Effect of Dietary Vitamin E Supplementation on Liver Oxidative Damage in Rats with Water-Immersion Restraint Stress

Yoshiji Ohta1, Koji Yashiro1, Koji Ohashi2, Yosuke HoriKoshi3, Chiaki Kusumoto1, Tatsuya Matsura3 and Kenji Fukuzawa4

1 Department of Chemistry, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan
2 Department of Clinical Biochemistry, Faculty of Medical Technology, Fujita Health University School of Health Sciences, Toyoake, Aichi 470-1192, Japan
3 Division of Medical Biochemistry, Department of Pathophysiological and Therapeutic Science, Tottori University, Yonago, Tottori 683-8503, Japan
4 Faculty of Pharmacy, Yasuda Women’s University, Asaminami-ku, Hiroshima 731-0153, Japan

(Received October 20, 2014)

Summary We examined how dietary supplementation of vitamin E protects against liver oxidative damage in rats with water-immersion restraint stress (WIRS). Before WIRS exposure, rats received a normal diet (ND) or vitamin E-supplemented diet (VESD) (500 IU α-tocopherol/kg diet) at a mean dose of 15 g/animal/d for 4 wk. The two diet groups had serum transaminases and lactate dehydrogenase activities and adrenocorticotropic hormone, corticosterone, and glucose levels to a similar extent. VESD-fed rats had higher liver α-tocopherol concentrations and lower liver ascorbic acid, total coenzyme Q9 (CoQ9), reduced CoQ9, reduced CoQ10, and lipid peroxide (LPO) concentrations than ND-fed rats. When the two diet groups were exposed to 6 h of WIRS, the serum liver cell damage index enzyme activities increased more greatly in ND-fed rats than in VESD-fed rats but the serum stress marker levels increased to a similar extent. The WIRS exposure caused no change in liver LPO concentration with the further increase in liver α-tocopherol concentration in VESD-fed rats but increased liver LPO concentration without changing liver α-tocopherol concentration in ND-fed rats. Upon the WIRS exposure, liver reduced glutathione concentration decreased with the further decrease in liver ascorbic acid concentration in VESD-fed rats and those concentrations decreased in ND-fed rats. The WIRS exposure recovered the decreased liver total CoQ9 and reduced CoQ9 concentrations in VESD-fed rats but decreased liver total CoQ9, reduced CoQ9, and reduced CoQ10 concentrations in ND-fed rats. These results indicate that dietary vitamin E supplementation protects against liver oxidative damage without affecting the stress response in rats with WIRS.

Key Words water-immersion restraint stress, rat liver, dietary vitamin E supplementation, antioxidants, oxidative damage

Water-immersion restraint stress (WIRS), which is characterized with both psychological stress and physical stress, is widely used to induce reproducible stress ulcers in experimental animals (1). Reactive oxygen species (ROS) and nitric oxide (·NO) generation, lipid peroxidation, and neutrophil infiltration in the gastric mucosal tissue play a critical role in the pathogenesis of WIRS-induced gastric mucosal lesions (2–5).

Iwai et al. (6) have reported that, in rats with 6 h of WIRS, the level of liver lipid peroxide (LPO), which is generated via ROS, increases with concomitant increases in the levels of serum LPO and liver cell damage markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) activities and that pre-administration of gamazumi fruit juice or (−)-epigallocatechin gallate, each of which possesses antioxidant activity, for 2 wk attenuates all these stress-induced changes. Furthermore, the same authors have reported that not only an increase in LPO level but also decreases in superoxide dismutase (SOD) activity and reduced glutathione (GSH) level occur without changes in catalase and glutathione peroxidase activities in the liver of rats with 6 h of WIRS and that pre-administration of gamazumi crude extract possessing antioxidant activity to rats with 6 h of WIRS for 2 wk attenuates the increased LPO level and the decreased SOD activity and GSH level in the liver (7). We have reported that, in the liver of rats exposed to WIRS over a 6-h period, cell damage occurs before the appearance of oxidative stress with disruption of antioxidant defense systems associated with decreased ascorbic acid (vitamin C) and GSH levels and SOD activity and enhanced lipid peroxidation, enhanced ·NO generation, and neutrophil infiltration in the tissue, although the vitamin E level does not change in the liver tissue (8). We have also shown that a single pre-administration of L-ascorbic acid protects against oxidative damage in the liver of rats with 6 h of WIRS by attenuating disrupted antioxidant defense systems and enhanced lipid peroxidation, enhanced ·NO generation, and neutrophil infiltration in
the liver tissue (9). In addition, our previous report has shown that α-tocopherol pre-administered once to rats with 6 h of WIRS attenuates liver cell damage, which is judged from the changes in serum ALT, AST, and LDH levels in the serum and liver tissue (10). Thus, it has been suggested that vitamin E, in addition to vitamin C, could play an important role in protecting against oxidative damage in the liver of rats with WIRS.

