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# Coadjustment of quercetin and hydrogen peroxide: the role of ROS in the cytotoxicity of quercetin

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Quercetin (QU) displays antioxidant and cell protective effects in most cell culture models, yet in some studies it is reported that QU shows prooxidant and cytotoxic effects. In order to explore the real role of ROS in QU's cytotoxicity, the cytotoxicity of QU and/or $H_2O_2$, as indicated by the proliferation and viability of HL-60 cells, was examined in this study. Both $H_2O_2$ and QU dose-dependently induced cell proliferation arrest and cell death. The cytotoxicity of QU could be diminished by the supplement of $H_2O_2$ in the culture medium, at the same time, the addition of QU also significantly attenuated $H_2O_2$-caused cytotoxicity. These results indicated that certain amounts of ROS are critical for the proliferation and viability of HL-60 cells, QU scavenged the necessary ROS, and hence led to the proliferation arrest and cell death; on the contrary, the excessive ROS, such as $H_2O_2$, are obviously harmful to HL-60 cells, under these conditions, QU protected cells through diminishing the excessive ROS *in vivo*. Thus QU exerted its effects on cells, including its cytotoxic and protective effects, mainly through its antioxidant activity. The malondialdehyde (MDA, an index of ROS level) assay further confirmed this conclusion, as the effects of QU, $H_2O_2$, or their combination on HL-60 cells were closely related with the variation of MDA amounts *in vivo*.

## 1. Introduction

The flavonoid quercetin (QU) possesses many biological effects, most of them are believed beneficial to cells through its antioxidant activity (Korkina and Afanasev 1997; da Silva et al. 1998; De Ruvo, et al. 2000; Feng et al. 2001; Inal et al. 2002; Lee et al. 2002; Glei et al. 2002; Lee et al. 2002; Lin et al. 2002; Choi et al. 2003). However, few studies showed that QU may also exhibit prooxidant and cytotoxic effects (Dickancaite et al. 1998; Wang et al. 1999). Obviously, the real role of reactive oxygen species (ROS) in the cytotoxicity of QU still needs to be clarified.

Since ROS are known as intracellular messengers (Thannickal and Fanburg 2000; Sauer et al. 2001), and the concentration of ROS is critically important for the cellular response to them (Burlacu et al. 2001; Kang et al. 2001), we suppose that the level of ROS *in vivo* before and after the treatment of QU may affect or even determine the final response of cells to QU. To clarify this is useful to further understand the mechanism of QU-induced cytotoxicity, and may provide information for the therapeutic use of QU.

Human leukemia cells (HL-60) have been proven to be sensitive to QU (Wang et al. 1999; Bestwick and Milne 2001; Lin et al. 2002), while $H_2O_2$ is the major mediator of oxidative stress and usually chosen as a suitable source of ROS for the study of their biological role (Lee et al. 2001; Slikker et al. 2001). In this study, to address our hypothesis, effects of QU and/or $H_2O_2$ on cell proliferation, viability and the lipid peroxidation (indicated by MDA level) were examined.

## 2. Investigations, results and discussion

### 2.1. The cytotoxicity of QU or $H_2O_2$

Proliferation arrest and cell death are the general cellular response to cytotoxins (Renis et al. 2000; Jadhav et al. 2000). Treating cells with either QU or $H_2O_2$ resulted in a dose- and time-dependent cell proliferation arrest in HL-60 cells (Fig. 1A and 1B). However, only the treatment of cells with QU at no less than 50 μM or $H_2O_2$ at no less than 200 μM caused the obvious increase of cell death (Fig. 1C), this increase of cell death also exhibited a time-dependent manner (Fig. 1D). These results indicated the cytotoxic potential of high concentrations of QU or $H_2O_2$ to HL-60 cells.

