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# Quercetin Prevents Glutathione Depletion Induced by Dehydroascorbic Acid in Rabbit Red Blood Cells

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Exposure of rabbit red blood cells to dehydroascorbic acid (DHA) caused a significant decline in glutathione content which was largely prevented by quercetin, whereas it was insensitive to various antioxidants, iron chelators or scavengers of reactive oxygen species. This response was not mediated by chemical reduction of either extracellular DHA or intracellular glutathione disulfide. In addition, the flavonoid did not affect the uptake of DHA or its reduction to ascorbic acid. Rather, quercetin appeared to specifically stimulate downstream events promoting GSH formation.

*Keywords*: Quercetin, flavonoids, dehydroascorbic acid, glutathione, erythrocytes

## INTRODUCTION

Quercetin (3,5,7,3′,4′-pentahydroxyflavone) is a plant polyphenolic flavonoid characterized by potent antioxidant[1–4] and iron-chelating properties.[1,5] It was shown to inhibit (a) the peroxidation of lipids induced by the attack of aqueous oxygen radicals[6] or initiated by ferric iron in the retina, purified rod segments and retinal pigment epithelium;[7] (b) DNA single strand breakage and cytotoxicity caused by *tert*-butylhydroperoxide;[8] (c) the oxidative alterations of membrane lipids and proteins induced by phenylhydrazine, acrolein, divicine and isouramil;[9] and (d) injury to cutaneous tissue-associated cell types induced by glutathione depletion elicited by buthionine sulfoximine (BSO) treatment.[10]

In the present study we report evidence of a novel effect of quercetin. Using an experimental system involving exposure of rabbit red blood cells (RBC) to DHA, we found that the resulting decline in GSH content was largely prevented by

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the flavonoid by a mechanism which appears to be independent of its antioxidant and iron-chelating properties. Erythrocytes take up extracellular DHA on the glucose transporter[11–13] and, once within the cell, DHA is rapidly reduced to ascorbate at the expense of GSH.[13–16] Under the experimental conditions utilized in this study, quercetin did not spare GSH by chemically reducing DHA or preventing its uptake and/or reduction to ascorbic acid but, rather, by promoting GSH formation.

## MATERIALS AND METHODS

### Materials

Enzymes, coenzymes, substrates, ascorbic acid, potassium ferricyanide, rutin, kaempferol and quercetin were purchased from Sigma-Aldrich (Milan, Italy) and from Boehringer Mannheim (Mannheim, Germany). All HPLC reagents were of HPLC grade.

### Dehydroascorbate Preparation

A 20 mM solution of DHA was prepared immediately prior to use by bromine oxidation of ascorbic acid, as reported by Waskho *et al.*[17]

### Rabbit Red Blood Cells

Rabbit whole blood was obtained from the marginal ear vein, using heparin as anticoagulant, and was immediately centrifuged at 3,000 rpm for 10 min at 4 °C. After removal of plasma, buffy coat and the upper 15% of the packed red blood cells, the cells were washed three times in 10 volumes of phosphate-buffered saline solution (PBS) which consisted of distilled deionized water containing 140 mM NaCl and 12.5 mM $NaH_2PO_4$ at pH 7.4. The packed erythrocytes were then resuspended in the same saline solution and incubated in the presence of DHA or those compounds reported in the legends of figures.

### Incubations of Cell-free-extract with DHA or Other Compounds

Washed red blood cells were lysed with 1 volume of cold water and maintained at 4 °C for 10 min. The cell-free extracts were then resuspended with 4 volumes of PBS with or without 2 mM DHA in the presence of 200 μM butylated hydroxytoluene (BHT), 10–100 μM *N,N'*-diphenyl-1,4-phenylendiamine (DPPD), 25–100 μM 1,10-phenanthroline, 50–100 μM desferoxamine mesylate (desferal), 50 μM-1 mM mannitol, 10–100 μM thiourea. The samples were incubated at 37 °C for various time intervals. Stock solutions of 20 mM quercetin or 10 mM 1,10-phenanthroline were dissolved in dimethyl sulfoxide (DMSO), 10 mM DPPD and 50 mM BHT in 95% ethanol and then diluted in PBS. At the treatment stage the final DMSO/ethanol concentration was never higher than 0.25%. Under these conditions DMSO and ethanol did not induce hemolysis nor did they affect GSH and hexokinase levels.

