Zhu, Dao-Li; Wang, Kang-Le; Chen, Pei-Lin; Li, Yuan
The c-Jun N-terminal Kinases (JNK) / Mitogen-activated Protein Kinase (MAPK) is Responsible for the Protection of Tanshinol (Danshensu) upon H\textsubscript{2}O\textsubscript{2}-Induced L\textsubscript{6} Rat Myoblast Cell Injury
Universidade Federal do Rio Grande do Sul
Porto Alegre, Brasil

Available in: http://www.redalyc.org/articulo.oa?id=289029240007

Acta Scientiae Veterinariae,
ISSN (Printed Version): 1678-0345
ActaSciVet@ufrgs.br
Universidade Federal do Rio Grande do Sul
Brasil
The c-Jun N-terminal Kinases (JNK) / Mitogen-activated Protein Kinase (MAPK) is Responsible for the Protection of Tanshinol (Danshensu) upon H$_2$O$_2$-Induced L$_6$ Rat Myoblast Cell Injury

Dao-Li Zhu, Kang-Le Wang, Pei-Lin Chen & Yuan Li

ABSTRACT

**Background**: Promoting L$_6$ rat myoblast cell (L$_6$ RMC) survival in the pro-apoptotic environment is critical to myoblast cell replacement for skeletal muscle degenerative disease therapy. Tanshinol (Danshensu), one of the principal bioactive components in salvia miltiorrhiza bunge, has been used widely in skeletal muscle system (SMS) diseases treatment and serves as an antioxidant to protect myoblast cells against oxidative stress. The present study was undertaken to investigate the protective effects of Tanshinol on L$_6$ RMC injury induced by hydrogen peroxide (H$_2$O$_2$). After challenge with 100 µM H$_2$O$_2$ for 1h, loss of cell viability and excessive apoptotic cell death were observed in cultured L$_6$ RMC, tanshinol treatment conferred protective effects against the loss of cellular viability in a concentration-dependent manner.

**Materials, Methods & Results**: L$_6$ rat myoblast cells (L$_6$ RMC) were maintained DMEM containing 4.5 g/L glucose and supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin on tissue culture flasks in a 37°C humidified atmosphere of 95% air and 5% CO$_2$. Cells were subcultured every 2-3 days. The cells were stained with SABC-Cy3 fluorescence and measured by counting the nuclei. L$_6$ RMC viability was determined by MTT assay in 96-well plates. The cells were counter-stained with H & E staining and Hoechst 33342 fluorescent staining. Cell cycle phase distribution and apoptosis rates were detected by flow cytometry. Western blot were repeated three times, and qualitatively similar results were obtained. L$_6$ RMC were pretreated with Tanshinol 375, 187.5, 93.75 mg/mL for 24 h, followed by treatment of 1000, 500, 100 µM H$_2$O$_2$ for 1h (P < 0.05) with viability by MTT method. Tanshinol 187.5 mg/mL pretreatment was protected in L6 RMC from H2O2-induced apoptosis with H&E staining and Hoechst 33342 fluorescent staining (P < 0.05). Tanshinol 375, 187.5, 93.75 mg/mL treatment showed the upregulation of Bcl-2 and the reduction of Bax antibody staining (P < 0.05) and western blot 375, 187.5, 93.75 mg/mL of three concentrations of Tanshinol of protection is different and 187.5 mg/mL is the best protection (P < 0.05) with flow cytometry. L6 RMC was treated with Tanshinol in the presence or absence of JNK and MAPK inhibitor, SP600125 and PD98059 at each 10 µM via modulation of JNK/MAPK signaling pathways.

**Discussion**: Tanshinol (Danshensu) of the principal bioactive components in salvia miltiorrhiza bunge was attenuated L$_6$ rat myoblast cells (L$_6$ RMC) apoptosis induced by H$_2$O$_2$ and the myoprotective effects of Tanshinol might be due to the activation of JNK/MAPK. L$_6$ RMC was potential to substitute for lost tissue, will survive, differentiate, and integrate into existing skeletal muscle networks and are very sensitive to increases of ROS which results in cell apoptosis. This study provided more data to support the antioxidant roles of Tanshinol in L$_6$ RMC, which are responsible for myogenesis for normal skeletal muscle functions in adults. These data demonstrate that the oxidative-induced calpain-dependent pathways could play a pivotal role in the depletion of the satellite cell pool leading to an impairment in regeneration events and the development of sarcopenia. Inhibition of JNK and MAPK with SP600125 (a JNK inhibitor) and PD98059 (a MAPK inhibitor) significantly increased H$_2$O$_2$-induced acute apoptosis. Therefore, adaptive cyoprotection through JNK/MAPK will be switched on upon H$_2$O$_2$ injury in L$_6$ RMC and the transient activation of JNK/MAPK signaling delays the H$_2$O$_2$-induced acute apoptosis.

