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The Effect of Cobalt (II) Chloride in the Viability Percentage and the Induced Hypoxia Inducible Factor - 1α of Human Adipose Mesenchymal Stem Cells (HAMSCs): An *In Vitro* Study

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ABSTRACT

Introduction: The preconditioning of HAMSCs using Cobalt (II) Chloride ($CoCl_2$) as a hypoxia mimicking agent (HMA) may provide the adaptive state of HAMSCs.

Objective: To examine the effect of $CoCl_2$ in the viability percentage and the induced Hypoxia Inducible Factor-1α (HIF-1α) expression of HAMSCs *in vitro*.

Methods: HAMSCs were thawed, cultured, and then sub-cultured from the laboratory cell stock. The Immunocytochemistry (ICC) methods with Fluorescein isothiocyanate (FITC) antibody labelling kit was used to validate the HAMSCs employing its Cluster of Differentiation (CD) expression such as CD73(+), CD105(+) and CD45(-). HAMSCs were sub-cultured and then co-cultivated with or without $CoCl_2$ in the different concentration (200µmol, 150µmol, 100µmol, 75µmol, 50µmol, 25µmol) for 24 hours. The cytotoxicity test was conducted by utilizing tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The formazan crystal was then observed, and the cell viability percentage was calculated. In examining $CoCl_2$ induced hypoxia in HAMSCs, the expression of HIF-1α applied ICC-FITC *in vitro*. Analysis of Variance (ANOVA) was performed to analyze the obtained data statistically ($p<0.05$).

Results: HAMSCs express CD73 and C105 positively, while HAMSCs expressed CD45 negatively. There is no significant different in cell viability percentage of HAMCs between groups with different concentrations of $CoCl_2$ ($p=0.99$; $p>0.05$). $CoCl_2$ successfully induces the hypoxia confirmed by HIF-1α positive expression of HAMSCs after 24 hours incubation *in vitro*.

Conclusion: $CoCl_2$ does not affect the viability percentage of HAMSCs and it can be used as a biocompatible HMA in HAMSCs *in vitro*.

Keywords: Cell Viability, Cluster of Differentiation Cobalt (II) Chloride, Human Adipose Mesenchymal Stem Cells, Hypoxia Inducible Factor-1α.

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INTRODUCTION

In the regenerative dental medicine, Mesenchymal Stem Cells (MSCs) based therapy is a promising and highly attractive approach to enhance the regeneration in the defective tissue.[1] MSCs secrete the beneficial growth factor that may help wound healing process.[2] MSCs have been known for having the potential differentiation ability into mesenchymal lineage, self-renewable with good controlled proliferation rate and genome stability *in vitro*.[3] MSCs express the osteogenic differentiation marker such as Runt-related transcription factor 2, Osteonectin, and Osteopontin expression.[4-6] MSCs also express the Aggrecan expression as chondrogenic differentiation marker that proves the multipotent ability of MSCs.[7] MSCs can be isolated from the various tissue source; such as from bone marrow[8], dental pulp of permanent or deciduous tooth[9,10], gingiva[11], hair follicle[12] and adipose tissue[13].

One of the promising MSCs sources is adipose tissue which can be easily isolated with minimum invasive procedure compared to iliac crest bone marrow.[14] Human Adipose mesenchymal stem cells (HADMSCs) provide the abundant number of stem cell that is potential for stem cell based therapy or tissue engineering approach.[12-13] In addition, HAMSCs may suitable for clinical use or experimental study investigation if the abundant amount MSCs are needed.[15] Despite the potentials and many advantages possessed by HAMSCs, the further investigation should be done in maintaining the HAMSCs' multipotent, proliferation, and stemness when HAMSCs were administered in the defective tissue. In the center of defective tissue, a hypoxic (oxygen deprived) niche is detected.[16] The hypoxia condition is found in the MSCs niche which states that MSCs is possibly adaptive to the oxygen restriction. The activation of intracellular mechanism by hypoxia condition may be responsible for cell apoptosis or adaptation in the niche.[17]