Vitamin E functions as a chain-breaking antioxidant for lipid peroxidation in cell membranes and also as a scavenger of ROS such as singlet oxygen and hydroxyl radical (11). Coenzyme Q (CoQ), a quinone derivative with an isoprenoid tail, not only exerts antioxidant action by itself but also supports the chain-breaking antioxidant action of vitamin E by reducing the vitamin E radical to vitamin E in the membranes (12–15).

It has been reported that dietary supplementation of vitamin E to rats for 6 wk causes increases in serum and liver vitamin E levels and liver reduced coenzyme Q9 (CoQ9red) and reduced CoQ10 (CoQ10red) levels without affecting the activities of liver antioxidant enzymes such as SOD, catalase, and glutathione peroxidase (16). Recently, we have reported that exposure of rats fed a normal diet and vitamin E-depleted diet for 4 wk to 6 h of WIRS reduces CoQred and CoQ10red levels in the liver tissue and that this vitamin E depletion enhances liver oxidative damage in rats with 6 h of WIRS through disruption of the liver non-enzymatic antioxidant defense system, although the vitamin E depletion causes increases in liver ascorbic acid and GSH levels in unstressed rats (17). However, there is no information on whether dietary vitamin E supplementation protects against WIRS-induced oxidative damage in the liver of rats.

In the present study, therefore, we examined the effect of 4-wk dietary vitamin E supplementation on liver oxidative damage in rats with 6 h of WIRS. It has been reported that oral supplementation of vitamin E for 28 d protects against gastric mucosal damage by reducing stress response, judging from the plasma levels of stress markers such as adrenocorticotropic hormone (ACTH), corticosterone, adrenaline, and noradrenaline in rats with 3.5 h of WIRS (18, 19). It is known that exposure of rats to 6 h of WIRS increases not only the serum or plasma levels of stress markers such as ACTH, corticosterone, adrenaline, noradrenaline, and glucose levels but also serum ascorbic acid and LPO levels (9, 10, 17, 20). It is also known that a single oral administration of vitamin E to rats with 6 h of WIRS has no effect on the serum levels of stress markers such as ACTH, corticosterone, and glucose but attenuates increased serum ascorbic acid and LPO levels (10, 21). Therefore, we examined the effect of dietary vitamin E supplementation on serum ACTH, corticosterone, glucose, ascorbic acid, and LPO concentrations in rats with WIRS.

MATERIALS AND METHODS

Materials. Chromatographically pure oxidized CoQ9 (CoQ9ox) and oxidized CoQ10 were kindly provided by Eisai Co. (Tokyo, Japan). l-Ascorbic acid, corticosterone, α,α′-dipyridyl, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB reagent), ethylenediaminetetraacetic acid (EDTA), GSH, 2-thiobarbituric acid, RRR-α-tocopherol (authentic standard) and RRR-δ-tocopherol (internal standard) used for vitamin E determination, and other chemicals were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). All chemicals used were of reagent grade and were not further purified.

Animals. Male Wistar rats aged 3 wk were purchased from Japan SLC Inc. (Hamamatsu, Japan). The animals were housed in cages in a ventilated animal room with controlled temperature (23 ± 2°C) and relative humidity (55 ± 5%) with 12 h of light (7:00 to 19:00). The animals were maintained with free access to rat chow; AIN-76A diet (Research Diets Inc., New Brunswick, NJ), and tap water for 1 wk. All animals received humane care in compliance with the Guidelines of the Management of Laboratory Animals in Fujita Health University. The animal experiment was approved by Institutional Animal Care and Use Committee (Protocol No. M14–02).

Feeding of normal diet and vitamin E-supplemented diet. Three-week-old rats were administered with a normal diet (ND) or vitamin E-supplemented diet (VESD) for 4 wk. The above-described AIN-76A diet was used as the ND and the composition of the ND was as follows: casein, 20.0%; cornstarch, 50.0%; cellulose, 5.0%; corn oil, 5.0%; mineral mixture, 3.5%; vitamin mixture, 1.0%; dl-methionine, 0.3%. This normal diet contained α-tocopherol acetate (50 IU/kg diet). The vitamin mixture did not contain vitamin C. The VESD was prepared by adding α-tocopherol acetate to the normal diet. This diet contained 500 IU α-tocopherol acetate per kg diet. The mean amount of each diet consumed per day was 15 g/animal, i.e., 7.5 IU/animal.

