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원 저

Protective Mechanism of *Hominis Placenta* Extract Against H_2O_2-Induced Apoptosis in PGT-β Cells

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PGT-β 세포주에서 紫河車 藥鍼液의 H_2O_2로 인한 Apoptosis에 대한 방어 기전

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목적 : 본 연구는 최근 임상에서 많이 사용하는 자하거 약침액이 과산화수소(H_2O_2)로 야기된 송과선 세포의 apoptosis에 있어서 세포 보호에 미치는 영향과 그 기전을 분석하였다.

방법 : 송과선 세포주에서 자하거 약침액의 H_2O_2로 인한 apoptosis에 대한 방어 기전을 관찰하기 위하여 면역세포화학법, 세포화학법 및 reverse transcription-polymerase chain reaction (RT-PCR)을 시행하였다.

결과 : 자하거 약침액 투여군에서는 nuclear factor kappa B (NFκB), inducible nitric oxide synthase (iNOS), nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase의 발현이 H_2O_2 투여군보다 감소하였다. RT-PCR에서는 caspase-3의 발현이 자하거 약침액 투여군에서 H_2O_2 투여군보다 억제되었다.

결론 : 이상의 결과를 통하여 자하거 약침액이 H_2O_2로 유발된 apoptosis에서 세포보호 효과가 있으며 그 기전은 iNOS와 caspase-3의 억제에 기인할 가능성을 시사한다고 하겠다. *(J Korean Oriental Med 2001;22(3):92-97)*

Key Words: *Hominis Placenta* extract, H_2O_2, Apoptosis, iNOS, Caspase-3

INTRODUCTION

In Oriental medicine *Hominis Placenta* extract derived from human placenta has been prescribed to some psychiatric behaviors such as anxiety, amnesia and manic[1]. It is also known that *Hominis Placenta* extract derived from human placenta affects brain monoamines and monoamine activity[2]. Recently Angelucci et al.[3] have reported that *Hominis Placenta* extract effectively modulates the growth of malignant and nonmalignant cells, and it has been shown to have cytotoxicity on malignant cells. However, few reports have been focused on the anti-oxidant effects of *Hominis Placenta* extract. Moreover, its role is not

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clearly defined yet. Thus, we examined the effects of human placental extract as an attempt to elucidate the possible underlying mechanism of its action. The purpose of this present study was to investigate by which mechanism *Hominis Placenta* extract prevents H_2O_2-induced apoptosis.

Free radicals from exogenous or endogenous sources damage tissue components. Since free radical tissue damage increases with age, the reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), nitric oxide (NO) and superoxides, have been implicated in the pathogenesis of aging-related diseases such as cardiovascular disease, cancer, arthritis and neurodegenerative diseases[4,5]. It is well known that free radicals have important roles in the pathophysiology of apoptosis[6,7]. The pineal gland is one of neuroendocrine organs related with the aging, the circadian rhythm, the immune system, cancer, and psychiatric disorders. It produces melatonin and is thought to be a vulnerable organ to exposure of free radicals[8-10].

In the present experiment, we used the immortalized pineal gland tumor cells PGT-β. The PGT-β cells were derived from the pineal tumor of a transgenic mouse by targeted tumorigenesis using the mouse tryptophan hydroxylase (TPH) promoter/SV-40 T-antigen[11]. And to determine the anti-apoptotic mechanisms of *Hominis Placenta* extract on H_2O_2-induced apoptosis, we have performed immunocytochemistry to estimate the level of expression of nuclear factor kappa B (NFκB) and inducible NOS (iNOS), nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase cytochemistry for the assessment of nitric oxide synthase (NOS) activity, and reverse-transcription polymerase chain reaction (RT-PCR) to measure the level of expression of apoptotic genes.