### 2.2. The coadjustment of QU and $H_2O_2$ on their cytotoxicity

In order to explore the real role of ROS in the cytotoxicity of QU, it was investigated whether the cytotoxicity of QU can be adjusted by $H_2O_2$, at the same while, the adjustment of QU on the effect of $H_2O_2$ was also studied. QU dose-dependently attenuated the proliferation arrest and cell death induced by $H_2O_2$ (Fig. 2A and 2B), indicating that QU exerted its antioxidant capacity in HL-60 cells. $H_2O_2$

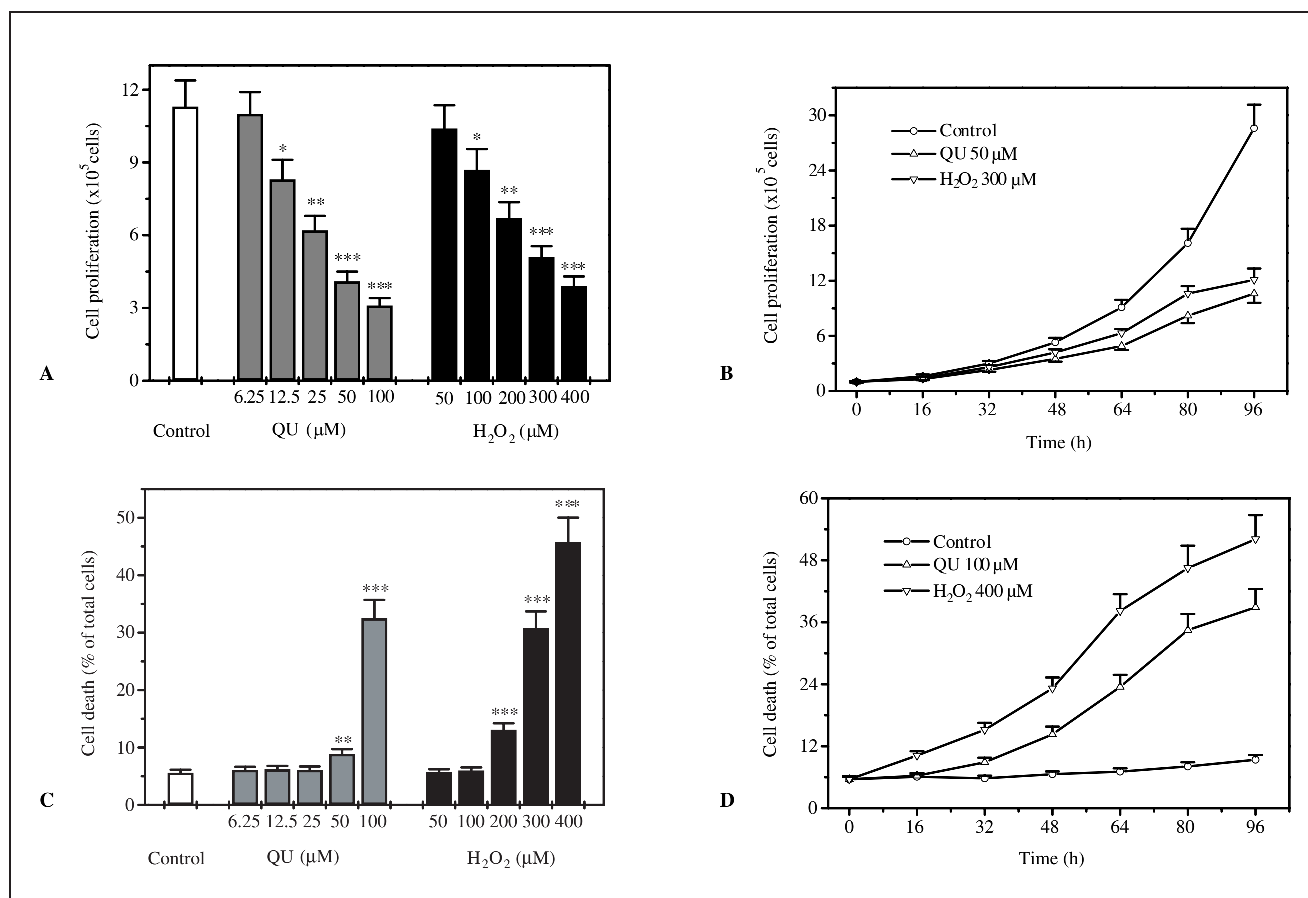


Fig. 1: Effect of QU and $H_2O_2$ on the proliferation and viability of HL-60 cells. HL-60 cells were incubated with different concentrations of QU or $H_2O_2$ for 72 h in A and C, with 50 μM of QU or 300 μM of $H_2O_2$ in B, or with 100 μM of QU or 400 μM of $H_2O_2$ in D for the indicated times. Means ± SD of three parallel experiments was indicated, n = 3 × 3 cultures per conditions, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* the control group in A and C

also significantly counteracted QU-caused cytotoxicity, showing that QU's cytotoxicity at least partially resulted from its antioxidant capacity (Fig. 2C and 2D).

Although in contrast to reports that QU triggers cells apoptosis while inducing ROS generation (Wang et al. 1999), the above conclusion is reasonable. This is because: 1) It is found important for all aerobic animals to keep a balance between ROS generation and scavenging *in vivo*, wheras excessively generating or scavenging ROS is harmful for the proliferation and viability of cells (Burdon 1995; McCord 1998; Martindale and Holbrook 2002); 2) The antioxidants EUK-134 (a superoxide dismutase/catalase mimetic), *N*-acetylcysteine, vitamin E or the anti-apoptotic gene Bcl-2 have been found to exert their cytotoxic effects on cell proliferation and viability by diminishing ROS (Nargi et al. 1999; Wedgwood and Black, 2003; Saitoh et al. 2003).

