Mitochondrial Dysfunction Secondary to Endoplasmic Reticulum Stress in Acute Myocardial Ischemic Injury in Rats

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background:
The relationship between endoplasmic reticulum and mitochondria during acute myocardial ischemic injury is still unclear. Our study aimed to define the dynamics of endoplasmic reticulum stress and mitochondrial dysfunction during acute ischemic injury.

Material/Methods:
A rat model of acute myocardial infarction and hypoxic cardiomyocytes were used in this study. Groups were set at 0 hours, 1 hour, 2 hours, 4 hours, and 6 hours after ischemic injury for both *in vivo* and *in vitro* studies. ATF6 and GRP-78 were examined to indicate endoplasmic reticulum stress. Cellular ATP and cytosolic levels of mitochondrial DNA and cytochrome *c* were detected to evaluate mitochondrial dysfunction. Caspase-3 was used for apoptosis analysis.

Result:
Our results showed that both mRNA and protein levels of ATF6 and GRP-78 were elevated from 1 hour after ischemic injury *in vivo* and *in vitro* ($P<0.05$). However, ATP levels were increased at 2 hours after ischemic injury and significantly decreased from 4 hours after ischemic injury *in vivo*, while ATP level of cultured cardiomyocytes decreased remarkably from 2 hours after ischemic injury ($P<0.05$). Cytosolic mitochondrial DNA levels began to increase from 2 hours after ischemic injury ($P<0.05$). Cytosolic levels of cytochrome *c* increased from 4 hours after ischemic injury. Additionally, both mRNA and protein expressions of caspase-3 started to significantly elevate at 6 hours after ischemic injury ($P<0.05$).

Conclusions:
The present study suggested that mitochondrial dysfunction was secondary to endoplasmic reticulum stress, which provides a novel experimental foundation for further exploration of the detailed mechanism after ischemic injury, especially the interaction between endoplasmic reticulum and mitochondria.

MeSH Keywords:
Endoplasmic Reticulum Stress • Membrane Potential, Mitochondrial • Myocardial Ischemia

Full-text PDF:
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Background

Coronary artery disease is the leading cause of death and will remains so for the next few decades [1]. In addition to its high mortality rate, it is also a leading cause of morbidity and loss of quality of life [2]. Acute myocardial infarction usually occurs due to primary coronary artery disease and there are many well-known risk factors [3]. Although some causes and risk factors have been gradually elucidated, the molecular mechanisms of cardiomyocytes during acute myocardial infarction are still unclear and efforts are still needed to salvage para-infarction heart muscle and prevent post-infarction complications.

Recently, an increasing number of studies have focused on the role of endoplasmic reticulum (ER) stress and mitochondrial dysfunction in acute myocardial infarction. It has been demonstrated that inhibition of endoplasmic reticulum stress can exert heart-protective effects after acute myocardial infarction [4]. Meanwhile, production of massive reactive oxygen species (ROS) is a well-known harmful factor to heart tissue, and inhibition of its production can limit mitochondrial damage and save heart tissue from damage [5]. In addition, mitochondria recently have been regarded as a promising target for cardio-protection in acute myocardial infarction [6]. However, the relationship between endoplasmic reticulum stress and mitochondrial dysfunction during acute myocardial infarction is still unclear. Both ATF6 and GRP-78 are 2 key proteins during the development of the endoplasmic reticulum stress. Dissociation of GRP78 and ATF6 could activated unfolded protein response [7,8]. It has been reported that the endoplasmic reticulum stress may result in mitochondrial damage and cell death, presenting as the production of ROS, releasing of mitochondrial DNA, and cleavage of caspases [9,10].

Many studies have revealed the possible relationships between the endoplasmic reticulum stress and mitochondrial damage in other pathophysiological scenarios [11,12]. However, in acute myocardial ischemic injury, these relationships have not been well-established, and insightful studies were imperatively needed. Therefore, in order to understand the pathophysiological processes during acute myocardial infarction and further explore the molecular mechanisms between endoplasmic reticulum and mitochondria, the present study aims to first define the time course of endoplasmic reticulum stress and mitochondrial dysfunction during acute ischemic injury.

