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Effect of geraniol on rat cardiomyocytes and its potential use as a cardioprotective natural compound

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ABSTRACT

Aims: Reactive oxygen species (ROS) are generated in the ischaemic myocardium especially during early reperfusion and affect myocardial function and viability. Monoterpenes have been proposed to play beneficial roles in diverse physiological systems; however, the mechanisms of action remain largely unknown. This study aims to assess the effect of monoterpene geraniol (GOH) on neonatal rat ventricular cardiomyocytes (NRVCs) subjected to oxidative stress.
Main methods: We used an in vitro model of hypoxia-reoxygenation. Cardioprotective (AMPK) and cardiotoxic (ERK1/2, ROS) signaling indicators were measured. Assays were performed by fluorogenic probes, MTT assays and Western-blots.
Key findings: We determined that the addition of GOH (5–200 μM) to cultured normoxic and hypoxic-NRVCs diminished the endogenous production of ROS in stressed cardiomyocytes. We observed that GOH treatment increased pAMPK levels and decreased pERK1/2 levels in cultured NRVCs.
Significance: This report suggests that GOH is a candidate cardioprotective natural compound that operates by blunting the oxidative stress signaling that is normally induced by hypoxia-reoxygenation.

1. Introduction

Acute myocardial infarction with subsequent left ventricular dysfunction and heart failure continues to be a major cause of morbidity and mortality in the Western world. Improvements in the clinical response to acute coronary events by reopening (reperfusion) of the blocked coronary circulation have dramatically reduced mortality, although many patients undergo some type of reperfusion injury that is characterized by oxidant production with increased reactive oxidant species (ROS) [1]. ROS induce multiple changes in cell structure and function that are associated with metabolic and signaling alterations linking cellular hypertrophy, cardiac remodeling, and myocardial cell death [2,3]. Pathways that were shown to be regulated by ROS involve p53 [4], Akt, mTOR [5], and mitogen-activated protein kinases (p38 kinase, JNK and ERK1/2) [6]. Nonetheless, some members of these complex signaling networks may either protect or injure the heart depending on the cellular context [2]. For example, a dual role of ERK1/2 activation comprises the anti-apoptotic pro-survival reperfusion injury salvage kinase (RISK) pathway [7] as well as the cardiotoxicity-linked hypertrophic events [2,8]. A parallel set of data indicates the existence of other signaling cascades activated by stress that serve a cardio-protective role. Among them, AMPK is an emerging central sensor of cellular energy and nutrient availability [9] and redox status [10]. Activation of the AMPK pathway reduces ROS levels [11].

Monoterpenes are phytochemicals that can be found in nearly all essential oils and are the most representative constituents. Monoterpenes have various pharmacological properties including antifungal, antibacterial, anticancer and also antioxidant [12]. The signaling pathways downstream of the beneficial effects of monoterpenes remain largely unknown. Geraniol (GOH) (3, 7-dimethyl-2, 6-octadien-1-ol) is an acyclic monoterpene mainly found in essential oil of palmarosa, ginger, lemon, lavender, orange and rose [13], and has been assessed by the Joint FAO/WHO Expert Committee on Food Additives as safe at current levels of intake [14]. Although there are numerous reports on the antitumoral, antioxidant and even anti-inflammatory properties of GOH [15–17], limited information exist on its effect (beneficial or not) on cardiac cells [18].

This study evaluates the effect of GOH in a cardiomyocytes culture that simulates the conditions of ischemia/reperfusion and evaluates the effect of GOH treatment by measuring AMPK, ERK1/2 and ROS signaling indicators. We conclude that GOH is a cardioprotective agent that blunts the production of ROS in cardiac cells.

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2. Materials and methods

2.1. Isolation of rat cardiac myocytes

NRVCs (Neonatal rat ventricular cardiomyocytes) were isolated using the neonatal rat cardiomyocyte isolation kit (Worthington) and cultured at 37 °C under 5% CO_2. In brief, ventricles were dissected from 1 to 2-day-old Sprague–Dawley rats, then digested overnight at 4 °C with trypsin. Digestion continued the following morning with collagenase for approximately 90 min at 37 °C. Cells were pooled, pre-plated for 90 min on an uncoated dish to remove fibroblasts, and seeded at 2×10^3 cells/well, in high-serum media (DMEM/F12 (1:1), 0.2% BSA, 0.1 mM ascorbic acid, 4 mg/L transferring, P/S 1%, supplemented with 10% horse serum and 5% FCS) on 384 well plates (Greiner 781,091) precoated with collagen I.

