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Low-dose vardenafil potentiates the protective effect of (-)-epigallocatechin gallate on cardiomyocytes

Kai Wang, Zhaoyang Fan, Peng Jin*, Dingkui Qin and Qizhen Du
The Key Laboratory for Quality Improvement of Agricultural Products of Zhejiang Province,
The College of Agricultural and Food Sciences, Zhejiang A & F University, Linan, China

Abstract: The major polyphenol (-)-epigallocatechin gallate (EGCG) of green tea shows well-known health benefits such as potential anti-cancer, anti-oxidation and ameliorating cardiovascular disease. This work aims to improve the bioactivity of EGCG on H9C2 cardiomyocytes by combination regimen of vardenafil and EGCG. The proliferative rates were significantly improved by 18.74%, 10.77% and 29.17% after 48 h with EGCG, vardenafil, and the combination of EGCG and low-dose vardenafil treatments, respectively. The treatments also increased the expression of the nitric oxide synthase (eNOS), and acutely stimulate production of vasodilators nitric oxide (NO) from 17.33μmol/L to 19.75, 20.87 and 24.47μmol/L in H9C2 cells. We further demonstrated that vardenafil also remarkably promoted EGCG to counteract H_2O_2-induced apoptotic damage in H9C2 by strengthening antioxidant defense systems and suppressing myocardial apoptosis. These results suggest that EGCG and low-dose vardenafil in combination may be a promising regimen to help prevent cardiovascular diseases.

Keywords: (−)-Epigallocatechin-3-gallate, vardenafil, cardiomyocytes, combination regimen, anti-apoptosis

INTRODUCTION

(-)-epigallocatechin-3-gallate (EGCG) is a natural polyphenolic catechin and widely distributed among green tea *Camellia sinensis* with constituted 40%-60% of polyphenol content (Du *et al.*, 2012; Nagle *et al.*, 2006). Numerous studies suggests that the health benefits from green tea consumption is mainly due to the contribution of EGCG strong antioxidant bioactivity (Mak, 2012). In particular, EGCG significantly promotes apoptosis activation to kill many types of cancer cells (Yang *et al.*, 2017), such as inducing cell cycle arrest, suppresses mitogen-activated protein kinases and blocks growth factor receptor signaling transduction (Hou *et al.*, 2004). However, these beneficial biochemical effects of EGCG are not unique to cancer cells, but also have significant health effects to ameliorate numerous pathophysiological processes, for example endothelial dysfunction and insulin resistance (Kim *et al.*, 2007). Studies show that EGCG is associated with ameliorate cardiovascular and metabolic diseases by improving endothelium-dependent vasodilation (Loke *et al.*, 2008). This capacity of EGCG probably mimics the insulin to augment metabolic and vasodilator actions. However, the underlying mechanisms remain unclear.

Vardenafil as phosphodiesterase type 5 inhibitor was approved for the treatment of erectile dysfunction in clinic. Pharmacodynamic studies have shown an interesting clinical report that vardenafil was a more potent acute pulmonary vasodilator on endothelial function (Jing *et al.*, 2011; Karasu-Minareci *et al.*, 2012; Mazo *et al.*, 2006). Inhibition of the cGMP-specific PDE-5 leads to an accumulation of cGMP enhancing the potent action of vasodilator nitric oxide (NO). Moreover, pharmacological experiments show that vardenafil can significantly potentiate the EGCG-induced CLL cell death (Kumazoe *et al.*, 2015). Therefore, whether the synergistic activation of EGCG by vardenafil potentiates the protective effect on the cardiovascular will need to be investigated.

In this work, we evaluated the combination effects of vardenafil and EGCG on the proliferation and anti-apoptosis in damage H9C2 cardiomyocytes. We demonstrate novel contributions of low-dose vardenafil in the stimulating actions of EGCG resulting from H9C2 cell proliferation and effectively attenuating apoptotic damage in cardiomyocytes. Our results provide novel insight into protective actions of EGCG and vardenafil to understand underlying biologic functions and interaction, and could also be as a promising substitute for the conventional therapies of cardiovascular disease.

MATERIALS AND METHODS

Materials

H9C2 cardiomyocytes were obtained from Shanghai Cells Bank, Institutes of Life Sciences of the Chinese Academy of Sciences. All culture materials were obtained from Gino biomedical technology co. LTD (Hangzhou, China). EGCG, vardenafil, assay kits, other antibodies and chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA).