To stop the reactions, samples were immediately placed in an ice bath, diluted 1:2 with water, and directly analyzed for GSH and hemoglobin content.

### Incubation of Rabbit Erythrocytes with DHA or Other Compounds

A 10% (v/v) red blood cell suspension prepared in PBS was incubated at 37 °C in a shaking water bath. Experiments were carried out in the presence of 2 mM DHA and quercetin and/or the compounds reported in the legends to the figures. To terminate the reaction, samples were immediately centrifuged at 3,000 rpm for 10 min at 4 °C and washed twice in 10 volumes of PBS. The packed cells were then lysed with 19 volumes of cold water and after 10 min in ice, the samples were analyzed for GSH, hemoglobin content and hexokinase activity.

### Hemoglobin

The hemoglobin (Hb) concentration was determined spectrophotometrically at 540 nm with Drabkin's solution as described by Beutler.[18]

### GSH and GSSG

The GSH and GSSG measurements were performed spectrophotometrically as described by Beutler.[18]

### Hexokinase

Rabbit red blood cell hexokinase (EC 2.7.1.1) was measured spectrophotometrically at 340 nm as described by Beutler.[18]

### GSH-Dependent DHA-reductase

GSH-dependent DHA-reductase (EC 1.8.5.1) was assayed as described by Wells *et al.*[19] at 37 °C in a system coupled with glutathione reductase (EC 1.6.4.2).

### Ferricyanide Reduction

Ferricyanide reduction by RBC was measured as reported by Avron and Shavit.[20] After preincubation with DHA or DHA + quercetin, 0.25 ml of 10% RBC were washed three times with 10 ml of PBS + 5 mM glucose (pH 7.4) and the final pellet (25 µl) was resuspended in 0.225 ml of PBS containing 1 mM potassium ferricyanide which was solubilized immediately prior to use. After 30 min at 37 °C, the cell suspensions were centrifuged at 3,000 rpm. The resulting supernatant was assayed in duplicate for ferrocyanide content using 1,10-phenanthroline as indicator, by measuring the optical density at 510 nm (extinction coefficient: 10,500 $M^{-1}$ $cm^{-1}$).

### Intracellular Content of Quercetin

In order to investigate the intracellular content of quercetin, RBC (10% v/v in PBS) were incubated in the presence of 50 µM quercetin for 60 min. At 5 min time intervals, aliquots of RBC suspension were collected and centrifuged at 3,000 rpm. The clear supernatant was extracted with ethyl acetate and erythrocytes were washed twice with PBS and lysed by freezing and thawing. The hemolysate was extracted with ethyl acetate.[9] The amount of quercetin was measured either spectrophotometrically at 340 nm or by HPLC method as previously described.[21]

Statistical analysis of the data was performed using Student's *t*-test.

## RESULTS AND DISCUSSION

Previous work from this[22] and other laboratories[11,12,15] demonstrated that DHA is rapidly taken up by red blood cells and that this event is followed by GSH-dependent reduction of DHA to ascorbate paralleled by a progressive decline in GSH.[22–24] The results illustrated in Figure 1A show the occurrence of this phenomenon at increasing time intervals of exposure of rabbit erythrocytes to 2 mM DHA. Interestingly, the addition of quercetin (50 µM) at the time of DHA exposure mitigates this response, whereas the flavonoid alone has hardly any effect on the GSH content in untreated erythrocytes. The effect of quercetin was concentration-dependent and the maximal inhibitory response at 90 min of incubation was achieved by a concentration of 25 µM (Figure 1B). As expected, the decline in GSH levels was paralleled by a time-dependent increase in the intracellular content of glutathione disulfide (GSSG) and this response was also mitigated by quercetin (50 µM) (Table I).

