# การกระตุ้นการหลั่งสารอักเสบภายใต้สภาวะพร่องออกซิเจนในเซลล์เนื้อเยื่อในฟันน้ำนมมนุษย์

นางสาว จรินทร โคธีรานุรักษ์

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# THE STIMULATION OF INFLAMMATORY CYTOKINE RELEASE UNDER HYPOXIC CONDITION IN HUMAN DECIDUOUS DENTAL PULP CELLS

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Pediatric Dentistry

Department of Pediatric Dentistry

Faculty of Dentistry

Chulalongkorn University

Academic Year 2010

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| Thesis Title | THE STIMULATION OF INFLAMMATORY CYTOKINE RELEASE UNDER HYPOXIAC CONDITION ON HUMAN DECIDUOUS DENTAL PULP CELLS |
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| Field of Study | Pediatric Dentistry |
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จรินทร โคธีรานุรักษ์ : การกระตุ้นการหลั่งสารอักเสบภายใต้สภาวะพร่องออกซิเจนในเซลล์เนื้อเยื่อในฟันน้ำนมมนุษย์ (THE STIMULATION OF INFLAMMATORY CYTOKINE RELEASE UNDER HYPOXIC CONDITION IN HUMAN DECIDUOUS DENTAL PULP CELLS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ทพญ.สุภาภรณ์ จงวิศาล, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ.ทพ.ดร.ประสิทธิ์ ภวสันต์, 63 หน้า.

**วัตถุประสงค์**: ออกซิเจนมีบทบาทสำคัญในการเหนี่ยวนำและกระตุ้นการทำงานของเซลล์ต่างๆ การศึกษาในครั้งนี้มีวัตถุประสงค์เพื่อศึกษาการตอบสนองของเซลล์เนื้อเยื่อในของฟันน้ำนมมนุษย์ในการหลั่งสารอักเสบภายใต้สภาวะพร่องออกซิเจน (ออกซิเจนร้อยละ 2) **วิธีการทดลอง**: นำเซลล์ที่ได้มาจากเนื้อเยื่อในของน้ำนมมนุษย์ในห้องปฏิบัติการมาเพาะเลี้ยงเพื่อศึกษาการเจริญเติบโตและการสร้างโปรตีนของเซลล์ภายใต้สภาวะออกซิเจนต่ำ เป็นระยะเวลา 6, 9, 12, และ 15 ชั่วโมง โดยใช้วิธีเมทตาบอลิก แอสเสส (เอ็มทีที) และ วิธีเอ็นไซม์ลิงค์ อิมมูโนซอร์เบนท์ แอสเสส (อีไลซา) ศึกษาระดับการแสดงออกของอาร์เอ็นเอนำรหัสใช้วิธีรีเวอร์ส ทรานสคิปชั่น โพลิเมอร์เลสเชน รีแอคชั่น (อาร์ที-พีซีอาร์) **ผลการทดลอง**: พบว่าเซลล์เนื้อเยื่อในโพรงฟันน้ำนมมนุษย์ภายใต้สภาวะออกซิเจนต่ำมีการแสดงออกของยีนและโปรตีนที่เกี่ยวข้องกับการอักเสบโดยเฉพาะอินเตอร์ลิวคิน- 6 (IL-6) สูงกว่าเซลล์ที่อยู่ในสภาวะปกติ (ออกซิเจนร้อยละ 20) โดยสามารถเริ่มพบความแตกต่างอย่างชัดเจนในกลุ่มเซลล์ที่อยู่ภายใต้สภาวะออกซิเจนต่ำเป็นระยะเวลา 12 ชั่วโมง การใส่โคบอลท์คลอไรด์สามารถเพิ่มระดับของการหลั่งอินเตอร์ลิวคิน- 6 ได้เช่นกัน นอกจากนี้สภาวะพร่องออกซิเจนยังสามารถกระตุ้นให้เกิดการแสดงออกที่เพิ่มขึ้นของยีนเร็กซ์-1 (Rex-1) โดยการใส่สารละลายอินเตอร์ลิวคิน-6 นิวทรอไลซิ่ง แอนติบอดี้ (IL-6 neutralizing antibody) สามารถลดระดับการแสดงออกของยีนเร็กซ์- 1 ได้ **วิจารณ์ผลการทดลอง**: การเพิ่มขึ้นของการหลั่งสารอักเสบโดยเฉพาะอินเตอร์ลิวคิน- 6 เป็นการตอบสนองที่ผ่านไฮปอกเซีย- อินดิวซิเบิล แฟคเตอร์-1$\alpha$ (HIF-1$\alpha$) และอินเตอร์ลิวคิน- 6 มีผลต่อการเพิ่มขึ้นของการแสดงออกของยีนเร็กซ์- 1 ในเซลล์เนื้อเยื่อในของฟันน้ำนมภายใต้สภาวะออกซิเจนต่ำ ซึ่งการตอบสนองของเซลล์ในการหลั่งสารอินเตอร์ลิวคิน- 6 ที่ค่อนข้างช้านี้น่าจะแสดงถึงการมีบทบาทของสารนี้ที่เกี่ยวข้องกับกระบวนการซ่อมแซมเซลล์ การเพิ่มจำนวนเซลล์หรือการแปรสภาพของเซลล์ เพื่อตอบสนองต่อสภาวะพร่องออกซิเจน

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KEYWORDS : HYPOXIA, STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH (SHED), HYPOXIA- INDUCIBLE TRANSCRIPTION FACTOR (HIF), VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF), INTERLEUKIN-6 (IL-6)

JARINTORN KOTHEERANURAK: THE STIMULATION OF INFLAMMATORY CYTOKINE RELEASE UNDER HYPOXIC CONDITION IN HUMAN DECIDUOUS DENTAL PULP CELLS. THESIS ADVISOR: ASSOC. PROF. SUPAPORN CHONGVISAL, D.D.S., M.Sc.,THESIS CO- ADVISOR: PROF. PRASIT PAVASANT, D.D.S., M.Sc., Ph.D., 63 pp.

**Objective**: Oxygen plays an important role in signal transduction and regulation of cell behavior. The aim of this study was to investigate the response of SHED to hypoxia (2%$O_2$) especially on the inflammatory cytokine release. **Materials and methods**: Dental pulp cells were obtained from exfoliated deciduous teeth. Cells were cultured and put under hypoxic condition (2%$O_2$) for 6, 9, 12, and 15 hours. The number of cells was determined by metabolic assay (MTT assay). The changes in RNA expression were examined by reverse transcription polymerase chain reaction (RT-PCR) and the amount of protein was measured using enzyme-linked immunosorbent assay (ELISA). **Result**: The results showed that 2% oxygen could significantly up-regulate IL-6 expression and secretion after 12 hours under hypoxia condition. Cobalt chloride ($CoCl_2$) could also increase IL-6 secretion in a dose-dependent manner. Hypoxia could up-regulate Rex-1 expression and the use of IL-6 neutralizing antibody demonstrated the role of IL-6 in Rex-1 induction. **Discussion**: The data from this study suggested that the HIF-1α dependent up-regulation of IL-6 expression is a late response. The hypoxia-induced IL-6 can induce Rex-1 expression in SHED and may play a role in cell tissues repair or cell proliferation/ differentiation in response to the low oxygen environment.

Department : Pediatric Dentistry Student's Signature .....

Field of Study : Pediatric Dentistry Advisor's Signature .....

Academic Year : 2010 Co-Advisor's Signature .....

# ACKNOWLEDGEMENTS

I sincerely thank and appreciate to my thesis advisor, Associate Professor Supaporn Chongvisal and my thesis co-advisor, Professor Dr. Prasit Pavasant for their suggestion, encouragement, attention and kindness throughout the course of my Master degree program. I would like to give thanks for my thesis committee members; Associate Professor Dr. Thippawan Tarapiwattananont, Associate Professor Dr. Siriruk Nakornchai and Lect. Dr. Wantida Sri-arj for their suggestion and kindness.

Sincere appreciation is expressed to the staffs of the Unit of Mineralized Tissue Laboratory for their kind advice and technical assistance. Moreover, I would like to thank the staff of the Department of Pediatric Dentistry, Faculty of Dentistry, Chulalongkorn University for their kindness and for collecting and preserving the extracted deciduous teeth.

Finally, I would like to thank sincerely to my family and my friends for their love, help, understanding and encouragement throughout the course of my program.

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| | |
|---|---|
| $CoCl_2$ | cobalt chloride |
| ELISA | enzyme- linked immunosorbent assay |
| hDDP cells | human deciduous dental pulp cells |
| HIF | hypoxia-inducible transcription factors |
| IL- 6 | interleukin- 6 |
| IL-1β | interleukin- 1β |
| $O_2$ | oxygen |
| RT-PCR | reversed transcription- polymerase chain reaction |
| SHED | stem cells from human exfoliated deciduous teeth |
| TNF-α | tumor necrosis factor- α |
| VEGF | vascular endothelial growth factor |

# CHAPTER I
# INTRODUCTION

## Background and Rationale

The level of tissue oxygenation is one of the factors that can affect the tissue homeostasis. In normal tissues, a hypoxic condition can be found associated with reduced blood flow. The significance of hypoxic condition in tissue homeostasis has been described in many organs including bone *(1-2)*, cartilage *(3)*, cornea *(4)* and vascular tissues *(5-6)*. For dental pulps, the structure of human teeth with narrow vascular openings at the apex of the roots makes it susceptible to the hypoxic condition. Hypoxic condition within the dental pulp tissue can frequently occur when the blood supply to tissues is disrupted as in the case of dental trauma, inflammation or during the use of local anesthetics containing vasoconstrictors *(7)*.

It has been demonstrated that oxygen can regulate the signal transduction and subsequently control the expression of target genes. Hypoxic condition is generally correlated with the up-regulation of vascular endothelial growth factor (VEGF), a potent angiogenic growth factor, leading to the increasing rate of new blood vessels formation *(6)*. In addition, correlation between hypoxia and tissue inflammation has also been reported *(8)*. During inflammation, up-regulation of pro-inflammatory cytokines such as interleukin (IL)-1$\beta$, IL-6, and tumor necrosis factor- $\alpha$ (TNF-$\alpha$) were observed *(9)*. In bone healing, osteoprogenitor cells, regardless of their origin, can differentiate into either osteoblasts or chondrocytes *(10)*. A study showed that low oxygen tension (5%$O_2$) or hypoxia resulted in cartilaginous matrix synthesis and high oxygen condition (35%$O_2$) induced mesenchymal tissue differentiation toward bone *(11)*, suggesting the effect of oxygen concentration on the bone formation and osteogenic differentiation.

The principal regulator of the transcriptional response to hypoxia is the hypoxia-inducible transcription factors (HIF) family of transcription factors *(12-13)*. In hypoxic condition, activation of HIF has been reported *(13-16)*. HIF will be trans-located into the

nucleus in hypoxic condition, bound to hypoxia-responsive elements (HRE) and regulated gene expression *(17)* such as vascular endothelial growth factor (VEGF), a key regulator of blood vessel growth (angiogenesis), erythropoietin, a cytokine required for the formation of red blood cells and tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis *(18)*.

Nevertheless, there are few reports regarding the responses of human deciduous dental pulp cells (hDDP cells) to hypoxia, or the molecular mechanisms responsible for resistance to hypoxia by pulp cells, and the effect of hypoxia on the up-regulation of inflammatory cytokines is still unclear. It has been reported that hDDP cells obtained stem cells characteristic and has been described as stem cells from human exfoliated deciduous teeth (SHED) *(19)*. Since the level of oxygen can influence the function and differentiation of cells, it is possible that oxygen level may influence cell behavior and might affect the differentiation or repairing activity of hDDP cells/ SHED.

The purpose of this study is to investigate the response of hDDP cells or SHED to the hypoxic condition, especially on the inflammatory cytokine release. In addition, the mechanism involved in the hypoxia-induced cytokine release will be examined.

## Research Questions

Can hypoxic condition stimulate the expression of the inflammatory cytokines in hDDP cells.

## Research Objective

To investigate the response of hDDP cells to hypoxic condition especially on the inflammatory cytokine release.

## Research Hypotheses

Hypoxic condition can stimulate the expression of the inflammatory cytokines in hDDP cells.

**Keywords**

Hypoxia, Human deciduous dental pulp cells (hDDP cells), Stem cells from human exfoliated deciduous teeth (SHED), Hypoxia- inducible transcription factor (HIF), Interleukin-6 (IL-6)

**Research Design**

Laboratory experimental study

**Limitation of the Study**

The results from this study may represent only the tendency of cell responses when facing hypoxic condition as the conventional tissue culture condition (used as a controlled condition) exposed to dental pulp cells is non-physiological atmospheric levels of oxygen (20%) and most cells are not normally exposed to such high $pO_2$ in vivo.

**Pre-agreement**

The hypoxic condition is the condition with 2% oxygen.

The controlled condition is the condition with 20% oxygen

**Expected Outcomes**

Hypoxia or ischemia is an important pathogenic factor for many body vital tissues including dental pulp cells. It has become apparent that hypoxia can greatly impact the development of inflammation through the regulation of oxygen- dependent gene expression *(20)*. Therefore, the better understanding about the inflammatory- related response of the SHED under the hypoxic condition will benefit both basic knowledge and clinical application.