Cell culture

H9C2 cardiomyocytes were cultured as previously described (Sun *et al.*, 2012; Xiao *et al.*, 2013). Dulbecco's

**Corresponding author:* e-mail: jinpeng@zafu.edu.cn

modified Eagle's medium (DMEM) supplemented with 4.5 g/L glucose, 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin. The cell monolayer cultures were performed in cell incubators with 95% air and 5% CO_2 at 37°C.

Cell viability assay

The effects of EGCG or vardenafil on H9C2 cell viability were assessed by the MTT assay as previously described (Yang *et al*., 2017). Dose-response effects on H9C2 cell proliferation were measured by adding increasing concentrations of EGCG (25, 50, 100 and 200μmol/L), vardenafil (1, 2, 4 and 8μmol/L) and EGCG-vardenafil (100 and 2μmol/L), respectively. The time-response effects were also analyzed after 24, 48 and 72h, respectively.

In some experiments, the NO production in H9C2 cell culture medium was measured with fluorescent dye 4,5-diaminofluorescein diacetate as described previously (Kim *et al*., 2007).

H_2O_2 treatment

H9C2 cell were seeded at densities of 5×10^4 cells/ml after 24 h culture. H9C2 were treated with various final concentrations of 540, 560, 580 and 600μmol/L H_2O_2 for 24 h. After pretreatment, the optimal H_2O_2-induced concentration was determined according to the damage effect by MTT assay. Two experiment groups were set to assess the impact of drug pretreatment and posttreatment on H_2O_2-induced damage H9C2. In A group, precultured H9C2 were treated with an EGCG (100μmol/L), vardenafil (2μmol/L) and combination of EGCG (100 μmol/L)-vardenafil (2μmol/L) without H_2O_2, respectively. In B group, precultured H9C2 were treated with an EGCG (100μmol/L), vardenafil (2μmol/L) and combination of EGCG (100μmol/L)-vardenafil (2μmol/L) with 560 μmol/L H_2O_2, respectively. The control was set as the same operation without drugs. After 24 h culture, B group were added EGCG (100μmol/L), vardenafil (2μmol/L) and combination of EGCG (100μmol/L)-vardenafil (2 μmol/L), respectively. The equal volume PBS buffer was added in the treatments of a group and control. Then the treatments of B group were further cultured 48 h and detected by MTT assay. Also, The A group cells were harvested at 48 after drug and H_2O_2 treatments and then detected by MTT assay.

Analytical procedures

Superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) activities, lipid peroxidation production Malonyldialdehyde (MDA) and caspases proteins were measured using a commercial detection kit. The expression levels of various signaling pathway proteins were investigated with immunoblotting. Protein bands were calculated by ImageJ software (National Institutes of Health, Bethesda, MD, USA). The activity of Caspase-3, -8 and -9 was measured fluorometrically at excitation 400 nm and emission at 505 nm.

STATISTICAL ANALYSIS

All the experiments were performed in triplicates and given as means ±SD (standard deviation). Significant differences among groups were determined using Dunnett's post-hoc test followed by One-way analysis of variance (SPSS software version 19.0). Values with $P< 0.05$ were considered significant.

RESULTS

Combination regimen of EGCG and vardenafil promoting H9C2 cell proliferation

To evaluate proliferation actions of EGCG and vardenafil, we treated H9C2 cells with EGCG concentrations of 25, 50, 100 and 200μmol/L, and vardenafil concentrations of 1, 2, 4 and 8μmol/L, respectively. As shown in fig. 1A, H9C2 showed a remarkable increase of the proliferation rate with EGCG stimulation ranging from 25 to 100 μmol/L. In particular, the highest proliferation value reached 18.74% at 48 h with EGCG of 100μmol/L.

This result showed that EGCG induced proliferation in H9C2 cells in a dose-dependent manner. However, EGCG of 200μmol/L induced proliferation of about 4.8% at 24 h and obviously lower than those of low-dose EGCG stimulation. The proliferation rate further decreased after 48 h, and even resulting in noticeably apoptosis at 72 h. Previous studies have demonstrated that high concentrations of EGCG could catalyze the production of hydrogen peroxide (H_2O_2), and promote pro-oxidant and potentially genotoxic events (Isbrucker *et al.*, 2006; Sugisawa and Umegaki, 2002) .

