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원 저

Preventive Effects of Hominis Placenta Extract on H_2O_2-Induced Apoptosis in Pineal Gland Cell Line

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초 록

紫河車 藥鍼液이 과산화수소로 유발된 송과선 세포의 Apoptosis에 대한 보호 효과

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목적 : 본 연구는 최근 임상에서 많이 사용하는 紫河車 藥鍼液이 과산화수소(H_2O_2)로 야기된 송과선 세포의 apoptosis에 있어서 세포 보호에 미치는 영향과 그 기전을 분석하였다.

방법 및 결과 : MTT assay를 이용하여 분석한 결과 처리 시간 및 농도에 따른 세포 독성의 효과가 H_2O_2 투여로부터 관찰되었다. 또한 자하거 약침액은 세포 증식 효과를 나타내었고 자하거 약침액을 전처치하고 H_2O_2를 처치하였을 때 세포 독성이 크게 감소되었다. DAPI staining에서 자하거 약침액 투여군은 H_2O_2 투여군에 비해 fragmentation이 억제되었다. TUNEL assay를 통하여 자하거 약침액 투여군은 H_2O_2 투여군에 비하여 TUNEL 양성세포의 발현이 감소되었다.

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Flow cytometry를 통하여 자하거 약침액 투여군은 H_2O_2 투여군에 비하여 세포주기 중 sub G1 분획의 증가가 억제되었다.

결론 : 이상의 결과를 통하여 자하거 약침액이 H_2O_2로 유발된 apoptosis에서 세포보호 효과가 있음이 확인되었다.

Key words : Hominis Placenta extract, H_2O_2, Apoptosis, Pineal gland cell line

Ⅰ. Introduction

Hominis Placenta extract is derived from human placenta. In Oriental medicine it has been applied to psychiatric disorders such as amnesia, anxiety and manic[7], and it has been shown to have clinical efficacy. Many researchers have reported on its effects of cytotoxicity[1]. Recently some studies have been reported that Hominis Placenta extract is effective in the treatment of learning difficulties[2]. However, few studies have been comed out about the anti-oxidant effects of Hominis Placenta extract. Moreover, its role is not clearly defined yet.

Hydrogen peroxide (H_2O_2) is one of the free radicals and a highly oxidative substance. H_2O_2-induced cytotoxicity occurs through the oxidation of proteins, lipids and nucleic acids; thus impairing cellular function and leading to cell death. Much investigation has been done on H_2O_2-induced apoptosis[3], but few studies have been made concerning the prevention of apoptosis.

The pineal gland is a neuroendocrine organ involved in aging, the circadian rhythm, the immune system, cancer, endocrine disorders and psychiatric disorders[5,6,10]. It expresses serotonin and is thought to be one of the organs vulnerable to free radicals.

In the present study, to determine the anti-apoptotic effects of Hominis Placenta extract solution on H_2O_2-induced apoptosis in the immortalized pineal gland tumor cells PGT-β, we have performed morphological analysis, DAPI staining assay, TUNEL assay and flow cytometric analysis.

Ⅱ. Materials and methods

1. Extracting procedure of Hominis Placenta

Hominis Placenta extract solution was obtained from Whasung Pharmacy (Geochang, Korea) and was prepared as followed: 200 kg of healthy placental chorionic parenchyma was obtained from full-term births, was rinsed and the tissue fat was removed using acetone. And it was vacuum dried to obtain about 14kg of skimmed particulate chorionic tissue. It was heated after treatment with pepsin, hydrochloric acid and purified water were added and the pH was readjusted to 1.8 using hydrochloric acid. The liquid phase was extracted and the supernatant was adjusted with 80 ℓ of purified water, and the solution was sterilized

by heat.

Activated carbon was added, then it was stirred and filtered. Ion exchange resin was added to the filtrate to reach pH 5 and it was filtered again. After the volume was adjusted to 100 l with purified water, it was filtered using a Millipore® filter. The filtrate was poured into washed and dried vials and then the liquid was capped and sterilized by 121℃ for 20minutes in an autoclave. From above step final concentration of Hominis Placenta extract was 1.7 mg/ml.