However, there still remains one conflict in Fig. 2C and 2D, i.e., at low concentrations of no more than 200 μM, $H_2O_2$ dose-dependently increased the counteraction against QU's cytotoxicity, whereas the effect of $H_2O_2$ against QU dose-dependently decreased when the concentrations of $H_2O_2$ reached more than 200 μM. This conflict may be explained as follows. At low concentrations, although most of them would probably be scavenged by QU, the added $H_2O_2$ diminished QU's cytotoxicity through supplying certain ROS necessary for cell viability. With the increase of $H_2O_2$, however, the antioxidant capacity of QU is exceeded, i.e., QU can not completely scavenge the added $H_2O_2$. Thus under these conditions, the effect of QU and high concentrations of $H_2O_2$, such as 300 or 400 μM $H_2O_2$, may be not due to the lack of ROS *in vivo* caused by QU, but due to the left ROS. In other words, the final concentration of ROS determines the response of cells to QU, $H_2O_2$, or their combination. This is probably true since the important role of ROS concentration in determining their biological roles has already been proven (Burlacu et al. 2001; Kang et al. 2001). In order to confirm this, the level of lipid peroxidation (one representative of ROS level *in vivo*) was studied.

### *2.3. The variation of MDA in vivo*

Lipid peroxidation (as indicated by MDA) results from the effect of ROS *in vivo*, and is regarded as indicator of *in vivo* ROS accumulation. Although treating cells with QU or $H_2O_2$ at high concentrations leads to similar effects on the proliferation and viability of cells, they caused inverse effects on lipid peroxidation *in vivo,* i.e., QU significantly decreased while $H_2O_2$ increased the amount of MDA (Fig. 3A), indicating an inverse role of ROS in the cytotoxicity of QU or $H_2O_2$. Coincidently, like in the cell proliferation and viability assay, both QU and $H_2O_2$ significantly counteracted their effects on the formation of lipid peroxidation (Fig. 3B and 3C). Simply, QU similarly diminished the cell proliferation arrest, cell death and lipid peroxidation triggered by $H_2O_2$, while $H_2O_2$ obviously attenuated QU-triggered cell proliferation arrest and cell death via increasing the decreased lipid peroxidation by QU. In addition, as we hypothesized, treating cells with a combination of high concentrations of $H_2O_2$ (no less than 200 μM), QU did not