Induction of WIRS. WIRS was induced in rats according to our previous reports (3, 4, 8–10, 17, 20, 21). Namely, before the onset of WIRS, 7-wk-old rats fed the ND or VESD were starved for 24 h, but they were allowed free access to water. The fasted rats were restrained in wire cages and immersed up to the depth of the xiphoid process in a 23°C water bath for 6 h. Rats without WIRS were starved for 24 h and allowed free access to water in the same manner as rats with WIRS.

Determinations of serum and liver components. All rats were weighed and then sacrificed under pentobarbital anesthesia, at which time blood was collected from the inferior vena cava. Serum was obtained from the collected blood by centrifugation. Immediately after sacrifice, each liver was perfused with ice-cold 0.9% NaCl through the portal vein and isolated. The isolated liver was washed in ice-cold 0.9% NaCl, wiped on a paper filter, and then weighed. The collected serum and livers were stored at −80°C until use. Serum ACTH was assayed using a commercial kit, ACTH ELA kit (Phoenix Pharmaceutical Inc., Burlingame, CA). Serum corticosterone was measured by the fluorometric method (22) using authentic corticosterone as a standard. Serum glucose was assayed using a commercial kit, Glucose-CII Test Wako. Serum alanine aminotransferase (ALT)
Supplemental Vitamin E and Stress-Induced Liver Oxidative Damage

Supplemental Vitamin E and Stress-Induced Liver Oxidative Damage

and aspartate aminotransferase (AST) were assayed using a commercial kit, Transaminase C II-Test Wako. Serum lactate dehydrogenase (LDH) was assayed using a commercial kit, LDH-Test Wako. These assay kits were obtained from Wako Pure Chemical Ind., Ltd. Serum α-tocopherol was assayed by the high-performance liquid chromatographic method with electrochemical detection using δ-tocopherol as an internal standard as described in our previous report (23). Serum ascorbic acid was assayed by the α,α′-dipyrindyl method (24) using L-ascorbic acid as a standard. Serum LPO was assayed by the fluorometric thiobarbituric acid method (25) using tetramethoxypropane as a standard. The concentration of serum LPO is expressed as that of malondialdehyde (MDA) equivalents.

Liver protein was assayed using a commercial Rapid Protein Assay kit (Wako Pure Chemical Ind., Ltd.). Bovine serum albumin was used as a standard in this protein assay.

Statistical analysis. All results obtained are expressed as means ± SD. The statistical analyses of the results were performed using a computerized statistical package (StatView). Each mean value was compared by two-way analysis of variance (ANOVA) and Fisher’s protected least significance (PLSD) for multiple comparisons as the post-hoc test. The significance level was set at \( p < 0.05 \).

RESULTS

There were no significant differences in serum ACTH, corticosterone, glucose, and ascorbic acid concentrations between ND rats (ND group) and VESD rats (VESD group) without stressful conditions (Fig. 1). Exposure to 6 h of WIRS caused significant increases in serum ACTH, corticosterone, glucose, and ascorbic acid concentrations in the ND and VESD group (Fig. 1). Although there were no differences in the increased serum ACTH, corticosterone, and glucose concentrations between the two stressed groups, the increased serum ascorbic acid concentration was significantly lower in the stressed VESD group than in the stressed ND group (Fig. 1).

There were no significant differences in serum ALT, AST, and LDH activities between ND-fed and VESD-fed rats without stressful conditions (Fig. 2). Exposure to 6 h of WIRS caused significant increases in serum ALT,
AST, and LDH activities in the ND and VESD groups but the increased serum ALT, AST, and LDH activities were significantly lower in the VESD group than in the ND group (Fig. 2).

There were no significant differences in body weight and liver weight between the ND and VESD groups before the application of stress (Fig. 3). Exposure to 6 h of WIRS had no significant effect on body weight or liver weight in the ND and VESD groups (Fig. 3).

