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# Resveratrol attenuates testicular apoptosis in type 1 diabetic mice: Role of Akt-mediated Nrf2 activation and p62-dependent Keap1 degradation

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ABSTRACT

Infertility is a common complication in diabetic men, mainly due to the loss of germ cells by apoptotic cell death. However, effective and safe approaches to prevent diabetic induction of testicular apoptosis for diabetic patients have not been available. Resveratrol (RSV), a group of compounds called polyphenols from plants, has been indicated its promising used clinically for cancers and cardiovascular diseases. Therefore, the present study aimed determining whether RSV attenuates type 1 diabetes (T1D)-induced testicular apoptotic cell death in a mouse model. We found that testicular apoptosis and oxidative stress levels were significantly higher in T1D mice than control mice. In addition, the phosphorylation level of metabolism-related Akt and GSK-3β was downregulated and Akt negative regulators PTEN, PTP1B and TRB3 were upregulated in the T1D group. These effects were partially prevented by RSV treatment. Nrf2 and its downstream genes, such as NQO-1, HO-1, SOD, catalase and metallothionein were significantly upregulated by RSV treatment. In addition, RSV-induced Nrf2 activation was found due to Keap1 degradation, mainly reliant on p62 that functions as an adaptor protein during autophagy. These results indicate that the attenuation of T1D-induced testicular oxidative stress and apoptosis by RSV treatment was mainly related to Akt-mediated Nrf2 activation via p62-dependent Keap1 degradation.

## 1. Introduction

The global increase in the prevalence of diabetes presents significant clinical challenges due to the high rates of diabetic complications and mortality associated with the disease. For instance, according to the National Diabetes Statistics, 30.3 million American individuals, representing 9.4% of the American population, had diabetes in 2015. Furthermore, it is estimated that about 193,000 Americans under the age of 20, representing approximately 0.24% of the American population, have been diagnosed with diabetes. (http://www.diabetes.org/diabetes-basics/statistics/#sthash.XrouwO0y.dpuf). It is known that diabetes is associated with pathological and functional damage to various organs, resulting in a variety of complications. Therefore, the development of efficient approaches to prevent or postpone the development of these complications is critical. Diabetes is significantly associated with infertility in males [1], but effective and safe approaches to prevent diabetic induction of testicular apoptosis for diabetic patients have not been available. Several mechanisms have been proposed for explaining the development of infertility in diabetic men [1–3]; however, germ cell loss may represent the direct and most important contributor to the loss of fertility in diabetic males [4–6].

Testicular apoptotic cell death, which occurs at low levels during normal spermatogenesis, is significantly increased under diabetic conditions [4,7–9]. There is increasing evidence demonstrating that

*Abbreviations:* RSV, resveratrol; STZ, streptozotocin; ROS, reactive oxygen species; RNS, reactive nitrogen species; SOD, superoxide dismutase; Nrf2, nuclear factor erythroid 2-related factor 2; Keap1, kelch-like ECH-associated protein 1; NQO-1, NAD(P)H:quinone oxidoreductase; HO-1, heme oxygenase 1; CAT, catalase; T1D, type 1 diabetes mellitus; MDA, malondialdehyde; 4-HNE, 4-hydroxynonenal; 3-NT, 3-nitrotyrosine; PTP-1B, protein tyrosine phosphatase-1B; TRB3, Tribbles Homologue 3; MT, metallothionein; qRT-PCR, quantitative real-time polymerase chain reaction; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; TdT, terminal deoxynucleotidyl transferase

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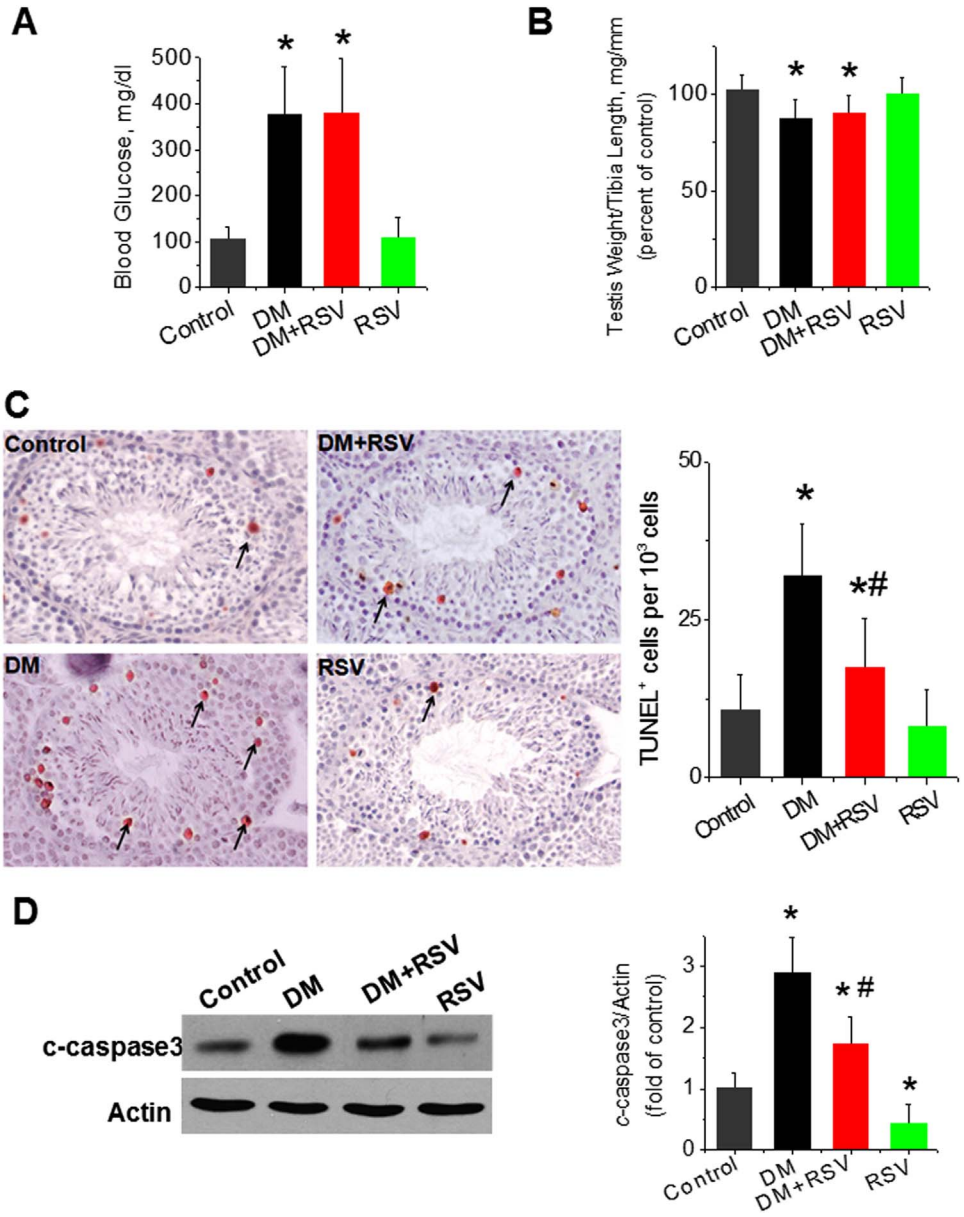


