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木香順氣湯이 인간의 장관상피세포내에서 항산화효과에 미치는 영향

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Effect of Mokhyangsungi-tang (MHS) on antioxidative ability in human intestinal epithelial cells

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목적 : 본 연구는 木香順氣湯이 인간의 장관상피세포 계열인 Caco-2 세포에서 항산화작용을 증진시키는 효과가 있는지 검증하기 위한 실험이다.
방법 : 배양된 인간장관 세포계열인 Caco-2 세포에서 세포의 사망은 trypan blue의 소실정도에 의해 평가했으며 H_2O_2는 표본산화제로 사용되었다.
결과 : H_2O_2에 노출된 세포들은 용량에 비례하여 세포 사망하는 결과를 보였다. 木香順氣湯은 H_2O_2에 의해 유발된 세포사망을 방지하였고, 0.05-1%의 농도범위에 걸쳐서 효과가 극대화되었다. 木香順氣湯과 강력한 항산화제인 DPPD는 H_2O_2에 의해 억제된 SOD의 활성에는 영향을 주지는 못했다. 그러나 H_2O_2에 의해 유발된 catalase, glutathione peroxidase, hydroperoxide 탈취효소의 활성이 감소되는 것을 억제하였다. 또한 H_2O_2에 의해 유발된 glutathione의 감소는 木香順氣湯과 DPPD에 의해 억제되었다. 木香順氣湯은 H_2O_2에 의해 유발된 ATP의 소실을 회복시켰지만 DPPD는 ATP 소실을 회복시키지 못하였다.
결론 : 이러한 결과로 볼 때 Caco-2세포에서 목향순기탕이 세포사망을 억제하는 것은 다른 기전을 통하여 항산화작용을 하는 것으로 볼 수 있다. 따라서 본 연구는 木香順氣湯이 반응성산소기에 의해 유발된 인체 위장관질환의 치료에 사용할 수 있을 가능성을 제시하고 있다.

Key Word : H_2O_2, Mokhyangsungi-tang, Caco-2 cell, antioxidant

I. INTRODUCTION

During aerobic metabolism, cells generate energy by reducing molecular oxygen to water. This reaction which is catalyzed by the mitochondrial enzyme cytochrome c oxidase, involves the transfer of four electrons to oxygen without formation of intermediates. However, partially reduced and more reactive oxygen molecules can be formed under a variety of circumstances that are associated with disease in human. Such activated oxygen species are increasingly recognized to be the mediator of the cell injury in these diseases[1-3].

Reactive oxygen species (ROS) have been implicated in the pathogenesis of various gastrointestinal diseases such as schema-reperfusion injury[4-6], certain types of drug-induced gastroenteropathy[7-10], narcotizing enterocolitis[11], experimental colitis[12-15], and inflammatory bowel diseases[16-19]. Thus, agents that efficiently scavenge ROS may protect the gastrointestinal damage induced by noxious chemicals[20,21].

Mokhyangsungi-tang (MHS), which is prescribed by Luo Tianyi (羅天益) a physician of Yuan (元) Dynasty, author of "Wei Sheng Bao Jian (衛生寶鑑)". Mokhyangsungi-tang is indicated to enhance the function of the stomach and to Regulating Stomach and Qi (和胃理氣), and to check upward adverse flow qi, and for treating symptoms such as abdominal fullness, abdominal distension[4-6], hypochondriac pain[4-7], stuffiness in the chest, oppressed feeling in the chest, constipation, abdomen pain. MHS is one of the most useful drugs and effective treatment for various gastrointestinal diseases.

This study was undertaken to determine whether MHS, an oriental

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medicine, exerts the protective effect against ROS-induced cell death and to examine whether its efficacy was associated with its antioxidant action in small intestine using the human-derived cultured intestinal epithelial cell line Caco-2 as a model. This cell line has been extensively employing in studies to characterize intestinal transport function[22,23].

