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Research Report

The role of ubiquinone supplementation on osteogenesis of non-vascularized autogenous bone graft

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ABSTRACT

Background: *Ubiquinone is one of food supplement which is known have positive effect in wound healing. However the study to evaluate the possible role of ubiquinone in bone healing in autogenous bone grafting after mandibular resection has not been studied. An in vitro study is required to evaluate whether ubiquinone or coenzyme Q-10 (CoQ10) has a positive effect on osteogenesis. Viability test of CoQ10 and a model of osteogenic-induced and hypoxic-condition mesenchymal stem cell culture were established to support the study.* ***Purpose:*** *The study was made to evaluate the role of ubiquinone in osteogenesis by analyzing the toxicity effect and the optimal dose of CoQ10 that might interfere in bone marrow derived mesenchymal stem cell (BM-MSC) that was dose in cell culture medium. The BM-MSC culture under hypoxia condition were also observed.* ***Method:*** *The toxicity and the optimum viability concentration of ubiquinone were observed using MTT assay. The osteogenic differentiation under hypoxic condition was done on BM-MSC in osteogenic medium that composed of ascorbic acid, glycerolphosphate and dexamethasone in hypoxia chamber for 21 days. Osteogenic differentiation and cellular hypoxia features were tested with immunocytochemical staining using anti-Runx2 and anti-HIF1α monoclonal antibody, respectively.* ***Result:*** *the maximum density value of 1.826 was found in the group of ubiquinone concentration of 75μM/ml, increasing of in concentration of ubiquinone resulted with the decrease of optical density of CoQ10. Statistic analysis using Anova showed with no significant difference among groups with various concentration. Immunocytochemical staining showed that Runx2 expression in 3% hypoxia group ($p<0.05$).* ***Conclusion:*** *Ubiquinone was found non toxic in its optimum dose of 75μM/ml, showed by optimum result in the expression of Runx2 and HIF1α further study is necessary to evaluate the angiogenic and osteogenic effect of ubiquione.*

Keywords: *Osteogenesis; ubiquinone; toxicity; hypoxia*

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INTRODUCTION

Mandibular defects after tumor resection needs reconstruction to restore mastication, speech and facial aesthetic. Gold standard of mandibular reconstruction is non-vascularized autogenous bone graft because of its osteogenic, osteoconductive, and osteoinductive properties. However, the result of bone healing with autogenous bone grafting depend largely on the integrity of surrounding periosteum since it is the source of mesenchymal stem cells (MSC) and blood vessels which is critical in initial graft healing.[1] Periosteum, however, is often defective in patients affliced with extramural tumors in which case the graft failed to grow or incorporate with the surrounding bone.[2] Increasing angiogenesis in such cases can be theoretically achieved by supplementing the patients with micronutrient that increase the expression of angiogenic factor, such as vascular endothelial growth factors (VEGF) by osteogenic-committed mesenchymal stem cell. Study showed that VEGF expression was regulated by hypoxia-inducible

factor 1 alpha (HIF-1α), a trancription factor responding to hypoxic condition.[3]

Ubiquinone, or coenzyme Q-10 (CoQ10), is one of essential co-enzymes which has antioxidant effect to protect cell membrane from oxidation by the reactive-oxygen species (ROS) and provides a regenerative effect, sustaining effective levels of antioxidants in damaged tissues.[4] With regards to that, a viability test of ubiquinone and a model of osteogenic and hypoxic-induced cell culture is required prior to the study. The expression of Runt-related transcription factor-2 (Runx2), the most important osteogenic trancriptional factor, was frequently used as a marker for osteogenic-committed MSC.

The aim of this study was to evaluate the role of ubiquinone in osteogenesis. The objectives of this current study are to analyze the toxicity and optimal dose of ubiquinone in bone-marrow derived mesenchymal stem cell (BM-MSC); osteogenic differentiation of BM-MSC culture under osteogenic medium; and hypoxia characterization of BM-MSC culture under hypoxic condition.

MATERIAL AND METHODS

The study used stock of BM-MSC culture which was isolated and expanded from human bone-marrow aspirates from iliac crestal bone. The processing and storage of BM-MSC culture were performed in Centre for Biomaterial Tissue Bank and Stem Cell, Dr. Soetomo General Hospital, Surabaya. The thawing procedure was as follows. The vial of cells was removed from dry ice, defrosted in 37^0 C water bath until ice in the vial was no longer visible. The vial was opened and content transferred to a sterile 15 mL tube then slowly added 10 mL of alpha MEM medium, and centrifuged for 10 minutes. The pellet was resuspended gently in the medium and then transferred into a coated culture flask and incubated at 37^0C and 5% CO_2.

