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Implantation of bovine hydroxyapatite and secretome with different oxygen concentration may improve massive bone defect regeneration: An experimental study on animal model

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Abstract

The most widely used biomaterials in the treatment of massive bone defects are allograft bone or metal implants. The current problem is that the availability of allographs is limited and metal implants are very expensive. Mass production of secretome can make bone reconstruction of massive bone defects using a scaffold more effective and efficient. This study aims to prove bone regeneration in massive bone defects using bovine hydroxyapatite reconstruction with normoxic and hypoxic secretome conditions using collagen type I (COL1), alkaline phosphate (ALP), osteonectin (ON), and osteopontin (OPN) parameters. This is an in vivo study using male New Zealand white rabbits aged 6–9 months. The research was carried out at the Biomaterials Center—Tissue Bank, Dr. Soetomo Hospital for the manufacturer of bovine hydroxyapatite (BHA) and secretome BM-MSC culture under normoxic and hypoxic conditions, and UNAIR Tropical Disease Institute for implantation in experimental animals. Data analysis was carried out with the one-way ANOVA statistical test and continued with the Post Hoc test LSD statistical test to determine whether or not there were significant differences between groups. There were significant differences between hypoxic to normoxic group and hypoxic to BHA group at day-30 observation using ALP, COL 1, ON, and OPN parameters. Meanwhile, there is only osteonectin parameter has significant difference at day-30 observation. At day-60 observation, only OPN parameter has significant differences between hypoxic to normoxic and hypoxic to BHA group. Between day-30 and day-60 observation, BHA and normoxic groups have a significant difference at all parameters, but in hypoxic group, there are only difference at ALP, COL 1, and ON parameters. Hypoxic condition BM-MSC secretome with BHA composite is superior and could be an option for treating bone defect.

Keywords

BHA, secretome, ALP, collagen 1, osteopontin, osteonectin

Introduction

In Indonesia, from 1997 to 2001, there was an increase in the need for biomaterials as much as 400%.[1] The need will continue to grow due to the increasing cases of bone damage due to trauma, tumors, congenital abnormalities, infections, and bone resorption due to complications of joint prostheses. Bone defects due to trauma, tumors, congenital abnormalities, and other diseases are still a major problem in the field of orthopedics and traumatology. Small bone defects can heal spontaneously, but in certain circumstances require a small bone graft.[2] In massive bone defects, spontaneous healing is not possible

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because large defects do not allow bone healing to occur. For massive bone defect reconstruction, a graft is needed to fill the bone defect.[3]

Currently, most of the biomaterials used in the treatment of massive bone defects are allograph bone or metal implants as fillers for bone defects. The availability of allographs is limited, depending on the availability of human bone donors, while metal implants (endoprosthesis) are very expensive, so many patients cannot afford them. This condition can result in disability and even amputation.[4] Allograft bone only has osteoconductive and slightly osteoinductive properties because it still contains growth factors contained in the organic components of bone, while the osteogenesis property is completely lost because all allograft bone cells have died.[5] The use of an allograph will provide benefits to the recipient because it avoids the occurrence of morbidity in patients due to taking autographs; the number is relatively more and also varies in shape and size; in addition to the above advantages, allographs have several weaknesses that must be considered, namely, having the risk of transmitting disease and causing immunogenic reactions.[6]

Massive bone defect reconstruction with tissue engineering by adding biomaterials with stem cells and/or growth factors gives great hope as an answer to the aforementioned problems. Bonegraf will serve as an osteogenesis, osteoinductive, and osteoconductive.[7] In the process of proliferation and differentiation of stem cells into osteoblast cells, it is characterized by the ability of the cells to produce alkaline phosphatase (ALP), bone sialoprotein (BSP), collagen type I (COL I), osteonectin (ON), and osteopontin (OPN) as well as mineralization.[8] In small bone defects, stem cells can be directly injected. Stem cells will proliferate and differentiate into osteoblasts and bone regeneration occurs.[9]

