The effect and underlying mechanism of Timosaponin B-II on RGC-5 necroptosis induced by hydrogen peroxide

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Abstract

Background: Necroptosis is an important mode of cell death, which is due to oxidant stress accumulation. Our previous study indicated that oxidant stresses could be reduced by Timosaponin B-II (TBII), a kind of Chinese herb Rhizoma Anemarrhenae monomer extraction. We wonder the possible effect of Timosaponin B-II, whether it can protect cells from necroptosis via reducing the oxidant stress, in RGC-5 following hydrogen peroxide (H2O2) insult.

Methods: RGC-5 cells were grown in DMEM, the model group was exposed in H2O2 with the concentration of 300 μM, and the experimental group was pre-treated with Timosaponin B-II at different concentrations (1 μM, 10 μM, 100 μM and 1000 μM) for 24 hrs. MTT assay was carried out to measure the cytotoxicity of H2O2, MDA concentration assay was executed to evaluate the degree of oxidative stress, TNF-α ELISA Assay was used to measure the concentration of TNF-α, finally, the degree of necrosis were analyzed using flow cytometry.

Results: We first constructed the cell injury model of necroptosis in RGC-5 upon H2O2 exposure. Morphological observation and MTT assay were used to evaluate the degree of RGC-5 death. MDA assay were carried out to describe the degree of oxidant stress. Annexin V/PI staining was used to detect necroptotic cells pre-treated with or without Timosaponin B-II following H2O2 injury. TNF-α ELISA was carried out to detect the TNF-α accumulation in RGC-5. Upon using Timosaponin B-II with concentration of 100 μM, the percentage of cell viability was increased from 50% to 75%, and the necrosis of cells was reduced from 35% to 20% comparing with H2O2 injury group. Oxidant stress and TNF-α was reduced upon injury which decreased the ratio of RGC-5 necroptosis.

Conclusion: Our study found out that Timosaponin B-II might reduce necroptosis via inhibition of ROS and TNF-α accumulation in RGC-5 following H2O2 injury.

Keywords: Retinal ganglion cells-5, Timosaponin B-II, TNF-α, Necroptosis, Oxidative stress
which has the similar finding with our previous work [13]. Additionally, our recent studies have also indicated that necroptosis occurs in retinal ganglion cells-5 (RGC-5) at an early stage following elevated hydrostatic pressure (EHP) in vitro [14] or hydrogen peroxide (H$_2$O$_2$) treatment (our unpublished data) detected by flow cytometry (a typical way to monitor necroptosis) [15].

Although some scientists recently have synthesized necroptosis inhibitors, such as Nec-3 [16], Nec-5 [17], Nec-7 [18], Nec-21 [19], etc., the mechanism still needs to be further clarified. Nec-1 is a synthetic small molecular compound which is the most commonly used in necroptosis inhibition [1]. So far, there are no reports about whether it has side effects when it is applied to animal or cellular models. Traditional Chinese herb rhizoma anemarrhenae is the dried rhizome of anemarrhe-naaphodeloidesbg. It contains lots of steroidal saponins including sarsasapogenin, markosapogenin, megotigenin, diosgenin and its glycosylated derivatives, like Timosaponin A-I, A-II, A-III, A-IV, B-I, B-II,C, D and Timosaponin E [20]. Li [21] indicated that Timosaponin B-II (Figure 1, the chemical structure of Timosaponin B-II, CAS number: 136656-07-0) might improve the impairment of learning and memory caused by cerebral ischemia in a dose-dependent manner. Lu [11] and Kim’s [22] research suggested that, Timosaponin B-II could not only inhibit the production of IL-1β and IL-6, but also TNF-α (one of important molecules which initiates necroptosis), these results suggested the effect might be related to Timosaponin B-II which has anti-inflammatory activity. Besides, Zhang [23] and Kaname [24] found Timosaponin E-I, E-II, B-II, B-III and A-III played an important role in superoxide dismutase-generation. Deng [25] demonstrated that culturing primary rat neurons (APβ25–35 insult model) with Timosaponin B-II at a certain range of concentrations showed remarkable anti-oxidative damage effects, which indicated Timosaponin B-II could remove oxygen radicals, and keep the intra-cellular redox reactions in a dynamic equilibrium [26]. Our previous study also showed that Timosaponin B-II participated in the protection of rat RGCs which was treated by FeCl$_3$ solution via anti-oxidation [27]. Of note, whether Timosaponin B-II could inhibit oxidative stress-induced RGCs necroptosis or not, it is still under investigation. Therefore, our present experiments focused on Timosaponin B-II’s potential roles in RGC-5 necroptosis suppression upon hydrogen peroxide (H$_2$O$_2$) treatment. Meanwhile, the effect of Timosaponin B-II on malondialdehyde (MDA, biomarker of oxidative stress) and TNF-α in RGC-5 has been investigated. Our study may give a better understanding of the protective effect of Timosaponin B-II upon neuronal injury, and provide the experimental basis of novel mechanism on cell death.