**Fig. 1.** ***Effects of RSV on T1D-induced testicular apoptosis.*** T1D was induced in mice by multiple low-doses of STZ at 50 mg/kg daily for 5 days. After the onset of hyperglycemia, diabetic and age-matched control mice were treated with or without RSV at 20 mg/kg every other day for 4 months. At the end of the treatment period, blood glucose levels (A), testis weight/tibia length ratios (B) were determined. Testicular apoptotic cell death was examined by TUNEL staining. TUNEL-positive cells were quantitatively analyzed (C). Testicular apoptosis expression was examined by western blotting assay for the expression of cleaved-caspase3 (D). Data are presented as mean ± SD (n = 6 at least in each group). *, $P < 0.05$ vs. control group; #, $P < 0.05$ vs. DM.

testicular apoptotic cell death, which may be induced by the administration of streptozotocin (STZ) in the type 1 diabetic (T1D) rat or mouse model, occurs predominantly via activation of the mitochondrion-mediated cell death pathway [4–6,9–11]. These studies indicate that oxidative stress and damage play a critical role in testicular cell death in diabetic individuals. Oxidative stress occurs in cells or tissues when the excessive generation of reactive oxygen or nitrogen species (ROS or RNS) overwhelms the endogenous antioxidant defense. Therefore, by increasing the antioxidant capacity of the testis tissue would be a potentially efficient approach for preventing and reducing the incidence of testicular apoptotic cell death, and consequently preventing the occurrence of infertility in diabetic males.

Nuclear Factor-Erythroid 2-Related Factor 2 (Nrf2), as a transcription factor, regulates basal and inducible transcription of genes encoding protective molecules against various oxidative stresses [12]. In response to a range of oxidative and electrophilic stimuli including ROS and/or RNS, heavy metals, and certain disease processes, Nrf2 is activated and mediates the induction of a spectrum of cyto-protective proteins including phase II enzymes, such as NAD(P)H: quinone oxidoreductase (NQO-1), catalase (CAT), and superoxide dismutase (SOD), and antioxidant proteins, such as heme oxygenase 1 (HO-1), through the antioxidant response element-dependent pathway. Deletion of the Nrf2 gene was found to cause an age-dependent testicular and epididymal oxidative stress, which disrupts spermatogenesis [13], suggesting a critical role for the transcription factor Nrf2 in preventing oxidative disruption of spermatogenesis. In our previous study, we have also demonstrated a critical role of Nrf2 in preventing diabetes-induced oxidative disruption of spermatogenesis both in T1D and type 2 diabetes (T2D) model [9,14].

The p62 localizes to sites of autophagosome formation and can associate with both the autophagosome-localizing protein LC3 and ubiquitinated proteins [15]. Therefore, p62 is considered to act as a receptor for ubiquitinated proteins, organelles, and microbes, which it sequesters into the autophagosome [16]. The p62 interacts with the Nrf2-binding site of Keap1 and competitively inhibits the Keap1-Nrf2 interaction, which is responsible for the expression of a battery of genes encoding antioxidant proteins and anti-inflammatory enzymes [17,18]. Nrf2 positively regulates p62 gene expression, implying a positive feedback loop [19].