2. Cell culture

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]. PGT-β cells were grown until 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), penicillin (10 units/ml) and streptomycin (10 mg/ml) (GibcoBRL, Grand Island, NY) at 37℃ in 5% CO_2, 95% air in a humidified cell incubator. The medium was changed every 2 days.

3. Cytotoxicity by H_2O_2

For the measurement of cytotoxicity, the 3-(4,5-dimethylthiozol-2-yl)-diphenyltetrazolium bromide (MTT) assay (Roche, Mannheim, Germany) was performed according to the manufacturers instructions. In brief, PGT-β cells were cultured in a 96-well plate (Corning Incorporated, Corning, NY) at a density of 5 x 10^3 cells per well. The cells were treated with varying concentrations of H_2O_2 (Junsei, Japan). After incubation for 1.5 or 3 hrs, the cells were incubated with the MTT labeling reagent for 4 hrs. Then the cells were incubated in the solubilization solution for 24 hrs to solve blue formazan crystals. Optical density (OD) was measured at 595 nm using a microtiter plate reader (Bio-Tek, Winooski, VT).

For determination of cell viability, percent viability was calculated as (absorbance of drug-treated sample/control absorbance) x 100. For morphological analysis, following a 1.5 or 3 hrs exposure to H_2O_2 (1, 10, 50 and 100 μM), PGT-β cells were observed by phase-contrast microscopy (Olympus, Japan).

4. Cell proliferation by Hominis Placenta extracts

For analysis of cell proliferation, PGT-β cells were treated with Hominis Placenta extract at concentrations of 1.7 x 10^{-5}, 1.7 x 10^{-4}, 1.7 x 10^{-3}, 1.7 x 10^{-2}, 1.7 x 10^{-1} and 1.7 mg/ml. After 24 hrs of treatment, the cells were harvested using trypsin-EDTA (Gibco BRL, Grand Island, NY). For measurement of cell proliferation, MTT assay was performed as described above.

5. Protection of cell death by Hominis Placenta extracts

To examine the preventive effects of Ho-

minis Placenta extract against cell death induced by H_2O_2, cells were pretreated with 0, $1.7x10^{-2}$, $1.7x10^{-1}$ and 1.7mg/ml of Hominis Placenta extract and incubated for 2hrs. Then the cells were grown for 1.5 or 3hrs in the presence of H_2O_2 (50μM). The cells were harvested for analysis of cell viability. For determination of the preventive effects, MTT assay was performed as described above.

6. DAPI staining

4,6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO) staining procedure was performed as previously described[8]. In brief, PGT-β cells were cultured on 4-chamber slides (Nalge Nunc International, Naperville, IL) at a density of $2x10^4$ cells/chamber. After treatment with Hominis Placenta extract and H_2O_2, the cells were washed twice with phosphate-buffered saline (PBS) and fixed by incubation in 4% paraformaldehyde (PFA; Sigma, St. Louis, MO) for 30 minutes. The fixed cells were washed twice with methanol and stained with 1 mg/ml DAPI solution for 30 minutes to label the nuclei. The apoptotic cells were observed with a fluorescence microscope (Zeiss, Oberkochen, Germany).

7. In situ detection of nuclear DNA fragmentation

For in situ detection of apoptotic cells, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) technique was performed using the ApoTag peroxidase in situ apoptosis detection kit (Intergen, Purchase, NT). In brief, PGT-β cells were cultured on 4-chamber slides at a density of $2x10^4$ cells/chamber. The cells were pretreated with Hominis Placenta extract (0, 1.7mg/ml) for 2hrs and then exposed to H_2O_2 (0, 50μM) for 3hrs. After the 3hrs exposure to H_2O_2, cells were fixed by incubating in 1% PFA for 10minutes. The fixed cells were incubated with digoxigenin-conjugated dUTP in a TdT-catalyzed reaction for 60 minutes in a humidified chamber at 37℃ and were immersed in stop/wash buffer for 10 minutes at room temperature. Then the cells were incubated with anti-digoxigenin antibody conjugated with peroxidase for 30 minutes. The DNA fragments were stained using 3,3′-diaminobenzidine (DAB; Sigma, St. Louis, MO) as the chromogen for the peroxidase.