**Methods**

**Cell culture**

The mouse retinal ganglion cell line (RGC-5) was contributed by Department of Ophthalmology, Second Hospital of Ji Lin University, China [28]. RGC-5 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, HyClone Laboratories, Inc. UT, USA) and supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Inc. UT, USA), 100 U/ml of penicillin and 100 μg/ml of streptomycin (HyClone Laboratories, Inc. UT, USA). The RGC-5 cells used in the experiment were with 2-3 passages post-thawed to minimize the variability in the assays based on our observations. The density of RGC-5 cells was around 80% in 6 ml culture media in 50 ml flask before insults (H$_2$O$_2$ treatment).

**Reagent**

The powder of Timosaponin B-II was provided by Professor Wan-Sheng Chen from Department of Pharmacology, School of Pharmacology, Second Military Medical University, Shanghai, China [29,30] and its purity is above 98%. Timosaponin B-II was dissolved in sterile normal saline (NS) at 2 mM in room temperature (24°C), H$_2$O$_2$ (Sigma-Aldrich, MO, USA) with 0.01 M PBS at 3 mM in store to yield a low concentration working solutions.

**Cell model construction and drug treatment**

Cells were equally divided into three groups randomly. The normal control group (CTL), the model group (H$_2$O$_2$, 300 μM), and the experimental group (H$_2$O$_2$ + Timosaponin B-II). The experimental group was pre-treated with Timosaponin B-II at different concentrations (1 μM, 10 μM, 100 μM and 1000 μM) for 24 hrs. No drugs were exposed to the normal control or the model group. After that, we used H$_2$O$_2$ of 300 μM for 12 hrs to get cell insult and randomly selected three in each group for morphological studies while the remaining cells were used for biochemistry studies. For morphological study, the pictures of the attached cells were captured using inverted microscope in 10 × objective (Olympus, CKX41, Tokyo, Japan).

![Figure 1 The chemical structure of Timosaponin B-II.](http://www.biomedcentral.com/1472-6882/14/459)
We have captured ten pictures at least in each group, and selected the typical graphics to illustrate.

**MTT assay**

Cytotoxicity of H$_2$O$_2$ model group and Timosaponin B-II pretreated group (1 μM, 10 μM, 100 μM and 1000 μM) were assessed in RGC-5 cells by measuring the amount of insoluble formazan formed in live cells based on the reduction of 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) salt (Nanjing Jian-Cheng Bio-engineering Institute, Jiangsu, China) according to the manufacturer’s protocol. The cells were seeded in 96-well plates with a density of 10$^4$ cells/well according to the manufacturer’s protocol. The cells were incubated for 24 hrs at 37°C and 5% CO$_2$. The cells were pretreated with different concentrations of Timosaponin B-II before H$_2$O$_2$ insult or H$_2$O$_2$ used alone and PBS as a negative control. Within 24 hrs pre-treatment of Timosaponin B-II, 50 μl of MTT labeling reagent (2 μg/ml) was added to each well. The cells were incubated at 37°C in a humidified atmosphere with 5% CO$_2$ for 4 hrs. Thereafter, 100 μl of the solubilization solution was added to each well and followed by incubation overnight at 37°C to dissolve formazan crystals. Absorbance was ultimately read using an ELISA plate reader (Bio-tek, ELx800, IL, USA) at a wavelength of 570 nm. Where, H$_2$O$_2$ model group and Timosaponin B-II pretreated group are mean absorbance of treated cells and negative control, respectively.

