Therapeutic Role of Resveratrol and Quercetin on Aortic Fibroblasts of *Psammomys obesus* After Oxidative Stress by Hydrogen Peroxide

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In our study, we propose to analyze the effects of resveratrol (RES) and quercetin (QRC) on proliferation markers, oxidative stress, apoptosis, and inflammation of aortic fibroblasts of *Psammomys obesus* after induced oxidative stress by hydrogen peroxide (H2O2). Fibroblasts were incubated in RES 375 μM and QRC 0.083 μM for 24 hours after exposure to H2O2 1.2 mM for 6 hours. We performed the proliferation rate, cells viability, morphological analyses, cytochrome c, Akt, ERK1/2, and p38 MAPK quantification. The redox status was achieved by proportioning of malondialdehyde, nitric monoxide, advanced oxidation protein products, carbonyl proteins, catalase, and superoxide dismutase activity. The inflammation was measured by TNFα, MCP1, and NF-kB assay. The extracellular matrix (ECM) remodeling was performed by SDS-PAGE. Stressed fibroblasts showed a decrease of cell proliferation and viability, hypertrophy and oncosis, chromatin hypercondensation and increase of cytochrome c release characteristic of apoptosis, activation of ERK1/2 and Akt pathway, and decreases in p38 MAPK pathways marking the cellular resistance. The redox state was disrupted by increased malondialdehyde, nitric monoxide, advanced oxidation protein products, carbonyl protein production, catalase and superoxide dismutase activity, and a decreased production of proteins including collagens. Inflammation state was marked by MCP-1, TNFα, and NF-kB increase. Treatment of fibroblasts stressed by RES and QRC inverted the oxidative stress situation decreasing apoptosis and inflammation, and improving the altered redox status and rearrangement of disorders observed in extracellular matrix. H2O2 induced biochemical and morphological alterations leading to apoptosis. An improved general condition is observed after treatment with RES and QRC; this explains the antioxidant and antiapoptotic effects of polyphenols.

Keywords: oxidative stress, apoptosis, inflammation, polyphenols, aortic fibroblasts, therapeutic

INTRODUCTION

Under normal conditions, the delicate balance between oxidants and antioxidants is maintained. This balance is disrupted by excessive production of free radicals leading to oxidative stress inducing a damage that has been linked to the pathophysiology of a number of illnesses such as...
cancer, cardiovascular disease, atherosclerosis, diabetes mellitus, and inflammatory diseases.\(^1\) Flavonoids such as quercetin (QRC) (3, 5, 7, 3', 4'-pentahydroxyflavone) and resveratrol (RES) (3, 4', 5-trihydroxystilbene) have an anti-inflammatory, anticarcinogenic, and antioxidant role, and are generally abundantly available in our food diets. Research has shown their role in the prevention of cardiovascular disease and cancer.\(^2\) Reactive Oxygen Speacies (ROS) are known to activate ERK1/2, p38 kinase, JNK, and Akt in many cell types including cardiac cells.\(^3,4\) In recent years, great attention has been paid to natural dietary antioxidants notably polyphenols.\(^5\) Flavonoids are polyphenols derivatives of plant origin that have a broad range of pharmacological properties. Many studies have found both proapoptotic and antiapoptotic effects in many of these compounds. In this context, we investigated whether RES and QRC had antioxidant and antiapoptotic properties. As part of our work, we proposed to study some properties of QRC and RES in cultured adventitial fibroblasts of Psammomys obesus submitted to an oxidative stress by hydrogen peroxide (H\(_2\)O\(_2\)). In this study, we treated fibroblasts with RES 375 µM and with QRC 0.083 µM for 24 hours after induced-oxidative stress by 1.2 mM H\(_2\)O\(_2\) for 6 hours. In this framework, morphological changes, the rate of cellular proliferation, cell viability, cytochrome c released, p38 MAPK, Akt, and ERK1/2 were evaluated. The assay of some markers of oxidative–antioxidant balance was determined, and in particular, one parameter of lipid peroxidation, malondialdehyde (MDA), nitric monoxide (NO), advanced oxidation protein products (AOPP), protein carbonyl, catalase (CAT), and superoxide dismutase (SOD) enzymatic activity contained in the intracellular compartments. Estimation of inflammation was assessed by quantification of TNF-\(\alpha\), MCP1, and NF-kB, and the remodeling of the extracellular matrix (ECM) was estimated based on the identification of type I and III collagen chains by SDS-PAGE.

**MATERIALS AND METHODS**

**Aortic fibroblasts of P. obesus**

Our study was performed on fibroblasts from aortic seeds P. obesus cultured using the technique of explants.\(^6\)

**Quercetin**

QRC (Sigma) was added to the culture medium with an amount of 0.083 µM for 24 hours.

**Resveratrol**

RES (RESVERATROL 40 mg, box/60 Tablets; Biovea, France) was added to the culture medium at 375 µM for 24 hours.