Material and Methods

Experimental protocols in the acute myocardial infarction rat model

We used 10-week-old Sprague Dawley (SD) male rats, weighting 220 to 250 g, purchased from Da-Shuo Biotech Company (Chengdu, China). All procedures followed the *Guide for the Care and Use of Laboratory Animals* and the protocols of the study were approved by the Ethic Committee of West China Hospital, Sichuan University.

All rats were incubated and ventilated by an animal ventilation with room air. The rats had a left thoracotomy through a left parasternal incision performed to expose the heart. After 20 minutes stabilization, except for the control group, rats were induced to acute myocardial infarction by ligating the left anterior descending coronary artery. After 1 hour, 2 hours, 4 hours, and 6 hours, rats were sacrificed by cervical dislocation and their hearts were harvested for further analysis. Each group included 5 rats.

Primary neonatal rat cardiomyocyte culture

Neonatal rat hearts were collected within 24 hours and the heart tissue was cut into small pieces, followed by digestion with 22.5 µg/L Liberase blendzyme 4 (Roche, Germany) at 37°C for 40 minutes. The isolated cells were pre-plated for 90 minutes to remove non-cardiomyocytes, after which cardiomyocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) in 24-well culture plates pre-coated with 1% gelatin (Sigma). After 48 hours of cell culture, cardiomyocytes were subjected to hypoxia condition with 5% O_2, 5% CO_2 and 90% N_2 for different time sets (0 hours, 1 hour, 2 hours, 4 hours, and 6 hours).

Cytosol extraction

Cytosol extraction was obtained by using the Mitochondria Isolation Kit for Tissue and Cultured Cells (KC010100, BioChain, USA). All procedures were strictly performed following the manufacturer's instruction. Briefly, myocardium or cultured cardiomyocytes were collected and washed twice with 10 mL ice-cold phosphate-buffered saline (PBS). Then the myocardium or cultured cardiomyocytes were homogenized and transferred into an Eppendorf tube, which was centrifuged at 600 g for 10 minutes at 4°C. The supernatant was carefully transferred into a new tube. The supernatant was centrifuged at 12 000 g for 15 minutes at 4°C to obtain the cytosol extraction, which is stored at –80°C for further analysis.

Real-time polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from myocardium or cultured cardiomyocytes by using TRIzol reagent (Sigma, USA). And cDNA was obtained by using the M-MLV reverse transcriptase kit (Invitrogen, USA). SYBR green PCR master mix was used in the PCR system following the manufactory's instruction (Bio-Rad Laboratories, USA). β-actin was used as a loading control. The primer sequences were as follows:
1) Atf6: forward: 5'-GATTTGATGCCTTGGGAGTC-3',

reverse: 5'-GGACCGAGGAGAGACAG-3';
2) Grp78: forward: 5'-AAGGTGAACGACCCCTAACAAA-3',
reverse: 5'-GTCACTCGGAGAATACCATTAACATCT-3';
3) caspase-3: forward: 5'-TGTCATCTCGCTCTGGTACG-3',
reverse: 5'-AAATGACCCCTTCATCACCA-3';
4) β-actin: forward: 5'-GACGGCCAGGTCATCACTAT-3',
reverse: 5'-CGGGATGTCAACGTCACACTT-3'.
Samples were amplified for 34 cycles using the CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, USA). The expression of Atf6, Grp78 or caspase-3 in relation to β-actin was determined.

ATP levels detection

ATP levels from myocardium and cultured cardiomyocytes were detected using a specific kit (Jiancheng Bioengineering Institute, China). All procedures were performed following the manufacturer's instruction. For the last step, the ATP level was detected by spectrophotometry at 636 nm. The detections were equilibrated by the total protein concentration.

Western blots

Protein expressions of cleaved-ATF-6, GRP-78, cleaved-caspase-3, and cytochrome *c* were assessed by western blots. The myocardium was homogenized, and the cultured cardiomyocytes were lysed at 4°C for protein extraction, which was subjected to bicinchoninic acid (BCA) assay for measuring protein contraction. 50 μg protein extraction was added into 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane. The membrane was incubated with specific primary antibodies against rat cleaved-ATF6, GRP-78, cleaved-caspase-3, cytochrome *c* and actin at 1: 1000 dilution overnight at 4°C, followed by incubation with horseradish peroxidase (HRP) conjugated secondary antibodies (Cell Signaling, USA) at room temperature for 1 hour. Protein bands were developed on an x-ray film. Densitometric ratios of cleaved-ATF6, GRP-78, cleaved-caspase-3, and cytochrome *c* to actin were obtained.