In principle, these results might simply be explained by chemical reduction of DHA or by an inhibitory effect of quercetin on DHA uptake, which in erythrocytes is mediated by facilitative hexose transporters.[11–14] The first possibility, however, appears to be unlikely since a 30 min incubation at 37 °C of 50 µM quercetin/2 mM DHA in PBS, pH 7.4, caused neither an appreciable

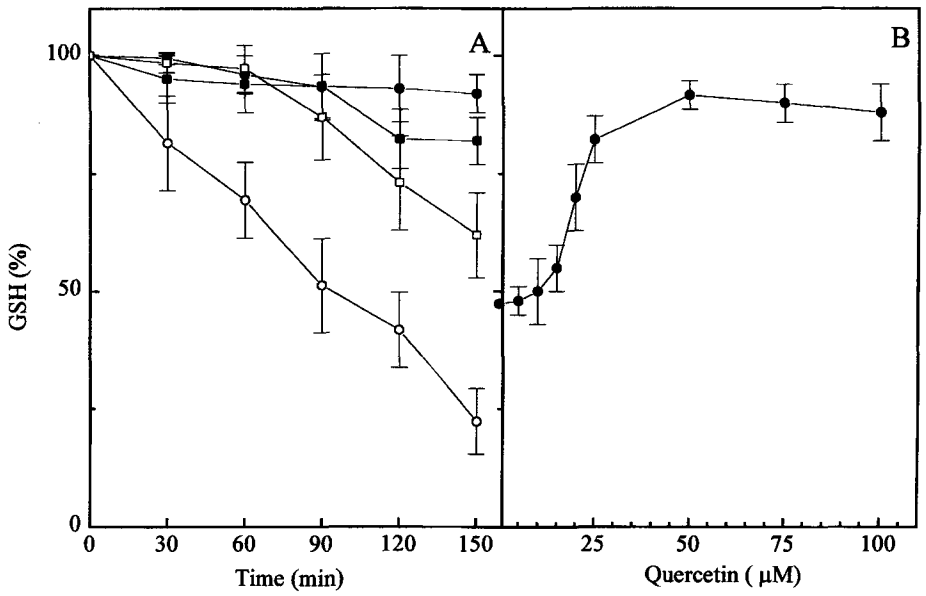


FIGURE 1 *Effect of quercetin on reduced glutathione levels in rabbit erythrocytes exposed to dehydroascorbic acid.* (A) Rabbit erythrocytes were resuspended (10% v/v) in PBS and incubated at 37 °C for increasing time intervals with 2 mM DHA (○), 50 μM quercetin (■), or 2 mM DHA/50 μM quercetin (□). The control sample is represented by a (●). GSH levels were measured as reported in Materials and Methods. The basal content of GSH corresponds to 7.5 ± 0.7 μmol/g Hb. (B) Rabbit erythrocytes were resuspended (10% v/v) in PBS and incubated at 37 °C with 2 mM DHA in presence of increasing concentrations of quercetin for 90 min. (●). GSH levels were measured as reported in Materials and Methods. All values are the means ± SD of at least five independent determinations.

TABLE I GSSG levels in DHA-treated RBC or hemolysate in the presence of quercetin

| | GSSG (nmol/ml) | | | |
|---|---|---|---|---|
| | RBC[a] | | Hemolysate[b] | |
| | 60 min | 120 min | 30 min | 120 min |
| DHA 2 mM | 48 ± 8 | 100 ± 10 | 75 ± 6 | 79 ± 6 |
| DHA 2 mM + Quercetin 50 μM | 20 ± 9 | 42 ± 8 | 67 ± 9 | 37 ± 7 |

[a]Rabbit red blood cells were resuspended (10% v/v) in PBS pH 7.4 and incubated at 37 °C for 120 min with DHA both in the absence or presence of quercetin. The basal content of GSSG corresponds to 3.7 ± 0.2 nmol/ml. Data are referred to ml of red blood cells. [b]Rabbit red blood cells were lysed with 1 volume of $H_2O$. The hemolysate was then diluted (1:5) with PBS pH 7.4 and incubated at 37 °C for 120 min with DHA, both in the absence or presence of quercetin. The basal content of GSSG corresponds to 0.4 ± 0.02 nmol/ml. Data are referred to ml of hemolysate. The GSSG level of the control samples did not show significant variation during the incubation time. Data are the means ± SD of three independent determinations.