II. MATERIALS and METHODS

MHS extract preparation

Table 1. Prescription of MHS

HERBAL NAME	SCIENTIFIC NAME	WEIGHT
Mok Hyang(木香)	Costi Radix	30g
Chang Chul(蒼朮)	Atractylodis Rhizone	30g
Cho Du Gu(草豆寇)	Alpiniae Frucyus	30g
Cho Gwa(草果)	Amani Costati Fructus	30g
Hu Bak(厚朴)	Magnoliac Cortex	30g
Cheong Pi(青皮)	Aurantii Pericarpium	30g
Ik Ji In(益智仁)	Amomi Amari fructus	30g
Jin Pi(陣皮)	Aurantii nobilis Pericarpium	30g
Taek Sa(澤瀉)	Alismatis Rhizoma	30g
Baek Bok Ryeong(白茯苓)	Hoelen	30g
Ban Ha(半夏)	Pinelliae Rhizoma	30g
Geon Gang(乾薑)	Zingiberis Rhizoma	30g
O Su Yu(吳茱萸)	Evoidiae Fructus	30g
Dang Gwi(當歸)	Angelicae gigantis Radix	30g
In Sam(人參)	Ginseng Radix	30g
Si Ho(柴胡)	Bupleuri Radix	30g
Seung Ma(升麻)	Cimicifugae Rhizoma	30g
	Total amount	510g

MHS (510 g of crushed crude drugs) was extracted with 3000 ml distilled water at 100°C for 2 hr and the total extract was evaporated under reduced pressure to give 60.4 g. The dried extract was dissolved in Hank's balanced salt solution (HBSS, Sigma Co. USA) just before use.

Culture of Caco-2 cells

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained by serial passages in 75-cm^2 culture flasks (Costar, Cambridge, MA). The cells were grown in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12, Sigma Chemical Co.) containing 10% fetal bovine serum at 37°C in 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 in DMEM/F12 medium containing 10% fetal bovine serum. All experiments started 3-4 days after plating when a confluent monolayer culture was achieved. Cells were treated with hydrogen peroxide (H_2O_2) in HBSS without serum in the presence or absence of MHS.

Measurement of cell death

Cells were grown to confluence in 24-well dishes, incubated in the HBSS containing H_2O_2 for 120 min at 37°C in 5% CO_2, and then harvested using 0.025% trypsin. Cells were incubated with 4% trypan blue solution. Cells failing to exclude the dye were considered nonviable, and the data are expressed as percentage of nonviable cells.

Measurement of superoxide dismutase (SOD) activity

SOD activity was assayed based on alkaline auto-oxidation. After preincubation, tissues were homogenized in a fresh deionized water and centrifuged at 3,000 rpm for 10 min. The 0.25 ml of supernatant was mixed with 0.4 ml of ice-cold extraction reagent (absolute ethanol/chloroform: 62.5/37.5, v/v) and vortex for 30 sec, centrifuged at 3,000 rpm for 5 min. The upper aqueous layer was used to measure absorbance changes at 525 nm by using SOD assay kit (Calbiochem Co. Ltd). Protein concentration was measured according to the method of Bradford[24].

Measurement of catalase activity

Catalase activity was determined with use of a method that tracks the decomposition of H_2O_2 at 240 nm[25]. Cells were homogenized in 50 mM potassium phosphate buffer at pH 7.0 and centrifuged at 12,000 rpm, 4°C for 5 min, the supernatant (0.1 ml) was mixed with 1 % Triton X-100 (0.9 ml) and let it 30 min at room temperature. Then, 0.1 ml of sample

was added to a quartz cuvette that contained 1ml of 59 mM H_2O_2 and 1.9 ml distilled water, absorbance changes were observed for 3 min as described above.

