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研究當歸在離體培養及活體動物模式對抗三級丁基過氧化氫誘發大鼠血管內皮及心肌細胞老化的作用機轉

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中　華　民　國 102 年 9 月 30 日

Abstract

Key words: ***Angelica sinesis*, *tert*-butyl hydroperoxide, aorta, heart, apoptosis, telomerase activity, p53**

Oxidative stress-induced endothelial dysfunction is one of the underlying causes for the pathogenesis of cardiovascular diseases. The *tert*-butyl hydroperoxide (*t*-BHP), a short-chain lipid hydroperoxide analog or a reactive oxygen species (ROS) generator, has been referred to generate cause cellular senescence in different systems. However, it remains a matter of debate whether there is a potential candidate for antagonizing the *t*-BHP-induced cytotoxicity and impaired cardiac function. *Angelica sinensis* (AS) extracts, a commonly used traditional Chinese Medicine, has multiple pharmacological activities. This study was to investigate whether *AS* extract could ameliorate the *t*-BHP induced toxicity in rat aortic endothelium by *in vitro* and *in vivo* study. Primary cultured endothelial cells (ECs) were treated with vehicle or *t*-BHP (50, 100, 250, 500, or 1,000μM). After incubation for 2 h of *t*-BHP followed by 22h treatment of 2% fetal bovine serum medium, exposure of ECs to lower doses of *t*-BHP (under 250μM) caused cellular senescence and apoptosis, and higher doses (higher than 500μM) initiated cell-cycle arrest, telomerase activity diminished, and cellular inflammation. Of note, these adverse effects (250μM of *t*-BHP) could be antagonized by pretreatment of *AS* extract (2.5μg/ml for 24h) via the p53-dependent mitochondrial signaling pathways. In terms of *in vivo* study, male rats at 6- or 24-week of age were administrated with vehicle or *t*-BHP (0.5, 1.0, or 2.0 mM /kg of body weight intraperitoneally (i.p.) daily). After 10 days, significantly increased TUNEL- and SA-β-gal-positive cells, provoked caspase-3 activities, and inhibition of telomerase activities in aorta and hearts from *t*-BHP-treated groups. In conclusion, these results suggest *t*-BHP impaired vascular cell survival at least partially by activating the p53-mediated signaling pathway and *AS* can counteract these actions of *t*-BHP to protect the aortic endothelium from *t*-BHP induced dysfunction.

中文摘要

關鍵字:當歸、三級丁基過氧化氫、主動脈、心臟、細胞凋亡、端粒酶活性、p53

氧化壓力所誘發的內皮細胞功能低下是導致形成心血管疾病的主要因素。三級丁基過氧化氫是一短鍊的脂質過氧化物或稱為活性氧產生劑，已知在很多不同的細胞或動物系統中會誘發細胞老化的現象。然而對於是否有能對抗三級丁基過氧化氫所引起的細胞毒性及造成心臟功能損傷的可能的候選物質，則仍待進一步研究。當歸萃取物是一種經常被使用的傳統中藥，具有相當多的藥理活性。本研究的目的即為以活體外及活體實驗探討當歸萃取物是否能減少三級丁基過氧化氫在大鼠動脈內皮細胞所造成的毒性。出及培養的內皮細胞分別處理空白實驗或不同劑量的三級丁基過氧化氫(50, 100, 250, 500, or 1,000μM)，在處理 22 小時僅含 2%胎牛血清的培養液後將細胞置換成含有三級丁基過氧化氫的細胞培養液 2 小時後，發現低於 250μM 濃度組會造成細胞老化現象及細胞凋亡作用，但是過高的濃度(500μM 以上)則會引起細胞週期停止、端粒酶活性降低以及細胞發炎等作用。值得注意的是，事先處理 2.5μg/ml 劑量的當歸萃取物 24 小時可以經由 p53 相關的粒線體訊息傳導路徑，減少劑量 250μM 的三級丁基過氧化氫所造成的毒性作用。在動物實驗方面，6 及 24 週大的公鼠分別以腹腔注射方式處理空白對照組及不同劑量的三級丁基過氧化氫(0.5, 1.0, or 2.0 mM/kg)。處理 10 天後，發現處理三級丁基過氧化氫的實驗組主動脈及心臟組織，顯著增加細胞凋亡及細胞老化標的物染色結果，細胞死亡活性增加以及端粒酶活性被抑制等作用。總結本研究結果，我們發現三級丁基過氧化氫造成血管細胞損傷，至少經由活化 p53 相關的訊息傳導路徑。而當歸萃取物可以減緩這些不良的作用以保護三級丁基過氧化氫造成心血管功能低下的傷害。

1. Introduction

Radix Angelica sinesis (AS), known as Danggui in Chinese (a commonly used traditional Chinese medicine), has exhibited a wide spectrum of pharmacological activities and been widely used to treat cardiovascular diseases for a long time[1, 2]. The potential biological activities of AS on the vascular system included stimulating human endothelial cell proliferation and promoting angiogenesis via p38 MAPK/ERK pathway [3], protecting against doxorubicin-induced chronic cardiotoxicity through eliminating the production of oxygen free radicals [4], preventing the ischemia-reperfusion induced myocardial injury by reducing oxidative stress and apoptosis [5].