# CHAPTER II

# LITERATURE REVIEW

## Hypoxia

Oxygen is essential in cells and tissues survival. The level of oxygen, high or low, can lead to a number of pathophysiological consequences. In normal condition, the oxygen partial pressure ($pO_2$) of inspired air is around 160 mmHg (20% $O_2$) at sea level. After water evaporation and diffusion in the lung, the level of $pO_2$ of the alveolar capillaries carrying towards organs and tissues can drop to around 104 mmHg (13% $O_2$) *(21)*. The level of tissue oxygenation is one of the factors that can affect the tissue homeostasis. Low oxygen tension in tissues or hypoxia is an inadequate oxygen perfusion of the tissues. It occurs when the blood cannot deliver adequate oxygen to the tissues or organs, which may result from pulmonary dysfunction, cardiovascular dysfunction, stroke, traumatic brain injury (TBI), disorders of the blood such as anemia that affect erythrocytes (red blood cells) or hemoglobin, and breathing disturbances such as apnea. Hypoxia also occurs in healthy individuals when breathing mixtures of gases with low oxygen content, e.g. while diving underwater, especially when using closed-circuit re-breather systems that control the amount of oxygen in the supplied air *(22)*.

Low oxygen concentration is an essential developmental and physiological stimulus that plays a key role in the pathophysiology of many important organs *(23)*. In arterial blood, interstitial oxygen tension ($pO_2$) is about 95 mmHg (~12%), and about 40 mmHg (~5%) in venous and capillary blood. In normal tissue, it is roughly in the range 4-8% *(24)*. In environments such as the poorly vascularized yellow fatty bone marrow of the elderly, or in wounds, infected tissues, inflamed tissues, fractured sites, and tumors, $pO_2$ could be lower *(2)*. Permanent tissue damage or tissue death results when hypoxia persists *(25)*. The significance of hypoxic condition in tissue homeostasis has been described in many organs including bone *(1-2)*, cartilage *(3)*, cornea *(4)* and vascular tissues *(5-6)*.

There is a gradient of oxygen concentration in the human body. The cells located nearby the blood vessels obtain oxygen more efficiently than the cells apart from these vessels. Therefore, hypoxic regions occur throughout the body. Hypoxia may involve only a defined organ or area, such as a region of the brain affected by stroke, or involves the entire body *(22)*. Most of the tissues or organs in the human body are hypoxic in comparison to the oxygen concentration in the air and it is normally within the range of 2-9% *(26)*. In some tissues, the oxygen level can be lower than 1%, thus make the oxygen level of the conventional tissue culture using atmospheric air about 2.5- 20 times higher. This tissue hypoxic condition is referred to 'physiological hypoxia', where the tissues do not necessarily activate the hypoxic response *(27)*.

When cells encounter hypoxic conditions, one of the major changes is their metabolic state. Under aerobic condition, cells utilize the tricarboxylic acid (TCA) cycle and oxidative phospholylation in the mitochondria as well as glycolysis for energy production. Since the oxidative phospholylation process requires oxygen, under hypoxic condition, they rely only on the glycolysis pathway for energy production *(28)*. Furthermore, they also decrease the metabolic rate, such as translation rate, to limit cellular activity *(29)*.

Hypoxic condition could be superior to maintain cells in a non- dividing, or at least, slow cycling, stage and to protect cells from the accumulation of reactive oxygen species (ROS) because of the decreasing in mitochondrial respiration due to limitation in oxygen. Excess amount of ROS can be detrimental to cells due to highly reactive molecules, such as, hydrogen peroxide, superoxide, and the hydroxyl radical *(30)*. In addition to energy deprivation, radical formation, in particular ROS generation contributes to hypoxia induced apoptosis *(31)*.

**Hypoxia- Inducible Transcription Factor (HIF)**

It has been demonstrated that oxygen can regulate the signal transduction and subsequently control the expression of target genes. In normal tissue, the hypoxic condition can be associated with the reduced or excessive blood flow and the activation of HIF subunits has been reported *(13-16)*.

HIF, a master regulator of cellular response to hypoxia is the primary source of metabolic energy that widely expressed in all eukaryotic cells and tissues *(18)*. HIF is a heterodimeric transcription factor, composed of one of the 3 oxygen- sensitive HIF- $\alpha$ subunits (HIF- 1$\alpha$, HIF- 2$\alpha$, and HIF- 3$\alpha$) and the oxygen- insensitive and constitutively expressed HIF- $\beta$ subunits *(32-33)*. While HIF- 1$\alpha$ and HIF- 2$\alpha$ subunits are structurally similar in their DNA binding and dimerisation domains, their adaptive responses to hypoxia are distinctive. A study suggests that HIF- 1$\alpha$ is ubiquitous and responds only to severe hypoxia whereas HIF- 2$\alpha$ is restricted to certain tissues and stabilized in relatively moderate hypoxia *(34-35)*. And the role of HIF- 3$\alpha$ is yet to be clearly defined. The $\beta$ subunit or arylhydrocarbon nuclear translocator (ARNT) is expressed independently of $pO_2$, whereas the $\alpha$ subunit is oxygen sensitive. HIF-$\alpha$ cannot be detected unless cells are challenged by hypoxia. In the presence of oxygen above a critical intracellular oxygen tension, HIF- $\alpha$ is rapidly degraded in the ubiquitin-proteasome dependent degradation *(18, 36)*.

In hypoxic condition, HIF- $\alpha$ subunits will be trans-located into the nucleus, dimerizes with HIF- $\beta$ subunit and regulate various gene expressions *(17)* such as vascular endothelial growth factor (VEGF), a potent angiogenic growth factor, leading to an increasing rate of new blood vessels formation *(6)*.

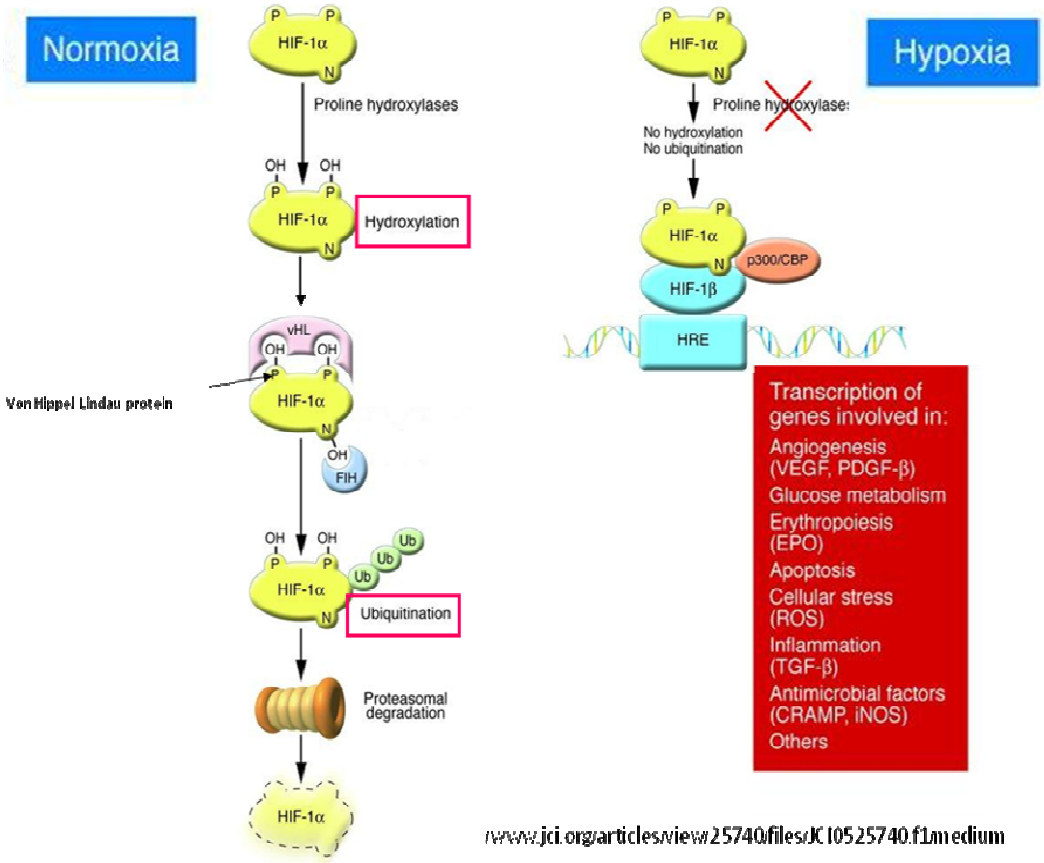


Figure 1. The degradation pathway of HIF-1 α

However, in some conditions, even with the present of oxygen, HIF can be detected. It has been well documented that cobalt, a transition metal, mimics hypoxia by causing the stabilization of HIF- α. Cobalt can inactivate the HIF-specific hydroxylase by occupying an iron- binding site on the proline hydroxylases *(37)*, or binding directly to the oxygen-dependent degradation (ODD) domain of HIF- α. Furthermore, cobalt also can inhibit the von Hippel- Lindau protein (pVHL) binding to HIF- α even when HIF- α is hydroxylated *(38)*.

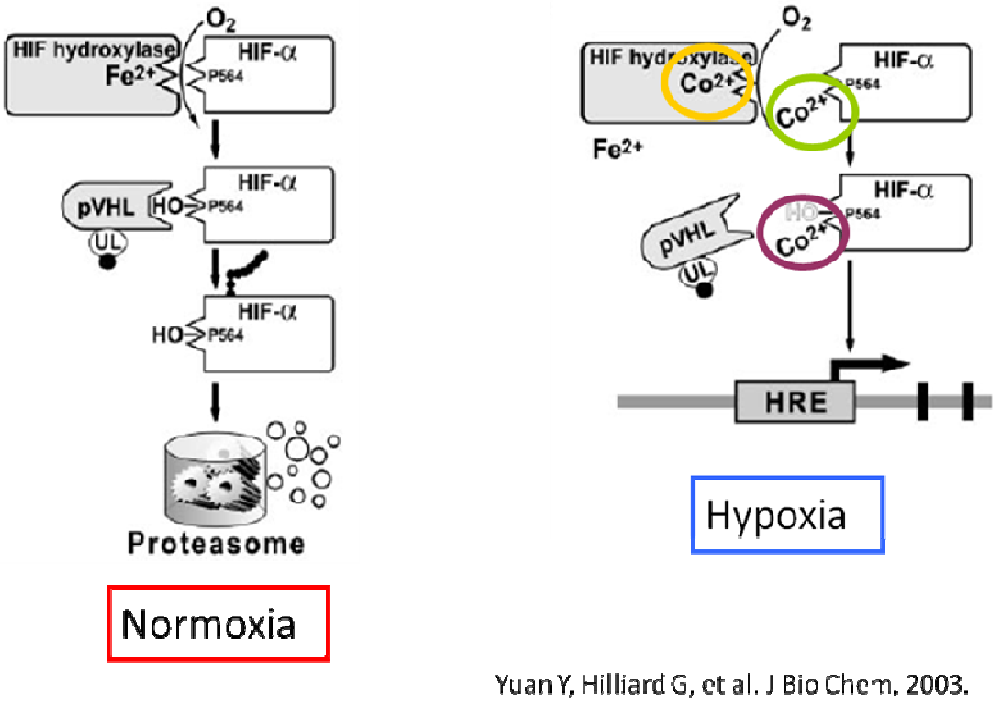


**Figure 2**. The proposed model for HIF- α stabilization by cobalt

## Hypoxia and Inflammation

Inflammation is a part of the complex biological responses of vascular tissues to harmful stimuli. It is characterized by the increase in blood flow to the affected tissues, resulting in tissues redness, swelling, heat, pain, and sometimes, immobility or loss of function *(39)*.

During inflammation, up-regulation of pro-inflammatory cytokines such as interleukin (IL)- 1β, IL- 6, and tumor necrosis factor - α (TNF- α) was observed *(9)*. IL-1β, and TNF- α are known to be the potent stimulants of many genes involved in inflammation and bone destruction *(40)*. IL-6 is a multifunctional cytokine with anti- inflammatory properties that affects multiple cell types *(41-42)*. IL- 1β and IL-6 have both been shown to regulate VEGF levels in a variety of tissues including blood- derived endothelial progenitor cell *(43)*, and pituitary adenoma cells *(44)*. In addition, IL- 6 was suggested to have angiogenesis induction either through its effects on VEGF expression *(45)* or by its direct stimulation in many cells such as endothelial cells *(43)*.

The correlation between hypoxia and tissue inflammation has been reported. Just as hypoxia can induce inflammation, inflamed lesions often become severely hypoxic. The likely causes of hypoxia during inflammation are the increasing of the cells metabolic activity resulting in elevated oxygen demand *(46)*, and the vasculopathy caused by extensive inflammation leading to poor perfusion and subsequently decreased oxygen supply *(47)*.

## Hypoxia in Dentistry

For dental pulps, the structure of human teeth with narrow vascular openings at the apex of the roots makes it susceptible to the hypoxic condition. Hypoxic condition within the dental pulp tissue can be frequently occurred when the blood supply to tissues is disrupted as in the case of inflammation, during the use of local anesthetics containing vasoconstrictors, or dental trauma *(7)*.