8. Flow cytometric analysis

Flow cytometric analysis was performed as previously described methods[9]. After treatment with Hominis Placenta extract and H_2O_2, the cells were washed twice with PBS. Then the cells were collected by centrifugation and washed in ice-cold PBS. Approximately 1 x 10^6 cells were suspended in PBS and then fixed in a cold 75% ethanol solution at 4℃ for 1 hr. Before analysis, cells were spun out of the ethanol solution and resuspended in 1㎖ PBS containing 100μg/ml propidium iodide (PI; Sigma, St. Louis, MO) and 1 mg/ml RNase (Sigma, St. Louis, MO). The stained cells were incubated for 30 minutes at 37℃ and were analyzed with a FACScan (Becton Dickinson,

San Jose, CA).

9. Statistical analyses

Statistical analyses were performed using the Statistical Package for Social Science software SAS (version 6.1.2). Data were analyzed by ANOVA (one-factor analysis of variance). $P < 0.05$ was considered to indicate statistical significance.

Ⅲ. Results

1. Assessment of H_2O_2-induced cytotoxicity

A dose- and time-related pattern was seen in the viability of PGT-β cells according to various concentrations of H_2O_2. Viability of cells treated with H_2O_2 for 12 hrs at concentrations of 1, 10, 50 and 100 μM were about 89, 86, 15 and 11% compared to that of the untreated cells (100%) respectively (Fig. 1).

2. Assessment of cell proliferation induced by Hominis Placenta extract

The viability of PGT-β cells when treated with various concentrations of Hominis Placenta extract is showen in Fig. 2. Viabilities of cells treated with Hominis Placenta extract for 24 hrs at concentrations of 1.7×10^{-5}, 1.7×10^{-4}, 1.7×10^{-3}, 1.7×10^{-2}, 1.7×10^{-1} and 1.7 mg/ml were about 96, 100, 108, 88, 102 and 128% compared to that of the untreated cells (100%) respectively.

3. Assessment of preventive effects against cell death of Hominis Placenta extract

The viability of PGT-β cells pretreated with Hominis Placenta extract for 2 hrs before exposure to H_2O_2 for 3hrs is shown in Fig. 3. Viability of cells at Hominis Placenta extract concentrations of 1.7×10^{-2}, 1.7×10^{-1} and 1.7mg/ml with H_2O_2 at a concentration of 50 μ M were about 32, 35 and 44% compared to

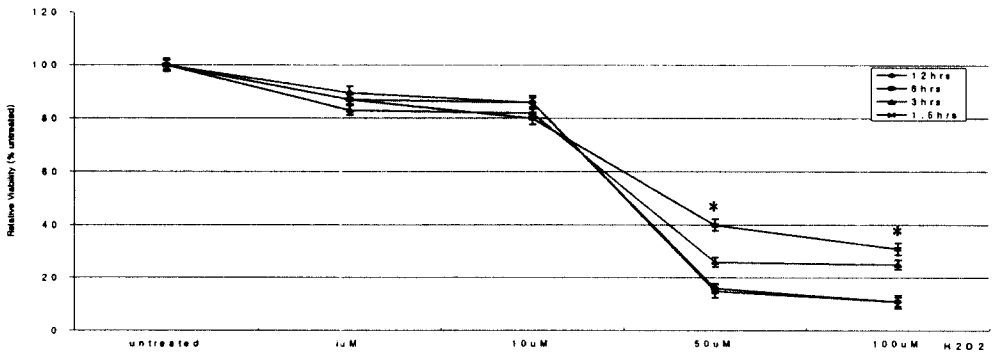

Fig. 1. Dose-and time dependent effect of H_2O_2 on PGT-β cell viability. Cellular viability was determine by an MTT assay. Relative viability (% control) is represented as a percentage absorbance of the sample with respect to the control. Results are represented as mean ± standard error (bars) for two independent experiments with a minimum of three cultures. Values significantly different from the corresponding control at $P < 0.05$ are indicated with asterisks.