Cytosolic mitochondrial DNA levels detection

The whole cytosolic DNA was obtained by using the DNeasy Blood & Tissue Kit (No. 69504, Qiagen, China) as previously described [13]. The mitochondrial DNA level was detected using the SYBR green PCR master mix and the PCR system the CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, USA). The primer sequences used to detect mitochondrial DNA (rat NADH dehydrogenase 1 gene) were as follows: CGCCTGACCAATAGCCATAA (forward); ATTCGACGTTAAAGCCTGAGA (reverse). All samples were analyzed 3 times for quality control.

Statistical analysis

All descriptive data was expressed as mean±standard error of the mean (SEM). Statistical studies were performed by GraphPad Prim 6 (GraphPad Software). One-way ANOVA followed by Bonferroni's test were used between multiple groups. $P<0.05$ was considered as statistical significance.

Results

As shown in Figures 1 and 2, both mRNA levels and protein levels of ATF6 and GRP-78 were significantly increased at the first hour and continued to increase *in vivo* and *in vitro* ($P<0.05$). The *in vivo* study revealed that the ATP levels were increased at the first 2 hours after ischemic injury and significantly decreased from 4 hours after ischemic injury ($P<0.05$). Intriguingly, ATP level of cultured cardiomyocytes decreased remarkably from 2 hours after ischemic injury ($P<0.05$). Both *in vivo* and *in vitro* studies showed cytosolic levels of mitochondrial DNA increased at 2 hours ($P<0.05$). While cytochrome *c* increased at 4 hours *in vitro* and 6 hours *in vivo* after ischemic injury ($P<0.05$).

In addition, caspase-3, as the central biomarker of cell apoptosis, was used to examine the apoptotic status of the cardiomyocytes. Significant increasing of mRNA expression of caspase-3 and protein expression of cleaved caspase-3 were found at 6 hours after ischemic injury *in vivo* ($P<0.05$). In addition, *in vitro* study also revealed that increased mRNA level of caspase-3 at 6 hours and protein level of cleaved-caspase-3 at 6 hours after ischemic injury ($P<0.05$).

Discussion

The present study first showed that after acute ischemic injury to cardiomyocytes, indicators for endoplasmic reticulum stress significantly increased at 1 hour, which demonstrated that endoplasmic reticulum stress occurred in the early stage of disease. In addition, ATP levels, which indicate energy metabolism were significant inhibited at 4 hours. In addition, mitochondrial DNA, which is a marker of mitochondrial damage, was elevated significantly at 2 hours, which demonstrated that the mitochondrial damage presented later than the endoplasmic reticulum stress. Our study implied that the mitochondrial dysfunction was secondary to the endoplasmic reticulum stress under ischemic insults.