MATERIALS AND METHODS

1. Extracting Procedure of *Hominis Placenta*

Hominis Placenta extract solution was prepared previously described[12]. The final concentration of *Hominis Placenta* extract was 1.7 mg/ml. In brief, *Hominis Placenta* extract was obtained from Whasung Pharmacy (Geochang, Korea). In brief, 200 kg of placental chorionic parenchyma obtained from full-term births was washed and the tissue fat was removed using acetone. About 14 kg of skimmed particulate chorionic tissue was obtained using vacuum drier. And it was treated with pepsin and hydrochloric acid. Purified water was then added and the hydrochloric acid was added until pH 1.8. After the liquid phase was extracted and the supernatant was adjusted with 80 l of purified water, the solution was sterilized by heat and filtered. Ion exchange resin was added to the filtrate until pH 5. After the final volume was adjusted to 100 l with sterile water, the final solution was filtered using a Millipore® filter. And then the liquid was sterilized by 121℃ for 20 minutes in an autoclave.

2. Cell Culture

PGT-β cells were grown until 70-80% confluent in Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GibcoBRL, Grand Island, NY), at 37℃ in 5% CO_2, 95% air in a humidified cell incubator. The medium was changed every 2 days. According to our previous results[12], in the present study *Hominis Placenta* extract solution at concentration of 1.7 mg/ml was pretreated for 2 hrs before exposure to 3 hr treatment of 50 μM H_2O_2.

3. Immunocytochemistry

Immunocytochemistry was performed using a

previously described avidin-biotin-peroxidase complex method (ABC kit, Vector Laboratories, Burlingame, CA)[13]. Briefly, PGT-β cells were cultured in 4-chamber slides (Nalge Nunc International, Naperville, IL) at a density of 2 x 10^4 cells/chamber. After treatment with *Hominis Placenta* extract and H_2O_2 (Junsei, Japan), the cells were washed twice with phosphate-buffered saline (PBS) and fixed by incubation in 4% paraformaldehyde (PFA) for 30 minutes. Cells were incubated with primary antibodies (NFκB, 1:500; iNOS, 1:500) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) overnight, and with biotinylated antibodies (1:100) for another 2 hrs and with ABC for a further 1 hr (1:100) at room temperature. For visualization of antibody reactivity, 3,3' -diaminobenzidine (DAB; Sigma, St. Louis, MO) as a chromogen was applied to the chambers for 5-10 minutes. Cells were washed three times with 0.1 M phosphate buffer (PB) containing 5-10% goat serum (Sigma, St. Louis, MO) for blocking nonspecific antibody reaction. Coverslips were mounted using Permount (Fisher Scientific, Far Lawn, NJ).

4. Nicotinamide Adenine Dinucleotide Phosphate (NADPH)-Diaphorase Cytochemistry

NADPH-diaphorase expression was measured according to the previously described cytochemical methods[14,15]. PGT-β cells were cultured in 4-chamber slides at a density of 2 x 10^4 cells/chamber. After treatment with *Hominis Placenta* extract and H_2O_2, the cells were stained for the detection of NADPH-diaphorase activity. In brief, PGT-β cells were washed with 50 mM PBS and then were fixed with a freshly prepared solution consisting of 4% PFA in 0.1 M PB at pH 7.4. The cells were incubated at 37℃ for 1-6 hrs in 0.1 M PB, pH 7.4, containing 0.3% Triton X-100 (Sigma, St. Louis, MO), 0.1 mg/ml nitroblue tetrazolium (Sigma, St. Louis, MO) and 0.1 mg/ml β-NADPH (Sigma, St. Louis, MO) in the dark place. The stained cells were rinsed three times with 50 mM PBS. The slides were air-dried overnight at room temperature, rinsed twice with distilled water and dried again. Coverslips were mounted using Permount.

5. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from PGT-β cells using RNAzol™ B (Tel-Test, Friendswood, TX) as the manufacturer' s instructions. The concentration of extracted RNA was determined by spectrophotometric measurement of absorbances at 260 nm and 280 nm and calculated the ratio of the optical densities, with a ratio of 1.0 (260/280) equivalent to 40 μg/ml of RNA. Single-strand cDNA was synthesized using a reaction mixture containing 2 μg of mRNA template, 1.0 μg of random primer (Promega, Madison, WI) and sterile H_2O for adjustment of the final volume to 10 μl; the template was then denaturated at 65℃ for 10 minutes and placed at room temperature for 5 minutes. Thirty U AMV reverse transcriptase (Promega, Madison, WI), AMV RT 5 X reaction buffer (250 mM Tris-HCl at pH 8.3, 250 mM KCl and 50 mM $MgCl_2$), 10 mM dNTP mix and 40 U RNasin (Ribonuclease inhibitor, Promega, Madison, WI) were added to the reaction mixture, which was then incubated at 42℃ for 2 hrs. The reaction mixture was then diluted to a final volume of 100 μl with sterile H_2O. Amplification of each cDNA species was conducted in a reaction mixture (total volume 40 μl) containing the reverse transcription reaction mixture, 10 X PCR buffer (20 mM Tris-HCl at pH 8.0, 100 mM KCl, 2 mM $MgCl_2$), 2.5 mM dNTP mixture, 10 pM each of the sense and antisense primers and 2.0 U Taq polymerase (TaKaRa Ex Taq, TaKaRa, Shiga, Japan). The primer sequences were generated from the information posted on Entrez (NCBI, NIH). The oligonucleotide primers corresponded to sequences located in different exons separated by at least one

intron in order to differentiate spliced mRNA PCR products from those of unspliced RNA or genomic DNA. The sequences of oligonucleotide primers used for PCR were as follows; caspase-3, 5' -TGTCAT CTCGCTCTGGTACG-3' (sense, bases 245-264) and 5' -CCGTGTATTGTGTCCATGCT-3' (antisense, bases 850-869); cyclophilin, 5' -ACCCCACCGTG TTCTTCGAC-3' (sense, bases 43-62) and 5' -CATTTGCCATGGACAAGATG-3' (antisense, bases 323-342). The reaction mixture was then incubated at 94℃ for 5 minutes to activate the polymerase, and amplification was performed over the following numbers of amplification cycles; caspase-3, 40 cycles; cyclophilin, 25 cycles. Each amplification cycle consisted of incubation at 94℃ 30 seconds, at 58℃ for another 30 seconds and at 72℃ for a final 30 seconds. A 5-minutes incubation at 72℃ was performed at the end of the procedure for a final extension reaction. The entire process was performed using a Perkin Elmer 9600 PCR machine. The 10 μl of PCR products were analyzed by gel electrophoresis using 1.0% agarose gels. The gels were stained with ethidium bromide (0.5 μg/ml). The PCR products were visualized using ultraviolet light, and photographed.

RESULTS

1. Immunocytochemistry

To determine the changes of NFκB and iNOS expression, immunocytochemistry were measured during the cell death of PGT-β. PGT-β cells pretreated with *Hominis Placenta* extract showed decreased expression of NFκB and iNOS, compared to that of the H_2O_2-only treated cells (Fig. 1).

2. NADPH-Diaphorase Cytochemistry

To detect the change of NOS activity, NADPH-diaphorase cytochemistry was measured during the cell death of PGT-β. It was found that NADPH-diaphorase was intensively expressed in the H_2O_2-only treated cells, but in the cells pretreated with *Hominis Placenta* extract the expression was decreased(Fig. 1).

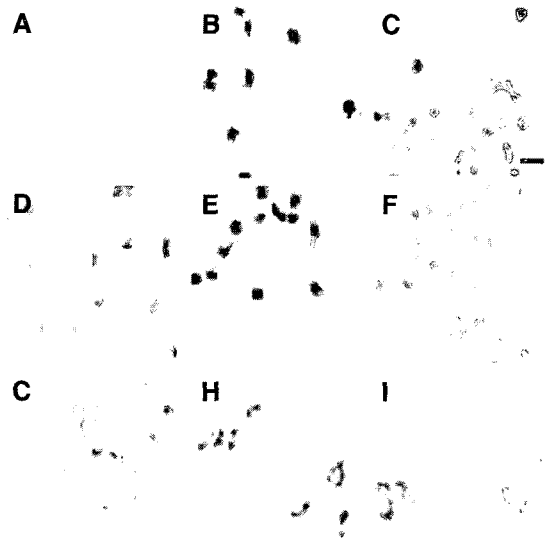

Fig. 1. Expression of NFκB, iNOS and NADPH-diaphorase.
NFκB (A: control, B: H_2O_2, C: *Hominis Placenta* extract), iNOS(D: control, E: H_2O_2, F: *Hominis Placenta* extract) and NADPH-diaphorase (G: control, H: H_2O_2, I: *Hominis Placenta* extract) was expressed highly in H_2O_2-treated cells, but in *Hominis Placenta* extract treated cells showed markedly decreased expression of those.

