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ORIGINAL ARTICLE

Cobalt Chloride as a Hypoxia Mimicking Agent Induced HIF-1α and mTOR Expressions of Human Umbilical Cord Mesenchymal Stem Cells

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ABSTRACT

Objective: To assess the effects of cobalt chloride ($CoCl_2$) as a hypoxia mimicking agent on human umbilical cord mesenchymal stem cells (hUCMSCs) expression of HIF-1α and mTOR for use in regenerative dentistry. **Material and Methods:** Human umbilical cord mesenchymal stem cells were isolated and then cultured. The characteristics of stemness were screened and confirmed by flow cytometry. The experiment was conducted on hypoxia (H) and normoxia (N) groups. Each group was divided and incubated into 24-, 48-, and 72-hours observations. Hypoxic treatment was performed using 100 μM $CoCl_2$ on 5th passage cells in a conventional incubator (37°C; 5% CO_2). Then, immunofluorescence of HIF-1α and mTOR was done. Data was analyzed statistically using One-way ANOVA and Tukey's HSD. **Results:** Significant differences were found between normoxic and hypoxic groups on HIF-1α ($p=0.015$) and mTOR ($p=0.000$) expressions. The highest HIF-1α expression was found at 48 hours in the hypoxia group, while for mTOR at 24 hours in the hypoxia group. **Conclusion:** Hypoxia using cobalt chloride was able to increase human umbilical cord mesenchymal stem cells expression of HIF-1α and mTOR.

Keywords: Umbilical Cord; Mesenchymal Stem Cells; Stem Cell Research; Hypoxia; Regeneration.

Introduction

Hypoxia is a condition where oxygen levels decrease in cells, tissues, or organs. The lowering of oxygen level could be caused by the decreased oxygen supply when there were decreased vasculature, healing process or inflammation [1]. Other than that, hypoxia can be contributed to pathological factors involving microorganisms and immune responses [2,3]. Every tissue and cell have a different ability to adapt into a new environment [4]. In dentistry, hypoxic conditions can be found in gingivitis, periodontitis, periapical lesions, and peri-implant mucositis or peri-implantitis, as caused by periodontopathic biofilms [1]. This hypoxia condition in periodontal diseases has a correlation to systemic problems [3].

Tissue-engineering therapy using stem cells is well developed because of many advantages, such as cell regeneration, growth factor signaling, ability to differentiate, and biocompatibility [5]. The umbilical cord, one of the stem cell sources, has a significant number of mesenchymal stem cells (MSC) that could be easily acquired and cultured. *In vitro*, hUCMSCs has high proliferation and growth [6]. Previous *in vitro* researches have tried to explore and optimize the use of hUCMSCs for future application in regenerative dentistry [7-10]. Other study also showed that the proliferation potential of hUCMSCs when cultured *in vitro* was higher than other sources such as bone marrow MSCs [11]. Many studies on *in vitro* culture and clinical application of MSC recommend to cultivate MSC under hypoxic conditions (1% to 10% O_2) [12]. Clinically, these will promote stem cell survival for application in hypoxic conditions, especially in regenerative dentistry, both for pediatric and adult patients.

Cobalt chloride ($CoCl_2$) as hypoxia-mimicking substance has been introduced and broadly used in many *in vitro* studies. Even though several researches have focused on the effect of hypoxia on dental derived stem cells, the effect of hypoxia on hUCMSCs need to be discussed. $CoCl_2$ solution is generally used as an alternative of modular incubator chamber [13]. Cobalt chloride is proven to improve the stemness of human dental pulp cells, this is an advantage as a method to increase the amount of cells without the loss of their capacity to differentiate in regenerative procedures [14]. $CoCl_2$ is water soluble which produced clear red solution when applied into cell culture to induce hypoxia [4]. $CoCl_2$ could mimic hypoxic condition through reduction or obstruction of HIF-1α degradation. The outcome of $CoCl_2$ was not significantly different from using hypoxic chamber [15,16] and in laboratory, it is hard to regulate and sustain steady oxygen tension [14]. Previous study with 100 μM of $CoCl_2$ concentration for hypoxia induction used other stem cells sources such as stem cells derived from human exfoliated deciduous teeth, adipose derived stem cells, and dental pulp stem cells [13,15,17], and one current study on hUCMSCs discuss a wide range of 10 μM and 250 μM but did not observe a longer time range [18].

