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• BASIC RESEARCH •

Effects of hydrogen peroxide on mitochondrial gene expression of intestinal epithelial cells

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Abstract

AIM: To study the effects of hydrogen peroxide on mitochondrial gene expression of intestinal epithelial cells in *in vitro* model of hydrogen peroxide-stimulated SW-480 cells.

METHODS: RNA of hydrogen peroxide-induced SW-480 cells was isolated, and reverse-transcriptional polymerase chain reaction was performed to study gene expression of ATPase subunit 6, ATPase subunit 8, cytochrome c oxidase subunit I (COI), cytochrome coxidase subuit II (COII) and cytochrome c oxidase subunit III (COIII). Mitochondria were isolated and activities of mitochondrial cytochrome c oxidase and ATPase were also measured simultaneously.

RESULTS: Hydrogen peroxide led to differential expression of mitochondrial genes with some genes up-regulated or down-regulated in a dose dependent manner. Differences were very obvious in expressions of mitochondrial genes of cells treated with hydrogen peroxide in a concentration of 400 μmol/L or 4 mmol/L. In general, differential expression of mitochondrial genes was characterized by up-regulation of mitochondrial genes in the concentration of 400 μmol/L and down-regulation in the concentration of 4 mmol/L. In consistence with changes in mitochondrial gene expressions, hydrogen peroxide resulted in decreased activities of cytochrome c oxidase and ATPase.

CONCLUSIONS: The differential expression of mitochondrial genes encoding cytochrome c oxidase and ATPase is involved in apoptosis of intestinal epithelial cells by affecting activities of cytochorme c oxidase and ATPase.

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INTRODUCTION

Hidden injuries of gut during early stage of severe burn may occur, and lead to endogenous translocation of intestinal endotoxin or bacteria. But factors contributing to gut barrier dysfunction are multiple and its mechanism is unclear[1-10]. Apoptosis of intestinal epithelial cells induced by excessive reactive oxygen species released by activated polymorphonuclear cells and vascular endothelial cells plays a role in the pathogenesis of intestinal mucosal dysfunction. The role of mitochondria in the development of apoptosis has been well clarified recently[11-15]. As we know, there are some proteins related to mitochondrial electron transport chain such as cytochrome coxidase subunits and ATP synthase subunits encoded by mitochondrial genome, which may be involved in mitochondrial injuries of intestinal epithelial cells. Our study focused on the effects of hydrogen peroxide on mitochondrial gene expression of intestinal epithelial 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 ℃ in a humidified 5 % CO_2, 95 % air incubator. Confluent cells were prepared for further studies.

Treatment and groups

Cells were treated with 4 mmol/L or 400 μmol/L hydrogen peroxide. Cells without any hydrogen peroxide were prepared as control.

Primers design

WWW primer picking software (primer3) from Whitehead Institute for Biomedical Research was used to design the primers of ATPase6, ATPase8, COI, COII and COIII genes encoded by mitochondrial genome in accordance with the latest Human Mitochondrial DNA "Cambridge" Sequence (Table 1). Housekeeping gene β-actin was regarded as internal control.

Table 1 Primers of ATPase6, ATPase8, COI, COII and COIII genes encoded by mitochondrial genome

Target genes	Sequence of base pairs	Size of PCR products(bp)
COI	5'-GTTGTAGCCCACTTCCAC-3'	222
	5'-CATCGGGGTAGTCCGAGTAA-3'	
COII	5'-TTCATGATCACGCCCTCATA-3'	187
	5'-TAAAGGATGCGTAGGGATGG-3'	
CO III	5'-AAAGCACATACCAAGGCCAC-3'	195
	5'-CTTCTAGGGGATTTAGCGGG-3'	
ATPase 6	5'-GCCCTAGCCCACTTCTTACC-3'	256
	5'-TTAAGGCGACAGCGATTTCT-3'	
ATPase 8	5'-CCCACCATAATTACCCCCAT-3'	102
	5'-TTTTATGGGCTTTGGTGAGG-3'	
β-actin	5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3'	661
	5'-CTAGAAGCATTGCGGTGGACGATGGAGGG-3'	

Isolation of total RNA

Total RNA was isolated by Tripure isolation reagent (Roche) according to manual provided by the kit. Any subject used in RNA isolation was treated with DEPC (Sigma) followed by vapor sterilization. A260/A280 of RNA samples were between 1.6 and 2.0.