**MDA concentration assay**

MDA levels in RGC-5 extractions were assayed using a commercial kit according to manufacturer’s instructions (Nanjing Jian-Cheng Biotechnical Co., Jiangsu, China) as in our previous study [27], the standard reference substance named tetraethoxypropane were used in 10 nmol/ml. Equal quantities (100 μg) of protein were loaded in each well and each analysis performed in duplicate.

**TNF-α ELISA assay**

A RGC-5 TNF-α concentration assay was performed using a commercial TNF-α ELISA kit (Invitrogen, CA, USA). The detailed processes were conducted according to the manual included in the kit. Positive control: the antibody tested in the kit was replaced by mouse TNF-α (provided in assay, concentration: 720 ng/L). Equal quantities of protein (80 μg) were analyzed in every tested well and the measurements were carried out by Bio-tek microplate-reader (ELx800, IL, USA). The percentage of TNF-α concentration in normal control group was set as 100%. All experiments were repeated at least twice.

Flow cytometry

The cells attached to the flasks were trypsinized followed by a gentle wash. The experimental group was pre-treated with Timosaponin B-II at 100 μM. The model group was treated with H$_2$O$_2$ at 300 μM. Resuspended the cells in 200 μl of 1x binding buffer, and then added 5 μl of 20 μg/ml Annexin V and 10 μl of 50 mg/ml PI, incubated at RT for 15 mins in the dark. After the cells were washed and analyzed by FACS Calibur (Becton, Dickinson Company, NJ, USA). The percentages of cells in each quadrant were analyzed using ModFit software (Verity Software House Topsham, NJ, USA). Statistical results of flow cytometry were conducted by calculating the PI+ cells numbers. All the results were repeated three times.

**Data analysis**

Figure panels were assembled by using Photoshop CC (Adobe, CA, USA). The data were analyzed by using SPSS 19.0 (SPSS, IL, USA). One-way analysis of variance (one-way ANOVA) was performed to test differences in average value between groups. All results were presented as mean ± SD. A value of $p < 0.05$ was considered statistically significant.

**Results**

**Timosaponin B-II could protect RGC-5 from H$_2$O$_2$ injury**

The normal RGC-5 cell graphic were shown in Figure 2A. The cells grew in apposite density and adherent well with axon-elongated and interweave each other to be a mesh, each of them presented as identical size and consistent morphology. The number of the cells decreased in H$_2$O$_2$ injury group (Figure 2B), and plenty of them were detached and floated in culture media. As shown in Figure C-E, the number of adherent cells increased following the incubated concentration of Timosaponin B-II increase, it is worth noting that the number of adherent cells in 1000 μM Timosaponin B-II treatment group is less than those in 100 μM Timosaponin B-II treatment group (Figure E and F). These results indicated that Timosaponin B-II could protect RGC-5 from H$_2$O$_2$ insult yield to the concentration manner, but the effect may reverse with higher concentrations of Timosaponin B-II in 1000 μM.

**Timosaponin B-II could increase RGC-5 viability upon H$_2$O$_2$ injury**

In order to evaluate the viability of RGC-5 in model group and Timosaponin B-II treatment group, we carried out MTT assay to detect cell viability. The statistical analysis of MTT result was shown in Figure 3. Comparing to normal control group (CTL), the cell viability was significantly decreased in model group and Timosaponin B-II treatment group. The cell viability kept gradually increasing...
upon elevating the concentration of Timosaponin B-II except 1000 μM Timosaponin B-II group. These results indicated that Timosaponin B-II could rescue RGC-5 viability, which was impaired by the H₂O₂.

**Figure 2** The morphological graphic of RGC-5 cell line insult with H₂O₂ after 12 hrs pretreating in different concentration of TBII by inverted microscope; A: Normal Control; B: Model group (H₂O₂ treatment); C: TBII pretreatment group (H₂O₂ + 1 μM TBII); D: TBII pretreatment group (H₂O₂ + 10 μM TBII); E: TBII pretreatment group (H₂O₂ + 100 μM TBII); F: TBII pretreatment group (H₂O₂ + 1000 μM TBII), Scale bar = 100 μm in A-E.