The *tert*-butyl hydroperoxide (*t*-BHP), one of the pro-oxidants as an additive in lubricants, bleaching and disinfectant is a free radical initiator underwent epoxidation of alpha-olefins in the presence of various choices of transition metal catalysts. *t*-BHP could induce the conversion of the major cellular antioxidant GSH to its oxidized form GSSG and is frequently used as an oxidative stress inducer, which triggers peroxyl and alkoxyl radicals to disrupt cellular membranes, produce highly reactive hydroxyl radicals, and damage cells by reacting with DNA, proteins, and lipids [6-8]. Recently, a growing number of evidences concerned that *t*-BHP not only elicited hepatotoxicity by inducing lipid peroxidation, oxidative stress, glutathione depletion, and capase-3 activation [6, 9-14], but also induced toxicity in rat astroglial cells [15], murine peritoneal macrophages [16], human corneal endothelial cells [17], and mouse brain tissue [18]. Most importantly, several studies also indicated that *t*-BHP is a potent inducer in modeling premature senescence involving an increased rate of telomere shortening, senescence-associated β-galactosidase (SA-β-gal) activity, and activated caspase-3 in human umbilical vein endothelial cells [7, 19] , human corneal endothelium [20], lens epithelium [8], and human fibroblast cells [21, 22].

Telomerase activity is associated with resistance to apoptosis and shortened telomere triggered by DNA damage would send cellular signals to induce a stated of irreversible cell growth arrest (cellular senescence) [23, 24]. Increase production of reactive oxygen species (ROS) and oxidative stress and progressive telomere shortening in human arteries has been considered to be associated with the pathogenesis of cardiovascular associated disorders, neurodegenetative diseases, and cancer [25-27]. The cyclin-dependent kinase inhibitors (CKIs) and apoptotic-related proteins (p53, p21, and Bax) are highly expressed in some senescent and terminally differentiated cells which telomerase activity is very low [28-31], which have been thought as oxidative stress indicators for their participating in the inductions of G1 phase into S phase, senescence, and apoptosis [22, 32].

Endothelial cells as an element of the vascular system are a suitable target of the cytotoxicity by oxidative stress and endothelial cell apoptosis is involved in the pathology of cardiovascular diseases (e.g. atherosclerosis) [27, 33]. Interestingly, previous studies indicated that *t*-BHP induced vasoconstriction in isolated rat thoracic aorta [34, 35], which can cause the blood flow be restricted or decreased. Although tyrosine kinase and cyclooxygenase pathways were suggested as mediators involved in the *t*-BHP induced vascular toxicity, it remains unclear whether *t*-BHP induces vascular dysfunction associated with aging. Thus, in the present study we demonstrate the dose-response

effects of *t*-BHP in endothelial dysfunction on apoptosis, cell cycle progression, senescence, and necrosis mediated pathways in rats by *in vivo* and *in vitro* experiments.

2. Materials and Methods

2.1. Reagents

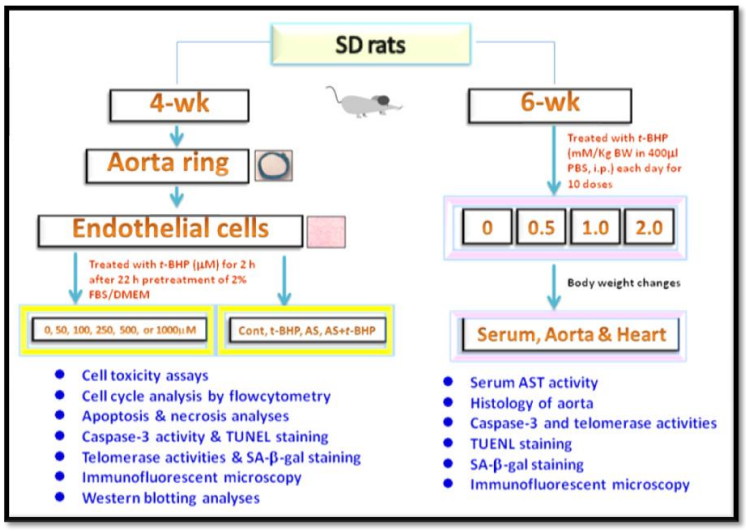
2.2. Isolation of rat aortic endothelial cell and in vitro experimental protocol

2.3. Animals and in vivo pharmacological treatment

2.4. Histopathological examinations of thoracic aorta rings and morphometric analysis

2.5. Measurement of serum alanine aminotransferase activities

2.6. Assessment of endothelial cell viability with MTT assay



2.7. Flow cytometric analysis by propidium iodide staining

2.8. Apoptosis and necrosis assays using fluorescence-conjugated Annexin-V (Annexin-V-FITC)/Propidium iodide staining

2.9. In-situ detection of apoptosis in thoracic aorta and endothelial cells

2.10. Determination of Caspase-3 activity in thoracic aorta and endothelial cells

2.11. Senescence-associated β-galactosidase staining in aorta and endothelial cells

2.12. Telomerase activity

2.13. Immunoblotting analysis

2.14. Immunofluorescent microscopy

2.15. Statistical analysis

3. Results

3.1. Effects of t-BHP on general observations andmorphometric parameters of thoracic aorta in experiment rats

At the end of the treatment period, all mice from each group were alive and normal. However, the dose-response effects of *t*-BHP administration on body weight of rats were observed. During the initial day of treatment, there was no significant difference in body weights in

