Clinical Research

Biomolecular changes on mice caput femur after human recombinant erythropoietin administration

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ABSTRACT

Introduction: Osteonecrosis is a process of death of bone tissue which also causes disturbance in the bone's blood vessel supply. Administration of recombinant human erythropoietin (rHuEPO) may affect cell proliferation by stimulating angiogenesis and it has been proven that cell proliferation during osteogenesis depends heavily on the formation of new blood vessels. Therefore, EPO has a role in bone regeneration.

Methods: This research is an experimental study using Randomized post-test control only group design. Rats were divided into 2 groups, P0 which received dexamethasone injection for 5 weeks and group P1 which received dexamethasone injection combined with rHuEpo for 5 weeks. On the last day of the fifth week, the femoral head of the rats were taken for assessment of VEGF (Vascular Endothelial Growth Factor), BMP-2 (Bone Morphogenic Protein-2), osteocytes, osteoblasts and adipocytes levels. The examination results were then analyzed by using SPSS.

Results: From the immunohistochemical and histopathologic examination and further SPSS analysis, it is shown that the number of osteocyte in the femoral heads of rats injected with dexamethasone and rHuEpo were significantly higher than those injected with dexamethasone alone ($p < 0.05$). This was also the case with the number of osteoblasts ($p < 0.05$). The expression of BMP-2 and VEGF were also significantly higher in rats injected with dexamethasone and rHuEPO ($p < 0.05$). However, the number of adipocytes in the femoral heads of the rats injected with dexamethasone and rHuEpo were significantly lower than those injected with dexamethasone alone ($p < 0.05$).

Conclusion: Administration of rHuEPO in rats induced with dexamethasone was shown to increase the number of osteoblast, osteocyte, BMP-2 expression, and VEGF, but the number of adipocyte was found to be lower than in rats injected with dexamethasone alone.

ABSTRAK

***Pendahuluan**: Osteonecrosis adalah proses kematian pada tulang yang juga menyebabkan gangguan suplai pembuluh darah tulang. Pemberian eritropoietin rekombinan manusia (rHuEPO) diketahui dapat mempengaruhi proliferasi sel dengan jalan menstimulasi angiogenesis. Proliferasi sel juga telah dibuktikan sangat bergantung pada pembentukan pembuluh darah baru. Oleh karenanya, EPO memiliki peran dalam regenerasi tulang.*

***Metode:** Penelitian ini adalah penelitian eksperimental yang dirancang menggunakan* Randomized post-test control only group design*. Sampel tikus dibagi menjadi 2 kelompok. Kelompok tikus P0 mendapat perlakuan injeksi deksametason selama 5 minggu, kelompok tikus P1 mendapat perlakuan injeksi deksametason selama 5 minggu dan pemberian rHuEpo selama 5 minggu. Hari terakhir pada minggu kelima, bagian tulang femur tikus diambil untuk dilakukan pemeriksaan kadar VEGF* (Vascular Endothelial Growth Factor), *kadar BMP-2 (Bone Morphogenic Protein-2), osteosit, osteoblas dan adiposit. Hasil pemeriksaan ini kemudian dianalisis menggunakan SPSS.*

***Hasil**: Dari pemeriksaan imuno histokimia dan histopatologi dan analisis SPSS selanjutnya menunjukkan bahwa jumlah selosteosit pada kaput femur tikus yang diinjeksi deksametason dan diberikan rHuEpo* (Recombinant Human Erithropoietin) *lebih tinggi dibandingkan tikus yang hanya diinjeksi deksametason dengan nilai yang berbeda nyata ($p<0,05$), begitu pula dengan jumlah selosteoblas ($p<0,05$). Ekspresi BMP-2 dan VEGF juga memiliki nilai yang lebih tinggi pada tikus yang diinjeksi deksametason dan diberikan rHuEPO dengan nilai signifikansi yang sama ($p<0,05$). Namun, jumlah sel adiposit pada kaput femur tulang tikus yang diinjeksi deksametason dan diberikan rHuEpo lebih rendah dibandingkan dengan tikus yang hanya diinjeksi deksametason dengan nilai signifikansi ($p<0,05$).*