When pulpal inflammation or pulpitis occurs, pulp tissue pressure is elevated *(48-49)* and blood flow in the pulp falls in direct proportion to any increase in pulp tissue

pressure *(50)*. The localized reduction in pulp blood flow, however, allows the accumulation of mediators of inflammation, which in turn, causes a spread in the elevation of tissue pressure, reducing pulp blood flow to a larger volume of pulp etc., leading to dull aching poorly localized pulp pain *(50-51)*. With proper treatment, the hypoxic condition produced by the reduction in blood flow in dental pulp is transcient, and pulpal viability is generally improved by re-oxygenation after an increase of pulpal blood flow *(52)*.

Hypoxia can also induce complex and cell type-specific pro- angiogenic responses. In human dental pulp cells, the expression of HIF- 1$\alpha$ and VEGF increased under hypoxic condition *(53)*.

Low $pO_2$ condition was found to be an effective treatment to amplify numbers of progenitor cells from human dental pulp as hypoxia can significantly increase the cell number *(54)* and the side population in dental pulp cells *(55)* and re-oxygenation improves the hypoxia-induced pulp cell arrest *(56)*.

# CHAPTER III

# EXPERIMENTAL DESIGN

## Population

Human deciduous dental pulp cells

## Samples

Human dental pulp tissues obtained from the sound deciduous teeth of healthy children 5- 12 years old with no systemic disease or local anesthetic allergy. The samples collections are done under the children's legal guardians' informed consents.

### Inclusion criteria

1. Human dental pulp obtained from the sound deciduous teeth of children 5- 12 years old.
2. Human dental pulp obtained from the sound deciduous teeth of healthy children with no systemic disease.
3. Human dental pulp obtained from the sound deciduous teeth of healthy children with no local anesthetic allergy.
4. Human dental pulp obtained from the sound deciduous teeth of healthy children with legal guardians' informed consents.

### Exclusion criteria

1. Human dental pulp obtained from the deciduous teeth with lesions.
2. Human dental pulp obtained from the sound deciduous teeth of healthy children with systemic disease.
3. Human dental pulp obtained from the sound deciduous teeth of healthy children allergic to local anesthetic agents.

4. Human dental pulp obtained from the sound deciduous teeth of healthy children without legal guardians' informed consents.

### Study group

Human deciduous dental pulp cells incubated at 37ºC for 16 hours at normal level of oxygen (20% oxygen) before expose to the 2% oxygen in a modular incubator chamber at 37ºC for another 6, 9, 12, and 15 hours.

### Control group

Human deciduous dental pulp cells incubated at 37ºC for 16 hours at 20% oxygen until the end of the experiments.

## Study variables

### Independent variables

Oxygen levels

### Dependent variables

The levels of inflammatory cytokines expressed

## Materials

1. Sterile Phosphate Buffer Saline: PBS
2. Distill water
3. Dulbecco modified Eagle medium: DMED, Sigma- Aldrich Co, St. Louis, MO, USA
4. 10% fetal bovine serum: FBS, Gibco, Grand Island, NY, USA
5. Penicillin, Gibco, Grand Island, NY, USA
6. Streptomycin, Gibco, Grand Island, NY, USA

7. Glutamine, Gibco, Grand Island, NY, USA
8. 35, 60 mm. cell culture dishes
9. 12- welled culture plates
10. TRIzol, Gibco, Grand Island, NY, USA
11. Chloroform
12. Isopropanol
13. Agarose
14. MTT ultrapure
15. Primers for 18S
16. Primers for interleukin-1β: IL-1β
17. Primers for interleukin-6: IL-6
18. Primers for interleukin-8: IL-8
19. Primers for vascular endothelial growth factor: VEGF
20. Primers for Rex-1
21. Primers for tumor necrosis factor- α: TNF- α
22. Nuclease free water
23. cDNA synthesis kits
24. Taq polymerase
25. Deoxyribonucleotidetriphosphate: dNTP
26. Cobalt chloride: $CoCl_2$, Sigma- Aldrich, St. Louis, MO, USA
27. Human IL-6 neutralizing antibody: R&D systems, MN, USA
28. Absolute ethanol
29. Methanol
30. Sealed plastic tubes 5 ml.
31. Microcentrifuge tubes 0.5, 1 ml.
32. PCR tubes 0.2 ml.
33. Disposable pipette tips 10, 200, 1000 µl.
34. Disposable latex gloves

Equipments

1. Modular incubator chamber, Billups- Rothenberg, CA, USA
2. Oxygen detector
3. Microplate Reader ELx800, Biotex
4. Nanodrop 2000, Thermo sciencetific
5. Enzyme- linked immunosorbent assay: ELISA kits for VEGF, IL-1β, IL-6, R&D systems, MN, USA
6. UV/Visible spectrophotometer
7. Vortex, Genie2, Scientific Industries, USA
8. Centrifuge, Sigma, 101, Western Germany
9. High speed centrifuge, Sorvall, Super T 21, Dupont Company, USA
10. Microcentrifuge, Hero lab, Microcen 13, Hero lab GmbH, Germany
11. Low speed rotor
12. Shake 'n' stack hybridization oven, Hybaid, HBOVCST220, Hybaid Limited, UK
13. Water bath
14. Heating block
15. Polymerase chain reaction: PCR, PCR system
16. Horizontal electrophoresis apparatus
17. Gel Documentation System
18. Analytical balance
19. Automatic pipets
20. Quartz cuvette, Optiglass Ltd., Russia
21. Disposable cuvettes
22. Stainless scissors
23. Stainless forceps
24. Phase contrast light microscope
25. Digital camera, Fujifilm Finepix S100
26. Digital camera, Canon EOS 500D

## Methodology/ Experimental Design

### Sample Collection

Deciduous pulp tissues were obtained from nearly exfoliated teeth from children attended the Department of Pediatric Dentistry, Chulalongkorn University. Informed consent was obtained from each patient's legal guardian before the extraction was performed. Immediately after tooth extraction, the tooth was kept in a sterile tube containing Dulbecco' s Modified Eagle' s Medium (DMEM) containing 10% serum.

### Cell Culture

The extracted tooth was extensively washed with sterile phosphate-buffered saline (PBS). Pulp tissue was removed from each extracted tooth using sterile forceps; cut into small pieces approximately 1x1 mm, and digested with type I collagenase for 1 hour at 4$^{o}$C. The digestion was centrifuged and the explants were placed on the culture dishes and cultured with Dulbecco' s Modified Eagle' s Medium (DMEM) containing 10% serum. After cells crawled out of the explants and reached confluence, they were subcultured at 1:3 ratios. The cells at passage 3-5 were used in the experiments.

### Hypoxic Condition

Hypoxic condition was performed using a modular incubator chamber (Billups-Rothenberg, CA, USA). Oxygen in the chamber was flushed with gas mixtures of 5% carbondioxide ($CO_2$) and balanced nitrogen (N2) until the oxygen level reached 2%.

The exact oxygen level for hypoxic condition both in vivo and in vitro is still unclear. Normally, HIF is activated at a cut-off point at approximately 5% $O_2$ (40 mmHg) *(57)* and most experiments performed with cultured cells are undertaken at between 1-2% $O_2$ (8-16 mmHg) *(21)*. In this experiment, we selected 2% $O_2$ to demonstrate the hypoxic condition.

## Techniques Used in the Study

### MTT Assay

This assay is based on the ability of viable cells to reduce a tetrazolium-based compound, a 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; USB Corporation), to a purplish formazan product. To determine cell number, the medium was replaced with MTT solution for 30 minutes at 37°C and the formazan product was dissolved in dimethylsulfoxide and read the absorbance at 570 nm. Cell proliferation was analyzed by the assay.

In brief, cells were seeded in the 24- welled plates at a density of 30,000 cells/ well. At the end of the culture period, the medium was replaced with MTT solution for 30 minutes at 37°C and the formazan product was dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich, Germany). The absorbance of the supernatant was evaluated with a Thermospectronic Genesis 10 UV-visible spectrophotometer at 570 nm.

The absorbance was converted into cell number using cell number standard curve. The cell number from the controlled group was marked as 1 fold and the cell number from the hypoxia group was divided by the number from the controlled group to make a fold difference.

### RT-PCR

RNA was extracted from hDDP cells using TRIzol reagent (Roche Diagnostic, Indianapolis, IN, USA) according to the manufacturer' s protocol. One μg of mRNA was converted to cDNA using the ImPromII Reverse Transcription System (Promega, Southampton, UK). A polymerase-chain reaction (PCR) was performed using Tag polymerase (Tag DNA Polymerase, Invitrogen, Brazil). Specific primers for 18S, VEGF, IL-1β, IL-6, IL-8, TNF- α and Rex-1 were used and the PCR reaction was performed in the DNA thermal cycler (Biometra GmH, Göttingen, Germany) and the amplified DNA was visualized by ethidium bromide fluorostaining (EtBr; Bio- Rad, USA).

| Primer | Sequences | Cycles |
|---|---|---|
| 18S | Forward : 5' GTGATGCCCTTAGATGTCC 3'<br>Reverse : 5' CCATCCAATCGGTAGTAGC 3' | 25 |
| VEGF | Forward : 5' CAAGGACAGCACATAGGAGA 3'<br>Reverse : 5' GGTGGGTGTGTCTACGGGAA 3' | 26 |
| IL-1β | Forward : 5' GGAGCAACAAGTGGTGTTCT 3'<br>Reverse : 5' AAAGTCCAGGCTATAGCCGT 3' | 35 |
| IL-6 | Forward : 5' CCTGAACCTTCCAAAGATGGC 3'<br>Reverse : 5' CTGACCAGAAGAAGGAATGCC 3' | 20 |
| IL-8 | Forward : 5' CGATGTCAGTGCATAAAGACA 3'<br>Reverse : 55' TGAATTCTCAGCCCTCTTCAAAAA 3' | 20 |
| TNF- α | Forward : 5' AAGCCTGTAGCCCATGTTGT 3'<br>Reverse : 5' CAGATAGATGGGCTCATACC 3' | 36 |
| Rex-1 | Forward : 5' AGAATTCGCTTGAGTATTCTGA 3'<br>Reverse : 5' GGCTTTCAGGTTATTTGACTGA 3' | 35 |

**Table 1** showed the sequences of the primers and the RT-PCR cycles used in the experiment

ELISA

Conditioned medium was collected to determine the levels of VEGF, IL-1β, and IL-6 using ELISA kits according to the manufacturer's protocols (R&D Systems, Inc., Minneapolis, MN, USA).

## Experimental Designs

### Effects of Hypoxia on hDDP Cells

Cell cultures were grown in 6-welled plates at the density of 25 x $10^5$ cells/ well at 37°C for 16 hours at normal level of oxygen (20%). Cells in the hypoxic group were then moved into a modular incubator chamber and incubated with 2% oxygen at 37°C. Alternative plates were cultured in the 20% oxygen and used as a controlled condition. Cells from three different donors were used in three separated experiments.

For morphological analysis, cells were exposed to the 2%$O_2$ for 6, 9, 12, and 15 hours. Cell morphology was observed under phase contrast microscope and photographs were taken under phase contrast light microscope using digital camera (Canon EOS 500D).

Cell number was determined by the MTT assay, and evaluated using standard curve. Enzyme-linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR) analysis was used to examine the secretion and expression of inflammatory cytokines, respectively.

### Cell Response to Re-oxygenation after Hypoxic Condition

Cell cultures were grown in 35mm. dishes at density of 25 x $10^5$ cells/ dish at 37°C for 16 hours at normal level of oxygen. Cells were then divided into 5 groups; group 1, cells were cultured in 2% $O_2$ for 1 hour and hen the media was changed and cells were cultures in 20% $O_2$ for another 11 hours, group 2 were cultured in 2% $O_2$ for 3 hours and hen the media was changed and cells were cultures in 20% $O_2$ for another 9 hours, group 3 were cultured in 2% $O_2$ for 6 hours and hen the media was changed and cells were cultures in 20% $O_2$ for another 6 hours, group 4 were cultured in 2% $O_2$ for 12 hours, and group 5 were cultured in 20% $O_2$ for 12 hours. (See figure 3.) ELISA and RT-PCR analysis were used to observe the secretion and expression of IL-6, respectively.