	[*t*-BHP] (mM /kg of body weight)			
	Cont	0.5	1.0	2.0
Body weight (BW)				
Baseline (g)	160.1±2.5	159.6±2.7	162.7±4.2	164.3±4.1
Final (g)	254.3±3.6	250.9±4.9	244.3±4.9	248.8±5.1
Gain (final vs. baseline, %)	59.0±2.1	57.3±2.7	50.6±2.5*	51.8±2.7*
Thoracic aorta				
Radius (μm)	2304.8±225.6	2636.9±122.2	2331.0±69.0	2376.2±90.4
Medial thickness (μm)	174.2±5.4	175.5±12.8	146.0±3.1*	158.2±2.9

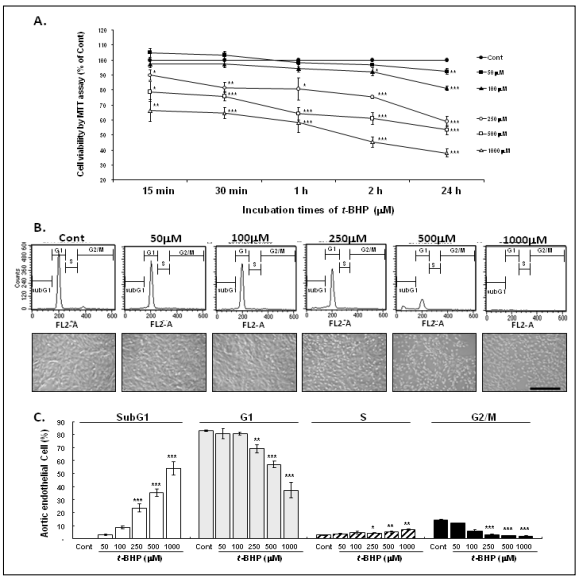
*p<0.05 versus Cont group

**p<0.01 versus Cont group

the 4 groups. After the 10-day experiment, no visibly difference in body weights was found, while the body weight gains were much less in animals treated with higher doses of *t*-BHP (1.0 and 2.0mM/kg BW) than the controls (Cont) (p=0.016 and p=0.050 vs. Cont, respectively) (Table 1). Additionally, the medial thickness of aortic rings was notably decreased by higher doses of *t*-BHP (2.0mM/kg BW vs. Cont, p=0.037), while the aortic ring display normal endothelial and vascular smooth muscle morphology in the control group. (Table 1 Effects of *t*-BHP on body weight changes and morphometric parameters of thoracic aorta in rats (n=12)).

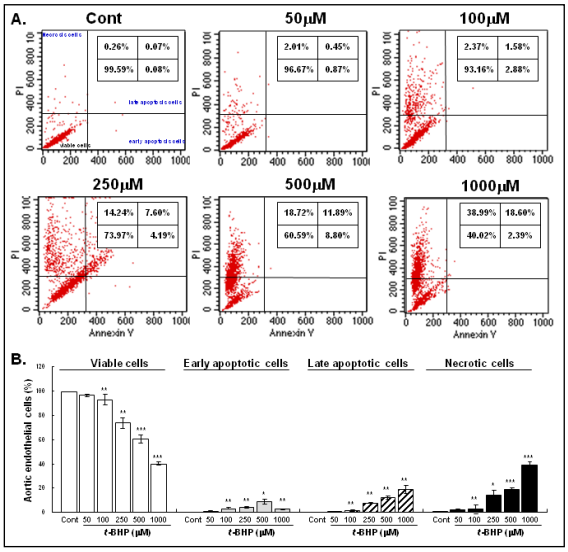
3.2. Effects of t-BHP on cell viability, cell morphology, and cell cycling in aortic endothelial cells

The cytotoxic effects of *t*-BHP on ECs were illustrated by viability tests as shown in Figure A. Treatment of *t*-BHP (50, 100, 250, 500, or 1,000μM) for 15min, 30min, 1h, 2h, and 24h significantly inhibited MTT viability of ECs in a time-dependent manner compared with the vehicle-treated cells (p<0.001 for each). Phase contrast microscopic study demonstrated that low doses of *t*-BHP (50 and 100μM) treatment for 24h had no significantly adverse effects on cell shape an size, whereas higher-doses of *t*-BHP (250, 500, or 1,000μM) caused reduction of cell numbers, rounding up of cell contours, and shringage of cell size with condensed and vacuolated nuclei (Fig. 1B), indicative of the insulting nature of shikonin on ECs. Furthermore, *t*-BHP-treated cells were analyzed by PI staining and flowcytometry. DNA distribution histograms showed that lower doses of *t*-BHP had no observable effects on cell apoptosis (Fig. 1B&C), as the proportion of apoptotic cell (sub-G1 phase) were similar to control cells. In contrast, when the concentrations of t-BHP were increased, the apoptotic index in cells elevated in a dose-dependent manner, associated with reduced G1- and G2/M- and increase subG1- and S-phase cell populations, indicating induction of cellular apoptosis and retardation of cell proliferation. (Fig. 1. Impacts of *tert*-butyl hydroperoxide (*t*-BHP) on inducing cytotoxicity and inhibiting cell cycle progression.)



