Protective Effects of Mitochondria-Targeted Peptide MTP-131 on Trabecular Meshwork Cells under Elevated Hydrostatic Pressure

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Abstract

Purpose: To investigate the effects of MTP-131 on trabeculum cells cultured under hydrostatic pressure

Methods: Glaucomatous human trabecular meshwork (GTM3) cells were divided into three groups: the MTP-131 group, the hydrostatic group, and the normal group. The MTP-131 group was pretreated with 1 µM MTP-131, and the cells were then cultured under 80 mmHg pressure for 24 hours. The normal group was cultured under normal atmospheric pressure for 24 h, and the hydrostatic group was cultured under 80 mmHg pressure for 24 h. The change in the mitochondrial membrane potential the generation of reactive oxygen species (ROS) and the release of cytochrome c from the mitochondria were analyzed with confocal microscopy. The generation of ROS and apoptosis were detected by flow cytometry. The expression of caspase-3 was detected by western blot analysis.

Results: In GTM3 cells, culturing under hydrostatic pressure resulted in a decrease in the mitochondrial membrane potential, an elevation of intracellular ROS and a release of cytochrome c from the mitochondria. Pretreatment with MTP-131 prevented the mitochondrial depolarization, decreased the intracellular ROS, prohibited the release of cytochrome c from the mitochondria and inhibited apoptosis. The path of apoptosis is caspase-3 independent.

Conclusion: MTP-131 can protect cultured trabecular cells from the damage caused by hydrostatic pressure.

Keywords: Glaucoma, Trabecular Meshwork, Mitochondria-Targeted Peptide, Hydrostatic Pressure, Apoptosis

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Introduction

As we all know that pressure is a crucial component of the cell's environment, it can have a major effect on the cell's function and vitality.[1] Furthermore the pressure in vivo changes in many pathological conditions, such as hypertension, emphysema, edema etc. And there are some studies reported that elevated pressures increased the levels of oxidative stress [indicated by reactive oxygen species (ROS)] in cells.[2] The ROS is able to oxidize the proteins, lipids and DNA, which may affect the cell's functions severely. So antioxidants may be used to protect the cells from damage caused by elevated pressure.

However, many traditional antioxidants have not performed as well in vivo as in vitro. In several clinical trials, many traditional antioxidants resulted in no significant difference compared with a placebo.[3-8] But these results are not enough to deny the prospects of antioxidants.[9] Two key factors in the performance of antioxidants are the time of administration and the time it takes to reach the injured tissue.[10,11] The failure of the traditional antioxidants may be due to their poor permeability. Many antioxidants cannot reach the location of ROS production promptly, and over 90% of the ROS are produced in the mitochondria.[12] The half-life of ROS is very short, approximately 10^{-9}s, and the ROS are produced and reacted with the surrounding materials very quickly.[13] This rapid turnover means that the antioxidants must enter the mitochondria very rapidly. The traditional antioxidants remain close to the cell membrane or cannot present an effective concentration within the mitochondria. Therefore, scientists have invented a number of new antioxidants. These new antioxidants directly target the mitochondria, such as Mito Q and Mito E.[14] MTP-131 is a new antioxidant that targets the mitochondria and is an oligopeptide with the amino acid sequence D-Arg-Dmt-Lys-Phe-NH$_2$ (Dmt=2′,6′-dimethyltyrosine).[15]

Above all elevated pressure will increase the ROS, the ROS is generated mainly from the mitochondria.[12] But the potential role of mitochondria and ROS generation in pressure induced injury is less well understood. So we perform this present study to detect the potential mitochondrial mechanism of injury induced by elevated pressure and whether the MTP-131 can protect the cells from the damage caused by elevated pressure.