**Methods**

**Antioxidant activity of QRC and RES by DiPhenylPicrylHydrazyl**

The antioxidant power of QRC and RES was tested using the DPPH method (1,1 diphenylpicrylhydrazyl 2 (DPPH); C18H12N5O6); It corresponds to the effective concentration, which reduces the initial concentration of DPPH by 50%, and also antiradical power (ARP equal to 1/EC50).\(^7\)

**Culture of adventitial fibroblasts**

Adventitial fibroblasts of P. obesus were put in culture using the explants technique.\(^6,8\) Explants were prepared after removing adventitia collageneaction at 0.1% (type IA, Sigma), incubated in DMEM (Gibco), and supplemented with 10% fetal calf serum (FCS) (Sigma), streptomycin (5 µg/mL), penicillin (50 IU/mL), and glutamine at 200 mM (Gibco). The explants were kept at 37°C under air:CO\(_2\) (95%:5%) atmosphere until they reached confluence. Then, they were trypsinized and subcultured. For the experiments, adventitial fibroblasts were used in the sixth passage. After trypsinization (0.1% of trypsin; Gibco), they were sown in plaques of 6 wells at 10\(_5\) cells/well. At confluence, the cells were exposed to H\(_2\)O\(_2\) 1.2 mM for 6 hours. A plaque of control cells was submitted to the same conditions with no addition of H\(_2\)O\(_2\). A Medium containing H\(_2\)O\(_2\) was eliminated, and the cells were reincubated with RES 375 µM or QRC 0.083 µM for 24 hours.

**Viability and apoptosis study**

**Study of the proliferation and viability.** After exposure to H\(_2\)O\(_2\), RES, and QRC, cells were trypsinized and the rate of proliferation was performed on the cellular suspension by counting using the Malassez cells. The rate of viability cells was estimated by trypan blue staining and presented as the percentage of living cells.

**Morphological study.** After various treatments, fibroblasts were washed with a phosphate-buffered saline (1X), then fixed in aqueous bath and colored with either a May Grunwald-Giemsat (V/V, 1/1) or with acridine orange (100 µg/mL). The observation was made with an inverted microscope (Zeiss) for May Grunwald-Giemsata coloring and a fluorescence microscope (Zeiss, blue filter) for the coloring with orange acridine. The measurement of the fluorescence (green) was performed on photographs taken by the fluorescence microscope after staining by acridine orange by logiciel (Mesurim).
Evaluation of cytochrome c, Akt, p38 MAPK, and ERK1/2. The cytochrome c, Akt, p38, and ERK1/2 assays were performed in the intracellular compartment (ICC) of control fibroblasts, fibroblasts exposed to H$_2$O$_2$, and those treated with RES or QRC, by the Elisa Kit method (Invitrogen) following the manufacture.

Redox status study

Proportioning of MDA. The MDA was determined in the ICC of fibroblasts under all experimental condition studies (in both absence and presence of H$_2$O$_2$, and after exposure to H$_2$O$_2$ and RES or QRC) by reaction to thiobarbituric acid in the presence of trichloroacetic acid (TCA). This reaction leads to the formation of a complex readable at 532 nm.9

Proportioning of NO. The formation of NO was evaluated by determination of nitrite and nitrate concentrations, which constitute the oxidative products of NO. Therefore, to be quantified, the nitrates would have to be reduced into nitrates by cadmium beforehand. The concentration thus measured represents the sum of nitrates and nitrites. The nitrites contained in the deproteinized and regenerated ICC of fibroblasts submitted to the different experimental conditions were quantified after adding the Griess reagent [0.1% N-(1 naphthyl) ethylenediamine dihydrochloride, 1% sulphanilamide, and 5% phosphoric acid]. The optic density reading was performed at 543 nm.

Evaluation of total proteins and collagens. The total collagens, contained in the ECC, underwent 2 successive 24-hour dialyzes (Serva, 8/32), respectively, against running water and acetic acid at 0.5 M at 4°C, in the presence of pepsin (Merck, at 200 mg/mL). Total proteins contained in the ICC reacted to Bradford10 reagent and resulted in the formation of a colored complex readable at 595 nm.

Determination of AOPP. Quantification of AOPP contained in ICC of samples was performed according to the method of Witko-Sarsat et al.11 The assay was performed by mixing ICC and potassium iodide (KI) 1.16 M and acetic acid for 2 minutes. Absorbance was read at 340 nm. The results were expressed in nanomoles of AOPP/mL of chloramines-T equivalents/μg of total proteins/10⁶ cell.

