

Role of mitochondrial dysfunction in hydrogen peroxide-induced apoptosis of intestinal epithelial cells

Jian-Ming Li, Hong Zhou, Qian Cai, Guang-Xia Xiao

Jian-Ming Li, Hong Zhou, Qian Cai, Guang-Xia Xiao, Institute of Burn Research, Southwest Hospital, Third Military Medical University, Chongqing 400038, China

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Correspondence to: Professor Hong Zhou, Institute of Burn Research, Southwest Hospital, Third Military Medical University, Chongqing 400038, China. zhoh64@mail.tmmu.com.cn

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Abstract

AIM: To study the role of mitochondrial dysfunction in hydrogen peroxide-induced apoptosis of intestinal epithelial cells.

METHODS: Hydrogen peroxide-induced apoptosis of human intestinal epithelial cell line SW-480 was established. Cell apoptosis was determined by Annexin-V and PI double-stained flow cytometry and DNA gel electrophoresis. Morphological changes were examined with light and electron microscopy. For other observations, mitochondrial function, cytochrome c release, mitochondrial translocation and membrane potential were determined simultaneously.

RESULTS: Percentage of apoptotic cells induced with 400 $\mu\text{mol/L}$ hydrogen peroxide increased significantly at 1 h or 3 h after stimulation and recovered rapidly. Meanwhile percentage of apoptotic cells induced with 4 mmol/L hydrogen peroxide increased with time. In accordance with these changes, we observed decreased mitochondrial function in 400 $\mu\text{mol/L}$ H_2O_2 -stimulated cells at 1 h or 3 h and in 4 mmol/L H_2O_2 -stimulated cells at times examined. Correspondingly, swelling cristae and vacuole-like mitochondria were noted. Release of cytochrome c, decreased mitochondrial membrane potential and mitochondrial translocation were also found to be the early signs of apoptosis.

CONCLUSION: Dysfunctional mitochondria play a role in the apoptosis of SW-480 cell line induced by hydrogen peroxide.

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INTRODUCTION

Hidden injuries of gut during the early stage of severe burn may contribute to early translocation of bacteria or its endotoxin. Although the mechanisms of gut barrier dysfunction postburn are unclear^[1-10], evidences recently indicate that apoptosis of intestinal epithelial cells after thermal injury may be one of possible factors leading to gut barrier dysfunction^[11,12]. Apoptosis of intestinal epithelial cells induced by excessive reactive oxygen

species released by activated polymorphonuclear leukocytes and vascular endothelial cells plays a role in the pathogenesis of dysfunction of intestinal mucosa. Besides, the role of mitochondrion in the development of apoptosis has been emphasized recently^[13]. We have found that differential expression of mitochondrial genes encoding cytochrome c oxidase and ATPase was involved in apoptosis of intestinal epithelial cells by affecting activities of cytochrome c oxidase and ATPase^[14]. So mitochondrial dysfunction may contribute to the apoptosis of intestinal epithelial cells. In the present study, possible relationship between mitochondrial dysfunction and apoptosis was studied in hydrogen peroxide-induced apoptosis model of SW-480 cells.

MATERIALS AND METHODS

Cell line and culture

Human intestinal epithelial cell line SW-480 stored routinely in our laboratory was cultured in RPMI1640 supplemented with 10 % (V/V) heat-inactivated newborn calf serum (Hyclone), 100 units/ml of penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine at 37 °C in a humidified 5 % CO_2 incubator. Confluent cells were prepared for further studies.

Treatment

Experimental cells were treated with 4 mmol/L or 400 $\mu\text{mol/L}$ hydrogen peroxide. Cells without stimulation by hydrogen peroxide were prepared as control.

Transmission electron microscopy

Samples were fixed, embedded and sectioned routinely. Ultrastructural changes of mitochondria were observed with transmission electron microscopy.

Assessment of apoptosis by Annexin-V and PI double-staining flow cytometry

Annexin-V and PI double staining kit (Roche) was used to assess apoptosis in hydrogen peroxide-stimulated SW-480 cells. Ten thousands of cells were counted, and data acquisition and analysis were performed in a Becton Dickinson FACS-can flow cytometer using the CellQuest software.

DNA fragmentation analysis

The DNA fragmentation pattern (DNA ladder) was demonstrated by agarose gel electrophoresis described previously^[15].

