Isorhamnetin protects endothelial cells model CRL1730 from oxidative injury by hydrogen peroxide

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Abstract: To explore the protective effect and mechanism of isorhamnetin against oxidative injury caused by H_2O_2 to endothelial cell strain CRL1730 of human umbilical vein. H_2O_2 and endothelial cell strain CRL1730 were used, as a model of injured endothelial cell. Three levels of crude drugs areorhamnetin, 22.8μg/ml, 11.4μg/mL and 5.7μg/mL was added to the injured cell strain CRL1730 respectively. The cell injury was measured in terms of necrotic rate, quantities of von Wilebr and factor (vWf) and thrombomodulin (TM), lactate dehydrogenase (LDH) and intracellular free calcium ions through flow cytometry, ELISA, fluorescent spectrometer and laser scanning confocal microscopy respectively. Isorhamnetins @ 11.4μg/mL and 5.7μg/mL has significantly decreased EC necrotic rate, while the increased vWf concentration due to oxidant (200μmol/L of H_2O_2) was significantly decreased by 5.7μg/mL versus 11.4 and 22.8μg/mL isorhamnetin. Also, the increased in TM and LDH in injured cells was reversed to normal level with 5.7 to 11.4μg/mL isorhamnetin. These results suggest that isorhamnetin protect the integrity of cell membranes. Similarly, H_2O_2 treatment of cells elicited the release of intracellular calcium, however, 5.7μg/mL and 11.4μg/mL isorhamnetin dramatically inhibited transient release of intracellular calcium. This suggests that isorhamnetin, at lower concentration, could inhibit the IP3-sensitive calcium pool from releasing calcium, protecting VECs from injury by H_2O_2. Traditional Chinese herbs, hippophaerhamnoides have been recognized as safe and as a source of flavonoids,with strong cardiovascular protection. The results of this study revealed that isorhamnetin produce a strong effect on some targetspresent in ECs and thus, provide a basis for the future work targeted towards endothelial cells protection.

Keywords: Isorhamnetin, endothelial cells, von Wilebrand factor, lactate dehydrogenase, calcium.

INTRODUCTION

Endothelial cells (EC) lines the interior of the blood vessels. These cells have the capability to change the properties of the biological reaction. The most essential functions these cells perform include blood pressure, vascular permeability and immune tune (Salvador *et al.*, 2016) and hence, vascular biology can be effectively changed via targeting. Wei *et al.* (2016) reported the presence of AMPK/PI3K-Akt/eNOS pathway in human EC, which responded to xenatides, an insulin analogue while, Winnik *et al.* (2016) described another pathway in EC, C/EBP-β-dependent rescue mechanism for superoxide dismutase (SOD_2) activity against oxidative stress. As well, Notch signaling pathways, are the underlying mechanisms in EC modifying hematopoietic stem cells (HSCs) activity. However, abnormal EC may change the physiology of the cells and thus, it appears that EC dysfunction has a direct relationship with the pathogenesis of many severe human diseases. Many factors, such as cytokines, active oxygen (H_2O_2) and endotoxins, can cause injury tovesicular endothelial cells (VEC). Exposure of VEC to H_2O_2 can induce lipid oxidative reactions of the unsaturated fatty acid in cell membrane, resulting in injury to cell membrane and ultimately cell death (Suzuki *et al.*, 1997). In view of the back ground knowledge, it is of great significance to find a drug with protective effect on endothelial cells.

Isorhamnetin, kind offlavonoids found in hippophaerhamnoides, with anti-tumor effect. Up to now, there are only few reports regarding isorhamnetin effect on cardiovascular system (Luo *et al.*, 2015) and yet, the role of isorhamnetin in VEC protection remains unknown. In this study, we used H_2O_2 as stress producing agent, against endothelial cell strain CRL1730 of human umbilical vein, because oxidative stress is a hallmark of endothelial dysfunction. We aimed to unravel the effects of isorhamnetinon endothelial function and the underlying mechanism. The present study underscored many findings related to a very important medical disease, cardio vasicular diseases. The new crude drugs in our experimentation have the ability to bind with many targets, present in the endothelial cells. It is also worth mentioning that hydrogen peroxide injured endothelial cells, act as a reasonable model of endothelial dysfunction, and might be used in future exploration.

