치의학석사 학위논문

과산화수소로 인한 인간치수줄기세포의 산화독성에 대한 인돌 3-아세트산의 영향

Indole-3-acetic acid prevents human dental pulp stem cells from hydrogen peroxide-induced oxidative toxicity

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Abstract

Indole-3-acetic acid prevents human dental pulp stem cells from hydrogen peroxide-induced oxidative toxicity

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The purpose of present study was to investigate the effect of Indole-3-acetic acid (IAA) on human dental pulp stem cells (hDPSCs) during hydrogen peroxide (H_2O_2)-induced oxidative toxicity. To validate this situation, we first assessed the effects of H_2O_2 on viability in hDPSCs at different concentrations ranging from 1-400 μM. The measurement of viable cells was determined after 24h treatment. To demonstrate the effect of IAA, the experimentation was also conducted same as the H_2O_2 experiment. Next, we tested the effects of IAA on H_2O_2-induced cytotoxicity in hDPSCs. To assess the effect of IAA as antioxidant, we tested IAA at various concentrations ranging from 1-400 μM after treatment of H_2O_2 at 180 μM and the cell cycle was also estimated.

As our results showed, H_2O_2 at 40 μM did not affect cell viability, whereas the

inhibitory effect started at 50 μM H_2O_2 and maximal performance was at 180 μM for 24 h. On the other hands, the treatment of IAA did not influence on the viability of hDPSCs. TheH_2O_2-induced cytotoxicity was strikingly inhibited in the presence of IAA and these effects were maximal at 150 μM. By the FACS analysis, the cell cycle in control group was seriously destroyed by H_2O_2 treatment through whole phase. Moreover, the apoptotic cells in control group were remarkably increased comparing with that in non-treat group. Interestingly, the damaged cell cycle by H_2O_2-induced cytotoxicity was recovered with the treatment IAA at 150 μM and the apoptotic cells were also decreased. In conclusion, the IAA was able to protect hDPSCs from H_2O_2-induced cytotoxicity via cell cycle restoration.

Key words: Indole 3-acetic acid (IAA), hydrogen peroxide (H_2O_2), Antioxidant, Supernumerary tooth, Dental pulp stem cell

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Contents

Ⅰ.Introduction

Adult stem cells (ASCs) are able to proliferate constantly and differentiate into multiple lineages. In contrast to pluripotent stem cells including embryonic stem cells and induced pluripotent stem cells, it has been reported that ASCs fail to develop teratoma formations in immunodeficient murine testes.[1)~3)] So, it is considered that ASCs may be safe stem cell source for incurable diseases.

Dental pulp stem cells (DPSCs) have recently been isolated from permanent teeth, and have similar characteristics to those of bone marrow–derived MSCs (BMSCs).[4)~7)] DPSCs are therefore regarded as an alternative source of MSCs. It has potential to differentiate into both mesenchymal lineage including odontoblasts, chondrocytes, myocytes, adipocytes, osteoblasts[4)~6),7)] and non-mesenchymal ectodermal lineage including neurons.[8)~12)] Moreover, DPSCs can be easily cultured in vitro and the cells exhibit a high proliferation rate compared to BMSCs which constitute the major supply of adult stem cells. Also, nonfunctional or useless supernumerary teeth, which are supposed to be extracted, can be sources for DPSCs, so that they are obtained less invasively.

Hydrogen peroxide (H_2O_2) has been well known as powerful inducer of oxidative stress which causes endothelial cell dysfunction, cellular injury, and vascular diseases.[13)~15)] It has been also documented that H_2O_2 is able to bring to cell-senescence and apoptosis.[16),17)] In dental field, H_2O_2 is generally used for tooth whitening both in professionally- and in self administered products (up to 35%) as original or in the form of carbamide peroxide.[18)~21)]. As the result of increasing demands for improving esthetics,

H_2O_2 tooth bleaching has been popularize. However, adverse effects have become evident; cervical root resorption, tooth sensitivity, ulceration of soft tissue and potential tumor promoter. Moreover, it has been also demonstrated that H_2O_2 is able to penetrate into pulp chambers via enamel and dentin.[22)~25)] Hence, dental pulp stem cells may be damaged such as senescence and apoptosis by H_2O_2-induced oxidative stress.