3.3. Effects of t-BHP on endothelial cell apoptosis and necrosis

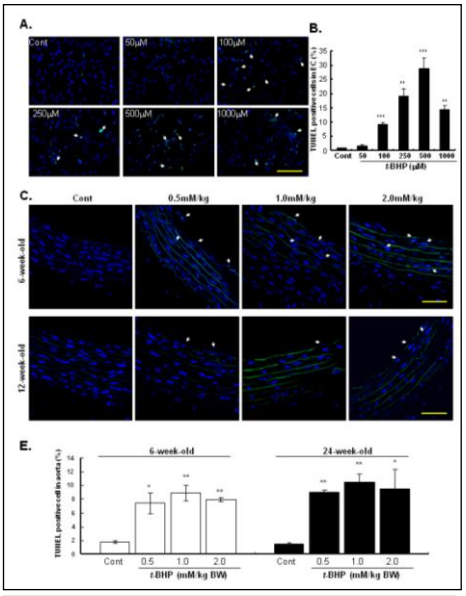
The cytotoxic mechanism of *t*-BHP was further differentiated by Annexin-V/PI-stained flowcytometry. Overall, proportion of viable cells (low PI, low annexin-V staining) decreased significantly in those treated with *t*-BHP at the doses hogher than 100μg/ml, as compared with untreated controls (Fig. 2A&B). Specifically, cells started to sucuumb to early (low PI, high annexin-V staining) and late (high PI, high annexin-V staining) apoptosis when exposed to *t*-BHP at the doses higher than



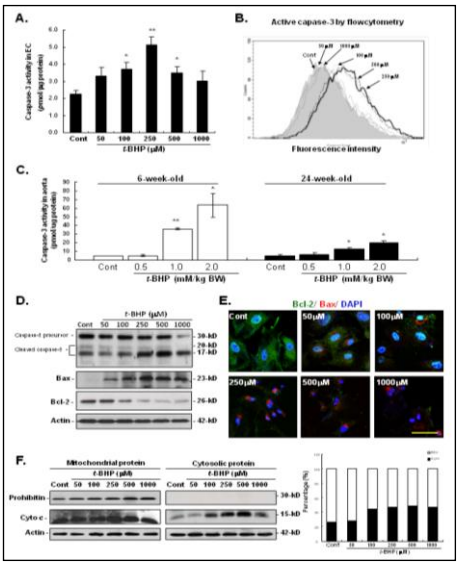
100μg/ml. Notebably, necrotic cells (high-PI/low annexin-V staining) at the doses higher than 100μg/ml were also found, suggesting that higher doses of *t*-BHP not only induced apoptosis but also trigger necrosis of ECs. (Fig. 2. Effects of *tert*-butyl hydroperoxide (*t*-BHP) on stimulating apoptosis and necrosis.)

3.4. Effects of t-BHP on capase-3 activity, apoptotic- and cell-cycle regulatory protein levels

Apoptosi of ECs and aorta (from 6-and 24-wk of rats) induced by *t*-BHP was further verified by TUNEL study, which demonstrated that cells with TUNEL-positive nuclei were scarce in the control group (Fig. 3A&C), whereas were prevalent when treated with *t*-BHP (Fig. 3B&E). (Fig. 3. Effects of *tert*-butyl hydroperoxide (*t*-BHP) on increase of TUNEL index).