Cobalt (II) Chloride ($CoCl_2$) is a chemical agent which is cheap and easy to obtain. It's also can be used to mimic the hypoxia condition. The Hypoxia Inducible Factor-1α (HIF-1α) expression in the cell culture reflects the hypoxia condition.[18] Despite there were previous studies in the use of $CoCl_2$ that mimic hypoxia condition, the biocompatible of $CoCl_2$ and its effect on HAMSCs culture should also be further examined. For that reason, this study is aimed to examine the effect of $CoCl_2$ in the viability percentage and the induced HIF-1α expression of HAMSCs *in vitro.*

MATERIALS AND METHODS

Study Design and Ethical Clearance Approval

This study was a post-test only control group design and an *in vitro* study in nature. The sample was selected blindly random. The Ethical Clearance Committee of Health Research from the Faculty of Dental Medicine, Universitas Airlangga was approved for this study design (number: 2.KE.017.02.2020).

Thawing, Culture, Subculture, Validation of Human Adipose Mesenchymal Stem Cells

The third passage of frozen HAMSCs was thawed, cultured, and then sub cultured from the Stem Cell Research and Development Center Universitas Airlangga laboratory's cell stock. A daily cell culture medium, α-Minimum essential medium (α MEM) (Gibco, Paisley, UK) was administered to the cell pellet and put in the cell culture plate with 100 mm diameter (Iwaki, Asahi, Japan) and then incubated at 37°C. The HAMSCs culture was examined for every 3-4 days until 80-90% cell confluency, then HAMSCs were split for further sub-cultures.[12,13] HAMSCs at the 4th sub culture was validated by using Immunocytochemistry (ICC) methods with Fluorescein isothiocyanate (FITC) antibody labelling kit to detect the Cluster of Differentiation (CD) expression such as CD73(+), CD105(+) and CD45(-) (BD Biosciences, USA) and then finally HAMSCs were observed by applying fluorescence microscope (Olympus, Tokyo, Japan) with 100x magnification.[19]

The Preparation of $CoCl_2$ for HAMSCs Co-Cultivation

For HAMSCs co-cultivation, $CoCl_2$ was prepared by using the diluted $CoCl_2$ red violet powder (Sigma Aldrich, US) with α-MEM (Gibco, Paisley, UK). This study employed the various $CoCl_2$ concentration as much as 200 μmol, 150 μmol, 100 μmol, 75 μmol, 50 μmol, 25μmol to be compared in this study.[18]

The MTT Assay in the Co-Cultivation of $CoCl_2$ and HAMSCs

The cytotoxicity was conducted by applying tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. At the fourth sub-culture of 5.10^3, HAMSCs was planted per well (n=6) in the 96-well microtiter plate (Iwaki, Japan) and co-cultivated with $CoCl_2$ in various concentrations (200 μmol, 150 μmol, 100 μmol, 75 μmol, 50 μmol, 25μmol) for 24 hours. MTT was administered per well of each group then continued by 3 hours of incubation. The dimethyl sulfoxide was added to stop the reaction process. The formazan crystal was observed, and the cell viability percentage was calculated. The automatic microplate reader at a wavelength of 595 nm (GloMax®Explorer, Wisconsin, USA) was utilized to detect the optical density (OD).

The Assessment of CoCl2 induced Hypoxia Inducible Factor-1α expression in the HAMSCs

At the fourth sub-culture of 10^4, HAMSCs was planted in the 24-well microtiter plate (Iwaki, Asahi, Japan) and co-cultivated with or without 100 μmol $CoCl_2$ in α-MEM then incubated for 24 hours. After the 24 hours incubation, the cell medium was removed and HAMSCs in each well (n=4) were washed with 1xPBS then fixed with 4% paraformaldehyde. To examine ($CoCl_2$) induced hypoxia HIF-1α the expression in HAMSCs, this study used ICC methods with polyclonal FITC antibody labelling kit *in vitro* (BD Biosciences, USA). The positive expression of HIF-1α in HAMSCs was observed by applying fluorescence microscope (Olympus, Tokyo, Japan) with 100x magnification.