Methods

Materials

The glaucomatous human trabecular meshwork (GTM3) cell line was provided by Professor Yuhao Pen (Glaucoma Research, Alcon Laboratory, Fort Worth, TX). The DMEM/F$_{12}$ Medium and trypsin were obtained from Gibco (Gibco, Invitrogen, Carlsbad, CA). The fetal bovine serum (FBS) was purchased from Gibco. The 2′,7′-Dichlorodihydrofluorescein diacetate (H$_2$DCFDA), TMRM, MitoTracker Deep Red and Hoechst 33342 were purchased from Molecular Probes (Molecular Probes, Invitrogen, Carlsbad, CA). The MTP-131 was kindly provided by Stealth Peptides International Inc. The monoclonal rabbit anti-human caspase-3 and monoclonal rabbit anti-human cytochrome c antibodies were purchased from CST (Cell Signal Technology, Boston, USA). The monoclonal mouse anti-human actin antibody was purchased from MultiSciences Biotech Co., Ltd. (Hangzhou, China). The FITC-Annexin V/PI Apoptosis detection kit was purchased from BD Pharmingen (New Jersey, USA). The western blot assay kit was purchased from Guangzhou Whiga Technology Co., Ltd. (Guangzhou, China). The fluorescent dye-conjugated goat anti-rabbit secondary antibody was obtained from Boster (Wuhan, China). All other materials were from Sigma Aldrich (St. Louis, MO, USA) unless otherwise indicated. The stock solutions of 1 mM MTP-131 were prepared in PBS.

Methods

Cell culture

GTM3 cells were cultured in 25 cm^2 flasks containing DMEM/F$_{12}$ supplemented with 15% FBS. GTM3 cells were divided into three groups: the hydrostatic group, the normal group and the MTP-131 group. The cells in the hydrostatic group were subjected to an elevated hydrostatic pressure of 80 mm Hg for 24 hours as reported.[2,16] The cells in the normal group were incubated under normal atmospheric pressure, and the cells in the MTP-131 group were pretreated with MTP-131 at a concentration of 1 μM and then

subjected to an elevated hydrostatic pressure of 80 mm Hg for 24 hours. All three groups were incubated in a conventional 5% CO_2 incubator at 37°C.

Pressure system

All hydrostatic pressures were stable and adjustable. The cells were subjected to elevated pressure in a pressure chamber, the chamber inlet of which was connected by a low-pressure, two-stage regulator to a gas cylinder of 5% co_2 and 95% air (Figure 1). Two levels of needle valve gas-pressure controllers provided a constant hydrostatic pressure, which was monitored by a pressure gauge.[2] It means that the pressure increases from P1 to P2, and the P3 is equal to P2. So there is no pressure gradient in the pressure chamber. We maintain its temperature at 37°C by placing it in a conventional incubator.

Furthermore, in Liu Quan's study, the statistical analysis showed no significant difference in the PH, Pco_2 and po_2 levels among the pressure-treated and control-cultured media.[2] And the configuration of our study is similar to that of Liu Quan .

Measurement of reactive oxygen species

First, GTM3 cells were plated onto petri dishes and cultured for 24 hours. Second, the cells were incubated with serum-free DMEM/F_{12} for another 24 hours. The cells of MTP-131 group were then pretreated with MTP-131. Third, the cells of the hydrostatic group and the MTP-131 group were both subjected to an elevated hydrostatic pressure for another 24h; the cells in the normal group were incubated under normal atmospheric pressure as a control at the same time. Finally, the cells from the three groups were incubated with freshly diluted H_2DCFDA at 37°C away from light. H_2DCFDA fluoresces green after oxidation. We used 5µM H_2DCFDA for 0.5 hour for the confocal microscopy and 1 µM for 0.5 hour for the flow cytometry.

For the flow cytometry, H_2DCFDA-labeled cells were rinsed twice with PBS and immediately analyzed by flow cytometry with 488 nm excitation and 530 nm emission wavelengths. Ten thousand cells were collected, and the data were expressed as the median fluorescence intensity in arbitrary units from the average of at least 3 separate experiments.

Measurement of mitochondrial membrane potential

We used TMRM to demonstrate changes in the mitochondrial membrane potential. The dye fluoresces red. The TMRM was freshly diluted in serum-free DMEM/F_{12} to a final concentration of 500 nM and was added to the three groups of cells (the hydrostatic group, the MTP-131 group, and the normal group). After incubation for 0.5 hour away from light, all the samples were rinsed twice in PBS and analyzed immediately with confocal microscopy.

