원 저

Effect of Juglans sinensis Dode extract on chemical hypoxia-induced cell injury in human glioma cells

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국문초록

胡桃藥鍼이 人間의 신경교종 細胞에 유발된 低酸素症에 대한 防禦效果

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이 論文은 활성 산소(ROS)의 作用을 규명하고 胡桃藥鍼液이 인간의 신경 교종 세포인 A172에서 化學的 低酸素症으로 유발된 세포 사멸에 대해 효능이 있는지를 硏究한 것이다. 化學的 低酸素症은 세포내 미토콘드리아의 전자 수송을 방해하는 antimycin A를 가진 배양세포에 의해 誘發하였다.

化學的 低酸素症에 노출된 細胞는 시간과 그 양에 따라서 세포 사멸의 結果가 다르게 나타난다. 화학적 저산소증에 의해서 ROS의 생산이 증가하는데 이것은 H_2O_2 消去 Catalase(과산화수소를 물과 산소로 분해하는 효소)에 의해 防止된다. Catalase는 화학적 저산소증에 의해 誘發된 세포 사멸을 방지하는데 비해 DMTU는 효과적이지 않다. 脂質에 녹는 산화방지제 DPPD와 물에 녹는 산화방지제 Trolox는 세포사멸을 방지하는데 效果가 없다. 胡桃藥鍼液은 그 量에 의존적으로 저산소증에 의해 유발된 세포 사멸을 방지하는 효과가 있다. 즉 화학적 저산소증으로 유도된 ROS의 발생을 막고, H_2O_2로 유도된 세포사멸을 방지하는데 이것은 화학적 저산소증과 H_2O_2 의해 유도된 세포사멸에 대해 胡桃藥鍼液이 防止效果가 있다는 것을 의미한다.

이러한 結果들은 H_2O_2가 지질 과산화와는 무관한 메카니즘으로 低酸素症으로 誘發된 세포사멸을 중재하고, 따라서 胡桃藥鍼液은 지질막의 과산화를 방지하기 보다는 ROS를 직접적으로 消去함으로써 방지 효과가 있다는 것을 의미한다. 더구나 化學的 低酸素症은 caspase와 무관한 메카니즘으로 apoptosis를 誘發한다.

Key words : *Juglans sinensis* Dode, Chemical hypoxia, Human glioma cells

* 이 논문은 2002년도 교내 연구비의 지원을 받아 이루어졌습니다
· 접수 : 2003년 1월 7일 · 수정 : 2003년 1월 15일 · 채택 : 2003년 1월 25일
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I. Introduction

Brain ischemia can be resulted from cerebrovascular diseases(transient ischemic attacks, stroke, and atherosclerosis of cerebral arteries), subarachnoid hemorrhage, and trauma. Focal cerebella ischemia still represents a major cause of morbidity and mortality. Reduction in the stores of tissue energy, i.e. glucose and oxygen, appears to be underlying cause of acute neuronal dysfunction induced by ischemia[1]. However, the mechanisms by which glucose deprivation and/or chemical hypoxia result in neuronal cell death have not been clearly understood.

Increased formation of reactive oxygen species(ROS) such as superoxide, hydrogen peroxide, and hydroxyl radical has been postulated to play an important role in the pathogenesis of ischemia-induced neuronal cell injury[1),2)]. Several potential sources of ROS include accumulation of eicosanoids, altered mitochondrial function, neutrophil accumulation, and activation of certain enzymes such as NO synthase and xanthine oxidase[2)].

Since the nervous system have a high content of polyunsaturated fatty acids, which are particularly susceptible to peroxidative attacks by ROS, resulting in lipid peroxidation[3),4)], the peroxidative breakdown of the lipid membrane has been suggested to be an important event in the cascade of reactions leading to neuronal death[5)]. Therefore, attempts have been made to protect cells from the ischemic neuronal injury using pharmacological agents that can scavenge or antagonize ROS[1)]. However, no agents with adequate efficacy in vivo are obtained at present.

In previous studies, we observed that *Juglans sinensis* Dode extract(JS) has a strong antioxidant action[6),7)]. Therefore, the present study was undertaken to determine (1) whether the cell death induced by chemical hypoxia is the results of ROS generation and (2) whether JS exerts the protective effect against the cell death in human glioma cells.