Auxins were classified as a plant hormone and the function of them has many different effects including growth, development and wound response.[26)~27)] Recently, it has been demonstrated that auxins are able to regulate senescence as antiaging.[28)~29)] in plant. Indole-3-acetic acid (IAA) is one of the most important member of the auxins and found to be synthesized naturally by plants.[30)] It has been confirmed that IAA is present not only in plants but also in animals, including mammals.[31)~32)] However, there have been few studies on the function of IAA in mammalian cells. Moreover, to date, no studies have delineated the protective effects of IAA in dental pulp stem cells.

In present study, we investigated the effects of IAA on hDPSCs during H_2O_2-induced oxidative toxicity, and determined its functions, antiaging and antioxidant by measurement of morphology, proliferation rate, survival rate, cell cycle and gene expression pattern.

Ⅱ. Materials and methods

Chemicals

All inorganic and organic compounds were obtained from Sigma-Aldrich Korea (Yong-in, Korea) unless indicated in the text

Isolation of human dental pulp cells (hDPSCs) and cell culture

To isolate human dental pulp tissue, mesiodens (maxillary central supernumerary teeth) (n=8) were extracted from children at the Department of Pediatric Dentistry in Dental Hospital of Seoul National University according to the guidelines provided by ethics committee (IRB No. S-D20100005). The extracted teeth were cut around the cemento-enamel junction using cutting disk to expose the pulp tissue as previously described [13]. The pulp tissue was gently separated from the crown and root using sterile endodontic file. The isolated tissue was digested in 1% (w/v) collagenase type I to generate single-cell suspensions. The cells were cultured in to 24-well plastic dishes and incubated at 37℃ in an humidified atmosphere containing 5% CO_2 in DPSC culture medium containing alpha-MEM-Eagle, 10% (v/v) fetal bovine serum (FBS; Life technologies, NY, USA) and 100IU/ml penicillin-100μg/ml streptomycin (Life technologies). The culture medium was refreshed once every 3 days to allow further growth. The adherent cells grown to more than 70% confluence were named as passage zero (P0) cells. When the cells were grown to 70% confluence, they were subcultured at 1/5 dilution for later passaging. The growth medium was replaced every 2 days until growth to proper confluence.

Cell Viability Assay

The effects of H_2O_2 or IAA on cellular viability were evaluated by MTT assay (Amresco, USA). Exponentially growing DPSCs were adjusted to 1.0 × 104 cells/cm^2 with culture medium, plated in 96-well plates (Corning, USA) at 200 μL/well and then incubated for 12 h according to routine procedure. After being treated with H_2O_2 or IAA (1-400 μM) and incubated for 24 h, 20 μL/well MTT (5 ug/ul) was added to each well. The medium was then removed after 4 h incubation and 100 μL/well sodium dodecyl sulfate (SDS) was added to dis-solve the reduced formazan product. Finally, the plate was read in an enzyme-linked immunosorbent micro- plate reader (Floustar Optima, USA) at 490 nm.

Flow Cytometry Analysis

The effect of H_2O_2 or IAA on cell cycle was evaluated by measuring the distribution of the cells in the different phases of the cell cycle by flow cytometry. This determination was based on the measurement of the DNA content of nuclei labeled with propidium iodide according to the method of Vindelov and Christensen (17), with slight modifications. Prior to cell cycle analysis, DPSCs were cultured with H_2O_2 or IAA at different concentrations ranging from 1-400 μM for 24h. The cultured cells were detached as a single cell by trypsin. Then, the cells were washed twice with PBS and fixed in cold 70% ethanol on ice. After 30 min, the cells were washed twice with PBS and resuspended in 220 ml PBS (1 x 10^6) with 50 μl RNase A solution (100ug/ml) for an additional 10 min. Finally, the cells were incubated with 180 μl of PI solution (50ug/ml) for 10 min. Analyses were performed by flow cytometry (FAC-SAria1; BD Biosciences, Erembodegem, Belgium) using the CellQuest software (BD Biosciences).