2.2. Cell culture and treatment

After 24 h, media was changed from the aforementioned high-serum medium to low-serum medium (DMEM/F12 (1:1), P/S 1%, 0.25% FCS) and maintained at 37 °C with 5% CO_2. The experimental design was as follows: 24 h incubation with 0–200 μM GH, followed by 3 h 150 μM H_2O_2 (±G) (Fig. 1A); or, 24 hs incubation with 0–200 μM GOH followed by 1 h hypoxia (3% O_2 using a Modular Incubator Chamber) and 30 min reoxygenation (room O_2 level, approx. 21% hereon called normoxia) (Fig. 1B). Control cells were incubated in normoxia for 1 h 30 min. GOH (98%) (Sigma, St. Louis, Mo.) stock was dissolved in DMSO (Analyticals Carlo Erba, Milan, Italy). Final concentration of the vehicle was 0.3% (v/v) in all GOH treated and parallel control cultures.

2.3. Measurement of ROS in live cells

Intracellular ROS measurement in live cells was measured at the beginning of reoxygenation using fluorescent probes. Cells were stained with 5 μM with CellROX® Green Reagents (Molecular Probes, Life technology, USA) and Hoechst 33342 by adding the probe to the culture medium and incubating at 37 °C for 30 min. The cells were then washed with PBS. The cell images were analyzed using a Kinetic imaging cytometry (KIC) instrument and software (Vala Sciences) to determine ROS levels and number of cells.

2.4. Cell viability

MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to determine cell viability after treatments. $IC_{50}$is the concentration of the tested compound required to produce 50% reduction in cell viability after an exposure time of 24 h. For IC_{50}, NRVCs were exposed to increasing concentrations of GOH ranging from 5 μM to 4 mM for 24 h. After treatment, cells were incubated in 384-well microtest plates with 0.5 mg/mL of MTT (Sigma Chemical Co.) in DMEM without phenol red, P/S 1%, 0.25% FCS at 37 °C for 3 h. The MTT was then removed leaving the formazan crystals at the bottom of the well. These crystals were dissolved in 50 μL of dimethyl sulfoxide (DMSO). The absorbance of formazan was measured at 560 nm with background subtraction at 640 nm. Cell viability was calculated as the percentage of control OD. Experiments were designed to use low GOH concentrations away from the toxic values.

2.5. Western blotting

Cardiomyocytes were incubated in DMEM:F12 without serum for 6 h (serum starvation), then cells were treated with GOH for several times (5, 10, 15 and 60 min). Cells were washed in cold PBS, collected with enzyme-free dissociation buffer and lysed with ice-cold RIPA buffer supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific, USA). Lysates were run on 10% SDS-tris glycinegels (Invitrogen) and transferred to 45 mm PVDF membranes, which were blocked and stained in Odyssey buffer 0.1% Tween and then probed with anti-phospho-ERK1/2 (Cell Signaling 9101, 1:1000), anti-ERK1/2 (Cell Signaling 9107, 1:2,000) and anti-phospho-AMPKα (Thr172) (Cell Signaling 2532, 1:1,000). Infrared labeled secondary antibodies were used at 1:10,000 dilutions and detection was performed with an Odyssey system (LICOR).

2.6. Statistics

All data are presented as mean ± standard error (SE). Statistical analyses were performed by means of the Student's *t*-test. Differences in the data were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effects of geraniol on cardiomyocytes in normoxic conditions

NRVCs were exposed to increasing concentrations of GOH ranging from 5 μM to 4 mM for 24 h and viability was assessed by MTT uptake (Fig. 2). Cell viability decreased slightly at 500 μM GOH, and markedly at 2 mM. The calculated IC_{50} value from the graph depicted in Fig. 1 was 1300 μM. GOH concentrations from 5 to 200 μM do not show significant changes in cell viability or cell number, therefore were considered as a non-toxic range of GOH appropriate for further experiments. These low concentrations of GOH caused a slight decrease in basal intracellular ROS levels that did not reach statistical significance ($p = 0.07$) (data not shown).