Resveratrol (RSV) is a group of compounds called polyphenols, with

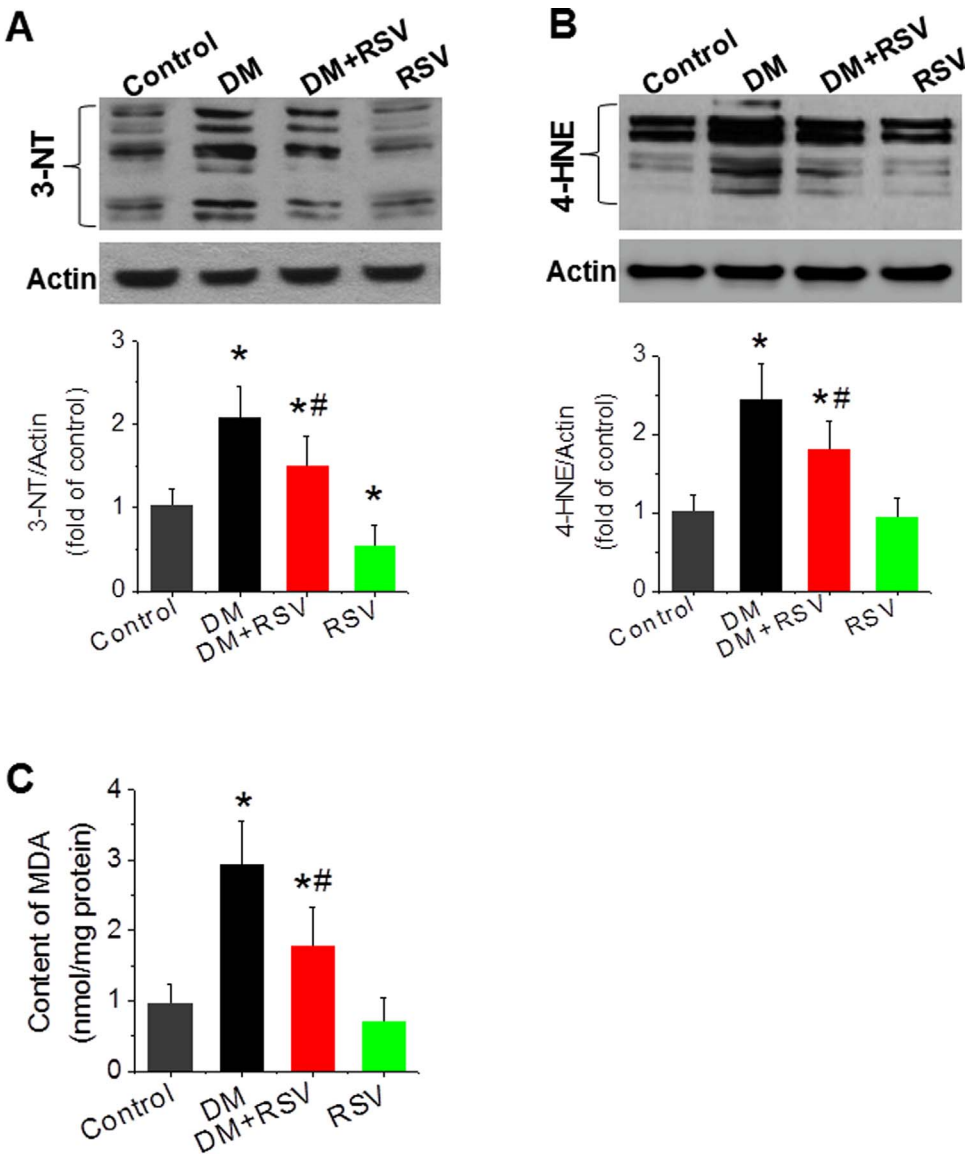


**Fig. 2.** ***Effects of RSV on T1D-induced testicular oxidative damage.*** T1D and control mice were subjected to the same treatment, as described in Fig. 1. Testicular oxidative damage was examined by western blotting assay for the expression of 3-NT as an index of protein nitration (A) and 4-HNE as an index of lipid peroxidation (B). Activity of MDA was confirmed by chemical quantification assay (C). Data are presented as means ± SD (n = 6 at least, in each group). *, $P < 0.05$ vs. control group; #, $P < 0.05$ vs. DM.

antioxidant properties and has been found in a wide range of foods especially grapes and red wine. During the last decade, RSV has been shown to possess wide spectrum of pharmacologic properties such as anti-inflammatory, anti-oxidative, anti-carcinogenic, anti-aging, neuroprotective and cardioprotective effects [20]. It has been reported that short-term treatment with RSV (2–8 weeks) has beneficial antidiabetic effects, mainly via reduction in blood glucose, lipid peroxidation, circulatory proinflammatory cytokines and apoptosis levels with concomitant enhancement of antioxidant defenses [21–25]. In vivo and in vitro studies have shown that RSV protects spermatocytes against lipid peroxidation and increases testicular sperm numbers and sperm motility. Also, it increases sperm production, reduces apoptosis in germinal cells, and protects against environmental toxins [26].

Whether RSV can prevent T1D-induced testicular apoptosis has not been addressed yet. In the present study, therefore, we investigated whether RSV supplementation could stimulate testicular antioxidant production and suppress diabetes-induced testicular apoptosis, using a T1D mouse model. In addition, we explored associated mechanisms related to Akt-mediated Nrf2 activation and p62-dependent Keap1 degradation.

## 2. Materials and methods

### 2.1. Animal models

All animal protocols were approved by the Animal Ethics Committee of Jilin University. Eight-week-old male FVB mice (Weitonglihua, Beijing, China) were acclimated in an air-conditioned room at 22 °C with a 12:12-h light-dark cycle and fed with standard rodent chow and tap water. They were injected intraperitoneally with multiple low doses of STZ (Sigma Aldrich, St. Louis, MO, USA) at 50 mg/kg daily for 5 days to induce T1D. Five days after the last injection of STZ, whole blood glucose obtained from mouse tail-vein was measured with a SureStep complete blood glucose monitor (LifeScan, Milpitas, CA, USA). Blood glucose levels of > 250 mg/dl were considered as diabetic. After the onset of diabetes, mice were divided into four groups: control (Ctrl, n = 6), resveratrol (RSV, n = 6), diabetes mellitus (DM, n = 7), and DM with RSV treatment group (DM/RSV, n = 7). RSV (Sigma Aldrich, St. Louis, MO, USA) supplementation was given by gavage at 40 mg/kg every other day for 4 months. Control and DM group mice were administered equal amounts of saline. At the time of sacrifice, two testes were harvested for the following studies.