Many studies have been carried out for the reconstruction of massive bone defects using various types of scaffolds and cells. Alizadeh-Osgouei and colleagues used rabbits with synthetic scaffolds of hydroxyapatite, titanium, and biodegradable polymer-bioceramic composite. Mesenchymal stem cells were used with a concentration of 2×10^7 cells. The results showed that the best results were on the hydroxyapatite scaffold.[10] If we can produce a lot of secretomes in vitro, then we can get a lot of growth factors needed for bone regeneration that are in those secretomes. With the mass production of a secretome that is useful for bone regeneration in vitro, we can make bone reconstruction in massive bone defects using a scaffold with the addition of an appropriate secretome to make bone regeneration more effective and efficient.[11] In one study, 5% of hypoxic conditions in stem cell culture could affect the condition of stem cells to stay younger (stemness). This condition of stemness will certainly maintain the nature of the stem cells to remain like the parent. With properties that match the parent, stem cells will be better able to provide useful protein production through the resulting secretome.[12]

In the process of mesenchymal stem cell differentiation into osteoblasts, RUNX2 is a very important gene for mesenchymal stem cells to the osteoblast lineage and actively directs the early stages of osteoblast differentiation. Osterix (OSX) begins to play an important role in differentiation toward osteoblasts following RUNX2-mediated mesenchymal condensation. During the differentiation process, RUNX2 is involved in the expression of bone matrix genes COL 1, osteopontin, and osteonectin.[13] Based on the above background, this study aims to prove bone regeneration in massive bone defects using bovine hydroxyapatite reconstruction with normoxic and hypoxic secretome conditions using COL 1, osteopontin, osteonectin, and ALP parameters within 30-day and 60-day observation.

Methods

Study design

This research was conducted using a pure experimental research design on experimental animals using a post-test-only control group design and a research design with a control group, as shown in Figure 1. Randomization was carried out by dividing into three groups: the BHA group, the BHA group with normoxic secretome, and the BHA group with hypoxic secretome. On the 30th day and 60th day, the three groups were seen through the parameters of COL 1, osteonectin, osteopontin, and ALP.

Synthesis of bovine hydroxyapatite (BHA)

The first process for BHA synthesis was dissection or cleaning of bovine bones from soft tissue, then cutting according to the required size and cleaned using 70% alcohol. Then, it was followed by washing with H_2O_2 solution and pasteurization, which was putting the bones into a water shaker and heated to a temperature of 60°C. After that, it was rinsed to remove the H_2O_2 solution. The next step is to soak the bone in a solution of N-Hexane to remove the remaining fat and wash it again. Bones are put into a furnace for deproteinase process with a furnace temperature of 1000°C for 2 h. The deproteinase (HA) bone was washed with distilled water and dried in an oven at 100°C for 1 h. The final process is to sterilize with 15kGγ gamma rays.

Normoxic BM-MSCs culture

Cells that have been arranged with a density of 105 cells/cm^2 in culture media consisting of -MEM plus 20% Fetal Bovine Serum (FBS), 100 U/ml penicillin, 100 mg/mL streptomycin were cultured on 10 cm Petri dishes in an