II. Materials and Methods

A. JS preparation

Juglans sinensis Dode was obtained from a local herb store, Pusan City, Korea. Crushed crude drug(300g) was extracted with distilled water at 100℃ for 4 hr and the total extractive was evaporated under reduced pressure to give 15.5g. The dried extract was dissolved in incubation medium just before use.

B. Culture of A172 cells

A172 cells were obtained from the American Type Culture Collection(Rockville, MD) and maintained by serial passages in 75-㎠ culture flasks(Costar, Cambridge, MA). The cells were grown in Dulbecco's modified Ea-

gle's medium(DMEM, Gibco, BRL) containing 10% heat inactivated fetal bovine serum(Gioco, BRL) at 37℃ in humidified 95% air/5% CO_2 incubator. When the cultures reached confluence, subculture was prepared using a 0.02 % EDTA-0.05% trypsin solution. The cells were grown on 24-well tissue culture plates and used 1~2 days after plating when a confluent monolayer culture was achieved.

C. Induction of chemical hypoxia

Chemical hypoxia was induced by incubating cells with antimycin A(AA), the inhibitor of mitochondrial electron transport, in a glucose-free medium as previously described[8].

D. Cell viability assay

The cell viability was evaluated using a MTT assay[9]. Tetrazolium salts such as MTT are metabolized by mitochondrial dehydrogenases to form a blue formazan dye and are therefore useful for the measurement of cell viability. The cells were gently washed with Hanks' balanced salt solution(HBSS, Sigma Co, USA), and exposed to AA. After washing the cells, culture medium containing 0.5mg/ml of MTT was added to each well. The cells were incubated for 2 hr at 37℃, the supernatant was removed and the formed formazan crystals in viable cells were solubilized with 110 μl of dimethyl sulfoxide. A 100 μl aliquot of each sample was then translated to 96-well plates and the absorbance of each well was measured at 550 nm with ELISA Reader(Bio-Tek instrument, EL,311). Data were expressed as a percentage of control measured in the absence of AA. Unless stated otherwise, the cells were treated with 0.1mM AA for 120 min. Test reagents were added to the medium 30min before AA exposure.

E. Measurement of lipid peroxidation

Lipid peroxidation was estimated by measuring total malondialdehyde(MDA) production, utilizing the lipid peroxidation assay kit(Calbiochem). Protein was measured by the method of Bradford[10].

F. Measurement of intracellular H_2O_2 generation

The intracellular generation of H_2O_2 was measured using 2', 7'-dichlorofluorescein diacetate(DCFH-DA)[11),12),13)]. The nonfluorescent ester penetrates into the cells and is hydrolyzed to DCFH by the cellular esterases. The probe(DCFH) is rapidly oxidized to the highly fluorescent compound 2', 7'-dichlorofluorescein(DCF) in the presence of cellular peroxidase and ROS such as hydrogen peroxide or fatty acid peroxides. Cells were cultured in 35-mm tissue culture petri dishes. The culture medium was removed, and the cells were collected from flasks using trypsin-EDTA solution. The cells were preincubated at 37℃ in fluorescent cuvette containing 3ml of glucose-free-HBSS with 20 μM DCFH-DA(from a stock solution of 20mM DCFH-DA in ethanol). After the preincubation, the cells were treated with antimycin A in the presence or absence of JS and incubated up to

30min during which the fluorescent intensity was monitored on a spectrofluorometer(SPEX 1681, SPEX Co., USA) with excitation wave length 485nm and emission wave length 530 nm. The net increase in DCF fluorescence (arbitrary units) was calculated by taking the difference between the values before and after addition of antimycin A.

G. Chemicals

Antimycin A, Trolox, catalase, hydrogen peroxide(H_2O_2), malondialdehyde tetraethylaceta, cisplatin, and Hoechst 33258 were purchased from Sigma Chemical(St. Louis, MO). *N,N'*-diphenyl-*p*-phenylenediamine(DPPD) and dimethylthiourea(DMTU) were purchased from Aldrich Chemical(Milwaukee WI). DCFH-DA was obtained from Molecular Probes (Eagene, OR, USA). All other chemicals were of the highest commercial grade available.

H. Statistical analysis

Data are expressed as mean±SEM. Comparison between two groups was made using the unpaired t test. Multiple group comparison was done using one-way analysis of variance followed by the Dunnett's test. $P<0.05$ were considered statistically significant.