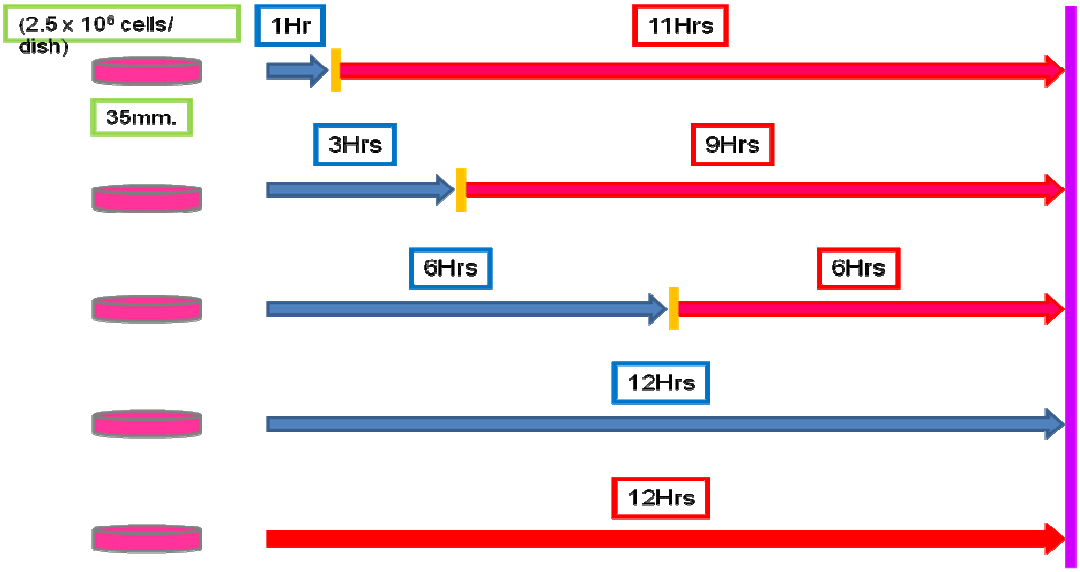


**Figure 3.** A study design for the "cell response to re-oxygenation after hypoxic condition" experiment.

### Role of HIF-1 in Hypoxia-induced IL-6

Cell cultures were grown in 6- welled plates at the density of 25 x $10^5$ cells/ well at 37°C for 16 hours at normal level of oxygen. Cobalt chloride ($CoCl_2$) (0, 50, 100, and 200 mM), an inorganic compound that can stabilize HIF-1α, thus mimics the hypoxic condition, was used. Cells were then cultured at normal level of oxygen for 12 hours. ELISA and RT-PCR analysis were used to observe the secretion and expression of VEGF, and IL-6. See figure 4 for summary of all studies.

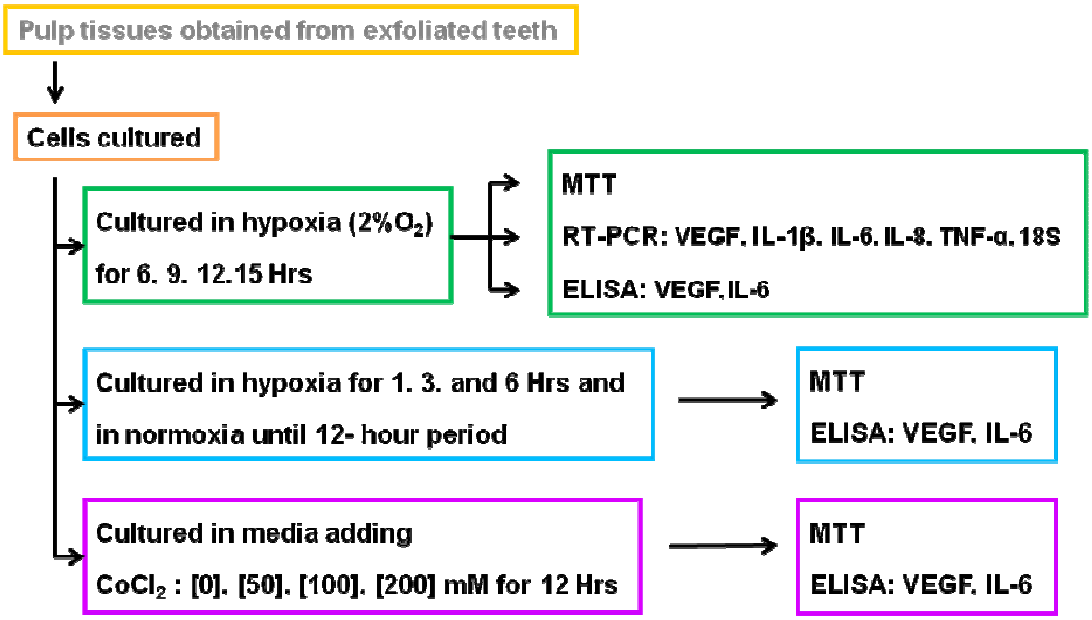


Figure 4. Summary of the study

## Statistic Analysis

All experiments were performed at least three times, with the data being represented as mean ± standard deviation (mean ± SD). Results of proliferation and hypoxia experiments between two groups (hypoxia and control) were analyzed by Student t- test ($p<0.05$)

## Ethical Considerations

1. The study was proposed for ethical approval from the ethical committee, Faculty of Dentistry, Chulalongkorn University.
2. The study process causes no harm to the patients as the extracted deciduous teeth used in the experiment are teeth that nearly exfoliated or the prolonged ones with the present of the permanent succidaneous teeth, which is the indication for tooth extraction in children. The extraction was performed under the standard protocol.
3. The legal guardians are informed about the details of the study process before signing the consent forms.

# CHAPTER IV
# RESULTS

## Effect of Hypoxia on Cell Morphology and Cell Number

### Cell Morphology

Human deciduous dental pulp cells were subjected to the hypoxia condition with 2% of oxygen. The morphology was observed under phase contrast microscope. As shown in Fig. 5, hDDP cells, when cultured in hypoxia displayed a similar appearance compared to the control culture. However, a slight size reduction and fewer cell extensions could be observed in hDDP cells cultured in hypoxic condition (2%$O_2$) for 15 hours.

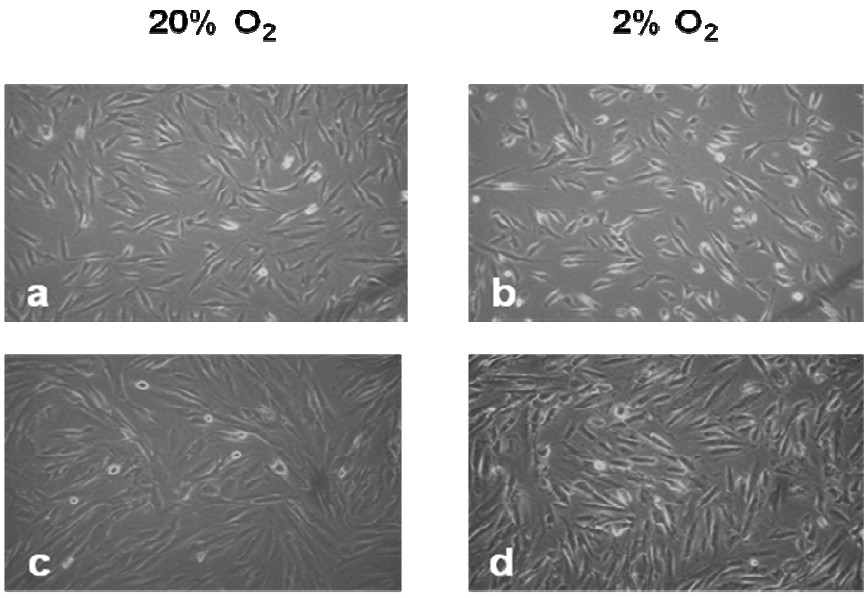


**Figure 5**. Photograph showed the morphology of cells in culture: Human deciduous dental pulp cells were cultured in normal (20% $O_2$; a, c) or hypoxic (2% $O_2$; b, d) conditions. Photographs were taken under phase contrast microscope (Magnification: 4x) after 9 (a, b), and 15 (c, d) hours. The results showed a slight change in cell morphology.

Cell Number

The cell number, as determined by the MTT assay revealed lower number of cells in hypoxic groups as showed in Fig. 6. Cells were cultured in normal condition for 16 hours and subjected to hypoxic condition for 6, 9, 12 and 15 hours. Generally, the normal duplication time for dental pulp cells is approximately 24-26 hours. Therefore, increasing cell number observed at 9 hours in Fig. 6 corresponded with the normal duplication time in control condition. In hypoxia, the constant cell number was observed from 6 to 15 hours suggesting that low level of oxygen might interfere with the cell division.

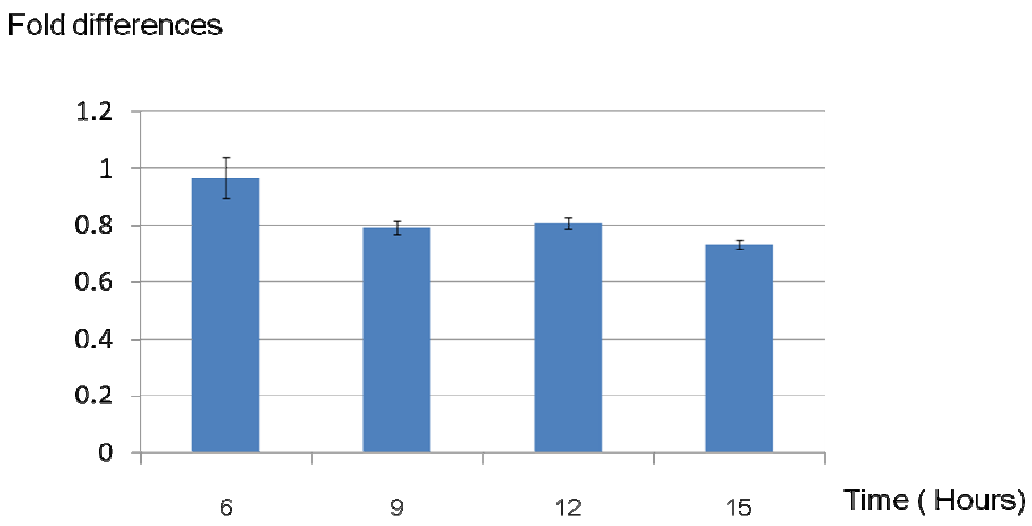


Figure 6. Hypoxia decreased the dental pulp cells growth. Primary dental pulp cells were grown for 16 hours before subjected to the condition of 20% (control) or 2 % (hypoxia) oxygen for another 6-15 hours. The cell number was determined by MTT assay. The numbers of cells in all controlled groups were marked as 1 fold. Data were shown as mean $\pm$ SD from three experiments.

**Hypoxia Induced Expression of VEGF and IL-6 in hDDP Cells**

To investigate the effect of hypoxia on inflammatory cytokines secretion, cells were cultured for 16 hours in normal condition; they were then subjected to hypoxic condition for another 12 hours. Total RNA was extracted and subjected to RT-PCR analysis. The VEGF expression was used as a positive control since the increasing expression of VEGF in cells underwent the hypoxic condition is well known. After 12 hours in hypoxia (2%$O_2$) the up-regulation of VEGF expression was detected as expected. Interestingly, hypoxia could markedly induce the expression of IL-6 in deciduous dental cells as compared to the control (20%$O_2$) as showed in Fig.7. Slight increase of IL-1β and IL-8 expression was also detected. However, the expression of TNF-α mRNA was decreased in hypoxia.

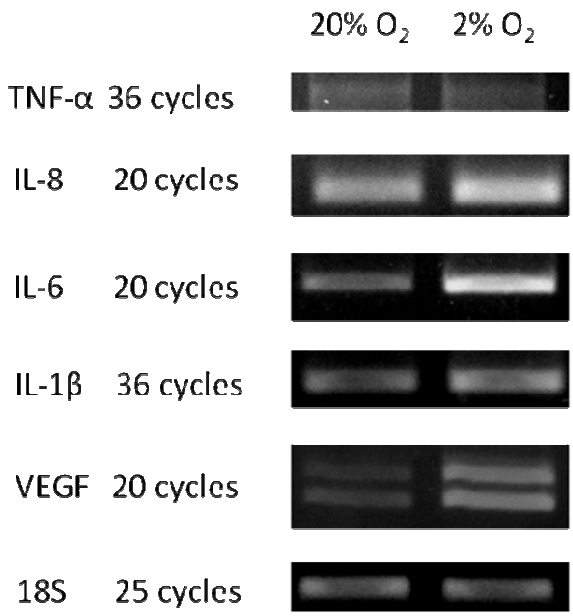


**Figure 7.** Micrograph showed the results from RT-PCR analysis. The expression of cytokines in hDDP cells underwent 12 hours under hypoxic condition was examined. Up-regulation of both VEGF and IL-6 and the slightly increased in the expression of IL-1β and IL-8 were observed. The expression level of 18S was used as an inner control.

**The Increase in the Level of IL-6 Protein after 12 Hours under Hypoxic Condition**

To confirm the effect of hypoxia on IL-6 expression, ELISA analysis was performed and the result of IL-6 secretion was shown in Fig. 8. Cell cultures were grown in 6-welled plates at the density of 25 x $10^5$ cells/ well at 37°C for 16 hours at normal level of oxygen (20%). Cells in the hypoxic group were then moved into a modular incubator chamber and incubated with 2% oxygen at 37°C for 6, 9, 12, and 15 hours. Alternative plates were cultured in the 20% oxygen and used as a controlled condition. The medium were then used to analyze the amount of IL-6 protein secreted. The amount of IL-6 in each experiment was normalized to cell number and the levels of IL-6 secretion in all controlled groups were marked as 1 fold. The numbers above the graph represent the mean of fold increase after hypoxic condition. The result showed the level of IL-6 secretion significantly increased after 12 hours in the hypoxic condition.