PCR experiment

Total RNA was extracted using the RNeasy mini kit (Qiagen), and M-MLV Reverse Transcriptase was used to synthesize cDNA according to the manufacturer's instructions. Real-time PCR was performed with an Applied Biosystems 7500HT system (Foster City, CA) using SYBR Premix Ex Taq (Takara). Real-time PCR was carried out in a volume of 20 ml, containing 1 ml reverse transcript product. Cycling conditions were 1 cycle of 95 ℃ for 30 s and 40 cycles of 95℃ for 5 s and 60 ℃ for 30 s. The Ct method was used to determine relative quantification of mRNA expression in samples, and fold change was determined as 2^{Ct}. The specific primer sequences representing pluripotency and three-germ layer differentiation marker genes are listed.

Statistical analysis

All values are expressed as mean ± s.e.m. To determine significance between two groups, comparisons were made using Student's t-test by Graphpad Prism Ver. 5.0 (Graphpad Soft- ware, San Diego, CA, USA). $P<0.05$ was considered to be significant.

Ⅲ. Results

The effects of H_2O_2 on viability in human dental pulp stem cells (hDPSCs)

In the first series of experiment, the effect of H_2O_2 on hDPSCs was examined. The morphology of cells culture with 80 μM H_2O_2 was not shown any differences comparing with control group (Fig. 1A, B). However, different from 80 μM and control group, the shrunk and suspended cells were appeared like damaged cells after 180 μM and 200 μM H_2O_2 (Fig. 1C, D). The MTT assay was also conducted for the viability. As our results, the rate of viability was significantly decreased at 180 μM and over (Fig. 1E).

The effects of IAA on viability in human dental pulp stem cells (hDPSCs)

To investigate the effect of IAA on hDPSCs, the morphology and viability were also verified. In contrast with H_2O_2 results, the treatment of IAA at various concentrations did not influence on the viability of hDPSCs (Fig. 2).

The effects of IAA on H_2O_2-induced cytotoxicity in human dental pulp stem cells (hDPSCs)

To examine the effect of IAA in H_2O_2-damaged hDPSCs IAA, we treated 180 μM H_2O_2 to hDPSCs for 24 h and then treated various concentrations of IAA for 24 h again, respectively. Compared to control group (0 μM IAA, Fig. 3A), 150 μM IAA treated group showed the highest survival rate (Fig. 3E). Morphology of cells was consistent with the result (Fig. 3C). Viable cells were observed more frequently in the group with 50 μM IAA than control group (Fig. 3B). Interestingly, the normal morphologies of cells

were observed to a level similar to negative control at 150 μM IAA. (Fig. 3C, D)

FACS analysis for estimating the cell cycle distribution

To confirm the IAA protective mechanism, the cell cycle distribution and apoptotic H_2O_2-damaged cell proportion were assessed by the FACS analysis. Comparing with negative control (NC) group, the cell cycle in control group (0 μM) was completely destroyed (fig. 4A,D). Moreover, the population of SubG1 phase was significantly increased in control group comparing with that in NC group, suggesting the apoptotic cells may be induced by H_2O_2 treatment. However, the population of SubG1 phase was decreased when H_2O_2-damaged cells were cultured with 50 μM IAA (Fig. 4B). Intriguingly, the damaged cell cycle was recovered to almost the same level as NC group by treatment of 150 μM IAA (Fig. 3C, E). As our results, it is suggested that IAA may be prevent from H_2O_2 damage, consistent with an earlier experiment in this paper.

PCR experiment

To ensure the effect of IAA on H_2O_2-induced apoptosis, the expression of apoptosis-related genes was examined. First, the expression of apoptosis-associated genes, *BAX* and *p53*, was investigated. As a result, *BAX* and *p53* gene expressions were significantly increased in control group (only 180 μM H_2O_2) comparing with that in negative control group. On the other hand, the gene expression of them was remarkably down-regulated in the group, treated with both 180 μM H_2O_2 and 150 μM IAA.

In contrast to apoptosis-related genes, the expression of anti-apoptotic gene, *BCL-2*, was decreased in control group comparing with that in negative control group.

Interestingly, different from control group, *BCL-2* expression was strikingly up-regulated in the group, treated with both 180 μM H_2O_2 and 150 μM IAA. Together, the results suggest that the treatment of IAA may be connected with the regulation of H_2O_2-inducedapoptosis-associated genes.

Ⅳ. Discussion

In present study, we investigated the effects of IAA on H_2O_2-damaged hDPSCs by morphology, viability, cell cycle and gene expression pattern.