Expression of ATPase6, ATPase8, COI, COII and COIII genes

Titan One Tube RT-PCR Kit (Roche) was applied according to manual for studying expression of mRNA of ATPase6, ATPase8, COI, COII and COIII genes. The PCR profile was as follows: a reverse-transcription reaction at 50 ℃ for 30 min and initial denaturation at 94 ℃ for 2 min, followed by 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 56 ℃ for 30 s, primer extension at 68 ℃ for 45 s and a final extension at 68 ℃ for 10 min. PCR products were separated by electrophoresis on a 1 % vertical agarose electrophoresis and observed under ultraviolet and stored in computer by gel imaging system. Relative density of genes was calculated by dividing density of respective gene with density of β-actin.

Isolation of mitochondria

Mitochondria were isolated as described previously[16]. Cells about 1×10^7 were typsined followed by washing for one time, and then cells suspended in ice-cold isolation buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4) were homogenized with a glass homogenizer for 10 strokes in ice. The supernatants was further centrifuged at 1 500×g for 10 min at 4 ℃, the pellet was suspended again in ice-cold isolation buffer and homogenized in ice. Two supernatants were mixed and centrifuged at 17 000 ×g for 10 min at 4 ℃. Fresh isolated mitochondria was suspended in isolation buffer, protein concentration were determined by Folin-Phen assay and adjusted to 4 mg/ml. Activities of cytochrome c oxidase and ATPase were measured within 6 h after isolation of mitochondria.

Cytochrome c oxidase activity

In 1 ml reaction volume, 70 μl reduced ferrocytochrome c, 10 μl isolation mitochondria (4 mg/ml) and 920 μl 10 mM K_2HPO_4 (pH 7.4) were mixed at room temperature, and the absorbance was measured every 30 seconds. The decreased absorbance represents activity of cytochrome c oxidase[17].

ATPase activity

Ten micro-liter of isolated mitochondria (4 mg/ml) and 1 ml of 10 % TCA were mixed in Eppendorf tubes at 37 ℃ for 60 min, and the reaction was terminated by incubation in ice. Then 250 μl of 0.02 mol/L ATP-Na_2 and 100 μl of 0.05 mol/L $MgCl_2$ were added respectively, and the reaction was ended by adding 1 ml of 10 % TCA after 5 min at 37 ℃, followed by centrifugation at 3 000 rpm for 15 min at 4 ℃. The contents of inorganic phosphorus in supernatants representing ATPase activity were determined[18].

Statistical analysis

Data were expressed as *mean* ±*SD*. To analyze the data statistically, student's *t* test was used to determine statistical differences, and $P<0.05$ was considered significant.

RESULTS

Expression of house-keeping gene b-actin mRNA

After treating with hydrogen peroxide, no significant changes of house-keeping gene β-actin mRNA could be observed, indicating its value as an internal control (Figure 1).

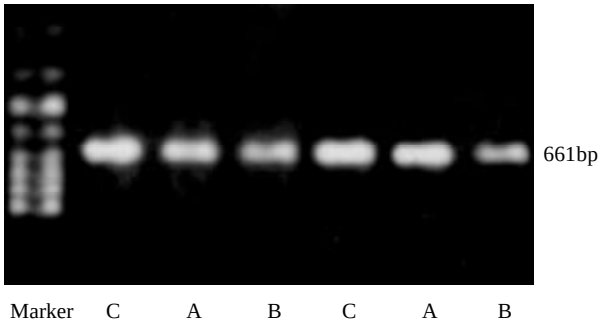

Figure 1 Effects of hydrogen peroxide on expression of homing gene β-actin mRNA.
C: control cells; A: 4 mM H_2O_2-treated cells (3 h); B: 400 mM H_2O_2-treated cells (3 h).

Expression of COI mRNA

Cells from normal control expressed certain amount of COI mRNA. Increasing expression of COI mRNA was found after treating with hydrogen peroxide in the concentration of 400 μmol/L, but no significant changes could be observed in the concentration of 4 mmol/L (Figure 2).

