Arch Pharm Res Vol 28, No 11, 1251-1256, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

Cytoprotective Effect of Green Tea Extract and Quercetin against Hydrogen Peroxide-Induced Oxidative Stress

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(Received April 30, 2005)

In this study, we evaluated the cytoprotective effects of antioxidative substances in hydrogen peroxide (H_2O_2) treated Mel-Ab melanocytes. Tested substances include selenium, quercetin, green tea (GT) extract, and several vitamins (ascorbic acid, Trolox, and folic acid). Of these, both quercetin and GT extract were found to have strong cytoprotective effects on H_2O_2-induced cell death. We also examined additive effects, but no combination of two of any of the above substances was found to act synergistically against oxidative damage in Mel-Ab cells. Nevertheless, a multi-combination of GT extract, quercetin, and folic acid appeared to prevent cellular damage in a synergistic manner, which suggests that combinations of antioxidants may be of importance, and that co-treatment with antioxidants offers a possible means of treating vitiligo, which is known to be related to melanocyte oxidative stress.

Key words: Oxidative stress, Reactive oxygen species, Cytoprotection, Antioxidant, Vitiligo

INTRODUCTION

Cellular damage is often associated with the generation of reactive oxygen species (ROS) and is implicated in the pathogenesis of many diseases. Major ROS include hydrogen peroxide (H_2O_2), the superoxide anion ($O_2^{\cdot-}$), the hydroxyl radical ($^{\cdot}OH$), and nitric oxide (NO). ROS are metabolized to water and oxygen by antioxidative enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and other glutathiones (Nordberg and Arner, 2001). Moreover, accumulations of ROS damage the integrity of various biomolecules including DNA, proteins, and lipids and are associated with a wide range of disorders in humans (Nordberg and Arner, 2001). In particular, oxidative stress has been suggested to be a cause of melanocyte disappearance in vitiligo, which is an acquired pigmentary disorder of the skin characterized by white spots (Boissy and Manga, 2004; Jimbow *et al.*, 2001; Schallreuter *et al.*, 1999). Recently, it was proposed that there might be a close correlation between melanocyte loss and redox imbalance, e.g., catalase, selenium, and glutathione levels were reported to be reduced in vitiligo patients (Beazley *et al.*, 1999; Bowers *et al.*, 1999; Giovannelli *et al.*, 2004). Thus, melanocyte loss in vitiligo may be related to increased oxidative stress and the subsequent induction of H_2O_2.

Accordingly, several researchers have tried to address this melanocyte loss by attempting to regulate antioxidant-related mechanisms (Bowers *et al.*, 1999; Montes *et al.*, 1992). However, although some vitiligo patients were found to respond positively in various trials involving the oral administration of folic acid, vitamin B_{12}, or vitamin C, the restoration of melanocyte loss in vitiligo remains a therapeutic objective (Boissy and Manga, 2004; Montes *et al.*, 1992).

In the present study, we aimed to determine whether melanocytes could be protected from oxidative stress by antioxidants, such as, green tea (GT) extract, quercetin, selenium, Trolox, folic acid, vitamin C, or B_{12}, or by their combinations. It has been reported that quercetin may restore the antioxidant enzyme system, e.g., it restored superoxide dismutase activity and glutathione content after oxidative damage has been induced by the environmental endocrine disrupter Aroclor 1254 (Mi and Zhang,

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2005). Moreover, GT extract was found to protect cellular membranes against *t*-butylhydroperoxide-induced oxidative damage (Saffari and Sadrzadeh, 2004). Furthermore, a combination of vitamin C and E showed a protective effect against ultraviolet radiation (Quevedo *et al.*, 2000; Smit *et al.*, 2004). However, the individual or combinatorial effects of such agents have not been thoroughly examined in terms of protecting melanocytes from oxidative stress. Therefore, we tested the individual effects of these antioxidants and investigated the possibility of their acting synergistically to protect Mel-Ab cells (an immortalized mouse melanocyte cell line) from H_2O_2. We found that both GT extract and quercetin protected Mel-Ab cells from H_2O_2. In addition, co-supplementation with a combination of GT extract/quercetin/folic acid increased this protective effect. These results suggest that the selection of anti-oxidants is important, and that the co-supplementation of antioxidants could possibly be used to treat cases of vitiligo.