Measurement of glutathione peroxidase activity

The glutathione peroxidase activity was measured by the method of Flohe and Gunzler[26] which uses an oxidation reaction of reduced glutathione by glutathione peroxidase coupled to the disappearance of NADPH by glutathione reductase using hydroperoxide as the substrate. The assay mixture contains 0.5 ml of 0.1 M phosphate buffer (pH 7.0), 1 mM EDTA, 1mM NaN_3, 0.1 ml sample supernatant, 0.1 ml of 100 mM reduced glutathione and 0.1 ml of 2.4 U/ml of glutathione reductase. After preincubation of the mixture for 10 min at 37°C, the reaction was started by adding 0.1 ml of 1.5 mM H_2O_2 and conversion of NADPH to NADP was monitored by a continuous recording of the change of absorbance at 340 nm for 5 min.

Measurment of reduced glutathione (GSH) content

The GSH content was measured using the DTNB-GSSG reductase recycling assay methods[44]. For the measurement of total glutathione (GSH plus oxidized glutathione, GSSG), cells were homogenized in 5 % 5-sulfosalicylic acid and centrifuged at 12,000 g for 5 min. A 0.025 ml aliquot of supernatant was mixed with 0.1 ml of 6 mM DTNB, 0.7 ml NADPH (0.248 mg/ml) dissolved in 6.3 mM EDTA and 143 mM sodium phosphate buffer (pH 7.5) and 0.175 ml water. The reaction was started by the addition of 0.008 ml glutathione reductase and rate of change in absorbance at 412 nm was monitored for 10 min and compared with a standard curve generated using various concentrations of GSH. For the measurement of GSSG, the free GSH was masked by adding 0.002 ml 2-vinylpyridine and 0.006 ml triethanolamine to 0.1 ml supernatant. After 60 min, the samples were assayed as described above using the DTNB recycling assay. The difference between the total glutathione and GSSG was taken as a measure of GSH.

Measurement of ATP content

ATP levels in cells were measured by a luciferin-luciferase assay. After an exposure to oxidant stress, cells were homogenized with 2 ml of 0.1 M perchloric acid containing 0.1% Triton X-100 and placed on ice. The homogenate was then diluted with 10 mM potassium phosphate buffer containing 4 mM $MgSO_4$ (pH 7.4) and 100 l of 20 mg/ml luciferin-luciferase was added to 10μl of diluted sample. Light emission was recorded at 20 s with a luminometer (MicroLumat LB96P, Berthold, Germany). Protein content was determined on an aliquot of cell suspension.

Reagents

NADPH, glutathione reductase, reduced glutathione (GSH), 2-vinylpyridine, and triethanolamine were purchased from Sigma Chemical (St. Louis, MO). N,N' -diphenyl-p-phenylenediamine (DPPD) was purchased from Aldrich Chemical (Milwaukee WI). All other chemicals were of the highest commercial grade available.

Statistical analysis

The data are expressed as mean± SE and the difference between two groups was evaluated using Student' s t-test. A probability level of 0.05 was used to establish significance.

III. RESULTS

Dose dependency of H_2O_2 cytotoxicity and effect of MHS in Caco-2 cells

Fig. 1 depicts the effect of various concentrations of H_2O_2 in the presence or absence of 0.1% MHS. When cells were exposed to 0.1-2 mM H_2O_2 for 120 min, the oxidant increased cell death in a dose-dependent manner. Cell death was 14.63±2.90% and significant statistically compared with the control (4.17±2.20%) at 0.1 mM concentration. When H_2O_2 concentration was increased to 2 mM, cell death was

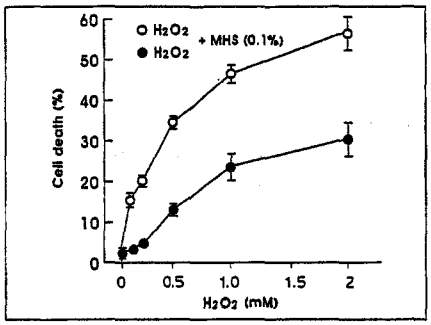

Fig. 1 Dose-dependency of H_2O_2 effect on cell death in Caco-2 cells. Cells were incubated for 120 min in medium containing various concentrations of H_2O_2 in the presence or absence of 0.1% Mokhyangsungi-tang (MHS). Cell death was measured by a trypan blue exclusion assay. Data are mean SE of four experiments.