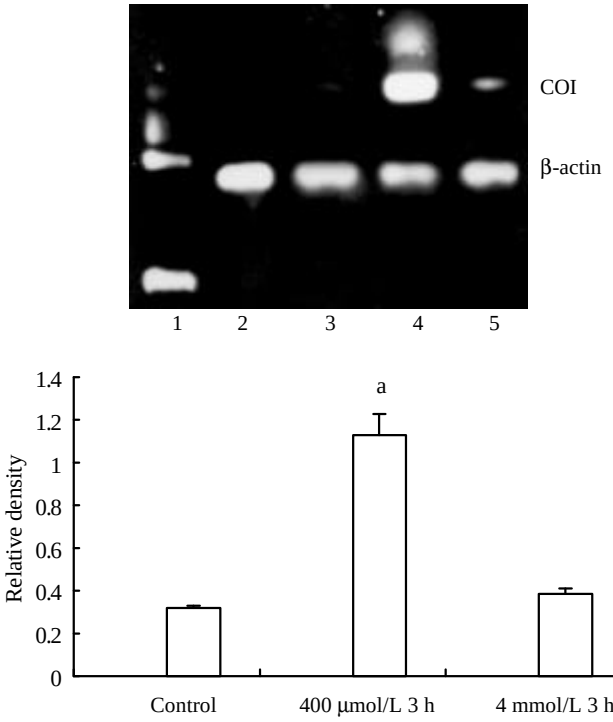

Figure 2 Effects of hydrogen peroxide on expression of COI mRNA ($^aP<0.05$, *vs.* normal control).
1 DNA marker: lDNA / Hind III, 2 positive control, 3 normal control, 4 400 μmol / L 3 h, 5 4 mmol / L 3 h.

Expression of COII mRNA

Weak expression of COII mRNA was found in control cells. Significant increase could be found in both 400 μmol/L and 4 mmol/L hydrogen peroxide-stimulated cells (Figure 3).

Expression of COIII mRNA

Stable expression of COIII mRNA was found in control cells. Significant decrease could be found in 400 μmol/L hydrogen peroxide-stimulated cells (Figure 4).

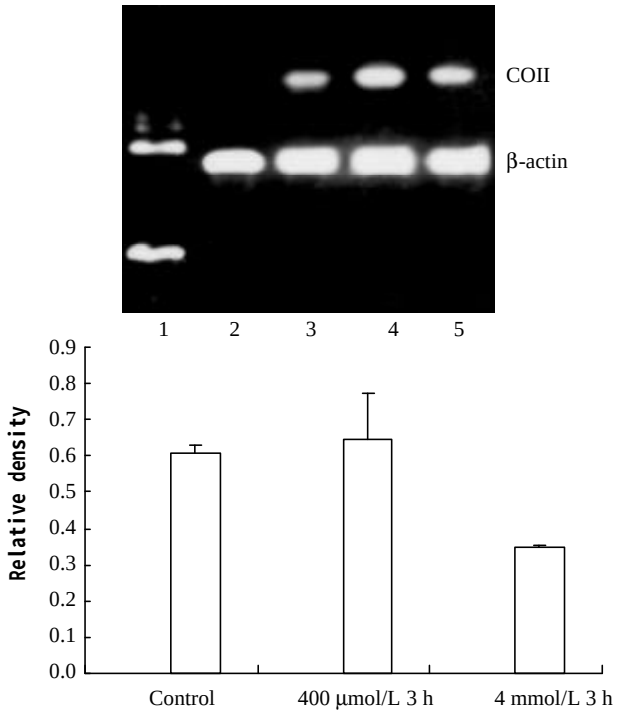

Figure 3 Effects of hydrogen peroxide on expression of COII mRNA ($^aP<0.05$, *vs* normal control).
1 DNA marker; lDNA / Hind III, 2 positive control, 3 normal control, 4 400 μmol / L 3 h, 5 4 mmol / L 3 h.