Measurement of cytochrome c release

We used confocal microscopy to measure the release of cytochrome c from mitochondria to cytoplasm as previously described.[17] Cells were seeded onto fisherbrand coverglass at a density of 2,000 cells/chamber. Then the cells culture were the same with the experiment of measurement of ROS. Freshly prepared MitoTracker Deep Red (200 nm) was added into cells and incubated at 37°C for half an hour. Then the cells were fixed with paraformaldehyde for 15 minutes and permeabilized with methanol for 5 minutes on ice. After being blocked with 5% BSA for half an hour, cells were incubated with monoclonal rabbit anti-human cytochrome c antibody (1:1000; CST) at 4°C overnight. After being given 3 rinses, cells were incubated with dye conjugated goat anti-rabbit IgG (1:64) for an hour at 37°C away from light. Finally cells were incubated with Hoechst 33342 for 5 minutes then washed and mounted with antifade fluorescence mounting medium. Then the cells were analyzed with confocal microscopy.

Measurement of cell apoptosis

Apoptosis was examined with a FITC-Annexin V apoptosis detection kit according to the manufacturer's protocol. Cells were grown in six-well plates at a density of 3×10^4 cells/well. Then, all the cells were cultured for 24h and synchronized for 24h. After the cells in the MTP-131 group were pretreated with MTP-131, both the hydrostatic and the MTP-131 groups were subjected to an elevated hydrostatic pressure for 24h. The cells in the normal group were incubated under normal atmospheric pressure as a control at the same time.

The cells were washed twice with cold PBS and then resuspended in 1× binding buffer at a concentration of 1×10^6 cells/ml. A volume of 100 μl of the suspension (1×10^5 cells) was transferred to a 5 ml culture tube. 5 ul of FITC-Annexin V and 5 μl of PI were added. The cells were gently vortexed and incubated for 15 min at RT (25°C) in the dark, and 400 μl of 1× binding buffer was then added to each tube. The samples were analyzed by flow cytometry within 1 hour.

Western blotting
For the western blot analysis, the GTM3 cells were lysed in lysis buffer and centrifuged; the supernatant containing cellular proteins was removed. The proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. After blocking with 5% BSA, the membranes were incubated with the primary antibodies (1:1000) for 24 hours at 4°C, followed by washing and incubation with the HRP-conjugated secondary antibodies for 2 hours at RT (25°C). The bound antibodies was detected with chemiluminescence (ECL system, BIO-RAD).

Statistical analysis
All assays were repeated at least three times, and the data were expressed as the mean±SE. We used One-way ANOVA to detect differences and the statistical significance was set at $p < 0.05$.
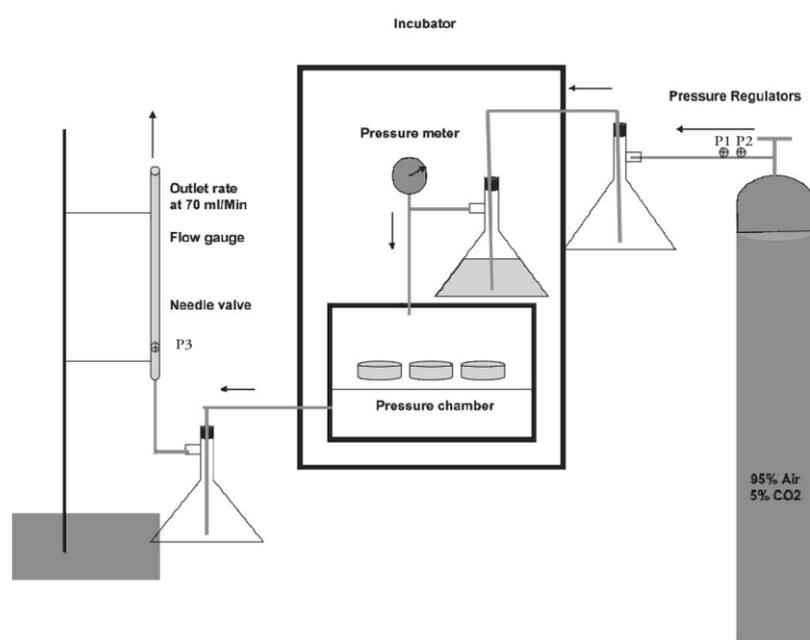

Figure 1. (cited from liu quan[2])

Results

Measurement of Reactive Oxygen Species
Figure 2-A shows that the level of endogenous ROS, indicated by the oxidation of H_2DCFDA in GTM3 cells, was significantly higher in the hydrostatic group than in the normal group, and the MTP-131 treatment considerably alleviated the ROS elevation induced by hydrostatic pressure. Figure 2-B and 2-C show that the total ROS level of the hydrostatic group increased by 9.53-fold compared with the normal group of GTM3 cells. However, when we pretreated GTM3 cells with MTP-131 at a concentration of 1 uM, the ROS level decreased to a nearly normal level even after exposure to hydrostatic pressure for 24 h.