formation of ascorbic acid (as measured by absorbance at 267 nm) nor a change in the absorption spectrum of quercetin (not shown). A similar observation, although using a different experimental approach, was recently made by Park and Levine.[25] The possibility that the observed effects of quercetin are causally linked to inhibition of DHA uptake also appears to be unlikely since the flavonoid fails to prevent additional effects mediated by DHA in erythrocytes, e.g. inhibition of hexokinase activity[22–24] and stimulation of ferricyanide reduction.[14,15] Indeed, as shown in Table II, a 60 min exposure to 2 mM DHA reduces hexokinase activity

TABLE II Effect of quercetin on DHA-induced inhibition of hexokinase and stimulation of ferricyanide reduction

| | Hexokinase[a] (U/g Hb) | Ferrocyanide[b] (μmol/ml RBC/30 min) |
|---|---|---|
| Control | 0.98 ± 0.1 | 1.05 ± 0.086 |
| Quercetin 50 μM | 0.92 ± 0.099 | 1.4 ± 0.7 |
| DHA 2 mM | 0.72 ± 0.05 | 4 ± 0.82 |
| DHA 2 mM + Quercetin 50 μM | 0.75 ± 0.09 | 3.9 ± 0.77 |

Rabbit red blood cells were exposed for 60 min at 37 °C to DHA both in the absence or presence of quercetin. [a]After incubations, RBC were washed twice with PBS and lysed 1:20 with $H_2O$. [b]At the end of the incubations, RBC were washed twice with 10 ml of PBS + 5 mM glucose and resuspended at 10% (v/v) with PBS containing 1 mM potassium ferricyanide for 30 minutes. Data are the means ± SD of four independent determinations.

by about 30%, both in the absence and presence of 50 μM quercetin. Under the same conditions, the 4-fold stimulation of ferricyanide reduction, an event mediated by intracellular ascorbic acid,[14–15] was also unaffected by the flavonoid (Table II).

Thus, these findings lead to the conclusions that quercetin is rapidly taken up by the RBC and has the ability to affect some processes involved in the regulation of GSH content in DHA-loaded erythrocytes. That quercetin rapidly penetrates the erythrocyte cell membrane was shown in a recent study[9] and confirmed by our findings indicating that as much as 90% of the flavonoid is found within the cell after only 5 min of exposure (not shown).

As summarized in the Introduction, quercetin displays potent antioxidant[1–4] and iron chelating properties.[1,5] In order to assess whether either one or both of these activities was responsible for its effects, the ability of the antioxidants BHT (200 μM) and DPPD (100 μM), the iron chelators 1,10-phenanthroline (25 μM) and desferoxamine mesylate (100 μM) and the oxygen radical scavengers mannitol (100 μM) and thiourea (100 μM) to prevent the GSH depletion mediated by DHA was tested in intact cells. As reported in Figure 2, this response was not affected by any of these treatments. The lack of effect of antioxidants, iron chelators and scavengers of reactive oxygen species does not appear to be due to inefficient cellular uptake since similar results were observed using cell-free extracts (not shown, see below).