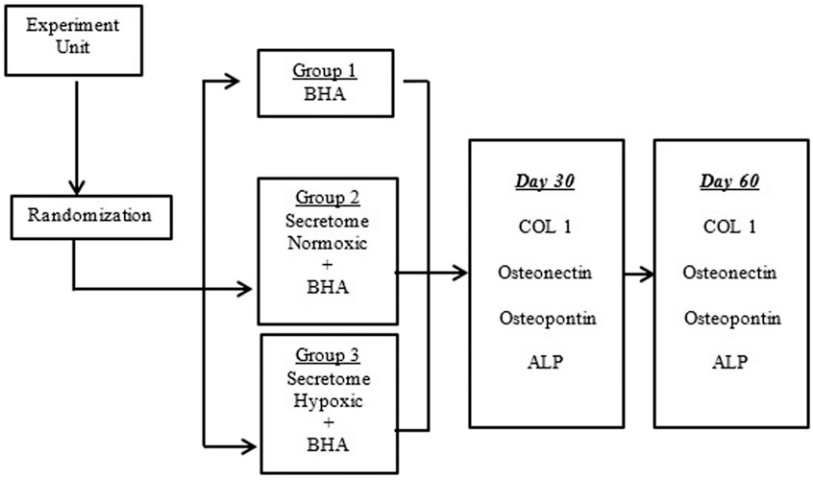

Figure 1. Research design chart.

incubator. With a temperature of 37°C and 5% CO_2, content was then selected based on the adhesion and proliferation of BM-MSCs on the base of tissue culture Petri dishes. Media replacement is done 2 times per week. BM-MSCs were separated from the media using 0.05% trypsin/0.53 mM EDTA and replated and rearranged at a density of 5 × 103 cells/cm^2 in the same culture medium as the first section. These BM-MSCs were then separated with trypsin to produce cells that would be used as germ cells on the studied scaffold. On the second day, the cells were washed, by taking the old medium and washing it with phosphate-buffered saline (PBS), then replacing it with the new medium. On the 7th day, when the cell growth was 80% confluent, splitting was carried out. The old medium was taken and discarded, then washed with 5 cc of PBS until it was clean of cell debris, then added with 2 cc of trypsin, and incubated for 5 min in the incubator. The addition of trypsin is done to break down the cells. After 5 min, 2 cc of -MEM growing medium was added and then resuspended to separate the fallen cells into single cells. The results of the resuspension were transferred to a sterile 15 mL disposable tube for centrifugation. The pellets obtained after the centrifugation process were then added to 10 cc of -MEM growing medium, resuspended and transferred to 2 Petri plates with a diameter of 10 cc, and incubated back into the incubator.[14]

Hypoxic BM-MSCs culture

Within 72 h, hypoxic preconditioning was administered to stem cell culture with several doses of O_2 concentration (21% and 1%) and several day of cultivation time until it reached the 4th passage. Cells from the BM-MSCs obtained from the phase 1 study were grown up to the 4th phase. Cells were taken aseptically and then placed in Petri dishes with a density of 2x10[5] cells/cm^2 in 10 Petri dishes with a diameter of 5 cm with α-MEM medium containing 20% FBS, BM-MSCs simulator supplements and antibiotics (100 U/ml penicillin/100µg/mL). Streptomycin) at 37°C, 5% CO_2 and 95% Nitrogen. Maintenance quiescence of BM-MSCs cells was carried out by administering hypoxic conditions with several doses of O_2 concentration (21% and 1%) by inserting cultured Petri dishes into a special incubator for hypoxic conditions (Modular Incubator Chamber) which was cultured for 14–21 days[14]

Secretome and BHA implantation into experimental animals

The secretome implantation process into the BHA is carried out in the following steps. BHA was soaked with normoxic secretome and hypoxic secretome in separate tubes for 24 h at room temperature. Then, the BHA composite with secretome can be processed further, namely, implantation into experimental animals. BHA composite implantation surgery with secretome into experimental animals was carried out in the operating room of the Institute for Tropical Diseases, Universitas Airlangga, Indonesia. The procedure is carried out as follows. The rabbits were anesthetized first by administering an injection of ketamine 20 mg/kg intramuscularly and xylazine 3 mg/kg intramuscularly; then the fur on the right front extremity was shaved. The incision is made layer by layer until it reaches the radius bone; then the radius bone and its periosteum are cut 1 cm long. In the treatment group, BHA composite implantation was performed with normoxic and hypoxic secretome, while in the control group, only BHA was given to replace the radius bone and fixed with 0.1 nylon thread. The surgical wound was sutured and splinted with a soft splint. Experimental

animals during the study were placed and free to move in the drum.[1]

Statistical analysis

Data collected from immunohistochemistry examination of COL I, ON, OPN, and ALP. The reliability test of 2 examinations was performed. Then, proceeding with the normality test, one-way ANOVA test on all data, and the LSD Post Hoc test were carried out to determine whether there were significant differences between groups.

