Application of Ginsenoside-Rg1 Could Not Ameliorate The Hyperoxic-Induced Vascular Endothelial Oxidative Stress and Apoptosis

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Research

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Abstract

**Background:** Oxygen therapy is necessary to preterm infants with respiratory distress. However, hyperoxia may cause bronchopulmonary dysplasia and retinopathy of prematurity due to suppression of vasogenesis and increase of cell death. Ginsenoside-Rg1, one of the active components of Ginsen, is shown as a proangiogenic factor of vascular endothelial cells. We evaluated whether application of Ginsenoside-Rg1 is able to improve hyperoxic-induced vascular endothelium injury.

**Materials and methods:** Human umbilical vein endothelial cells (HUVECs) were cultured under room air and 60% oxygen for 72 hours, respectively. Gensenoside-Rg1 was added to the medium at 0, 75, 150, 300nM. HUVECs proliferation, oxidative stress and apoptosis under normoxic and hyperoxic conditions were assayed by Western Blot.

**Results:** Under hyperoxia (60% O\(_2\)), HUVECs proliferation and levels of vascular endothelial growth factor (VEGF) were significantly decreased after ginsenoside-Rg1 treatment. Interestingly, both levels of glucocorticoid receptor (GR) and glutathione peroxidase (GPx) were increased after 72 hr Ginsenoside-Rg1 treatment, but no changed under room air control. The levels of oxidative stress-induced Bax and cytochrome c and apoptosis-related active caspase 3 and poly ADP-ribose polymerase were significantly increased after ginsenoside-Rg1 treatment under hyperoxic condition.

**Discussion and conclusion:** In HUVECs model, Ginsenoside-Rg1 is unable to overcome the major hyperoxic-induced vascular endothelium injury. It might aggravate oxidative stress and endothelial apoptosis caused by oxygen toxicity. However, both elevated levels of GR and GPx indicate that Ginsenoside-Rg1 could be involved in vascular signaling and the regulation of oxidative stress under hyperoxia. Further investigation of Rg1 effects under hyperoxia is required.

**Background**

Oxygen administration is necessary to preterm infants with respiratory stress, but hyperoxia is shown to be correlated with several diseases in preterm infants [1], such as bronchopulmonary dysplasia (BPD) and retinopathy of prematurity (ROP) [2]. Hyperoxia-induced lung injury is characterized by influx of inflammatory cells, increased pulmonary permeability, endothelial and epithelial cell death [3]. Vascular endothelial cells appear to be one of the major targets of hyperoxic injury. From the pathophysiology, hyperoxia disrupts the survival and functions of endothelial cells, which contributes to the development of diseases [4]. Hyperoxia also alters vascular endothelial growth factor (VEGF) signaling pathway in both BPD and ROP [1], and high concentration oxygen administration releases reactive oxygen species (ROS), which is believed to cause oxidative stress. Moreover, hyperoxic microenvironment is associated with multiple alternations in the extracellular and intracellular milieu of vascular endothelium that oxidative stress injury may act as inducers of apoptosis and cell death.

Ginseng, the root of *Panax ginseng*, has been used for centuries in China as a component of Chinese traditional medicine [5]. It could be used as a tonic to combat stress agents, or a medicine to improve
cardio-pulmonary function. The molecular compositions of ginseng have been studied extensively. Ginsenosides are the constituents that responsible for the actions of ginseng, and Rg1 is among the most abundant and active components. Recent in vitro studies showed that Ginsenoside-Rg1 is a proangiogenic factor of vascular endothelial cells [6]. In endothelial cell model, Ginsenoside-Rg1 is capable of regulating VEGF through activation of glucocorticoid receptor (GR) and vascular modeling via endothelial nitric oxide synthase (eNOS) [5–10]. Whether Ginsenoside-Rg1 could regulate vascular endothelial signaling under hyperoxic condition needs to be ascertained.

Literatures concerning the application of Ginsenoside-Rg1 in protection of hyperoxia-induced vascular endothelium injury are limited. We hypothesize that the treatment of Ginsenoid-Rg1 could involve in vascular endothelium signaling under hyperoxic condition. The vascular endothelial oxidative stress and cellular apoptosis were carefully evaluated.