***Kesimpulan:** Pemberian rHuEPO pada tikus yang diinjeksi deksametason terbukti dapat meningkatkan jumlah sel osteoblas, sel osteosit, ekspresi BMP-2, VEGF, dan jumlah sel adiposit ditemukan lebih rendah dibandingkan tikus yang hanya diinjeksi deksametason.*

Keywords: Osteonecrosis, erythropoietin, osteocyte, osteoblast, BMP-2, VEGF, adipocyte

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INTRODUCTION

Osteonecrosis (ON) is the process of bone death which causes disruption of the vascular supply of bone. Besides idiopathic causes, osteonecrosis may also becaused by trauma (the most frequent cause) and non-trauma (systemic conditions), such as alcoholism, steroid therapy, haematological diseases, and Systemic Lupus Erythematosus (SLE).[1] There are two categories of Osteonecrosis of the Femoral Head (ONFH), traumatic and non-traumatic. ONFH leads to pathological and clinical manifestations due to malfunction or interruption of blood supply of the femoral head resulting in necrosis in bone marrow and osteocyte cells.[2]

Studies in mice and humans showed that dexamethasone that is given in certain doses and independent of timewill trigger differentiation of the stem cells obtained from the 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 suppress the differentiation of BMCS.[3,4,5]

Dexamethasone has been shown to increase the expression of mRNA of Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ) and lower the mRNA expression of Cbfa1. Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ) and Core Binding Factor Alpha 1(Cbfa1) are important transcription factors in the differentiation of pluripotent cells into adipogenic and osteogenic cells. These findings support the idea that dexamethasone inhibits adipogenesis and osteogenesis. In addition, several studies also stated that dexamethasone can interfere with angiogenesis by suppressing the production of Vascular Endothelial Growth Factor (VEGF).[3,6,7]

After a century of study of erythropoietin (EPO), the structure, the production, and how hematopoietic produced the EPO has successfully been described in detail, while the disposal and degradation of EPO could not be understood thoroughly. Erythropoietin (EPO) is a glycoprotein hormone that mainly regulates the production of red blood cells, and is produced mainly by the kidney in adults and the liver during fetal life. The use of recombinant human EPO (rHuEPO) has been approved by the Food and Drug Administration (FDA) and is now widely used for the treatment of anaemia 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 regulating the process is still unclear. One study reported that 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. 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 cell surface of BMP receptors (BMPRs).[9]

It is envisaged that EPO can affect cell proliferation by way of 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 in bone repair process. Interestingly, EPO has a genetic and functional similarity to VEGF, so it has a similar role in bone repair. EPO has also been reported to stimulate tissue regeneration after injury to the skin and myocardial infarction through the VEGF pathway. EPO administration upregulates the expression of VEGF during the initial phase of healing of bone defects. In addition, EPO administration is also associated with an increase in the number of blood vessels in osteotomy gap in 2 weeks.[10,11,12]

METHODS

The study was conducted from February 2018 to March 2018 at Veterinary PathologyLaboratory, Veterinary Faculty of Udayana University, Bali. The aim of this study is to strengthen the theory of management of osteonecrosis using rHuEPO through the role of VEGF and BMP-2.

This study is an experimental study designed withrandomized post-test control only group.The study population was maleWistar Rat whichhad been conditioned in cages environment and food. The use of experimental animals needed certificate of Eligible Research Ethics.