Determination of cytochrome c release by cell immunocytochemistry

Cells were grown on glass cover slips. After treated with 4 mmol/L or 400 $\mu\text{mol/L}$ hydrogen peroxide, samples were fixed in 10 % neutral formaldehyde solution for 30 min with PBS rinsing for several times. Then, cells were stained by overnight incubation with 100-fold diluted rabbit anti-human cytochrome c polyclonal antibody (Oncogene) at 4 °C, followed by extensive washing with phosphate-buffered saline and a 30 min incubation with biotin-binding goat anti-rabbit antibody. After another 30 min incubation with horseradish peroxidase conjugated ovalbumin, the specimens were colorized and photographed.

MTT assay

Mitochondrial function was assessed by MTT (3, (4,5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide) assay as described previously^[16]. Cells were cultured in 96-well plates 5 000 cells for each well at 37 °C in a humidified 5 % CO₂ incubator. Confluent cells were prepared for further studies. After treated with hydrogen peroxides and washed with phosphate-buffered saline, cells were incubated with MTT (2 µg/ml, Sigma) and RPMI1640 medium without serum at 37 °C for 1 h and dissolved with dimethyl sulfoxide. The absorbance at 570nm, which represented the total mitochondrial function, was recorded.

Measurement of mitochondrial membrane potential

Cells were grown on glass cover slips. After treated with 4 mmol/L or 400 µmol/L hydrogen peroxide, cells were incubated with Rhodamine 123 (1 µmol/L, Molecular probe) and RPMI1640 medium without serum at 37 °C for 30 min, fluorescence intensity was determined by confocal microscope (Leica) with fixed parameters, cells in three random-selected visual fields from each group were scanned and analysed.

Mitochondrial translocation assay

As described previously, cells were seeded in chambered cover slips and preincubated overnight at 37 °C in a humidified 5 % CO₂ air incubator. After the cells were treated with hydrogen peroxide, mitochondria were stained with Rhodamine 123 for 30 min at 37 °C before analysis. The distribution of mitochondria was analyzed with a Zeiss TSTN confocal microscope^[17].

Statistical analysis

Data were summarized as mean ±SD. Student's *t* test was used for multiple comparisons between groups. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Effects of hydrogen peroxide on apoptosis of SW-480 cells

Percentage of apoptotic cells induced with 400 µmol/L hydrogen peroxide increased significantly at 1 h or 3 h after stimulation and recovered rapidly. Meanwhile percentage of apoptotic cells induced with 4 mmol/L hydrogen peroxide increased with the time, which indicated that the irreversible changes had taken place (Table 1).

Table 1 Percentage of apoptotic cells induced with hydrogen peroxide at different time after stimulation

Group	0 h	1 h	3 h	6 h	12 h	24 h
400 µmol/L	10.93±0.63	19.47±0.36 ^a	19.81±1.82 ^a	12.32±1.67	13.61±1.24	12.72±0.72
4 mmol/L	10.93±0.63	20.84±1.47 ^a	32.25±1.37 ^a	39.48±4.26 ^a	57.91±9.82 ^a	69.05±11.62 ^a

^a*P*<0.05 vs preceding time point (0 h).

Table 2 MTT absorbance (570nm) of SW-480 cells stimulated by hydrogen peroxide

Group	1 h	3 h	6 h	12 h	24 h
Control	1.971±0.101	1.996±0.013	1.867±0.008	2.087±0.126	2.189±0.178
4mmol/L	0.864±0.116 ^a	0.756±0.023 ^a	0.612±0.006 ^a	0.518±0.035 ^a	0.373±0.043 ^a
400mmol/L	1.588±0.005	1.277±0.300 ^a	1.778±0.098	1.599±0.214	1.899±0.031
200mmol/L	1.626±0.262	1.914±0.046	1.941±0.032	1.787±0.033	1.962±0.149
100mmol/L	1.683±0.070	1.973±0.048	1.933±0.094	1.901±0.097	2.079±0.081
50mmol/L	1.865±0.122	1.974±0.080	2.077±0.077	1.876±0.053	1.922±0.048

^a*P*<0.05 vs control at corresponding time point.

DNA fragmentation analysis

DNA ladder in both 4 mmol/L and 400 µmol/L H₂O₂-stimulated groups were clearly observed by DNA fragmentation assay at 1h or 3h after stimulation (Figure 1).