Evaluation of protein carbonyl. The carbonylated proteins were performed in the ICC of samples according to the procedure described by Levine et al.12 ICC of samples was added to 2,4-dinitrophenylhydrazine 10 Mm prepared in Hcl 2.5 M, incubated at a dark room temperature for 1 hour, and swirled every 15 minutes. After precipitation of proteins by addition of TCA 20%, four rinsing of pellets were performed; one with TCA 10% and three with ethanol/ethyl acetate (v/v). Washings were achieved by mechanical disruption of the pellets in the washing solution using a small spatula, and the mixture was centrifuged for 5 minutes at 6000 rpm. The resulting precipitates were dissolved in the guanidine hydrochloride 6 M at 37°C for 15 minutes. The optic density was read at 370 nm. The results were expressed in nanomoles of carbonyl groups per mL/μg of total proteins/10⁶ cell using a molar extinction coefficient of 22,000 M⁻¹ cm⁻¹.

Evaluation of the enzymatic activity of CAT. The enzymatic activity of CAT was determined using the method of Clairborne.13 The principle of this method lies in the degradation of hydrogen peroxide (H$_2$O$_2$) in the presence of CAT at 25°C. The K-phosphate buffer (KH$_2$PO$_4$ 50 mM; pH 7) was added to freshly prepared H$_2$O$_2$ (0.33 mM) and to ICC of samples. The absorbance reading was performed at 240 nm after the first minute and second minute. The enzymatic activity of CAT was evaluated in IU·min⁻¹·μg⁻¹ of total proteins/10⁶ cells.

Evaluation of the enzymatic activity of SOD. Evaluation of SOD was performed in the ICC of samples using the procedure adopted by Beauchamp and Fridovich.14 Reagent A (50 mM of K-phosphate buffer (pH 7.8), methionine 13 mM, NBT 75 μM, EDTA 0.1 mM, and riboflavin 4 μM) was added to ICC of samples. The photoreaction of riboflavin and O$_2$ was performed by exposing the mixture of reagent A and samples to a light bulb (15 Watt) for 10 minutes. NBT was reduced to formazan by superoxide anions (transformed to H$_2$O$_2$ by SOD). The reduction of NBT to blue colored formazan by superoxide radicals was measured at 560 nm. The values were expressed in IU/10⁶ cells. One unit of SOD activity was fined as the amount of enzyme required to cause 50% inhibition of the NBT reduction.

TNFα, MCP1, and NF-kB evaluation

TNFα, MCP1, and NF-kB rates were performed in the ICC of controls fibroblasts, fibroblasts exposed to H$_2$O$_2$, and in those submitted to H$_2$O$_2$, RES, or QRC, using Elisa Kit method (Invitrogen) and according to the manufacturer’s directions.

ECM remodeling

Collagens were separated by electrophoresis in vertical polyacrylamide gel at 10% and in denaturing conditions to separate the α chains of collagen by breaking disulfide bonds.15

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STATISTICAL ANALYSIS

Our results correspond to the affected averages of the standard deviation, which are analyzed by the test of Student.

RESULTS

Antioxidant activity of RES and QRC

The antioxidant (antiradical) power of RES and QRC corresponds to the effective concentration that reduced the DPPH by 50%. EC50 of RES was evaluated at 375 μM, and EC50 of QRC was evaluated at 0.083 μM (Figure 1).

Viability and apoptosis study

Our results show that the proliferation rate increased, respectively, with 19% and 65% in fibroblasts exposed to H2O2 and treated with RES and QRC compared with fibroblasts incubated in H2O2. This rate decreased with 83% in cells stressed by H2O2 versus pilot cells. After trypan blue staining, our results show that the rate of cell viability was, respectively, 89% and 91% in fibroblasts submitted to H2O2 and treated by RES and QRC compared with 69% in fibroblasts exposed to H2O2 and 98% in controls fibroblasts (Figure 2).

The quantification of cytochrome c contained in the ICC of fibroblasts submitted to H2O2 showed significant increases compared with control cells (P ≤ 0.01). In contrast, fibroblasts submitted to H2O2 and treated with RES and QRC showed a, respectively, nonsignificant and little significant decrease in cytochrome c released versus fibroblasts incubated in the presence of H2O2 (P > 0.05; P ≤ 0.05), and this level remained little significantly increased versus nonstressed cells (P ≤ 0.05) (Figure 3).

Morphologic analysis

After adding H2O2 to fibroblasts, we noted an increase in cell size compared with the corresponding reference (fibroblasts controls), a marked compensatory hypertrophy, and a vacuolization of the cytoplasm sign of oncosis. After treatment with RES or QRC, we noted a decrease in the size of cells and reduced oncosis compared with those exposed to H2O2 (board I).

After coloring with acridine orange, the cells observed under a fluorescence microscope showed that, compared with the corresponding controls fibroblasts, which showed a low level of fluorescence, those subjected to H2O2 produced an important fluorescence in the perinuclear area, marking hypercondensation of chromatin and showing a budding of the plasma membrane. After exposure of fibroblasts to H2O2 and treatment with RES and QRC, we observed a slight improvement in the morphology of the cells with a decrease in the perinuclear fluorescence and budding of the plasma membrane (board II, Figure 3).