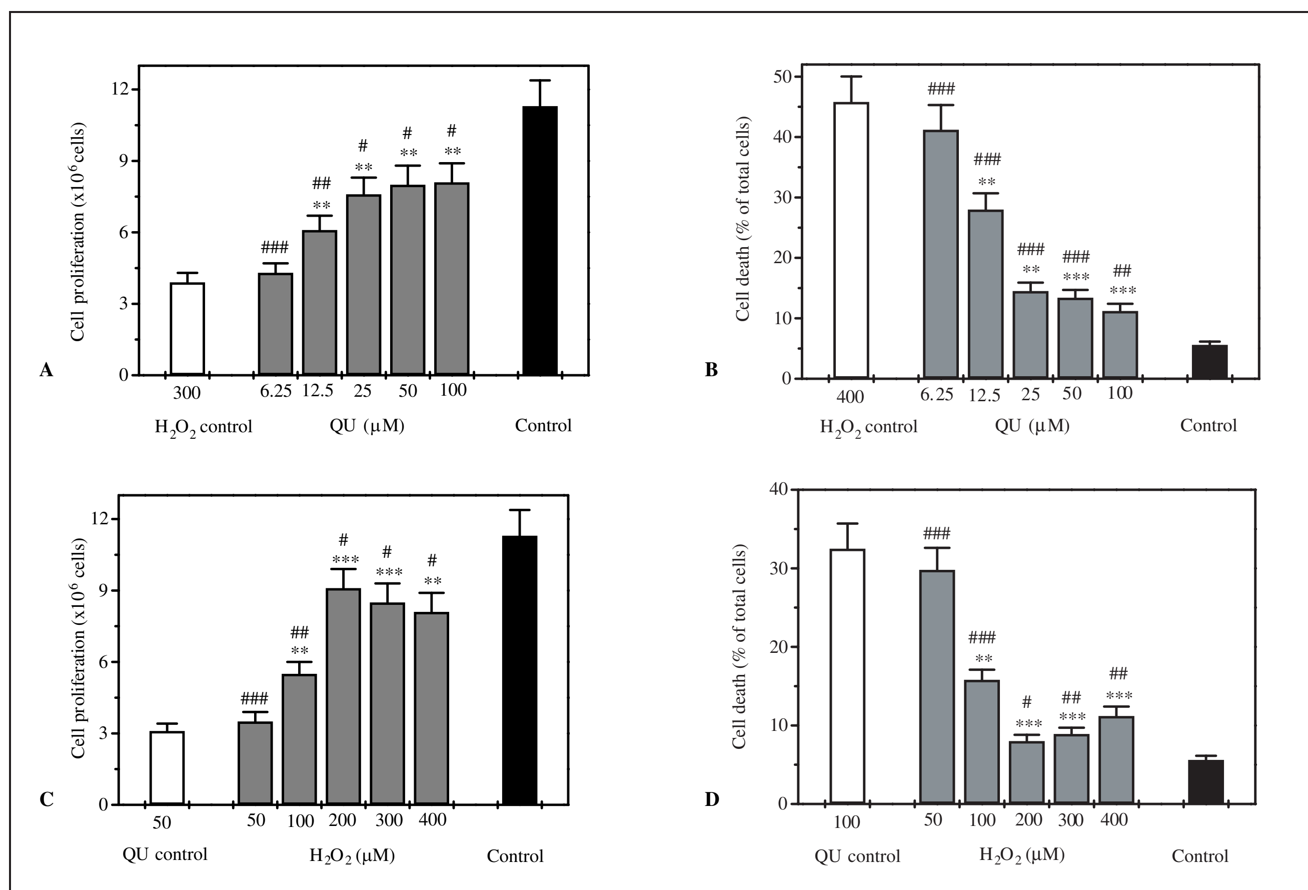


Fig. 2: Coadjustment between QU and $H_2O_2$ on the proliferation and viability of cells. Except the control group, cells were treated with QU or $H_2O_2$ in the presence of 300 µM $H_2O_2$ (A), 400 µM $H_2O_2$ (B), 50 µM QU (C), or 100 µM QU (D) for 72 h, respectively. A and B, QU dose-dependently attenuated $H_2O_2$-induced cell proliferation arrest and cell death. C and D, the effect of $H_2O_2$ on QU-triggered variation of cell proliferation and viability. Means of three parallel experiments are displayed (means ± SD), n = 3 × 3 cultures per conditions, **, *** indicates $p < 0.01$ or $p < 0.001$ as compared with the $H_2O_2$ or QU control, while #, ##, ### indicate $p < 0.05$, $p < 0.01$, or $p < 0.001$ as compared with the control group

completely abolish the effect of $H_2O_2$. Thus the effect of QU and $H_2O_2$ combination under these conditions is mainly ascribed to the excess of $H_2O_2$.

As we have noticed, lower concentrations of QU or $H_2O_2$ were selected as the positive control in the cell proliferation assay, and a short treating time was used in the MDA assay. Different concentrations of QU or $H_2O_2$ were used to diminish the possible influence of higher cell death on the proliferation assay, i.e., 50 µM QU or 300 µM $H_2O_2$ were used in the proliferation assay, while 100 µM QU and 400 µM $H_2O_2$ were used in the cell viability assay or the MDA assay. The relatively short time (4 h) used in the MDA assay is long enough for the lipid peroxidation due to the rapid reaction of ROS.

In conclusion, QU is a novel antioxidant, which exerts the cytotoxic or protective effect through scavenging the necessary or excessive ROS in HL-60 cells. Considering the automatic generation of ROS and the low superoxide dismutase activity in tumor cells (Oberley 1982; Burden 1994), and the involvement of ROS in lots of pathogenesis, such as carcinogenesis and the occurrence of neurodegenerative diseases (Datta et al. 2000; Floyd and Hensley 2002; Deshpande and Irani 2002), QU may be particularly useful for the anti-oxidant therapy for these diseases.