Results

The first step in this study was to perform a bone marrow aspiration. Prior to the aspiration, the rabbit was anesthetized by giving ketamine injection and then the rabbit's radius was implanted using a secretome with bovine hydroxyapatite as shown in Figure 2.

On day 30 and day 60 after implantation surgery, immunohistochemistry examination of ALP, COL I, osteonectin, and osteopontin was performed and data analysis was performed as shown in Table 1. Normality test is useful for determining the data that has been collected is normally distributed or taken from a normal population. In the normality test, if $p > 0.05$, then the sample is normally distributed. If $p < 0.05$, then the sample is not normally distributed. The parameters of COL 1, ON, OPN, and ALP with observation time of 30 days and 60 days were normally distributed because they had p value >0.05 and could be continued with homogeneity test.

The results in Table 1 are in the COL 1, osteonectin, osteopontin, and ALP groups with the BHA, normoxic, and hypoxic groups at 30 days of observation having a p value < 0.05 indicating a significant difference between the BHA, normoxic, and hypoxic groups. The results showed that there were significant differences in the groups, namely, in the COL 1, osteonectin, osteopontin, and ALP groups. Meanwhile, in the 60-days observation, only the osteopontin parameter had a p value of 0.05, which is 0.003, this indicates that there is a significant difference in the osteopontin parameter with the BHA, normoxic, and hypoxic groups. Meanwhile, in the COL 1, ALP, and osteonectin group parameters, $p > 0.05$; this indicates that there is no significant difference with the BHA, normoxic, and hypoxic groups.

In the COL 1 parameter, it was found that there was no significant difference between the BHA and normoxic groups, while the BHA group with hypoxic and the normoxic and hypoxic group obtained significant differences. The results of this COL I study are shown in Figure 3, namely, the thickness of COL I in the BHA group.

Based on Table 1 through the Post Hoc LSD test on the 30-day observation ALP parameters, it is known that there is no significant difference between the BHA group and normoxic, while the BHA group with hypoxic and the normoxic and hypoxic group obtained a significant difference. Meanwhile, in the 60-day observation, there were no significant differences between the BHA, normoxic, and hypoxic groups. The results of this ALP study are shown in Figure 4, namely, ALP expression in the hypoxic BHA group. Green arrows indicate ALP-expressing osteoblasts and red arrows indicate non–ALP-expressing osteoblasts.

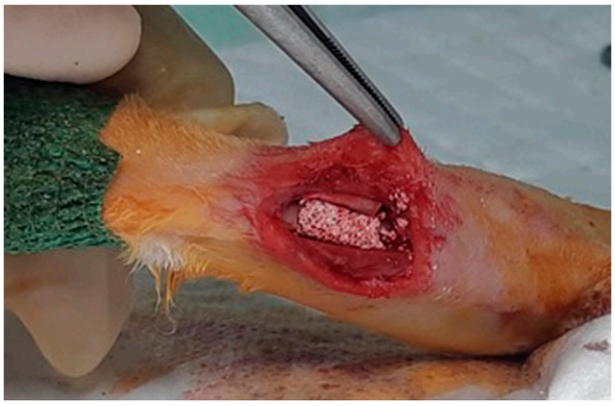

Figure 2. Secretome with bovine hydroxyapatite implantation at radius bone of white rabbit.