Redox status study

Quantification of MDA

In the ICC of fibroblasts incubated in H2O2, we noted highly significant increases of MDA levels compared with control fibroblasts (P < 0.0001). The treatment of Akt, ERK1/2, and MAPK 38 rate contained in fibroblasts incubated in H2O2 showed, respectively, both significant (P ≤ 0.01) increases and little significant (P ≤ 0.05) decreases compared with the corresponding controls. However, in fibroblasts exposed to H2O2 and treated with RES and QRC, we noted that AKT, ERK1/2, and MAPK 38 rates showed, respectively, little significant (P ≤ 0.05), significant reduction, and significant (P ≤ 0.01) increases compared with those incubated in H2O2 (Figures 4, 5, and 6).
cells incubated in H2O2 with RES showed a highly significant decrease in MDA levels versus those exposed to H2O2 alone ($P \leq 0.0001$), and it was little significantly increased compared with control fibroblasts ($P \# 0.05$). Fibroblasts treated with QRC after H2O2 incubation showed a highly significant decrease in MDA levels versus those incubated in H2O2 alone ($P \leq 0.0001$), and these levels remained very significantly increased compared with control fibroblasts ($P \leq 0.001$) (Table 1). These results indicate an antioxidant effect of these 2 molecules.

**NO rate**

The rate of total nitrites evaluated in the ICC of fibroblasts subjected to H2O2 indicates a highly significant increase with 197% compared with the corresponding reference of controls ($P < 0.0001$). The fibroblasts treated with RES and QRC after H2O2 show, respectively, a little significant (8%, $P \leq 0.05$) and a very significant decrease in the rate of NO (26%, $P < 0.001$) versus those stressed with H2O2, and it was highly significantly increased compared with control fibroblasts ($P < 0.0001$) (Table 1).

**CAT enzymatic activity**

The evaluation of the CAT enzymatic activity in the ICC of fibroblasts after exposure to H2O2 showed a very significant increase compared with control cells ($P < 0.001$). After treatment of fibroblasts with RES and QRC, we observed a significant decrease in CAT activity versus fibroblasts incubated in H2O2 ($P \leq 0.01$), and it was higher, however, compared with control fibroblasts (Table 1).

**SOD enzymatic activity**

The quantification of SOD enzymatic activity of fibroblasts exposed to H2O2 showed a little significant increase compared with control cells ($P \leq 0.05$). A little significant decrease of SOD activity was observed after treatment of fibroblasts with RES and QRC versus fibroblasts stressed by H2O2 alone ($P \# 0.05$), and it was higher, however, compared with control fibroblasts (Table 1).

**Total proteins and total collagens rate**

Total collagens contained in ECC were evaluated in the context of the analysis of ECM remodeling by SDS-PAGE. The quantity of total collagens after exposure to H2O2 showed a significant reduction compared with the control ($P \leq 0.01$). Treatment of fibroblasts exposed to H2O2 with RES and QRC showed a nonsignificant increase compared with fibroblasts incubated in H2O2, and this increase is of 2% and 5% ($P \geq 0.05$), respectively, and this amount remained significantly decreased compared with controls, and this reduction was of 30% and 28% respectively after treatment with RES and QRC ($P \leq 0.01$) (Table 2).

Total proteins were evaluated within the frame of their use in the evaluation of AOPP, of proteins carbonyl, and the enzymatic activity of CAT. The quantity of total proteins of ICC after 6 hours incubation in H2O2 showed very significant decreases compared with the control.
FIGURE 3. Rates of cytochrome c (ng·mL⁻¹·10⁶ cells⁻¹) and percentage of cellular fluorescence (%) in adventitious fibroblasts of *P. obesus* incubated in the presence of hydrogen peroxide to 1.2 mM for 6 hours and treated with RES 375 μM and QRC 0.083 μM for 24 hours. Values are mean of cytochrome c rate, which affected the standard deviation (*) and percentage of cellular fluorescence. Cells subjected to H₂O₂ versus control cells; cells subjected to H₂O₂ and treated, respectively, with RES and QRC versus control cells (†). Cells subjected to H₂O₂ and treated, respectively, with RES and QRC versus cells subjected to H₂O₂.

**Board I** - May Grunwald-Giemsa (MGG) staining of *P. obesus* aortic fibroblasts, incubated in the presence of hydrogen peroxide to 1.2 mM for 6 hours and treated with RES at 375 μM and QRC at 0.083 μM for 24 hours (G × 100). (A) Controls aortic fibroblasts. (B, C) Controls fibroblasts incubated in the presence of RES and QRC, respectively. (D) Aortic fibroblasts incubated in the presence of 1.2 mM H₂O₂ for 6 hours, showing cytoplasm vacuolization (red arrows), hypercondensation of chromatin (yellow arrows), formation of apoptotic bodies (blue arrows), and disruption of the plasma membrane (orange arrows). (E, F) Aortic fibroblasts exposed to 1.2 mM H₂O₂ for 6 hours and treated with RES and QRC, respectively, showing an improvement of cellular state, increased of nuclei in division (green arrows) and reappearance of cellular prolongements (blue arrows).
Fibroblasts treated with RES and QRC, and showed a little significant increase compared with fibroblasts incubated in H$_2$O$_2$; these increases are 16% and 19% (P < 0.05), respectively, and these amounts remain not significantly decreased compared with controls; this reduction was 8% and 5%, respectively, after treatment with RES and QRC (P ≤ 0.01) (Table 2).