The Ratswere subjected to adjustment to the place and the food. The food was changed from vegetables to rat food extract containing20-25% protein, 5% fat, 40-50% starch, 5% crude fiber. Every day, the rats were fed with a diet of 12-20 grams rat food extract. Drinking waterwas

provided 80-100 cc/kg per day, drinking water remained available ad libitum. Each group of rats occupied a cage made of wood or bamboo and the cage was kept clean, sheltered from the wind, rain and direct sunlight, with ambient temperature of about 15-20°C (Smith and Mangkoewidjojo, 1988). The cages of both groups of ratwere keptin the Veterinary Laboratory,Veterinary Faculty of Udayana University with a size of 30x20 cm and given a normal diet in the form of pellets and water twice a day. This research was conducted in the morning at 09.00 am using 32 male Wistar rats about 12 weeks of age.The first group,group P0,was treated with dexamethasone 1mg/kg/weight intramuscularly 2 times a week for 5 weeks without administration ofrHuEpo. The second group, group P1, was treated with dexamethasone 1 mg/kg/weight 2 times a week for 5 weeks and rHuEpo 500 unit/kg/day for 5 weeks.Each rat wasweighed every week throughout the study.On the last day of the fifth week, the rats wereeuthanized with ketamine and then interstitial part of the femur of the rats weretaken at the medial proximal and distal side forassessment of VEGF, BMP levels 2 and osteocytes, osteoblasts and adipocytes levels. The rest of the rats' body wereburned.

The data collected was analyzed with a statistical program, SPSS for Windows version 22.0.Data analysis was conducted with the following steps: Descriptive analysis, Normality test by the Shapiro-Wilk test to determine whether the sample data was derived from a population of normal distribution, Homogeneity analysis by Levene test to determine whether the data variance was homogeneous.Inferential analysis: if the data had normal distribution (the value of $\alpha = 0.05$), it was conducted independent t-test,if the distribution wasnot normal (the value of $\alpha = 0.05$), it was conductednon-parametric test with Mann Whitney U-Test.

RESULTS

The data from the experimental studies of osteonecrosis of the femoral head of the rats exposed to corticosteroids combined withrHuEPOwas compared to the data from the rats exposed to corticosteroids alone for 5 weeks.The samples were taken for examination in the laboratory followed by data processing and analyzed to evaluate and answer the hypotheses and the research objectives. Thedata wasaresult of quantitative calculation of osteocytes, osteoblasts, BMP-2, VEGF and adipocytes in trabecular bone using visual field microscope with a magnification of 200 times and 400 times combined with a digital camera in order to gave digital image. There were36 rats randomlygrouped (simple random sampling) into 2: 1.Control group (P0)was injected with dexamethasoneintramuscularly 1 mg/kg, 2 times a week for 5 weeks. 2. Treatmentgroup (P1)was injected withdexamethasone intramuscularly 1 mg/kg, 2 times a week for 5 weeks,combined with rHuEPO 500 U/kg/day for 5 weeks.

At the end of the study, five animals were terminated and then the proximal part of the femur was taken and the number of osteocytes, osteoblasts, BMP-2, VEGF and adipocytes were calculated. The results obtained in the form of quantitative data. The data obtained by examination under themicroscope with a pre determined histopathology

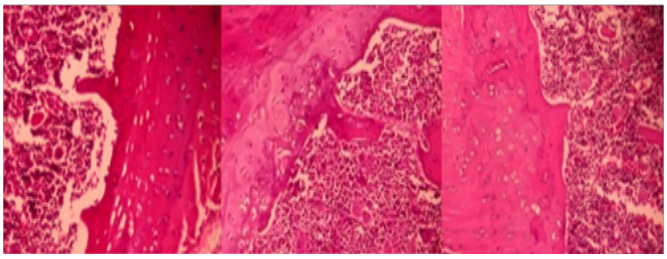

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Figure 1. Results of histopathological examination ofosteocytes, osteoblasts and adipocytes in the control group