**Timosaponin B-II reduced MDA production in RGC-5**

The statistical analysis of MDA level is shown in Figure 4. The level of MDA in the experimental group (except 1000 μM Timosaponin B-II treatment group) kept decreasing...
gradually compared to the model group upon H$_2$O$_2$ insult after increasing the concentration of Timosaponin B-II, but it remained at higher level than those in normal control group (CTL). In 100 μM Timosaponin B-II treatment group, the level of MDA decreased significantly compared with H$_2$O$_2$ insult group (p < 0.05). These results indicated that Timosaponin B-II remarkably inhibited oxidative stress which is usually induced by H$_2$O$_2$ in RGC-5. Nevertheless, the capacity of anti-oxidant was limited, because the oxidative stress remained higher than normal control group in Timosaponin B-II treatment group.

Timosaponin B-II inhibited TNF-α production in RGC-5
The statistical analysis of ELISA assay for TNF-α level was described in Figure 5. The level of TNF-α in the experimental group (except 1000 μM Timosaponin B-II treatment) was gradually decreasing compared to the model group after increasing the concentration of Timosaponin B-II, but it remained at higher levels compared to those in normal control group. These results indicated that Timosaponin B-II remarkably inhibited TNF-α production. Nevertheless, the effect of Timosaponin B-II was impaired to a certain extent since the level of target peptides was still higher than those in normal condition.

Timosaponin B-II may decrease the rate of RGC-5 necrosis
Upon 300 μM H$_2$O$_2$ insult, necrosis in RGC-5 occurred in our study. Therefore, the cells were treated under this condition with the addition of Timosaponin B-II at different concentrations. After that, we analyzed cellular necrosis by using flow cytometry with PI/Annexin V double staining, and detected whether it could decrease the rate of necrosis in RGC-5 with pre-treatment of Timosaponin B-II upon H$_2$O$_2$ insult. These results showed that the ratio of necrosis cells is 30.3% (Figure 6B), the percentage decreased to 22.8% upon adding Timosaponin B-II (100 μM, Figure 6C). Meanwhile, statistical analysis indicated that there were significant changes in the rate of PI-positive RGC-5 in Timosaponin B-II pretreatment group compared with normal control group and H$_2$O$_2$ model group (Figure 6D). These results indicated that RGC-5 necroptosis in the early stage may decrease with Timosaponin B-II usage.
Figure 6 The flow Cytometry analysis of RGC-5 cell line insult with H$_2$O$_2$ after 12 hrs pretreating in different concentration of TBII. A: Normal Control; B: Model group (H$_2$O$_2$ treatment); C: TBII pretreatment group (H$_2$O$_2$ + 100 μM TBII); D: Statistical analysis; *vs CTL: p < 0.01; *vs Model group: p < 0.01.
Discussion

At present, mechanism of cell death is one of the hot-spot in the field of life science research. As far as we are concerned, compared to apoptosis and autophagy, the researchers did not pay enough attention to necrosis due to the traditional viewpoint that it cannot be modulated and intervened. Necroptosis is the latest cell death mode which was conceptualized in last century in mid-1980s [31]. Since then, it started to catch more and more attention [32-34]. We speculate that damaged cells could be rescued if necroptosis can be intervened at an early stage, and it will also help us find a better strategy for rational interventional therapy in the future.

Chinese herb has a wealth of resources from natural materials with lower side effects, more economical and other advantages, etc. However, it also has the disadvantages such as its complicated composition which cannot be identified easily. Timosaponin B-II is a monomer which is extracted from Chinese herb *rhizoma anemarrhenae*, it has multiple pharmacological effects, including anti-inflammatory [21,35], anti-diabetic [36], anti-oxidative stress [23,26,27], anti-senile dementia [37], learning ability or memory improvement [21,35] and anti-diabetic [36], anti-oxidative stress [23,26,27], anti-senile dementia [37]. Since then, it started to catch more and more attention [32-34]. We speculate that damaged cells could be rescued if necroptosis can be intervened at an early stage, and it will also help us find a better strategy for rational interventional therapy in the future.

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