MATERIALS AND METHODS

Materials

H_2O_2, quercetin, vitamin C, Trolox (a water-soluble vitamin E analogue), selenium, vitamin B_{12}, and folic acid were purchased from Sigma (St. Louis, MO). Green tea (GT) extract were obtained from Bioland (Chonan, Korea).

Cell cultures

The Mel-Ab cell line is a mouse-derived spontaneously immortalized melanocyte cell-line that produces large amounts of melanin (Dooley *et al.*, 1994). Mel-Ab cells were incubated in DMEM supplemented with 10% fetal bovine serum (FBS), 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 1 nM cholera toxin (CT), 50 μg/mL streptomycin, and 50 U/mL penicillin at 37°C in 5% CO_2. All conditioned culture media used in this experiment were serum-free.

Exposure to H_2O_2

In order to evaluate the response of melanocytes to oxidative-stress, cells were exposed to H_2O_2. Briefly, cells were seeded into 6-well plates (2×10^5 cells/well). After serum starvation for 24 h (DMEM with 0.1% BSA), cells were cultured in serum-free DMEM (with 0.1% BSA) at different H_2O_2 concentrations (0.612, 1.2, 2.4, 6.1, and 12.2 mM) for 24 h. Cell viability was then assessed by crystal violet assay (Dooley *et al.*, 1994).

Cell viability assay

After removing culture media, cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at RT and rinsed four times. The crystal violet retained by adherent cells was extracted with 95% ethanol, and absorbance was determined at 590 nm using an ELISA reader (TECAN, Salzburg, Austria).

Treatment of antioxidants

To investigate the cyto-protective activity of each substance (Fig. 2), cells (2×10^5 cells/well) were seeded into 6-well plates. After serum starvation for 24 h, cells were pretreated with GT extract (10 or 100 μg/mL), selenium (1 or 10 μM), quercetin (10 or 100 μM), vitamin C and vitamin B_{12} (0.1 or 1 mM), Trolox (20 or 100 μg/mL) or folic acid (0.1 or 1 mM) for 2 h. H_2O_2 (2 mM) was then added to each well and incubated for 24 h. Cell viabilities were determined by crystal violet assay (Dooley *et al.*, 1994).

Statistical analysis

Statistical analysis was performed using the Student's *t*-test. $P<0.05$ and $P<0.01$ were taken to indicate statistical significance as shown in the figure legends.

RESULTS

The viability of melanocytes against oxidative stress

To investigate the viabilities of melanocytes in the presence of oxidative stress, we used immortalized mouse Mel-Ab cells and H_2O_2 as means of administering oxidative stress (Nordberg and Arner, 2001). To determine the appropriate H_2O_2 treatment concentration, Mel-Ab cells were exposed to different concentrations of H_2O_2 for 24 h, and cell viabilities were determined by crystal violet staining. Based on the cell viability data shown in Fig. 1, a

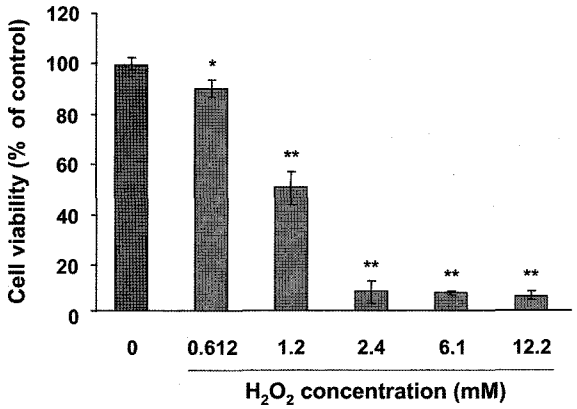

Fig. 1. Determination Mel-Ab cell viabilities after treatment with H_2O_2 at different concentrations. After serum starvation, Mel-Ab cells were incubated with H_2O_2 at the indicated concentrations (0, 0.612, 1.2, 2.4, 6.1, and 12.2 mM) for 24 h. Cell viabilities were determined by crystal violet staining. Relative viabilities were quantified using an ELISA reader. Data are expressed as means±S.D. of three independent experiments. *, $P<0.05$; **, $P<0.01$ compared with untreated Mel-Ab cells as assessed by the Student's *t*-test.