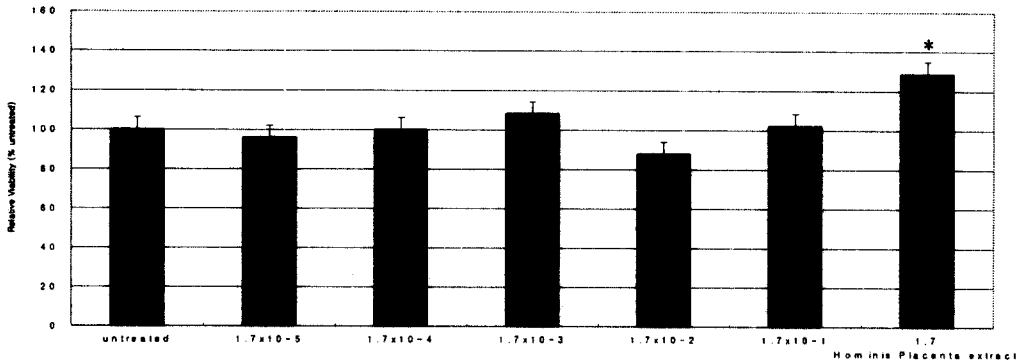

Fig. 2. The effect of Hominis Placenta extracts on PGT-β cell viability. Cellular viability was determine by an MTT assay. Relative viability (% control) is represented as a percentage absorbance of the sample with respect to the control. Results are represented as mean ± standard error (bars) for two independent experiments with a minimum of three cultures. Values significantly different from the corresponding control at $P < 0.05$ are indicated with asterisks.

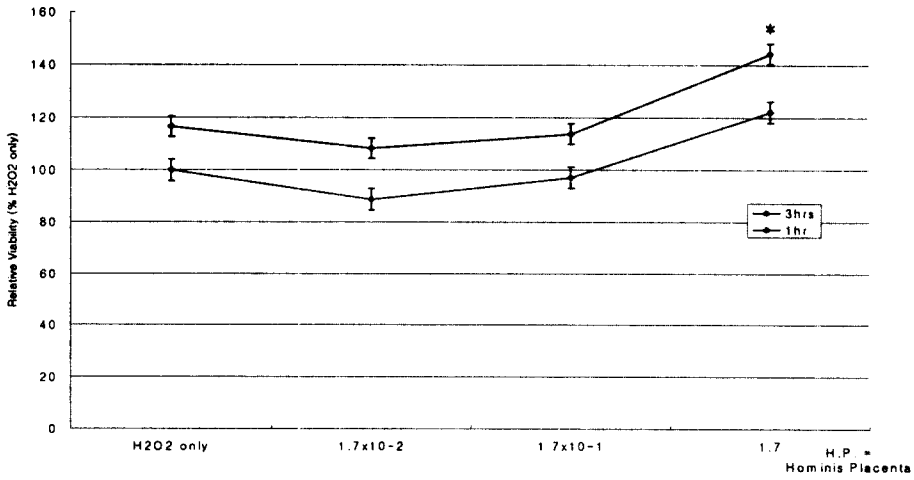

Fig. 3. The protection effect of Hominis Placenta extracts on PGT-βcell viability. Cellular viability was determine by an MTT assay. Relative viability (% control) is represented as a percentage absorbance of the sample with respect to the control. Results are represented as mean ± standard error (bars) for two independent experiments with a minimum of three cultures. Values significantly different from the corresponding control at $P < 0.05$ are indicated with asterisks.

that of the H_2O_2 only treated cells (36%) respectively.

4. Morphological analysis

For analysis of morphological changes, cells were examined by phase-contrast microscopy. PGT-β cells pretreated with Hominis Placenta extract showed decreased features of apo-