HIF-1α was initially thought to be the key oxygen sensing subunit, which could upregulate the secretion of fibroblast growth factor (FGF)-2, vascular endothelial growth factor (VEGF), angiopoietin-1, angiopoietin-2, transforming growth factor (TGF)-b, and platelet derived growth factor (PDGF)-b, under hypoxia condition [19]. HIF-1α activation would affect self-renewal, proliferation, and colony forming activity of MSCs [20]. Mammalian target of rapamycin (mTOR) controls metabolism and cell growth in response to nutrient, including O_2. mTOR activation would lead to stem cells growth and quiescence [21]. mTOR pathway has a crucial role as an integrator and sensor of a great variation of environmental signs that are associated to metabolic quiescence, cell cycle arrest, and biosynthetic requirements [18].

Cultured hUCMSCs in hypoxic condition is expected to improve proliferation of the cells through HIF-1α and mTOR. MSC culture proved that some cells proliferation under low O_2 pressure compared to normal atmospheric pressure. hUCMSCs that is being cultured under 2,5% or 5% O_2 concentration showing HIF-1α in

significant amount [22]. The other marker which plays a chief role in cells growth, survival, metabolism and proliferation is mTOR [23]. This study assessed the effects of cobalt chloride as hypoxia mimicking agent on human umbilical cord mesenchymal stem cells (hUCMSCs) towards HIF-1α and mTOR expressions at 24, 48, and 72 hours for future use in regenerative dentistry.

Material and Methods

Ethical Clearance

This study was approved by The Health Research Commission for Ethical Clearance, Universitas Airlangga, Faculty of Dental Medicine (630/HRECC.FODM/XII/2021).

Isolation of hUCMSCs

Isolation, expansion, and culture of umbilical cord was done according to previously published method [24]. Umbilical cord was provided from healthy donor with Caesarean operation without complications conducted at the Integrated Surgical Center RSUD Dr. Soetomo, Surabaya. The donor has approved and signed the informed consent. Healthy donor was chosen because based on our unpublished study, health status of the donor would affect the quality of the umbilical cord and the stem cells.

Hypoxic Treatment on hUCMSCs using $CoCl_2$

Hypoxia on hUCMSCs was done at passage 5. Hypoxic condition was done using chemical inducer (Hypoxia mimicking agent) Cobalt chloride hexahydrate solution ($CoCl_2$) in concentration of 100 µM was applied in the culture media to induce hypoxia [25]. Then, hUCMSCs was incubated for 24, 48, and 72 hours. For the control groups hUCMSCs were incubated in normoxia condition (37°C; 5% CO_2).

Immunofluorescence Test of HIF-1α and mTOR Expression

Cultured hUCMSCs from treatment groups were examined with the following procedures: fixation buffer 200 µL was added, then incubation was done for 20 minutes at 4°C, and rinsed with flowing buffer solution. Cells were permeabilized by addition of perm buffer, then incubated for 20 minutes 4°C and washed with flowing buffer solution again. After cells were fixated and/or permeabilized, the first antibody was being put inside and incubated for 25 minutes at 4°C, then, the cells were rinsed with flowing buffer solution. After the first antibody was attached to the cells, the second antibody was introduced and incubated for another 25 minutes at 4°C. The cells were washed again with flowing buffer solution. The antibody used were FITC HIF-1α (Bioss Antibodies, USA) to observe the expression of HIF-1α and FITC mTOR (Bioss Antibodies, USA) to observe the expression of mTOR. Immunofluorescence imaging was seen as green colored luminescence and captured using fluorescence microscope at 200× magnification (Olympus CKX53, Japan).