H_2O_2 concentration of 2 mM was chosen for the following experiments.

Protective effects in H_2O_2-treated Mel-Ab cells

To study cyto-protective effects, Mel-Ab cells were pretreated for 2 h with GT extract, selenium, quercetin, vitamin B_{12}, vitamin C, Trolox, or folic acid, and then exposed to 2 mM H_2O_2 for 24 h. The viabilities of H_2O_2-treated Mel-Ab cells reduced to about 5% of those not treated with H_2O_2 after this time. However, GT extract (100 μg/mL, 68%) or quercetin (100 μM, 67%) markedly protected cells from the H_2O_2, and vitamin C (1 mM, 14%) was observed to have minor protective effect. The other substances, including Trolox and folic acid, had no significant protective effect on H_2O_2-treated cells (Fig. 2). These results indicate that both GT extract and quercetin protect Mel-Ab cells against H_2O_2-induced toxicity.

Effects of combinations of antioxidants on H_2O_2-induced oxidative stress

In order to study the synergistic effects of GT extract, quercetin, and vitamin C against H_2O_2-induced oxidative damage, H_2O_2-treated Mel-Ab cells were treated with different combinations of these agents. Fig. 3 shows the different combinations of these compounds tested with 2 mM H_2O_2. Compared with cells treated with GT extract alone, co-treatment with GT extract in combination with quercetin, vitamin C, or folic acid showed no significant synergistic effect (Fig. 3A). Next, we tested combinations of quercetin with other substances, and found that quercetin showed also no significant effect in combination with vitamin C or folic acid (Fig. 3B), and neither did the triple combination of quercetin/folic acid/vitamin C (Fig. 3B). Although vitamin C alone was observed to have a weak protective effect (Fig. 2), it showed no additive effect in combination with folic acid (Fig 3C), GT extract, or quercetin (Fig. 3A and B). Interestingly, the GT extract/quercetin/folic acid combination significantly prevented cellular damage (Fig. 3D). These results suggest that the selection and combination of antioxidants is important in terms of increasing cellular resistance to oxidative stress.

DISCUSSION

H_2O_2 is a source of ROS and acts as an activator of oxidative stress in affected cells (Nordberg and Arner, 2001). In the present study, we used a relatively high concentration of H_2O_2 (2 mM) because this level is known to have an obvious effect on Mel-Ab melanocyte viability. Melanocyte death has been well demonstrated in vitiligo (Gauthier *et al.*, 2003), although the mechanism of melanocyte death is unknown, various stimuli including localized trauma, autoimmune predisposition, and oxidative stress have been suggested as etiologies (Gauthier *et al.*, 2003). Several reports have suggested that oxidative stress in vitiligo might be due to an imbalance of the oxidant-antioxidant system, or to excessive quantities of ROS, such as, H_2O_2, hydroxyl radicals, and superoxide anions (Boissy and Manga, 2004; Nordberg and Arner, 2001; Sies, 1997).

In this study, we investigated the protective effects of potential antioxidants. We found that both GT extract and quercetin had a significant cyto-protective effect on H_2O_2-treated Mel-Ab cells. Moreover, these results agree with