Through the Post Hoc LSD test on the osteonectin parameter of 30-day observation, there were statistically significant differences between the groups, namely, the BHA group with hypoxic, the BHA group with normoxic and the normoxic group with hypoxic. Meanwhile, in the 60-day observation, there were no significant differences between the BHA, normoxic, and hypoxic groups. The results of this osteonectin study are shown in Figure 5. Green arrows indicate osteonectin-expressing osteoblast, and red arrows indicate non osteonectin-expressing osteoblasts.

Based on Table 1 on the parameters of osteopontin observation for 30 days, it is known that there is no significant difference between the BHA group and normoxic, while the BHA group with hypoxic and the normoxic and hypoxic group obtained a significant difference. At 60 days of observation through the Post Hoc test of Multiple Games-Howell comparisons in Table 1, it is known that there is a significant difference between groups, namely, the BHA group with hypoxic and the normoxic group with hypoxic, which is statistically significant. The results of this osteopontin study are shown in Figure 6. Green arrows indicate osteopontin-expressing osteoblasts, and red arrows indicate non osteopontin-expressing osteoblast.

Furthermore, an analysis was carried out to determine the difference between the 30-day and 60-day observation on the parameters of COL 1, ALP, osteonectin and osteopontin with the BHA, normoxic and hypoxic groups as shown in

Table 1. Comparatives analysis of parameters in various groups at 30 and 60 days.

Parameter	Group	Day 30			Day 60		
n	SD.	*p*-value	*n*	SD.	*p*-value		
ALP	BHA	7	22.53 ± 4.557	< 0.001[1,2]	7	3.68 ± 1.693	0.064[1]
	Normoxic	7	19.23 ± 1.825		5	1.80 ± 0.786	
	Hypoxic	7	11.70 ± 2.841		7	2.54 ± 1.069	
COL I	BHA	7	2.81 ± 0.246	0.005[1,2]	7	3.46 (2.87–4.39)	0.275[3]
	Normoxic	7	2.74 ± 0.288		5	3.67 (3.55–3.88)	
	Hypoxic	7	3.17 ± 0.099		7	3.65 (3.46–5.64)	
Osteonectin	BHA	7	7.93 ± 1.404	<0.001[1,2]	7	3.15 (1.95–4.10)	0.059[3]
	Normoxic	7	12.38 ± 2.580		5	2.1 (1.2–4.45)	
	Hypoxic	7	20.39 ± 2.391		7	1.7 (1.55–2.75)	
Osteopontin	BHA	7	11.13 ± 1.033	0.009[1,2]	7	5.21 ± 1.529	0.003[5,6]
	Normoxic	7	10.61 ± 0.500		5	5.39 ± 1.828	
	Hypoxic	7	9.39 ± 1.186		7	9.84 ± 3.043	

[1]ANOVA.
[2]LSD.
[3]Kruskal–Wallis.
[4]Mann–Whitney.
[5]Brown–Forsythe.
[6]Games-Howell.

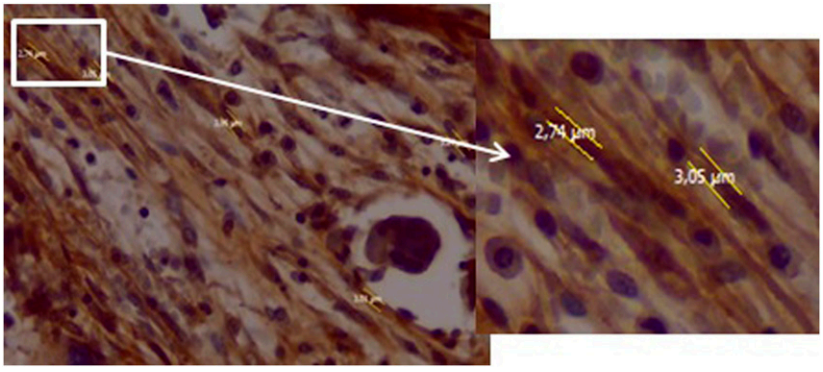

Figure 3. The results of the thickness of the COL I group BHA.