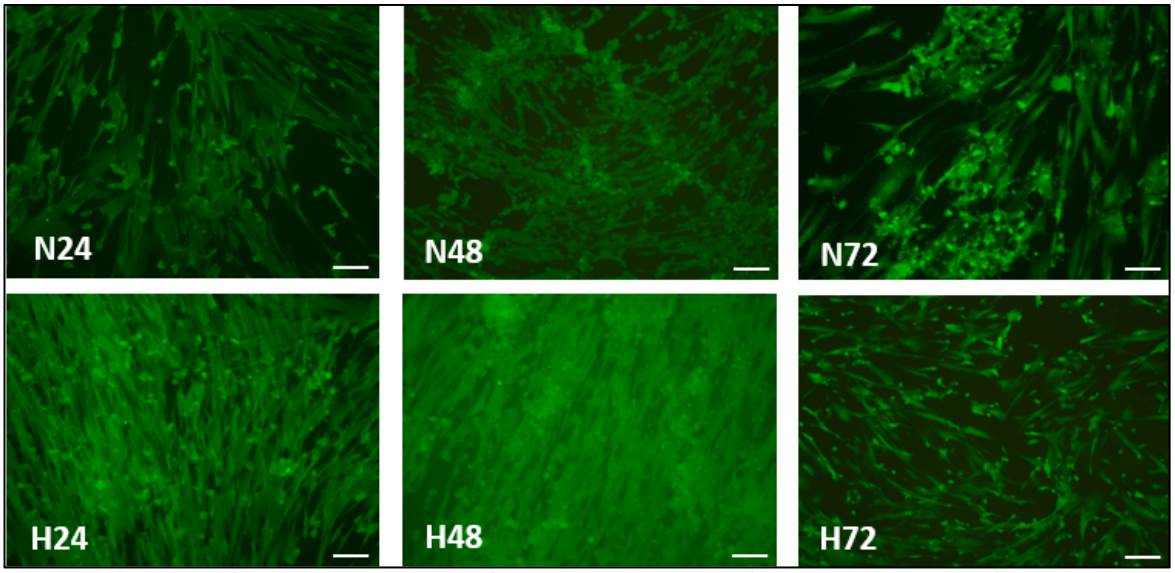
Statistical Analysis

Statistical analysis was performed using SPSS software (SPSS, USA). Data were analyzed statistically using One-way ANOVA and Tukey's HSD with level of significance $\alpha = 0.05$. The data was tested for normality using Shapiro-Wilk.

Results

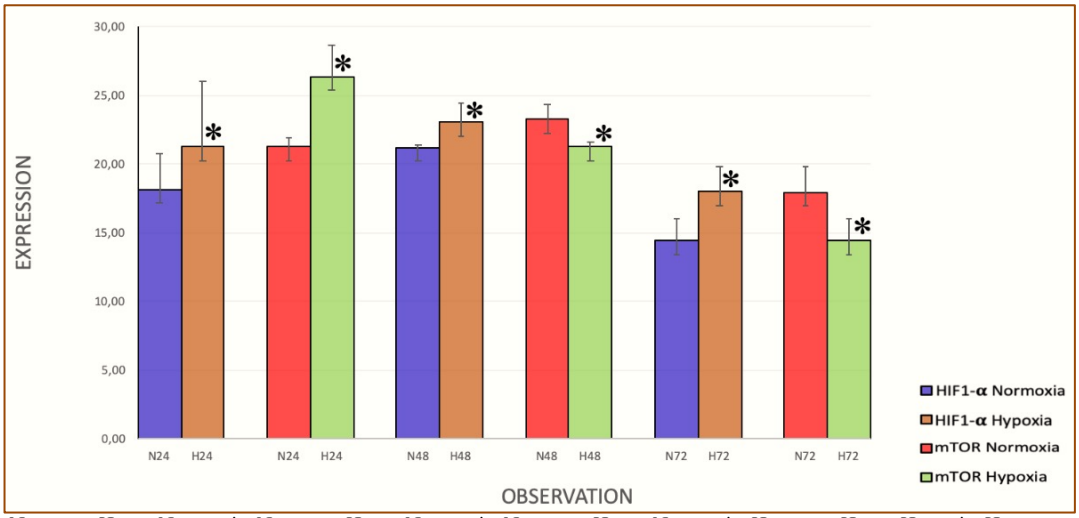
HIF-1α Expression

HIF-1α expression under normoxia and hypoxia conditions in 24-, 48-, and 72 hours can be seen in Figures 1 and 2. Data analysis of HIF-1α expression in the study can be seen in Table 1. The data were distributed normally ($p>0.05$). Homogeneity test showed the variants of those groups were homogeny ($p>0.05$). One-way ANOVA test showed $p = 0.015$ ($p<0.05$) which meant there were significant differences between HIF-1α count on control and treatment groups. From multiple comparison Tukey HSD test, there were significant differences of expression between groups. Significant difference was found among the control and treatment groups. The highest HIF-1α expression was found in hypoxia 48 hours group (H48). From the data analysis, HMA $CoCl_2$ significantly increase the expression of HIF-1α in 24-, 48- and 72 hours.



N24 = 24 Hours Normoxia; N48 = 48 Hours Normoxia; N72 = 72 Hours Normoxia; H24 = 24 Hours Hypoxia; H48 = 48 Hours Hypoxia; H72 = 72 Hours Hypoxia. Scale Bar: 200 µm.

Figure 1. Comparison of HIF-1α expression on hUCMSCs between control and treatment groups. HIF-1α expression on hUCMSCs marked with the presence of green colored fluorescence.



N24 = 24 Hours Normoxia; N48 = 48 Hours Normoxia; N72 = 72 Hours Normoxia; H24 = 24 Hours Hypoxia; H48 = 48 Hours Hypoxia; H72 = 72 Hours Hypoxia; *Statistically Significant ($p<0.05$).

Figure 2. Bar chart showing mean and standard deviation of HIF-1α and mTOR expressions.