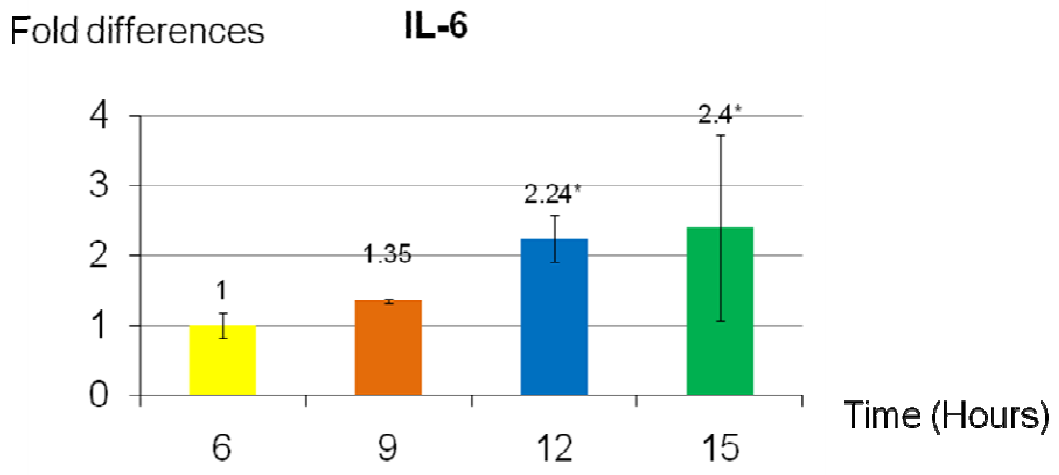


**Figure 8.** Graph showed the results from ELISA analysis of secreted IL-6 from hDDP cells under hypoxic condition from 6 to 15 hours. Data was shown as mean ± SD ($p<0.05$) from three separated experiments. * represents the significance at $p<0.05$.

**Hypoxia-Induced IL-6 can be Observed after 1 Hour under Hypoxic Condition**

To examine the minimum time required in hypoxia for IL-6 induction, hDDP cells were seeded and cultured in various time periods from 1-12 hours in hypoxia followed by normoxia until reached a total time of 12 hours in culture. For example, cells were exposed to hypoxia for 1 hour followed by normoxia for another 11 hours.

The result in Fig. 9 showed cells cultured in hypoxia for 1, 3 and 6 do not induced IL-6 secretion. Only 12 hours under the hypoxia did the significant increased on IL-6 secretion in hDDP cells was observed, consistent with the result from Fig. 8.

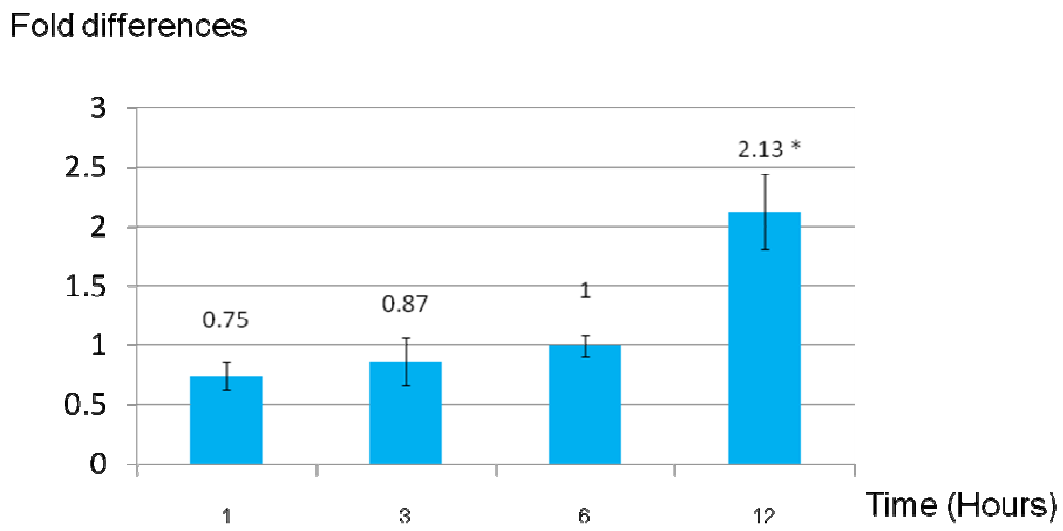


**Figure 9.** Graph showed the effect of hypoxia for 1, 3, 6, and 12 hours on hDDP cells. The significant increase in IL-6 secretion could be observed only at 12 hours under hypoxia. Data was shown as mean $\pm$ SD ($p<0.05$) from three separated experiments. * represents the significance at $p<0.05$.

However, after cells were moved from hypoxic condition into normoxic condition with new medium, the level of IL-6 secretion started to increase above control as showed in Fig. 10. From the result, the condition of 1 hour in hypoxia followed by 11 hours in normoxia showed the highest and significant level of IL-6 secretion (Lane 1 in Fig.10). In contrast, cells cultured in 3 and 6 hours in hypoxia followed by 9 and 6 hours in normoxia did not increase the IL-6 secretion compared to the control. (Lanes 2 and 3 in Fig. 10). Graph in fig.11 showed the summary of cells cultured in hypoxia for 1 hour

followed by 11 hours in normoxia compared to the control culture for 1 and 12 hours in hypoxia.

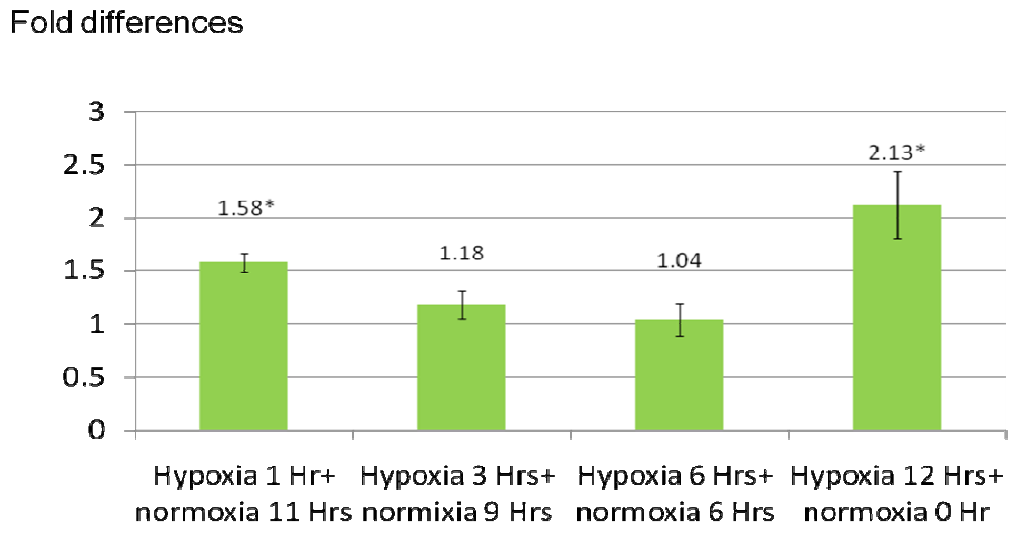


**Figure 10.** The graph showed the effect of re-oxygenation on the IL-6 secretion. Cells were subjected to hypoxia for 1, 3, and 6 hours and the medium were changed before returning them to the normoxic condition until the end of study period.

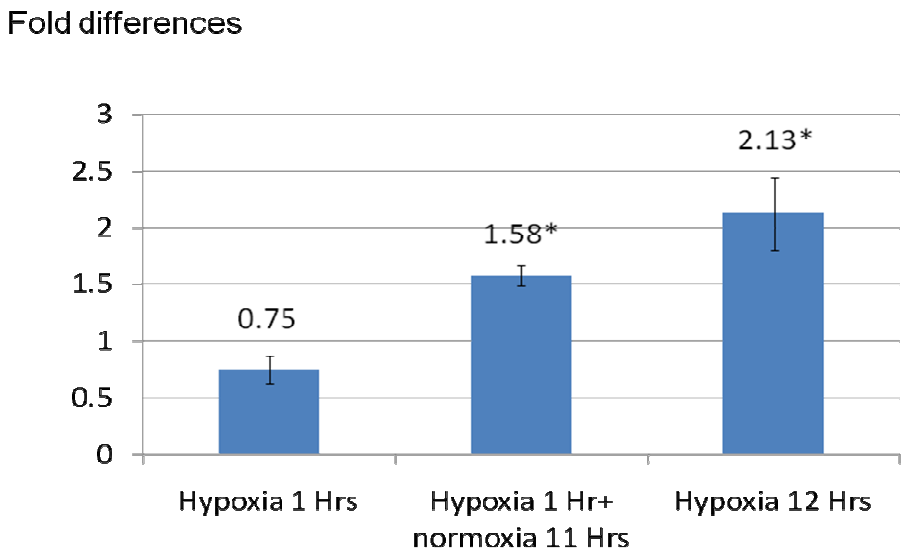


**Figure 11.** Graph showed the level of IL-6 secretion as determined by ELISA. The result indicated that one hour of hypoxia was sufficient to induced IL-6 secretion significantly at 12 hours.

* represents the significance at $p<0.05$.

**HIF-1 was Involved in Hypoxia-Induced IL-6 and Hypoxia Induces VEGF and IL-6 Expressions via HIF-1α**

It is well documented that of hypoxia-inducible transcription factors (HIF) is activated and stabilized in hypoxic condition. Activated HIF will be trans-located into the nucleus, bound to hypoxia-responsive elements (HRE) and regulated gene expression. Activation of HIF-1α activity could be mimicked by cobalt chloride ($CoCl_2$), an inorganic compound that can stabilize HIF-1α. Therefore, this compound can be used to confirm the role of HIF in gene regulation. The results in Fig. 12 indicated that $CoCl_2$ could up-regulate the expression of IL-6 in a dose-dependent manner. The expression of VEGF was used as a control since VEGF is one of the targets of HIF-1. The result supports that HIF-1 also regulates IL-6 expression in hypoxic condition. The levels of VEGF and IL-6 secretion in the controlled groups (0 mM $CoCl_2$) were marked as 1 fold and the levels of VEGF and IL-6 in the experiment groups (50, 100, 200 mM $CoCl_2$) were compared with the controlled groups.

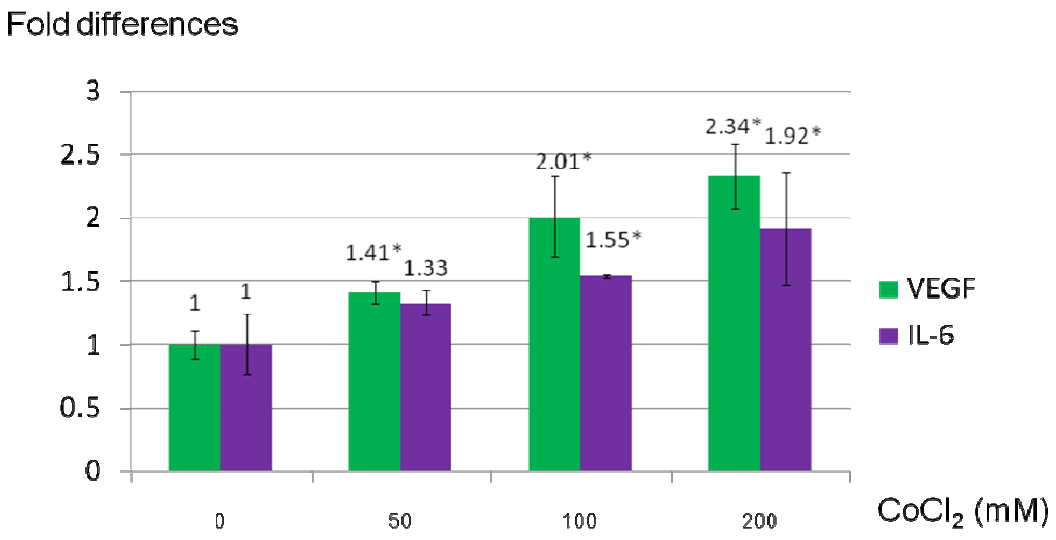


**Figure 12.** hDDP cells were treated with 50, 100 and 200 mM of $CoCl_2$ for 12 hours. ELISA analysis revealed that $CoCl_2$, a hypoxia mimetic agent, could increase both VEGF and IL-6 secretion. Data was shown as mean ± SD ($p<0.05$) from three separated experiments. * represents the significance at $p<0.05$.

**Hypoxia Up-Regulates Rex-1 Expression via IL-6 Secretion**

In order to understand more about the certain effect of hypoxia on hDDP cells, apart from the inflammatory cytokines genes, some other possibly related genes were also screened. Interestingly, the RT-PCR analysis showed the up-regulation of Rex-1, a marker of embryonic stem cells and believed to play role in maintaining the stemness of the cells, expression after cultured in hypoxic condition as showed in Fig. 13.