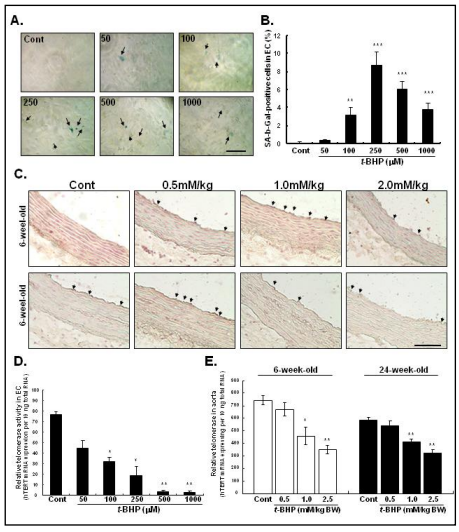


To define the cellular mechasnism responsible for *t*-BHP's proapoptotic action, various crucial apoptosis-regulating mediators were surveyed. Caspase-3 activity was found markedly up-regulated in ECs treated with *t*-BHP (doses higher than 200μg/ml), as compared with the control group (Fig. 4A&B), suggesting that *t*-BHP activated caspase-3-mediated apoptotic signaling in a dose-dependent manner. Similar results also obesrved in aorta from *t*-BHP treated groups (6- and 24-wk) (Fig. 4C). As shown in Fig. 4D, *t*-BHP not only activated the caspase-3 cleavage, but also decreased the abundance of anti-apoptotic Bcl-2 and increased the content of pro-apoptotic Bax in ECs in dose-dependent manner. Immunifluorescent results also showed that the content of Bcl-2 and Bax were similar with the immunoblotting results (Fig. 4E). Additionally, immunoblotting analysis of protein fractions from mitochondria and cytosol further demonstrated that cytosolic fraction of cytochrome c (Cyto c) was 26.98% of the total contents (cytosolic plus mitochondrial) in the controls, increased to 44.18, 37.57, 48.29 and 47.00% in *t*-BHP-treated cells (at the doses of 100, 250, 500, and 1000μg/ml), indicating that *t*-BHP induced redistribution of cyto c from mitochondria to cytosol (Fig. 4F). (Fig. 4. Effects of *ter*t-butyl hydroperoxide (*t*-BHP) on activation of caspase-3 and apoptotic related protein expressions.)



3.5. Effects of t-BHP on cellular senescence and telomerase activity

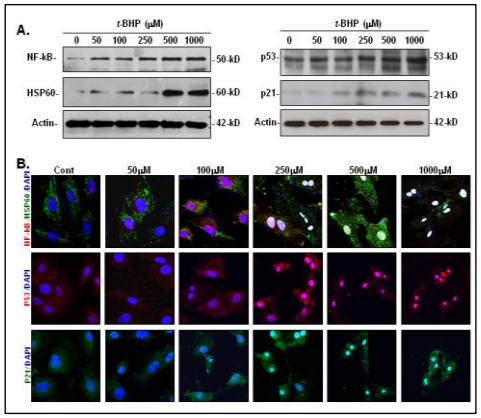
We first examined SA-β-gal activity in endothelial cells (ECs). When ECs were treated with *t*-BHP, these morphological features of cellular senescence emerged (green color) (Figure 5A). Quantitative analyses showed that numbers of positively-SA-β-gal stained cells were increased and then declined along with the doses of *t*-BHP



increased (Fig. 5B). The SA-β-gal activity was also examined in thoracic aortas obtained from *t*-BHP-treated groups (6- and 24-wk of rats) (Fig. 5C). The SA-β-gal-positive cells were predominately located on the luminal surface of aorta from the rat treated with higher doses of *t*-BHP.

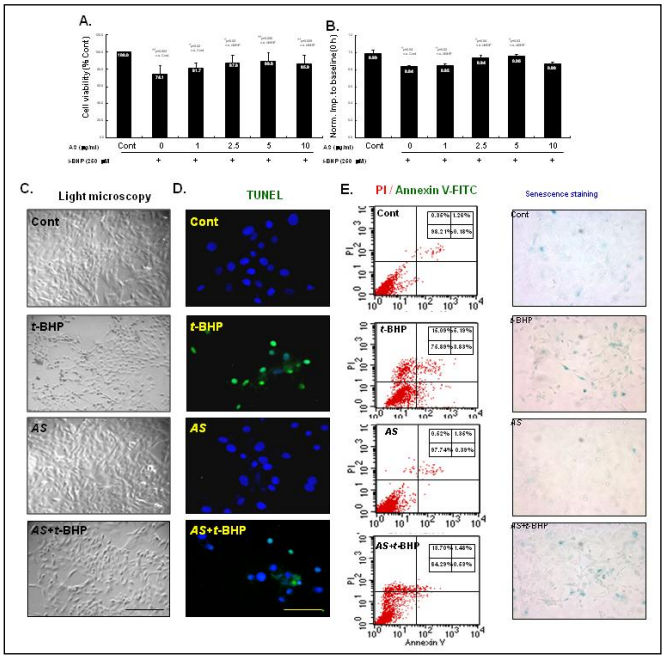
To further determine the role of telomerase in the senescence-stimulating action of *t*-BHP on endothelial cells and aortic tissues, telomerase activity (hTERT mRNA expression to total mRNA) was also determined by the TRAP assay. Results showed that telomerase activity was significantly reduced in endothelial cells treated with *t*-BHP in a dose-dependent way (Fig. 5D). Finally, we examined telomerase activity in aorta and results indicated that the effective dose of *t*-BHP to induce aortic senescence in rat should be higher than 1.0mM/kg BW (Fig. 5E). (Fig. 5. Effects of *tert*-butyl hydroperoxide (*t*-BHP) on senescent phenotype and telomerase activity.)