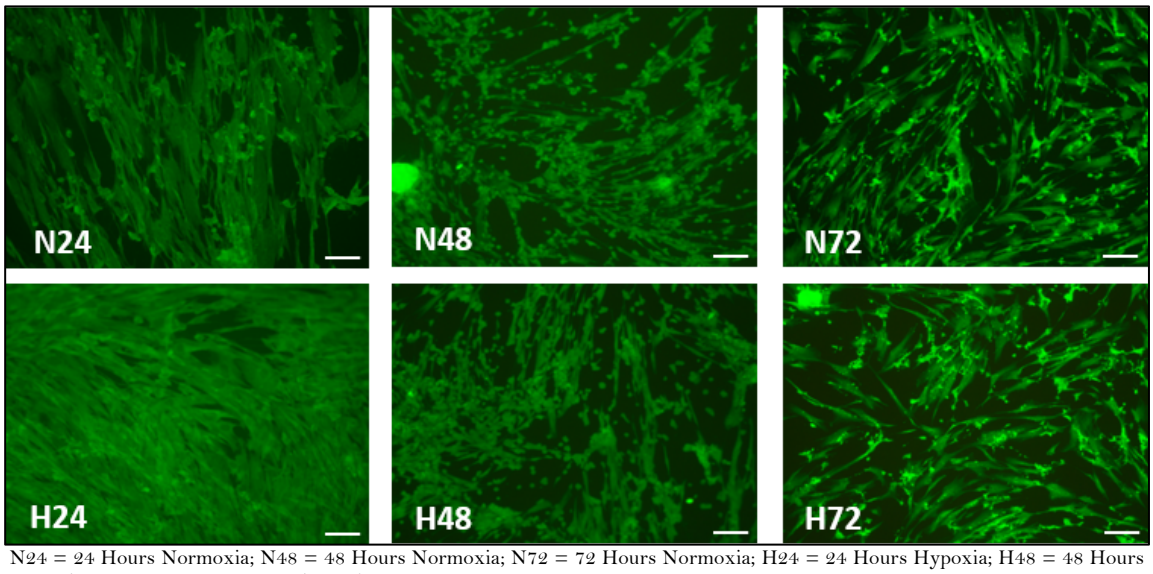
Table 1. Tukey HSD test results between the control and treatment groups of HIF-1α and mTOR expressions.

Groups	N	HIF-1α Expression Mean (SD)	p-value	mTOR Expression Mean (SD)	p-value
N24	3	18.19 ± 2.62	0.015*	21.29 ± 0.65	0.000*
N48	3	21.24 ± 0.18		23.28 ± 1.05	
N72	3	14.45 ± 1.57		17.98 ± 1.84	
H24	3	21.27 ± 4.82		26.41 ± 2.30^	
H48	3	23.06 ± 1.45^		21.27 ± 0.30	
H72	3	17.99 ± 1.84		14.45 ± 1.57	

*Significant at α = 0.05 (One-way ANOVA); ^Highest average score (Tukey HSD).

mTOR Expression

mTOR expression under normoxia and hypoxia conditions in 24-, 48-, and 72 hours can be seen in Figures 2 and 3. Data analysis of mTOR expression in the study can be seen in Table 1. The data were distributed normally (p>0.05). Homogeneity test showed the variants of those groups were homogeny (p>0.05). One-way ANOVA test showed p=0.000, which meant there were significant differences between mTOR count on control and treatment groups. From multiple comparison Tukey HSD test, there were significant differences of expression between groups. The highest expression was found on H24 group. Based on the data analysis, HMA $CoCl_2$ significantly increase mTOR expression from 24 hours treatment group.



N24 = 24 Hours Normoxia; N48 = 48 Hours Normoxia; N72 = 72 Hours Normoxia; H24 = 24 Hours Hypoxia; H48 = 48 Hours Hypoxia; H72 = 72 Hours Hypoxia. Scale Bar: 200 µm.

Figure 3. Comparison of mTOR expression on hUCMSCs between control and treatment groups. mTOR expression on hUCMSCs marked with the presence of green colored fluorescence.

Discussion

This study used human umbilical cord mesenchymal stem cells because MSCs from umbilical cord have multipotent properties as an alternative treatment option for many diseases and abnormalities, easy to culture, has broad application and beneficial for *in vivo* applications [26]. In this study, one donor is sufficient to provide the cells for assessments, as hUCMSCs are easily cultured and highly expandable in a relatively short time [27,28]. *In vitro* researches were conducted to study the complex processes in application and preservation of stem cells [13,14,17]. In this study, $CoCl_2$ concentration of 100 µM was used and the observation is expanded from 24, 48 to 72 hours.