GRP-78-initiated unfolded protein response plays a critical and corrective role in endoplasmic reticulum stress, in which ATF6 is a key factor in the unfolded protein response [14,15]. It is reported that acute ischemic injury could cause significant endoplasmic reticulum stress, which induced cardiomyocytes
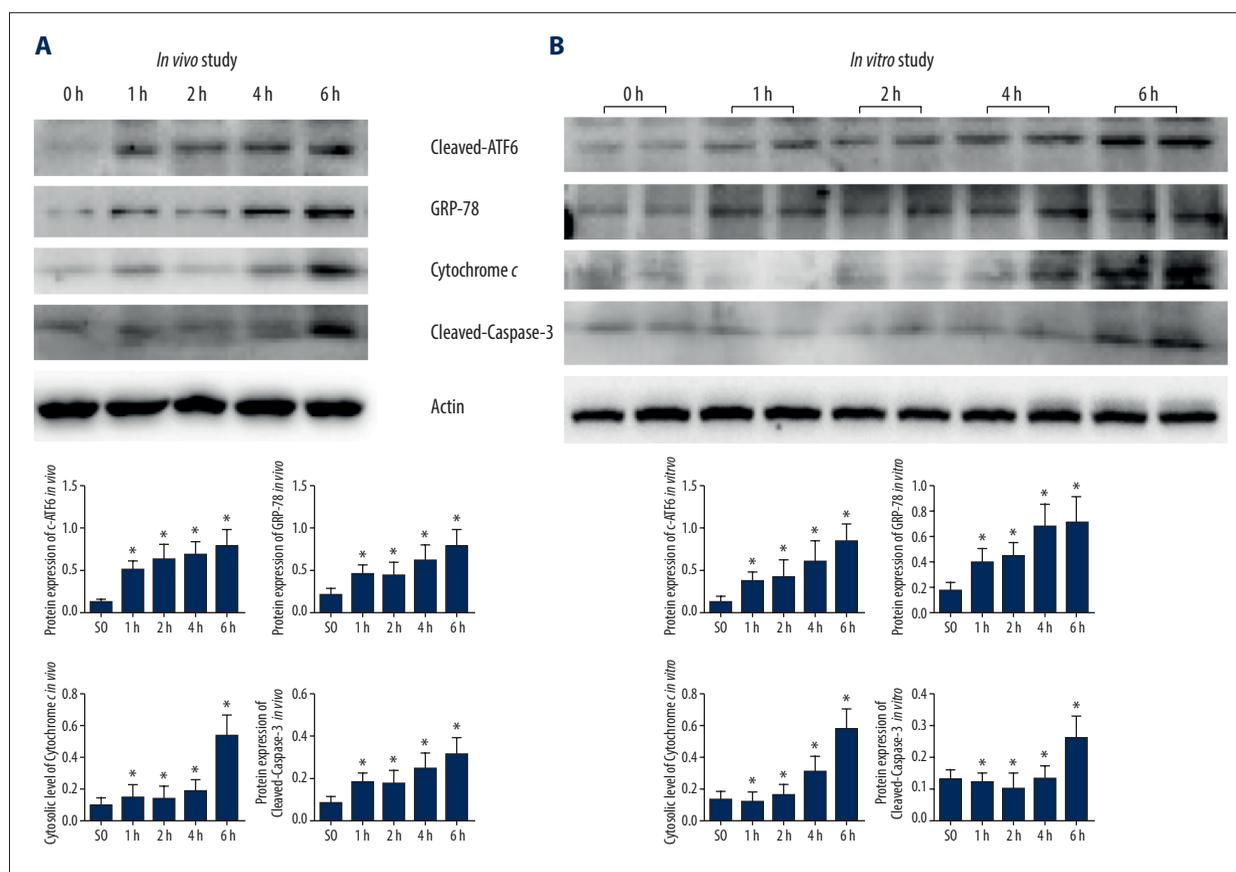
Figure 1. *In vivo* (**A**) and *in vitro* (**B**) western blot images and quantified analyses of Cleaved-ATF6, GRP-78, cytochrome *c*, cleaved-caspase-3, and actin. * $P<0.05$ versus SO. # $P<0.05$ versus 2 hours. N=4–6. SO group – sham-operation, or 0 hours group.

apoptosis through endoplasmic reticulum stress-related apoptotic pathways [16]. Recently, increasing studies have demonstrated that inhibition of endoplasmic reticulum stress had great benefits on the ischemic injury cardiomyocytes [17,18]. Therefore, targeting endoplasmic reticulum stress might be a compromising strategy for treating acute myocardial infarction.

Additionally, many studies have focused on acute myocardial infarction-induced mitochondrial dysfunction. As a major energy-producing organism in the cardiomyocyte, the mitochondria are essential for contraction through constant oxidative phosphorylation [19]. Under ischemic insults, multiple signaling pathways are activated to cause the uncoupling of the electron transport chain, opening of the mitochondrial permeability transition pore, and releasing of cytochrome *c*, which induces mitochondrial dysfunction-mediated cell death [5,20].