To further evaluate proliferation in H9C2 cells, vardenafil was tested at various concentrations and shown in fig. 1B. The low-dose vardenafil clearly promoted H9C2 cell growth at 24 h, and reached the highest proliferation rate of 10.77% at 48 h with 2μmol/L. Higher-dose vardenafil of 8μmol/L also reached proliferation rate of 6% at 48 h and 4% at 72 h, respectively. Our data indicated that vardenafil did not induce H9C2 cell damage or apoptosis at high-dose of 8μmol/L. However, Considering for no more benefit, excessive costs and adverse risks with higher dose of vardenafil (Venhuis and De, 2012), such a very low dose of vardenafil for combination regimen was administered for the following study.

Moreover, the proliferation effect of EGCG combination with vardenafil (EGCG-Var) in H9C2 cells was assessed. As shown in fig. 1C, this combination regimen significantly stimulated cell proliferation in H9C2 cells. Compared with EGCG group (100μmol/L) and vardenafil group (2μmol/L), 100μmol/L EGCG combined with 2 μmol/L vardenafil significantly potentiated the proliferation effect in H9C2 cells with 29.17% after 48 h treatment. Similarly, this combination regimen of EGCG and vardenafil was also reported to significantly induced cell death in CLL cells (Kumazoe *et al*., 2015).

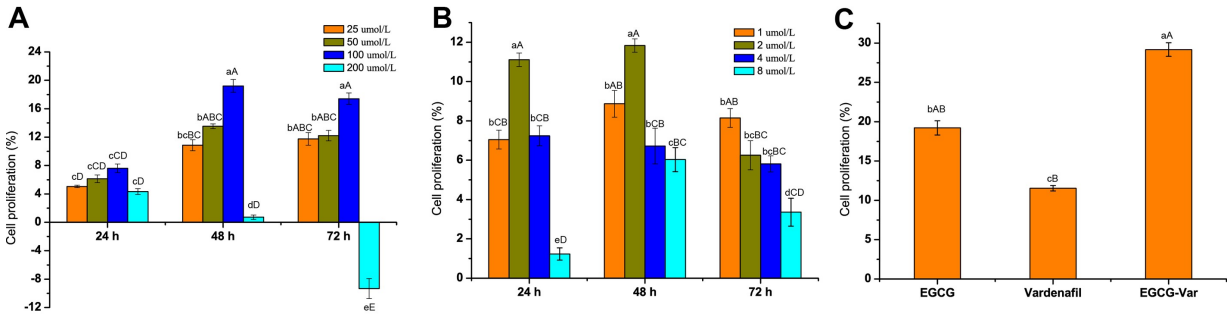

Fig. 1: Effects of EGCG (A), vardenafil (B), and combination of EGCG-vardenafil (C) on H9C2 cell proliferation. H9C2 were treated with various concentrations of EGCG (25, 50, 100 and 200 umol/L) or Vardenafil (1, 2, 4 and 8 umol/L), respectively. Data represent the means ± SD from three independent experiments. Significant differences within each group by different treatments are indicated by lowercase letters ($P< 0.05$) and capital letters ($P < 0.01$).