Ⅲ. Results

A. Effects of chemical hypoxia and JS in human glioma cells

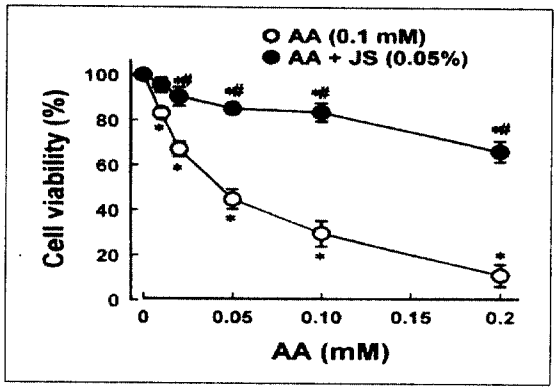

Fig. 1. Dose-dependency of cell death in human glioma cells subjected to chemical hypoxia. Cells were exposed to various concentrations of antimycin A(AA) for 120 min in the presence or absence of 0.05% *Juglans sinensis* Dode extract (JS). The cell viability was measured by a MTT reduction assay. Data are mean±SE of six experiments.

* $p<0.05$ compared with control ;
\# $p<0.05$ compared with AA alone.

In order to determine the potency of antimycin A on cell death, the cells were exposed to various concentratiens of antimycin A for 120min. As shown in <Fig. 1>, antimycin A caused the loss of cell viability over concentration range of 0.01~0.2mM in a dose-dependent manner. A significant loss of cell viability was present at 0.2mM antimycin A and approximately 89% of cells had died at 0.2mM. When cells were treated with antimycin A in the presence of 0.05% JS, however, the cell viability was significantly incr-eased.

<Fig. 2> depicts the time course of chemical hypoxia-induced cell death. The cells were

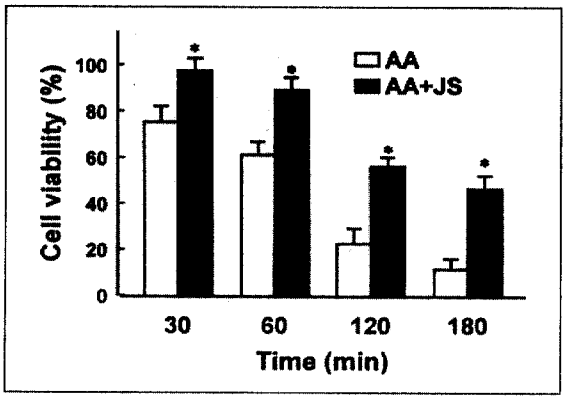

Fig. 2. Time courses of cell death in human glioma cells subjected to chemical hypoxia. Cells were exposed to 0.1 mM antimycin A(AA) for 0–180 min in the presence or absence of 0.05% *Juglans sinensis* Dode extract(JS). The cell viability was measured by a MTT reduction assay. Data are mean±SE of five experiments.

* $p<0.05$ compared with AA alone.

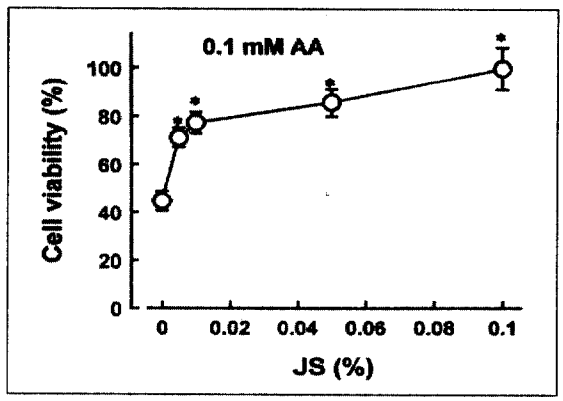

Fig. 3. Protective effect of *Juglans sinensis* Do-de extract(JS) on chemical hypoxia- induced cell death in human glioma cells. Cells were exposed to 0.1 mM antimycin A(AA) for 120 min in the presence or absence of various concentrations of *Juglans sinensis* Dode extract(JS). The cell viability was measured by a MTT reduction assay. Data are mean±SE of four experiments.