**AOPP production**

The quantity of AOPP contained in fibroblasts incubated in H$_2$O$_2$ showed an increase of 593% versus controls, and this increase was considered highly significant (P < 0.0001). Proportioning of AOPP in fibroblasts exposed to H$_2$O$_2$ and treated with both RES and QRC showed a highly significant decrease compared with those exposed to H$_2$O$_2$, we recorded 26% and 53% (P < 0.0001), respectively, and this amount remains highly significant increased versus controls, we recorded 412% and 226%, respectively, (P < 0.001) (Table 2).

**Protein carbonyl quantity**

Proportioning of protein carbonyl in control fibroblasts after their exposure to RES and QRC showed very significant decreases versus fibroblasts incubated without polyphenols, we found 41% and 53% (P < 0.001), respectively. Evaluation of protein carbonyl contained in fibroblasts exposed to H$_2$O$_2$ showed an increase of 504% versus controls, and this increase was highly significant (P < 0.0001). Both evaluations of protein carbonyl in fibroblasts exposed to H$_2$O$_2$ and treated with RES and QRC showed a highly significant decrease compared with those exposed to H$_2$O$_2$, we noted 35% and 65% (P < 0.0001), respectively, and this amount remains highly significant increased compared with controls, we noted 292% and 110% (P < 0.0001), respectively (Table 2).

**TNFα, MCP1, and NF-κB rates**

Evaluation of the quantity of TNFα, NF-κB, and MCP1 contained in the ICC of fibroblasts exposed to H$_2$O$_2$ showed both significant (P ≤ 0.01) and very significant (P < 0.001) increase compared with the corresponding reference of controls. In fibroblasts treated with RES and QRC, TNFα, NF-κB, and MCP1 rates showed respective little significant (P ≤ 0.05) and both significant (P ≤ 0.01) decreases with RES treatment and, respectively, little significant (P ≤ 0.05), significant
(P ≤ 0.01), and very significant (P < 0.001) decreases with QRC treatment versus those incubated in H2O2 (Figures 7, 8, and 9).

**ECM remodeling**

Collagen contained in the ECC separated by SDS-PAGE-revealed bands was quantified by densitometry. In our study, we performed the typing of collagens I and III contained in the ECC of adventitial fibroblasts under different experimental conditions by extrapolation on a standard curve known molecular weight. Control cells showed 2 similar bands, which represent 2 molecular weights 120 kDa and 112 kDa corresponding, respectively, to the α1 chain of collagen I and III, and α2 chain of collagen I. Addition of QRC to control fibroblasts caused an increase in the condensation of the bands of collagens, sign of compensatory effect. After incubation in H2O2, these two bands were poorly expressed. Treatment of fibroblasts with RES and QRC after H2O2 induced the visualization of the same bands (α1 chains of collagens I and III, and α2 chain of collagen I), and the treatment of fibroblasts with QRC caused an increase in the condensation of the collagen bands compared with those treated with RES (Figure 10).
DISCUSSION

In this study, we analyzed the antioxidant and antiapoptotic effects of RES and QRC on aortic fibroblasts of *P. obesus* in cultures after their exposure to hydrogen peroxide.

At high concentrations, H$_2$O$_2$ induced cell damages and important injuries such as cytoplasm vacuolization, chromatin condensation, increases of cell size and cytochrome c release, fragmentation of nuclei, and membrane budding responsible for the loss of cellular viability by apoptotic pathway compared with the corresponding reference of controls. Treatment of stressed fibroblasts with polyphenols (RES and QRC) could reverse stress situation and produce improvement of physiological condition. Cao et al.$^{16}$ showed that the treatment of retinal pigmented epithelial cells, preincubated in H$_2$O$_2$, with QRC caused an increase of cell proliferation and cell viability. Martin and Andriantsihoinaina$^{17}$ showed that polyphenols, in general, are able to block apoptosis, and QRC blocks the release of cytochrome c. Our results join those of Zheng et al.$^{18}$ which showed that RES pretreatment could inhibit the ROS production in human lens epithelial cells exposed to H$_2$O$_2$. Our results join those of Dudley et al.$^{19}$ who noted that RES protects ischemic heart by inducing survival signs, by reduction of infarcted areas (cardiac hypertrophy and oncosis) and apoptotic

Table 1. MDA (malondialdehyde) and NO (nitric oxide) levels, CAT (catalase) and SOD (superoxide dismutase) enzymatic activity evaluation in the aortic fibroblasts of *Psammomys obesus* exposed to 1.2 mM H$_2$O$_2$ for 6 hours and treated with RES (resveratrol) 375 $\mu$M and QRC (quercetin) 0.083 $\mu$M for 24 hours.