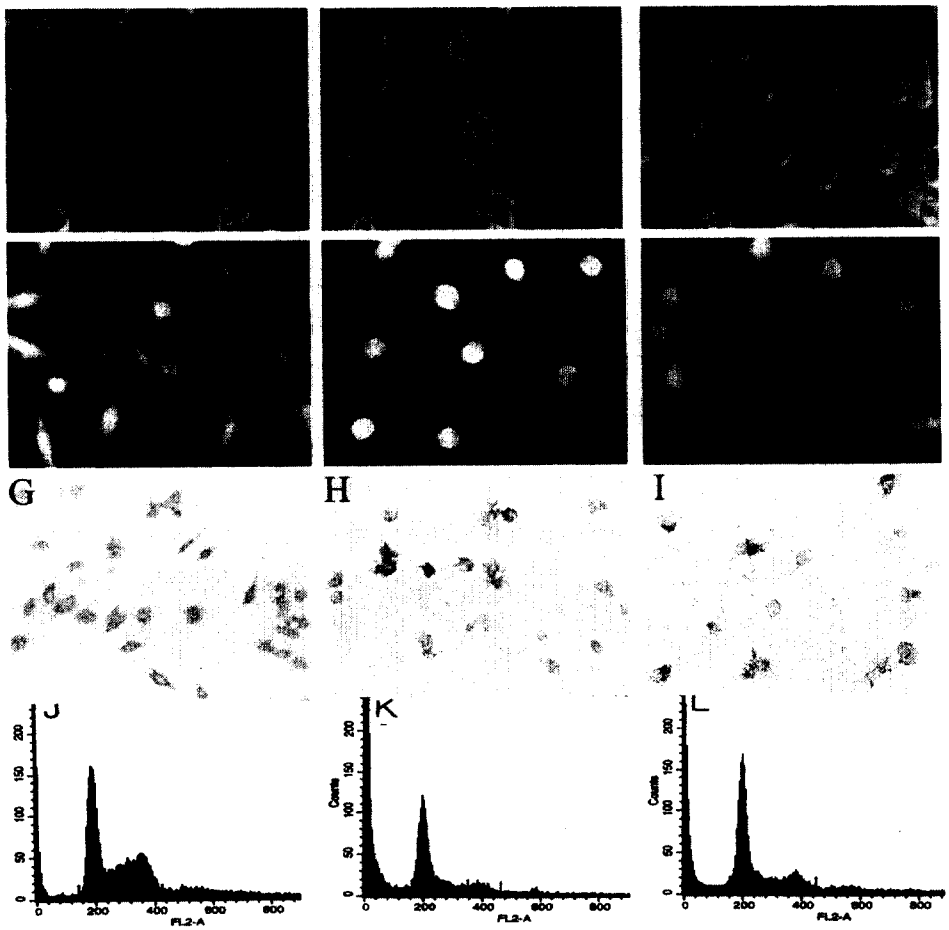

Fig. 4. Effect of Hominis Placenta extracts on the morphology of PGT-β cells. Phase-contrast microscopy revealed much cell shrinkage, retraction of processes, irregularity of shapes (B: H_2O_2), and significantly decreased cell shrinkage, retraction of processes (C: Hominis Placenta extracts), but not in control cultures (A: control). All pictures were taken with 100 x magnification. Scale bars represent 100 μm. Effect of Hominis Placenta extracts on the biochemistry of PGT-β cells. Much condensed nuclei, DNA fragmentation was shown (D: DAPI H_2O_2, G: TUNEL H_2O_2), and significantly decreased condensed nuclei, DNA fragmentation (E: DAPI Hominis Placenta extracts, H: TUNEL Hominis Placenta extracts) but not in control cultures (F: DAPI control, I: TUNEL control). All pictures were taken with 100 x magnification. Scale bars represent 100 μm. Effect of Hominis Placenta extracts on the flow cytometry of PGT-β cells. It was shown that there was an increased proportion of cells in the sub-G1 phase among the H_2O_2-treated cells (K, 58 %) compared to that of the control (J, 11 % of total cells). But in the Hominis Placenta extracts-treated cells the proportion of cells in the sub-G1 phase much decreased compared to that of the H_2O_2-treated cells (L, 44% of total cells).

ptosis, such as cell shrinkage, intracytoplasmic vacuoles, retraction of processes, cytoplasmic condensation, and irregularity in shape, compared to that of the H_2O_2-only treated cells (Fig. 4).

5. Biochemical analyses

Next, to examine the biochemical findings of apoptosis induced by H_2O_2 in PGT-β cells, DAPI staining analysis and TUNEL assay were performed. As shown in Fig. 4, PGT-β cells pretreated with Hominis Placenta extract showed decreased features of apoptosis, such as chromatin condensation and internucleosomal DNA fragmentation, compared to that of the H_2O_2-only treated cells. By flow cytometric analysis, it was shown that PGT-β cells pretreated with Hominis Placenta extract showed decreased proportion of cells in the sub-G_1 phase (44% of total cells) compared to that of the H_2O_2-only treated cells (58% of total cells) and untreated cells (11% of total cells) (Fig. 4).