3.6. Effect of t-BHP on expression of inflammatory-, apoptosis- and cell cycle-regulating proteins



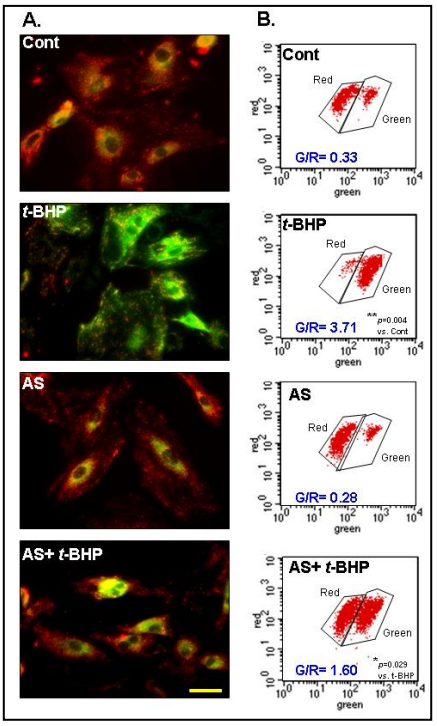
To determine the mechanism through which t-BHP triggered necrosis, apoptosis and cellular senescence, expression of inflammatory, cell cycle-related and apoptosis-mediating proteins were measures. Abundance of NF-kB and HSP60 as well as p53 and p21, as assessed by immunoblotting study, was obviously increased in cells treated with *t*-BHP in a dose-dependent manner (Fig. 6A). Besides, both p53 and p21 were marked upregulated and co-localized in *t*-BHP-treated cells as examined by immunofluorescent analyses (Fig. 6B). (Fig. 6. Effects of *tert*-butyl hydroperoxide (*t*-BHP) on inflammatory, apoptotic and cell cycle regulatory protein expressions.)

3.7. tert-butyl hydroperoxide (t-BHP) impeded whereas Angelica sinesis (AS) extracts preserved viability, apoptosis, necrosis, and cellular senescence in endothelial cells



Dose-response trypan blue exclusion assay demonstrated that *t*-BHP at the concentration of 250μM reduced viability to 75% at 24h of exposure. Pre-treatment with AS 22hrs prior to *t*-BHP at the doses of 1, 2.5, 5, and 10μg/ml sustained cell viability (Fig. 7A). Similar preservative effects of AS on EC viability were also reflected by MTT assay (Fig. 7B). As AS administered 22hr prior to *t*-BHP at the dose of 2.5μg/ml offered the best protective effects, this timing and concentration were used for subsequent experiments. To determined whether AS preserves cell viability through intervening in the process of apoptosis, necrosis, or cellular senescence, ECs of all four groups were assessed by light microscopy, TUNEL assay, PI/Annexin

V-stained flow cytometry, and SA-β-gal activity. Results showed that the pre-treatment fo AS could ameliorate the *t*-BHP induced shrinkage of cell size, rounding up of cell contours, and reduction of cell numbers (Fig. 7C). With similar results, TUNEL study revealed that cells with TUNEL-positive nuclei were prevalent when treated with *t*-BHP and were suppressed significantly though not complemently by AS pretreatment (Fig. 7D). Again, results from flowcytometry showed that the proportion of apoptotic and necrotic cells were significantly maintained by AS pretreatment, though still a level higher than the control (Fig. 7E). The SA-β-gal activity was further examined and results indicated that SA-β-gal-positive cells were antagonized significantly though not complemently by AS pretreatment (Fig. 7F). (Fig. 7. Effects of Angelica sinensis extract (AS) and *tert*-butyl hydroperoxide (*t*-BHP) on endothelial cell (EC) proliferation, cytotoxicity, apoptosis, and cellular senescence.)