**Keywords**: tanshinol, cell apoptosis, cell viability, SMS treatment, L$_6$ RMC, JNK / MAPK.
INTRODUCTION

Tanshinol (Danshensu) is one of the principal bioactive components derived from the root of Salvia miltiorrhiza bunge, a traditional Chinese herbal which has been widely used in the treatment of skeletal muscle diseases including Duchenne muscular dystrophy [19], Myotonic dystrophy and myotonic myopathy [9], Polymyositis and dermatomyositis [20], and Amyotrophic lateral sclerosis [2]. It has been reported Tanshinol has various pharmacological activities including antioxidant, anti-inflammation and myoblast-protective effects on various types of cells [14].

As a natural antioxidant, Tanshinol penetrates through the blood-brain barrier [8] and the myoblast protective effects of Tanshinol have been evidenced both in vivo and in vitro [18]. Tanshinol significantly reduced the infarct volume and ameliorated the deficits in muscle weakness and malgia symptoms caused by transient MCAO [17]. Further study showed the Tanshinol attenuated cognitive deficits and muscle damage caused by chronic cerebral hypoperfusionin rats [25]. However, whether Tanshinol protects L6 rat myoblast cell (L6 RMC) from oxidative-induced apoptosis and related signaling pathways involved in this process is still unclear. In the present study, we used cultured L6 RMC to characterize hydrogen peroxide (H2O2)-induced L6 RMC apoptosis and related cell signaling pathways. The present study was designed to determine the protective effects of Tanshinol on L6 RMC injury from oxidative stress-induced apoptosis by activating JNK / MAPK, regulating the Bcl-2 family, including Bcl-2 and Bax expression.

MATERIALS AND METHODS

Cell culture and treatments

L6 rat myoblast cells (L6RMC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/L glucose and supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin on substrate-free tissue culture flasks in a 37°C humidified atmosphere of 95% air and 5% CO2. Cells were allowed to adhere to the culture flasks surface and grown to approximately 70-80% confluence at which point cells were washed with Ca2+ and Mg2+ free phosphate buffered saline (PBS) and media replaced. Cells were subcultured every 2-3 days.

Immunocytochemistry staining and MTT assay of Cell viability

L6 RMC cultured in 12-well dishes were incubated with monoclonal Desmin, Myosin, Bax and Bcl-2 antibodies and with SABC-Cy3 fluorescence, fluorescent images were captured by an Nikon inverted microscope (Nikon, Japan) [10]. The distribution of nuclei in L6 RMC was measured by counting the nuclei in at least 10 different locations selected randomly.

L6 RMC were cultured in 10% FBS+DMEM +100 U/mL penicillin and 100 U/mL streptomycin in 96-well plates at 1x10⁴ cells/well for 1 day. For H2O2 -induced injury, cells were incubated with different concentrations of H2O2 (1000, 500, 100, 50, 10, 5, and 0 µM) for 1h, then subjected to MTT assay. For Tanshinol mediated protection assay, L6 RMC were pretreated with Tanshinol (1500, 750, 375, 187.5, 93.75, 46.875 and 0 mg/mL) 24 h before subjected to H2O2 (100 µM) injury for 1 h. Then the culture medium were replaced with fresh medium containing 5mg/mL MTT for 4 h at 37.0ºC. The medium was replaced by 150 µL/well DMSO to resolve the formazan crystals. The optical density (A values that is the OD values) was measured at an emission wave length of 570 nm and reference wave length of 630 nm. The data were expressed as a percent of control value and means ± SD of three experiments and six wells included in each group.

Hematoxylin and Eosin (H&E) staining and fluorescent staining assay

L6 RMC cultured in 12-well dishes after each treatment were fixation for 10 min with 4.0% paraformaldehyde and subsequently washed with PBS for 3 times. Cells were incubated in hematoxylin for 10 min, rinsed with tap water for 5 min and incubated in eosin for 30 s. The staining was dehydrated sequentially with 75%, 95% and 100% ethanol for 2-5 min each. Nuclei were counter-stained with Hoechst 33342.