Statistical Analysis

The obtained data in this study was described as average ± standard deviation (SD). In this study, Statistical Package for Social Science (SPSS) 20.0 version (Chicago, Illinois, USA) was employed to analyze the data statistically. Analysis of Variance (ANOVA) was conducted to compare the data between group ($p<0.05$).

RESULTS

In this study, the cultured HAMSCs are confirmed as MSCs. HAMSCs were expressed in CD73 and C105 positively. In comparison, HAMSCs did not expressed CD45 (see Figure 1). The formazan crystal is found after the MTT reaction in each group (see Figure 2A). The macroscopic of HAMSCs after MTT reaction in the 96-culture plate that reflects the red violet can be seen in the Figure 2B. There is no significant difference in the cell viability percentage of HAMCs between groups with different concentration of $CoCl_2$ ($p=0.99$; $p>0.05$) (see Figure 2C). The administration of 100μmol $CoCl_2$ has been successfully induced the hypoxia condition confirmed by HIF-1α positive expression in HAMSCs *in vitro* as shown in the Figure 3.

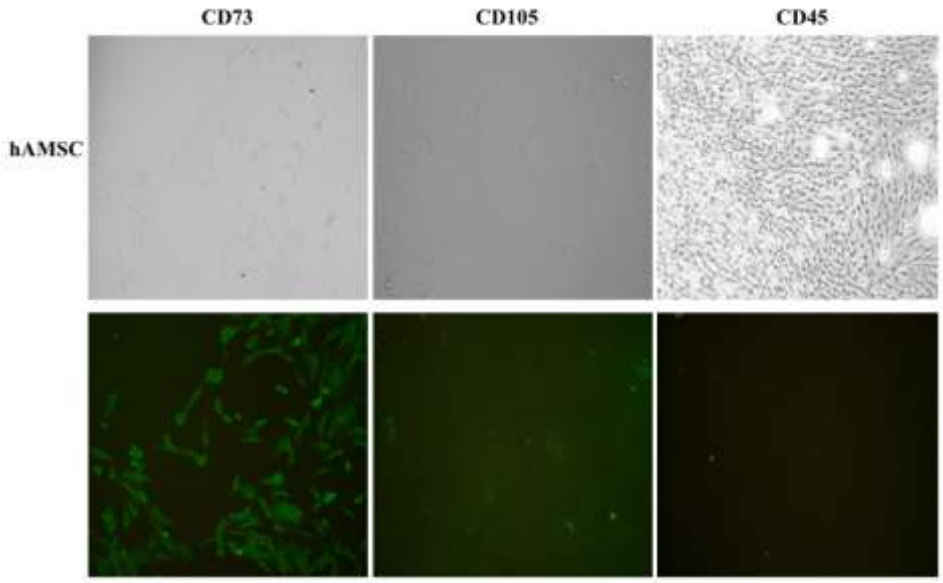

Figure 1: The Positive expression (green color) of HAMSCs in CD73 and C105 but not in CD45 expression. The expression of CD in HAMSCs are observed by applying fluorescence inverted microscope (Olympus, Japan) with 100x magnification.