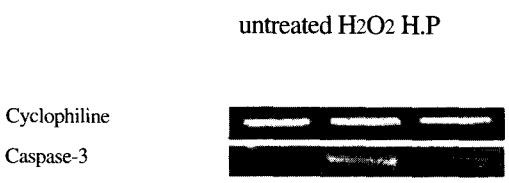

Fig. 2. Expression of *caspase-3* mRNA.
In H_2O_2-treated cells, the mRNA expression of *caspase-3* were increased but mRNA level of *caspase-3* in *Hominis Placenta* extract-treated cells were decreased markedly.

3. Expression of *caspase-3* mRNA

To detect the expression of the genes involved in apoptosis, the mRNA expression of caspase-3 was evaluated during the cell death of PGT-β using RT-PCR. It was found that the mRNA expression of caspase-3 was intensively expressed in the H_2O_2-only treated cells, but the expression was decreased in the cells pretreated with *Hominis Placenta* extract(Fig. 2).

DISCUSSION

We have performed immunocytochemical and cytochemical analyses to assess the changes in NOS activity. Icreases in NOS activity were seen in cells treated with 50 μM H_2O_2. PGT-β cells pretreated with *Hominis Placenta* extract showed decreased expression of NFκB, iNOS and NADPH-diaphorase, compared to that of the H_2O_2-only treated cells (Fig. 1). NO acts as a mediator that plays a role in neurotransmission, long term potentiation, depression, brain development[16] and in the cardiovascular, immune and nervous systems[17,18]. NO is synthesized by a family of enzymes that are collectively called NOS (EC 1.14.13.49). Three isoforms (nNOS, iNOS and eNOS) of NOS have been identified; these enzymes were found to be heme-containing flavoproteins employing L-arginine as a substrate and requiring NADPH, flavin adenine dinucleotide and tetrahydrobiopterin as cofactors[19,20]. NADPH-diaphorase converts a soluble tetrazolium salt to an insoluble formazan. It has been reported that NADPH-diaphorase accounts for the NOS activity in neural tissue[21,22] because NADPH-diaphorase-positive cells are the same as those containing NOS[15]. The transcription factor NFκB was identified as a protein complex consisting of a p65 subunit and a p50 subunit. NFκB plays a critical role in the expression of NOS genes, especially in the induction of an iNOS gene by lipopolysaccharides[23]. Among apoptotic genes caspase-3 is a novel type of gene that accelerates cell death by acting as a proapoptotic factor. Apoptotic genes closely interact with the mitochondria and lead to the release of the electron transporter cytochrome c. Cytochrome c activates a set of proteases called caspases that proteolyze cellular components[24]. In the H_2O_2-treated cells, release of cytochrome c may activate caspase-3, whose expression may have been enhanced by H_2O_2, and finally lead to DNA fragmentation. The *Hominis Placenta* extract-treated cells revealed a decreased level of caspase-3 mRNA (Fig. 2). Maybe *Hominis Placenta* extract interrupt the apoptotic pathway and result in decreased occurrence of apoptotic features.

These results suggest that the preventive effect of *Hominis Placenta* extract against H_2O_2-induced apoptosis might result from transcriptional regulation (inhibition) of iNOS and the apoptosis-related gene caspase-3. And these suggest that NOS and caspase-3 are involved in the mechanism of prevention of H_2O_2-induced apoptosis in PGT-β, and that *Hominis Placenta* extracts could increase the DNA repair capability of cells and protect from cell death due to oxidant stress and facilitate the recovery of cellular viability. The defined molecular mechanism of *Hominis Placenta* extract is still unknown. Further long-term studies are needed to elucidate the precise molecular mechanism of *Hominis Placenta* extract on free radical damage.

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