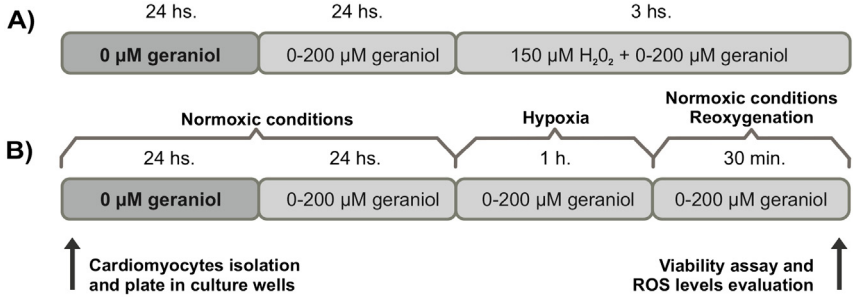

Fig. 1. Outline of experimental protocol. To evaluate the effect of GOH on neonatal rat ventricular cardiomyocyte's viability and redox state, we treated the cells with 150 μM H_2O_2 for 3 h (A) or with an in vitro protocol of simulated ischemia/reperfusion (B). GOH at 5, 50, and 200 μM, or its vehicle (0.3% DMSO) was present 24 h before treatment and during the peroxide or hypoxia-reoxygenation (H/R) treatment. H/R consisted in incubating the cells for 1 h at low levels of O_2 (3%) followed by 30 min incubation under normoxic conditions. Cardiomyocytes were incubated in high-serum media during plating (dark grey squares) or low-serum media for the rest of the experiment (light grew squares).

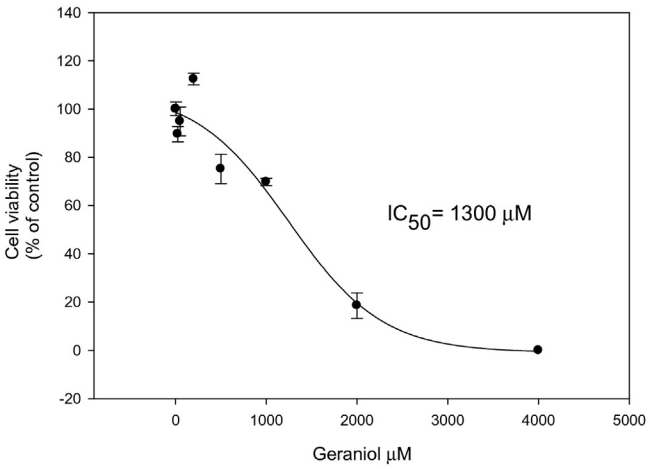

Fig. 2. Neonatal rat ventricular myocytes were incubated with a 5 µM–4 mM concentration range of geraniol for 24 h under normoxic conditions and cell viability was assessed by the MTT test. Cell viability is expressed as percent respect to vehicle (DMSO) control. Each point on the curve was calculated from the mean value ± SD of 8 replicate wells per dose performed in 3 separate experiments. IC50: concentration of geraniol required to produce 50% reduction in cell viability.

3.2. Cytoprotective effect of geraniol against H_2O_2-induced oxidative stress

To gain insights on the protective effect of non-cytotoxic concentrations of GOH on oxidative cell damage induced by H_2O_2, NRVCs were treated with increasing concentrations of GOH (5–200 µM) for 24 h previous to H_2O_2 exposure. H_2O_2 treatment was 150 µM for 3 h and was chosen because it caused a 50% decrease in cell viability by MTT assays ($p < 0.05$). H_2O_2 treatment also resulted in a significant accumulation of intracellular ROS ($p = 0.0041$) with respect to non-treated cells (Fig. 3). However, NRVCs treated with GOH before and during H_2O_2 exposure showed a dose-dependent decrease in the intracellular ROS levels and improved viability compared to H_2O_2-treated cells alone ($p < 0.05$). Therefore, GOH has cytoprotective effects in part by blunting ROS elevation.