Apoptosis rates detected by flow cytometry and Western blot analysis

Cell cycle phase distribution was analyzed by a FACS Calibur Flow Cytometer (BD Biosciences, San Jose, CA, USA) with propidium iodide (PI) staining, as described previously [11]. Tanshinol group 375, 187.5, 93.75 mg/mL were pretreated for 24 h, then 100 µM H2O2 damage for 1 h. Model group 100 µM H2O2 damage and normal group L6 RMC were used as positive and negative controls.
Cells were respectively pretreated with 10 µM SP600125 and 10 µM PD98059 for 2 h followed by inhibitors with 187.5 mg/mL Tanshinol for 30 min. L₆RMC were pretreated with 187.5 µg/mL Tanshinol for 24h then subjected to 100 µM H₂O₂ for 1h, the expression changes of Bax and Bcl-2 which are related to apoptosis pathways were checked. Western blot analyses were repeated three times, and qualitatively similar results were obtained.

Statistical analysis

All data were expressed as means ± SD (x ± s). The data were evaluated statistically by analysis of SPSS 17.0 statistics software followed by the t test for paired observations. In all cases, P < 0.05 was considered statistically significant.

RESULTS

Upon visual inspection (Figure 1.A,B), the formation of L₆RMC was found to be significantly increased in the presence of Tanshinol (1500, 750, 375, 187.5, 93.75, 46.875 mg/mL) for 24h compared to the control cells. We observed a significant positive effect of all the tested Tanshinol on L₆RMC proliferation, with the number of cells being increased by 375, 187.5, 93.75 mg/mL of Tanshinol.

To determine whether Tanshinol plays a role in protecting L₆ rat myoblast cells (L₆RMC) from oxidative stress-induced cell death, L₆RMC were pre-treated with Tanshinol (1500, 750, 375, 187.5, 93.75, 46.875 and 0 mg/mL) for 24 h, followed by H₂O₂ (100 µM) injury for 1h with cell viability by MTT method (Figure 2. A, B, C, D, E). Hematoxylin and Eosin (H&E) staining and Hoechst 33342 fluorescent staining were performed (Figure 3.A,B).

To confirm the signal mechanisms of Tanshinol (Danshensu) in the inhibition of H₂O₂-mediated L₆RMC apoptosis, expression levels of survival and apoptotic marker proteins were estimated by Western blot analysis. As expected, oxidative stress of H₂O₂ stimulation was increased the Bax level (Figure 4. B, C), decreased Bcl-2 level (Figure 4. A, C). Tanshinol pretreatment attenuated H₂O₂-induced Bax level in L₆RMC, and reversed the decreased levels of Bcl-2 significantly, which may contribute to the increased cell viabilities (Figure 4. A-C).

Phosphorylation of JNK/MAPK induced by L₆RMC was decreased markedly in the presence of SP600125/PD98059 as shown in Figure 5. A1. Accordingly, in the presence of SP600125 and PD98059, Tanshinol-mediated myoprotection of L₆RMC from apoptosis induced by H₂O₂ was abolished (Figure 5. A2), while SP600125 and PD98059 treatment alone did not induce L₆RMC apoptosis and had no effects on L₆RMC protection. L₆RMC were treated with 375, 187.5, 93.75 mg/mL Tanshinol for 24h, then from apoptosis induced by 100 µM H₂O₂ for 1h. The cellular DNA content was analyzed by flow cytometry to detect changes in the cell cycle distribution (Figure 5. B).

![Figure 1](image)

Figure 1. (A) L₆RMC cells were induced to proliferation in GM (10 µm, 20 µm) in the absence (A1) or presence of Tanshinol (1500, 750, 375, 187.5, 93.75, 46.875 mg/mL) for 24 h (A1-7, 10 µm, 20 µm). (B) A values (i.e. the OD values) (n = 3, P < 0.05 versus control).
The c-Jun N-terminal Kinases (JNK) / Mitogen-activated Protein Kinase (MAPK) is Responsible for the Protection...

Figure 2. (A) The Myogenic of L.RMC was identified by Desmin, Myosin staining in A1, A2 and nuclei were counter-stained with Hoechst 33342 (10 µm, 20 µm, Figure A3). (B) MTT assays. 1: normal cell; 2: 1000 µM H$_2$O$_2$; 3: 500 µM H$_2$O$_2$; 4: 100 µM H$_2$O$_2$; 5: 50 µM H$_2$O$_2$; 6: 10 µM H$_2$O$_2$; 7: 5 µM H$_2$O$_2$. 8: L.RMC was assessed after exposure to 0, 1000, 500, 100, 50, 10, 5 µM H$_2$O$_2$ for 1 h. (C) L.RMC were pretreated with Tanshinol 375, 187.5, 93.75 mg/mL for 24h, followed by treatment of 1000, 500, 100 µM H$_2$O$_2$ for 1 h. 1: normal cell; 2: respectively 1000, 500, 100 µM H$_2$O$_2$; 3: 375 mg/mL + 1000, 500, 100 µM H$_2$O$_2$; 4: 187.5 mg/mL + 1000, 500, 100 µM H$_2$O$_2$; 5: 93.75 mg/mL + 1000, 500, 100 µM H$_2$O$_2$ (n = 3, P < 0.05 versus control$^a$ and P < 0.05 versus H$_2$O$_2$ group$^b$).