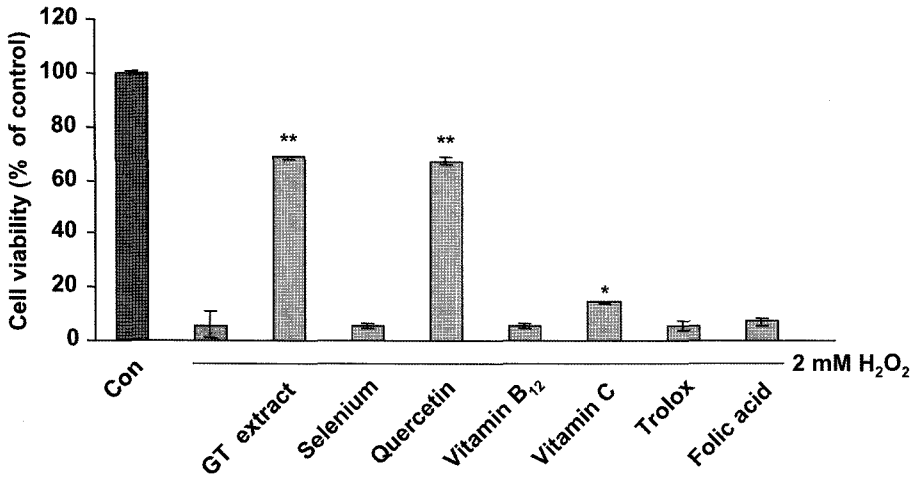

Fig. 2. Protection of Mel-Ab cells from oxidative stress by GT extract or quercetin. After serum starvation, Mel-Ab cells were treated with; GT extract (100 μg/mL), selenium (10 μM), quercetin (100 μM), vitamin C, and B_{12} (1 mM), Trolox (100 μg/mL), or folic acid (1 mM) and then treated with 2 mM H_2O_2. Cell viabilities were determined by crystal violet staining. Relative viabilities were quantified using an ELISA reader. Data are expressed as the means±S.D. of three independent experiments. *, P <0.05; **, P <0.01 compared with H_2O_2 only-treated Mel-Ab cells as assessed by the Student's t test.