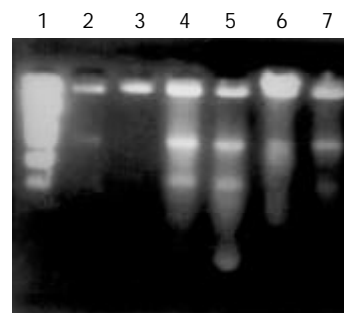


Figure 1 Hydrogen peroxide induced apoptosis of SW-480 cells. 1: PBR322/Hinf I (75, 154, 220, 221, 298, 344, 396, 504, 517, 1632); 2, 3: cells from normal control; 4: 4 mmol/L H₂O₂-stimulated cells (1 h); 5: 4 mmol/L H₂O₂-stimulated cells (3h); 6: 400 µmol/L H₂O₂-stimulated cells (1 h) 7: 400 µmol/L H₂O₂-stimulated cells (3 h).

MTT assay

The MTT assay is based on the conversion of MTT (light yellow) to formazan (blue) by the mitochondrial enzyme succinate dehydrogenase and has been widely used as an indicator of cellular respiration and viability^[3]. We observed decreased mitochondrial function in 400 µmol/L H₂O₂-stimulated cells at 1 h or 3 h after stimulation and in 4 mmol/L H₂O₂-stimulated cells at times examined (Table 2). Interestingly, these changes were in accordance with the apoptosis induced by hydrogen peroxide.

Ultrastructural changes of mitochondria

Swelling cristae and vacuole-like mitochondria were found in hydrogen peroxide -treated cells.

Translocation of mitochondria

Mitochondria were observed to be evolved from an originally scattered, bipolar and nearly symmetric distribution to the asymmetric clustered state in the majority of cells treated with H₂O₂ (Figure 2).