It is well-known that mitochondrial dysfunction-induced massive ROS production and cytochrome *c* release are responsible for apoptosis and necrosis, which have negative effects on adjacent cardiomyocytes and expands the infarct area [21]. Growing evidence has identified the inflammatory properties of free mitochondrial DNA, and that mitochondrial dysfunction or

cell injury could result in releasing mitochondrial DNA from the mitochondria [22,23]. In addition, our previous study showed elevated circulatory mitochondrial DNA levels in patients with acute myocardial infarction [24].

In the present study, we found that mitochondrial dysfunction occurred later than endoplasmic reticulum stress under acute ischemic injury, which implied that mitochondrial dysfunction was secondary to endoplasmic reticulum stress. A study revealed a relationship between endoplasmic reticulum and mitochondria-regulated synthesis of proteins in the endoplasmic reticulum, and production of ATP in the mitochondria and apoptosis [25]. Another study demonstrated that endoplasmic reticulum stress could affect not only mitochondrial biosynthesis, but also mitochondrial function, in which mitochondrial-associated membrane (MAM), acting as the physical connecting site between the endoplasmic reticulum and the mitochondria, plays a critical role [26]. It has been suggested that endoplasmic reticulum stress could induce mitochondrial dysfunction and cell death through the disruption of Ca^{2+} homeostasis [27]. Deniaud et al. found that endoplasmic reticulum, as the storage of Ca^{2+}, could release massive Ca^{2+} after endoplasmic reticulum stress, which could cause
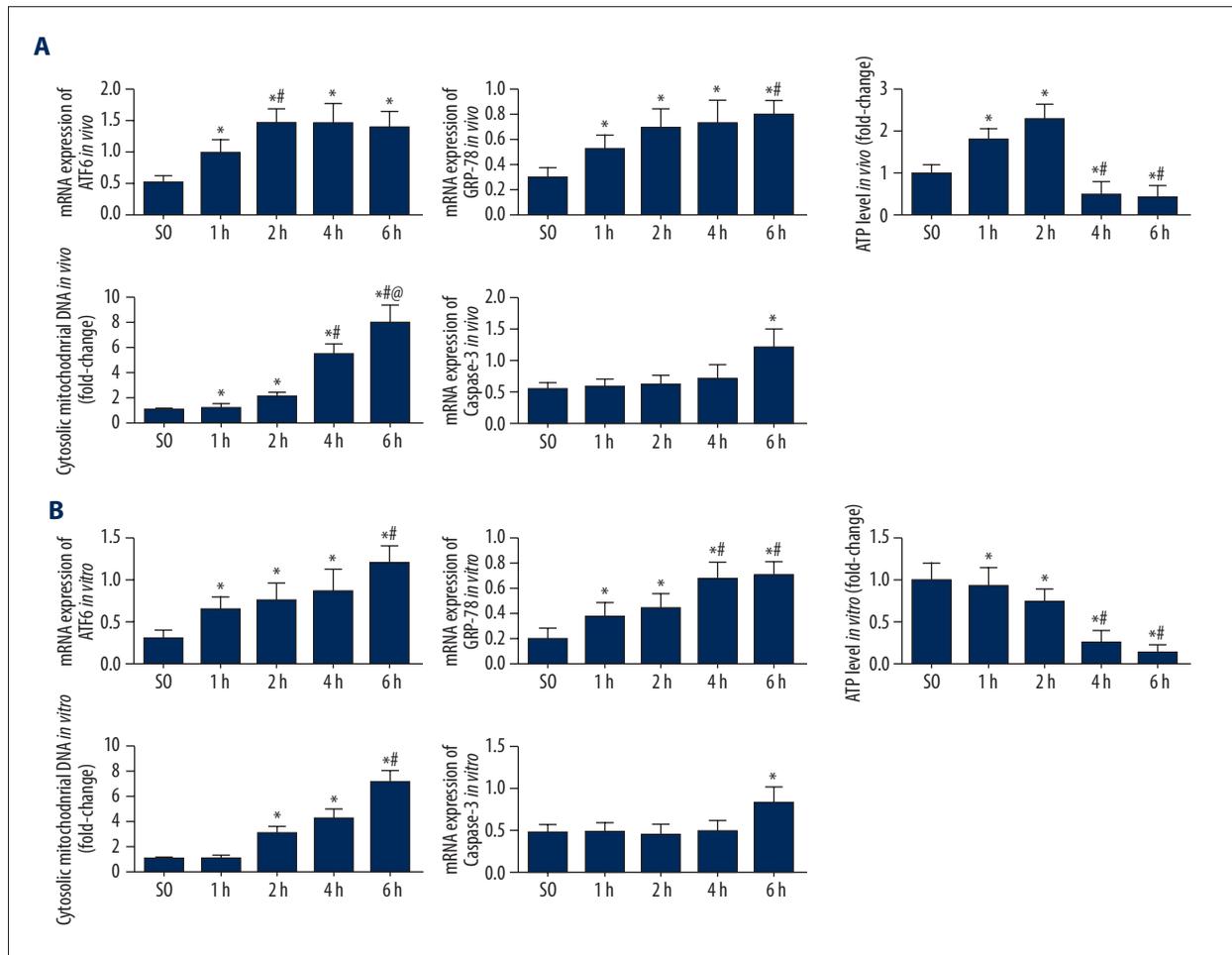
Figure 2. mRNA levels of ATF6, GRP-78 and caspase-3, ATP level and cytosolic mitochondrial DNA level *in vivo* (**A**) and *in vitro* (**B**). * $P<0.05$ versus SO. # $P<0.05$ versus 2 hours. @ $P<0.05$ versus 4 hours. N=4–6. SO group – sham-operation, or 0 hours group.