Materials And Methods

Study design

In order to evaluate the effects of Ginsenoside-Rg1 on HUVECs under hyperoxia, various concentrations of Ginsenoside-Rg1 were added to endothelial cells cultured under normoxia and hyperoxia in the experiment period. Cell proliferation and protein production will be measured. In clinical condition, Fick's first law is used to describe the exchange of oxygen in the lung capillaries, where oxygen passes from the alveoli through the alveolar membrane and entering the blood stream. Fick's law can also be applied to cell culture in a similar manner if the oxygen concentration at the air-culture medium interface is considered similar to the oxygen concentration of gas at the surface of the alveolar membrane, and oxygen right above the cell monolayer is paralleled to the oxygen concentration in the blood stream [11]. Thus the culture atmosphere was set at 60% O₂, which is similar to the oxygen percentage administrated to preterm infants with respiratory distress syndrome.

Materials

Cell culture: Primary human umbilical vein endothelial cells (HUVEC, CRL-1730™) were used in our study. The cells were purchased from ATCC (Manassas, VA). Culture medium M199 and endothelial cell growth supplement (ECGS) were purchased from Sigma. Fetal bovine serum (FBS) was from Gibco.

Ginsenoside-Rg1: Experimental reagent Ginsenoside-Rg1 is a reference compound, with purity around 97.7%, purchased from the Division of Chinese Material Medica and Natural Products, National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Public Health, China. A stock solution of Ginsenoside-Rg1 50 mM was prepared in sterile double distilled H₂O and stored at -80°C.

Antibodies: β-actin was used as a loading control. Anti-β-actin antibody was obtained from Millipore (Temecula, CA). Antibodies for VEGF, GR, GPx (Glutathione Peroxidase), Bax, Cyt c (cytochrome c), PARP (Poly ADP-ribose polymerase), caspase 3 were purchased from Cell Signaling Technology, Beverly, MA,
USA. Cell proliferation was measured by MTT colorimetric assay (CytoSelect™ MTT Cell Proliferation Assay, CELL BIOLABS, INC.).

**Methods**

**Cell culture**

HUVECs were cultured in medium M199 with 10% FBS, 25μg/ml ECGS, and 1% penicillin/streptomycin in an incubator at 37°C. After seeding, the cells were stabilized in incubator under room air over night before experiment. In the experiments, cells were divided into normoxia and hyperoxia groups. In normoxia group, cells remained in incubator supplied with 95% room air (approximately 20% O\(_2\) and 5% CO\(_2\)). In hyperoxia group, cells were transferred to a chamber supplied with mixed air containing 60% O\(_2\), 35% N\(_2\), and 5% CO\(_2\). Culture medium was saturated with mixed air previously before experiments.

The duration for experiments were 24, 48, and 72 hours after cells were exposed to normoxia or hyperoxia. In hyperoxia group, NexBiOxy Hypoxia/Hyperoxia System (NexBiOxy, Taiwan) was used to monitor and maintain the oxygen concentration in the culture chamber.

**Ginsenoside-Rg1 administration**

According to references, 0, 75, 150, 300nM of Ginsenoside-Rg1 were used in our experiments [6]. Various concentrations of Ginsenoside-Rg1 were prepared and added to the medium just before experiment. Medium was changed every day to keep Ginsenoside-Rg1 within half-life period.

**Western blot**

HUVECs were washed in phosphate-buffered saline and then extracted with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton, 0.1% SDS, and 0.5% sodium deoxycholate) and protease inhibitors. Lysates were centrifuged at 12,000 rpm for 5 min, and the resulting supernatant was collected. The extracted protein was quantized by protein assay. Aliquots (25 μg) of cellular lysate was separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membrane. After blocking with 5% BSA, blots were incubated with primary antibodies and then corresponding secondary antibodies. An enhanced chemiluminescence kit (Amersham, Piscataway, NJ) was used for immunodetection. The protein bands were quantified using the Image J software (NIH, Bethesda, MD).