Hydrogen peroxide (H_2O_2) is used to bleach or sterilize the tooth surface during dental practice. However its high reactivity causes cytotoxicity when it is penetrated into pulp chamber through dentinal tubules. Like H_2O_2, other ROS from various dental materials including resin restorative material, resin adhesives and resin cement can induce cytotoxicity.[33)~35)] In addition, these oxidative stresses can affect not only cell apoptosis but also mineralization of tooth via affecting cell differentiation and DSPP protein.[35)~38)] Although these series of problem, there is little research has been conducted about cell protection against oxidation. To develop the solution, this study is performed using indole 3-acetic acid. IAA is the most common, naturally occurring plant hormone and a member of auxin family. Recently, auxin has been demonstrated that they are able to regulate cell senescence in plant.[28)~29)] And the possibility that IAA as radical scavenger is proposed.[39)~41)] IAA is natural hormone and presents in both plants and animals, so it is easily accessible and can be regarded safe. In this study, it is proven that IAA does not show any positive or adverse effect on normal cells at various concentrations (0-400 μM).

On the other hand, from a certain extent concentration (80 μM), H_2O_2 shows cytotoxic effects that decreasing the number of normal cells and promoting apoptosis. From the experiment, we were also able to find out, H_2O_2 exert maximal inhibitory effect at 180 μM.

To demonstrate the effect of IAA at optimal condition, we treated IAA on destructed hDPSCs with 180 μM H_2O_2 for 24h. However, IAA shows anti-oxidative effect against H_2O_2–damaged hDPSCs and its protective effect maximum at 150 μM. Although its mechanisms are not fully understood, it is expected that IAA preclude apoptosis by recovering normal cell cycle. FACS analysis shows that the apoptotic cell proportion is strikingly decreased, and the outline of normal cell cycle is recovered. PCR experiment also means that apoptosis is induced in H_2O_2–damaged hDPSCs and inhibited by IAA treatment.

Therefore, apply of IAA can play an important role in cell protection against oxidative stress. Moreover anti-oxidative effect of IAA in hDPSC can be applied very usefully as hDPSCs are considered attractive stem cell sources in dental science. It may be contributed to regeneration of tooth-perio complex.

However, still, further study is needed. First of all, this study is conducted *in vitro*, so there can be differences in real tooth. And the mechanisms are not clear, so some questions are not answered yet. For example, IAA shows maximal protective effect at 150μM but the effect is decreased as the concentration higher.

In summary, H_2O_2 promotes apoptosis and disturbances cell cycle in hDPSCs. However, IAA is able to protect hDPSCs from H_2O_2-induced cytotoxicity via cell cycle restoration

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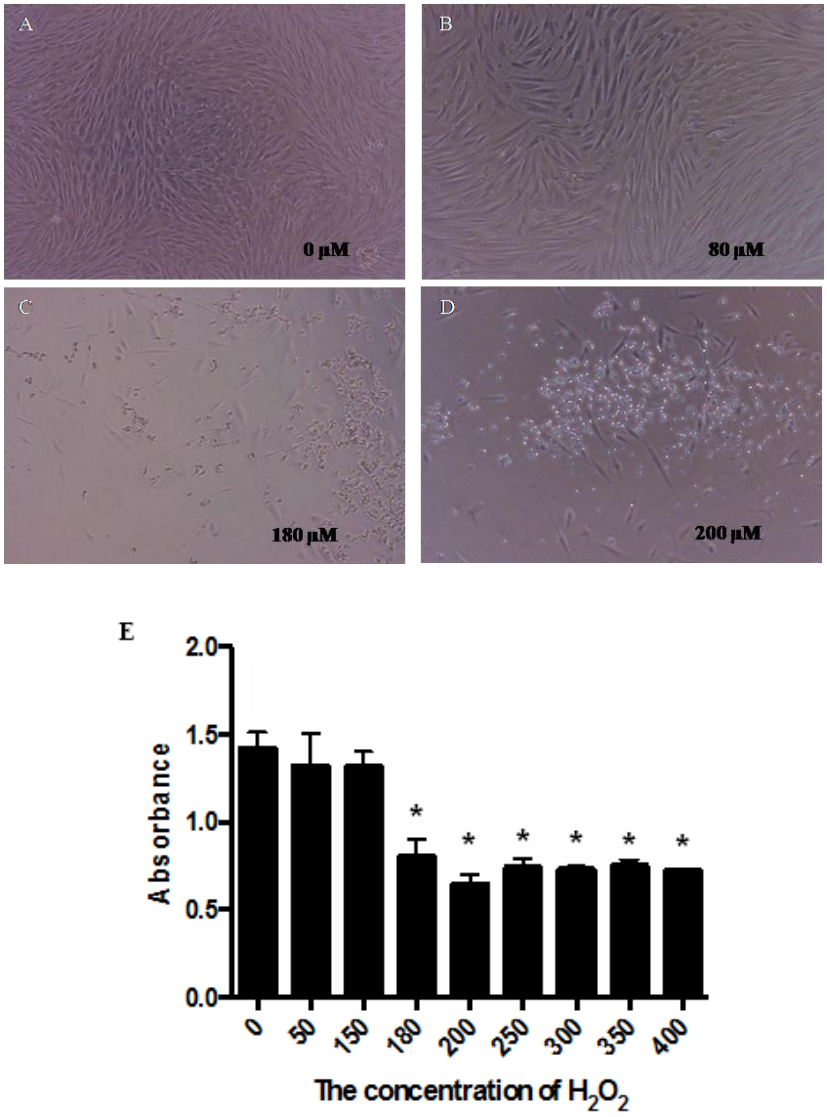