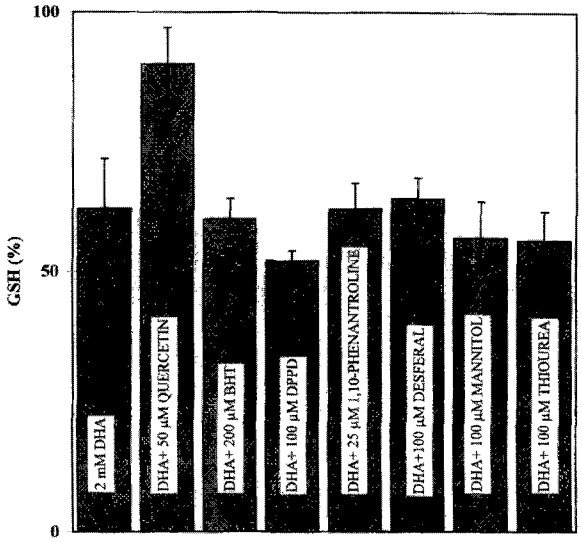


FIGURE 2 *Effect of quercetin, antioxidants, iron-chelators or oxygen radical-scavengers on DHA-induced depletion of GSH in rabbit erythrocytes.* Rabbit red blood cells were resuspended (10% v/v) in PBS and incubated at 37 °C for 60 min with 2 mM DHA in the presence of the compounds reported in the figure. The incubation with those compounds in the absence of DHA did not modify the GSH levels. Results represent the means ± SD of at least three separate experiments, each performed in duplicate.

Thus, quercetin appears to specifically spare GSH depletion induced by DHA in rabbit erythrocytes via a non-antioxidant and non-iron-chelating mechanism.

It is well established that the net amount of GSH which can be measured at a given time of exposure to a GSH-oxidizing agent is the result of dynamic processes in which the tripeptide is on the one hand consumed with concomitant

formation of GSSG whereas, on the other hand, it is being formed by the action of glutathione reductase. The same events are to be expected in erythrocytes loaded with DHA since, while various mechanisms leading to conversion of DHA to ascorbate have thus far been described,[13,14,26–29] reduction of DHA at the expense of GSH clearly takes place under the conditions utilized in the present study. Indeed, the robust decline in GSH erythrocyte content mediated by DHA (Figure 1) via a mechanism insensitive to antioxidants or iron chelators (Figure 2) could not be otherwise explained. Whether this event is mediated by chemical or enzymatic reactions is still a matter of debate since either one, or both, of these mechanisms have been reported to occur.[14,30,31] Regardless of the nature of these mechanisms, however, an inhibitory effect of quercetin on GSH-dependent DHA reductase activity would obviously result in prevention of GSH depletion. Although the reported ability of quercetin to inhibit various oxido-reductases[32,33] makes it a likely mechanism, the results illustrated in Table III in fact demonstrate that quercetin fails to inhibit DHA reductase activity. The same results were obtained regardless of whether the flavonoid was given to the red blood cells during DHA exposure or added directly to the assay mixture.

TABLE III Effect of quercetin on GSH-dependent DHA-reductase activity

| | DHA-reductase activity (%) |
|---|---|
| Control[a] | 100 |
| DHA 2 mM[a] | 95 ± 2 |
| Quercetin 50 μM[a] | 97 ± 1 |
| DHA 2 mM + Quercetin 50 μM[a] | 98 ± 2 |
| Basal[b] | 100 |
| Basal + Quercetin 50 μM[b] | 97 ± 3 |

[a]After 90 min of treatment at 37 °C with the compounds reported above, the rabbit red blood cells were washed twice with PBS and lysed 1:20 with cold $H_2O$. 20 μl aliquots of the cell-free extract were used for the DHA-reductase assay as described in Materials and Methods. The basal level of GSH-dependent DHA reductase corresponds to 10.8 ± 0.3 U/g Hb. [b]The basal mixture was composed of 0.1 M $KH_2PO_4$/0.1 M $Na_2HPO_4$ pH 7.5, 1 mM GSH, 0.3 mM NADPH, 2 mM DHA, 1.2 U/ml GSSG reductase, 20 μl hemolysate. Quercetin was added directly to the assay mixture. The data are the means ± SD of at least five independent determinations.

The above findings that quercetin does not affect the reduction of ferricyanide (Table II) provide an additional indication that the flavonoid does not affect recycling of DHA to ascorbate. It is well established that ferricyanide, while not being taken up by the cells, effectively oxidizes intracellular ascorbic acid via a transmembrane oxidoreductase enzyme;[34,35] formation of ferrocyanide therefore provides an indirect measure of intracellular ascorbic acid content.[13–15] As a consequence, the lack of effect of quercetin on this response mediated by DHA loading, strongly suggests that the flavonoid does not affect DHA reductase activity.