HIF-1α acted as the main regulator of genes that could be induced in hypoxic condition. Main target gene of HIF-1α is connected to angiogenesis, proliferation, survival of the cell, and metabolism [19,20]. Higher HIF-1α would maintain hUCMSCs' stemness and decrease cell aging, where quiescent adult stem cells sustain a slow cycling state to evade cellular damage and to safeguard tissue regeneration capacity [29]. A study shown the potential when modulating nutrition sensing and pathway response to improve survival and therapeutic features of MSCs. Several metabolic regulators are pleiotropic and have more and unwanted effect against cell properties, so it is important to understand intracellular tissue and metabolism to do rational approach when applying stem cell in tissue engineering for optimal therapeutic effect [30].

The amount of HIF-1α expression based on the result in group with hypoxic $CoCl_2$ treatment showed higher fluorescence than in control group without hypoxic treatment, at 24 hours, 48 hours, and 72 hours. But, the highest expression in hypoxic treatment was found at 48 hours. The lowest HIF-1α expression was found at 72 hours. This score was parallel with previous study that shown the stabilization of HIF-1α protein on low oxygen condition. HIF-1α activity depended on oxygen level and HIF-1α target is set under normoxic or hypoxic condition could be a potential therapy target [31]. Another study also shown that in hypoxic condition, HIF-1α increased survival and suppressed apoptosis of MSCs. This study elaborated protective effect of HIF-1α against MSCs survival under hypoxic condition [32].

Overall, both control and treatment groups, the highest expression of HIF-1α was found at 48 hours. HIF-1α expression at 24- and 72-hours control group only have slight differences. Highest increase could be seen on 48 hours control group. HIF-1α expressions are higher on hypoxic treatment group than the normoxic control group. The highest expression is at 24 hours and the difference is significant between control and treatment groups. The lowest expression is at 48 hours. Hypoxic condition made most eukaryotic cells changed their main metabolism strategy from more dominant mitochondrial respiration into glycolysis escalation to be able to maintain ATP level. Deprogramming of metabolism that was induced by hypoxia was the key to fulfill cellular energy needs during acute hypoxic condition. On transcription level, this metabolic switch could be arranged by few ways including HIF-1α [33]. HIF-1α played an important role in hypoxic signal transduction process physiologically and pathologically. Gene target from activation of HIF-1α has many functions, such as glycolysis, glucose transport, erythropoiesis, and angiogenesis. A lot of these genes involved in proliferation, survival, and differentiation of the cells, such as EPO, VEGF, and many others [33].

mTOR expression on normoxic control group was higher than in hypoxic treatment group at 48 and 72 hours, whilst lower expression was found at 24 hours control group compared to 24 hours hypoxia treatment group. The highest expressions of mTOR in this study was at 24 hours hypoxic group. On the other hand, the lowest mTOR expression was at 72 hours hypoxic group. Previous study showed that mTOR function is regulated by hypoxic condition [34]. The result of this study was in line with study about hypoxic effect on migration and proliferation of umbilical cord blood MSCs. Hypoxic treatment increased mTOR phosphorylation at 24- and 48-hours incubation. However, mTOR expression at 48 and 72 hours were decreased. This would explain that prolonged exposure to hypoxia would lead to decreased expression of mTOR, as cells need oxygen for their metabolism. Other possibility is the reduced number of cells or other factors which need further exploration. After 24 hours of hypoxic treatment, fluorescence amount of p-mTOR (Ser2481 and Ser2448) increased to 356% and 153% from the control group [35].

mTOR expression at 48 hours control group was increased and then decreased at 72 hours control group. The highest score on the control group was at 48 hours group, whereas on control group the 72 hours

showed the lowest mTOR expression. mTOR played a vital role in regulating growth, proliferation, and cell survival that depended on nutritional condition, stress signal, and growth factor [36]. mTOR is one of the proteins which mechanism affected by hypoxic condition. Hypoxic condition affected almost all aspects of cellular life, from cytokine secretion, mitogen, and extracellular matrix modulator. Hypoxic condition on mTOR could stimulate autophagy [37]. Research on amnion-derived MSCs (AD-MSCs) showed *in vitro* hypoxic condition increased trophoblast proliferation and autophagy which could be observed from escalation of mTOR expression [36].

mTOR expression at 24 hours hypoxia group was higher than 24 hours control group. This range was the highest between the normoxic and hypoxic group. On the other hand, the lowest expression was found at 48 hours. The 72 hours normoxic and hypoxic have the lowest expressions of mTOR. The result of previous study indicated that hypoxic condition increased proliferation and stem cell migration through FN-IN β1 expression that passed through PI3K/Akt, HIF-1α, and mTOR pathway. This research shown mTOR expression reached maximum after being given hypoxic treatment for more than 120 minutes. Besides that, HIF-1α expression also started to increase after hypoxic treatment for 6 hours [38]. A study on placental chorionic plate-derived MSCs (CP-MSCs) also shown that hypoxic treatment induced stem cell factors (SCF) expression on CP-MSCs. Especially on SCF/c-kit pathway that increased self-renewal activity of CP-MSCs through paracrine / autocrine mechanism by autophagy which kept the balance of death and survival activity. Autophagy was an effective way to preserve healthy stem cell population by balancing elimination of abnormal cell with normal cell proliferation. Furthermore, this activity happened on the higher level on CP-MSCs if being compared with BM-MSCs by regulating mTOR phosphorylation. This research also shown that HIF-1α affected mTOR activity in hypoxic condition [39].

