterinary Laboratorium of Fakultas Kedokteran Hewan Universitas Udayana with a size of 30x20 cm and given normal diet in the form of pellets and water twice a day. Research conducted in the morning at 09.00 am. Using 36 rat with a type of male Wistar rat between the ages of 12 weeks. Rat were divided into two groups, Group P0 treated in the form of injection dexamethasone 1 mg / kg / weight intramuscularly 2 times a week for 5 weeks without administration rHuEpo dose 500 unit/kg/day. This group were treated in the form of injection P1 dexamethasone 1 mg/kg/weight 2 times a week and given rHuEpo dose 500 unit/kg/day for 5 weeks. Each mouse weighted every week throughout the study. On the last day of the fifth week, the rat euthanized with ketamine, then interstitial part of the femur rat was taken at medial proximal side and distal to examination levels of VEGF, BMP levels 2 and osteocytes, osteoblasts and adipocytes. The rest of the rat's body was burned. The data collected will be analyzed with a statistical program SPSS for Windows version 22.0.

Results

This is an experimental study of osteonecrosis of rat femoral head due to exposure to corticosteroids. One group was given rHuEPO for 5 weeks and compared with no provision of rHuEPO. The samples were examined in the laboratory, and then the data was processed and analyzed. This data set consists of quantitative calculation of the number of osteocytes, osteoblasts, BMP-2, VEGF and adipocytes in trabecular in one visual field with the magnification of 200 and 400 times. Data was also captured using digital camera. At the end of the 5th week, specimens were harvested. The proximal part of the femur was taken and the number of osteocytes, osteoblasts, BMP-2, VEGF and adipocytes were evaluated. Specimens were examined under a microscope by a predetermined histopathology procedure, and quantitative data was recorded. The histopathology features are shown in the figures 1,2 and 3 below.