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MATERIALS AND METHODS

Materials

Human umbilical vein endothelial cell strain CRL1730 was purchased from China Medical University Tumor Research Institution. The cells were cultured in DMED high glucose medium with 20% fetal bovine serum at 37C, 5% CO2. Isorhamnetin standard was obtained from Qingzhe Medical Technology Development Company (Nanjing, China); von Wilebr and factor (vWf) ELISA kit from Xitang Biological Technology Company (Shanghai,China); thrombomodulin (TM) ELISA kit from Adlitteram Diagnostic Laboratories Company (USA, lot No: 071015); Lactate dehydrogenase (LDH) kit from Jiancheng Biological Engineering Institution (Nanjing, China); Iodization proplodine (PI) from Sigma (Germany).

Equipments

Enzyme label meter SUNRISE RC from TECAN (Swizerland); flow cytometry FACS Calibur from BD Company (USA); Microscope with auto-photomicrograph equipment AX70 from OLYMPUS (Japan); and Laser scanning confocal microscope LEICA TCS SP5 from Leica Company (Germany).

Methods

Necrotic experiment

The growth response of the H_2O_2 stress CRL1730 cells was evaluated in response to various level of isorhamnetin. CRL1730 (100µL) cell solution (in logarithmic phase) was added to 96-well plate while the cell densities in the cell solution was $3x10^4$/ well. The cell lines solution was culture in 5% CO2 $37C^0$ for 8h. After this incubation time, the unattached cells from wells were removed while the live cells were remained attached with walls of the well. This was followed by the addition of 150µL of DMEM that contained 200µmol/L of H_2O_2 and isorhamnetin of 22.8µg/mL (H group), 11.4µg/mL (M group), 5.7µg/mL (L group) and 0.0µg/mL (Positive group). The cells 96-well plate was incubated for another 48 hrs and the experiment was repeated five times and growth inhibition rate was calculated from necrotic result (by PI staining method).

Elisa analysis

CRL1730 (100µL) cell solution was added to 96-well plate while the cell densities in the cell solution was $3x10^4$/ well. The cell lines solution was culture in 5% CO_2, 37C for 8h. After this incubation time, the unattached cells from well were removed while the live cells were remained attached with walls of the well. This was followed by the addition of 150µL of DMEM that contained 200µmol/L of H_2O_2 and isorhamnetin of 22.8µg/mL (H group), 11.4µg/mL (M group), 5.7µg/mL (L group) and 0µg/mL (Positive group). The cells 96-well plate was incubated for another 48h and the unattached cells were discarded, but the attached cells to the walls we added pancreatine enzymes. The cells thus digested by this enzyme were used for vWf, TM and LDH through ELISA.

Intracellular free calcium ions analysis

The growth response of the H_2O_2 stress CRL1730 cells was evaluated in response to isorhamnetin. CRL1730 (100µL) cell solution was added to 96-well plate while the cell densities in the cell solution was $3x10^4$/ well. The cell lines solution was culture in 5% CO_2, 37^oC for 8h. After this incubation time, the unattached cells from well were removed while the live cells were remained attached with walls of the well. This was followed by the addition of 150µL of DMEM that containedisorhamnetin of 0µg/mL (Positive Control), 22.8µg/mL (H group), 11.4µg/mL (M group) and 5.7µg/mL (L group) were supply by 150µL of DMEM. The cells 96-well plate was incubated for another 48h and the unattached cells were discarded. The attached cells were added pancreatine enzymes. The digested cells were added with 100µl of carrier solution with Fluo-3 and AM Ester of concentration of10µmol/L, followed by incubatation at 37^oC for 45minutes. The carrier solution was removed and 1.0mL of non-serum culture media was added. Under confocal microscope, Time Series scan was performed to produce sequential images for the control. After scanning of three images, within 10s, H_2O_2 was added. Under laser scanning confocal microscope, it was measured again. The changing percentage of fluorescent intensity was measured as:

Fluorescent Intensity (%) = (Fx / F0)×100%

Where,

Fx= Fluorescent intensity of the fluorescent complex at the x min after stimulation

F0= Basal value of fluorescent intensity before drug stimulation

STATISTICAL ANALYSIS

SPSS 13.0 software was used for statistical analysis. A *P*-value less than 0.05 was considered as significant.