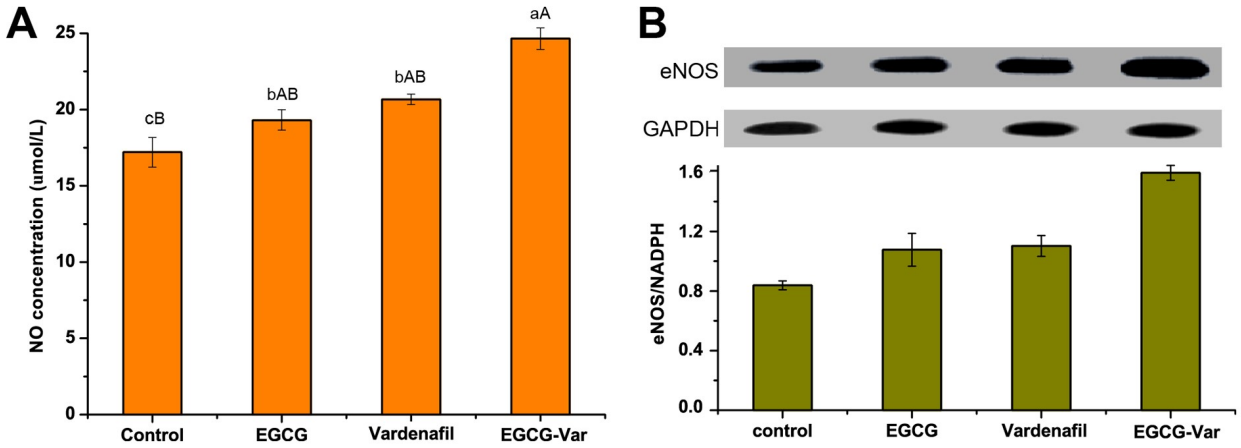

Fig. 2: Effects of EGCG or ardenafil on NO concentration (A) and eNOS protein level (B) of H9C2 cells. EGCG-Var: combination of EGCG and vardenafil. Data represent the means ± SD from three independent experiments. Significant differences within each group by different treatments are indicated by lowercase letters ($P< 0.05$) and capital letters ($P < 0.01$).

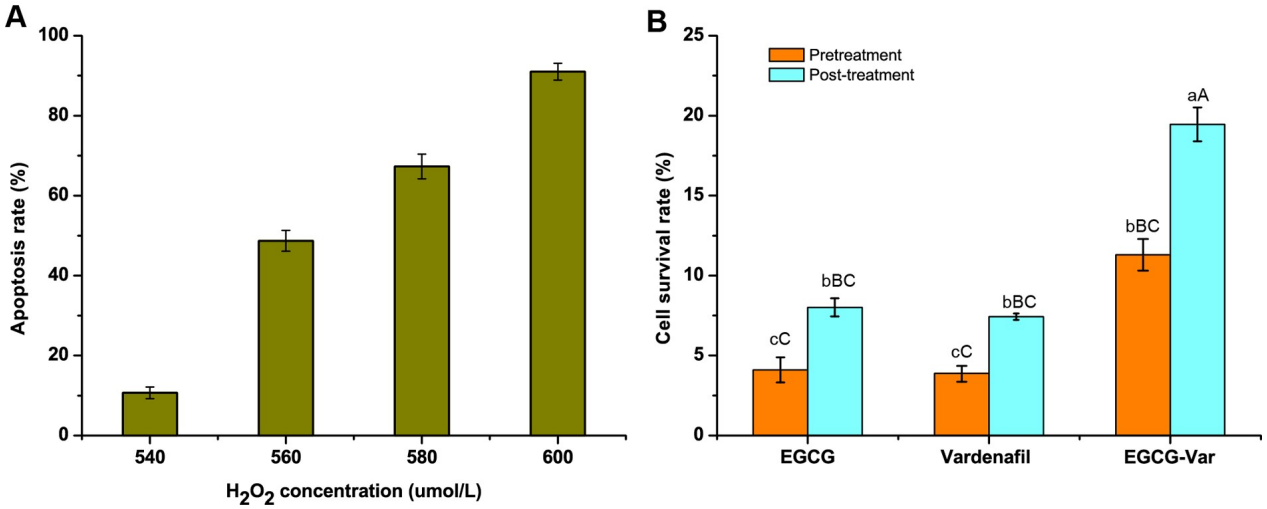

Fig. 3: Effects of EGCG, vardenafil and their combination on H_2O_2-induced damage of H9C2 cell. (A) effects of various H_2O_2 concentration on H9C2 apoptosis; (B) effects of pretreatment or post-treatment by drugs on the cell survival rate. Data represent the means ± SD from three independent experiments. Significant differences among different treatments are indicated by lowercase letters ($P< 0.05$) and capital letters ($P < 0.01$).