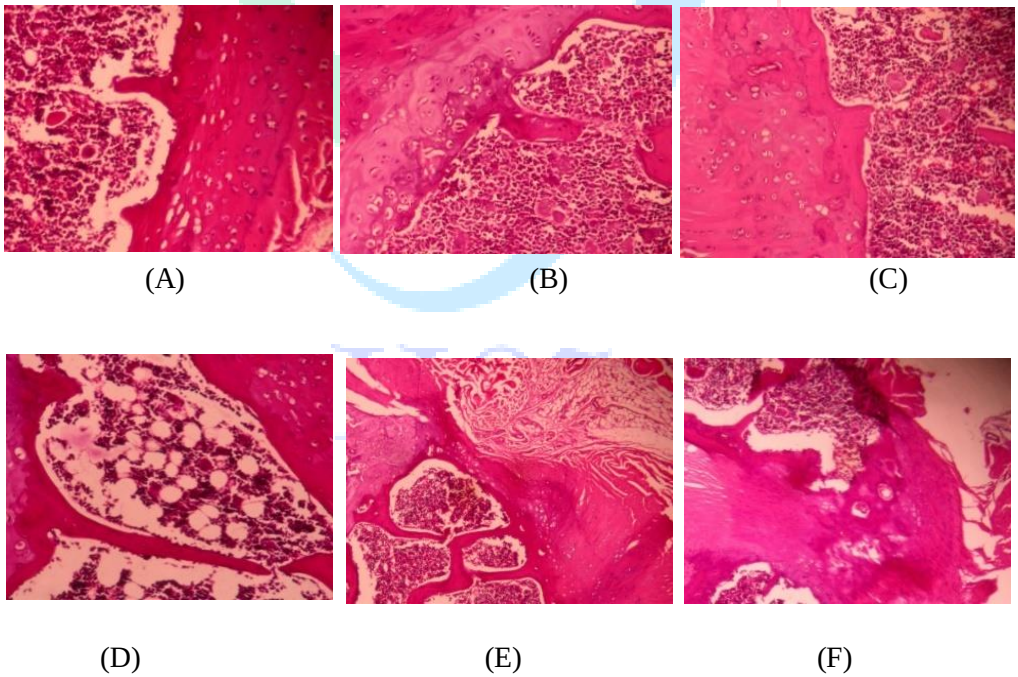
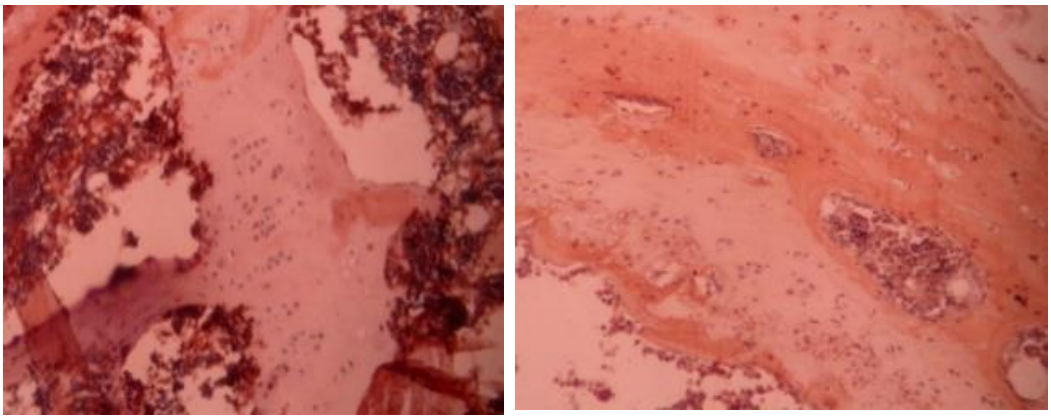
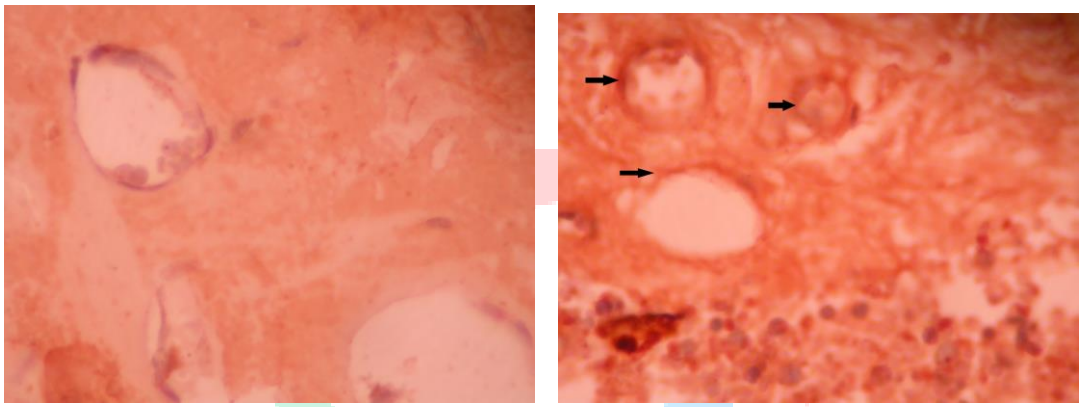


Figure 1: Histopathological examination of osteocytes, osteoblasts, and adipocytes in the control group (A), (B), (C) and in the treatment group (D), (E), (F) respectively



(A) (B)

Figure 2: The immunohistochemical examination of BMP-2 in the control group (A) and in the treatment group (B)



(A) (B)

Figure 3: The immunohistochemical examination of BMP-2 in the control group (A) and in the treatment group (B)

The quantative data include the number of osteocytes, osteoblasts, adipocytes and VEGF count and also the expression of BMP-2 as shown in table 1 and 2. The number of osteocytes count in the control group (105.4 ± 3.59) is lower than the treatment group (160.6 ± 7.62) (P = 0.000). Similar results can be seen in terms of number of osteoblast cells in the control group (203.8 ± 10.01) and treatment group (299.6 ± 8.58) (P = 0.000). Meanwhile, the average number of adipocytes cells in the control group (151.7 ± 5.10) is higher than the treatment group (60.2 ± 5.60) (P = 0.000). As for VEGF, the control group (4.31 ± 1.53) has lower value than the treatment group (7.56 ± 1.71) (P = 0.000). All statistical analysis was based on 95% CI. This means that the numbers of osteocytes, osteoblasts, adiposity and VEGF in control and treatment group were significantly different.