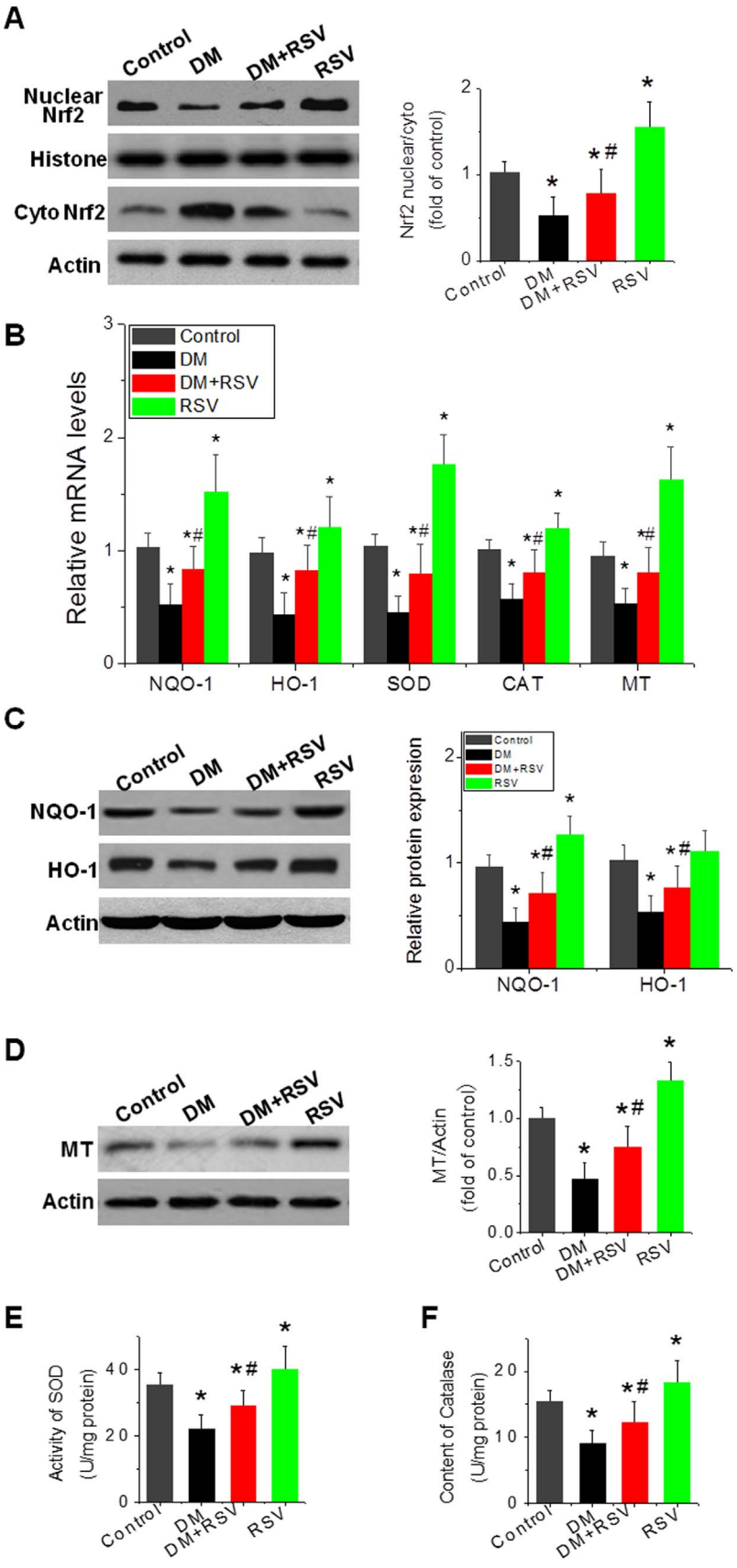


**Fig. 3.** ***Effects of RSV on testicular Nrf2 expression and function.*** T1D and control mice were subjected to the same treatment, as described in Fig. 1. The expression of Nrf2 was detected by western blotting assay (A), for which the ratio of nuclear Nrf2/cytosolic Nrf2 was presented. mRNA levels of NQO-1, HO-1, SOD, CAT and MT were detected by qRT-PCR (B). Testicular expression of NQO-1 (C), HO-1 (C) and MT (D) were measured by western blotting assay. Activity of SOD (E) and CAT levels (F) were assayed using the corresponding quantification kits. Data are presented as means ± SD (n = 6 at least, in each group). *, $P < 0.05$ vs. control group; #, $P < 0.05$ vs. DM.