In general, this study elucidated that in hypoxic condition, the expression of HIF-1α increased higher than in normoxic condition and mTOR expression on hypoxic condition also increased on early treatment if compared to in normoxic condition. Latest research shown that HIF-1α and mTOR were the main pathway to connect metabolism affected by hypoxia and proliferation and migration of hUCMSCs [35]. This study proven that there is a connection between HIF-1α and mTOR. The possibility to use hUCMSCs at 48 hours of hypoxia pretreatment where both HIF-1α and mTOR were at the highest expression, as this is important factors in stem cell application, mainly in hypoxia microenvironment. This study used one donor for homogeneity, but this can also be the limitation of this study. The evaluation of hypoxia on heterogenous hUCMSCs from multiple donors can be suggested for future studies. Current study using hUCMSCs from one donor is sufficient to positively affect implant osseointegration [40]. More donor might be required if there is a need for massive hUCMSCs productions or trials and for heterogeneity purposes. This research only focuses on the expression of HIF-1α and mTOR, therefore needed further researches to find other factors or expression as to optimize hUCMSCs use in regenerative dentistry.

Conclusion

$CoCl_2$ that is applied to human umbilical cord mesenchymal stem cells (hUCMSCs) could increase HIF-1α and mTOR expressions. The highest expression of HIF-1a was at 48 hours hypoxia and the lowest expression was at 72 hours hypoxia. Hypoxia increased mTOR expression at 24 hours and reached the lowest expression at 72 hours.

Authors' Contributions

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GH	https://orcid.org/0000-0002-6620-1302	Methodology, Investigation, Writing - Review and Editing and Supervision.

All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

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None.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

References

[1] Mendes RT, Nguyen D, Stephens D, Pamuk F, Fernandes D, Hasturk H, et al. Hypoxia-induced endothelial cell responses-possible roles during periodontal disease. Clin Exp Dent Res 2018; 4(6):241-248. https://doi.org/10.1002/cre2.135

[2] Devine DA, Marsh PD, Meade J. Modulation of host responses by oral comensal bacteria. J Oral Microbiol 2015; 7:26941. https://doi.org/10.3402/jom.v7.26941

[3] Celik D, Kantarci A. Vascular changes and hypoxia in periodontal disease as a link to systemic complications. Pathogens 2021; 10(10):1280. https://doi.org/10.2290/pathogens10101280

[4] Wu D, Yotnda P. Induction and testing of hypoxia in cell culture. J Vis Exp 2011; 54:2899. https://doi.org/10.3791/2899

[5] Misawa MYO, Huynh-Ba G, Villar GM, Villar CC. Efficacy of stem cells on the healing of peri-implant defects: systematic review of preclinical studies. Clin Exp Dent Res 2016; 2(1):18-34. https://doi.org/10.1002/cre2.16

[6] Chao YH, Wu HP, Chan CK, Tsai C, Peng CT, Wu KH. Umbilical cord-derived mesenchymal stem cells for hematopoietic stem cell transplantation. J Biomed Biotechnol 2012; 2012:759503. https://doi.org/10.1155/2012/759503

[7] Kuntjoro M, Prasetyo EP, Cahyani F, Kamadjaja MJK, Hendrijantini N, Laksono H, et al. Lipopolysaccharide's cytotoxicity on human umbilical cord mesenchymal stem cells. Pesqui Bras Odontopediatria Clín Integr 2020; 20:e0048. https://doi.org/10.1590/pboci.2020.153

[8] Prasetyo EP, Kuntjoro M, Cahyani F, Goenharto S, Saraswati W, Juniarti DE, et al. Calcium hydroxide upregulates interleukin-10 expression in time dependent exposure and induces osteogenic differentiation of human umbilical cord mesenchymal stem cells. Int J Pharm Res 2021; 13(1):140-145. https://doi.org/10.31838/ijpr/2021.13.01.023

[9] Kuntjoro M, Agustono B, Prasetyo EP, Salim S, Rantam FA, Hendrijantini N. The effect of advanced glycation endo products (AGEs) on human umbilical cord mesenchymal stem cells (hUCMSCs) with regard to osteogenesis and calcification. Res J Pharm Technol 2021; 14(8):4019-4024. https://doi.org/10.52711/0974-360X.2021.00696