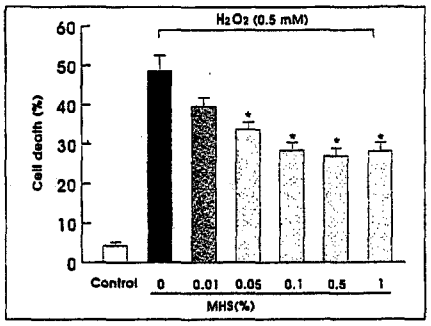

Fig. 2 Dose-dependency of Mokhyangsungi-tang (MHS) effect on H_2O_2-induced cell death in Caco-2 cells. Cells were incubated for 120 min in medium containing 0.5 mM H_2O_2 in the presence or absence of various concentrations of MHS. Cell death was measured by a trypan blue exclusion assay. Data are mean SE of five experiments. *p<0.05 compared with H_2O_2 alone.

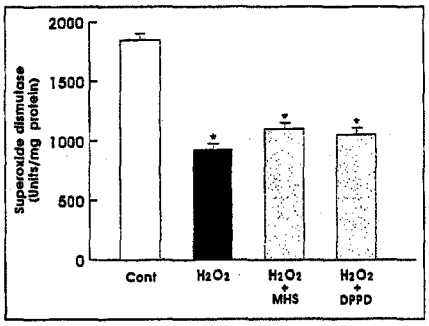

Fig. 3 Effects of Mokhyangsungi-tang (MHS) and N,N' -diphenylphenylene diamine (DPPD) on H_2O_2-induced inhibition of superoxide dismutase activity in Caco-2 cells. Cells were incubated for 120 min in medium containing 0.5 mM H_2O_2 in the presence or absence of 0.1% MHS and 0.01 DPPD. Data are mean SE of five experiments. *p<0.05 compared with the control.

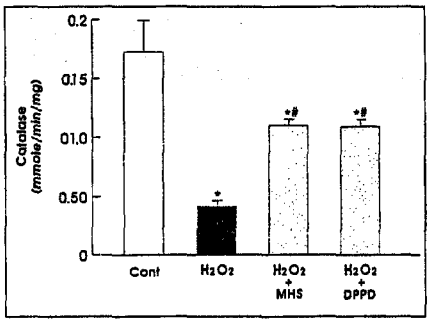

Fig. 4 Effects of Mokhyangsungi-tang (MHS) and N,N' -diphenylphenylene diamine (DPPD) on H_2O_2-induced inhibition of catalase activity in Caco-2 cells. Cells were incubated for 120 min in medium containing 0.5 mM H_2O_2 in the presence or absence of 0.1% MHS and 0.01 DPPD. Data are mean SE of five experiments. *p<0.05 compared with the control; #p<0.05 compared with H_2O_2 alone.

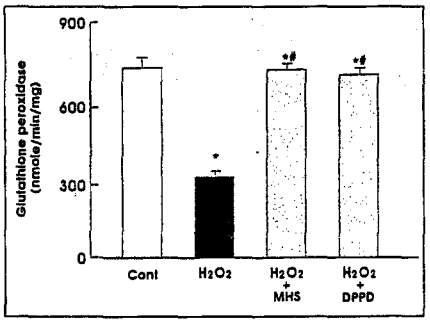

Fig. 5 Effects of Mokhyangsungi-tang (MHS) and N,N' -diphenylphenylene diamine (DPPD) on H_2O_2-induced inhibition of glutathione peroxidase activity in Caco-2 cells. Cells were incubated for 120 min in medium containing 0.5 mM H_2O_2 in the presence or absence of 0.1% MHS and 0.01 DPPD. Data are mean SE of five experiments. *p<0.05 compared with the control; #p<0.05 compared with H_2O_2 alone.