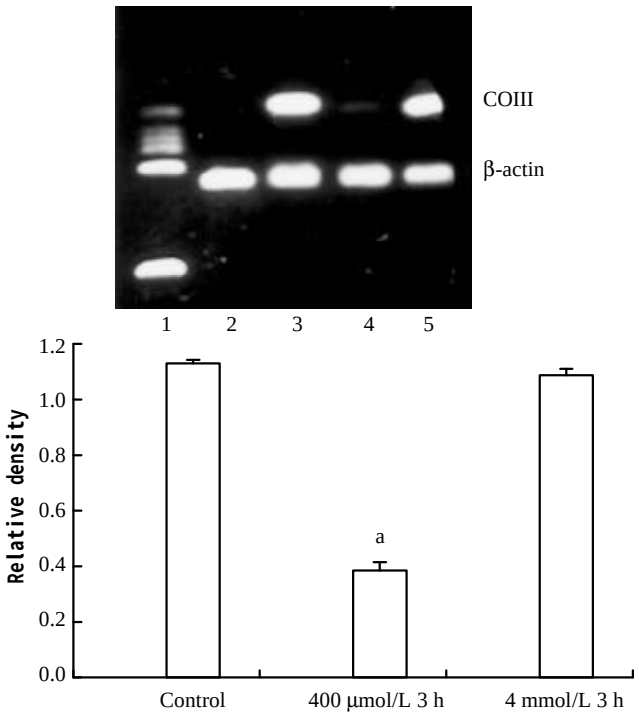

Figure 4 Effects of hydrogen peroxide on expression of COIII mRNA ($^aP<0.05$, *vs* normal control).
1 DNA marker: lDNA / Hind III, 2 positive control, 3 normal control, 4 400 μmol / L 3 h, 5 4 mmol / L 3 h.

Expression of ATPase6 mRNA

Expression of ATPase6 mRNA increased significantly in 400 μmol/L hydrogen peroxide stimulated-cells, meanwhile decreased expression could also be found in 4 mmol/L hydrogen peroxide-stimulated cells (Figure 5).

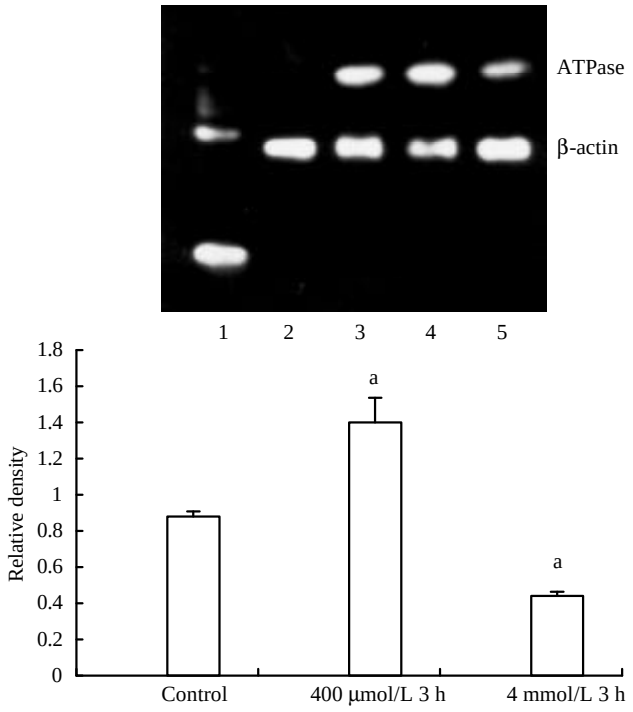

Figure 5 Effects of hydrogen peroxide on expression of ATPase6 mRNA ($^aP<0.05$, *vs* normal control).
1 DNA marker: lDNA / Hind III, 2 positive control, 3 normal control, 4 400 μmol / L 3 h, 5 4 mmol / L 3 h.

Expression of ATPase8 mRNA

Stable expression of ATPase8 mRNA was found in control cells. No significant increase could be found in 400 μmol/L hydrogen peroxide-stimulated cells. Decreased expression of ATPase8 mRNA in 4 mmol/L hydrogen peroxide-stimulated cells was found (Figure 6).