Measurement of mitochondrial membrane potential
Figure 3 shows that the hydrostatic group had lower mitochondrial membrane potential than the normal group. Furthermore, pretreatment with MTP-131 largely alleviates the decrease of mitochondrial membrane potential induced by hydrostatic pressure.

Measurement of cytochrome c release

Figure 4 shows that hydrostatic pressure induced the release of cytochrome c from mitochondria. And pretreatment with MTP-131 could relieve the release of cytochrome c from mitochondria.

Measurement of cell apoptosis

We used the FITC-Annexin V apoptosis detection kit to detect cell apoptosis with flow cytometry. Figure 5 shows that cell apoptosis increased by 3.58 (23.8/5.2-1) folds in GTM3 cells after exposure to hydrostatic pressure. In cells pretreated with MTP-131 (1 µM), the apoptosis rate was nearly equal to the normal group even after exposure to hydrostatic pressure (80 mmHg) for 24 h. There is

significant difference between the hydrostatic and MTP-131 groups ($*$ p<0.05).

Apoptotic analysis

Figure 6 indicates that there is no significant difference in the expression of the caspase-3 protein among the three groups (the normal group, the hydrostatic group, and the MTP-131 group). Although the hydrostatic pressure induced an increase in cell apoptosis and cell apoptosis usually occurs with the activation of caspase-3. Therefore, we propose that the cell apoptosis in our experiment occurred through a caspase-3 independent pathway, and the specific mechanism deserves further study.
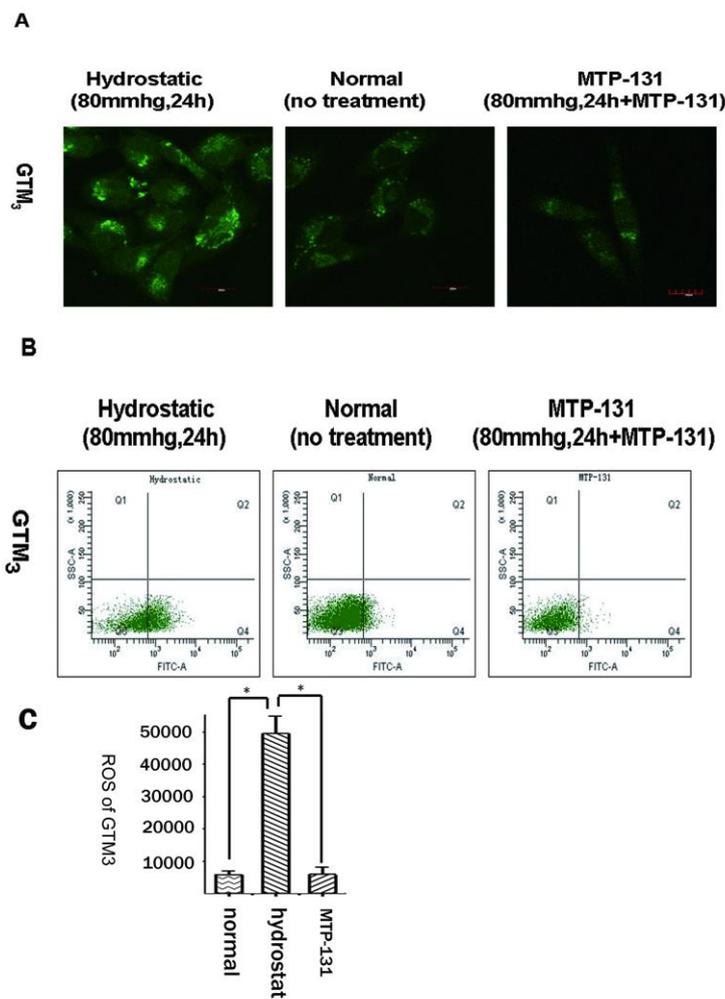