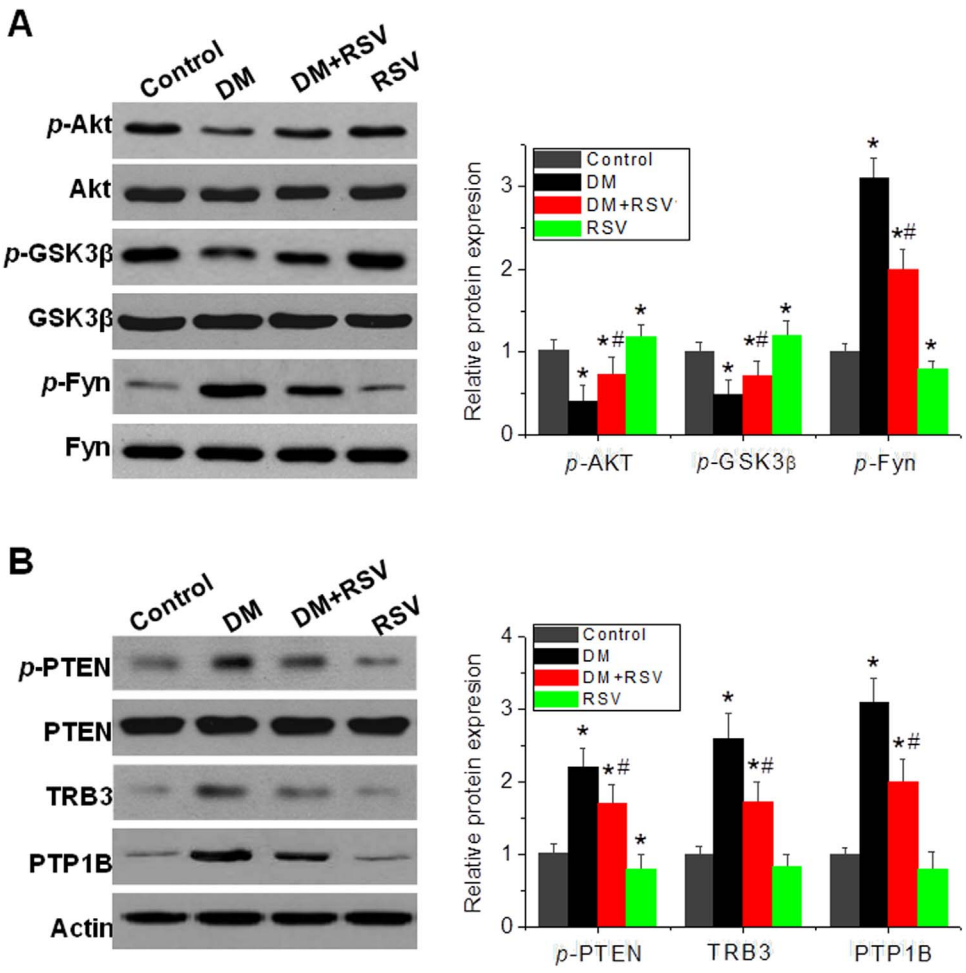


**Fig. 4.** ***Effects of RSV on testicular Akt expression and function.*** T1D and control mice were subjected to the same treatment, as described in Fig. 1. Testicular expression of phosphorylated and total Akt, GSK-3β and Fyn were measured by western blotting assay (A). The expression of PTEN, TRB3 and PTP1B (negative regulators of Akt) were also measured by western blotting assay (B). Data are presented as means ± SD (n = 6 at least, in each group). *, P < 0.05 vs. control group; #, P < 0.05 vs. DM.

### 2.2. Biochemical quantification assays

SOD activity, as well as CAT and malondialdehyde (MDA) levels in testicular tissues were assayed using the relevant quantification kits (A001-1, A007-1 and A003-1, Jiancheng, Nanjing, China).

### 2.3. Western blotting

The testicular tissues were homogenized in lysis buffer and proteins were collected by centrifuging at 12,000 × *g* at 4 °C [4]. Cytoplasmic and nuclei components of testicular cells were isolated using nuclei isolation kit (NUC- 201, Sigma, MO, USA), according to the manufacturer's protocol. Western blots were performed according to our previous studies [4]. Briefly, the proteins were fractionated on 10% SDS-PAGE gels, and then were transferred to a nitrocellulose membrane. The membrane was blocked with a 5% non-fat dried milk for 1 h and incubated overnight at 4 °C with the following antibodies: anti-cleaved caspase3, anti-phospho-Akt (Ser473), anti-Akt, anti-Kelch-like ECH-associated protein 1 (Keap1), anti- HO-1, anti-phospho-GSK3β (Ser9), anti-GSK3β, anti-LC3II (1:1000, Cell Signaling, Beverly, MA, USA), anti- NQO-1, anti-Fyn, anti-phospho-Fyn (Thr12) (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-3-nitrotyrosine (3-NT, 1:2000, Chemicon, Temecula, CA, USA), anti- Nrf2, anti-metallothionein (MT), anti-p62 (1:1000, Abcam, Cambridge, MA, USA), anti-protein tyrosine phosphatase-1B (PTP1B, 1:2000, BD Biosciences, Rockville, MD, USA), anti-4-hydroxynonenal- Michael adducts (4-HNE), and anti-Tribbles Homologue 3 (TRB3) (1:1000, Calbiochem, La Jolla, CA, USA), respectively. After removal of unbound antibodies using Tris-buffered saline (pH 7.2) containing 0.05% Tween 20, membranes were incubated with the secondary antibody for 1 h at room temperature. Antigen-antibody complexes were visualized using an enhanced chemiluminescence detection kit (Thermo Scientific, Barrington, IL, USA). In order to determine loading, blots were stripped using stripping buffer (Sigma Aldrich, St. Louis, MO, USA) and reprobed for β-actin as loading control of total protein. Histone was used as loading control of nuclei proteins. Quantitative densitometry was performed on the identified bands using a computer-based measurement system, as employed in previous studies [4].

### 2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted with TRIzol reagent (Invitrogen). RNA concentration and purity were quantified using a Nanodrop ND-1000 spectrophotometer. Complementary DNA (cDNA) was synthesized from total RNA using the RNA PCR kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. qRT-PCR was carried out in 20 µl of reaction buffer consisting of 10 µl of TaqMan Universal PCR Master Mix, 1 µl of primer, and 9 µl of cDNA. Amplification was performed in duplicate for each sample, using the ABI 7300 Real-Time PCR system. TaqMan primers for metallothionein (Cat. # 4351372, Carlsbad, CA, USA), NQO-1, HO-1, SOD, CAT, and the β-actin control were purchased from Applied Biosystems (Cat. # 4331182, Carlsbad, CA, USA). The fluorescence intensity of each sample was measured to monitor amplification of the target gene. The comparative cycle time method was used to normalize the amount of target to an endogenous reference (β-actin) and relative to a calibrator ($2^{-\Delta\Delta Ct}$).