* $p<0.05$ compared with the absence of JS.

exposed to 0.1mM antimycin A in the presence or absence of 0.05% JS for 0~180min. Exposure of cells to antimycin A resulted in the loss of cell viability and the significant loss of cell viability was present 30min after exposure, with cell death increasing up to 180min. After 180min, approximately 88% of cells had died. However, addition of JS significantly prevented the chemical hypoxia-induced loss in the cell viability. In subsequent experiments, the cells were treated with 0.1 mM antimycin A for 120min.

B. Dose-dependency of JS protection in chemical hypoxia-induced cell death

In order to determine the potency of the protective effect of JS, the cells were exposed to antimycin A in the presence of various concentrations of JS. As shown in <Fig. 3>, JS prevented the cell death in a dose-dependent manner, with a significant protection at 0.005%. JS did not exert any effect in the control cells untreated with antimycin A(data not shown).

C. Effect of JS on chemical hypoxia-induced ROS generation

In order to determine if chemical hypoxia increases ROS production, we measured the ROS generation in cells subjected to chemical

hypoxia using DCFH-DA. The results are depicted in <Fig. 4>. Exposure of cells to antimycin A induced an increase in the generation of ROS. This increase was significantly blocked by addition of catalase. This may support the notion that hydrogen peroxide is the principle ROS responsible for the oxidation of DCFH to DCF[11),14)]. When cells were exposed to chemical hypoxia in the presence of 0.05% JS, the DCF fluorescence decreased to almost control level, suggesting that JS can scavenge directly H_2O_2. Catalase and JS did not affect DCF fluorescence in control cells (data not shown).

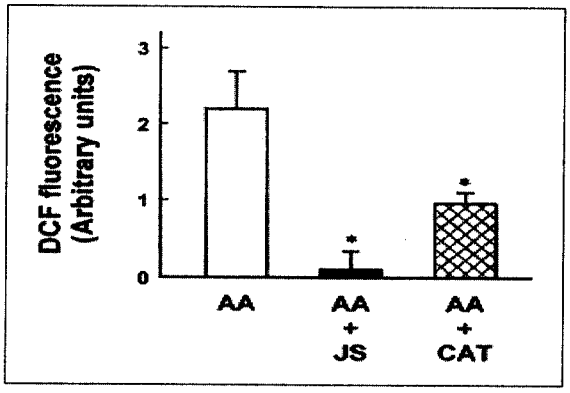

Fig. 4. Effects of catalase(CAT) and *Juglans sinensis* Dode extract(JS) on generation of reactive oxygen species in human glioma cells exposed to chemical hypoxia. The net increase in DCF fluorescence(arbitrary units) was calculated by subtracting the values for the control cells from the corresponding values for antimycin A-treated cells in the presence or absence of CAT (800 Units/ml) and JS(0.05%). Data are mean± SE of four experiments.

* $p<0.05$ compared with AA alone.

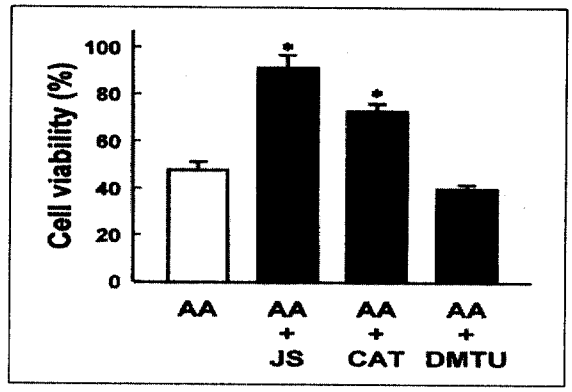

Fig. 5. Effects of reactive oxygen species scavengers on chemical hypoxia-induced cell death in human glioma cells. Cells were exposed to 0.1 mM antimycin A(AA) for 120 min in the presence or absence of 800 Units/ml catalase(CAT), 30 mM dimethylthiourea(DMTU), and 0.05% *Juglans sinensis* Dode extract(JS). The cell viability was measured by a MTT reduction assay. Data are mean±SE of five experiments.

* $p<0.05$ compared with AA alone.