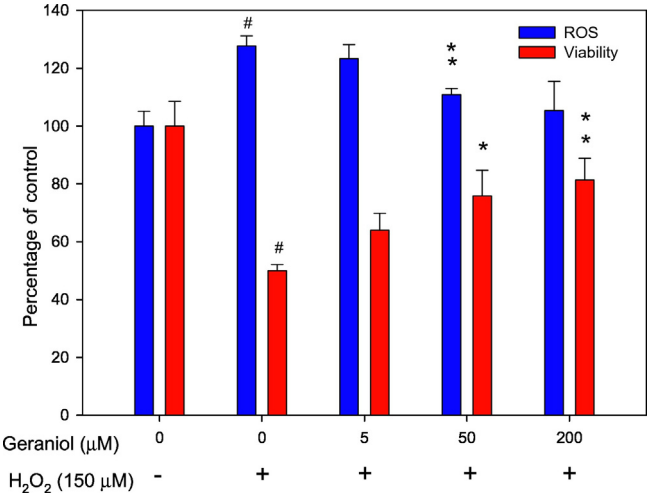

Fig. 3. Intracellular ROS levels and cytoprotective effects of geraniol in cardiomyocytes exposed to oxidative stress condition induced by H_2O_2. Neonatal rat cardiomyocytes were treated with geraniol (5, 50 and 200 µM) for 24 h before and during H_2O_2 treatment (150 µM, 3 h). Cellular viability was evaluated spectrophometrically by MTT test, whereas intracellular ROS levels were determined using Cell ROX Green reagent and the image analysis obtained in the KIC instrument. Data are expressed as the mean ± SE of quadruplicate measurements from three independent experiments. (#) $p < 0.01$ vs. non-treated cells (control); (*) $p < 0.05$, (**) $p < 0.01$ vs. H_2O_2-treated cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Effects of geraniol in ROS levels on cardiomyocytes subjected to hypoxia-reoxygenation (H/R)

Cardiomyocytes were placed into a hypoxic chamber for 1 h followed by 30 min reoxygenation in normoxia conditions in order to simulate in vitro an ischemia/reperfusion event. Our results show that after the H/R treatment, culture cells pre-incubated with all the concentrations of GOH evaluated, exhibited a significant reduction in ROS levels ($p < 0.05$) (Fig. 4A). However, cardiomyocytes incubated with GOH only during H/R (without pre-incubation period) showed a significant decrease only at 50 µM GOH (Fig. 4B). Cell viability did not have statistical changes with any GOH treatment (Fig. 4A–B).

3.4. Effect of geraniol in pAMPK and pERK levels

To assess the effect of GOH on the activation pattern of AMPK and ERK1/2, we used phosphorylation-specific antibodies that recognize the activated form of these kinases. To control for equal protein loading, blots were re-probed with an antibody detecting total ERK1/2 protein and/or tubulin. Our results showed that cardiomyocytes incubated with GOH increased pAMPK levels in the first 15 min with a maximum level after 10 min, and then they started to decrease and reached pre-treatment values at 60 min (Fig. 5A). On the other hand, pERK1/2 levels showed a constant decrease during the first hour of treatment that was statistical significant at 15 and 60 min after GOH was added to the medium (Fig. 5B–C).

4. Discussion

Myocardial infarction, stroke and peripheral vascular disease continue to be among the most frequent causes of debilitating disease and death, and share in common that the tissue or organ undergo episodes of ischemia/reperfusion [19]. Up to date there is no previous report on the direct effects of GOH (a secondary metabolite produced by many plants) on cardiac cells subjected to ischemia/reperfusion.

We focused on the possible antioxidant properties of GOH to counteract the toxic effects induced by the high levels of ROS generated after a simulated ischemia/reperfusion or by exposure to H_2O_2-oxidative stress. We demonstrated that treatment with low concentrations of GOH (5–200 µM) reduced intracellular ROS levels in rat cardiomyocytes subjected to both experimental conditions (H/R and H_2O_2). Cells also produced less intracellular ROS when they were pre-treated with the GOH monoterpene for 24 h than when it was added at the beginning of the experiment, thus indicating that GOH exerts a preconditioning or priming protecting role. Therefore, part of the protective role might be mediated from a direct radical-scavenging capacity of GOH [13,16,20] and part from preconditioning via the activation of the antioxidant system [17,21]. It has been shown that pre-treatment with GOH before application of oxidative stressors in different cells types (mouse skin, rat alveolar macrophages, rat liver) restored the activity of antioxidant enzymes such as catalase, glutathione reductase, glutathione peroxidase, glucose-6-phospahte dehydrogenese, and superoxide dismutase [17,21,22].

We report here that GOH activates AMPK in cardiomyocytes. AMPK works as a cellular energy sensor for cardiovascular protection. It stimulates ATP-generation and promotes cell survival pathways in cardiomyocytes subjected to oxidative stress conditions [23]. The mechanism for AMPK activation is still unknown. Previously, Kim et al. [24] showed that GOH activates AMPK in prostate tumor cells. A recent review [25] lists >100 plant-derived natural products that are AMPK activators, but the mechanisms of activation for most of them are also unknown. Some non-monoterpenes phyto compounds were shown to inhibit the synthesis of ATP in different cellular models [25]. Considering