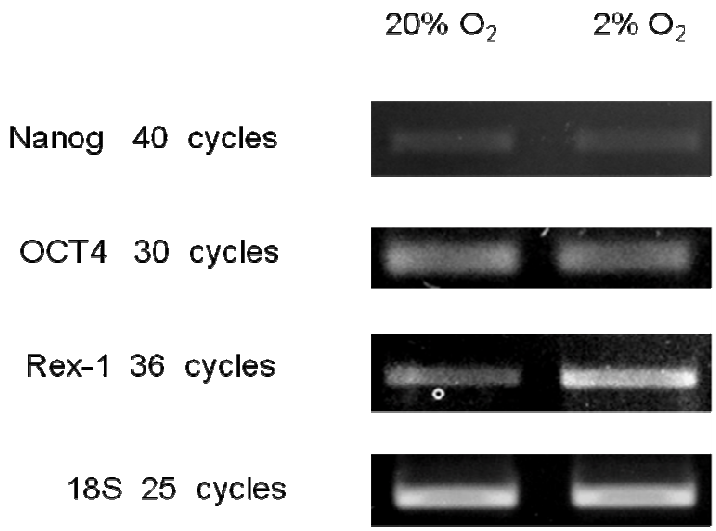


**Figure 13.** This figure showed the RT-PCR analysis of Rex-1, OCT4, and Nanog expressions from hDDP cells underwent hypoxia for 12 hours. 18S was used as an inner control. Hypoxia could up-regulate the expression of Rex-1 in hDDP cells.

The results from Fig. 13 revealed the influence of hypoxia on the expression of Rex-1 in hDDP cells. To further clarify the relationship between the up-regulation of IL-6 and Rex-1, hDDP cells were cultured in hypoxic condition in the presence or absence of IL-6 neutralizing antibody. Shed were cultured in 6- welled plates at the density of 25 x $10^5$ cells/ well at 37°C for 16 hours at normal level of oxygen. The cultures were treated with IL-6 neutralizing antibody (2μg/ml) for 1 hour prior to and subjected to hypoxic condition for another 12 hours. The expression of Rex-1 was determined by RT-PCR analysis.

The results in Fig. 14 confirmed the effect of hypoxia on Rex-1 expression. Interestingly, application of IL-6 neutralizing antibody could attenuate the induction of Rex-1 expression under hypoxic condition, suggesting the involvement of IL-6 in Rex-1 induction.

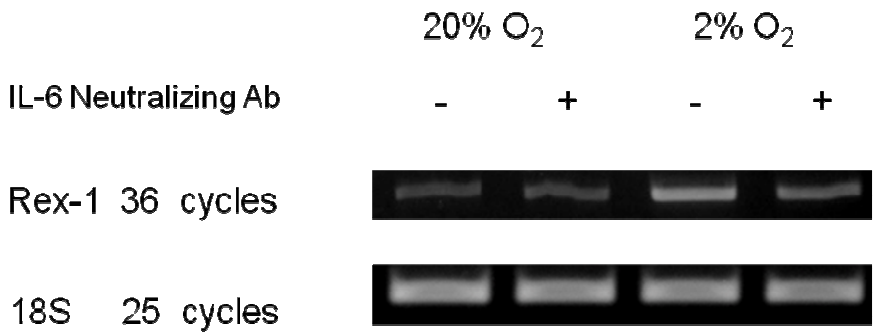


**Figure 14.** RT-PCR analysis showed the expression of Rex-1 in hDDP cells. Cells were subjected to hypoxic condition for 12 hours and RNA was extracted and processed for RT-PCR analysis. When compared the results from lane 1 and 3, expression of Rex-1 increased in hypoxic condition. Application of IL-6 neutralizing antibody suppressed the hypoxia-induced Rex-1 as shown in lane 3 and 4.

# CHAPTER V
# DISCUSSION, CONCLUSION, AND SUGGESTION

## Discussion

In this study, the results demonstrated that the level of oxygen could influence gene expression in human deciduous dental pulp cells (hDDP cells). Data from the study showed the influence of oxygen level on hDDP cells responses. When cells were cultured in the low oxygen level, some morphological changes such as size reduction and absence of cell extension could be observed. Furthermore, 12 hours of hypoxia could up-regulate the level of IL-6 expression and secretion via HIF-1$\alpha$ and IL-6 was found to have an effect on the up-regulation of Rex-1 expression under hypoxic condition.

Oxygen is an essential factor in the survival of all cells and tissues. The level of oxygen, high or low, can lead to a number of pathophysiological consequences. *(21)*. For example, if oxygen delivery to cells is insufficient for the demand (hypoxia), hydrogen will be shifted to pyruvic acid converting it to lactic acid. Lactic acid build up (in tissues and blood) is a sign of inadequate mitochondrial oxygenation, which if severe or prolonged, could lead to cell death *(58)*. Hypoxia in which there is complete deprivation of oxygen supply is referred to as anoxia.

In arterial blood, interstitial oxygen tension ($pO_2$) is about 95 mmHg (~12%), and about 40 mmHg (~5%) in venous and capillary blood. In normal tissue, it is roughly in the range 4- 8% *(24)* and being 23.2 mmHg (equivalent to 3% $O_2$) in rat incisor pulp *(59)*.

HIF, a master regulator of cellular response to hypoxia is the primary source of metabolic energy that widely expressed in all eukaryotic cells and tissues *(18)*. HIF-$\alpha$ can be detected in many cell culture systems at a cutoff of around 5% (40 mmHg). However, most experiments performed with cultured cells are undertaken at between 1-2% oxygen (8-16 mmHg), which can be considered hypoxic but not as in many tumor cells *(21)*.

Since HIF-1α is highly inducible and its activation is largely specific to hypoxic condition, it is ideally suited as an assay target for such screening strategy. The data from the cobalt chloride experiment in this study showed the increasing in IL-6, as well as VEGF proteins secretion in a dose-dependent manner. The results from the study indicated that the level of oxygen we used (2% $O_2$) performed a hypoxic condition that stabilized HIF-1α.

**Hypoxia and Cell Survival**

The result from MTT assay (Fig. 6) showed the decreasing of cells number after being cultured under hypoxic condition. A study showed that the combination of a lack of oxygen and a lack of nutrients causes energy deprivation and severe hypoxia can lead to apoptotic cell death *(60)*. Apoptosis, a process of programmed cell death can be induced in response to hypoxia. HIF- 1α is also involved in hypoxia- induced apoptosis. Hypoxia in combination with hypoglycemia reduces proliferation and increases apoptosis in wild- type embryonic stem cells (ES), but not in ES cells with inactivated HIF- 1α genes *(61)*. Furthermore, HIF-1 can initiate apoptosis by inducing high concentrations of pro-apoptotic proteins such as BCL- 2/ adenovirus E1B 19 kDa interacting protein 3 (BNIP3), and can cause stabilization of the related tumor-suppressor protein p53. However, during hypoxia, anti-apoptotic proteins, such as inhibitor of apoptosis protein 2 (IAP-2), can be induced, whereas the pro-apoptotic proteins, such as Bax can be down- regulated *(31)*. In this study, as 10% fetal bovine serum (FBS) was added to the media we used, the decreasing in cell number as seen from MTT assay may be the result from lack of oxygen alone. Apart from the level of oxygen, the survival of cells may depend on other factors as well. As our result on the effect of hypoxia on cell survival showed the decrease of cell number under hypoxic condition whereas another study on human dental pulp cells suggested that hypoxia is an effective treatment to amplify number of cells *(62)*. However, the dental pulp cells in that study were obtained from third molars of adult patients, and the study was observed in a longer period of 14 days. Furthermore, the medium was changed every 2-3 days implicating a short-term of re-oxygenation, which can improve cell viability and cell

proliferation *(52)*. Further study on the factors effecting cell survival under hypoxic condition may need to be done.

**Hypoxia and inflammation**

Inflammation is a part of complex biological responses of vascular tissues to harmful stimuli such as infection, pressure, hypoxia etc. and angiogenesis is an essential component of inflammation and its resolution. VEGF, a major regulator of both physiologic and pathologic angiogenesis *(63)* was found to participate in the revascularization of hypoxic tissues *(64-66)* including dental pulp cells *(53, 67)*. Moreover, the pathologic picture in hypoxic tissue injury shares features with the inflammatory response, including production of pro-inflammatory cytokines. However, a study in mesenchymal cells under hypoxic condition found the release of IL-1β and TNF-α from hypoxia/SD-stimulated mesenchymal cells was unexpectedly undetectable unless ATP or lipopolysaccharide was present, suggesting that IL-1β and TNF-α are not responsible for the paracrine effects of mesenchymal cells under ischemic conditions *(68)*.

In this study, the 12 hours under hypoxia (2%$O_2$) could increase the up-regulation of some cytokines as showed in the RT-PCR analysis (Fig. 8). Hypoxia could markedly increase IL-6 mRNA expression as well as VEGF, a positive control in the study. However, hypoxia did not affect much in the expression of IL-1β and TNF-α, the 2 major inflammatory cytokines known to be the potent stimulants of many genes involved in inflammation and bone destruction *(40)*, as only slight increases in IL-1β and IL-8 and no alternate of TNF-α expression could be observed.

After 12 hours under hypoxic condition, hDDP cells showed marked increases of IL-6 in both expression and protein secretion. IL-6 is a multifunctional cytokine that affects a variety of biological functions, including immunoglobulin production, acute phase reaction, inflammation, and plasmacytomegagenesis, by regulating cell growth, differentiation, and survival *(69-71)*.The data from RT-PCR analysis (Fig. 7) and the result

from ELISA analysis (Fig. 8) showed significant increase of IL-6 after 12 hours under hypoxic condition. These data indicated that hypoxia increased IL-6 mRNA expression and protein secretion in hDDP cells at 12 hours under such condition. Hypoxia- induced expression of IL-6 is not limited to hDDP cells.

The increases in transcription, translation and release of the IL-6 gene product after the exposure to hypoxia can also be observed in other cells such as endothelial cells *(72)*, and smooth muscles cells *(73)*. Besides, the role of IL-6 in non-inflammatory process has also been reported. Accumulation of IL-6 can lead to production of adenosine by alkaline phosphatase and subsequent protection from ischemic injury *(74)*. Furthermore, IL-6 also plays a pivotal role in a variety of CNS functions such as induction and modulation of reactive astrogliosis, pathological inflammatory responses and neuroprotection *(75)*.

**Hypoxia- Induced IL-6 can be observed after 1 Hour under Hypoxic Condition**

The hypoxia-induced IL-6 protein release which could be detected after 12 hours might be considered as a late response when compared with the release of VEGF protein at 6 hours under hypoxic condition. Interestingly, the data showed in Fig. 11 indicated that 1 hour of hypoxia was enough to increase IL-6. However, the up-regulation of the expression detected was at 12-houred time. The data from Fig. 9 and Fig. 10 showed the effect of re-oxygenation on the secretion of IL-6 in hDDP cells. The period of normoxia time seemed to have an effect on the level of IL-6 secretion. In Fig. 9, the level of IL-6 detected was higher in the group that underwent the longer period of time under hypoxic condition. However, after being brought back to normoxia, the level of IL-6 was higher in the group that cells underwent hypoxic condition for shorter of time but stayed longer under normoxia as showed in Fig. 10. From the results, it is possible that if given enough time in re-oxygenation after cells undergo the hypoxic condition, the level of IL-6 can be up-regulated in hDDP cells as 1 only hour of hypoxia together with 11 hours of normoxia could significantly increase the level of IL-6 secretion.

**Hypoxia Induces VEGF and IL-6 Expression via HIF-1α**

It has been very well documented that under hypoxic condition, VEGF is regulated via HIF *(6)*. However, the up-regulation of IL-6 in hypoxia was still unclear. In this study, cobalt, a transition metal that can stabilize HIF, in the form of cobalt chloride ($CoCl_2$) was used to perform hypoxia-mimicking condition. The expression of VEGF was used as a control since VEGF is one of the targets of HIF-1. The results in Fig. 12 indicated that culture of hDDP cells in the presence of $CoCl_2$ (0-200 mM) could up-regulate the expression of IL-6 in a dose-dependent manner, indicating that both VEGF and IL-6 are HIF-1α- dependent cytokines.

The fact that hypoxia induced the release of IL-6 but not the major inflammatory cytokines such as IL-1β, and TNF-α in dental pulp cells suggests the possibility that IL-6 release from hypoxia induction may participate in the non-inflammatory mechanism. And the delay in IL-6 secretion suggests the possibility that IL-6 may participate in the tissue repair after the damage caused by low oxygen level.

Recent studies showed that low oxygen concentration represent an important regulatory mechanism that allows the balance between self- renewal and differentiation of stem cells and influences proliferation, differentiation and maturation of committed progenitors *(76-78)*. In addition, low oxygen concentration ($1\%O_2$) and IL-6 synergize could enhance the maintenance of hematopoietic stem cells from mouse bone marrow and the effect was correlated to the induction of VEGF *(79)*.

**Hypoxia Up-Regulates Rex-1 Expression via IL-6 Secretion**

Stem cells have been characterized at the transcriptional level by the identification of stem cell specific marker genes. Rex-1 or zinc-finger protein-42 (Zfp42) , an embryonic stem cells marker that was believed to play a role in maintaining the stemness of the cells is highly expressed in both mouse *(80-81)* and human embryonic stem (ES) cells *(82-83)*. It is one of the important gene markers used to identify human stem cells *(84)*. It was first identified in mouse F9 teratocarcinoma cells and its expression was found to decrease during cell differentiation following the addition of

retinoic acid (RA) *(85)*. And Rex-1 mRNA levels decrease in response to the initiation of differentiation, independent of the presence of RA *(81)*. It is believed that the Rex-1 gene and REX1 protein play crucial roles in regulating ES cell differentiation into different lineages *(86-88)*. Furthermore, REX1 also regulates the proliferation/ differentiation of human mesenchymal stem cells (hMSCs) through the suppression of p38 MAPK signaling via the direct suppression of MKK3 *(89)*.