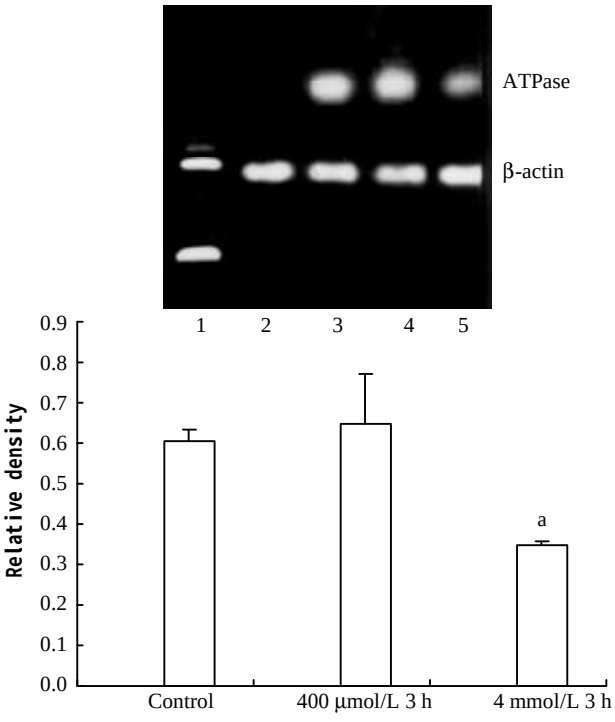

Figure 6 Effects of hydrogen peroxide on expression of ATPase8 mRNA ($^aP<0.05$, *vs*. normal control).
1 DNA marker: lDNA / Hind III, 2 positive control, 3 normal control, 4 400 μmol / L 3 h, 5 4 mmol / L 3 h.

Activity of cytochrome c oxidase

Activities of cytochrome c oxidase decreased significantly in both 400 μmol/L and 4 mmol/L hydrogen peroxide-stimulated cells (Figure 7).

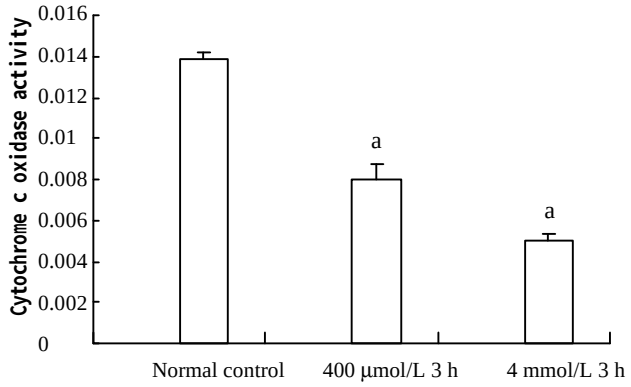

Figure 7 Activity of cytochrome c oxidase in hydrogen peroxide-stimulated SW-480 cells ($^aP<0.05$, *vs.* normal control).

ATPase activity

Although only ATPase activity in 4 mM hydrogen peroxide-stimulated cells decreased significantly on the view of statistics, the trend is very obvious (Figure 8).

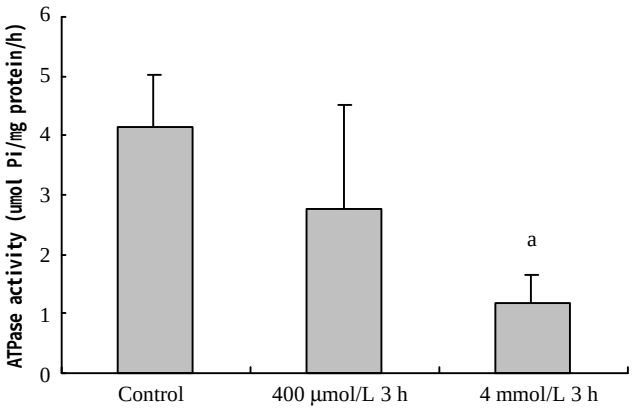

Figure 8 Activity of ATPase in hydrogen peroxide-stimulated SW-480 cells ($^aP<0.05$, *vs.* normal control).

DISCUSSION

The role of mitochondrion in the pathogenesis of apoptosis has been well defined. Mitochondrion, a kind of organelle controlling growing, breeding and dying of eukaryocyte, embodies its functions through: (1) production of ATP; (2)production of ROS, which regulates nuclear gene expression; (3) link between cytoplasm and nucleus; (4)sensitive response to stimulator or signals outside cytoplasm; (5) triggering of cell death[19, 20]. Evidences show the central role of mitochondria in the development of apoptosis. Many stimulators like ROS, Ca^{2+} and cytokines lead to activation of caspases by inducing release of cytochrome C and thereby trigger a cascade in apoptosis[11].