Serum α-tocopherol concentration was significantly higher in the VESD group than in the ND group without stressful conditions; the serum α-tocopherol concentrations the VESD group was 1.9-fold higher than that in the ND group (Fig. 4A). Exposure to 6 h of WIRS had no significant effect on serum α-tocopherol concentrations in the ND or VESD groups (Fig. 4A). Before the stressful conditions, serum LPO concentration in the VESD group was significantly lower in that in the ND group (Fig. 4B). Exposure to 6 h of WIRS caused a significant increase in serum LPO concentration in the ND and VESD groups, but the increased serum LPO concentration in the VESD group with WIRS was not significantly different from that in the ND group without WIRS, although the stressed VESD group had significantly higher serum LPO concentration than the unstressed VESD group (Fig. 4B). Liver α-tocopherol concentration was significantly higher in the VESD group than in the ND group before the stressful conditions; the liver α-tocopherol concentration in the VESD group was 5.9-fold higher than that in the ND group (Fig. 4C). Exposure to 6 h of WIRS did not affect the liver α-tocopherol concentration in the ND group but further increased the liver α-tocopherol concentration in the VESD group significantly; the liver α-tocopherol concentration in the stressed VESD group was 9.1-fold higher than that in the stressed ND group (Fig. 4C). Liver LPO concentration was significantly lower in the VESD group than in the ND group under stress-free conditions (Fig. 4D). Exposure to 6 h of WIRS caused a significant increase in liver LPO concentration in the ND group, but no increase in liver LPO concentration in the VESD group (Fig. 4D). In addition, the liver LPO concentration in the stressed VESD group was not significantly different from that in the unstressed VESD group and was approximately 60% of that in the stressed ND group (Fig. 4D).

Under stress-free conditions, the VESD group had a significantly lower liver ascorbic acid concentration than the ND group (Fig. 5A). Exposure to 6 h of WIRS reduced the liver ascorbic acid concentration in the ND group significantly and further reduced the liver ascorbic acid concentration in the VESD group with WIRS, although the liver ascorbic acid concentration in the
stressed ND group was significantly higher than that in the stressed VESD group (Fig. 5A). Under stress-free conditions, there was no significant difference in liver GSH concentration between the ND and NDVE groups (Fig. 5B). Exposure to 6 h of WIRS reduced the liver GSH concentration in the ND and VESD groups significantly, but the liver GSH concentration in the stressed ND group was significantly higher than that in that in the stressed VESD group (Fig. 5B). Without stressful conditions, the VESD group had a significantly lower liver CoQ10red concentration than the ND group (Fig. 4C). Exposure to 6 h of WIRS reduced the liver CoQ10red concentration in the ND group significantly but had no significant effect on the liver CoQ10red concentration in the VSED group, although the liver CoQ10red concentration in the stressed VESD group was significantly higher than that in the stressed ND group (Fig. 5C).

Under stress-free conditions, total CoQ9red and CoQ9ored concentrations and the ratio of CoQ9red to total CoQ9 in the liver of the VESD group were significantly lower than those in the liver of the ND group, although there was no significant difference in liver CoQ9ox concentration between the two groups (Fig. 6). Exposure of rats in the ND group to 6 h of WIRS reduced the liver total CoQ9red, CoQ9red, and CoQ9ored concentrations significantly, although there was no significant change in the ratio of CoQ9red to total CoQ9 in the liver (Fig. 6). Exposure of rats in the VESD group to 6 h of WIRS...
increased the liver total CoQ9 red, CoQ9 red, and CoQ9ox concentrations significantly and further significantly decreased the ratio of CoQ9 red to total CoQ9 in the liver, although the liver total CoQ9 red and CoQ9 red concentrations in the stressed VESD group were not significantly different from those in the unstressed ND group (Fig. 6).

DISCUSSION

The present study has clearly shown that dietary vitamin E supplementation protects against hepatic oxidative damage in rats exposed to WIRS for 6 h without affecting the stress response. In the present study, ND-fed rats with 6 h of WIRS showed increases in the serum levels of stress makers such as ACTH, corticosterone, and glucose, as shown in our previous reports (9, 10, 17, 21). In rats fed the VESD for 4 wk, exposure to 6 h of WIRS also increased the serum levels of ACTH, corticosterone, and glucose. The increased serum ACTH, corticosterone, and glucose concentrations in VESD-fed rats with WIRS were not significantly different from those in ND-fed rats with WIRS. These results indicate that dietary vitamin E supplementation for the period of 4 wk does not affect the stress response via the hypothalamus-pituitary-adrenal axis or the sympathetic-adrenomedullary system in rats with 6 h of WIRS. In contrast, Aziz Ibrahim et al. (18) and Mohd Fahami et al. (19) have shown that when rats pre-administered α-tocopherol (60 mg/kg body weight) through oral intubation once daily for 28 d are exposed to WIRS for 3.5 h, WIRS-induced increases in plasma ACTH, corticosterone, adrenaline, and noradrenaline concentrations are reduced, although WIRS-induced gastric mucosal damage is attenuated at the same time. We have shown that the same dietary vitamin E supplementation as used in the present study attenuates gastric mucosal lesions in rats with 6 h of WIRS (30). Thus, there is a clear difference in the effect of vitamin E on stress response in rats with WIRS between dietary vitamin E pre-administration and repeated vitamin E pre-administration through oral intubation for the same period. However, the reason for this difference is unclear at present. Accordingly, further study is needed to clarify the vitamin E administration route-related difference in the effect of long-term vitamin E pre-administration on stress response in rats with WIRS.