Figure1. The effect of H_2O_2 in human dental pulp stem cells (hDPSCs). (A) The morphology of hDPSCs cultured without H_2O_2 as control. The morphologies of hDPSCs cultured at 80 μM (B), 180 μM (C) and 200 μM (D) were confirmed. (E) The viability of hDPSCs was also assessed after H_2O_2 at different concentrations ranging from 1-400 μM. The viability of hDPSCs was significantly decreased after treatment of H_2O_2 at 180 μM and above. N = 4, *P< 0.05.

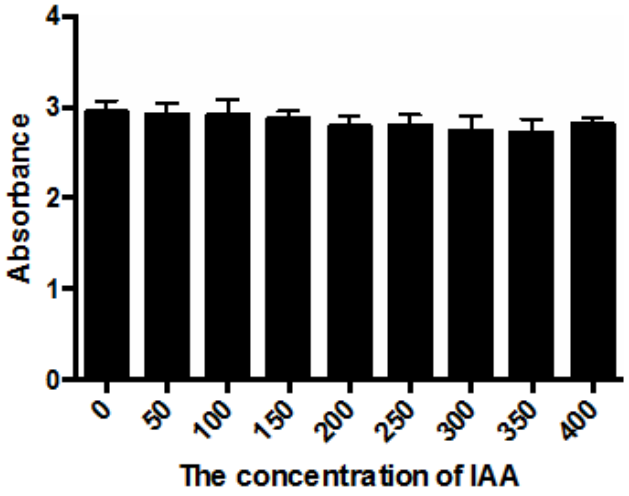

Figure2. The effect of IAA in human dental pulp stem cells (hDPSCs). The viability of hDPSCs was measured after IAA at different concentrations ranging from 1-400 μM. IAA itself did not influence on the viability of hDPSCs. N = 4