D. Effects of radical scavengers and JS on chemical hypoxia-induced cell death

Chemical hypoxia with antimycin A or cyanide in neuronal cells induces ROS production[15),16),17)], which may mediate cell death[17)]. Thus, to determine if ROS is involved in chemical-induced cell death, the effect of ROS scavengers on the cell viability was examined. As shown in <Fig. 5>, the H_2O_2 scavenger catalase(800 Units/ml) showed a significant protective effect against antimycin A-induced cell death. JS also exerted the protective effect and the extent of the protective effect by 0.05% JS was larger than that of 800 Units/ml

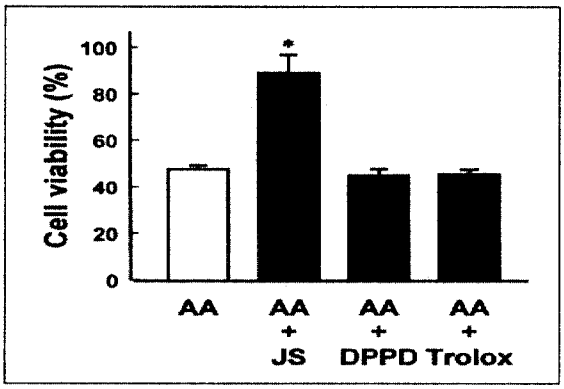

Fig. 6. Effects of antioxidants on chemical hypoxia-induced cell death in human glioma cells. Cells were exposed to 0.1 mM antimycin A(AA) for 120 min in the presence or absence of 20μM DPPD, 0.5 mM Trolox, and 0.05% *Juglans sinensis* Dode extract(JS). The cell viability was measured by a MTT reduction assay. Data are mean±SE of five experiments.

* $p<0.05$ compared with AA alone.

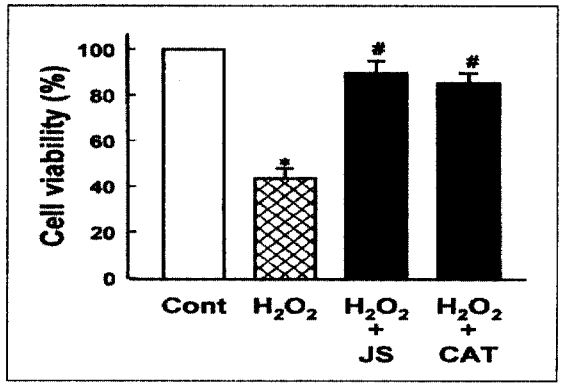

Fig. 7. Effects of catalase(CAT) and *Juglans sinensis* Dode extract(JS) on H_2O_2-induced cell death in human glioma cells. Cells were exposed to 0.5 mM H_2O_2 for 120 min in the presence or absence of 800 Units/ml CAT and 0.05% JS. The cell viability was measured by a MTT reduction assay. Data are mean±SE of five experiments.

* $p<0.05$ compared with control(Cont) ;
# $p<0.05$ compared with H_2O_2 alone.

catalase. However, the hydroxyl radical scaven-ger DMTU failed to protect cells from death.

E. Comparison of antioxidants and JS on chemical hypoxia-induced cell death

In order to clarify if lipid peroxidation is involved in chemical hypoxia-induced cell death and the protective effect of JS is attributed to antioxidant action, the effect of well-known antioxidants on antimycin A-induced cell death was examined and compared with that of JS. The results are summarized in <Fig. 6>. Interestingly, the lipid-soluble antioxidant DPPD and water soluble antioxidant Trolox were not effective in preventing chemical hypoxia-induced cell death, unlike JS, indicating that antimycin A-induced cell death is not associated with lipid peroxidation and the protective effect of JS is not due to antioxidant action.

F. Effect of JS and catalase on H_2O_2-induced cell death

Since JS inhibits H_2O_2 generation induced by chemical hypoxia, it was examined if JS could prevent H_2O_2-induced cell death in human glioma cells. As shown in <Fig. 7>, 0.5 mM H_2O_2 resulted in approximately 60% cell death, which was almost completely prevented by 0.05% JS and 800Units/ml catalase.

Ⅳ. Discussion

The present study demonstrated that chemical hypoxia causes cell death in a dose- and time-dependent manner in human glioma cells <Figs. 1 and 2>. Exposure of cells to antimycin A induced a significant increase in DCF fluorescence, which was inhibited by the H_2O_2 scavenger catalase <Fig. 4>. These results are consistent with the notion that the principle ROS responsible for the oxidation of DCFH to DCF is H_2O_2[14,18]. Catalase also prevented antimycin A-induced cell death <Fig. 5>, suggesting that H_2O_2 is involved in the pathogenesis of chemical hypoxia-induced cell injury in human glioma cells. Involvement of ROS in the cell death induced by chemical hypoxia has been reported in neuronal models intoxicated wit' cyanide[17),19)].