**Statistical analysis**

Each experiment was repeated for at least three times. Data were expressed as means ± S.D. Statistical comparisons were carried out by one-way ANOVA for multiple groups or t test for two groups. Significance was accepted at the p ≤ 0.05 level and was marked with * in the figures.
Results

Impairment of HUVECs proliferation under 60% O₂ but no influence under room air

We firstly evaluated the influence of O₂ in HUVECs proliferation. Under room air condition, the total number of HUVECs increased gradually in a time dependent fashion in response to Ginsenoside-Rg1 stimulation (Fig 1a). Conversely under 60% O₂ condition, the total number of HUVECs was significantly decreased in response to Rg1 treatments (Fig 1b). This indicates the detrimental proliferation of HUVECs was triggered by Ginsenoside-Rg1 under 60% O₂, whereas no influence under room air.

Levels of VEGF

Under room air, the levels of VEGF were significantly elevated in response to Ginsenoside-Rg1 treatment in a time-depend fashion (Fig 2a). Under 60% O₂ condition, Ginsenoside-Rg1 significantly decreased the levels of VEGF in a time-depend fashion compared with room air control (Fig 2b). The inhibition of VEGF expressions indicating the Ginsenoside-Rg1 could damper the potential vasogenesis under hyperoxic condition.

Levels of GR

Under room air condition, the levels of GR were significantly elevated in response to Ginsenoside-Rg1 treatment (Fig 3a). Under 60% O₂ condition, Ginsenoside-Rg1 elevated the levels of GR at 72hr compared with room air control (Fig 3b). The elevation of GR expressions indicating the Ginsenoside-Rg1 could upregulate vascular signal transduction under hyperoxic condition.

Levels of GPx

In order to know the potential anti-oxidative stress after Ginsenoside-Rg1 treatment, we isolated cytosolic proteins from HUVECs.

Under room air condition, the levels of GPx did not change in response to Ginsenoside-Rg1 treatment. Under 60% O₂ condition, the levels of GPx were significantly elevated in response to Rg1 treatment. Treatment with increasing concentrations of Rg1 (75, 150, and 300nM) increased the level of GPx. The stimulatory effect of Ginsenoside-Rg1 on GPx production was in a dose- and time-dependent fashion. Result indicates Ginsenoside-Rg1 treatment possesses the capability of anti-oxidative stress under hyperoxic condition.

Levels of Bax and Cytochrome c (Cyt c)

To determine the status of oxidative stress, we isolate cytosol Bax and Cyt c and confirmed overexpression after Ginsenoside-Rg1 treatment. Under 60% O₂ condition, the levels of Bax (Fig 5a, 5b) and Cyt c (Fig 6a, 6b) increased significantly when compared with those in the room air control. Neither
Bax nor Cyt c was changed under room air condition, indicating Ginsenoside-Rg1 treatment showed no influencece in oxidative stress.

**Levels of active Caspase 3 and Poly ADP-ribose polymerase (PARP)**

We next sought to determine whether vascular endothelium apoptosis could be influenced after Ginsenoside-Rg1 treatment, the Western blot was performed. Under 60% O$_2$ condition, the levels of active Caspase 3 (Fig 7a, 7b) and PARP (Fig 8a, 8b) increased significantly when compared with those in the room air control. The levels of active Caspase 3 (cleaved 17 and 19 kDa) were not changed under room air condition. Similar to active Caspase 3, immunoblotting analysis of PARP showed that the C-terminal 85 kDa PARP apoptotic fragment was not significantly generated under room air condition, indicating Ginsenoside-Rg1 treatment has no influence in vascular endothelium apoptosis.

**Discussion**

Ginseng has been used as a tonic remedy in Chinese traditional medicine for over 2000 years. In recent years, many beneficial effects of ginsenoside-Rg1, one of the major active ingredients of ginseng, have been reported. It stimulates glucose uptake, relieves oxidative stress, and suppresses adipocyte development and possible neuroprotective role as well [12]. It is a potent proangiogenic factor of vascular endothelial cells and stimulator of VEGF expression in human umbilical vein endothelial cells [6]. We hypothesize that the treatment of Ginsenoid-Rg1 could involve in vascular endothelium signaling under hyperoxia.