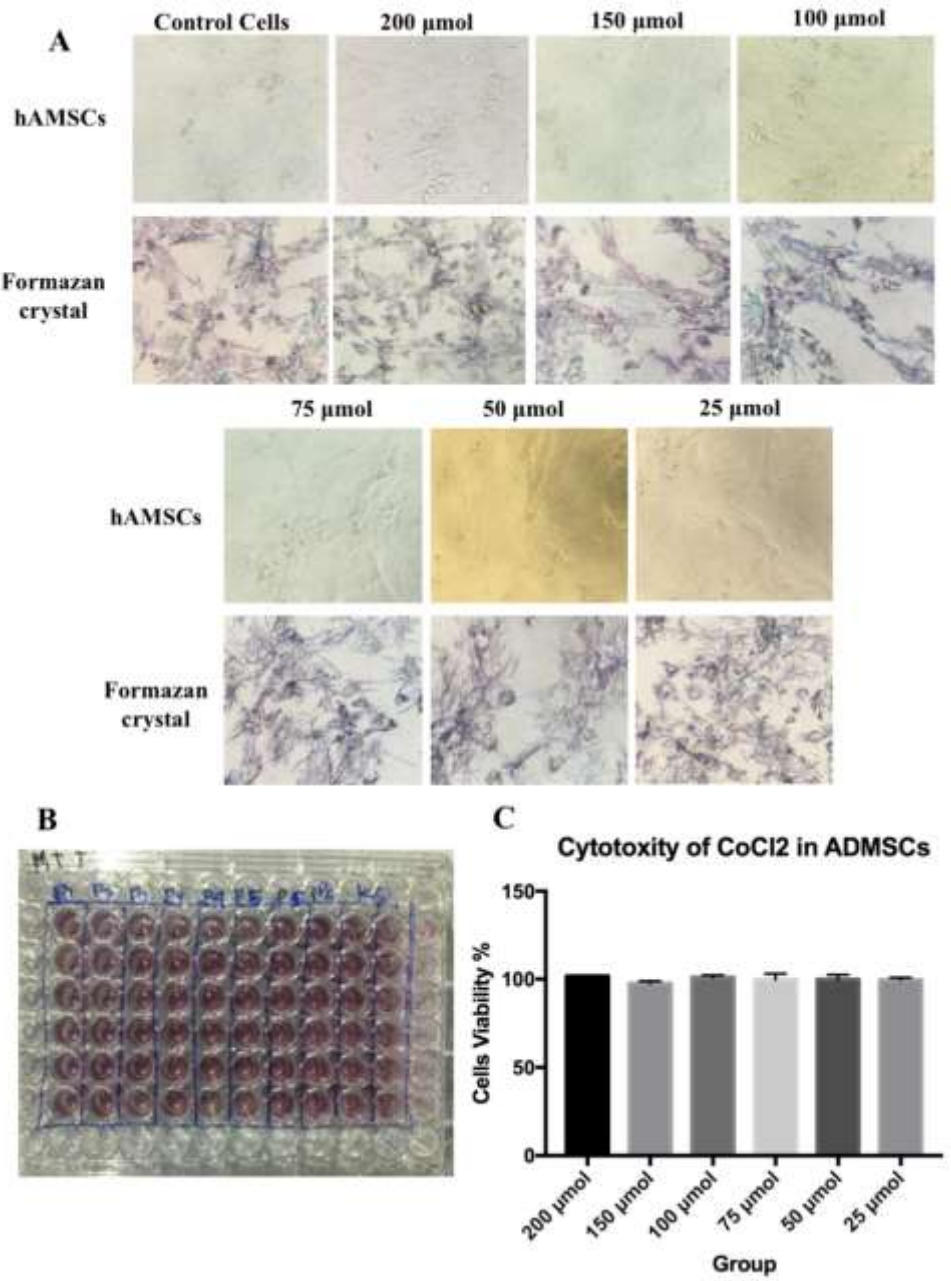

Figure 2: A. The detected formazan crystal after MTT reaction in each group, observed by utilizing the inverted microscope (Olympus, Tokyo, Japan) with 40x magnification. B. The macroscopic of HAMSCs after MTT reaction in the 96-culture plate that reflects the red violet color. The average and SD of HAMSCs viability percentage in each group with no statistically different ($p>0.05$).