The function of IL-6 in hypoxia-induced Rex-1 expression was suggested from this study. 12 hours under hypoxia could up-regulate the Rex-1 expression in hDDP cells. Moreover, IL-6 neutralizing antibody could attenuate the induction of Rex-1 expression under hypoxic condition, suggesting the role of IL-6 in Rex-1 induction.

A study has addressed another facet of IL-6 activity on stimulating the proliferation and differentiation of hematopoietic cells *(90)*. And osteoblast-like cells have been demonstrated that hypoxia results in decreased cellular proliferation, decreased expression of proliferating cell nuclear antigen, and increased alkaline phosphatase (a marker of osteoblast differentiation) *(91)*. The results from this study showed the possibility that hypoxia-induced IL-6 could induce Rex-1 expression in hDDP cells and may played a role in cell tissues repair or cell proliferation/ differentiation in response to the low oxygen environment.

## Conclusion

Human deciduous dental pulp cells (hDDP cells) or stem cells from human exfoliated deciduous teeth (SHED) altered their behavior in hypoxic condition (2%O2). Morphological changes such as size reduction and absence of cell extension could be observed in hDDP cells cultured in hypoxic condition (2%$O_2$) for 15 hours. In addition, data from the MTT assay suggested that low level of oxygen might interfere with the cell number.

12 hours under hypoxia (2%$O_2$) could markedly increase the up-regulation of VEGF and IL-6 mRNA expression, slightly increase the expression of IL-1$\beta$, and IL-8 but did not alter TNF-$\alpha$ expression, as shown in RT-PCR analysis. The level of IL-6 secretion significantly increased after 12 hours under hypoxic condition. The time- coursed study indicated that the minimum time exposure to hypoxic condition required for IL-6

induction was 1 hour. Culture of hDDP cells in the presence of $CoCl_2$ (0-200 mM) up-regulated the expression of both VEGF and IL-6 mRNA in a dose-dependent manner, indicating that both VEGF and IL-6 are HIF-1α- dependent cytokines.

Interestingly, the expression of Rex-1 increased after cultured in hypoxic condition. To clarify the role of IL-6 in hypoxia- induced Rex-1 expression, cells were treated with IL-6 neutralizing antibody for 1 hour prior to the hypoxic treatment. The result clearly indicated that IL-6 neutralizing antibody could attenuate the induction of Rex-1 expression under hypoxic condition, suggesting the role of IL-6 in Rex-1 induction.

## Suggestion

In summary, this study has demonstrated the effects of hypoxia on cell morphology and number in hDDP cells. Hypoxia can also up-regulate the expression and the secretion of VEGF and IL-6 via HIF-1α. IL-6 does play an important role in Rex-1 expression. Moreover, the late response of hypoxia-induced IL-6 and the expression of Rex-1 may participate in the tissue repair after the damage caused by low oxygen level. For a better understanding and the development of new therapeutic strategies for cells and tissues under critical stress, further investigations should be emphasized on the roles of the specific cytokines and the mechanisms involved in the response of dental pulp cells to hypoxia. Moreover, the effects of hypoxia on other dental pulp cells such as those from the permanent and the effects of different levels of oxygen for hypoxic condition should also be further investigated.

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# Appendix

## Cell Number

**Descriptive Statistics**

| group1 | | N | Mean | Std. Deviation | Minimum | Maximum |
|---|---|---|---|---|---|---|
| CCe6 | Cell | 3 | 29.1600 | 1.63000 | 27.53 | 30.79 |
| HCe6 | Cell | 3 | 28.2400 | 2.15000 | 26.09 | 30.39 |
| CCe9 | Cell | 3 | 34.1800 | 4.15000 | 30.03 | 38.33 |
| HCe9 | Cell | 3 | 27.0800 | .37000 | 26.71 | 27.45 |
| CCe12 | Cell | 3 | 35.1200 | 2.66000 | 32.46 | 37.78 |
| HCe12 | Cell | 3 | 28.2367 | 4.85760 | 23.29 | 33.00 |
| CCe15 | Cell | 3 | 40.6267 | 1.79339 | 38.69 | 42.23 |
| HCe15 | Cell | 3 | 27.0867 | 7.31864 | 18.76 | 32.50 |

**One-Sample Kolmogorov-Smirnov Test**

| group1 | | | Cell |
|---|---|---|---|
| CCe6 | N | | 3 |
| | Normal Parameters(a,b) | Mean | 29.1600 |
| | | Std. Deviation | 1.63000 |
| | Most Extreme Differences | Absolute | .175 |
| | | Positive | .175 |
| | | Negative | -.175 |
| | Kolmogorov-Smirnov Z | | .303 |
| | Asymp. Sig. (2-tailed) | | 1.000 |
| HCe6 | N | | 3 |
| | Normal Parameters(a,b) | Mean | 28.2400 |
| | | Std. Deviation | 2.15000 |
| | Most Extreme Differences | Absolute | .175 |
| | | Positive | .175 |
| | | Negative | -.175 |
| | Kolmogorov-Smirnov Z | | .303 |
| | Asymp. Sig. (2-tailed) | | 1.000 |
| CCe9 | N | | 3 |
| | Normal Parameters(a,b) | Mean | 30.2300 |
| | | Std. Deviation | 2.00749 |
| | Most Extreme Differences | Absolute | .206 |
| | | Positive | .206 |
| | | Negative | -.186 |
| | Kolmogorov-Smirnov Z | | .357 |
| | Asymp. Sig. (2-tailed) | | 1.000 |

| | | | |
|---|---|---|---|
| HCe9 | N | | 3 |
| | Normal Parameters(a,b) | Mean | 26.4133 |
| | | Std. Deviation | 1.48669 |
| | Most Extreme Differences | Absolute | .340 |
| | | Positive | .243 |
| | | Negative | -.340 |
| | Kolmogorov-Smirnov Z | | .588 |
| | Asymp. Sig. (2-tailed) | | .879 |
| CCe12 | N | | 3 |
| | Normal Parameters(a,b) | Mean | 35.1200 |
| | | Std. Deviation | 2.66000 |
| | Most Extreme Differences | Absolute | .175 |
| | | Positive | .175 |
| | | Negative | -.175 |
| | Kolmogorov-Smirnov Z | | .303 |
| | Asymp. Sig. (2-tailed) | | 1.000 |
| HCe12 | N | | 3 |
| | Normal Parameters(a,b) | Mean | 28.2367 |
| | | Std. Deviation | 4.85760 |
| | Most Extreme Differences | Absolute | .182 |
| | | Positive | .179 |
| | | Negative | -.182 |
| | Kolmogorov-Smirnov Z | | .315 |
| | Asymp. Sig. (2-tailed) | | 1.000 |
| CCe15 | N | | 3 |
| | Normal Parameters(a,b) | Mean | 37.2933 |
| | | Std. Deviation | 4.52948 |
| | Most Extreme Differences | Absolute | .288 |
| | | Positive | .209 |
| | | Negative | -.288 |
| | Kolmogorov-Smirnov Z | | .498 |
| | Asymp. Sig. (2-tailed) | | .965 |
| HCe15 | N | | 3 |
| | Normal Parameters(a,b) | Mean | 31.9367 |
| | | Std. Deviation | 4.66680 |
| | Most Extreme Differences | Absolute | .375 |
| | | Positive | .273 |
| | | Negative | -.375 |
| | Kolmogorov-Smirnov Z | | .650 |
| | Asymp. Sig. (2-tailed) | | .792 |

a Test distribution is Normal.
b Calculated from data.

# T-Test

**Group Statistics**

| | group1 | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Cell | CCe6 | 3 | 29.1600 | 1.63000 | .94108 |
| | HCe6 | 3 | 28.2400 | 2.15000 | 1.24130 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| Cell | Equal variances assumed | .149 | .720 | .591 | 4 | .587 | .92000 | 1.55771 | -3.40490 | 5.24490 |
| | Equal variances not assumed | | | .591 | 3.728 | .589 | .92000 | 1.55771 | -3.53215 | 5.37215 |

# T-Test

**Group Statistics**

| | group1 | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Cell | CCe9 | 3 | 30.2300 | 2.00749 | 1.15902 |
| | HCe9 | 3 | 26.4133 | 1.48669 | .85834 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| Cell | Equal variance assumed | .154 | .715 | 2.646 | 4 | .057 | 3.81667 | 1.44225 | -.18765 | 7.82099 |
| | Equal variance not assumed | | | 2.646 | 3.686 | .062 | 3.81667 | 1.44225 | -.32560 | 7.95893 |

# T-Test

**Group Statistics**

| | group1 | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Cell | CCe12 | 3 | 35.1200 | 2.66000 | 1.53575 |
| | HCe12 | 3 | 28.2367 | 4.85760 | 2.80453 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| Cell | Equal variance assumed | .723 | .443 | 2.153 | 4 | .098 | 6.88333 | 3.19749 | -1.99432 | 15.76099 |
| | Equal variance not assumed | | | 2.153 | 3.100 | .117 | 6.88333 | 3.19749 | -3.10844 | 16.87511 |

# T-Test

**Group Statistics**

| | group1 | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Cell | CCe15 | 3 | 37.2933 | 4.52948 | 2.61510 |
| | HCe15 | 3 | 31.9367 | 4.66680 | 2.69438 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| Cell | Equal variance assumed | .024 | .885 | 1.427 | 4 | .227 | 5.35667 | 3.75479 | -5.06829 | 15.78163 |
| | Equal variance not assumed | | | 1.427 | 3.996 | .227 | 5.35667 | 3.75479 | -5.07196 | 15.78529 |

The Increase in the Level of IL-6 Protein after 12 Hours under Hypoxic Condition

**Descriptive Statistics**

| group2 | | N | Mean | Std. Deviation | Minimum | Maximum |
|---|---|---|---|---|---|---|
| C6 | IL6a | 3 | 1.6527 | .98712 | .86 | 2.76 |
| H6 | IL6a | 3 | 1.5600 | .51069 | 1.00 | 2.00 |
| C9 | IL6a | 3 | .7500 | .25000 | .50 | 1.00 |
| H9 | IL6a | 3 | 1.0667 | .20817 | .90 | 1.30 |
| C12 | IL6a | 3 | 2.1133 | .52166 | 1.52 | 2.50 |
| H12 | IL6a | 3 | 4.9933 | .71598 | 4.23 | 5.65 |
| C15 | IL6a | 3 | 2.3333 | .61101 | 1.80 | 3.00 |
| H15 | IL6a | 3 | 5.4333 | .55076 | 4.90 | 6.00 |

**One-Sample Kolmogorov-Smirnov Test**

| group2 | | | IL6a |
|---|---|---|---|
| C6 | N | | 3 |
| | Normal Parameters(a,b) | Mean | 1.6527 |
| | | Std. Deviation | .98712 |
| | Most Extreme Differences | Absolute | .294 |
| | | Positive | .294 |
| | | Negative | -.212 |
| | Kolmogorov-Smirnov Z | | .508 |
| | Asymp. Sig. (2-tailed) | | .958 |
| H6 | N | | 3 |
| | Normal Parameters(a,b) | Mean | 1.5600 |
| | | Std. Deviation | .51069 |
| | Most Extreme Differences | Absolute | .260 |
| | | Positive | .197 |
| | | Negative | -.260 |
| | Kolmogorov-Smirnov Z | | .450 |
| | Asymp. Sig. (2-tailed) | | .988 |
| C9 | N | | 3 |
| | Normal Parameters(a,b) | Mean | .7500 |
| | | Std. Deviation | .25000 |
| | Most Extreme Differences | Absolute | .175 |
| | | Positive | .175 |
| | | Negative | -.175 |
| | Kolmogorov-Smirnov Z | | .303 |
| | Asymp. Sig. (2-tailed) | | 1.000 |
| H9 | N | | 3 |
| | Normal Parameters(a,b) | Mean | 1.0667 |

| | | | |
|---|---|---|---|
| | | Std. Deviation | .20817 |
| | Most Extreme Differences | Absolute | .292 |
| | | Positive | .292 |
| | | Negative | -.212 |
| | Kolmogorov-Smirnov Z | | .506 |
| | Asymp. Sig. (2-tailed) | | .960 |
| C12 | N | | 3 |
| | Normal Parameters(a,b) | Mean | 2.1133 |
| | | Std. Deviation | .52166 |
| | Most Extreme Differences | Absolute | .321 |
| | | Positive | .229 |
| | | Negative | -.321 |
| | Kolmogorov-Smirnov Z | | .555 |
| | Asymp. Sig. (2-tailed) | | .917 |
| H12 | N | | 3 |
| | Normal Parameters(a,b) | Mean | 4.9933 |
| | | Std. Deviation | .71598 |
| | Most Extreme Differences | Absolute | .226 |
| | | Positive | .190 |
| | | Negative | -.226 |
| | Kolmogorov-Smirnov Z | | .391 |
| | Asymp. Sig. (2-tailed) | | .998 |
| C15 | N | | 3 |
| | Normal Parameters(a,b) | Mean | 2.3333 |
| | | Std. Deviation | .61101 |
| | Most Extreme Differences | Absolute | .253 |
| | | Positive | .253 |
| | | Negative | -.196 |
| | Kolmogorov-Smirnov Z | | .438 |
| | Asymp. Sig. (2-tailed) | | .991 |
| H15 | N | | 3 |
| | Normal Parameters(a,b) | Mean | 5.4333 |
| | | Std. Deviation | .55076 |
| | Most Extreme Differences | Absolute | .191 |
| | | Positive | .191 |
| | | Negative | -.182 |
| | Kolmogorov-Smirnov Z | | .330 |
| | Asymp. Sig. (2-tailed) | | 1.000 |

a Test distribution is Normal.
b Calculated from data.