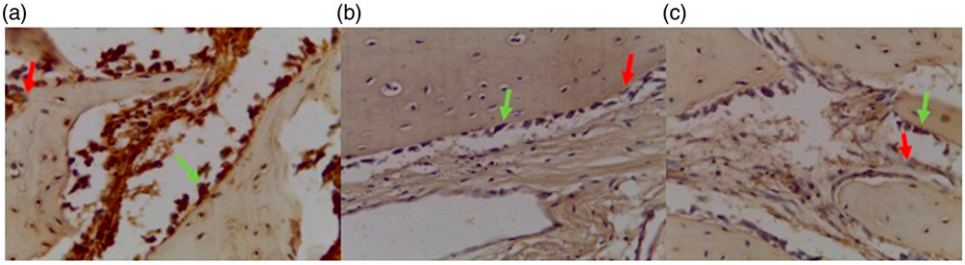

Figure 4. (a) Results of ALP expression in the 30-day observation group BHA; (b) the results of ALP expression in the BHA group with normoxic secretome at 60 days observation; (c) the results of ALP expression in the BHA group with hypoxic secretome at 30 days observation.

Figure 7. The results obtained were in the BHA group; there was an effect on the observation time 30 days and 60 days were significant on the parameters of COL 1, ALP, osteonectin, and osteopontin. Meanwhile, in the BHA group with the addition of normoxic secretome, the results also showed a significant effect on the parameters of COL 1, ALP, osteonectin, and osteopontin. In the BHA group with the addition of a hypoxic secretome on the osteopontin

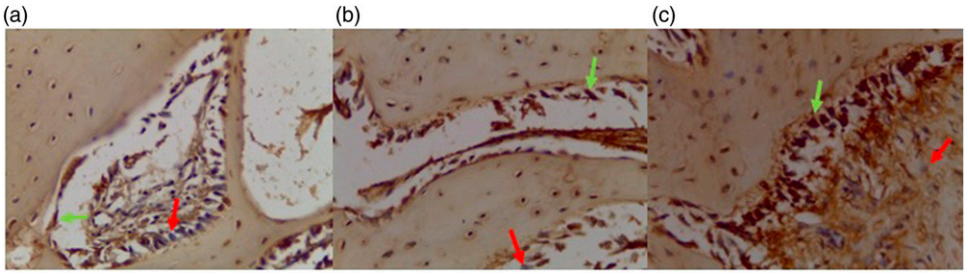

Figure 5. (a) The results of osteonectin expression in the 60-day observation group BHA; (b) the results of osteonectin expression in the BHA group with normoxic secretome for 30 days observation; (c) the results of osteonectin expression in the BHA group with hypoxic secretome at 30 days observation.

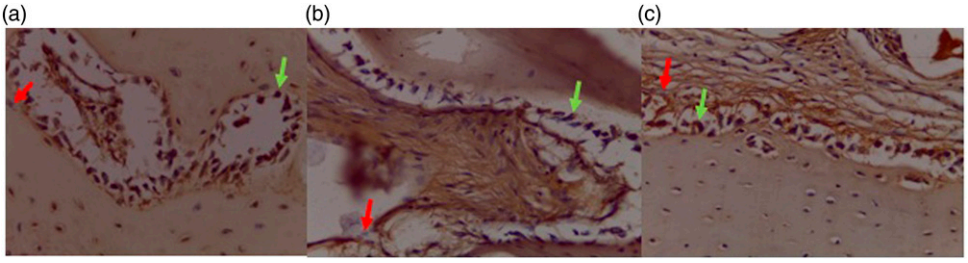

Figure 6. (a) Results of osteopontin expression in the 30-day observation group BHA; (b) the results of osteopontin expression in the BHA group with normoxic secretome at 60 days observation; (c) the results of osteopontin expression in the BHA group with hypoxic secretome at 60 days observation.