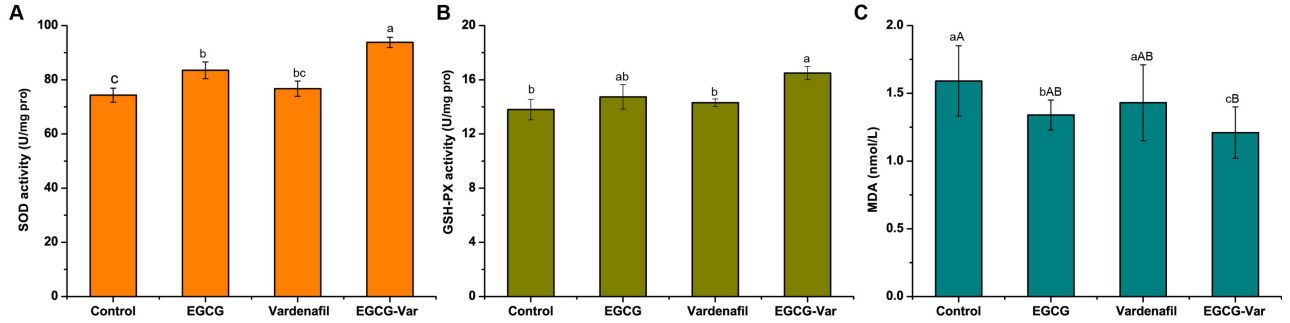

Fig. 4: Effects of EGCG and their combination on reductase/peroxidase system of H9C2 cells. (A) SOD activity, (B) GSH-PX activity, MDA level (C). Data represent the means ± SD from three independent experiments. Significant differences versus control are indicated by lowercase letters ($P< 0.05$) and capital letters ($P < 0.01$).

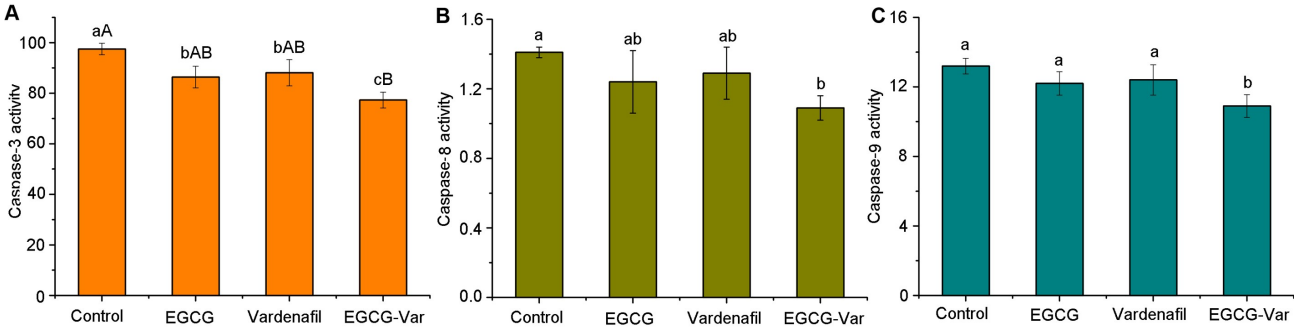

Fig. 5: Effects of EGCG and their combination on apoptosis proteins Caspase-3 (A), Caspase-8 (B) and Caspase-9 (C) activities of H9C2 cells. Data represent the means ± SD from three independent experiments. Significant differences versus control are indicated by lowercase letters ($P< 0.05$) and capital letters ($P < 0.01$).