significantly increased concentration of Ca^{2+} in the mitochondria. Intriguingly, inducing accumulation of Ca^{2+} in the mitochondria without endoplasmic reticulum stress failed to result in the apoptotic status of mitochondria [28].

Conclusions

From our preliminary data, we need to further explore the interaction between endoplasmic reticulum stress and mitochondrial dysfunction and identify the critical components, like some calcium channel proteins and small genetic regulators, in the ischemic injury. In summary, the present study first revealed the time-dependent variations of endoplasmic reticulum stress and mitochondrial dysfunction after the insult of ischemic injury, which suggested that mitochondrial dysfunction was secondary to endoplasmic reticulum stress. Our study provided a novel experimental foundation for further exploring the detail mechanism after ischemic injury, especially the interaction between endoplasmic reticulum and mitochondria, which could help identify more pharmacological targets for rescuing the myocardium after ischemic injury.

Conflict of interest

None.

References:

1. Kullo IJ, Fan X, Ding K: Genetic risk, lifestyle, and coronary artery disease. N Engl J Med, 2017; 376: 1192–93

2. Harrington RA: Targeting inflammation in coronary artery disease. N Engl J Med, 2017; 377: 1197–98

3. Moran AE, Roth GA, Narula J, Mensah GA: 1990–2010 global cardiovascular disease atlas. Glob Heart, 2014; 9: 3–16

4. Wei C, Wang Y, Li M et al: Spermine inhibits endoplasmic reticulum stress-induced apoptosis: A new strategy to prevent cardiomyocyte apoptosis. Cell Physiol Biochem, 2016; 38: 531–44

5. Akhnokh MK, Yang FH, Samokhvalov V et al: Inhibition of soluble epoxide hydrolase limits mitochondrial damage and preserves function following ischemic injury. Front Pharmacol, 2016; 7: 133

6. Ertracht O, Malka A, Atar S, Binah O: The mitochondria as a target for cardioprotection in acute myocardial ischemia. Pharmacol Ther, 2014; 142: 33–40

7. Soto-Pantoja DR, Wilson AS, Clear KY et al: Unfolded protein response signaling impacts macrophage polarity to modulate breast cancer cell clearance and melanoma immune checkpoint therapy responsiveness. Oncotarget, 2017; 8: 80545–59

8. Kammoun HL, Chabanon H, Hainault I et al: GRP78 expression inhibits insulin and ER stress-induced SREBP-1c activation and reduces hepatic steatosis in mice. J Clin Invest, 2009; 119: 1201–15

9. Okumura N, Kitahara M, Okuda H et al: Sustained activation of the unfolded protein response induces cell death in fuchs' endothelial corneal dystrophy. Invest Ophthalmol Vis Sci, 2017; 58: 3697–707