Culture of BM-MSC of 80-90% confluence was harvested using typsination, the cells were replaced in wells containing 2.5 x 10^4 cells per well and incubate under 37^0 C, 5% CO_2 for 24 hours. Samples of ubiquinone with dose of 10, 20, 50, 75, 100μM were added into the wells and incubate for 20 hours. Tetrazolium dyes composed of 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium (MTT) were then added to the wells and incubate for 4 hours and finally dimethyl sulfoxide (DMSO) was supplemented to increase the solubilizaton of formazan formed. The color changes in the well was analyzed with ELISA reader using 595 nm wave length.

BM-MSC from passages 4 were detached with trypsin solution and seeded into 24-well microplate, with density of 2 x 10^7 cell/cm^2, which were divided into experimental and control group consisting of 10 wells per group. The wells in experimental group was given osteogenic medium, composed of minimum essential medium Eagle - alpha modification, or α-MEM, supplemented with ascorbic acid, glycerol phosphate and dexamethasone for osteogenic differentiation study. The wells from control groups were given normal medium (α-MEM). Both of culture groups were incubated at 37^0 C, 5% CO_2 and 95% air for 21 days. The medium in both groups were changed every 2 to 3 days. After 21 days, 5 culture wells from each group were kept in specific hypoxia chamber (Modular Incubator Chamber) for 3 days at 37^0 C, 5% CO_2 and 3% oxygen concentration, while the remainders were maintained in normal oxygen (normoxic) condition. After 3 days, the cells in the osteogenic medium as well as normal medium, were detached from petri disk and seeded into 24-well plate containing 10% formaldehyde for cell fixation.

Osteogenic differentiation was confirmed using anti human Runx2 monoclonal antibody, while hypoxic conditioned culture was evaluated using anti human HIF1α monoclonal antibody. The methods for immunocytochemical staining was as follows. Slides was washed with phosphate buffered saline (PBS) with pH 7.4 for 5 minutes, then dipped in H_2O_2 3% to block the endogenous peroxides and washed 3 times with PBS. Blocking unspecific protein done using 10% fetal bovine serum (FBS) containing 1% Triton X-100 and 0.02% NaN3, then washing with PBS 3 times. The slides were incubated in anti-Runx2 monoclonal antibody overnight at 40^0C. The slides were washed with PBS 3 times, incubated with universal link secondary antibody for 2 hours at room temperature and washed again 3 times, and incubated with Streptavidine horse radish peroxidase (HRP) for 40 minutes at room temperature, after washing with PBS 3 times the slides were given diamino benzidine (DAB) and incubated for 10 minutes and washed with distilled water for 5 minutes. Counterstaining was done using Mayer Haematoxylin followed by incubation for 10 minutes, and washing with PBS and rinsing with distilled water, the slides were then let to dry at room temperature for microscope reading. The cells positive for Runx2 expression were confirmed by the brown colour in the cell cytoplasm. The cells positive for HIF1α were also confirmed by brownish cell cytoplasm. The cell counting were done manually by two investigators using zigzag method under inverted light microscope with x400 magnification. The result of MTT assay, and immunocytochemical staining of Runx2 and HIF1α expression were analyzed statistically using one-way Anova ($p<0.05$). Assumption of distribution normality and homogenity of the variances was made prior to Anova testing.

RESULT

The result of MTT assay showed that the mean of optical density in the evaluated doses of CoQ10 increased along with the increase in its dose. The maximum density value of 1.826 was found in the group of dose of 75 μM/ml. Further increase in the dose of CoQ10 resulted in decrease in the optical density (Figure 1).

Distribution normality test using Kolmogorov Smirnov showed that all the groups had the value above 0.05

(p>0.05) confirming the normal distribution of the data. Homogenity of the variance test showed that significance was above 0.05 (p = 0.779; p>0.05) indicating the use of one-way Anova. Anova test showed that there was no significant difference among the groups which mean that CoQ10 was not toxic and the optimum viability dose for further studies was 75 µM/ml.

Culture of BM-MSC in osteogenic medium under normal and 3% hypoxic condition for three days showed that the cell density and number of osteoblast-like cells were higher in those under normal condition compared to hypoxic condition (Figure 2).

Expression of Runx2 and HIF-1α were evaluated under normoxia and 3% hypoxia within 24 hours. Positive expression of HIF-1α was identified by brown cytoplasm while expresion of Runx2 by blue cell nucleus, positive double staining indicated osteoblast-like cells under hypoxia (Figure 3). Data in Table 2 showed that there were significant differences both in Runx2 and HIF1α expression between normoxia and hypoxia group (p<0.05).