<table>
<thead>
<tr>
<th></th>
<th>MDA (mumole/10$^6$ cell)</th>
<th>NO ($\mu$M/10$^6$ cell)</th>
<th>CAT (UI/min/ $\mu$g protein/10$^6$ cell)</th>
<th>SOD (UI/10$^6$ cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.018 ± 0.004</td>
<td>9.40 ± 0.05</td>
<td>0.03 ± 0.01</td>
<td>3.09 ± 0.76</td>
</tr>
<tr>
<td>C+RES</td>
<td>0.015 ± 0.002</td>
<td>8.29 ± 1.41</td>
<td>0.36 ± 0.23</td>
<td>2.54 ± 0.63</td>
</tr>
<tr>
<td>C+QRC</td>
<td>0.013 ± 0.004</td>
<td>4.49 ± 0.13</td>
<td>0.21 ± 0.18</td>
<td>2.85 ± 0.62</td>
</tr>
<tr>
<td>+H$_2$O$_2$</td>
<td>0.611 ± 0.043*</td>
<td>27.91 ± 1.08*</td>
<td>1.58 ± 0.99†</td>
<td>17.95 ± 4.44‡</td>
</tr>
<tr>
<td>+H$_2$O$_2$+RES</td>
<td>0.15 ± 0.0941‡</td>
<td>25.55 ± 2.76*‡</td>
<td>0.65 ± 0.70‡†</td>
<td>15.05 ± 3.72‡</td>
</tr>
<tr>
<td>+H$_2$O$_2$+QRC</td>
<td>0.057 ± 0.0121‡</td>
<td>20.43 ± 1.50*‡</td>
<td>0.32 ± 0.22‡**</td>
<td>13.95 ± 4.65‡</td>
</tr>
</tbody>
</table>

Values are averages of MDA, NO, CAT and SOD assigned standard deviation.
†Cells subjected to H$_2$O$_2$ versus control cells, cells subjected to H$_2$O$_2$ and treated with resveratrol (RES) and quercetin (QRC) respectively versus control cells.
‡Cells subjected to H$_2$O$_2$ and treated with resveratrol and quercetin respectively versus cells subjected to H$_2$O$_2$. C, control cells.

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Our results show that treatment of fibroblasts stressed with RES does not decrease the amount of cytochrome c released; this could be explained by the low antioxidant power of RES comparatively with QRC, which is a powerful antioxidant that causes the decrease of cytochrome c released after exposure of fibroblasts to H2O2 versus those exposed to H2O2. Polyphenols (green tea epigallocatechin-3-gallate) could protect H2O2-induced apoptosis in retinal pigment epithelial cells by decreasing cytochrome c release.\textsuperscript{20} QRC is widely used as an antioxidant known to inhibit oxidative stress by inhibiting subsequent series of biochemical changes and apoptotic cascade.\textsuperscript{21,22}

Table 2. Total proteins and total collagens, AOPP (advanced oxidation protein products) and protein carbonyl rates in cultured aortic fibroblasts of \textit{Psammomys obesus} in the presence of 1.2 mM H2O2 for 6 hours and treated with RES (resveratrol) 375 \(\mu M\) and QRC (quercetin) 0.083 \(\mu M\) for 24 hours. (C; control cells).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total proteins ((\mu g/10^6) cell)</th>
<th>Total collagens ((\mu g/10^6) cell)</th>
<th>AOPP (nmole/mL/(\mu g) proteins/(10^6) cell)</th>
<th>Proteins carbonyl (nmole/mL/(\mu g) proteins/(10^6) cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>447.9 (\pm) 18.5</td>
<td>495.5 (\pm) 39.1</td>
<td>0.57 (\pm) 0.12</td>
<td>19.73 (\pm) 0.90</td>
</tr>
<tr>
<td>C+RES</td>
<td>416.9 (\pm) 50.9</td>
<td>435.0 (\pm) 5.1</td>
<td>0.45 (\pm) 0.04</td>
<td>11.53 (\pm) 0.62</td>
</tr>
<tr>
<td>C+QRC</td>
<td>506.7 (\pm) 18.6</td>
<td>521.0 (\pm) 9.9</td>
<td>0.42 (\pm) 0.03</td>
<td>9.26 (\pm) 0.37</td>
</tr>
<tr>
<td>+H2O2</td>
<td>357.0 (\pm) 32.1*</td>
<td>410.1 (\pm) 20.3\†</td>
<td>3.95 (\pm) 0.09\‡</td>
<td>119.12 (\pm) 1.09\§</td>
</tr>
<tr>
<td>+H2O2+RES</td>
<td>414.1 (\pm) 54.1\§</td>
<td>418.0 (\pm) 38.0\¶†</td>
<td>2.85 (\pm) 0.08*#</td>
<td>77.37 (\pm) 1.57\‡**</td>
</tr>
<tr>
<td>+H2O2+QRC</td>
<td>423.7 (\pm) 31.8\¶††</td>
<td>429.3 (\pm) 24.8\¶†</td>
<td>1.90 (\pm) 0.14*#</td>
<td>41.42 (\pm) 0.35\‡**</td>
</tr>
</tbody>
</table>

Values are averages of total proteins, total collagens, AOPP and protein carbonyl rates assigned standard deviation.