10. Bronner DN, Abuaita BH, Chen X et al. Endoplasmic reticulum stress activates the inflammasome via NLRP3- and caspase-2-driven mitochondrial damage. Immunity, 2015; 43: 451–62

11. Arruda AP, Pers BM, Parlakgul G et al: Chronic enrichment of hepatic endoplasmic reticulum-mitochondria contact leads to mitochondrial dysfunction in obesity. Nat Med, 2014; 20: 1427–35

12. Tang C, Koulajian K, Schuiki I et al: Glucose-induced beta cell dysfunction in vivo in rats: Link between oxidative stress and endoplasmic reticulum stress. Diabetologia, 2012; 55: 1366–79

13. Qin C, Gu J, Qian H et al: Potential mechanism of post-acute aortic dissection inflammatory responses: The role of mtDNA from activated platelets. Cardiology, 2016; 135: 228–35

14. Casas C: GRP78 at the centre of the stage in cancer and neuroprotection. Front Neurosci, 2017; 11: 177

15. Lewy TG, Grabowski JM, Bloom ME: BiP: Master regulator of the unfolded protein response and crucial factor in flavivirus biology. Yale J Biol Med, 2017; 90: 291–300

16. Wang S, Wang Z, Fan Q et al: Ginkgolide K protects the heart against endoplasmic reticulum stress injury by activating the inositol-requiring enzyme 1alpha/X box-binding protein-1 pathway. Br J Pharmacol, 2016; 173: 2402–18

17. Shi ZY, Liu Y, Dong L et al: Cortistatin improves cardiac function after acute myocardial infarction in rats by suppressing myocardial apoptosis and endoplasmic reticulum stress. J Cardiovasc Pharmacol Ther, 2017; 22(1): 83–93

18. Jian L, Lu Y, Lu S, Lu C: Chemical chaperone 4-phenylbutyric acid protects H9c2 cardiomyocytes from ischemia/reperfusion injury by attenuating endoplasmic reticulum stress-induced apoptosis. Mol Med Rep, 2016; 13: 4386–92

19. Jendrach M, Mai S, Pohl S et al: Short- and long-term alterations of mitochondrial morphology, dynamics and mtDNA after transient oxidative stress. Mitochondrion, 2008; 8: 293–304

20. Wang Y, Liu J, Ma A, Chen Y: Cardioprotective effect of berberine against myocardial ischemia/reperfusion injury via attenuating mitochondrial dysfunction and apoptosis. Int J Clin Exp Med, 2015; 8: 14513–19

21. Wang K, Liu CY, Zhang XJ et al: miR-361-regulated prohibitin inhibits mitochondrial fission and apoptosis and protects heart from ischemia injury. Cell Death Differ, 2015; 22: 1058–68

22. Zhang Q, Raoof M, Chen Y et al: Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature, 2010; 464: 104–7

23. Qin C, Liu R, Gu J et al: Variation of perioperative plasma mitochondrial DNA correlate with peak inflammatory cytokines caused by cardiac surgery with cardiopulmonary bypass. J Cardiothorac Surg, 2015; 10: 85

24. Qin C, Gu J, Liu R et al: Release of mitochondrial DNA correlates with peak inflammatory cytokines in patients with acute myocardial infarction. Anatol J Cardiol, 2017; 17(3): 224–28

25. Kim SH, Kim KY, Yu SN et al: Silibinin induces mitochondrial NOX4-mediated endoplasmic reticulum stress response and its subsequent apoptosis. BMC Cancer, 2016; 16: 452

26. Kemter E, Frohlich T, Arnold GJ et al: Mitochondrial dysregulation secondary to endoplasmic reticulum stress in autosomal dominant tubulointerstitial kidney disease – UMOD (ADTKD-UMOD). Sci Rep, 2017; 7: 42970

27. Ahn C, An BS, Jeung EB: Streptozotocin induces endoplasmic reticulum stress and apoptosis via disruption of calcium homeostasis in mouse pancreas. Mol Cell Endocrinol, 2015; 412: 302–8

28. Deniaud A, Sharaf el dein O, Maillier E et al: Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis. Oncogene, 2008; 27: 285–99