[10] Prasetyo EP, Kuntjoro M, Goenharto S, Juniarti DE, Cahyani F, Hendrijantini N, et al. Calcium hydroxide increases human umbilical cord mesenchymal stem cells expressions of apoptotic proteaseactivating factor-1, caspase-3 and caspase-9. Clin Cosmet Investig Dent 2021; 13:59-65. https://doi.org/10.2147/CCIDE.S284240

[11] Li T, Xia M, Gao Y, Chen Y, Xu Y. Human umbilical cord mesenchymal stem cells: an overview of their potential in cell-based therapy. Expert Opin Biol Ther 2015; 15(9):1293-1306. https://doi.org/10.1517/14712598.2015.1051528

[12] Ejtehadifar M, Shamsasenjan K, Movassaghpour A, Akbarzadehlaleh P, Dehdilani N, Abbasi P, et al. The effect of hypoxia on mesenchymal stem cell biology. Adv Pharm Bull 2015; 5(2):141-149. https://doi.org/10.15171/apb.2015.021

[13] Bhandi S, Kahtani AA, Mashyakhy M, Alsofi L, Maganur PC, Vishwanathaiah S, et al. Modulation of the dental pulp stem cell secretory profile by hypoxia induction using cobalt chloride. J Pers Med 2021; 11(4):247. https://doi.org/10.3390/jpm11040247