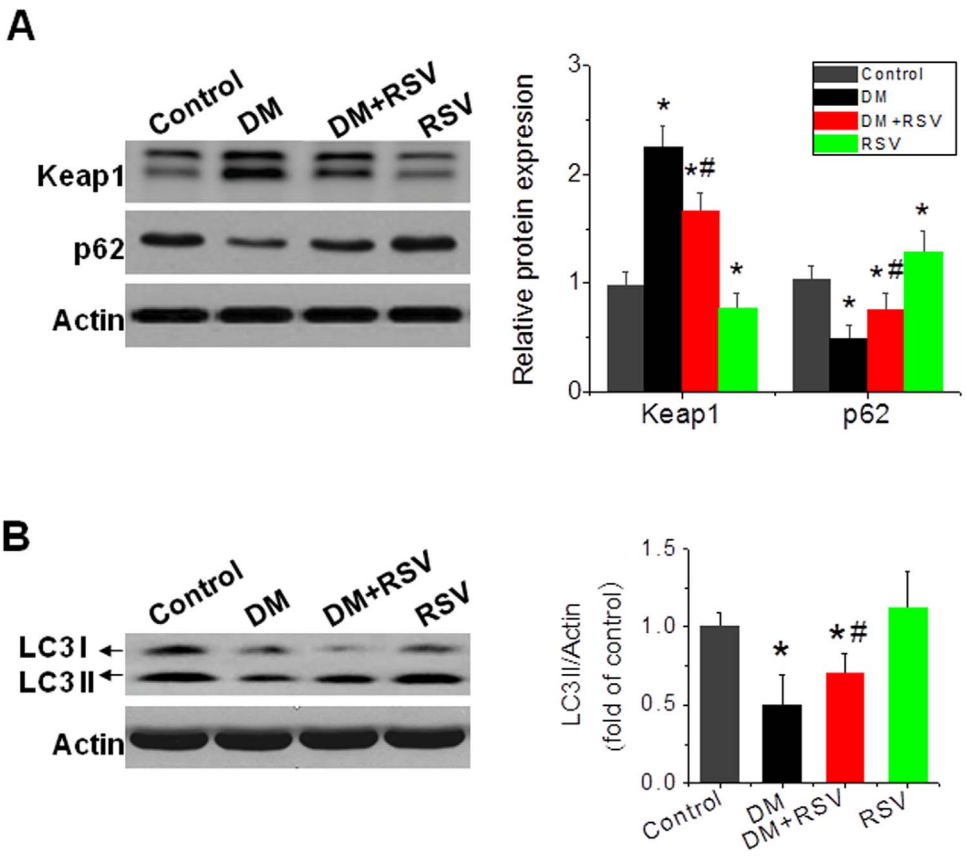


**Fig. 5.** ***Effects of RSV on testicular autophagic Keap1 degradation.*** T1D and control mice were subjected to the same treatment, as described in Fig. 1. Testicular expression of Keap1 (A), p62 (A), and LC3II (B) were measured by western blotting assay. Data are presented as means ± SD (n = 6 at least, in each group). *, P < 0.05 vs. control group; #, P < 0.05 vs. DM.

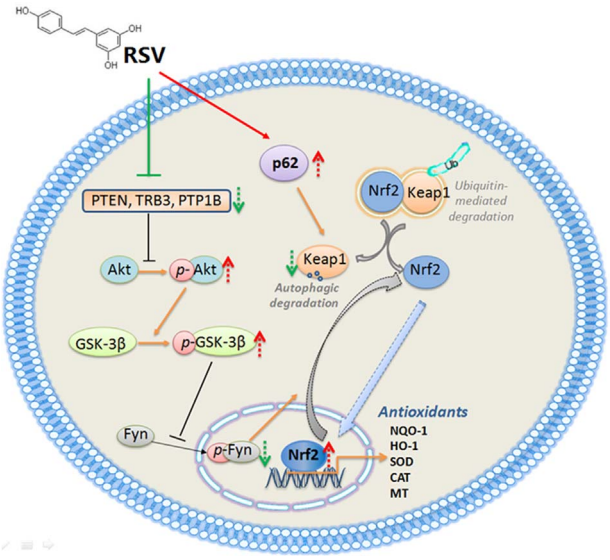


**Fig. 6.** ***Working hypothesis for the mechanism by which RSV protects the testis from diabetes.*** The working hypothesis for the mechanisms underlying RSV-induced attenuation of testicular apoptosis in T1D mice are illustrated in schematic diagram.

### *2.5. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay*

Testis tissue was fixed in 10% formalin, embedded in paraffin, and sectioned at 5 μm thickness. The slides were stained for TUNEL using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Boster, Wuhan, China), as described in previous studies [9,14]. Briefly, each slide was deparaffinized and rehydrated, and treated with proteinase K (20 mg/l) for 15 min. The endogenous peroxidase was inhibited using 3% hydrogen peroxide for 5 min, and incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-11-dUTP for 1 h. The TdT reaction was carried out in a humidified chamber at 37 °C. Then, 3, 3-diaminobenzidine chromogen was added. Hematoxylin was used for counterstaining. For the negative control, TdT was omitted from the reaction mixture. Under microscope, apoptotic cell death was quantitatively observed by counting the TUNEL-positive cells which were stained brown among 200 cells selected randomly from transections of ten seminiferous tubules by morphology from each one of the three slides for each mouse. The apoptotic cells and total cells were counted only from spermatogonia, primary spermatocytes, and secondary spermatocytes identified by morphology. However, the spermatid and spermatozoa were not counted for the quantification analysis. Results have been presented as TUNEL-positive cells per 1000 cells.

### *2.6. Statistical analysis*

Data were collected from repeated experiments and presented as means ± S.D. One-way analysis of variance was used to determine significant differences. Differences between groups were analyzed using the post hoc Tukey's test, with Origin 7.5 laboratory data analysis and graphing software. Statistical significance was considered as $P < 0.05$.

## 3. Results

### *3.1. T1D model and effects of RSV on testicular cell death*

T1D was induced by multiple low doses of STZ at 50 mg/kg daily for 5 days. After the onset of hyperglycemia, diabetic and age-matched control mice were treated with or without RSV at 40 mg/kg every other day for 4 months. At the end of the treatment, as shown in Fig. 1A, although diabetes group was associated with the significantly increased

level of blood glucose (Fig. 1A, fasting), RSV treatment did not affect its variation in the normal and diabetes groups. Diabetes was associated with a significant decrease in the testis weight/tibia length ratio (Fig. 1B); however, this was not affected by RSV treatment. Testicular apoptosis was examined by TUNEL staining for TUNEL-positive cells (Fig. 1C) and by western blotting assay for cleaved-caspase3 (Fig. 1D). Diabetes was associated with a significant increase in testicular apoptotic cell death, as shown by TUNEL staining, as well as cleaved-caspase3 levels. RSV treatment was found to significantly, but incompletely prevent the diabetes-induced apoptotic effect. These results suggest that T1D-induced testicular apoptotic cell death could be significantly attenuated by RSV treatment.