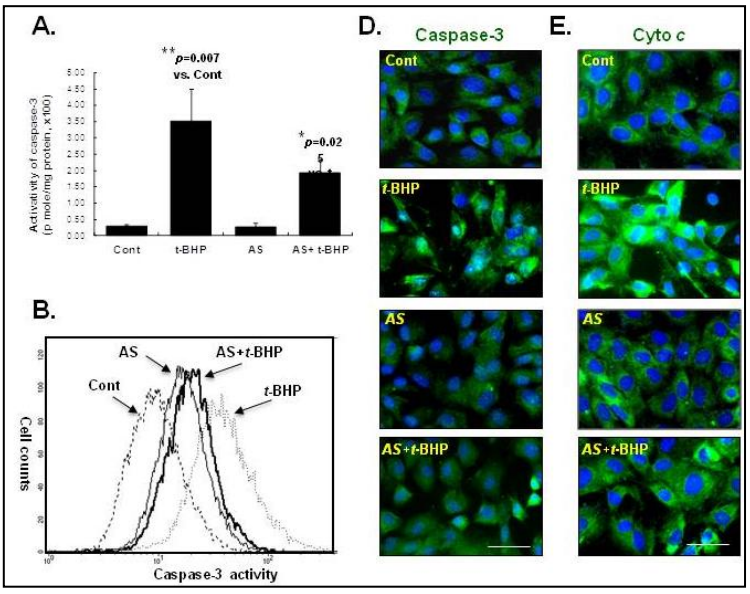


3.8. AS mitigated t-BHP-induced disruption of mitochondrial membrane potential $\Delta\Psi_m$

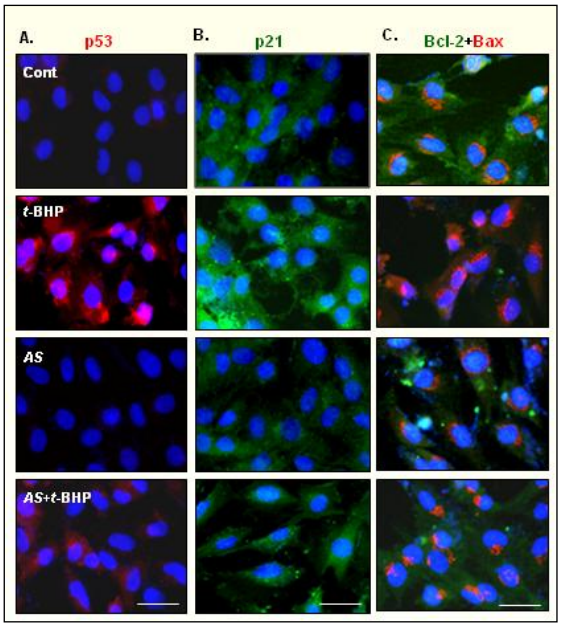
Mitochondrion is the key organelle that orchestrates the energy supply of cells and the mechanics of cellular apoptosis. Loss of mitochondrial membrane potential $\Delta\Psi_m$ causes membrane depolarization and triggers a cascade of mitochondrion-dependent apoptotic signaling [36]. JC-1 staining showed that *t*-BHP depolarized $\Delta\Psi_m$ as reflected by scanty red-color aggregates and plenty green-color monomers, while AS pretreatment abated $\Delta\Psi_m$ collapse in ECs (Fig. 8A). Flow cytometric analysis confirmed that t-BHP significantly increased the percentage of JC-1 positive (high green-low fluorescent) cells, or mitochondrial membrane potential-depolarized cells, while AS pretreatment lessen this effect (Fig. 8B). (Fig. 8. Effects of *tert*-butyl hydroperoxide (*t*-BHP) and *Angelica sinensis* extract (*AS*) on the mitochondrial cross-membrane electrochemical gradient DCm of endothelial cells (EC) by JC-1 staining.)

3.8. AS attenuate the pro-apoptotic effects of t-BHP on caspase-3 activity, cytochrome c release, p53- and p21-relocalization, and Bcl-2 family protein balance

Disruption of mitochondrial membrane permeability triggers release of mitochondrial cytochrome c to the cytosol, which in turn activates the apoptotic effector caspase-3 to induce cell apoptosis [37]. Caspase-3 activity was significantly upregulated in cells treated with t-BHP and was also depressed by *AS* pretreatment (Fig. 9A&B). As shown in Fig. 9D&E, the contents of cleaved caspase-3 and cytochrome c were increased in cytosol from cells treated with



t-BHP, but were less abundant in those pretreatment by *AS*. (Fig. 9. Effects of Angelica sinensis extract (AS) and *tert*-butyl hydroperoxide (*t*-BHP) on caspase-3 activation and immunolocalizations of activae-caspase-3 and cytochrome c.)



p53 is a tumor suppressor factor that responds to extrinsic stimuli by translocating to mitochondira with Bcl-2 family proteins, and activating downstream caspases in the mitochondrion-dependent apoptotic pathway to either cell cycle arrest, cellular senescence, or apoptosis of cells [38]. Pre-administration of *AS* ahead of *t*-BHP notably reduced the protein expressions of p53 and p21 in ECs and almost brought them back to the normal levels (Fig. 10A&B). Additionally, *t*-BHP decreased the abundance of anti-apoptotic Bcl-2 and increased the content of pro-apoptotic Bax in ECs, while AS pretreatment neutralizes these events (Fig. 10C). In conclusion, our results indicate that *t*-BHP activated while *AS* suppressed mitochondrion-dependent apoptotic cascades and cell cyclearrest in ECs. (Fig. 10. Effects of Angelica sinensis extract (AS) and *tert*-butyl hydroperoxide (*t*-BHP) on cell cycle regulator and apoptotic related protein expressions.)

4. Discussion

Endothelial dysfunction is considered to be a key event in the evolution of cardiovascular diseases. In this study, we demonstrated the p53/p21 and p16 mediated-pathways were activated in *t*-BHP induced vascular endothelial cell dysfunction in rats. It is reflected by the upregulation of p53 and p21 expression, comprising the p53/p21 pathway, and upregulation of p16 expression, exemplifying the p16 pathway. Our results also showed that *t*-BHP-treated endothelial cells upregulated p53 and p21expressions to induce apoptosis and senescence (detected with two techniques; annexin V staining, cleaved caspases-3 analysis, and decreased telomerase activity).