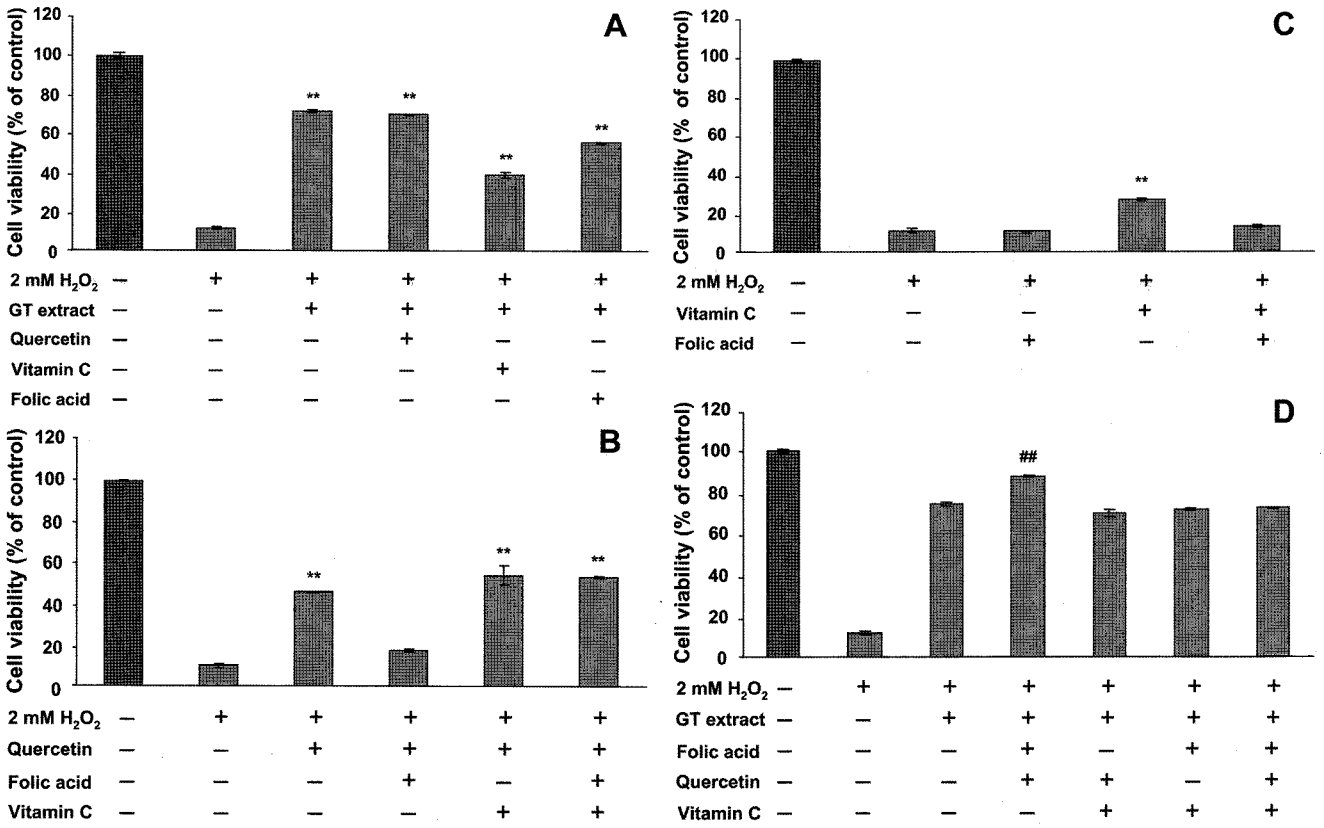

Fig. 3. Protective effects of cytoprotective antioxidants in combination. Mel-Ab cells were treated as described in Fig. 2, with the following; GT extract in A, quercetin in B, vitamin C in C, and with different combinations in D, which were used at the following concentrations; GT extract (100 μg/mL), quercetin (100 μM), vitamin C (100 μM), and folic acid (1 mM). Cell viabilities were determined using crystal violet assays. Relative viabilities were quantified using an ELISA reader. Data are expressed as the means±SD of three independent experiments. **, P <0.01 compared with H_2O_2 only-treated Mel-Ab cells, ##, P <0.01 compared with H_2O_2 and GT extract-treated Mel-Ab cells, as assessed by the Student's t test. –; not treated, +; treated.

those of previous studies, namely, that GT extract and quercetin exhibit oxidant scavenging activities under stress-induced conditions (Kessler *et al.*, 2003; Onuki *et al.*, 2005; Saffari and Sadrzadeh, 2004). Moreover, (-)-epigallocatechin gallate (EGCG), a major component of GT extract, and quercetin were reported to protect various types of cells from carcinogenesis (Brusselmans *et al.*, 2005; Katiyar *et al.*, 2001; Katiyar and Mukhtar, 1997; Park *et al.*, 2005). However, the molecular mechanisms of the protective effects of GT extract and/or quercetin are unclear. Nevertheless, it has been reported that EGCG is a powerful antioxidant and that it can inhibit lipid peroxidation and protect cell membrane-bound ATPases against oxidative stress (Saffari and Sadrzadeh, 2004). Moreover, quercetin has been reported to protect cells from oxidative damage by increasing the intracellular antioxidant level and reducing lipid peroxidation (Mi and Zhang, 2005). To elucidate the antioxidative mechanisms of GT extract and/or quercetin, further studies are required. Although in the present study, supplements other than GT extract and quercetin had either minimal or no protective effect, it has been reported that vitamin C may function as an antioxidant and that thus it may diminish cancer risk (Valko *et al.*, 2004). Trolox, a membrane stabilizer, was reported to show typical neuroprotective activity *via* an antioxidant mechanism, and to protect neurons from amyloid β (Aβ) toxicity by modulating the wingless (Wnt) signaling pathway (Chatterjee *et al.*, 2005; Quintanilla *et al.*, 2005; Yoon *et al.*, 2003). Moreover, several studies have shown that basal levels of selenium, folic acid, or vitamin B_{12} might have therapeutic roles in patients with vitiligo (Beazley *et al.*, 1999; Naziroglu *et al.*, 2004). Furthermore, when folic acid, vitamin C, or vitamin B_{12} were administered to vitiligo patients (Montes *et al.*, 1992), they were found to protect against ultraviolet B-induced DNA damage in the epidermis (Placzek et al., 2005). These reports suggest that antioxidants have different protective mechanisms *in vivo*.

In summary, GT extract and quercetin were found to protect melanocytes against H_2O_2-induced oxidative damage, whereas neither GT extract nor quercetin in combination with vitamin C or folic acid showed a synergistic

effect. Interestingly, however, the triple combination GT extract/quercetin/folic acid prevented the cellular damage induced by H_2O_2 in a synergistic manner, which suggests that effective combinations of antioxidants should be investigated versus ROS types. These results indicate that the selection of antioxidants may have important effects against given oxidative stressors. Thus, we suggest that co-supplementation with these antioxidants may offer an adjunctive therapeutic approach in vitiligo.

ACKNOWLEDGEMENT

This study was supported by a grant (#02-PJ1-PG1-CH11-0001) from the Good Health R&D Project, Ministry of Health and Welfare, Republic of Korea.

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