Thus, these findings demonstrate that the effects of quercetin are downstream to GSH oxidation resulting from DHA recycling to ascorbate and this notion is consistent with the results of experiments using cell-free extracts. Under these conditions, (DHA 2 mM) produced a robust and extremely fast depletion of GSH that was maximal after only 5 min and the residual GSH levels remained unchanged during an additional 120 min of incubation (Figure 3). Interestingly quercetin (50 μM), while not affecting the early GSH consumption, promoted a slow and progressive formation of GSH. Consistent with these findings are the results reported in Table I which indicate that, under similar experimental conditions, the GSSG levels were basically identical at 30 or 120 min of incubation and decreased in a time-dependent fashion in the presence of quercetin. Other flavonoids (rutin or kaempferol) failed to promote GSH formation when used at the same concentration at which quercetin was shown to be effective (Figure 3). The use of hemolysates presents the important advantage of allowing direct access of DHA and other substances to substrates and/or enzymes. Thus, while rutin and kaempferol are more hydrophilic than quercetin,[9] their lack of effect

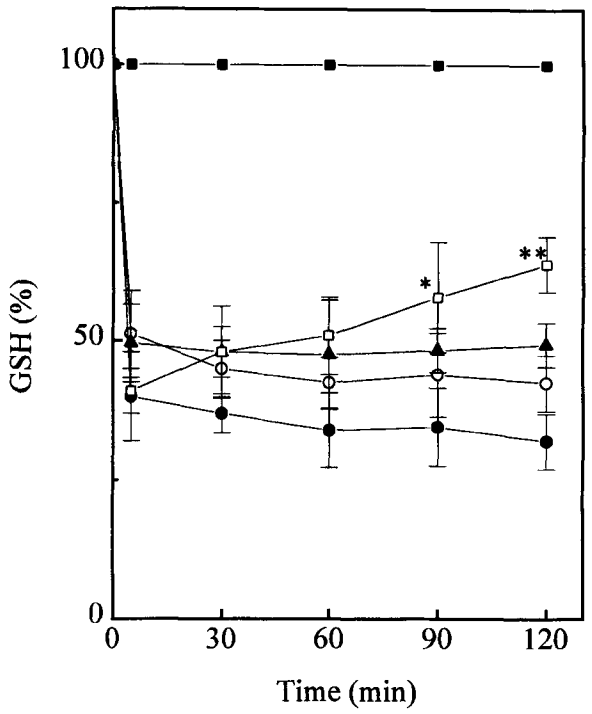


FIGURE 3 *Effect of quercetin, rutin and kaempferol on GSH levels in rabbit erythrocyte cell-free-extract exposed to 2 mM DHA.* Rabbit red blood cells were lysed with 1 volume of $H_2O$. The hemolysate was then diluted with PBS and incubated at 37 °C for 120 min with 2 mM DHA (●); 2 mM DHA/50 μM quercetin (□); 2 mM DHA/50 μM rutin (○); 2 mM DHA/50 μM kaempferol (▲). GSH levels were measured as reported in Materials and Methods. Incubations with quercetin, rutin and kaempferol alone did not modify GSH levels. The control sample is represented by (■). All values are the means ± SD of at least five independent determinations. $^*p < .05$; $^{**}p < .01$.

in this system cannot be attributed to the inability of these compounds to reach critical concentrations required to promote GSH formation and lead to the conclusion that the action of quercetin is not simply mediated by its flavonoid basic structure.

Along the same lines, the observation that BHT (200 μM), DPPD (10–100 μM), 1,10-phenanthroline (25–100 μM), desferoxamine mesylate (50–100 μM), mannitol (100 μM-1mM) and thiourea (10–100 μM) failed to prevent the early GSH depletion and to promote GSH formation (not shown) in hemolysates treated with 2 mM DHA, demonstrates that this lack of effect is not due to inefficient cellular uptake but, rather, that the antioxidant, iron-chelating and radical-scavenging properties of quercetin[1–5] are not involved in the action of this molecule on GSH formation (see above).

The experimental results thus far presented demonstrate that quercetin spares the GSH depletion induced by DHA in rabbit erythrocytes by acting downstream to DHA uptake and strongly suggest that the effects of the flavonoid are more likely to depend on stimulation of GSH formation (Figure 3 and Table I) than on inhibition of GSH oxidation.