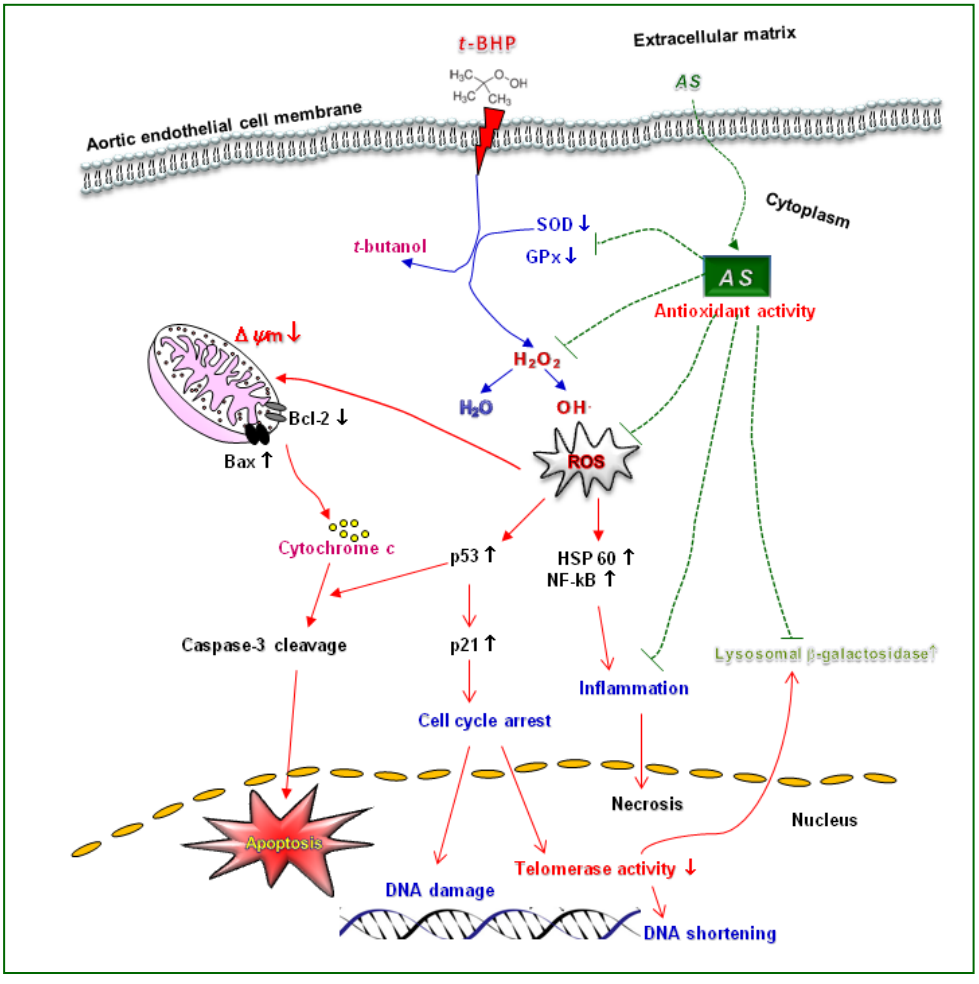
Apoptosis triggered by cell death receptors leads to the activation of caspases. These factors include cytochrome *c* which, in combination with the cellular Apaf-1, lead to activation of caspase-9, which then activates effectors caspases, such as caspase-3 [39]. Senescence is characterized to be a permanent growth arrest, in which cells remain metabolically active but are fully refractory to mitogenic stimuli. A broad range of cellular changes and functional alterations accompany senescence process. The consequence of senescence and senescence-associated changes have been suggested to contribute to the loss of normal organ function [19].

Senescence cells are believed to survive for years under appropriate tissue culture environment, and they are resistant to apoptosis. On the basis of this observation, it would appear that senescence and apoptosis are two independent outcomes in cells subjected to overthreshold stresses. Even though the same stressor is often capable of inducing either senescence or apoptosis, and these two

outcomes do share some important elements in their signaling cascades, as exemplified by the activation of p53 tumor suppressor, it is generally thought that in a given cell, only one pathway will be activated [29]. Confusion still exits in understanding the roles of the p16/Rb and p53/p21 signaling pathways in mediating the senescence process in general and premature senescence in particular. Little is known about the interaction between these two pathways, especially at the senescence-initiation stage [29].

5. Conclusion

In conclusion, this study indicates that for endothelial cells treated with the cytotoxic agent *t*-BHP, AS extracts could effectively preserve cell viability through relieving oxidative stress, preventing mitochondrial function, and inhibiting p53-mediated cell cycle arrest, apoptosis, and cellular senescence. Altogether, results from these experiments provide us better understandings of the antagonizing effects of AS on food additives *t*-BHP induced cardiovascular fucntion and structure changes via inflammation, changes of mitochondrial biogenesis and apoptosis, and enable us to discover an innovative, therapeutic approach to the prevention of oxidative stress induced-premature senescence of vascular endothelium and cardiomyocytes. Further researches are necessary to investigate the possible effects and detailed molecular protective mechanism played by *AS* against *t*-BHP-induced biochemical and physiological cardiac dysfunctions. (Fig. 11. Schematic diagram summarizing the possible mechanism for *Angelica sinesis* to counteract the oxidative, pro-apoptotic, and cellular senescent actions of *tert*-butyl hydroperoxide in endothelial cells.)



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