# T-Test

**Group Statistics**

| | group2 | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| IL6a | C6 | 3 | 1.6527 | .98712 | .56991 |
| | H6 | 3 | 1.5600 | .51069 | .29484 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| IL6a | Equal variances assumed | 1.913 | .239 | .145 | 4 | .892 | .09273 | .64166 | -1.68881 | 1.87428 |
| | Equal variances not assumed | | | .145 | 2.999 | .894 | .09273 | .64166 | -1.94970 | 2.13516 |

# T-Test

**Group Statistics**

| | group2 | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| IL6a | C9 | 3 | .7500 | .25000 | .14434 |
| | H9 | 3 | 1.0667 | .20817 | .12019 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| IL6a | Equal variances assumed | .013 | .914 | -1.686 | 4 | .167 | -.31667 | .18782 | -.83815 | .20482 |
| | Equal variances not assumed | | | -1.686 | 3.873 | .169 | -.31667 | .18782 | -.84496 | .21163 |

# T-Test

**Group Statistics**

| | group2 | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| IL6a | C12 | 3 | 2.1133 | .52166 | .30118 |
| | H12 | 3 | 4.9933 | .71598 | .41337 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| IL6a | Equal variances assumed | .238 | .651 | -5.631 | 4 | .005 | -2.88000 | .51146 | -4.30003 | -1.45997 |
| | Equal variances not assumed | | | -5.631 | 3.657 | .006 | -2.88000 | .51146 | -4.35421 | -1.40579 |

# T-Test

**Group Statistics**

| | group2 | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| IL6a | C15 | 3 | 2.3333 | .61101 | .35277 |
| | H15 | 3 | 5.4333 | .55076 | .31798 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| IL6a | Equal variances assumed | .080 | .791 | -6.527 | 4 | .003 | -3.10000 | .47493 | -4.41861 | -1.78139 |
| | Equal variances not assumed | | | -6.527 | 3.958 | .003 | -3.10000 | .47493 | -4.42419 | -1.77581 |

Hypoxia-Induced IL-6 can be observed after 1 Hour under Hypoxic Condition

**Descriptive Statistics**

| group | | N | Mean | Std. Deviation | Minimum | Maximum |
|---|---|---|---|---|---|---|
| C12 | IL6 | 3 | 160.0333 | 1.32885 | 158.65 | 161.30 |
| H12 | IL6 | 3 | 340.5500 | 1.76918 | 338.65 | 342.15 |
| H1N11 | IL6 | 3 | 253.3467 | 3.75032 | 249.32 | 256.74 |

**One-Sample Kolmogorov-Smirnov Test**

| group | | | IL6 |
|---|---|---|---|
| C12 | N | | 3 |
| | Normal Parameters [a,b] | Mean | 160.0333 |
| | | Std. Deviation | 1.32885 |
| | Most Extreme Differences | Absolute | .202 |
| | | Positive | .184 |
| | | Negative | -.202 |
| | Kolmogorov-Smirnov Z | | .349 |
| | Asymp. Sig. (2-tailed) | | 1.000 |
| H12 | N | | 3 |
| | Normal Parameters [a,b] | Mean | 340.5500 |
| | | Std. Deviation | 1.76918 |
| | Most Extreme Differences | Absolute | .234 |
| | | Positive | .192 |
| | | Negative | -.234 |
| | Kolmogorov-Smirnov Z | | .405 |
| | Asymp. Sig. (2-tailed) | | .997 |
| H1N11 | N | | 3 |
| | Normal Parameters [a,b] | Mean | 253.3467 |
| | | Std. Deviation | 3.75032 |
| | Most Extreme Differences | Absolute | .234 |
| | | Positive | .192 |
| | | Negative | -.234 |
| | Kolmogorov-Smirnov Z | | .405 |
| | Asymp. Sig. (2-tailed) | | .997 |

a. Test distribution is Normal.

b. Calculated from data.

# T-Test

**Group Statistics**

| | group | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| IL6 | C12 | 3 | 160.0333 | 1.32885 | .76721 |
| | H12 | 3 | 340.5500 | 1.76918 | 1.02144 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| IL6 | Equal variances assumed | .293 | .617 | -141.307 | 4 | .000 | 180.51667 | 1.27748 | -184.064 | -176.970 |
| | Equal variances not assumed | | | -141.307 | 3.712 | .000 | 180.51667 | 1.27748 | -184.175 | -176.859 |

# T-Test

**Group Statistics**

| | group | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| IL6 | C12 | 3 | 160.0333 | 1.32885 | .76721 |
| | H1N11 | 3 | 253.3467 | 3.75032 | 2.16525 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| IL6 | Equal variances assumed | 2.487 | .190 | -40.621 | 4 | .000 | -93.31333 | 2.29716 | -99.69126 | -86.93541 |
| | Equal variances not assumed | | | -40.621 | 2.494 | .000 | -93.31333 | 2.29716 | -101.538 | -85.08860 |

HIF-1 was Involved in Hypoxia-Induced IL-6 and Hypoxia Induces VEGF and IL-6 Expressions via HIF-1$\alpha$

**Descriptive Statistics**

| group3 | | N | Mean | Std. Deviation | Minimum | Maximum |
|---|---|---|---|---|---|---|
| V0 | CoCl2 | 3 | .4367 | .03512 | .40 | .47 |
| V50 | CoCl2 | 3 | .5093 | .03495 | .48 | .55 |
| V100 | CoCl2 | 3 | .7683 | .02021 | .75 | .79 |
| V200 | CoCl2 | 3 | .8807 | .00115 | .88 | .88 |
| IL0 | CoCl2 | 3 | .5100 | .01000 | .50 | .52 |
| IL50 | CoCl2 | 3 | .5667 | .04163 | .52 | .60 |
| IL100 | CoCl2 | 3 | .7567 | .08021 | .68 | .84 |
| IL200 | CoCl2 | 3 | .9413 | .01026 | .93 | .95 |

**One-Sample Kolmogorov-Smirnov Test**

| group3 | | | CoCl2 |
|---|---|---|---|
| V0 | N | | 3 |
| | Normal Parameters(a,b) | Mean | .4367 |
| | | Std. Deviation | .03512 |
| | Most Extreme Differences | Absolute | .204 |
| | | Positive | .185 |
| | | Negative | -.204 |
| | Kolmogorov-Smirnov Z | | .354 |
| | Asymp. Sig. (2-tailed) | | 1.000 |
| V50 | N | | 3 |
| | Normal Parameters(a,b) | Mean | .5093 |
| | | Std. Deviation | .03495 |
| | Most Extreme Differences | Absolute | .272 |
| | | Positive | .272 |
| | | Negative | -.201 |
| | Kolmogorov-Smirnov Z | | .471 |
| | Asymp. Sig. (2-tailed) | | .980 |
| V100 | N | | 3 |
| | Normal Parameters(a,b) | Mean | .7683 |
| | | Std. Deviation | .02021 |
| | Most Extreme Differences | Absolute | .232 |
| | | Positive | .232 |
| | | Negative | -.192 |
| | Kolmogorov-Smirnov Z | | .402 |

| | | | |
|---|---|---|---|
| | Asymp. Sig. (2-tailed) | | .997 |
| V200 | N | | 3 |
| | Normal Parameters(a,b) | Mean | .8807 |
| | | Std. Deviation | .00115 |
| | Most Extreme Differences | Absolute | .385 |
| | | Positive | .385 |
| | | Negative | -.282 |
| | Kolmogorov-Smirnov Z | | .667 |
| | Asymp. Sig. (2-tailed) | | .766 |
| IL0 | N | | 3 |
| | Normal Parameters(a,b) | Mean | .5100 |
| | | Std. Deviation | .01000 |
| | Most Extreme Differences | Absolute | .175 |
| | | Positive | .175 |
| | | Negative | -.175 |
| | Kolmogorov-Smirnov Z | | .303 |
| | Asymp. Sig. (2-tailed) | | 1.000 |
| IL50 | N | | 3 |
| | Normal Parameters(a,b) | Mean | .5667 |
| | | Std. Deviation | .04163 |
| | Most Extreme Differences | Absolute | .292 |
| | | Positive | .212 |
| | | Negative | -.292 |
| | Kolmogorov-Smirnov Z | | .506 |
| | Asymp. Sig. (2-tailed) | | .960 |
| IL100 | N | | 3 |
| | Normal Parameters(a,b) | Mean | .7567 |
| | | Std. Deviation | .08021 |
| | Most Extreme Differences | Absolute | .200 |
| | | Positive | .200 |
| | | Negative | -.184 |
| | Kolmogorov-Smirnov Z | | .346 |
| | Asymp. Sig. (2-tailed) | | 1.000 |
| IL200 | N | | 3 |
| | Normal Parameters(a,b) | Mean | .9413 |
| | | Std. Deviation | .01026 |
| | Most Extreme Differences | Absolute | .269 |
| | | Positive | .199 |
| | | Negative | -.269 |
| | Kolmogorov-Smirnov Z | | .466 |
| | Asymp. Sig. (2-tailed) | | .982 |

a Test distribution is Normal.
b Calculated from data.

# T-Test

**Group Statistics**

| | group3 | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| CoCl2 | V0 | 3 | .4367 | .03512 | .02028 |
| | V50 | 3 | .5093 | .03495 | .02018 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| CoCl2 | Equal variances assumed | .009 | .927 | -2.540 | 4 | .064 | -.07267 | .02860 | -.15209 | .00675 |
| | Equal variances not assumed | | | -2.540 | 4.000 | .064 | -.07267 | .02860 | -.15209 | .00675 |

# T-Test

**Group Statistics**

| | group3 | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| CoCl2 | V0 | 3 | .4367 | .03512 | .02028 |
| | V100 | 3 | .7683 | .02021 | .01167 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| CoCl2 | Equal variances assumed | .694 | .452 | -14.178 | 4 | .000 | -.33167 | .02339 | -.39662 | -.26672 |
| | Equal variances not assumed | | | -14.178 | 3.193 | .001 | -.33167 | .02339 | -.40363 | -.25971 |

# T-Test

**Group Statistics**

| | group3 | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| CoCl2 | V0 | 3 | .4367 | .03512 | .02028 |
| | V200 | 3 | .8807 | .00115 | .00067 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| CoCl2 | Equal variances assumed | 4.937 | .090 | -21.886 | 4 | .000 | -.44400 | .02029 | -.50033 | -.38767 |
| | Equal variances not assumed | | | -21.886 | 2.004 | .002 | -.44400 | .02029 | -.53111 | -.35689 |

# T-Test

**Group Statistics**

| | group3 | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| CoCl2 | IL0 | 3 | .5100 | .01000 | .00577 |
| | IL50 | 3 | .5667 | .04163 | .02404 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| CoCl2 | Equal variances assumed | 5.694 | .075 | -2.292 | 4 | .084 | -.05667 | .02472 | -.12530 | .01197 |
| | Equal variances not assumed | | | -2.292 | 2.230 | .136 | -.05667 | .02472 | -.15318 | .03985 |

# T-Test

**Group Statistics**

| | group3 | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| CoCl2 | IL0 | 3 | .5100 | .01000 | .00577 |
| | IL100 | 3 | .7567 | .08021 | .04631 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| CoCl2 | Equal variances assumed | 3.903 | .119 | -5.286 | 4 | .006 | -.24667 | .04667 | -.37623 | -.11710 |
| | Equal variances not assumed | | | -5.286 | 2.062 | .032 | -.24667 | .04667 | -.44177 | -.05157 |

# T-Test

**Group Statistics**

| | group3 | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| CoCl2 | IL0 | 3 | .5100 | .01000 | .00577 |
| | IL200 | 3 | .9413 | .01026 | .00593 |

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| CoCl2 | Equal variances assumed | .045 | .843 | -52.137 | 4 | .000 | -.43133 | .00827 | -.45430 | -.40836 |
| | Equal variances not assumed | | | -52.137 | 3.997 | .000 | -.43133 | .00827 | -.45431 | -.40836 |

# BIOGRAPHY

Miss Jarintorn Kotheeranurak was born on 13th February 1982 in Bangkok. She graduated with D.D.S. (Doctor of Dental Surgery) from the Faculty of Dentistry, Chulalongkorn University in 2006, and had worked at Takhli hospital in Nakornsawan province for 2 years. She started her Master degree program in Pediatric Dentistry at Graduate School, Chulalongkorn University in 2009.