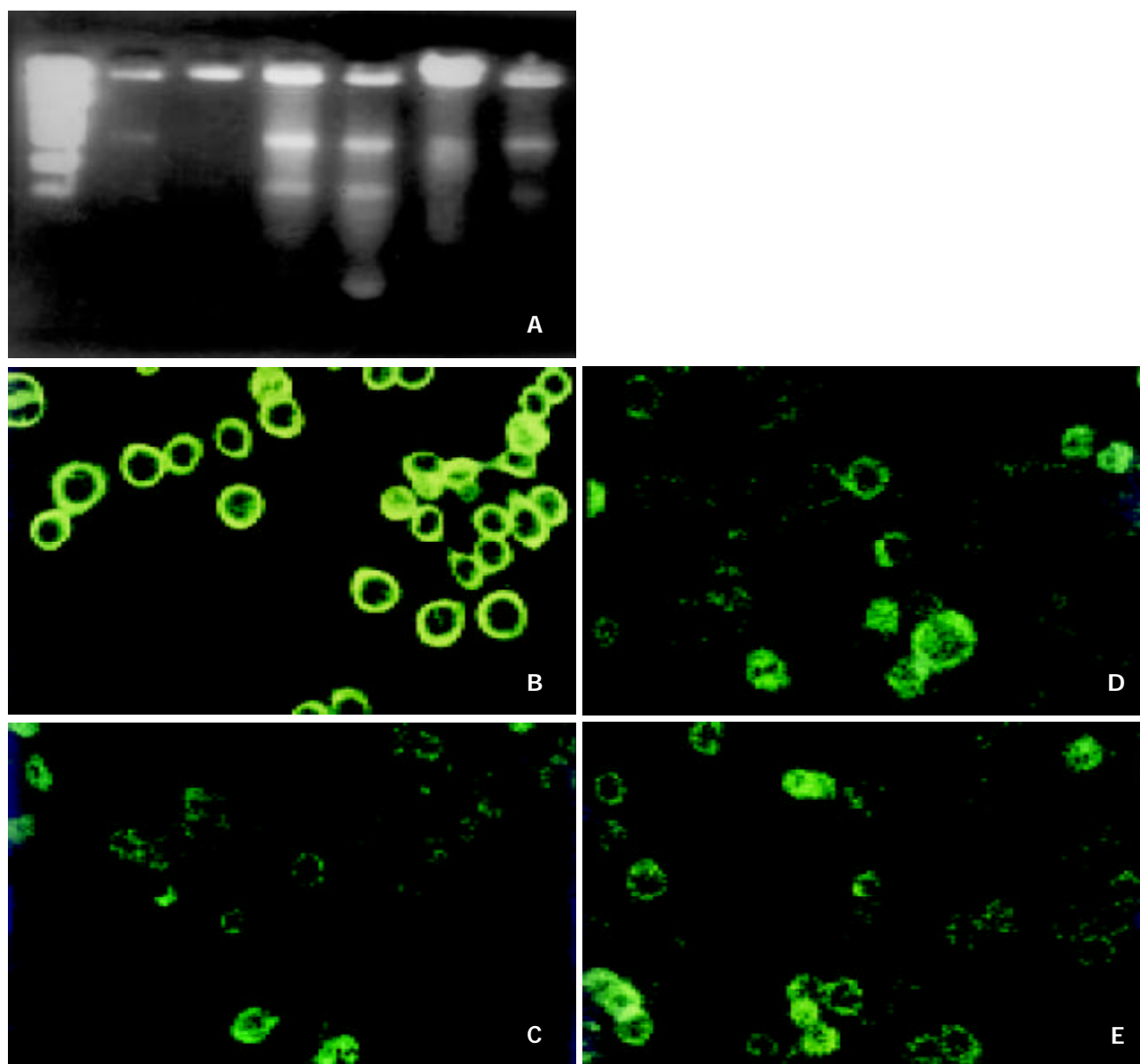


Figure 3a Changes of mitochondrial membrane potential induced by hydrogen peroxide. (A) cells from normal control; (B) 400 $\mu\text{mol/L}$ H_2O_2 -stimulated cells (1 h); (C) 400 $\mu\text{mol/L}$ H_2O_2 -stimulated cells (3 h); (D) 4 mmol/L H_2O_2 -stimulated cells (1h); (E) 4 mmol/L H_2O_2 -stimulated cells (3 h).

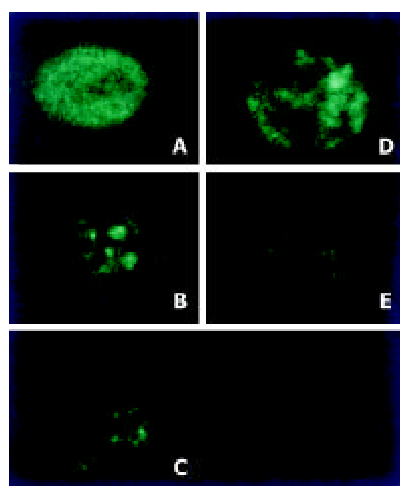


Figure 2 Hydrogen peroxide induced translocation of mitochondria. (A) cells from normal control; (B) 400 $\mu\text{mol/L}$ H_2O_2 -stimulated cells (1 h). (C) 400 $\mu\text{mol/L}$ H_2O_2 -stimulated cells (3 h); (D) 4 mmol/L H_2O_2 -stimulated cells (1 h); (E) 4 mmol/L H_2O_2 -stimulated cells (3 h).

Changes of mitochondrial membrane potential induced by hydrogen peroxide

Decreased mitochondrial membrane potential was observed in cells at 3 h after 400 $\mu\text{mol/L}$ H_2O_2 stimulation and at 1h after 4 mmol/L H_2O_2 treatment (Figure 3a, 3b).

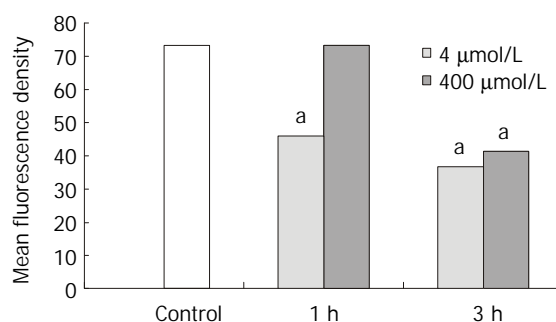


Figure 3b Effects of hydrogen peroxide on mitochondrial membrane potential of SW-480 cells (* $P < 0.05$ vs control).

Cytochrome c Release

Cytochrome c release could be found in both 400mmol/L and 4mmol/L H₂O₂-stimulated cells 30 min after stimulation by immunochemistry assay (Figure 4).