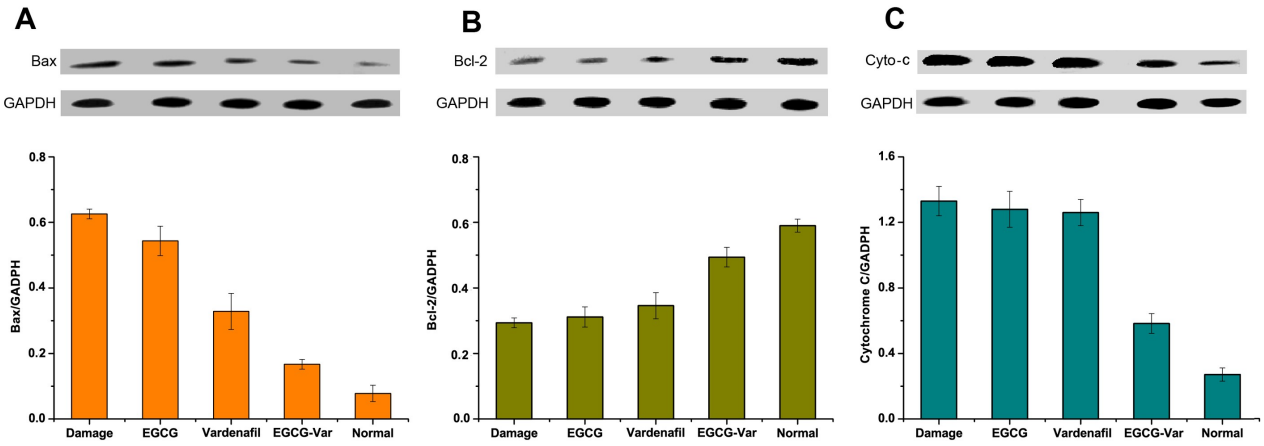

Fig. 6: Protein expression levels analysis of Bax (A), Bcl-2 (B) and cytochrome C (C) by Western blotting. Data represent the means ± SD from three independent experiments.

Effect of vardenafil on EGCG-Stimulated production of NO from H9C2 cell

Endogenous vasomotor factor NO plays an important role in the suppression of cell proliferation and platelet aggregation, and the regulation of vascular tone(Higashi *et al.*, 2009). To evaluate the relevance of vardenafil on the NO-dependent vasodilator actions of EGCG, we inquired whether the combination treatment of EGCG and vardenafil can directly enhance the production of NO from H9C2 cells in primary culture. As shown in fig. 2A, compared with the control, EGCG, and vardenafil groups stimulated the production of NO in H9C2 cell from 17.33 μmol/L to 19.75μmol/L and 20.87μmol/L, respectively. Moreover, Treatment with EGCG and vardenafil in combination resulted in greater production of NO, with a value of 24.47μmol/L compared with that of vardenafil or EGCG alone. Previous reporter has showed the eNOS in cardiovascular homeostasis is regulated by changes in

eNOS gene expression levels (Lorenz *et al.*, 2004). To investigate whether EGCG and vardenafil in combination can influence eNOS protein expression, we further analyzed the expression levels of eNOS in H9C2 cells after treatments by west-blot. The eNOS protein was slightly increased with vardenafil or EGCG alone treatment compared with control. As expected, EGCG and vardenafil in combination in H9C2 cell resulted in the significantly increase of eNOS expression (fig. 2B). This result was consistent with the NO levels (fig. 2A).

Suppresses H_2O_2-induced apoptotic damage in H9C2 cardiomyocytes

Previous studies had demonstrated that excessive reactive oxygen species (ROS) play an important role in the development and pathogenesis of cardiovascular diseases (Dhalla *et al.*, 2000), is also a major cause of myocardial apoptosis (Xiao *et al*., 2013). *In vitro* H_2O_2-induced H9C2 cells apoptosis model are widely used to study the mechanism of cardiomyocyte apoptosis (Von *et al.*, 1999). As shown in fig. 3A, treatment of H9C2 cells with H_2O_2 in various concentrations (540, 560, 580 and 600μmol/L) led to decreased cell viability in a dose-dependent manner, with the apoptosis rates of 11.11%, 47.16%, 67.26% and 89.70% respectively. Therefore, H_2O_2 concentration of 560μmol/L was selected as the lethal dose-50 dose for mimicking H9C2 cell apoptosis.

Furthermore, we investigated the effects of EGCG and vardenafil on H_2O_2-induced cardiomyocyte apoptosis. As shown in fig. 3B, we first examined the cell viability of pretreated H9C2 cardiomyocytes with H_2O_2 in A group experiment. After treatment with EGCG, vardenafil, and EGCG and vardenafil in combination increase the cell viability at roughly 4.42%, 3.65% and 10.78%, respectively (fig. 3B). Subsequently, we further investigated the effect of pretreatment with EGCG and vardenafil on the viability of H9C2 in B group H_2O_2-induced apoptosis experiment. Exhilaratingly, EGCG, vardenafil, and EGCG and vardenafil in combination treatments significantly improved the cell viability at roughly 7.98%, 7.22% and 18.76%, respectively. These results demonstrated the combination regimen of EGCG and vardenafil were more favorably suppressed H_2O_2-induced cell death in H9C2 cells than EGCG or vardenafil alone. Especially the pretreatment with drugs led to the higher viability from 10.78% to 18.76% compared with the pretreatment with H_2O_2.