\*Cells subjected to H2O2 and treated with resveratrol and quercetin respectively versus cells subjected to H2O2.

\#Cells subjected to H2O2 versus control cells, cells subjected to H2O2 and treated respectively with RES and QRC versus control cells.

In our study, we observed that H2O2 induced an important increase in ERK1/2 level, and this could be explained by the activation of both proapoptotic ERK pathway in cells remaining alive and antiapoptotic ERK pathway in more resistant cells after H2O2-induced oxidative stress because the response of cells is depended on their ability to adapt and to resist changes they may undergo. Our results may reflect differences in duration and amplitude of the oxidant stress compared with other studies, where higher concentrations and/or longer exposures were common conditions, which we observed as profoundly cytotoxic to aortic fibroblasts \textit{Psammomys} cells. Activation of ERK1/2 generally promotes cell survival; but under...
In certain conditions, ERK1/2 can have proapoptotic functions. In this study, we demonstrated that H2O2 activated PI3K-Akt pathway and decreased the p38 MAPK. Finally, we demonstrated that aortic fibroblasts treated with RES and QRC after H2O2 exposure induced decreases of Akt, explained by an alleviation of cells after external contribution of antioxidants (polyphenols), and we saw maintenance of activation of antiapoptotic Akt pathway and decreases of ERK1/2, which could be explained by decreases of proapoptotic ERK pathway when the antiapoptotic ERK pathway was retained. We also observed increases of p38 MAPK; this could be explained by the strengthened growth and the migration of resistant cells after the improvement of oxidative stress conditions.

Our results are in line with those of King et al, which suggested that RES significantly reduced intracellular ROS accumulation and decreased H2O2-induced ERK1/2 activation in retinal pigment epithelial cells. RES generated a survival signal by inducing the activation of p38MAP kinase β and Akt, and inhibition of p38MAP kinase α. Results of this study are consistent with those of Zheng et al, which demonstrated that after pretreatment of human lens epithelial cells, exposed to H2O2, with RES, the peak time of phosphorylation of p38 still occurred at...
1 hour, but the decrease was maintained for only 2 hours. The magnitude and duration of p38 MAPK signal transduction are key determinants of its biological effects. Activation of p38 MAPK occurs within minutes in response to most stimuli and is transient. This suggests that p38 MAPK functions as a biological switch that must be downregulated both under basal conditions and during adaptation.\(^{25-27}\)

The quantity of NF-kB, TNFα, and MCP1 contained in the ICC of fibroblasts after exposure to H\(_2\)O\(_2\) increased compared with control cells decreased after treatment with RES and QRC versus those exposed to H\(_2\)O\(_2\). Our results join those of Awai-Kasaoka et al,\(^{28}\) which showed that H\(_2\)O\(_2\)-induced NF-kB activation increased after a long exposure to H\(_2\)O\(_2\). NF-kB is an important nuclear transcription factor that mediates the inflammatory response caused by oxidative stress.\(^{29}\) RES might interact with the ankyrin domain of IKB (inhibitor of NF-kB) and prevent the activation of NF-kB and the inhibition of the inflammatory response.\(^{30}\) At low levels of cytochrome c release, superoxide signal may activate protective mechanisms, such as the activation of the redox-sensitive transcription factor NF-kB that controls the expression of several protective genes such as MnSOD.\(^{31,32}\) Our results regarding TNF-α production agreed with those of Bureau (2006),\(^{33}\) which showed that TNF-α increased after exposure of microglia cells to lipopolysaccharides (LPS) (source of oxidative stress) and decreased after pretreatment with RES and with QRC. According to (Zhang et al),\(^{34}\) after oxidative stress, RES restored endothelial function and caused vascular protective activity by inhibition of tumor necrosis factor α (TNFα). According to Wang et al,\(^{35}\) consumption of a high-fat diet was correlated with increased oxidative stress and chronic inflammation; they showed increases of MCP-1 rates. RES protected against high-fat diet-induced oxidative stress and
inflammation; they showed decrease in the rate of MCP-1.35