RESULTS

Morphology of endothelial cells

Fig.1 describes detailed morphology of the endothelial cells (human umbilical vein). Cells were found in various shapes and forms such as polygons, and spindles, with clear boundary. The nucleus was found to be round or elliptical in structures in which chromatins were loosely packed. After cultivation for about half an hour, cells started to adhere and grow on vascular wall. The H_2O_2 treatment, as can be seen (fig. 1), cells show black granules, where vascuoles could be seen inside cells. Nuclear membrane became unclear and with time, more cells became round in shape. This phenomena eventually detached cells from vascular wall and some cells were

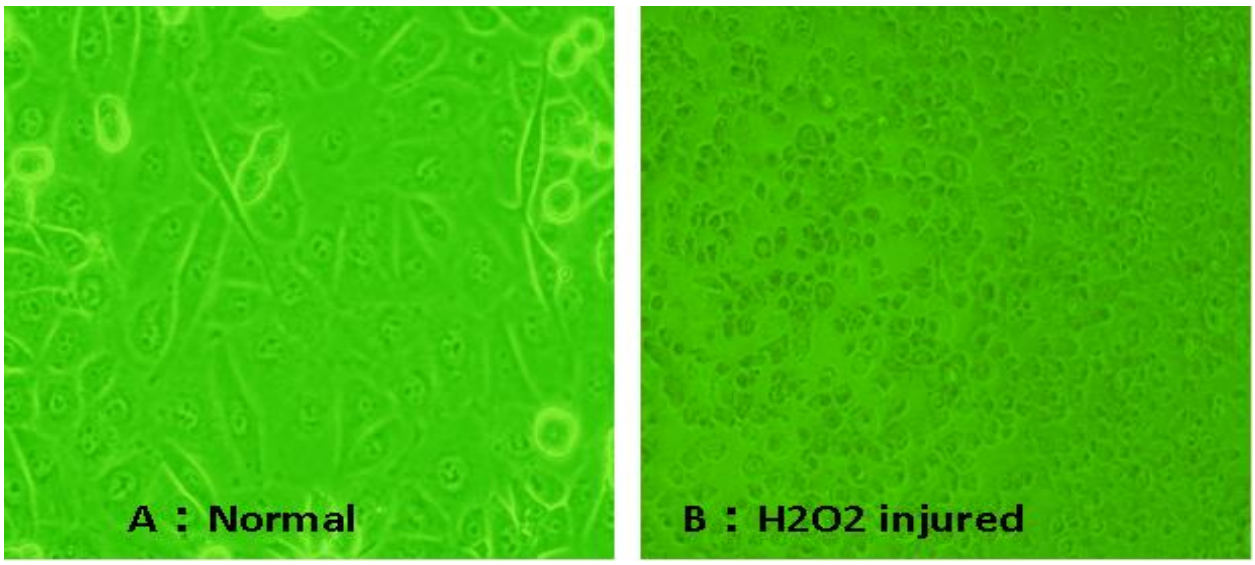

Fig. 1: Morphology of CRL1730 cells

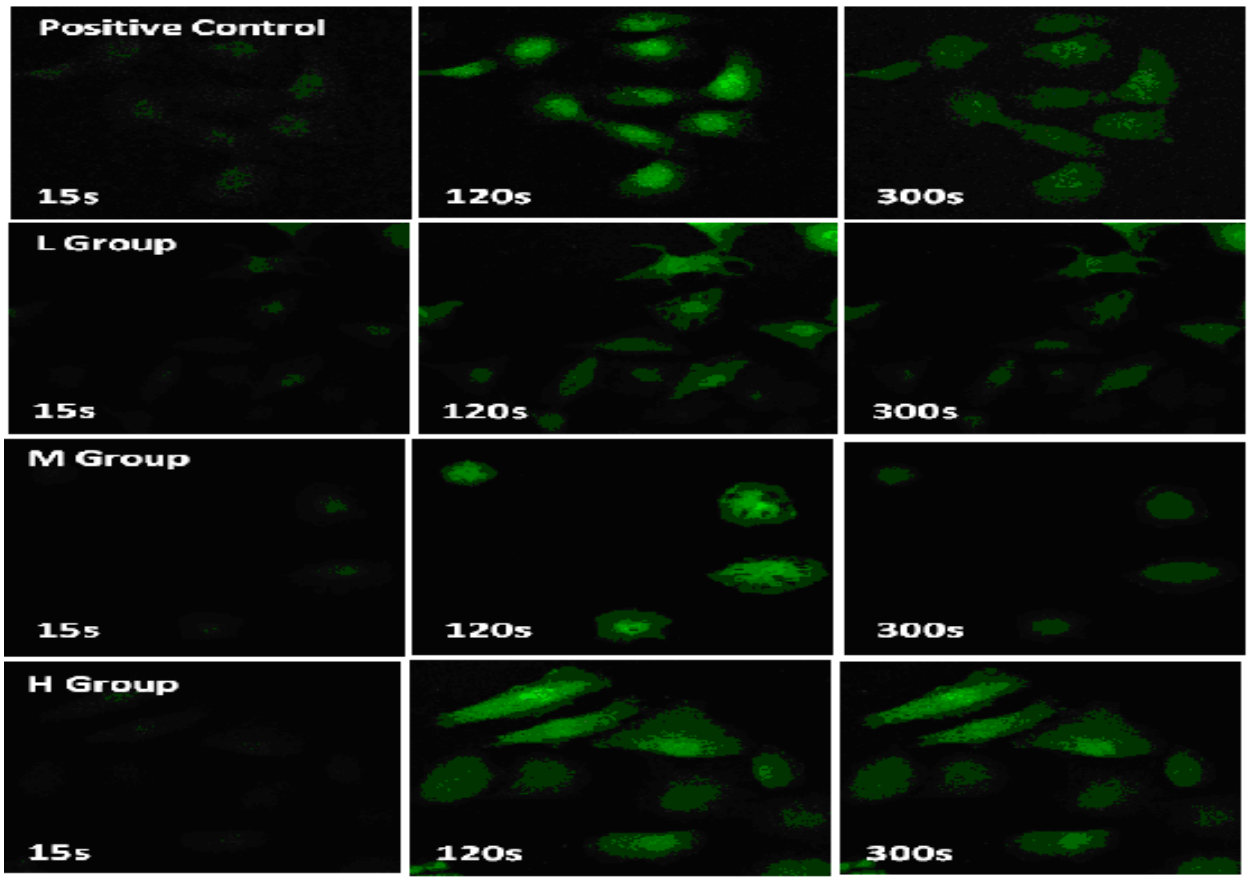

Fig. 2: Change of calcium ions to Isorhamnetin over time

broken into pieces while the attached cells shrank in size (fig.1).

Protective effect of isorhamnetin on H_2O_2 injured cells

The necrotic rate in endothelial cells is shown in table 1. Positive control and H group cells displayed significantly higher necrotic rate ($P<0.01$) compared with M group and L group cells. Moreover, necrotic rate was similar among M group and L group and were lower than those cells treated with high and medium level isorhamnetin (22.8μg/ml ISOR+200μmol/L H_2O_2; 11.4μg/ml ISOR +200μmol/L H_2O_2) respectively. Similarly, average

Table 1: The influence of Isorhamnetin on necrotic rate

Group	Necrotic rate (%)	Average fluorescent intensity
Positive Control	28.36±10.14	2255.76±73.44
H group	22.58± 0.65	2272.87±53.57
M Group	16.59± 3.15**	2084.59±69.84*
L Group	13.06± 1.47**	2112.60±84.91*