In this study, Ginsenoside-Rg1 was administrated to HUVECs, which were cultured under room air and 60% O$_2$ (hyperoxia) respectively. The vascular cell proliferation, oxidative stress, and apoptosis were carefully evaluated. Under room air, Ginsenoside-Rg1 is able to enhance the proliferation and VEGF production of HUVECs in a time- and concentration- dependent fashion when compared with those of hyperoxic conditions. Ginsenoside-Rg1 has been reported to activate the glucocorticoid receptor (GR), which is an important factor in inducing angiogenesis [5, 13]. Consistently, our results show that Ginsenoside-Rg1 is able to increase the levels of GR under room air. Furthermore, the highest level of GR was observed under hyperoxic condition for 72hr treatments. Several researches showed that the angiogenesis effect of Ginsenoside-Rg1 was expressed with VEGF production through the GR-related signaling pathway in HUVECs [5, 7, 13]. Ginsenoside-Rg1 diminished the levels of VEGF whereas increased the levels of GR under hyperoxic O$_2$, indicating the GR-dominant signaling. Ginsenoside-Rg1 has been reported as an agonist ligand of GR to induce NO production from endothelial nitric oxide synthase [9], or induce anti-inflammatory function through GR [20, 21]. Under hyperoxia, GR-dominant signaling after Ginsenoside-Rg1 treatment may involve in vascular anti-inflammatory mechanism.

Glutathione peroxidase (GPx), which is an antioxidant enzyme class, belongs to the selenoprotein family and protects cells from oxidative damage. The enzyme accomplishes this through the reduction of lipid hydroperoxides to alcohols and through the reduction of free hydrogen peroxide to water. Under room air,
the production of GPx showed no significant change. However, under hyperoxic condition, the GPx production increases significantly while HUVECs treated with Ginsenoside-Rg1 in a time- and dosage-dependent pattern. These results may indicate that Ginsenoside-Rg1 is able to trigger the antioxidant defense mechanism to protect the cells from hyperoxia-induced oxidative cellular damage. Some other researches also show that Ginsenoside-Rg1 has the potential against oxidative stress mediated cell damage through the glutathione system [24-26].

Hyperoxia is reported to cause cell death of vascular endothelial cells [14]. Cell death from hyperoxic injury may occur through an apoptotic pathway, which includes accumulation of reactive oxygen intermediates and inflammation [15-18]. Under room air, Ginsenoside-Rg1 showed no significant changes on the levels of oxidative stress-related molecules and preapoptotic molecules respectively. Nevertheless, under hyperoxia, the protein levels of Bax, active caspase3, Cytochrome c and PARP are increased along with the increased concentrations of Ginsenoside-Rg1. Bax is a pre-apoptotic member of the Bcl-2 protein family that resides in the outer mitochondrial membrane. Several researches showed that Bax could induce caspase activation and cell apoptosis via Cytochrome c released from mitochondria [27, 28]. These results indicate that higher doses of Ginsenoside-Rg1 may aggravate the apoptosis of HUVECs under hyperoxic condition. Furthermore, to see whether the release of Cytochrome c is a consequence of hyperoxia-induced apoptosis, higher levels of the cleaved 85 kDa PARP apoptotic fragment were observed. The activation of PARP, which known to result in a specific programmed cell death pathway involving the release of apoptosis inducing factor [19], is also seen in our results. The significant activation of PARP along with the increase of Ginsenoside-Rg1 dosage under hyperoxia shows the possibility of aggravating vascular endothelial cell death.

**Conclusion**

Under hyperoxic 60% O₂, Ginsenoside-Rg1 was unable to improve vascular endothelial cell proliferation and VEGF production, indicating Ginsenoside-Rg1 is unable to overcome the major hyperoxic injury of vascular endothelium. Furthermore, under hyperoxia, oxidative stress-induced Bax and cytochrome c, and preapoptosis-related active caspase 3 and PARP showed that Ginsenoside-Rg1 might aggravate the hyperoxia-induced vascular endothelium injury. However, both elevated levels of GR and GPx indicate that Ginsenoside-Rg1 could involve in vascular signaling and the regulation of oxidative stress under hyperoxia. Further investigation of Ginsenoside-Rg1 effects under hyperoxia is required.