Table 1: The mean difference of number of osteocytes, osteoblasts and adipocytes and VEGF between the control and treatment groups

Variables	Group		Mean difference	95% CI	p value
	Treatment with PDGF & HA (n = 16)	Control without PDGF & HA (n = 16)			
Osteocytes	160,6 ± 7,62	105,4 ± 3,59	55,1	50,8 – 59,4	0,001
Osteoblasts	299,6 ± 8,58	203,8 ± 10,01	95,8	89,1 – 102,6	0,001
Adipocytes	60,2 ± 5,60	151,7 ± 5,10	-91,5	-95,3 – (-87,6)	0,001
VEGF	7,56 ± 1,71	4,31 ± 1,53	3,2	2,0 – 4,4	

Table 2: The expression of BMP-2 between the control and treatment groups

BMP-2	Group		Total	P Value
	Control	Treatment		
Weak expression	11 68.8%	5 31.3%	16 100,0%	0,001
Medium expression	2 12.5%	14 87.5%	16 100,0%	0,001

Table 2 shows that expression of BMP-2 in the control group (n = 11 (68.8%)) is weaker were than in the treatment group (n = 5 (31.3%)). Weak expression of the BMP-2 was found in the control group (n = 2 (12.5%)) compared with the treatment group (n = 14 (87.5%)). Using the Chi Square test, we found that BMP-2 between the two groups has a significance level of > 0.05.

Discussion

In this study, we administered rHuEPO to determine the effect on the number of osteocytes, osteoblast, BMP-2, VEGF and adipocytes in cases of osteonecrosis of the head of the femur induced with dexamethasone.

Effect of Erythropoietin on Osteocyte Cells of The Femoral Head with Osteonecrosis Induced by Dexamethasone

In this study, the higher number of osteocytes cells in rats treated by rhuEPO was proven to be significantly different. Previous study showed that the osteogenic potential of EPO is due to its ability to suppress inflammation and to down-regulate NF-kB. Therefore, there are no side effects in terms of translation to clinical practice, namely, to increase hemoglobin levels to the extreme.6

Effect of Erythropoietin on Osteoblast Cells of The Femoral Head with Osteonecrosis Induced by Dexamethasone

The higher number of osteoblasts cells in rats treated by rhuEPO was proven to be significantly different. EPO was thought to be able to stimulate the production of BMPs, especially BMP2 and BMP6. The results of the production of BMPs and HSCs were the induction of osteogenic progenitor cells to differentiate into osteoblasts and the stimulation of the production of cartilage through interaction with the cell surface BMP receptors (BMPRs).16

Effect of Erythropoietin on the Expression of BMP-2 on The Osteonecrosis Induced by Dexamethasone

Some studies have shown that EPO can improve bone healing, yet the mechanism regulating the process is still unclear. According to one study, EPO has a role in the regeneration of new bone by stimulating the JAK-STAT signaling pathway in HSCs through Epo-R. This process stimulates the production of BMPs, especially BMP2 and BMP6. BMPs and HSCs can also induce osteogenic progenitor cells to differentiate into osteoblasts and stimulate the production of cartilage through interaction with the cell surface BMP receptors (BMPRs).16

Effect of Erythropoietin Against VEGF in The Osteonecrosis Induced by Dexamethasone

It is envisaged that Epo may affect cell proliferation by stimulating angiogenesis. It has been demonstrated that the proliferation of cells during osteogenesis is highly dependent on new blood vessel formation. VEGF is a potent angiogenic and osteogenic growth factors during bone repair process.9

Effect of Erythropoietin Against Adipocyte Cells in The Osteonecrosis Induced by Dexamethasone

In this study, the number of adipocytes is fewer in rats given rHuEPO. Results from a recent study demonstrated that EPO receptor is expressed on the surface of BMSCs and through this, EPO can promote the proliferation of BMSCs in acute renal failure. Recent research has also found that EPO stimulates the mobilization of BMSCs during bone tissue damage and stimulate differentiation of BMSC in the process of osteogenesis.29

Conclusion

Based on research results of rat administration rHuEPO in exposed dexametason research

conclusions obtained that administration rHuEPO can prevent the incidence of osteonecrosis in the rat's femoral head exposed to dexamethasone. From the conclusions, rHuEPO can be used as a basis for prevention osteonecrosis caput femur caused by long-term corticosteroid use. However, studies are needed using different samples or sample more to get clinical effects, especially in humans.

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