## 3. Experimental

### 3.1. Reagents

Quercetin, trypsin, trypan blue, 1,1,3,3-tetramethoxypropane and thiobarbituric acid were purchased from Sigma (Sigma, St. Louis, MO), RPMI-1640 was purchased from Gibco (Gibco, Santa Clara, CA). All other reagents are of analytical grade.

### 3.2. Cell culture and treatment

Human leukemia cells (HL-60) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (equivalent to 100 units/ml and 100 mg/ml, respectively) at 37 °C in a humidified atmosphere containing 5% $CO_2$. After culturing the cells ($1 \times 10^5$ cells/ml) for 24 h, the culture medium was aspirated and replaced with new medium containing quercetin and/or $H_2O_2$ where indicated, after that, the different cultures were replaced with fresh medium containing the corresponding reagents every 24 h.

### 3.3. Determination of cell proliferation and viability

Cells at $1 \times 10^5$/ml were cultured for 24 h, then treated with QU and/or $H_2O_2$ at the indicated concentrations, three dishes for each of differently treated cells were collected every 16 h in the first 4 days, the total and dead cells were counted respectively using the trypan blue stain exclusion method under a phase-contrast microscope.

### 3.4. Malondialdehyde (MDA) assay

After different treatment, HL-60 cells were collected and suspended in the lysis buffer (Cousens et al. 1979). The suspension was collected after pipetting the cell mixture up and down for 20 times. The MDA amount in the obtained suspension was immediately evaluated using a colorimetric assay as described previously (Ohkawa et al. 1979). All the analyses were performed in triplicate.