**Abbreviations**

BPD: bronchopulmonary dysplasia, Cyt c: cytochrome c, ECGS: endothelial cell growth supplement, eNOS: endothelial nitric oxide synthase, FBS: fetal bovine serum, GPx: glutathione peroxidase, GR: glucocorticoid receptor, HUVECs: Human umbilical vein endothelial cells, PARP: Poly ADP-ribose polymerase, ROP: retinopathy of prematurity, ROS: reactive oxygen species, VEGF: vascular endothelial growth factor.
Declarations

Ethics approval and consent to participate:
Not applicable. Since there is no patient involvement, no ethics approval and consent required.

Consent for publication:
Not applicable.

Availability of data and materials:
all datasets generated and analysed during the current study are not publicly available because not yet published, but are available from the corresponding author on reasonable request.

Competing interests:
There are no financial or non-financial competing interests.

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Authors’ contribution:
Ren-Huei Fu: research idea, literature review, protocol design, experiment processing, data analysis and discussion, manuscript writing.

Chi-Nan Tseng: literature review, protocol design, data analysis and discussion, manuscript discussion.

Yu-Hsueh Cho: research idea, protocol design, experiment processing, data collecting, analysis, and discussion.

Yen Chu: research idea, literature review, protocol design and improvement, experiment guiding and processing, data analysis and discussion, manuscript writing and refinement.

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**Figures**

*Figure 1 (a), (b). (a) Room air (20% O2)*

![Graph showing proliferation under room air condition](image1)

*Figure 1 (b) 60% O2*

![Graph showing proliferation under 60% O2 condition](image2)

**Figure 1**

Rg1 downregulates HUVECs proliferation under 60% O2. HUVECs exposed to room air or 60% O2 in paired chambers and treated with various concentrations of Rg1 for 24, 48 and 72hr, respectively. Cell proliferation was determined by colorimetry based on the uptake of MTT. (a) Under room air condition, the total number of HUVECs increased gradually in a time dependent fashion in response to Rg1 stimulation. (b) Under 60% O2 condition, the total number of HUVECs was significantly decreased in response to increasing concentrations of Rg1 (75, 150, and 300nM) stimulation. *P<0.05 vs. Control.
Figure 2

Rg1 does not induce VEGF production in HUVECs under 60% O2. HUVECs exposed to room air or 60% O2 in paired chambers and treated with various concentrations of Rg1 for 24, 48 and 72hr, respectively. The levels of VEGF were determined by Western blot. β-actin was used as the loading control. The signal intensities were determined by densitometry. Data are shown as mean ± SD of three independently experiments. (a) Under room air condition, the levels of VEGF were significantly elevated in response to Rg1 stimulation. Treatment with increasing concentrations of Rg1 (75, 150, and 300nM) increased the level of VEGF. The stimulatory effect of Rg1 on VEGF production on HUVECs was time-dependent. (b) Under 60% O2 condition, Rg1 significantly decreased the levels of VEGF compared with control. The inhibitory effect of Rg1 on decreased VEGF production was time-dependent. *P<0.05 vs. Control.
Figure 3

Rg1 induces GR production in HUVECs under 60% O2. HUVECs exposed to room air or 60% O2 in paired chambers and treated with various concentrations of Rg1 for 24, 48 and 72hr, respectively. The levels of GR (glucocorticoid receptor) were determined by Western blot. β-actin was used as the loading control. The signal intensities were determined by densitometry. Data are shown as mean ± SD of three independently experiments. (a) Under room air condition, the levels of GR were significantly elevated in response to Rg1 stimulation. Treatment with increasing concentrations of Rg1 (75, 150, and 300nM) increased the levels of GR. (b) Under 60% O2 condition, Rg1 elevated the levels of GR compared with control. Treatment with increasing concentrations of Rg1 (75, 150, and 300nM) elevated significantly the level of GR at 72hr. *P<0.05 vs. Control.
Figure 4