The anti-apoptotic effects of EGCG and vardenafil specifically related to antioxidant defense systems

Antioxidant defense systems rapidly scavenge ROS to inhibit NO degradation in the vasculature, for example, super oxide dismutase (SOD) dismutates O_2^- to H_2O_2, and then following eliminated to water by glutathione peroxidase (GSH-PX) and catalase. (Higashi *et al*., 2009). Therefore, the effects of EGCG and vardenafil on the antioxidant capacity of H9C2 cells were further investigated. As shown in fig. 4A, after pretreatment of EGCG, vardenafil and EGCG and vardenafil in combination, the SOD enzyme activity was improved from 74.58U/mg of control group to 83.87, 76.62 and 93.78U/mg, respectively. The vardenafil-treatment group shown almost no improvement in SOD activity compared with control. In contrary, EGCG and vardenafil in combination led to a significant improvement of SOD activity by 25.7%. Consistent with previous reports, suggests that vardenafil can effectively improve EGCG antioxidant capacity and SOD activity *in vivo*.

Analysis of another main antioxidant defense enzyme GSH-PX, shown the activity had a slight increase in H_2O_2-damaged H9C2 with EGCG or vardenafil alone. As expected, GSH-PX activity increased by 15% with EGCG-vardenafil in combination treatment compared with control (fig. 4B). Moreover, we further detected the effects of EGCG and vardenafil on the contents of lipid peroxidation production Malonyldialdehyde (MDA) in damaged H9C2 cell. As shown in fig 5C, all drug-treated groups could reduce the production of MDA, whereas EGCG and EGCG-vardenafil in combination similarly reduced the MDA levels from control of 1.6 to 1.3 and 1.21nmol/mL, respectively. This indicator indicates oxidative damage of H9C2 by H_2O_2 was relieved after EGCG and vardenafil treatments. Taken together, these results suggest that EGCG-vardenafil in combination could remarkably activate the SOD and GSH-PX of antioxidant defense system, and decrease the contents of MDA, scavenge oxidative free radical to protect the anti-oxidative damage of H9C2 cardiomyocytes.

Insight into effects of EGCG or vardenafil on antiapoptotic and proapoptotic proteins

To identify the effects of EGCG or vardenafil-stimulated apoptosis on signal transduction pathways, we examined the activation of initiator Caspase-8, -9 and effector Caspase-3 in H_2O_2-treated H9C2 cells with or without pretreatment of drugs. As shown in fig. 5, we found that EGCG or vardenafil pretreatment could attenuate H_2O_2-induced activation of Caspase-9 (fig. 5A) and Caspase-8 (fig. 5B). Caspase-8 is a key inducer of the death receptor-dependent apoptotic pathway and Caspase-9 is critical for mitochondria-dependent apoptosis. When activated, both can lead to subsequently triggering the activation of Caspase-3 and then the execution phase of apoptosis. Consequently, our assays indicate that the activation level of Caspase-3 was also down-regulated due to the activation inhibition of upstream critical inducers in two apoptotic pathways (fig. 5C). As an exception, EGCG-vardenafil in combination exhibited more obvious effect on inhibiting the Caspase-8, -9 and -3 cleavage than those of EGCG or vardenafil alone. Our results further confirmed that EGCG and vardenafil may act on suppressing H_2O_2-induced apoptosis specifically through the intrinsic and extrinsic apoptotic pathway.

To further reveal the apoptosis mechanism, classic regulatory proteins, such as apoptosis suppressor Bcl2 (Kluck *et al.*, 1997), proapoptotic protein Bax (Salomons *et al.*, 1997) and cytochrome c, involved in the antiapoptotic and proapoptotic effectors (Chipuk *et al.*, 2010) were evaluated by Western blotting. As shown in fig. 6, treatment with EGCG or vardenafil in H9C2 cells down-regulated the Bax protein expression (fig. 6A), whereas increased the Bcl2 expression level (fig. 6B). EGCG treatment resulted in decreasing Bax: Bcl2 ratio by 22.6%, and vardenafil treatment resulted in decreasing Bax: Bcl2 ratio by 55.3% compared with the H2O2-induced apoptosis group. In particular, the combination treatment of EGCG and vardenafil remarkably decreased the Bax: Bcl2 ratio from 2.17 to 0.36 with 78.8% down-regulation, which was very close to the ratio (0.31) of normal cell group (fig. 6A, 6B). These results indicated that the decreased ratio of Bax:Bcl2 proteins may be responsible for the anti-apoptosis (Zhao *et al.*, 2001) and cell rejuvenation in H9C2 cardiomyocytes. Furthermore, to determine the mitochondrial downstream apoptosis events, cytochrome c release in the cytosol which triggers caspase cascade activation was investigated. Western blotting results showed that cytochrome c was detected in the cytosolic fractions with EGCG or vardenafil treatment (fig. 6C). Cytosolic cytochrome c (Cyt-c) decreased by 6.7% for EGCG, 5.9% for vardenafil, and 58.2% for EGCG-vardenafil treatments compared with apoptosis group, respectively.