Release of mitochondrial apoptosis-inducing substance caused by collapse of mitochondrial membrane potential and open of mitochondrial permeability transition pore controlled by Bcl 2 family is the key step to initiate apoptosis. Mitochondrial respiration depends on maintenance of mitochondrial membrane potential.

Now the relationship between DNA injuries and apoptosis has been recognized[21]. Although mitochondrial genome was sequenced in 1981[22], there is little study on function of mitochondrial genes. Thirteen proteins encoded by mitochondrial genome make up parts of enzymes involving mitochondrial oxidative phosphorylation. Without protection of histone, mitochondrial genome is much easier to be injured compared with nuclear genome. Our research also confirmed this (results to be published). Many researches indicated that oxidative stress led to mutation of mitochondrial genes[23-32]. The relationship between mutation of DNA and expression of genes needs to be well investigated.

Hydrogen peroxide led to differential expression of mitochondrial genes with some genes up-regulated or down-regulated in a dose-dependent manner. Differences were very obvious in expression of mitochondrial genes of cells treated with hydrogen peroxide in the concentration of 400 μmol/L or 4 mmol/L. In general, differential expression of mitochondrial genes was characterized by up-regulation of mitochondrial genes in the concentration of 400 μmol/L and down-regulation in the concentration of 4 mmol/L. In consistence with changes of mitochondrial gene expression, hydrogen peroxide led to similar decreased activities of cytochrome C oxidase and ATPase. Differential expression reflected the difference in the sensitivity of gene responding to oxidative stress, which may relate greatly to cellular physiological process next. In rat lung epithelial cells treated with hydrogen peroxide for for 8 or 24 h, the differential display of genes showed that mutation of genes from mitochondrial genome encoding NADH dehydrognase subunit 5, subunit 6 and 16 S ribosome could be detected; the results also suggested that mitochondrial genes played a role in the control of apoptosis[33]. Researches showed that there were some links between mitochondrial dysfunction and injuries of mitochondrial DNA or expression of mitochondrial genes in hydrogen peroxide-stimulated vascular endothelial cells and smooth muscle cells[34].

Meanwhile we also found a large scale of point mutation of mitocondrial gene such as ATPase8 subunit in 4 mmol/L hydrogen peroxide treated SW-480 cells, suggesting its correlation with down-regulation of ATPase expression.

It is considered now that the mitochondrial permeability transition (MPT) plays a key role in the pathogenesis of apoptosis and necrosis[35-38]. If MPT initiates rapidly with depletion of ATP, cells undergo necrosis, and if MPT occurs without depletion of ATP, cells evolved into apoptosis. So we may conclude that complete inhibition of ATPase would lead to necrosis and partially inhibition of ATPase would lead to apoptosis. Consistent with this conclusion, our study proved a partially inhibition of ATPase in both 4 mmol/L and 400 μmol/L hydrogen peroxide-treated SW-480 cells, suggesting its potential role in apoptosis. But there are still many problems to be clarified, including the relationship between different expressions of mitochondrial genes, activities of cytochrome C oxdiase and ATPase, oxidative phosphorylation and regulation of apoptosis.

Studies including ours showed that mitochondrial genes played the role in apoptosis. To establish mtDNA deletion cells (ρ^0 cells) provided in our studies more direct evidences[39-42].

It is unclear about the pathway leading to increasing expression of mitochondrial genes. Defects of mitochondrial energy supply may be one of the compensatory mechanisms leading to increasing expression of mitochondrial genes[43].

Our results showed that differential expression seemed to be incompatible with decreasing activities of cytochorome C oxidase or ATP synthase. To answer this question, we should

pay attention to mitochondrial proteins encoded by the nuclear genome, which form main parts of mitochondrial proteins. For example, only subunits 6 and 8 of ATP synthase were encoded by mitochondrial genome, which may also be controlled by some nuclear transcriptional factors[44-46]. Meanwhile most parts of ATPase were encoded by the nuclear genome. Cross-talk between mitochondrial genes and nuclear genes will be the next key or difficult question to be answered[47-50]. It will become available to study cross-talk between mitochondrial genes and nuclear genes from the view of genome and proteome by the developing techniques of genome and proteome.

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