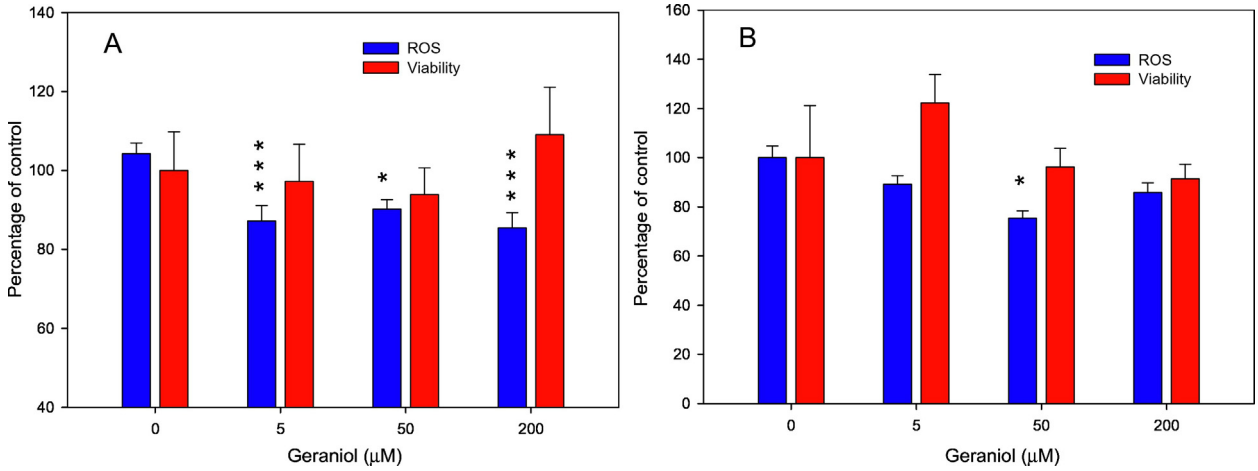

Fig. 4. Effect of geraniol on neonatal rat cardiomyocytes subjected to hypoxia conditions (1 h under 3% O_2) and reoxygenation (30 min under room O_2 level). (A) cells treated with 0–200 μM of geraniol in culture medium during 24 h pre-incubation and during hypoxia-reoxygenation. (B) cells treated with 0–200 μM of geraniol in culture medium only during the hypoxia-reoxygenation period. Cellular viability was evaluated spectrophotometrically by MTT test, whereas intracellular ROS levels were analyzed using fluorogenic probes and the image analysis obtained in the KIC instrument. Data are expressed as the mean ± SE of quadruplicate measurements from three independent experiments. (*) $p < 0.05$, (***) $p < 0.001$ vs. control. The average of absolute control values of the absorbance (570 nm–630 nm) for MTT assays was 0.09 and the mean signal intensity for ROS assays was 437.

that a decrease in the ATP/AMP ratio triggers the activation of AMPK, it is possible that GOH works in this manner. Here we evaluated the effect of GOH in cardiomyocytes incubated in non-oxidative stress conditions in order to avoid possible secondary activation by ROS produced during the H/R or by H_2O_2. We observed a short and transient (up to 15 min) induction of AMPK phosphorylation. This result suggests that GOH could intensify the expected AMPK activation in an oxidative stress situation, and could explain the increase viability of cardiomyocytes incubated with GOH and H_2O_2 compared to cells incubated only with H_2O_2 (Fig. 3). Although it is known that ROS levels are higher in mitochondria undergoing H/R and that the use of antioxidants during such event prevent cell death [1], we currently lack any insight on how monoterpenes could control mitochondrial ROS levels.

On the other hand, GOH significantly decreased pERK1/2 levels after 15 min of incubation time. The reason for this is currently unclear but goes along with observations of authors reporting that GOH significantly suppressed the Ras/Raf/ERK1/2 signaling pathway in tumor cells. Ras proteins require prenyl groups synthesized by the mevalonate pathway