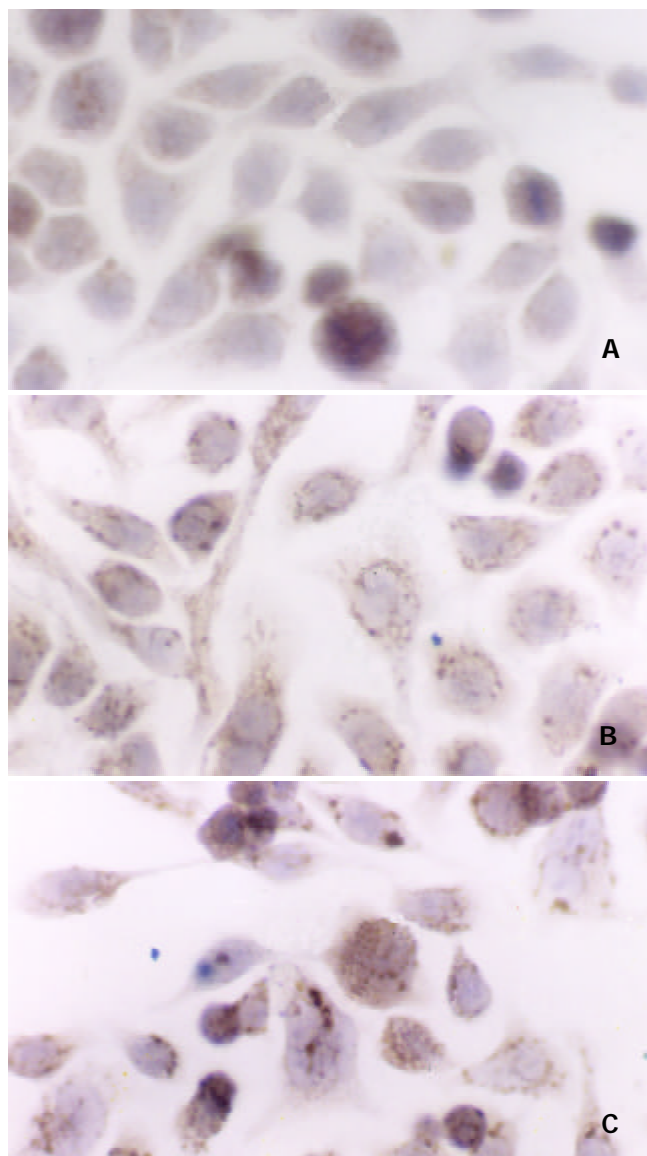


Figure 4 Cytochrome c release in SW-480 cells induced by hydrogen peroxide (S-P×400). A: control cells, B: 400 μmol/L H₂O₂-treated cells (30 min), C: 4 mmol/L H₂O₂-treated cells (30 min).

DISCUSSION

Metabolism of intestinal mucosal epithelium is so active that it is very sensitive to changes of energy supplies in normal conditions^[11]. Many researches have demonstrated that gut is a sensitive organ to be injured postburn^[18-22]. Intestinal mucosal injury can be caused by excessive reactive oxygen species (ROS) released by polymorphonuclear leukocytes and vascular endothelial cells, which is also involved in translocation of intestinal bacteria and its endotoxin.

Our results showed that hydrogen peroxide could lead to injuries of intestinal epithelial cells in the concentration of both 400 μmol/L and 4 mmol/L. From the results of DNA ladders and flow cytometry, apoptosis could be considered one of the main mechanisms for the injury. This indicated that the *in vitro* model of hydrogen peroxide-stimulated SW-480 cells used in the present study could be used to investigate mitochondrial dysfunction in apoptosis of intestinal epithelial cells, and it

also might be helpful to study the role of mitochondria in ROS-induced injuries of intestinal epithelial cells and to clarify mechanism of gut barrier dysfunction.

Role of mitochondria in the pathogenesis of apoptosis has been well defined^[23-27]. Mitochondria, a kind of organelle controlling growing, breeding and dying of eukaryocyte, perform their functions by production of ATP, production of ROS, ROS are also known as signals regulating gene expression and triggering of cell death^[28,29]. Many stimulators like ROS, Ca²⁺ and cytokines could activate caspases by inducing cytochrome c release.