Figure 3. H&E staining and Hoechst 33342 fluorescent staining (10µm, 20µm). 1: uninjured group; 2: 500 µM H$_2$O$_2$; 3: 200 µM H$_2$O$_2$; 4: 100 µM H$_2$O$_2$; 5: 187.5 mg/mL Tanshinol; 6: 187.5 mg/mL Tanshinol + 500 µM H$_2$O$_2$; 7: 187.5 mg/mL Tanshinol + 200 µM H$_2$O$_2$; 8: 187.5 mg/mL Tanshinol + 100 µM H$_2$O$_2$; 9: Quantification data were the mean ± SD (n = 3, P < 0.05 versus control$^b$).
D.L. Zhu, K.L. Wang, P.L. Chen & Y. Li. 2014. The c-Jun N-terminal Kinases (JNK) / Mitogen-activated Protein Kinase (MAPK) is Responsible for the Protection...

**Figure 4.** Tanshinol treatment showed the upregulation of Bcl-2 (A, C), and the reduction of Bax (B, C). (A) Bcl-2 antibody staining. 1: 375 mg/mL Tanshinol + 100 µM H₂O₂; 2: 187.5 mg/mL Tanshinol + 100 µM H₂O₂; 3: 93.75 mg/mL Tanshinol +100 µM H₂O₂; 4: normal control; 5: negative control; 6: 100 µM H₂O₂ damage model; 7: Quantitative analysis of Bcl-2 staining cell numbers. (B) Bax antibody staining. The other as above. (C) Western blot: 1: normal control; 2: 100 µM H₂O₂ injury; 3: 375 mg/mL Tanshinol + 100 µM H₂O₂; 4: 187.5 mg/mL Tanshinol +100 µM H₂O₂; 5: 93.75 mg/mL Tanshinol + 100 µM H₂O₂. Tanshinol treatment showed the upregulation of Bcl-2 (C3) and the reduction of Bax (C2). The mean ± SD (n = 3, *P* < 0.05 versus control and *P* < 0.05 versus H₂O₂ group.)

**Figure 5.** (A) Rat L₆RMC were pretreated with or without SP600125, PD98059 for 1h, then subjected to Tanshinol for 24 h followed by exposure to 100µM H₂O₂ for 1h. Phosphorylation of JNK/MAPK upon Tanshinol stimulation was shown in A1. The percentage of apoptotic L₆RMC was determined in A2. (B) Cell cycles status at different treatments were appeared with Peak 1 appear after DNA break: subdiploid peak; Peak 2: DNA peak during G1 mean diploid peak; Peak 3: G2+M peak. B1: normal control (Ap=0.00%); B2: 500 µM H₂O₂ (Ap=32.45%); B3: 100 µM H₂O₂ (Ap=26.67%); B4: 375 mg/mL Tanshinol +100 µM H₂O₂ (Ap=14.09%); B5: 187.5 mg/mL Tanshinol + 100 µM H₂O₂ (Ap=4.96%); B6: 93.75 mg/mL Tanshinol + 100 µM H₂O₂ (Ap=6.73%). B7: The cell cycle of L₆RMC; B8: Apoptosis rate was compared in each group. The mean ± SD (n = 3, *P* < 0.05 versus control, *P* < 0.05 versus H₂O₂ group and *P* > 0.05 versus H₂O₂ group).
DISCUSSION

Myoregeneration is a relatively recent concept that includes myogenesis, myoplasticity, and myoregeneration-implantation of viable cells as a therapeutical approach. Impaired myogenesis and myoplasticity have been reported in skeletal muscles of patients suffering from most peripheral neuropathy [13] or myasthenia gravis and progressive muscular dystrophy [15] which are associated with low endogenous protection. Bursts of intracellular ROS result in cell apoptosis, which is a process regulated by cell signaling pathways and leads to cell death accompanied by the reduction of the total cell volume, general compaction of cell organelles and DNA fragmentation. L5RMC, potential to substitute for lost tissue, will survive, differentiate, and integrate into existing skeletal muscle networks [16] and are very sensitive to increases of ROS which results in cell apoptosis. H2O2 is one of the ROS generated during cellular metabolism. There is evidence that antioxidant mechanisms might be important not only for the survival of L5RMC in disease conditions, but may also define their fate [12]. Many antioxidants are particularly known to provide protection from ROS-mediated cellular damage. Tanshinol is well known as a scavenger of ROS in extracellular environments and for its cytoprotective effect on other cell types against the oxidative damage of ray irradiation [4]. As a natural antioxidant, the myoprotective effects of Tanshinol have been evidenced both in vivo and in vitro [5]. This study provided more data to support the antioxidant roles of Tanshinol in L5RMC, which are responsible for myogenesis for normal skeletal muscle functions in adults.