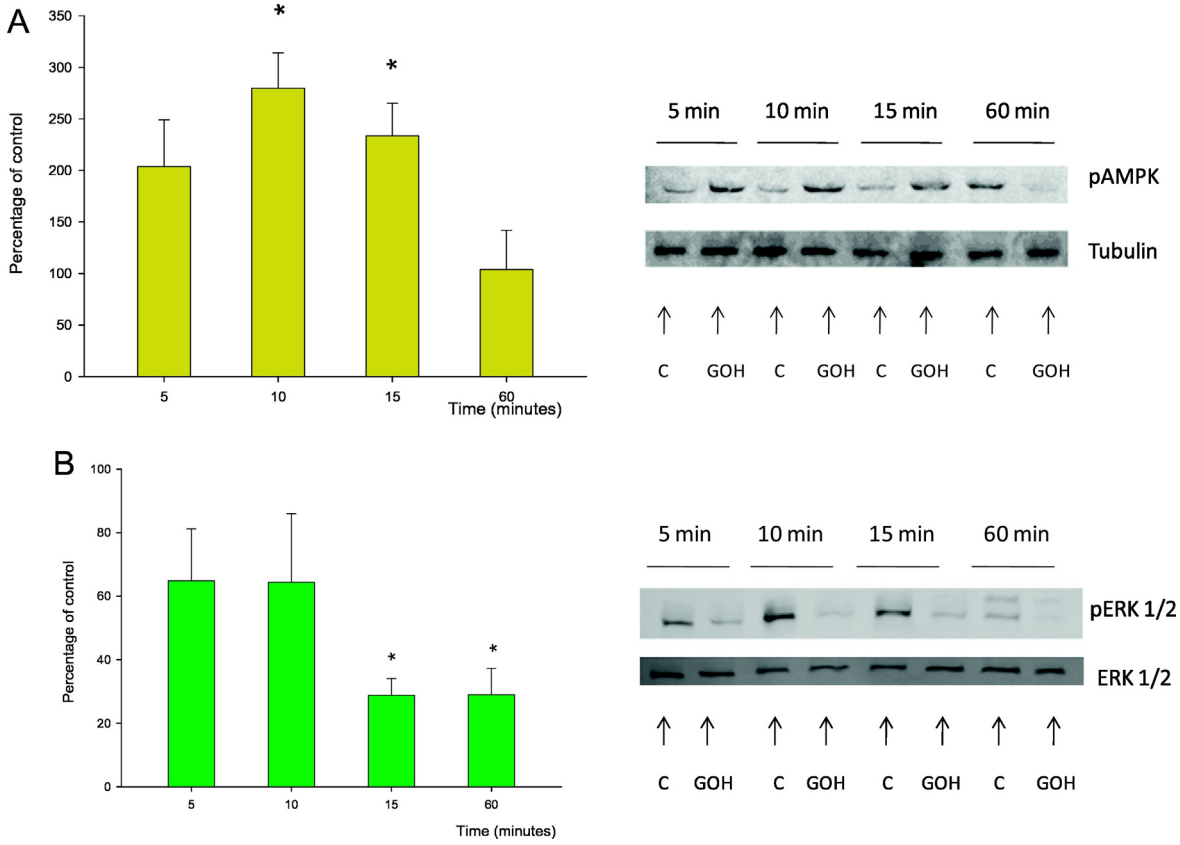

Fig. 5. Effect of geraniol on AMPK (A) and ERK (B) phosphorylation levels in ventricular cardiomyocytes from neonatal rats. Data are expressed as the mean ± SE of triplicate measurements from 3 independent experiments. Tubulin signals were used for normalization. (*) $p < 0.05$ vs. control. C: control, GOH: geraniol.

(MV) and prenyltransferase activity [26] in order to anchor to the cell membrane and trigger the cascade of MAPK/ERK pathway. GOH was shown to decrease membrane bound Ras levels [27,28] and to inhibit HMGCR, the main regulating enzyme of MV [28,29]. Still there are not reports on GOH's effect on prenyltransferase enzymes. Other mechanisms such as a reduction in intracellular calcium levels by GOH could also result in lower ERK activation. GOH has been shown to decrease cytosolic calcium levels in nervous tissues of diabetic rats [30] and to inhibit L-type calcium channels in mouse cardiomyocytes and guinea pigs heart tissue [18]. Cardiac function and viability are tightly regulated by calcium cellular levels. Both H/R processes and high concentrations of H_2O_2 induce changes in $Ca(2+)$ homeostasis in cardiomyocytes [31]. Calcium overload produces hypercontraction of cardiac muscle, opening of mitochondrial transition pore complex and activation of proteases, mechanisms that can result in cell death. Further research is necessary to deepen the study of GOH effect on the mechanisms of $Ca(2+)$ regulation and its potential use as calcium channel blocker.

5. Conclusions

The present work demonstrated that the monoterpene geraniol is a natural compound that decreases ROS levels in cardiomyocytes subjected to oxidative stress situations and modifies the phosphorylated state of two signaling proteins that are tightly implicated in the regulation of cell survival. Based on all exposed in this work, we propose that geraniol has a potential cardioprotective effect. Further understanding of its molecular mechanisms is essential in order to exploit its therapeutic efficacy.

Conflict of interests

The authors report no declarations of interest.

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