Our results showed that the apoptotic cells were characterized with swelling or vacuole-like mitochondria. It was considered previously that the apoptotic cells manifested condensed chromatin but intact mitochondria. Now much more evidences found that significant changes of mitochondria such as swelling, megamitochondria^[30], mitochondrial pyknosis and disrupted out-membrane have been taken place in many apoptosis models^[31]. Mitochondrial pyknosis was characterized with decreased size and condensed matrix of mitochondria. In apoptotic model of sympathetic neuron triggered by nerve growth factor (NGF) deprivation, the transition from normal to condensed morphology could be reversible following readdition of NGF to the neuron culture^[25]. In addition, the mitochondrial distribution within cells was profoundly affected during apoptosis. Mitochondria were normally dispersed throughout the entire cell. However, during the apoptosis triggered by tumor necrosis factor (TNF-α) a perinuclear clustering of mitochondria could be observed. Both mitochondrial condensation and perinuclear clustering occurred following production of the Bcl-2-related proapoptotic protein Bax in many cell types^[25].

Evidence showed that the spatial distribution of mitochondria evolved from an originally scattered, bipolar or nearly symmetric distribution to an asymmetric, clustered distribution in the majority of the cells within 1 h of treatment of L929 cells with TNF^[17]. Study also indicated that hydrogen peroxide could not lead to the mitochondrial translocation as TNF did^[17]. Interestingly, we found that hydrogen peroxide could induce mitochondrial translocation and massive aggregation by confocal microscopy and 3-D reconstruction technique, which was accompanied by decrease of mitochondrial membrane potential. So our results suggested that mitochondrial translocation may play a role in reactive oxygen species (ROS)-induced injuries of intestinal epithelial cells. As some researches proposed, the condensation of mitochondria may play roles in the inducing of cytochrome c release, generating high ATP levels in energy dependent apoptotic events and facilitating the translocation of mitochondrial proteins to the nucleus. Its mechanism is still uncertain.

The mitochondrial transmembrane potential has been found to be decreased in many apoptosis models, which indicates the opening of a large conductance channel known as the mitochondrial PT pore^[32-36]. PT pore opening results in a volume dysregulation of mitochondria due to the hyperosmolality of the matrix, which causes the matrix space to be expanded. Because the mitochondrial inner membrane with its folded cristae possesses a larger surface area than the outer membrane, this matrix volume expansion can eventually cause the outer membrane rupture, releasing caspase-activating proteins located within the intermembrane space into the cytosol.

We observed that hydrogen peroxide could result in the collapse of mitochondrial membrane potential, if we related it with the release of cytochrome c, we may get the conclusion that hydrogen peroxide caused the release of cytochrome c from mitochondria to cytosol followed by the increased permeability mitochondrial membrane and the opening of mitochondrial

PT pore, which initiated cascade reaction of apoptosis events. This idea has been confirmed by some studies^[37,38].

Recent progress in studies on apoptosis has revealed that cytochrome c is a pro-apoptotic factor^[39]. It is released from its places on the outer surface of the inner mitochondrial membrane at early steps of apoptosis and, combining with some cytosolic proteins, activates conversion of the latent apoptosis-promoting protease pro-caspase-9 to its active form^[39]. Our results also indicated that cytochrome c was released early in hydrogen peroxide-stimulated SW-480 cells.

Our results found that the morphological and functional changes of mitochondria appeared in SW-480 cells treated with hydrogen peroxide and correlated with development of cell apoptosis. Decreased mitochondrial membrane potential or early release of cytochrome c would be the early signs of apoptosis, which suggested mitochondrial dysfunction might be the key event in the development of apoptosis. We also observed mitochondrial translocation, which was reported in TNF-stimulated L929 cells but not in hydrogen peroxide-stimulated cells^[33]. Mitochondrial translocation suggested that cytoskeleton be involved in apoptosis induced by hydrogen peroxide.

Many researches indicated that oxidative stress led to mutation of mitochondrial genes^[40-50]. Researches showed that there are some links between mitochondrial dysfunction and injuries of mitochondrial DNA or abnormal expression of mitochondrial genes in hydrogen peroxide-stimulated vascular endothelial cells and smooth muscle cells^[50]. Our results also indicated that mitochondrial genes were involved in apoptosis of SW-480 cells. Injuries of mitochondrial genes may contribute to early mitochondrial dysfunction. Relationship between response of mitochondrial genes and dysfunctional mitochondria would be the next problem to be answered.

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