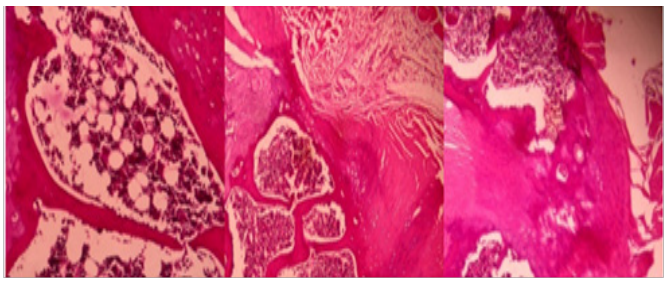

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Figure 2. Results of histopathological examination of osteocytes, osteoblasts and adipocytes in the treatment group

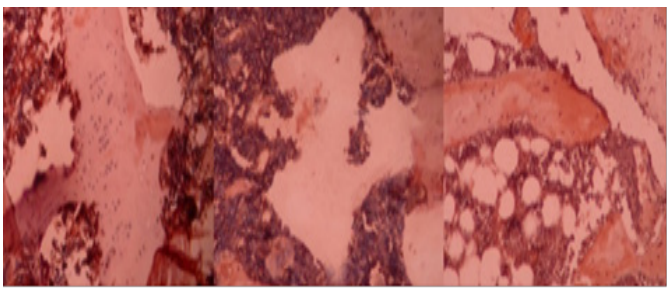

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Figure 3. The results of immunohistochemicalexamination of BMP-2 in the control group

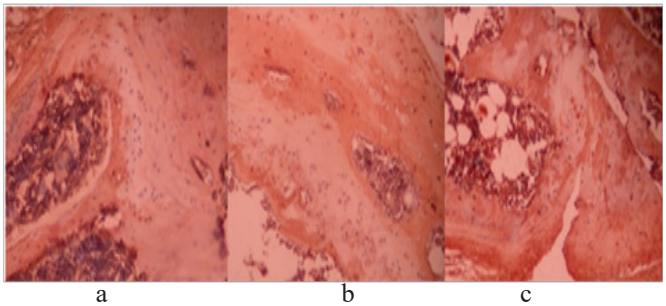

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Figure 4. The results of immunohistochemical examination of BMP-2 in the treatment group

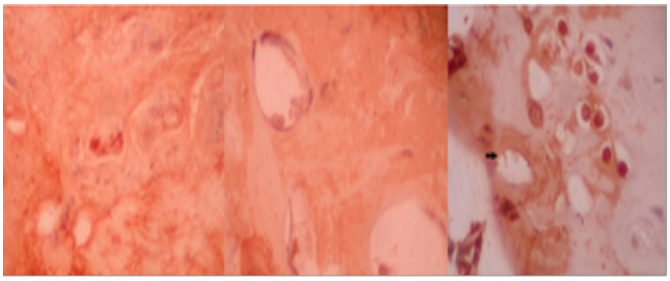

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Figure 5. The results of immunohistochemical examination of VEGF in control group

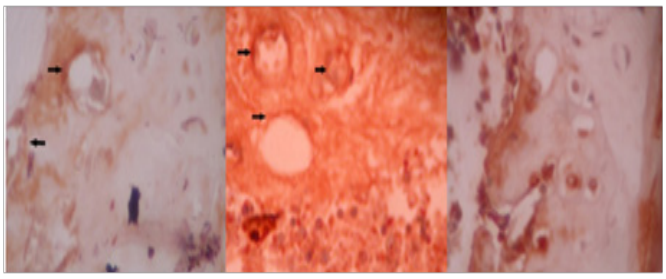

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Figure 6. The results of immunohistochemical examination of VEGF in Treatment Group

Descroptive Analysis

Descriptive data analysis aims to gain a clearer picture of the distribution and the standard deviation of each variable of the study. From the distribution shown inthe Table 1, it can be seen that the total number of research subjects are 32 subjects, which aredivided into control and treatment groups. Each group contains 16 subjects (50% each).