Mean ±SD value with star (*) in a column is significantly different at P value less than 0.005; n=5

Table 2: Effect of Isorhamnetin on inflammatory biomarkers

Group	vWf (mU/ml)	TM (ng/ml)	LDH(U/L)
Positive Control	9.60±1.38	58.39±18.79	1931.93±159.51
H group	8.59±2.32	40.38±14.12*	973.18± 80.07**
M Group	6.25±2.11	34.71±11.66**	753.51±132.20**
L Group	2.69±1.75**	34.28±11.23**	719.03±159.34**

Mean ±SD value with star (*) in a column is significantly different at P value less than 0.005; n=5

fluorescent intensity was also increased in positive control and H group compared with M group and L group but non-statistical significance was found (P>0.05) between M group and L group.

Effect of isorhamnetin onvWF level

The effect of Isorhamnetin on vWf level is presented in table.2 which shows that vWF concentration significantly increased (p<0.01) except, group-5 where this effect is reversed significantly. Isorhamnetinat lowest level demonstrated inhibitory effects versus higher level.

Effect of isorhamnetin on TM level

The level of TM is presented in table. 2. The results shows that TM level was significantly (p<0.01) reduced in H group, M group and L group compared with Positive control. Furthermore, Isorhamnetin has profound effect at medium and low concentration versus higher concentration (H group).

Effect of isorhamnetin on LDH level

The level of LDH enzymes were found to be higher in M group (p < 0.01; 1931.93±159.51) compared with medium and low concentration isorhamnetin treated groups as shown in table 2.

Protective effects of isorhamnetin oncalcium ions

The effect of the crude drugs is presented in fig. 2. The results showed that medium level isorhamnetin administration resulted in protection of the cells and thus, released limited calcium ions compared to others at 15second exposure.

The effect of the crude drug is presented in fig. 2. The results show that low and medium concentration of crude drugs was found effective in reducing calcium release from VEC versus control and high concentration of drugs.

The concentration of intracellular calcium ions in endothelial cells started to increase and reached the peak around 120seconds, followed by reduction. However, the concentration of intracellular calcium ions remained above the initial value. The concentrations of intracellular calcium ions of H group and M group were significantly decreased than positive control. The concentration of intracellular calcium ions in the L group was maintained at high level within 300seconds.

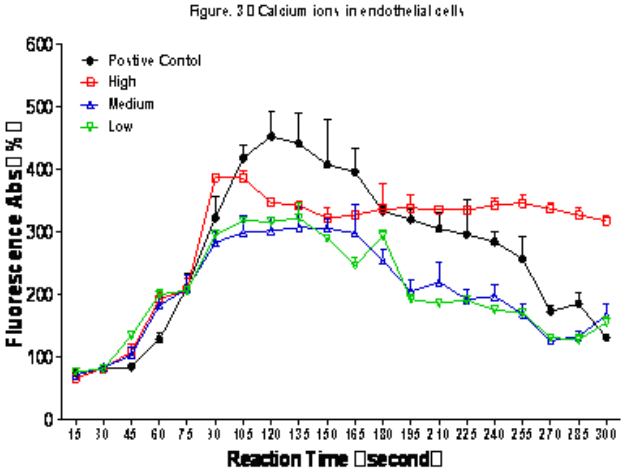

Fig. 3: Effect of isorhamnetin on intracellular calcium induced by H_2O_2

DISCUSSION

The CRL1730 cells used in this experiment was from human umbilical vein which presents the normal growth and function features of general vascular endothelial cell (Hughes 1996).