[14] Laksana K, Sooampon S, Pavasant P, Sriarj W. Cobalt chloride enhances the stemness of human dental pulp cells. J Endod 2017; 43(5):760-765. https://doi.org/10.1016/j.joen.2017.01.005
[15] Nugraha AP, Prasetyo EP, Kuntjoro M, Ihsan IS, Dinaryanti A, Susilowati H, et al. The effect of cobalt (II) chloride in the viability percentage and the induced hypoxia inducible factor - 1 of human adipose mesenchymal stem cells (HAMSCs): An in vitro study. Syst Rev Pharm 2020; 11(6):308-314. https://doi.org/10.31838/srp.2020.6.49
[16] Nugraha AP, Ihsan IS, Dinaryanti A, Hendrianto E, Susilowati H, Prasetyo EP, et al. Cobalt (II) chloride in enhancing hypoxia inducible factor-1a expression of gingival derived mesenchymal stem cells in vitro. Res J Pharm Technol 2021; 14(5):2639-2642. https://doi.org/10.52711/0974-360X.2021.00465
[17] Chen Y, Zhao Q, Yang X, Yu X, Yu D, Zhao W. Effects of cobalt chloride on the stem cell marker expression and osteogenic differentiation of stem cells from human exfoliated deciduous teeth. Cell Stress Chaperones 2019; 24(3):527-538. https://doi.org/10.1007/s12192-019-00981-5
[18] Moniz I, Ramalho-Santos J, Branco AF. Differential oxygen exposure modulates mesenchymal stem cell metabolism and proliferation through mTOR signaling. Int J Mol Sci 2022; 23(7):3749. https://doi.org/10.3390/ijms23073749
[19] Vishwakarma A, Karp J. Biology and Engineering of Stem Cell Niches. New York: Elsevier; 2017.
[20] Park IH, Kim KH, Choi HK, Shim JS, Whang SY, Hahn SJ, et al. Constitutive stabilization of hypoxia-inducible factor alpha selectively promotes the self-renewal of mesenchymal progenitors and maintains mesenchymal stromal cells in an undifferentiated state. Exp Mol Med 2013; 45(9):e44. https://doi.org/10.1038/emm.2013.87
[21] Leontieva OV, Natarajan V, Demidenko ZN, Burdelya LG, Gudkov AV, Blagosklonny MV. Hypoxia suppresses conversion from proliferative arrest to cellular senescence. Proc Natl Acad Sci 2012; 109(33):13314-13318. https://doi.org/10.1073/pnas.1205690109
[22] Lavrentieva A, Majore I, Kasper C, Hass R. Effects of hypoxic culture conditions on umbilical cord-derived human mesenchymal stem cells. Cell Commun Signal 2010; 8:18. https://doi.org/10.1186/1478-811X-8-18
[23] Saxton RA, Sabatini DM. mTOR Signaling in growth, metabolism, and disease. Cell 2017; 168(6):960-976. https://doi.org/10.1016/j.cell.2017.02.004
[24] Prasetyo EP, Widjiastuti I, Cahyani F, Kuntjoro M, Hariyani N, Winoto ER, et al. Cytotoxicity of calcium hydroxide on human umbilical cord mesenchymal stem cells. Pesqui Bras Odontopediatria Clín Integr 2020; 20:e0044. https://doi.org/10.1590/pboci.2020.141
[25] Teti G, Focaroli S, Salvatore V, Mazzotti E, Ingra L, Mazzotti A, et al. The hypoxia-mimetic agent cobalt chloride differently affects human mesenchymal stem cells in their chondrogenic potential. Stem Cells Int 2018; 2018:3237253. https://doi.org/10.1155/2018/3237253
[26] Ding DC, Chang YH, Shyu WC, Lin SZ. Human umbilical cord mesenchymal stem cells: A new era for stem cell therapy. Cell Transplant 2015; 24(3):339-347. https://doi.org/10.3727/096368915X686841
[27] Cordero CB, Santander GM, Gonzalez DU, Quezada A, Silva CI, Vasquez C, et al. Allogenic cellular therapy in a mature tooth with apical periodontitis and accidental root perforation: A case report. J Endod 2020; 46(12):1920-1927. https://doi.org/10.1016/j.joen.2020.04.007
[28] Wang L, Ott L, Seshareddy K, Weiss ML, Detamore MS. Musculoskeletal tissue engineering with human umbilical cord mesenchymal stromal cells. Regen Med 2011; 6(1):95-109. https://doi.org/10.2217/rme.10.98
[29] Suda T, Takubo K, Semenza GL. Metabolic regulation of hematopoietic stem cells in the hypoxic niche. Cell Stem Cell 2011; 9(4):298-310. https://doi.org/10.1016/j.stem.2011.09.010
[30] Liu Y, Ma T. Metabolic regulation of mesenchymal stem cell in expansion and therapeutic application. Biotechnol Prog 2015; 31(2):468-481. https://doi.org/10.1002/btpr.2034
[31] Cimmino F, Avitabile M, Lasorsa VA, Montella A, Pezone L, Cantalupo S, et al. HIF-1 transcription activity: HIF1A driven response in normoxia and in hypoxia. BMC Med Genet 2019; 20:37. https://doi.org/10.1186/s12881-019-0767-1
[32] Ho SS, Hung BP, Heyrani N, Lee MA, Leach JK. Hypoxic preconditioning of mesenchymal stem cells with subsequent spheroid formation accelerates repair of segmental bone defects. Stem Cells 2018; 36(9):1393-1403. https://doi.org/10.1002/stem.2853
[33] Kierans SJ, Taylor CT. Regulation of glycolysis by the hypoxia-inducible factor (HIF): implications for cellular physiology. J Physiol 2021; 599(1):23-37. https://doi.org/10.1113/JP280572
[34] Brugarolas J, Lei K, Hurley RL, Manning BD, Reiling JH, Hafen E, et al. Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. Genes Dev 2004; 18(23):2893-2904. https://doi.org/10.1101/gad.1256804
[35] Lee HJ, Ryu JM, Jung YH, Oh SY, Lee SJ, Han HJ. Novel pathway for hypoxia-induced proliferation and migration in human mesenchymal stem cells: Involvement of HIF-1α, FASN, and mTORC1. Stem Cells 2015; 33(7):2182-2195. https://doi.org/10.1002/stem.2020
[36] Chu Y, Chen W, Peng W, Liu Y, Xu L, Zuo J, et al. Amnion-derived mesenchymal stem cell exosomes-mediated autophagy promotes the survival of trophoblasts under hypoxia through mTOR pathway by the downregulation of EZH2. Front Cell Dev Biol 2020; 8:545852. https://doi.org/10.3389/fcell.2020.545852
[37] Blagosklonny MV. Hypoxia, MTOR and autophagy converging on senescence or quiescence. Autophagy 2013; 9(2):260-262. https://doi.org/10.4161/auto.22783

[38] Lee SH, Lee YJ, Han HJ. Role of hypoxia-induced fibronectin-integrin β1 expression in embryonic stem cell proliferation and migration: Involvement of PI3K/Akt and FAK. J Cell Physiol 2011; 226(2):484-493. https://doi.org/10.1002/jcp.22358

[39] Lee Y, Jung J, Cho KJ, Lee SK, Park JW, Oh IH, et al. Increased SCF/c-kit by hypoxia promotes autophagy of human placental chorionic plate-derived mesenchymal stem cells via regulating the phosphorylation of mTOR. J Cell Biochem 2013; 114(1):79-88. https://doi.org/10.1002/jcb.24303

[40] Kuntjoro M, Hendrijantini N, Prasetyo EP, Legowo D, Sitalaksmi RM, Agustono B, et al. Human umbilical cord mesenchymal stem cells accelerate and increase implant osseointegration in diabetic rats. J Appl Oral Sci 2023; 31:e20220375. https://doi.org/10.1590/1678-7757-2022-0375