In the presence of H2O2, fibroblasts showed increases in MDA rate compared with reference. Our results suggested a decrease in MDA levels after treatment with RES and QRC. Our results are in line with those of Jamshizadeh and Rezaeian Mehrabadi,36 which reported that, after incubation of erythrocytes in H2O2 after H2O2 exposure, the level of thiobarbituric acid reactant substances decreased compared with that measured after incubated in H2O2 alone. Lipid peroxidation can be a consequence of oxidative deterioration of membrane polyunsaturated fatty acids by H2O2-generated free radicals.37,38 RES and QRC can improve the oxidative stress-mediated remodeling by scavenging the ROS generated.39 The oxidative stress we induced by H2O2 led to an increase of NO production, and the treatment of fibroblasts exposed to H2O2 with RES and QRC showed a decrease of NO rate compared with fibroblasts incubated in H2O2. The activity dysregulation of NO synthase (NOS) and NO metabolism seems to be responsible for various heart diseases.40 In normal conditions, polyphenols (RES and QRC) activated and stimulated eNOS, responsible for NO production while in oxidative stress and in inflammatory situation; they inhibited iNOS and reduced the deleterious effects of NO.41,42

Our results join those of Bi et al,43 which showed that exposure of microglia cells to RES after a diet rich in LPS caused a decrease in NO. One of the reasons for decreased levels of NO is that NO in conjunction with thiols and oxygen radicals generates nitrotyrosine.44 According to De La Lastra and Villegas,45 RES is able to reduce the activity of iNOS, and Gregory and Kelly46 showed QRC, in vivo and in vitro, to inhibit the production of NO and expression of NOS.

Our results indicated that CAT and SOD activity in fibroblasts incubated in H2O2 increased compared with the corresponding controls, and we suggested that the cells remaining alive after H2O2 exposure were those that resisted to H2O2 because they were able to induce enzymes against oxidative stress. In our study, another possibility was that the enzymatic activity was directly activated by H2O2. Our results show a decrease in CAT and SOD activity in cells exposed to H2O2 and treated with RES and QRC. Our results join those of Roig et al47 and Djordjevic et al48; after 3 hours of cell incubation in H2O2, the CAT and SOD activity increased, but after treatment with flavonoids, their activity decreased. CAT played a pivotal role in adaptation, cytoprotection, and inhibition of apoptosis in fibroblasts after repetitive H2O2 stress.49 Flavonoids stimulated the transcription and expression of genes of certain antioxidant enzymes such as CAT which causes detoxification of cells cultured in the presence of H2O2.50

In our results, we found decreases of total protein rates (in ICC) and total collagens (in ECC) in fibroblasts incubated in H2O2. Besides, we have shown that treatment of fibroblasts with RES and QRC after their exposure to H2O2 led to a slight increase in the amount of total collagens in ECC. This increase was not significant, and it can be explained by the high dose of H2O2 used, application time of oxidizing agent (H2O2), and exposure time of RES and QRC being not sufficient to completely reverse the damage caused by H2O2. Of all proteins contained in the ECM, collagens represent the largest proportion produced by adventitious fibroblasts. Our results join those of Galicka et al51 that showed that incubation of human skin fibroblasts in H2O2 (0.3 μM) caused a decrease in protein and collagen, and the pretreatment of cells with an antioxidant completely prevented these alterations. Study of Vayalil et al52 demonstrated that chronic exposure of mouse skin to UV (source of oxidative stress) induced expression of metalloproteinases, which was involved in the degradation of types I and III collagen fragments generated by collagenases and type IV collagen of the basement membrane. According to this same study and that of Kim et al,53 oral administration of epigallocatechin gallate (green tea polyphenols) markedly inhibited expression of these MMP and protected from the decrease of dermal collagen in in vivo mouse skin. In our study, we observed that H2O2 induced increases in the amount of AOPP and protein carbonyls, and that RES and QRC treatment of fibroblasts stressed by H2O2 decreased this rate. Vayalil et al52 showed that in vitro treatment with green tea polyphenols of human skin fibroblast cells caused inhibition of H2O2-induced advanced oxidation and carbonylation of proteins, thus indicating the role of polyphenols as antioxidants.

CONCLUSIONS

The level of ROS is a key determinant of fibroblasts phenotype through the differential activation of multiple kinase signaling pathways and provides a mechanism by which a stimulus may exert diverse effects on fibroblast hypertrophy and apoptosis. Flavonoids provided beneficial effects on human health, associated with an improved antioxidant response in vivo and in vitro. The oxidative stress induced by hydrogen peroxide to cultured adventitious fibroblasts induced a decrease in cell proliferation and cell viability, an increase of oncosis and apoptosis accompanied by increases in cytochrome c release. Our results show that
P3K-Akt/NF-kB, ERK1/2, and p38 MAPK signaling pathways were primary oxidative stress response pathways, shown to be involved in the mechanism of recovery from cellular morphologic changes after H2O2 treatment. Moreover, after H2O2, we noted an increase of MDA responsible for the loss of cell viability after damages caused to membranes, an increase of NO, very significant metabolic disorders of the ECM, a decrease in total protein and collagen production, and an increase of AOPP and protein carbonyls. We also noticed an increase of antioxidant enzymatic system, such as SOD and CAT with the purpose of scavenging ROS and adapting cells to oxidative stress situation produced by H2O2. Treatment of aortic fibroblasts stressed with RES and QRC prevented these alterations.

REFERENCES


Antioxidant and Antiapoptotic Role of Polyphenols