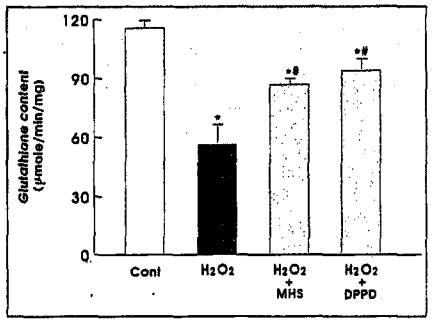

Fig. 6 Effects of Mokhyangsungi-tang (MHS) and N,N' -diphenylphenylene diamine (DPPD) on H_2O_2-induced reduction of glutathione content in Caco-2 cells. Cells were incubated for 120 min in medium containing 0.5 mM H_2O_2 in the presence or absence of 0.1% MHS and 0.01 DPPD. Data are mean SE of five experiments. *p<0.05 compared with the control; #p<0.05 compared with H_2O_2 alone.

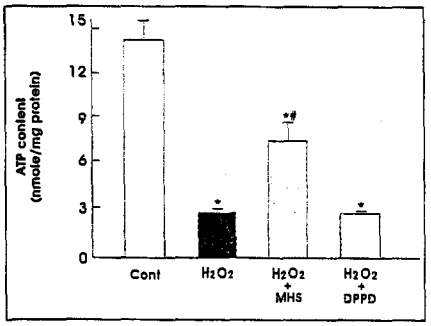

Fig. 7 Effects of Mokhyangsungi-tang (MHS) and N,N' -diphenylphenylene diamine(DPPD) on H_2O_2 -induced ATP depletion in Caco-2 cells. Cells were incubated for 120 min in medium containing 0.5 mM H_2O_2 in the presence or absence of 0.1% MHS and 0.01 DPPD. Data are mean SE of five experiments. *p<0.05 compared with the control; #p<0.05 compared with H_2O_2 alone.

57.05 3.61%. However, H_2O_2-induced cell death was significantly prevented by addition of 0.1% MHS.

Dose-dependency of protective effect of MHS against H_2O_2-induced cell death in Caco-2 cells

In order to evaluate dose-dependency of the protective effect of MHS, H_2O_2-induced cell death was examined in the presence of various concentrations of MHS. The cell death was significantly prevented by MHS of concentrations higher than 0.05%. Cell death was 46.83±3.98% in cells treated with 0.5 mM H2O2, which was decreased to 30.09 3.15% by 0.05% MHS. When MHS concentrations were 0.1 and 0.5%, cell death was 25.93±2.09 and 23.88 3.01%, respectively. However, MHS concentration was increased to 1%, the protective effect was not increased compared with 0.5% (Fig. 2).

Effect of MHS on H_2O_2-induced alterations in antioxidant enzyme activity

In order to determine whether the protective effect of MHS on oxidant-induced cell injury is due to changes in activities of antioxidant enzymes, the effects of oxidants and MHS on activities of antioxidant enzymes were examined in cells exposed to H_2O_2 in the presence or absence of MHS. The effect of MHS was compared with that of a potent antioxidant DPPD.

1) Superoxide dismutase activity

Exposure of cells to 0.5 mM H_2O_2 resulted in a significant inhibition of superoxide dismutase activity, a scavenging enzyme of superoxide. The activity in the control was 1744.70±76.60 Units/mg protein and decreased to 906.89±54.39 Units/mg protein in the presence of 0.5 mM H_2O_2. The effect of H_2O_2 on the superoxide dismutase activity was not affected by addition of MHS (Fig. 3). Similar results were observed by the antioxidant DPPD. The concentration of DPPD used in the present study was similar to or higher than those that prevent completely oxidant-induced cell injury in renal proximal tubular cells[27] and renal cortical slices[28].