Besides the antioxidants role of Tanshinol, FATPE including ferulic acid (F), hydroxyl safflor yellow A (A), tanshinol (T), protocatechualdehyde (P) and paoniflorin (E) were measured in blood after oral FATPE, was found in decreasing infarct size, myocardial apoptosis and caspase-3 activity of Sprague-Dawley rats with acute myocardial infarction (AMI) were induced by coronary occlusion [21]. The clinical therapeutic effects of Tanshinol on traumatic intracranial hematoma (TICH) were observed and the intracranial hematoma absorption, hemorheological changes, and changes in coagulation index espre- and post-treatment were evaluated [7]. Potential neuroprotective effects of tanshinones IIA (16 mg/kg) readily penetrated the blood brain barrier reaching a peak concentration of 0.41 nmol/g brain wet weight 60 min after intraperitoneal injection and decreased slowly over several hours, twenty-four hours after middle cerebral artery occlusion, brain infarct volume was reduced by 30% and 37% following treatment respectively [3]. All these reports provide evidence that Tanshinol can be used for most central nervous system and peripheral nervous system myopathy treatment.

H2O2-induced inflammation is a mixture response and the involved apoptotic pathways may favor either cell death or differentiation. These data demonstrate that the oxidative-induced calpain-dependent pathways could play a pivotal role in the depletion of the satellite cell pool leading to an impairment in regeneration events and the development of sarcopenia [26]. Using immuno-histochemistry, Western blot and flow cytometry, we found a high expression of Bcl-2 in normal muscle, whereas the expression was found to be lower in the presence of an inflammatory as well as a dystrophic muscle disease [22]. It takes time, usually some days, our findings suggest that a positive feedback control between STIM1/SOCE and NFATc3 is required for efficient induction and progression of myoblast differentiation [27]. In this study, we treated L5RMC for a short time, therefore, the properties “stem” of L5RMC should remain unchanged and the apoptotic pathways favor only cell death not differentiation.

It should be noted that H2O2 preconditioning confers adaptive cytoprotection against oxidative stress-induced injury in cells including L5RMC and skeletal muscle satellite cells [6]. The results showed that H2O2 induced L5RMC apoptosis and selectively, it transiently activated JNK/MAPK in a concentration-dependent manner and returned to basal level with in 1h [1]. Inhibition of JNK with SP600125 (a JNK inhibitor) significantly increased H2O2-induced acute apoptosis. Therefore, adaptive cyoprotection through JNK/MAPK will be switched on upon H2O2 injury in L5RMC, and the transient activation of JNK/MAPK signaling delays the H2O2-induced acute apoptosis [23]. H2O2 alone failed to phosphorylate MAPK in L5RMC at a 1h time point in this study. It is possible that different pathways are activated because of Tanshinol pharmacological properties, for example, Tanshinol influences JNK in the lipopolysaccharide (LPS) induced acute lung injury (ALI) and immune-modulating property [24]. We can not exclude the possibilities of other pathways which mediate the effects at this stage, and further studies are needed for investigation. Our data showed that Tanshinol protected L5RMC from oxidative stress-induced apoptosis by activating JNK/MAPK, regulating the Bcl-2 family, including Bcl-2 and Bax expression.
D.L. Zhu, K.L. Wang, P.L. Chen & Y. Li. 2014. The c-Jun N-terminal Kinases (JNK) / Mitogen-activated Protein Kinase (MAPK) is Responsible for the Protection...


REFERENCES


Acknowledgements. The Building Special Project of Nantong University Doctor’s Spot, No. 05024276 and The Funded Projects Training Objects of Nantong University Teaching Teacher Cultivation Project, No. 03080542.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Author contributions. Dao-Li Zhu conceived and designed this study, conducted experiments, and wrote manuscript. Kang-Le Wang, Pei-Li Chen and Yuan Li participated in experimentation, analyzed experimental data and performed statistical analysis. All authors have read the manuscript and agreed to submit to the journal.