### 3.2. Effect of RSV treatment on T1D-induced testicular oxidative damage

We have previously demonstrated that T1D-induced testicular apoptotic cell death occurs mainly due to testicular oxidative stress and damage [9]. Here, we additionally demonstrate significant increases in testicular 3-NT (Fig. 2A) and 4-HNE (Fig. 2B) accumulation as indices of protein nitration and lipid peroxidation, respectively, by western blotting assay. The increased lipid peroxidation was further confirmed by chemical assay of reactive aldehydes such as MDA, which represents the end products of lipid peroxidation (Fig. 2C). RSV treatment significantly reduced testicular 3-NT accumulation in the control group and prevented diabetes-induced testicular accumulation of both protein nitration (3-NT) and lipid peroxidation (4-HNE and MDA).

### 3.3. Possible mechanisms by which RSV prevents testicular oxidative stress and damage

The expression of Nrf2 was detected by western blotting assay (Fig. 3A). The ratio of nuclear Nrf2 to cytoplasmic Nrf2 was utilized as an index of Nrf2 transcription. Accordingly, the mRNA levels of Nrf2-downstream target genes (NQO-1, HO-1, SOD, CAT and MT) were evaluated by qRT-PCR (Fig. 3B), the expression of NQO-1 (Fig. 3C), HO-1 (Fig. 3C) and MT (Fig. 3D) were measured by western blotting assay. Additionally, biochemical assays were performed for SOD activity (Fig. 3E) and CAT content (Fig. 3F). The ratio of nuclear Nrf2 to cytosolic Nrf2 was decreased in DM group, and increased in DM/RSV group, suggesting that the transcription activity of Nrf2 was increased. In line with this result, the mRNA levels of NQO-1, HO-1, SOD, CAT and MT were significantly decreased in the DM group, significantly increased in the RSV group, and the changes in DM/RSV group were between DM and control group (Fig. 3B). Consistent with the mRNA profiles, the protein levels of NQO-1, HO-1 and MT were significantly decreased in DM group, significantly increased in RSV group, and the changes in DM/RSV group were between DM and control group (Fig. 3C and D). SOD activity and CAT content were also significantly decreased in DM group, significantly increased in RSV group, and the changes in DM/RSV group were between DM and control group (Fig. 3E and F). This suggests that RSV may stimulate the production of Nrf2-mediated downstream antioxidants to protect against diabetes-induced damage, as shown in Fig. 3.

In order to investigate the mechanisms by which RSV stimulates Nrf2 function under diabetic conditions, the expression of phosphorylated and total Akt, GSK-3β and Fyn (Fig. 4A) were measured by western blotting assay. In parallel with the results shown in Fig. 3, RSV treatment increased the phosphorylation levels of Akt and GSK-3β in non-diabetic mice. However, phosphorylated levels of Akt and GSK-3β were decreased in diabetic mice, the changes in DM/RSV group were between DM and control group (Fig. 4A). RSV treatment significantly decreased Fyn phosphorylation in diabetic mice, but only slightly decreased Fyn phosphorylation in non-diabetic mice (Fig. 4A). The expression of PTEN, TRB3 and PTP1B (Fig. 4B), three typical Akt negative regulators, were examined by western blotting assays. The results showed that diabetes significantly increased the *p*-PTEN/PTEN expression ratio, and TRB3 and PTP1B expression (Fig. 4B). RSV treatment did not elicit significant effects in the non-diabetic group, but, a significant reduction, albeit partial, in the expression of *p*-PTEN/PTEN, TRB3 and PTP1B in the diabetic group (Fig. 4B).

In addition, the testicular expression of Keap1 was examined by western blot assay (Fig. 5A). Keap1 was significantly increased in DM group, significantly decreased in RSV group, and the changes in DM/RSV group were between DM and control group (Fig. 5A). In order to further explore the mechanisms of keap1 degradation, p62 expression, examined by western blot assay (Fig. 5A) was found significantly decreased in DM group, and significantly increased in RSV group as compared to control. The change of p62 in DM/RSV group was between DM and control group (Fig. 5A). As a vital protein of autophagic pathway, LC3II was also examined by western blot assay (Fig. 5B). LC3II was significantly decreased in DM group, not significantly increased in RSV group, and the changes in DM/RSV group were between DM and control group (Fig. 5B).

## 4. Discussion

Diabetes is a global health problem due to its serious complications. The number of young patients with either T1D or T2D is increasing dramatically [27,28]. Therefore, infertility of these young diabetic patients has become a concern [2,3]. The main contributor to diabetes-induced infertility is the loss of germ cells due to increased apoptotic cell death [1,4–6]. Several studies in T1D animal models, including our own [4,9,29], have indicated that male germ cell death is predominantly mediated by the mitochondrial cell death pathway. Therefore, to develop an appropriate strategy for the prevention of testicular germ cell lost will be an essential approach to preserve or improve the fertility of young or adult males.

Majority of chemo anti-diabetic drugs have been synthesized based on recognized target genes or proteins are often recognized to have severe side effects due to cytotoxicity on normal non-target cells. Among diverse groups of phytochemicals, RSV was not only proven for their strong chemo-preventive effects in various types of human tumors [30], but also as a well-known antioxidant and phenolic compound, involving in preventing oxidative damage in various pathological conditions [31,32]. These research outcomes suggested that RSV governs redox homeostasis, inflammation, cell proliferation and death [30–32].