2) Catalase and glutathione peroxidase activity

Catalase and glutathione peroxidase catalyze the reduction of H_2O_2 to water and oxygen[29]. Thus, if MHS increases activities of these enzymes, H_2O_2-induced cell injury would be prevented. To test this possibility, alterations in activities of these enzymes were examined in cells exposed to H_2O_2 and MHS. Exposure of cells to 0.5 mM H_2O_2 resulted in a marked reduction in the catalase activity from 0.172 0.03 to 0.035 0.01 mmole/min/mg, which returned to 0.102 0.01 mmole/min/mg in the presence of MHS. H_2O_2-induced inhibition of the enzyme activity was also prevented by DPPD (0.101 0.01 mmole/min/mg) (Fig. 4).

The activity of glutathione peroxidase enzyme in H_2O_2-treated cells was 356.48 35.42 nmole/min/mg, which was approximately 48% of the control (737.41±57.52 nmole/min/mg. Such changes were significantly restored by MHS and DPPD to 3.97±0.38 mmole/mg protein/min (Fig. 5).

Intracellular reduced glutathione (GSH) is an essential component of cellular defense against oxidants and various other types of toxic and pathologic stresses in various cell types including liver cell[30-32]. Thus, alterations in GSH content were examined in cells exposed to H_2O_2 in the presence or absence of MHS. As shown in Fig. 6, exposure of cells to 0.5 mM H_2O_2 resulted in a reduction of the GSH content (54.29±9.23 vs. 112.44±5.50 mole/mg protein in the control). However, the decline of

GSH content by H_2O_2 was attenuated by MHS (81.29±2.46 mole/mg protein). Similar a protective effect was observed by DPPD.

Effect of MHS on H_2O_2-induced ATP depletion

It has been demonstrated that oxidants cause ATP depletion as an early response in various cell types[33-35]. In the present, the effect of MHS on oxidant-induced ATP depletion was examined. As shown in Fig. 7, H_2O_2 at 0.5 mM reduced ATP concentration to approximately 21% of control (2.90±0.27 vs. 13.77±1.72 nmole/mg protein in the control). When cells were treated with H_2O_2 in the presence of 0.1% MHS, ATP content returned to 6.44±1.17 nmole/mg protein. However, the H_2O_2-induced ATP depletion was not altered by DPPD (2.39±0.05 nmole/mg protein).

IV. DISCUSSION

Reactive oxygen species (ROS), in particular the hydroxyl radical, can react with all cell macromolecules such as lipids, proteins, nucleic acids, and carbohydrates[36] ROS are constant products of normal aerobic cell metabolism. Thus, auto-oxidation of various organic substances such as hydroquinones produces super oxide anion. Membrane -bound cytochromes (P450 and b5) located in the endoplasmic reticulum generate super oxide anion by auto-oxidation. The cytochromcatalyzed generation of superoxide anion from oxygen is particular active in an environment in which the concentrations of electron donor, such as NADPH and NADH, are relatively high. Arachidonic acid metabolisms of cytooxygenase and 5-lipoxygenase pathways, which are involved in a variety of fundamental cell functions, also produce ROS. However, cells, which are under constant threat of the toxic effect of ROS, are furnished with highly effective devices to eliminate these toxic oxygen species. A major mechanism of this elimination is the antioxidant enzyme cascade[37] Super oxide dismutase (SOD), catalase, and glutathione peroxidase serve to eliminate primary products of partially reduced oxygen[45].

Mokhyangsungi-tang (MHS), which is prescribed by Luo Tianyi (羅天益) a physician of Yuan (元) Dynasty, author of "Wei Sheng Bao Jian (衛生寶鑑)". MHS is indicated to enhance the function of the stomach and to Regulating Stomach and Qi (和胃理氣), and to check upward adverse flow qi, and for treating symptoms such as abdominal fullness, abdominal distension, hypochondriac pain, stuffiness in the chest, oppressed feeling in the chest, constipation, abdomen pain. Mokhyangsungi-tang is one of the most useful drugs and effective treatment for various gastrointestinal diseases. Mok Hyang(木香), Hu Bak(厚朴), Cheong Pi(青皮), Jin Pi(陣皮), which are the compents of MHS have been used to promote circulation, to smooth the flow of qi and digestion. Cho Du Gu(草豆寇), Ik Ji In(益智仁) used to remove food stagnancy and soothe the spleen. Chang Chul(蒼朮), Ban Ha(半夏), which are the compents of MHS have been used to pomote digestion, to remove dampness and phlegm. Geon Gang(乾薑) and O Su Yu(吳茱萸) used to dispel cold and warm the lower-jiao. Baek Bok Ryeong(白茯苓), Taek Sa(澤瀉), which are the components of MHS have been used to diuretic turbidity yin. Seung Ma(升麻), Si Ho(柴胡) have been used to promote clarity yang, Dang Gwi(當歸) used to nourish the blood for qi[48].