Table 2 shows the average number of variables in each treatment group. The number of osteocytes in the control group showsa lower number(105.4 ± 3.59) compared to the treatment group administered with erythropoietin(160.6 ± 7.62). Lowerresults can also be seen in the number of osteoblasts in the control group (203.8 ± 10.01) compared to the treatment group (299.6 ± 8.58). However, the average number of adipocytes in the control group is higher (151.7 ± 5.10) compared to the treatment group (60.2 ± 5.60). The result for VEGF is lower in the control group with a value of (4.31 ± 1.53) compared to the treatment group (7.56 ± 1.71).

Table 1. Frequency distribution of research subjects

	Osteo-cytes	Osteo-blasts	BMP-2	VEGF	Adipo-cytes
Control	16 (50%)	16 (50%)	16 (50%)	16 (50%)	16 (50%)
Treat-ment	16 (50%)	16 (50%)	16 (50%)	16 (50%)	16 (50%)
Total	16 (50%)	16 (50%)	16 (50%)	16 (50%)	16 (50%)

Table 2. Average number of osteocytes, osteoblasts and adipocytes in thecontrol and

	Variable	Mean	Standard Deviation (SD)
Osteocytes	Control	105.4	3.59
	Treatment	160.6	7.62
Osteoblasts	Control	203.8	10:01
	Treatment	299.6	8.58
Adipocytes	Control	151.7	5.10
	Treatment	60.2	5.60
VEGF	Control	4.31	1.53
	Treatment	7.56	1.71

Analysis of Inferential

This analysis aims to generalize the results to the population. Statistical inferenceused in this study was independent t-test.

Normality and Homogenety Test

The normality test used wasthe Shapiro-Wilk test and the variant homogeneity test used was Levene's test.

Table 3 shows the data for osteocytes, osteoblasts, adipocytes and VEGF normal distribution, where the value of $p > 0.05$.

In Table 4, the results of Levene's homogeneity test showed that the osteocytes, osteoblasts, adipocytes and VEGF are homogeneous ($p > 0.05$).

Table 3. Shapiro-Wilk Normality Test Results

Variable	Category	Shapiro-Wilk		
		statistics	Df	Sig
Osteocytes	Control	0.970	16	0.832
	Treatment	0.934	16	0.279
Osteoblasts	Control	0.940	16	0.351
	Treatment	0.896	16	0.070
Adipocytes	Control	0.942	16	0.379
	Treatment	0.971	16	0.861
VEGF	Control	0.895	16	0.067
	Treatment	0.902	16	0.086

Table 4. Levene's Homogenitiy Test Results

	Levene's Test		
	F	T	Sig
Osteocytes	2.73	26.19	0000
Osteoblasts	8.38	7.29	0000
Adipocytes	0.17	-48.28	0000
VEGF	0.090	5.65	0000

Table 5.Significanttest data result by the Independent T-test for the control and treatment groups

	Independent T-test					
	Df	Sig. (2 tailed)	Mean difference	Standard Error diff	Lower (CI 95%)	Upper (CI 95%)
Osteocytes	30	0000	55.1	2,107	50.8	59.4
Osteoblasts	30	0000	95.8	3.297	89.1	102.6
Adipocytes	30	0000	-91.5	1,895	-95.3	-87.6
VEGF	30	0000	3.2	0575	2.0	4.4

Independent T-test

Table 5 shows the mean difference value of osteocytes at 95% CI is 55.1 (50.8-59.4) and P = 0.000, osteoblasts at95% CI is 95.8 (89.1-102.6) and P = 0.000, adipocytes inCI 95% is at -91.5 (-95.3-87.6)) and P = 0.000, and VEGF at 95% CI is 3.2 (2.0-4.4) and P = 0.000. This means that there is significant difference amongthe osteocytes, osteoblasts, adipocytesand VEGF in the control and the treatment groups

Chi Square Test

From Table 6 , it is shownthat weak expression of BMP-2 is greater in the control group (11(68.8%)) than in the treatment group (5(31.3)), while mediumexpression of the BMP-2 in the control group is much less(2 (12.5%)) compared to the treatment group (14 (87.5%)).The results of Chi Square test obtained for BMP-2 has significance level of (p> 0.05).