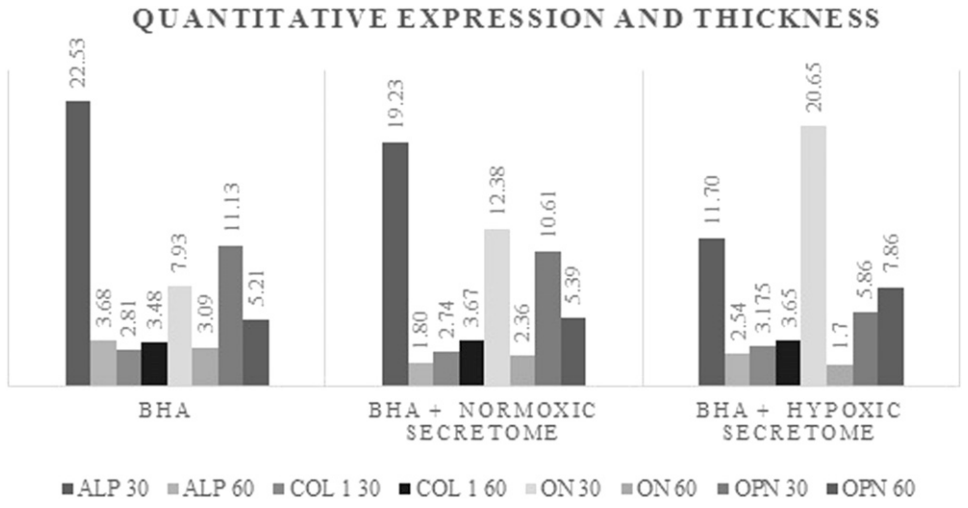

Figure 7. Quantitative expression and thickness of parameters in various groups between 30 and 60 days

parameter, the results showed that there was no significant effect on the observation time of 30 days and 60 days, but there is significant difference in ALP, COL 1, and osteonectin parameters.

Discussion

In this study, one-way ANOVA test in the COL 1, ALP, osteonectin, and osteopontin parameters shows a significant difference ($p < 0.05$). Post Hoc LSD test was continued and the results obtained BHA compare to BHA with normoxic secretome; there is no significant difference in 3 parameters, which are COL 1, ALP, and osteopontin. While there is significant difference in COL 1, ALP, osteonectin, and osteopontin between BHA compare to BHA with hypoxic secretome and BHA with normoxic secretome compare to BHA with hypoxic secretome ($p < 0.05$). Setyowardoyo has suggested that hypoxic conditions lead to controlled

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proliferation. Although hypoxic conditions caused slower proliferation, the process of MSCs formation in hypoxic culture was faster than that of normoxic culture. Slow proliferation is the process of breeding and population of stem cells in the body to maintain their existence through the ability to multiply slowly. This ability can be done repeatedly, even though to be unlimited, and can be maintained for a relatively long time in vivo.[2]

In hypoxic cultures, protein levels in the secretome were higher than in normoxic cultures. Hypoxic cultured stem cells produce more growth factors. Hypoxic can trigger chondrogenic differentiation of human adipose tissue-derived mesenchymal stem cells (hASCs), can down-regulate osteogenesis, and inhibit endochondral ossification with low expression in RUNX2 and COL1 resulting in inhibition of the expression of COL 1.[15] Measurements in the two groups, namely, COL 1, showed that the normoxic group had a lower score and thickness in the normoxic group than in the hypoxic group. Under normoxic conditions, the cells had high proliferation, but these cells directly differentiated into progenitor cells, causing loss of pluripotency which was indicated by the absence of OCT4 and SOX2 expression.[2] Meanwhile, in hypoxic conditions, the condition resembles stem cells and is in a quiescent state, so that cells with hypoxic conditions are still able to maintain multiple properties which are characterized by the expression of OCT 4 and SOX2.[16]