Figure 2-A. H_2DCFDA fluorescence labeling, examined with confocal microscopy, shows the increased fluorescence intensity of ROS in the GTM3 cell lines after exposure to hydrostatic pressure. MTP-131 largely alleviates the increase in fluorescence intensity of ROS induced by hydrostatic pressure. Scale bar, 10 µm. **2-B.** Distribution of H_2DCFDA fluorescence intensity in the GTM3 cell line examined with flow cytometry. The hydrostatic group has stronger H_2DCFDA fluorescence intensity than the MTP-131 group. **2-C.** The relative amount of total H_2DCFDA fluorescence intensity in the hydrostatic and MTP-131 groups compared with the normal group. There is a significant difference between the hydrostatic and MTP-131 groups ($*$ p<0.05).
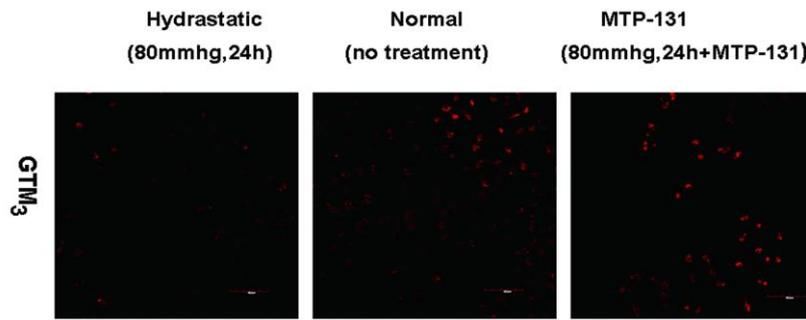
Hydrastatic
(80mmhg,24h)

Normal
(no treatment)

MTP-131
(80mmhg,24h+MTP-131)

GTM₃

Figure 3. Mitochondrial membrane potential was detected with TMRM fluorescence and examined using confocal microscopy. The MP is lower in the hydrostatic pressure group than in the MTP-131 group, and the MP in MTP-131 group is nearly equal to that in normal group. Scale bar, 10 µm.

Hydrostatic
80mmhg,24h

Normal
no treatment

MTP-131
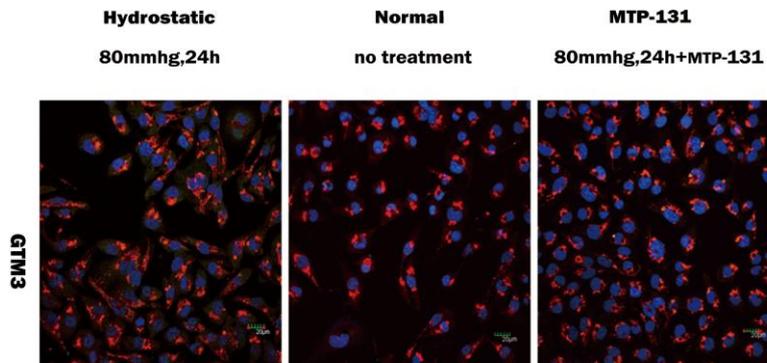**80mmhg,24h+MTP-131**

GTM3

Figure 4. Figure 4 shows that in normal group cytochrome c staining (green) and mitochondria staining (red) are overlapping, indicating colocalization of cytochrome c and mitochondria. After treatment with hydrostatic pressure for 24 hours, some cytochrome c is observed in the cytoplasm, indicating hydrostatic pressure induces the release of cytochrome c from the mitochondria. And pretreatment with MTP-131 can relieve the release of cytochrome c.
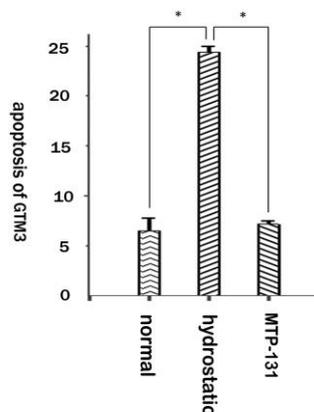
Figure 5. FITC-Annexin V staining shows that there is a large increase in cell apoptosis in the hydrostatic group compared with the MTP-131 group, and the amount of cell apoptosis in the MTP-131 group is nearly equal to that in the normal group.
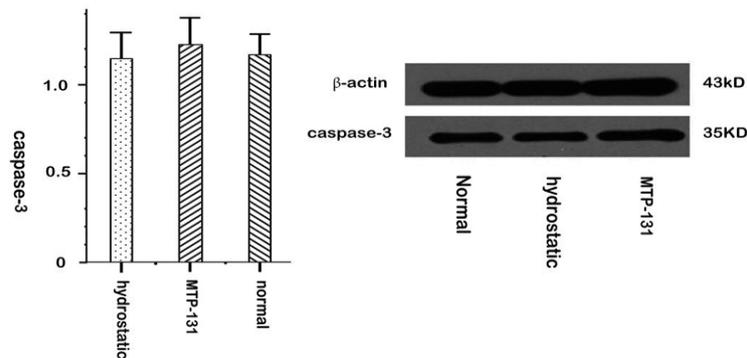
Figure 6. There is no significant difference in the expression of the caspase-3 protein among the three groups (normal group, hydrostatic group, and MTP-131 group) (p>0.05).