The possibility that quercetin mediates chemical reduction of intracellular GSSG was therefore investigated. In these experiments, however, a 60 min incubation (PBS, pH 7.4) of increasing concentrations of GSSG (0.2–2 mM) in the presence of 50 μM quercetin, neither promoted formation of detectable levels of GSH nor caused a

change in the absorption spectrum (200–430 nm) of quercetin (not shown).

Thus, these results indicate that the flavonoid, while promoting reduction of GSSG in hemolysates in which depletion of GSH was caused by DHA (Figure 3), fails to directly reduce GSSG in a saline solution. As a consequence, some component(s) of the hemolysate appear(s) to be necessary in order to allow quercetin to mediate its effects. The observation that addition of exogenous GSSG to a hemolysate promoted a time-dependent formation of GSH and that this response was significantly increased by quercetin (50 μM, Figure 4) is consistent with this notion.

In conclusion, the results presented in this study demonstrate that quercetin spares the depletion of GSH induced by DHA and strongly suggest that this response is mediated by a mechanism involving activation of some process resulting in the enzymatic reduction of GSSG to GSH.

It is well established that human erythrocytes take up extracellular DHA on the glucose transporter[11–14] and convert it to ascorbate.[13–16,26–31] Once DHA has been reduced to ascorbate, the molecule is trapped within the cells, because ascorbate is not an effective substrate for this transporter.[12] This ability to regenerate ascorbate from DHA may contribute to maintenance of ascorbate concentrations and to removal of DHA produced in areas of oxidative stress in the vascular bed.

DHA is present in very low amounts under physiological conditions, although elevated levels of DHA are found in the tissues of some individuals during the aging process[36] and in patients affected by diabetes,[37,38] rheumatoid arthritis,[39] cataract[40–42] and in general in conditions characterized by deficiencies in intracellular reductants (i.e. GSH) and/or a decreased ability to reduce DHA to ascorbic acid.

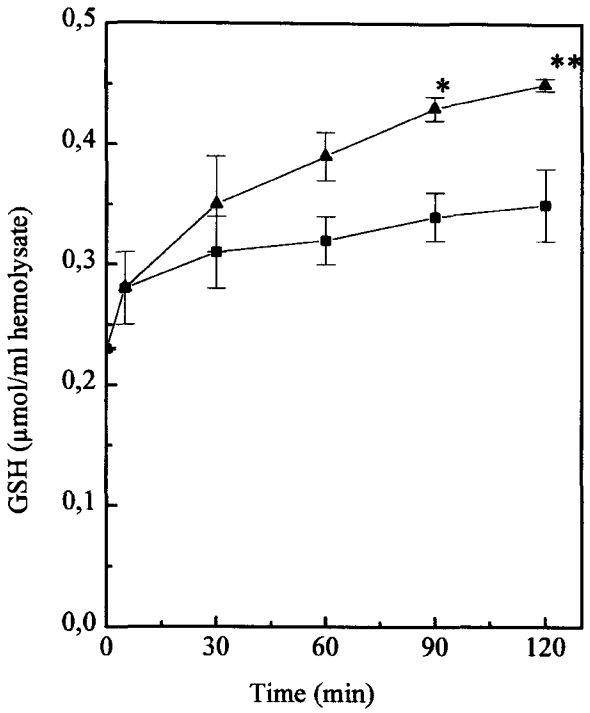


FIGURE 4 *Effect of quercetin on GSSG reduction in rabbit erythrocyte cell-free extract.* Rabbit red blood cells were lysed with 1 volume of $H_2O$. The hemolysate was then diluted with PBS and incubated at 37 °C in the presence of 0.2 mM GSSG (■) or 0.2 mM GSSG/50 μM quercetin (▲). The data are the means ± SD of at least three independent determinations. *$p < .05$; **$p < .01$.

Thus, the results presented in this study lead us to propose quercetin as a potential therapeutic agent for all of the above pathological conditions in which both the antioxidant properties of the flavonoid and its ability to promote GSH formation should mediate beneficial effects.

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