### 3.5. Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA post-hoc Bonferroni), and p values less than 0.05, 0.01, or 0.001 were denoted as *, **, or ***, respectively.

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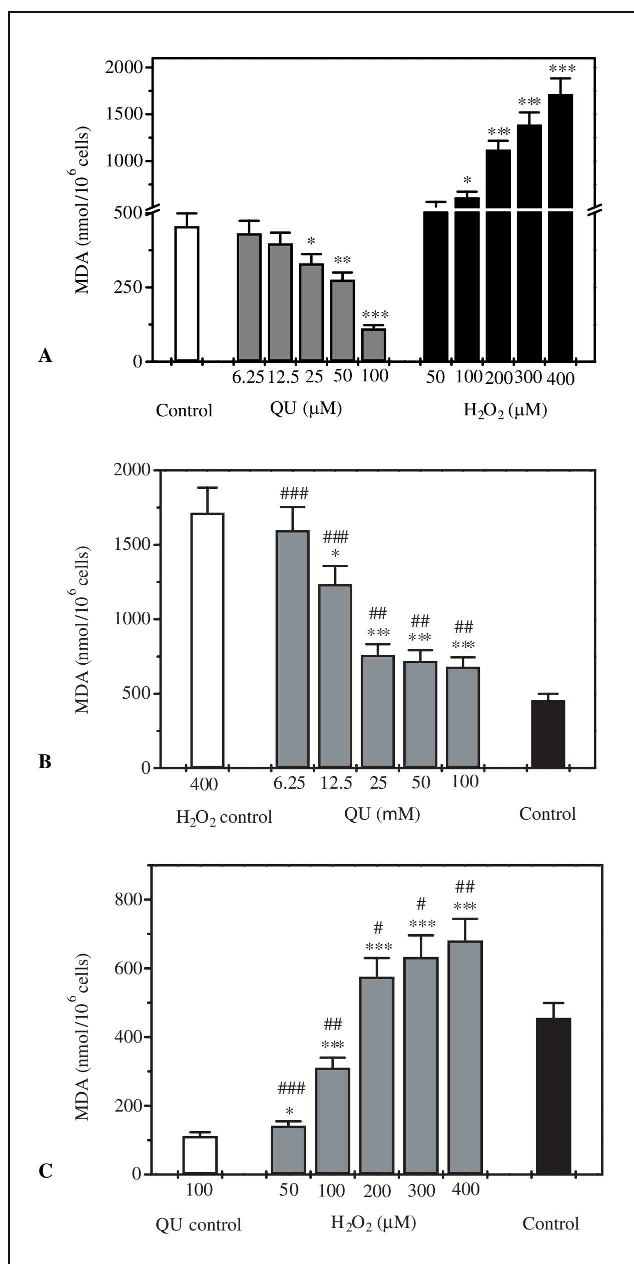


Fig. 3: Effect of QU and/or $H_2O_2$ on the peroxidation of lipid (MDA) in HL-60 cells. Cells were treated for 4 h in these assays. In B and C, except the control group, cells were treated with QU or $H_2O_2$ in the presence of 400 μM $H_2O_2$ (B) or 100 μM QU (C), respectively. A, QU or $H_2O_2$ dose-dependently decreased or increased the amount of MDA in HL-60 cells. B, QU dose-dependently attenuated $H_2O_2$-induced MDA increase. C, $H_2O_2$ abolished the inhibition of MDA triggered by QU. Means of three parallel experiments are displayed (means ± SD), n = 33 cultures per conditions, *, **, *** indicates $p < 0.05$, $p < 0.01$ or $p < 0.001$ as compared with the control group (A). In B and C, *, *** indicates $p < 0.05$ or $p < 0.001$ as compared with the $H_2O_2$ or QU control, while #, ##, ### indicate $p < 0.05$, $p < 0.01$, or $p < 0.001$ as compared with the control group

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