Among beneficial anti-diabetic actions of RSV, its antioxidant effect seems to be best documented. A variety of animals and human studies have shown that short-term RSV administration for diabetic subjects (up to two months), could enhance the antioxidant defense, reduce lipid and protein oxidation and decrease apoptosis rate [21,23,33]. Our data also are in line with previous studies in which RSV treatment significantly reduced testicular 3-NT accumulation in the control group and prevented diabetes-induced testicular accumulation of both 3-NT as well as end products of lipid peroxidation (4-HNE and MDA).

Diabetes was associated with a significant increase in testicular apoptotic cell death, as shown by TUNEL staining, as well as cleaved-caspase3 levels. RSV treatment was found to significantly, but not incompletely prevent the diabetes-induced apoptotic effect. The incomplete prevention of testicular apoptotic cell death may be significantly increased by properly increasing its dose. These results suggest that T1D-induced testicular apoptotic cell death could be significantly attenuated by RSV treatment. Although diabetes group was associated with significantly increased blood glucose levels (Fig. 1A, fasting), RSV treatment did not affect these variables in the normal and diabetes groups. Diabetes was associated with a significant decrease in the testis weight/tibia length ratio (Fig. 1B); however, this was not affected by RSV treatment. Therefore, there is no evidence to show the preventive effect of RSV on testicular cell death under diabetic conditions.

The ratio of nuclear Nrf2 to cytosolic Nrf2 was decreased in DM group, and increased in DM/RSV group (Fig. 3A), suggesting that the

transcription activity of Nrf2 was increased. The protein levels of NQO-1, HO-1 and MT were significantly decreased in DM group, SOD activity and CAT content were also significantly decreased in DM group, RSV treatment was found to significantly, but not completely, prevent the diabetes-induced oxidative effect (Fig. 3C-F). These data suggest that RSV may stimulate the production of Nrf2-mediated downstream antioxidants to protect against diabetes-induced damage, as shown in Fig. 3.

PI3K/Akt, a multifunctional signaling pathway that is associated with cell proliferation, anti-apoptosis and cellular defense, has been reported to regulate Nrf2 [34,35]. Akt is the primary mediator of PI3K-initiated signaling pathway. It commonly acts as an anti-apoptotic signaling molecule in many different cell death paradigms. In recent years, it is believed that the PI3K/Akt pathway creates a survival signal against oxidative stress-induced injuries by regulating Nrf2 expression in numerous cells. We demonstrated that RSV-induced upregulation of Nrf2-dependent antioxidant function likely occurs via the suppression of Akt negative regulators PTEN, TRB3 and TPT1B, which prevents the inhibition of Akt phosphorylation. Mechanistically, phosphorylated Fyn by active GSK-3β could translocate into the nucleus to export the nuclear Nrf2 to cytosol for its ubiquitination and degradation [36–38]. In line with this, we found that activated (phosphorylated) Akt phosphorylates (inactivates) GSK-3β, and the phosphorylated GSK-3β inhibits the phosphorylation of Fyn and prohibits the translocation of Fyn into the nucleus, where phosphorylated Fyn exports Nrf2 from nuclei to cytosol, resulting in ubiquitin-mediated degradation of Nrf2 (Fig. 4A and B).

The Keap1-Nrf2 system is currently recognized as one of the major cellular defense mechanisms against oxidative and electrophilic stresses [39–41]. Under quiescent conditions, Nrf2 is constitutively degraded through the ubiquitin-proteasome pathway. Keap1 is an adaptor of the ubiquitin ligase complex that targets Nrf2. Keap1 contains multiple highly reactive cysteine residues, upon stimulation of cells with ROS, the reactive cysteine residues undergo oxidation and form an intramolecular disulfide bond [42,43]. It is generally accepted that the modification of critical cysteines Cys257, Cys273, and Cys288 causes a conformational change of Keap1 and leads to the release of Nrf2 from Keap1 and subsequent Nrf2 nuclear translocation and activation [44,45].

P62 is a multifaceted adaptor protein that performs diverse biological functions by interacting with many other proteins and promoting protein aggregation [46]. Mice with deficient autophagy exhibit elevated p62 protein levels in their livers, and the level of the p62 protein is generally considered to correlate inversely with autophagy activity [17,47]. However, during inflammation, p62 can be transcriptionally upregulated and its enhanced expression can be correlated with increased autophagy activity [48,49]. It was reported that p62 binds to the Kelch domain of Keap1 via a motif designated the Keap1-interacting region (KIR), which resembles the ETGE motif of Nrf2, and that it thereby competes with Nrf2 for binding to Keap1 [19].

Indeed, our study provided direct evidence demonstrating that RSV treatment induces p62 expression (Fig. 5A) and increases the level of association of p62 with Keap1 as well as subsequent Keap1 degradation, which showed that RSV treatment was found to significantly, but not completely, prevent the diabetes-induced Keap1 upregulation (Fig. 5A). Degradation of Keap1 results in an increase in the Nrf2 transcription (Fig. 3A). However, degradation of Keap1 is not necessary for Nrf2 activation. P62 was identified as another protein that activates Nrf2 by disrupting the Keap1-Nrf2 interaction in autophagy-deficient cells [17,18].

In summary, here we found for the first time that RSV protects T1D-induced germ cell apoptotic death along with the increase in testicular Nrf2 expression and function. The working hypothesis for the mechanisms underlying RSV-caused attenuation of testicular apoptosis in T1D mice are illustrated in schematic diagram (Fig. 6). However, whether Nrf2 up-regulation and activation is the pivotal role in the testicular protection from diabetes remains further confirmed with Nrf2 gene knock mice. In addition, we demonstrated the up-regulation of Nrf2 expression and function was associated with RSV suppression of Akt negative regulators. Further, we demonstrated that the up-regulation of Nrf2 expression and function was also attributable to RSV's capacity to promote the p62-dependent autophagic degradation of Keap1. Whether these two associated phenomenon are the direct mechanism responsible for RSV's stimulation of Nrf2 remain further defined.

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## Conflicts of interest

No conflicts of interest. All authors takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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