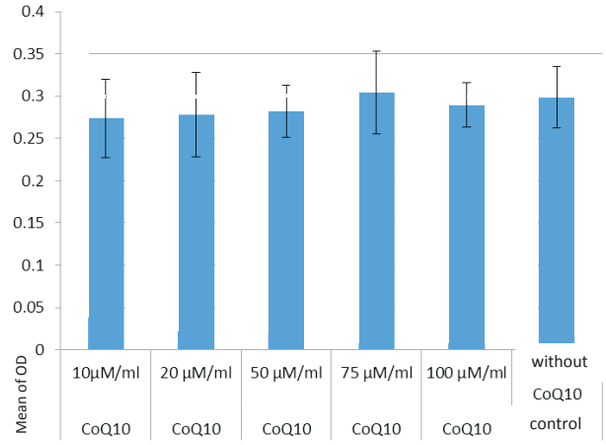

Figure 1. The result of MTT assay. The mean of optical density of Co-enzyme Q10 with various doses.

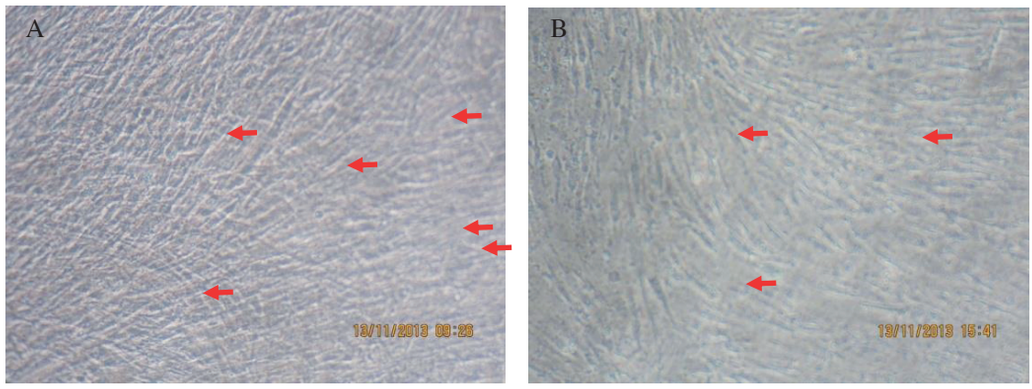

Figure 2. Morphology observation of *osteoblast-like* cells culture in osteogenic medium at 21 days, showing higher density in culture under normal condition (A) compared to hypoxic condition (B)(red arrow pointing to osteoblast-like cells, inverted microscope, x100 magnification).

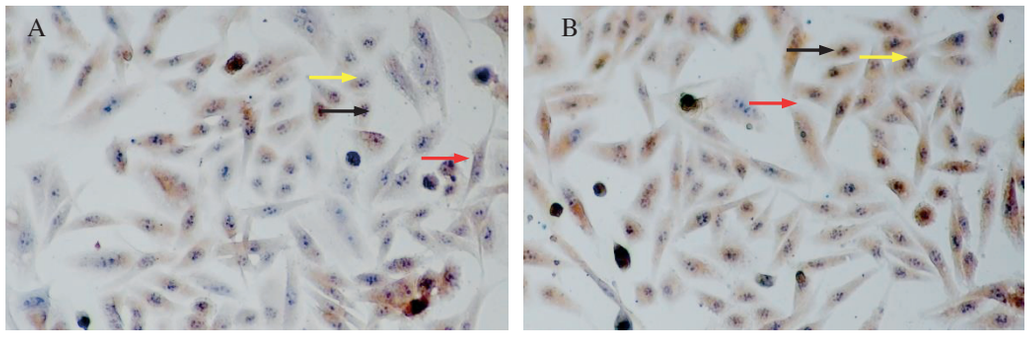

Figure 3. Distribution of osteoblas-like cells showing expression of HIF-1α (yellow arrow head) and Runx-2 (red arrow head) under normoxic (A) and hypoxic condition (B) (inverted microscope, 400x).

Table 1. Optic density of MTT assay result based on various concentratiion of co-enzyme Q10

	CoQ10 10μM/ml	CoQ10 20 μM/ml	CoQ10 50 μM/ml	CoQ10 75 μM/ml	CoQ10 100 μM/ml	(Control) without CoQ10
1	0. 241	0.252	0.282	0.344	0.297	0.280
2	0.295	0.299	0.305	0.259	0.290	0.292
3	0.358	0.368	0.288	0.279	0.290	0.353
4	0.257	0.261	0.306	0.298	0.331	0.279
5	0.232	0.226	0.222	0.265	0.278	0.331
6	0.259	0.263	0.290	0.381	0.250	0.257
N	1.642	1.669	1.693	1.826	1.736	1.792
Π	0.273666	0.278166	0.282166	0.304333	0.289333	0.298666

Note: the optimum optical density number of 1.826 was demonstrated in CoQ10 concentration of 75 μM/ml group.