Table 6. Crosstabulation expression of BMP-2 in the control group and the treatment group

Cross tabulation			
BMP-2	Group		Total
	Control	Treatment	
Weak expression	11	5	16
	68.8%	31.3%	100.0%
Expression Medium	2	14	16
	12.5%	87.5%	100.0%

Table 7. Significance test data by Chi Square for the control and treatment groups

Chi Square		
Variables	Df	Asymp. Sig. (2-sided)
BMP-2 (person Chi Square)	1	0.001

DISCUSSION

In this study, rHuEPO was injected to rats to determine itseffect on the number of osteocytes, osteoblast, adipocytes,BMP-2, and VEGF in cases of osteonecrosis of the femoral head induced by dexamethasone.

Effect of erythropoietin on osteocytesin osteonecrosis induced by dexamethasone

In this study, the higher number of osteocytes in rats treated withrhuEPO 500 mg/kg/day for 5 weeks isproven to be significantly different from the control group without rHuEPO administration, with p = 0.000. From a previous study, it is reported that osteogenic potential of EPO is derived from the ability to suppress inflammation and down-regulation of NF-kB. However, another research found no decrease in osteogenic effect.

Effect of erythropoietin on osteoblastsin osteonecrosis induced by dexamethasone

In this study, the number of osteoblasts is higher in rats administered withrHuEPO 500 U/kg/day for 5 weeks and proven to be significantly different fromthe control group without rHuEPO administration, with p = 0.000.This may be caused by EPO that can stimulate the production of BMPs, especially BMP-2 and BMP-6.[14]The production of BMPs and HSCs can then induce osteogenic progenitor cells to differentiate into osteoblasts and stimulate the production of cartilage through the interaction with the cell surface of BMP receptors (BMPRs).[15,16]

Effect of erythropoietin on the expression of bmp-2 in osteonecrosis induced by dexamethasone

In this study, the expression of BMP-2 is higher in rats treated withrHuEPO 500 U/kg/day for 5 weeks and proven to be significantly different fromthe control group without rHuEPO administration, with p = 0.001. Some studies have shown that EPOcan improve bone healing, yet the mechanisms regulating the process is still unclear. One study reported that EPO has a role in the regeneration of new bone.EPO activates JAK/STAT signaling in hematopoietic stem cells (HSCs) through the EPO receptor (EPO-R) leading to the production of bone morphogenetic protein 2 (BMP2), especially BMP2 and BMP6. The results from the production of BMPs can induce osteogenic progenitor cells to differentiate into osteoblasts and stimulates the production of cartilage through interaction with the cell surface of BMP receptors (BMPRs).[16,17,18]

Effect of erythropoietin onvegf in the osteonecrosis induced by dexamethasone

In this study, VEGF expression ishigher in rats treated withrHuEPO 500 U/kg/day for 5 weeks and proven to be significantly different fromthe control group without rHuEPO administration, with p = 0.000.EPOcanaffect cell proliferation by stimulating angiogenesis.[19] 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 in bone repair process.[20,21,22]

Effect of erythropoietin onadipocytes in osteonecrosis induced by dexamethasone

In this study, the number of adipocytes is fewer in rats treated withrHuEPO 500 U/kg/day for 5 weeks and provento be significantly different fromthe control group without rHuEPO administration, with p = 0.000.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.[23,24,25] Recent research has also found that EPO stimulates the mobilization of BMSCs into bone tissue damage and stimulate differentiation of BMSCs in osteogenesis.[26,27,28]

CONCLUSION

Based on this research results, rHuEPO administration in rats inducedwith dexamethasone can prevent the incidence of osteonecrosis of the femoral heads. Therefore, it is concluded thatrHuEPOcan be used as a basis for prevention of osteonecrosisof caput femur caused by long-term corticosteroid use. However, further studies are needed by using different samples or with greater number of samplesin orderto get clinical effects, especially in humans.

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