H_2O_2 can be converted to hydroxyl radical, a more potent oxidant, via the Fenton/Haber-Weiss reactions in the presence of iron[20)]. However, the hydroxyl radical scavenger DM TU did not prevent the antimycin A-induced cell injury <Fig. 5>.

Since lipid peroxidation has been considered to be an evidence for oxidant-induced cell injury[21)], the chemical hypoxia-induced cell death may be attributed to lipid peroxidation. However, the lipid soluble(DPPD) and water-soluble(Trolox) antioxiants all were ineffective in preventing the cell death induced by chemical hypoxia <Fig. 6>. These results suggest that the chemical hypoxia-induced cell death is mediated by a lipid peroxidation-independent mechanism in human glioma cells. Although H_2O_2 is responsible for the chemical hypoxia-induced cell death, antioxidants could be not effective. A previous study demonstrated that H_2O_2 induces the cell death via lipid peroxidation-independent mechanism in human glioma cells[22)]. Similar data are reported in cultured endothelial cells[3),4)] and murine fibroblasts[23)].

Juglans sinensis Dode, herbal medicine used in the present study is Walnut in english name. Functions of this herb are nourish the kidney, strengthen the waist and knees, warm the lung to relieve asthma and invigorate the intestines. They are used in the treatment of low back pain, frequent urination, weakness of both legs, chronic cough, asthma, constipation due to dryness or anaemia and stones in the urinary tract, etc. The cotyledons are used in the treatment of cancer. Walnut has a long history of folk use in the treatment of cancer, some extracts from the plant have shown anticancer activity. So aging has something to do with kidney function from the viewpoint of oriental medicine that *Juglans sinensis* Dode have widely been applied as treatment for senile disease such as aging, impotance, low back pain, frequent urination, weakness of both legs, arthritis, Alzheimer's disease and so on, which are closely related with reactive oxygen species[6),7),24)].

In the present study, it was evaluated whether JS prevents chemical hypoxia-induced

cell death in human glioma cells. JS prevented the cell death in a dose-dependent manner <Fig. 3>. In previous study, since JS was observed to exert a strong antioxidant action in renal cells[6),7)], the protective effect of JS could be attributed to its antioxidant action. However, since the antioxidants were not effective in preventing chemical hypoxia-induced cell death in human glioma cells, it is unlikely that antioxidant action of JS is responsible for the protective effect.

The resent study demonstrated that H_2O_2 is involved in the pathogenesis of chemical hypoxia-induced cell injury in human glioma cells, consistent with the data reported in neuronal models intoxicated with cyanide[17),19)]. JS inhibited an increase in DCF fluorescence induced by antimycin A<Fig. 5> and prevented H_2O_2-induced cell death <Fig. 7>. These results indicate that JS exerts the protective effect against the chemical hypoxia-induced cell death by scavenging H_2O_2, like catalase. Although the active ingredient of JS scavenging H_2O_2 remains to be defined, JS may be developed as a drug to prevent hypoxia-induced neurotoxicity in future clinical approaches. Natural plant-derived products continue to play a striking role in all forms of pharmaceutical care[25)].

In conclusion, ROS mediates chemical hypoxia-induced cell death via lipid peroxidation-independent mechanism. JS protected cells from chemical hypoxia-induced cell death by scavenging directly H_2O_2 in human glioma cells.

V. Conclusion

1. *Juglans sinensis* Dode extract(JS) prevented the cell death the chemical hypoxia-induced loss in the cell viability in a dose-dependent manner with a significant protection at 0.005%.

2. JS can scavenge directly H_2O_2 Catalase and JS did not affect DCF fluorescence in control cells.

3. JS and the H_2O_2 scavenger, catalase, showed a significant protective effect against antimycin A-induced cell death. However, the hydroxyl radical scavenger DMTU failed to protect cells from death.

4. The lipid-soluble antioxidant DPPD and water soluble antioxidant Trolox were not effective in preventing chemical hypoxia-induced cell death, unlike JS, indicating that the protective effect of JS is not due to antioxidant action.

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