MHS is indicated in the treatment of patients with anorexia, constipation, abdominal pain, abdominal distension due to hypo function of the spleen and stomach with obstruction in the channels.

The present study demonstrated that MHS exerts protective effect against H_2O_2-induced cell death in human intestinal cells using Caco-2 cells. Such beneficial effects were associated with enhancement of activities of catalase and glutathione peroxidase but not superoxide dismutase. MHS also increased oxidant -induced reduction of GSH. The protective effect of MHS was similar to that of DPPD, a potent antioxidant. These results suggest that MHS exerts the protective effect against H_2O_2-induced cell death in

Caco-2 cells. However, the effect of MHS on oxidant-induced ATP depletion was different from that of DPPD. MHS prevents significantly H_2O_2-induced ATP depletion, but DPPD did not affect the ATP depletion. Therefore, MHS may prevent H_2O_2-induced cell death through a mechanism other than protection of lipid peroxidation. Biological membranes contain a large amount of polyunsaturated fatty acids, which are particularly susceptible to peroxidative attacks by oxidants, resulting in lipid peroxidation[38,39]. Therefore, lipid peroxidation has been used as indirect marker of oxidant-induced cell injury[1]. However, whether lipid peroxidation is involved in the pathogenesis of oxidant-induced cell injury in renal tubules remains controversial. Previous studies suggested that lipid peroxidation plays a critical role in oxidant-induced renal cell injury[40,41], but the present study showed that H_2O_2-induced ATP depletion is not altered by DPPD. Dissociation of lipid peroxidation from oxidant-induced cell injury has been shown in other studies[1,42,43].

In the present study, MHS prevented H_2O_2-induced inhibition of activities of catalase and glutathione peroxidase. These enzymes induce reduction of inorganic and organic peroxides. Thus, MHS exerts the protective effect against H_2O_2-induced cell death by scavenging the oxidant. Although the precise mechanism of the MHS protection remains to be explored, the results of the present study suggest that MHS may be useful in treatment and prevention of gastrointestinal injuries mediated by ROS.

V. CONCLUSION

This study was undertaken to determine whether Mokhyangsungi-tang (MHS) increases the antioxidative ability in Caco-2 cells, a human intestine cell line. Cell death was evaluated by trypan blue exclusion. Hydrogen peroxide (H_2O_2) were used as a model oxidant.

1. Exposure of cells to H_2O_2 resulted in the cell death in a dose-dependent fashion. MHS prevented H_2O_2-induced cell death over concentration range of 0.05-1%.

2. H_2O_2 inhibited the activity of superoxide dismutase, which was not affected by MHS and a potent antioxidant N,N' -diphenyl-p-phenylenediamine (DPPD).

3. MHS and DPPD prevented H_2O_2-induced inhibition of activities of catalase and glutathione peroxidase, the enzymes scavenging hydroperoxides.

4. H_2O_2 decreased reduced glutathione content and its effect was prevented by MHS and DPPD.

5. MHS restored significantly ATP depletion by H_2O_2, but DPPD did not affect the ATP depletion.

These results indicate that MHS prevents cell death induced by oxidants in Caco-2 cells possibly by a mechanism other than an antioxidant action. In addition, the present study suggests that MHS may play a therapeutic role in the treatment of human gastrointestinal diseases mediated by ROS.

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