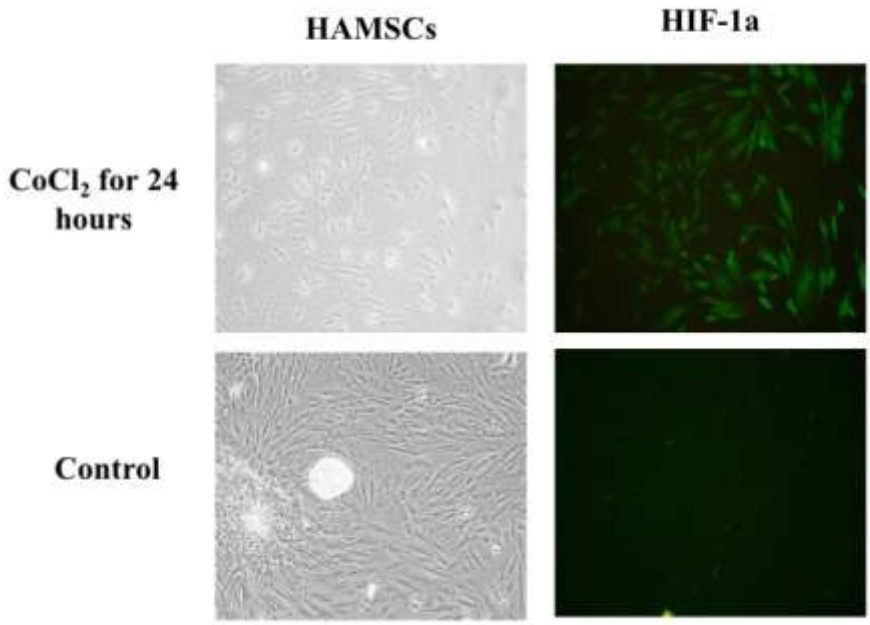

Figure 3: The comparison of HAMSCs treated with 100μmol $CoCl_2$ for 24 hours incubation expressed the HIF-1α positively (green color) and the control (without $CoCl_2$) observed by using fluorescence inverted microscope (Olympus, Japan) with 100x magnification.

DISCUSSION

HAMSCs are used in this study because it can be easily isolated and expanded in numerous quantities *in vitro*.[15] The isolated HAMSCs are confirmed as MSCs because the CD73 and CD90 are expressed positively but negatively expressed in CD45. This finding is in line with Rantam *et al*. and Sari *et al*. study that mentioned that the HAMSCs positively expressed the CD of MSCs markers.[12,13] Based on the standardization of MSCs by International Society for Cell therapy, there are minimal criteria needed to be fulfilled; such as (1) the cultured MSCs should adherence into the culture plate with spindle form or fibroblast-like cells morphology, (2) the cluster differentiation (CD) 105, 73, and 90 MSCs surface marker should be expressed. In addition, lack of expression of CD45 should be found.[20] Another source of MSCs such as from gingiva and dental pulp also shows the positive expression of CD73, CD90, CD105 but negative expression in CD45 for the MSCs confirmation.[19,21]

The material biocompatibility is an important thing that must be examined when using a material for treatment purpose. In this study, HAMSCs are treated with various concentration of $CoCl_2$ as HMA. MTT assay is done for the cytotoxicity test on HAMSCs under $CocC_2$ effect. MTT assay forms the formazan crystal with blue purple color. The color intensity of formazan crystal can be assessed by using OD in spectrophotometry. There was no significant difference in percentage of HAMSCs viability treated with various concentration of $CoCl_2$ (200 μmol, 150 μmol, 100 μmol, 75 μmol, 50 μmol, 25μmol) for 24 hours. The administration of $CoCl_2$ has no significant effect on cell viability as mentioned in Teti et al. study.[22] The result shows that $CoCl_2$ is a biocompatible HMA for cell culture. Based on the previous study, $CoCl_2$ is commonly used as HMA in the cell culture.[18] This is in line with this study results that HAMSCs treated with 100μmol $CoCl_2$ successfully induces HIF-1α *in vitro*. $CoCl_2$ is mimicking the hypoxia condition through the decreased or blocked HIF-1α degradation. The effects of $CoCl_2$ as HMA are not significantly different compared to the use of hypoxic chamber that decreases the oxygen levels.[18] The hypoxia condition may enhance the stemness of HAMSCs.[15] The hypoxia condition in the MSCs induced by $CoCl_2$ can enhance the osteogenic and chondrogenic potential differentiation.[22,24] Pretreatment of HAMSCs with $CoCl_2$ as HMA should be considered for MSCs based regenerative therapy in the dental medicine field.

CONCLUSION

The $CoCl_2$ is a biocompatible HMA that can be used on HAMSCs in *vitro*. Further studies are still needed to reveal the novelty of HAMSCs treated with $CoCl_2$ for regenerative dental medicine purpose.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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