Discussion

In the present study, we showed that GTM3 cells subjected to elevated hydrostatic pressure resulted in a decrease in mitochondrial membrane potential, an elevation of intracellular ROS and a release of cytochrome c from mitochondria. However, pretreatment with MTP-131 prevented mitochondrial depolarization, decreased the level of intracellular ROS, prohibited the release of cytochrome c from mitochondria and inhibited cell apoptosis. And the path of cell apoptosis is caspase-independent.

Our study showed that cells subjected to elevated pressures resulted in increase in their levels of oxidative stress, which is consistent with the results of Quan Liu.[2] Makoto Ishikawa also reported that elevated pressures of 10 and 25 mmHg resulted in no abnormal changes in the retinas ex vivo, but when they increased the pressure to 50 mmHg, the axonal swelling was found in the nerve fiber layer of a retina.[17] Chen min's previous study proved that MTP-131 could protect GTM3 cells from oxidative stress.[18] Our study showed that MTP-131 could alleviate ROS generation and cell apoptosis resulting from elevated pressure. Thus, we were easily able to attribute the protective effect of MTP-131 on GTM3 cells from elevated pressure to it's antioxidative capacity.

In the present study, it shows that the mitochondria are a primary target of injury caused by elevated pressure. In normal conditions, cytochrome c exists in the mitochondrial intermembrance space and

transfers electrons from mitochondria complex III to IV, resulting a few loss of electrons from electron transport chain (ETC). And the few loss of electrons from ETC will result in few ROS generation.[19] But in pathological conditions, the cytochrome c is released into cytoplasm and there are bulk loss of electrons and great ROS generation. And then the ROS generation will promote the release of cytochrome c from the mitochondria. Finally the cells are in a vicious circle between ROS generation and the release of cytochrome c. And in normal conditions, the cytochrome c in mitochondria exists in two forms: free-form or bound by cardiolipin.[20] About 85% of the cytochrome c is bound by cardiolipin.[21] The oxidization of the cytochrome c –cardiolipin bonds could liberate the cytochrome c bound by cardiolipin. The detachment of bound cytochrome c makes it possible for free cytochrome c to diffuse into the cytosol after the permeabilization of outer mitochondrial membrane.[22] So the ROS play an important role in the release of cytochrome c. Then we can refer that the protective effect of MTP-131 on prohibiting the release of cytochrome c are mainly resulted from its antioxidative capacity directly targeting the mitochondria.

In our study, we did not detect any increase in the expression of protein caspase-3 when cell apoptosis increased in the hydrostatic group. In this result, we differ from Chen Min's study. Chen Min's study detected an increase of caspase-3 when cell apoptosis increased.[17] In Chen Min's study, oxidative stress was produced by 200 uM H_2O_2, and there was

evidence showed that the concentrations of H_2O_2 affected the cell apoptosis pathway. The H_2O_2 produced by glucose oxidase appeared at a rate of approximately 1-2 UM/min in vivo, while the cell apoptosis induced by glucose oxidase was caspase-independent.[23] The literature on glucose oxidase suggests that the increased speed of ROS in the circumstances of our study may be more likely to glucose oxidase in vivo. Whether the cell apoptosis of the TM cell in vivo is caspase-independent requires further study.

Conclusion

The present study had shown that mitochondria played an important role in oxidative injury caused by elevated pressure. And as a mitochondria targeted antioxidants, MTP-131 is able to relieve the damage to the TM cells that was caused by elevated pressure.

Acknowledgements

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