Vascular von Wille brand factor (vWf) is a large molecular glycoprotein synthesized by VEC. Under physiological conditions, it is stored in the Weibei-Palade body of endothelial cell. However, when blood vessel is injured, vWf are released which enhanced coagulation reaction (Denis 1994) by reacting with coagulation factor VIII (Kang *et al.*, 2001). The more the concentration of

this protein, the more severe is vascular endothelium injury. Also, increased level of vWf is a risk factor for vascular lesion. Our findings show that vWf concentrations arise in response to oxidants (200μmol/L of H_2O_2) which causes oxidation of methionine residues in vWF (Fu *et al.,* 2011). However, this effect was significantly reversed in case of 5.7μg/mL isorhamnetin treated cells versus those cells treated with medium and high concentration isorhamnetin (11.4 and 22.8μg/ml), respectively.

Thrombomodulin (TM) is a cofactor for thrombin activating protein exhibiting both activity of anti-coagulation and anti-fibrolysis (Shingo *et al.,* 2002). It released to extra cellular fluid in response to cells injury. Therefore, TM is also a molecular marker for injury of endothelial cells. In this experiment, H_2O_2 with final concentration of 200μmol/L was added into endothelial cellsculture media, the cells were injured and TM released, resulting in significant increase in TM proteins in culture media. We found that lower concentrations of isorhamnetin could greatly reduce the level of TM, indicating that isorhamnetin at these concentrations (5.7 to 11.4μg/mL) could protect the integrity of cell membrane. Although, higher concentration of isorhamnetin (22.8μg/mL) also had protective actions but the effect was low. Miao *et al*. (2013) showed that the effect of high and moderate dose flavonoid is identical.

Lactase dehydrogenase (LDH) can be synthesized by endothelial cell and exists in cytoplasm. Injured cells have high membrane permeability and thus secrete LDH. As the degree of injury becomes severe, the amount of leakage increases, too. About 24hours after action of H_2O_2 on endothelial cell, lipid per oxidation in cell membrane increases, this has led to damage of the cell membrane. In positive control, the level of LDH was significantly increased. However, isorhamnetin could reduce the level of LDH, indicating its protective effect on the integrity of cell membrane. These findings are in collaboration with Huang *et al.* (2016) who reported that cellular injury is decreased when lactate dehydrogenase level decreased.

Furthermore, calcium is vital signal transduction factor in biological process. The elevated level of calcium in cytoplasm is primarily due to the release of intracellular calcium and the influx of extracellular calcium. It has been testified that the former can only induce temporary intracellular calcium increase, while the latter can result in continuous intracellular calcium increase (Parekh and Penner 1997). Many injury factors such as oxidative stress, hypoxia, and re-perfusion after hypoxia can all cause intracellular calcium overload and subsequent cell injury (Liu *et al.,* 2003; Siflingerbirnboim *et al.,* 1996; Suzuki *et al.,* 1997). In this study, we observed that H_2O_2 could temporarily elevate intracellular calcium in ECs, indicating intracellular calcium promoting release mechanism. However, isorhamnetin at concentrations of 5.7μg/mL and 11.4μg/mL could significantly inhibit such transient release of intracellular calcium. This suggests that isorhamnetin, at lower concentration, could inhibit the IP3-sensitive calcium pool from releasing calcium, protecting vascular endothelial cells from injury by H_2O_2. Moreover, some researches believed that the protective effect of isorhamnetinis associated with the regulative expressions of NO synthase and LOX-1 (Bao and Lou 2006a) genes, as well, the drugs might also enhance the expression of super oxide dismutase (Bao and Lou 2006b; Saito *et al.,* 2004; Zielińska *et al.,* 2001) enzyme, an antioxidant enzyme that protect cells integrity against cell injury. And hence, it can be speculated that isorhamnetin may achieve its anti-oxidation effect through many ways.

CONCLUSION

The low toxicity of traditional Chinese herbs is attracting more and more attention. It has been recognized that flavonoids have strong cardiovascular activity, for instance, flavonoids in hippophaerhamnoides have dramatic anti-thrombosis effect. In this study, the effect of isorhamnetin, a kind of flavonoids, was evaluated through a H_2O_2-induced cell injury model. Its protective mechanism was discussed and some of targets of the drug were tested, in order to provide a theoretical basis for exploring drugs with protective effect on endothelial cells.

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