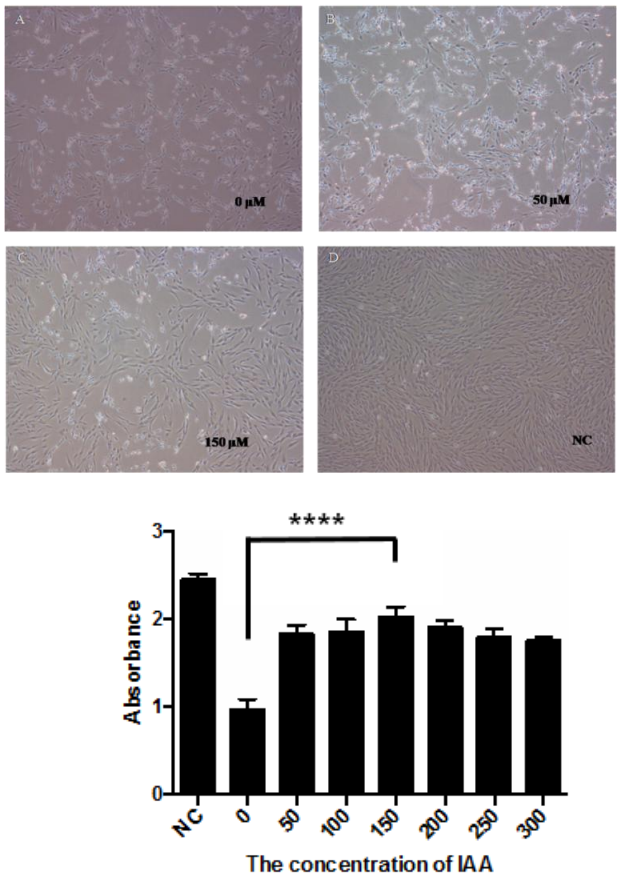

Figure3. The morphology and viability of H_2O_2-damaged human dental pulp stem cells (hDPSCs) were assessed after treatment of IAA at various concentrations ranged from 1 to 300 µM. The morphologies of H_2O_2-damaged hDPSCs (180 µM) were confirmed after treatment of IAA at 0 µM (A), 50 µM (B) and 150 µM (C). (D) The morphology of hDPSCs cultured without both H_2O_2 and IAA as negative control (NC). (F) The viability of H_2O_2-damaged hDPSCs was also assessed after treatment of IAA at different concentrations ranging from1-300 µM. The H_2O_2-induced cytotoxicity was significantly attenuated in the presence of IAA, and its maximum effect was occurred at 150 µM. N = 4, ****P< 0.0001.

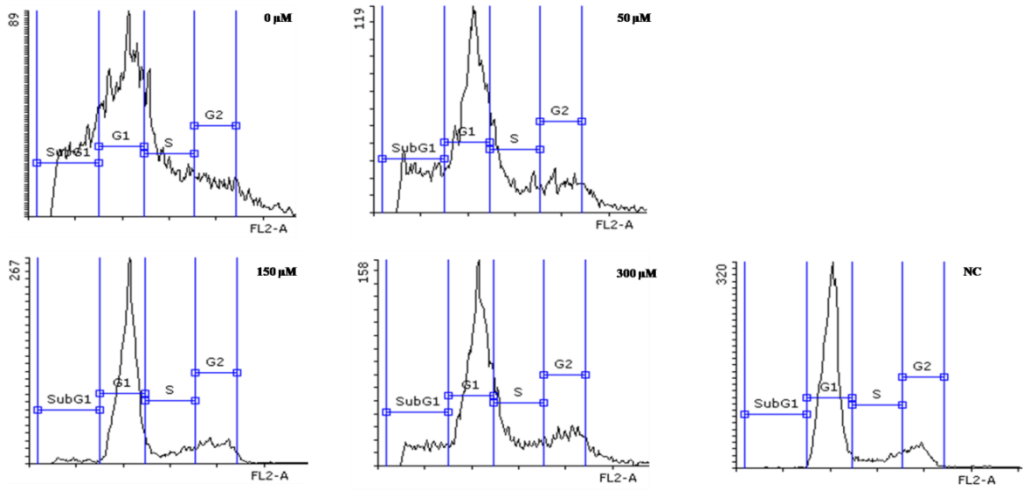

Figure4. The analysis of cell cycle and apoptotic cells in H_2O_2-damaged human dental pulp stem cells (hDPSCs) with treatment of IAA. In control group (0 μM), normal cell cycle was remarkably destroyed by H_2O_2 treatment (180 μM), and the number of apoptotic cells (SubG1) was also increased when compared with negative control (NC) group. Interestingly, the damaged cell cycle by H_2O_2-induced cytotoxicity was recovered by the treatment of 150 μM IAA. The number of apoptotic cells was also decreased by the treatment.