Table 2. Desriptive data of Runx-2 expression in 3% hypoxia and normoxia groups

Gene expression	Groups	Mean	± Std. Deviation	Sig. (2-tailed)
Runx2	Normoxia	83.20	3.768	0.000
	Hypoxia	45.60	4.561	
HIF1α	Normoxia	10.20	2.280	0.000
	Hypoxia	31.60	1.817	

DISCUSSION

Ubiquinone is one of essential co-enzymes which can be detected in mitochondria of every type of cell especially in heart, kidney and liver.[5] It has antioxidant effect to protect cell membrane from oxidation by the Reactive Oxygen Species (ROS) produced in response to cutanoeus injuries such as burn wound. It also provides a regenerative effect, sustaining effective levels of antioxidants in damaged tissues [4] although its concentration in the body tends to decrease with age, the peak of which is at 20 and drop dramatically as much as 68% at 40.[6] Health articles reported that was generally used as food supplements to increase immune system in immune-deficiency patients, Alzheimer and Parkinson, cardiovascular diseases, endocrine disease, and anti-aging. The positive effect of ubiquinone in wound healing may, logically, be attributable to induction of angiogenesis. This in vitro study was made to prove this rationale.

Bone healing after reconstruction of mandibular bone defects with autogenous bone graft according to remodelling cycle were divided into three stages, i.e. activation, resorption, and new bone phase.[7] The most important step in early activation phase was new blood vessels formation or angiogenesis in order to provide means for transportion of oxygen, nutrition, and biomolecules which were necessary for further healing into the implanted graft.[8] The process of angiogenesis was induced by expression of angiogenic factors, such as VEGF, by endothelial cells, osteoblast, and MSC either in the graft or surrounding tissue. Studies had proved that VEGF expression was induced by regional hypoxic condition.[9] HIF-1α is transcription factor that regulates genes involved in the response to hypoxia, some of which promote neovascularity. VEGF is one of the genes upregulated by HIF-1α and is the primary cytokine related to angiogenesis. Study showed that HIF-1α expression resulted in increased VEGF expression.[3]

The activation phase in bone healing was characterized by osteogenic differentiation of MSC residing at the bone graft and periosteum overlying the graft. The osteogenic differentiation was usually induced by bone morphogenic proteins (BMP). The osteogenic committed MSC were characterized by increased expression of trancriptional factor (Runx2).[10]

In view of the above, this study used angiogenic and osteogenic parameters, i.e. HIF-1α and Runx2, respectively and used MSC as research subjects of an in vitro model of osteogenic and hypoxic-induced cell culture. BM-MSC was used in this study because MSC derived from bone marrow was found to have the highest osteogenic capacity therefore was strongly suggested for such studies.[11]

The result of the study showed that expression of Runx2 by osteoblast-like cells was significantly higher in normoxic than hypoxic osteogenic medium, as opposed to HIF-1α expression which was significantly lower in normoxic compared to hypoxic condition. This result was in accordance with in vitro study which demonstrated that mesenchymal stem cell under hypoxic condition showed up-regulation of VEGF and down-regulation of core binding factor-alpha 1 or Cbfa-1/Runx2.[9] Some studies also exhibited that over-expression of VEGF inhibited osteogenesis in vitro and ectopic bone formation in vivo[12] and inhibited in vitro osteogenic differentiation of MSC induced by BMP2.[13] The result of these studies suggested that there was inverse relationship between angiogenic and osteogenic signalling either in vitro or in vivo.

The result of the study also showed that hypoxic condition in this procedure could induce expression of

hypoxic transcription factor HIF-1α. The result confirmed that the culture of osteoblast-like cells obtained from the hypoxic procedure in this study were suitable for further in vitro angiogenesis study because it could mimic the hypoxic condition to which the osteoblast in non-vascularized autogenous bone graft might be exposed.

The conclusion of the study was that ubiquinone was not toxic with the optimum viability concentration for further studies being 75 μM/ml, BM-MSC culture in the osteogenic medium was osteoblast-like cells as they overexpressed Runx2 and exhibited hypoxic feature as they overexpressed HIF1α and underexpressed Runx2. It was also suggested that the osteoblast-like cells culture in this study was appropriate for further in vitro study to evaluate the angiogenic and osteogenic effects of ubiquinone.

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