Ⅳ. Discussion

Since the monoaminergic neurotransmitter systems in the brain play an critical role in the processes of memory and learning[2], we examined the effects of human placental extract as an attempt to elucidate the possible underlying biochemical 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.

It is known that H_2O_2 induces apoptosis in cell cultures and in vivo. It has been reported that pineal gland cells, upon oxidation by superoxide radicals, may produce endotoxins, which may be contributing factors in neurodegeneration[13]. However, the report included no information regarding the prevention of superoxides-induced apoptosis. Apoptosis is a genetically controlled programmed cell death mechanism serving physiologic and homeostatic functions[12]. It is defined by a number of features, including decrease in cell size, condensation of the cytoplasm, blebbing of the plasma membrane, collapse of the chromatin, fragmentation of DNA into oligonucleosome-length and apoptotic bodies. Ultimately, the apoptotic bodies are phagocytosed and degraded by neighboring cells. It is a normal process during development and a morphologically distinct form of cell death which is involved in the pathogenesis and pathophysiology of several known human diseases, such as autoimmune dysfunction, cancer, stroke and neurodegenerative diseases[4].

Free radicals are unstable chemical entities that contain an unpaired electron and are in general very reactive. The reactive oxygen species (ROS), such as H_2O_2, NO and superoxides, are generated even under normal conditions and have been implicated in the pathogenesis of aging, cancer and various diseases including ischemic injury, arthritis and Alzheimer's disease. To date free radicals and

apoptosis are known to perform very important roles in physiological phenomena and the pathophysiology of various diseases.

In this study, we reported that pineal gland tumor cells PGT-β undergo apoptosis upon treatment with pharmacological concentrations of H_2O_2. A dose related pattern was shown in the viability of PGT-β cells according to various concentrations of H_2O_2. From MTT assay, the cytotoxicity of H_2O_2 was apparent at a concentration of 50μM. These results indicate that H_2O_2 induced cell death in PGT-β cells.

Viabilities of cells treated with Hominis Placenta extract for 24 hrs at concentrations of 1.7mg/ml increased compared to that of the untreated cells. These results provide that Hominis Placenta extracts at concentrations of $10^{-2}\mu g/m\ell$ proliferate PGT-β cells. The viability of PGT-β cells pretreated with Hominis Placenta extract for 2hrs before exposure to H_2O_2 for 3hrs was apparent compared to that of the H_2O_2 only treated cells. These results indicate that Hominis Placenta extracts protect H_2O_2-induced cell death in PGT-β cells.

H_2O_2-induced apoptosis of PGT-β cells was determined according to the morphological and biochemical criteria of apoptosis. Through morphological analysis, it was shown that cells undergoing H_2O_2-induced apoptosis exhibit classical visual markers of apoptosis, such as cell shrinkage, cytoplasmic condensation, and irregularity in shape. Biochemical analyses revealed the occurrence of DNA fragmentation and accumulation of cells in the sub-G_1 phase upon treatment with H_2O_2. However, in the PGT-β cells pretreated with Hominis Placenta extract reduced nuclear fragmentation and sub-G_1 phase fraction were observed. Thus, Hominis Placenta extract will be a useful drug in basic studies of the treatment of free radical-related and neurodegenerative diseases.

The definitive molecular mechanism of the preventive action of Hominis Placenta extract are still unknown. However, this study has demonstrated that Hominis Placenta extract prevents H_2O_2-induced apoptotic cell death in PGT-β cells. For the elucidation of the precise molecular mechanism of the preventive effects of Hominis Placenta extract on H_2O_2-induced damage in the human pineal cell lines, further studies involving signal pathways and apoptosis-related genes are called for.

V. Conclusions

To determine whether Hominis Placenta extract prevents H_2O_2-induced apoptosis, we have performed morphological and biochemical analyses for the detection of apoptotic phenomena in the pineal tumor cell line PGT-β. The results as follows:

1. H_2O_2 induced cell death in a dose-dependent pattern.
2. Hominis Placenta extract prevented H_2O_2-induced apoptosis.
3. Hominis Placenta extract decreased occurrence

of apoptotic features than H_2O_2-induced apoptosis by morphological study and biochemical analysis (DAPI staining assay, TUNEL assay and flow cytometric analysis).

VI. References

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