Rg1 induces glutathione peroxidase production in HUVECs under 60% O2. HUVECs exposed to room air or 60% O2 in paired chambers and treated with various concentrations of Rg1 for 24, 48 and 72hr, respectively. The levels of glutathione peroxidase (GPx) were determined by Western blot. β-actin was used as the loading control. The signal intensities were determined by densitometry. Data are shown as mean ± SD of three independently experiments. (a) Under room air condition, the levels of GPx did not change in response to Rg1 stimulation. (b) Under 60% O2 condition, the levels of GPx were significantly elevated in response to Rg1 stimulation. Treatment with increasing concentrations of Rg1 (75, 150, and 300nM) increased the level of GPx. The stimulatory effect of Rg1 on GPx production was time-dependent. *P<0.05 vs. Control.
Figure 5

Rg1 increases Bax production in HUVECs under 60% O2. HUVECs exposed to room air or 60% O2 in paired chambers and treated with various concentrations of Rg1 for 24, 48 and 72hr, respectively. The levels of Bax were determined by Western blot with β-actin used as the loading control. The signal intensities were determined by densitometry. Data are shown as mean ± SD of three independently experiments. (a) Under room air condition, the levels of Bax did not change in response to Rg1 stimulation. (b) Under 60% O2 condition, the levels of Bax were significantly elevated in response to Rg1 stimulation. Treatment with increasing concentrations of Rg1 (75, 150, and 300nM) increased the level of Bax. The stimulatory effect of Rg1 on Bax production was time-dependent. *P<0.05 vs. Control.
Figure 6

Rg1 increases cytochrome c production in HUVECs under 60% O2. HUVECs exposed to room air or 60% O2 in paired chambers and treated with various concentrations of Rg1 for 24, 48 and 72hr, respectively. The levels of cytochrome c (Cyt c) were determined by Western blot. β-actin was used as the loading control. The signal intensities were determined by densitometry. Data are shown as mean ± SD of three independently experiments. (a) Under room air condition, the levels of Cyt c did not change in response to Rg1 stimulation. (b) Under 60% O2 condition, the levels of Cyt c were significantly elevated in response to Rg1 stimulation. Treatment with 300nM Rg1 increased the level of Cyt c. The stimulatory effect of Rg1 on Cyt c production was time-dependent. *P<0.05 vs. Control.
Figure 7

Rg1 increases proapoptotic active caspase 3 production in HUVECs under 60% O2. HUVECs exposed to room air or 60% O2 in paired chambers and treated with various concentrations of Rg1 for 24, 48 and 72hr, respectively. The levels of active caspase 3 were determined by Western blot with β-actin used as the loading control. The signal intensities were determined by densitometry. Data are shown as mean ± SD of three independently experiments. (a) Under room air condition, the levels of caspase 3 did not change in response to Rg1 stimulation. (b) Under 60% O2 condition, the levels of caspase 3 were significantly elevated in response to Rg1 stimulation. Treatment with increasing concentrations of Rg1 (75, 150, and 300nM) increased the level of active caspase 3. The stimulatory effect of Rg1 production was time-dependent. *P<0.05 vs. Control.
Figure 8

Rg1 increases proapoptotic PARP production in HUVECs under 60% O2. HUVECs exposed to room air or 60% O2 in paired chambers and treated with various concentrations of Rg1 for 24, 48 and 72hr, respectively. The levels of PARP were determined by Western blot. β-actin was used as the loading control. The signal intensities were determined by densitometry. Data are shown as mean ± SD of three independently experiments. (a) Under room air condition, the levels of Poly (ADP-ribose) polymerase (PARP) did not change in response to Rg1 stimulation. (b) Under 60% O2 condition, the levels of PARP were significantly elevated in response to Rg1 stimulation. Treatment with increasing concentrations of Rg1 (75, 150, and 300nM) increased the level of PARP. The stimulatory effect of Rg1 on PARP production was time-dependent. *P<0.05 vs. Control.