DISCUSSION

This study demonstrates that EGCG effectively stimulates the proliferation and repairs the oxidative damage of H9C2 cardiomyocytes with low-dose vardenafil (fig. 1 and fig. 3). Furthermore, the phosphodiesterase type 5 inhibitor vardenafil synergizes with EGCG to suppress H_2O_2-induced apoptotic damage in H9C2, which was able to significantly reduce the occurrence of worsening events. These combination effects in cardiomyocytes may contribute to protect from cardiovascular diseases and complications.

In this work, we showed that vardenafil significantly enhanced the synergistic effect of EGCG on cell viability of H9C2 myocardial cell. Some evidences such as promoting the proliferation, NO-dependent vasodilator actions and protecting H9C2 myocardium from H_2O_2-induced damage were observed. EGCG has endothelial-dependent vasodilator actions (Kim *et al*., 2007), which as a natural activator of endothelial cell eNOS, acutely increases production of NO to ameliorate endothelial function by PI 3-kinase/Akt-dependent pathway (Lorenz *et al*., 2004). EGCG, also increases fatty acid oxidation by mimicking insulin actions (Murase *et al*., 2005). Vardenafil has induced pulmonary vasodilatation (Karasu-Minareci *et al*., 2012) and its signaling pathways mediating metabolic actions had also been demonstrated. Our results clearly demonstrated that vardenafil could enhance the EGCG-stimulated expression of eNOS, and then increase approximately 25% of NO production in H9C2 cells (fig. 2). It may indicate EGCG-mediating vasodilator signaling pathways are shared with that of vardenafil (Karasu-Minareci *et al*., 2012).

However, the actions of EGCG and vardenafil on H9C2 cells are very different from that on the tumor cells (Yang and Wang, 2013). Significant synergistic action between EGCG and vardenafil (Yang and Wang, 2013) was found in inducing apoptosis in multiple myeloma cell lines (U2GG, ARH-77, and RPMl 822G) and chronic lvmphocvtic leukaemia cells, and in inhibiting the tumor in mice from MDA-MB-231-RFP cells since the 67-kDa laminin receptor (67LR) overexpress in various types of cancer (Kumazoe *et al.*, 2013; Kumazoe *et al*., 2015). In this work, EGCG signaling pathways activates the antioxidant defense system, such as SOD, GPx and catalase, scavenges ROS in H9C2 cells (fig. 4), resulting in inhibition of NO degradation (Higashi *et al*., 2009). Vardenafil further enhances the amplification of NO-mediated vasodilation by inhibiting the cGMP degradation (Mazo *et al*., 2006). In the case of combination regimen of EGCG and vardenafil, the protective mechanisms of H_2O_2-induced apoptotic damage H9C2 cells may be attribute to alleviate the oxidative damage of cell membrane by reducing ROS (fig. 4). Overall, the homeostasis of oxidative stress in cardiovascular cells was possibly realized via activating the antioxidant defense systems and suppressing apoptosis. Furthermore, the anti-apoptosis mechanism was also involved in the mitochondria-dependent intrinsic apoptotic pathway (Circu and Aw, 2010) by regulating the apoptosis signaling (Danial and Korsmeyer, 2004), including the down-regulation of Bax:Bcl2 ratio, cytochrome c release from mitochondria and then significantly reduced the activation of subsequent downstream caspases (fig. 6). Nevertheless, the interacting molecular mechanisms of combination regimen remain unclear and need to further explore.

CONCLUSION

The important bioactive component (-)-Epigallocatechin gallate (EGCG) from green tea improved the bioactivity of EGCG on H9C2 cardiomyocytes by combination regimen of vardenafil and EGCG. These results suggested that combining EGCG and low dose vardenafil in functional foods provides a more promising strategy to prevent cardiovascular disease.

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