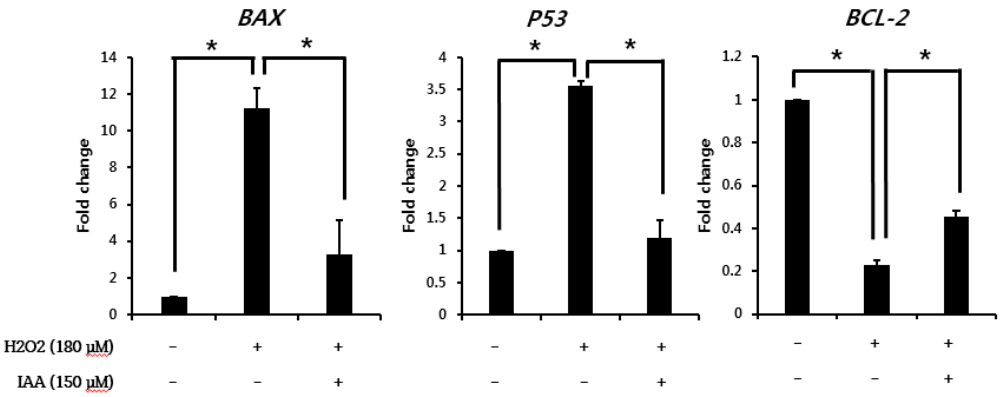

Figure5. The gene expression pattern of hydrogen peroxide (H_2O_2)-damaged hDPSCs was measured by RT-qPCR in IAA treated and non-treated group. The expression level of apoptotic related genes, *BAX* and *P53*, was increased by the treatment of H_2O_2 (180 μM), while the gene expression of them in H_2O_2-damaged hDPSCs was significantly decreased by the treatment of IAA (150 μM). Reverse tendency was observed about the expression of anti-apoptotic associated gene, *BCL-2*. It was increased after treatment of IAA (150 μM), but down-regulated by the treatment of H_2O_2 (180 μM) in H_2O_2-damaged hDPSCs. N = 4, **P*< 0.005

국문초록

과산화수소로 인한 인간치수줄기세포의 산화독성에 대한 인돌 3-아세트산의 영향

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치의학과

이 연구의 목적은 과산화수소에 의한 산화적 독성 상황의 인간치수유래줄기세포에 대한 인돌 3-아세트산의 효과를 알아보는 데 목적이 있다.

이를 증명하기 위해 먼저 인간치수줄기세포의 생존능력에 대한 1-500 μM 의 다양한 농도의 과산화수소 단독의 효과를 알아보았다. 생존한 세포의 수 측정은 과산화수소 처리 24시간 후에 행하였다. IAA 의 단독의 효과를 알아보기 위해 과산화수소와 동일한 과정의 실험을 IAA 로 수행하였다. 그리고 과산화수소가 유발한 세포독성 상태의 인간치수줄기세포에 IAA 를 처리했을 때 나타나는 변화를 살펴보기로 하고 180 μM 의 과산화수소를

처리한 인간치수줄기세포에 1-300 μM 의 IAA 를 처리한 뒤 살아있는 세포 수를 측정하고 세포주기의 양상을 확인하였다.

실험 결과에 쓰여 있듯이, 인간치수줄기세포에 다양한 농도의 과산화수소를 처리하고 24시간이 지난 후 세포 생존력을 측정하자, 40 μM 이하의 과산화수소는 세포 생존력에 영향을 주지 않았고 50 μM 이상의 농도에서 세포 저해효과가 나타나서 180 μM 농도에서 최대저해효과를 보였다. 반면 IAA 를 처리한 인간치수줄기세포들은 IAA 의 농도가 변해도 생존력에 별다른 변화를 보이지 않았다. 그러나 과산화수소가 유발한 세포독성에 대해서는 150 μM 의 IAA 가 뚜렷한 방어효과를 보였다. FACS 분석 결과를 보면, 과산화수소를 단독 처리한 컨트롤 그룹의 세포들은 세포주기의 모든 단계가 심각하게 망가진 양상을 보였으며 아무 처리도 하지 않은 그룹에 비해 많은 수의 세포자살이 관찰되었다. 흥미롭게도 150 μM 의 IAA 를 처리하자, 과산화수소가 유발한 세포독성으로 망가진 세포주기가 회복되고 세포자살이 현저하게 감소되는 모습이 관찰되었다.

결론적으로, IAA 는 과산화수소가 유발한 세포독성 상태의 인간치수줄기세포를 세포주기의 회복을 통해 보호할 수 있었다.

주요어: 인돌 3-아세트산 (IAA),과산화수소 (H_2O_2), 항산화작용, 과잉치, 치수줄기세포

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