The results of this study at the observation time of 60 days showed that there was a significant difference in the osteopontin parameters of the BHA to hypoxic secretome and the normoxic to hypoxic group. Meanwhile, the COL 1, ALP, and osteonectin parameters did not show any significant differences in the BHA, normoxic, and hypoxic groups. Both in vitro and in vivo approaches confirm the key role of hypoxic conditions in the survival of hematopoietic stem cells (HSCs). In vitro culture of mammalian HSCs under hypoxic conditions maintains cell quiescence and increases the transplantation potential of these cells when performed in vivo in recipient mice. In an in vivo context, hypobaric hypoxic mice exhibited increased proliferation and mobilization of BM HSCs associated with lineage changes characterized by increased megakaryocyte–erythrocyte progenitors.[17] Tsai and colleagues in their studies also found that the number of ALP cells was lower than in all normoxic groups due to inhibition of differentiation in hypoxic conditions. The hypoxic culture observation showed the lowest ALP production. Hypoxic is a condition that can prevent mesenchymal stem cells from differentiating into osteoblasts so that mesenchymal stem cells maintain their originality. Mesenchymal stem cells in their secretome still produce IGF, TGF, BMP2 so that some cells are still trying to differentiate into osteoblasts.[18] Grayson and colleagues found that osteonectin levels in hypoxic conditions increased significantly at the end of the induction period. Hypoxia has been reported to suppress adipogenic differentiation which explains why a higher number of multipotent cells at the start of the induction period only resulted in slightly higher LPL expression than hypoxic cells.[15]

The results of data analysis on the effect of treatment for 30 days and 60 days showed that there was an effect on the BHA group and the BHA group with normoxic secretome on parameters COL 1, ALP, osteopontin, and osteonectin. Meanwhile, in the BHA group with hypoxic secretome, the effect of 30 days and 60 days of observation was shown in the results with parameters COL 1, ALP, and osteonectin. The osteopontin parameter did not show any effect from the 30-day and 60-day observation. Hypoxic around the bone defect triggers the osteogenic differentiation of the precursor cells and promotes bone regeneration. Inducing hypoxia in precursor cells has been reported to promote the healing of bone defects. In addition, hypoxia enhances osteogenesis–angiogenesis via VEGF signaling during bone defect healing.[19] Lee and colleagues found in their studies that sustained hypoxia inhibited most markers of osteogenic differentiation, including RUNX2. The results showed that hypoxia which too short (1 day) or too long (5 and 7 days) periods failed but for 3 days strongly increased the osteogenic differentiation of the precursor cells. This suggests that hypoxia has an important optimal duration that induces bone regeneration in the healing process.[20] Differentiation under hypoxic conditions showed an increase in the expression of chondrogenic genes and proteins, such as an increase in SOX5, 6, and 9, aggrecan, and type II collagen.[21]

Osteopontin parameter in 30-day observation, the results obtained in the one-way ANOVA test were significantly different ($p < 0.05$). Osteopontin is an extracellular matrix protein produced by various types of cells, such as osteoblasts, osteoclasts, T lymphocytes, NK cells, and epithelial cells. Osteopontin affects normal physiological processes including bone resorption, wound healing, tissue remodeling, and vascularization. Osteopontin has also been shown to be involved in all stages of cancer development, tumor invasion, angiogenesis, and metastasis. $CoCl_2$-induced hypoxic can upregulate osteopontin mRNA and protein expression in osteosarcoma cells.[22] Osteopontin is involved in processes related to cell adhesion and cell-matrix attachment and is maximally expressed at the onset of mineralization and is associated with mineralization formation processes.[23]

Conclusion

Secretome combined with bovine hydroxy apatite as scaffold will have a better results in bone regeneration. Hypoxic secretome with BHA was superior to normoxic sceretome with BHA or BHA alone for speeding up of bone

healing process. Hypoxic secretome will be an option for treating bone defect.

Declaration of conflicting interests

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