In the present study, there was no difference in serum ascorbic acid concentration under stress-free conditions between ND-fed and VESD-fed rats. Exposure to 6 h of WIRS caused a significant increase in serum ascorbic acid concentration in ND-fed rats, as reported previously (9, 17). A significant increase in serum ascorbic acid concentration was also found in VESD-fed rats with 6 h of WIRS. However, the increase in serum ascorbic acid concentration was significantly less in VESD-fed rats with WIRS than in ND-fed rats with WIRS. We have suggested that an increase in ascorbic acid level in the serum of rats with WIRS could be, at least in part, due to the release of ascorbic acid from the liver tissue (9). Nakano and Suzuki (31) also have suggested that, in rats with restraint stress, an increase in ascorbic acid level in the serum is derived from the liver tissue. In the present study, however, WIRS-induced liver cell damage was less in VESD-fed rats than in ND-fed rats, as described below. Therefore, it is assumed that the less damaged
liver of VESD-fed rats with WIRS does not release ascorbic acid accumulated in the liver into the bloodstream more easily than the damaged liver of ND-fed rats with WIRS. In addition, it has been shown in rats with a single administration of corticotropin (ACTH) that ascorbic acid is released from the adrenal gland into the bloodstream (32). It has also been shown that human adrenal glands secrete ascorbic acid in response to ACTH (33). As described above, there was no difference in the serum level of ACTH between ND-fed and VESD-fed rats. Therefore, it is suggested that vitamin E supplementation to rats before the onset of WIRS could suppress the release of ascorbic acid from the liver into the bloodstream rather than the ACTH-mediated release of ascorbic acid from the adrenal gland into the bloodstream under WIRS exposure, resulting in a less increased ascorbic acid level in the serum.

In the present study, serum ALT, AST, and LDH activities, indices of liver cell damage, in rats fed the VESD for 4 wk were not significantly different from those in rats fed the ND for the same period. These results indicate that 4-wk dietary vitamin E supplementation has no effect on the liver tissue integrity of rats. Exposure of ND-fed rats to 6 h of WIRS caused apparent increases in serum ALT, AST, and LDH activities, as reported previously (6–10). The stress-induced increases in serum ALT, AST, and LDH activities were attenuated in VESD-fed rats. These results indicate that dietary vitamin E supplementation affords an efficient protection against WIRS-induced liver cell damage in rats.

There were no differences in body weight and liver weight between ND- and VESD-fed rats under feeding of each diet at a daily mean dose of 15 g per animal for 4 wk. These results indicate that VESD-fed rats have the same growth as ND-fed rats. Exposure to 6 h of WIRS did not affect the body weight or liver weight in VESD-fed rats or in ND-fed rats. Our previous report showed that apparent necrotic changes did not occur in the liver tissue of rats with 6 h of WIRS, although significant, but not so high, increases in serum ALT and AST activities occurred in the liver tissue (8), as found in the present study. Taking this finding and the WIRS-induced increases in serum ALT, AST, and LDH levels found in the present study into consideration, it seems that liver damage occurring in ND-fed rats with 6 h of WIRS is not so severe. Therefore, it can be thought that WIRS exposure has no effect on body weight or liver weight in VESD-fed or ND-fed rats.

When serum and liver \( \alpha \)-tocopherol concentrations in rats fed VESD for 4 wk were compared with those in rats fed ND under stress-free conditions, the serum and liver \( \alpha \)-tocopherol concentrations were significantly higher in VESD-fed rats than in ND-fed rats. In addition, VESD-fed rats showed a more marked change in \( \alpha \)-tocopherol concentration in the liver tissue than in the serum. Exposure to 6 h of WIRS did not affect serum \( \alpha \)-tocopherol concentration either in ND-fed rats, as reported previously (10, 17), or in VESD-fed rats. In contrast, exposure to 6 h of WIRS did not cause any change in serum \( \alpha \)-tocopherol concentration in ND-fed rats, as reported previously (10, 17), but caused further increase in liver \( \alpha \)-tocopherol concentration in VESD-fed rats. The reason why the liver \( \alpha \)-tocopherol concentration was higher in VESD-fed rats with WIRS than in VESD-fed rats without stress is unclear at present. However, maintaining the largely accumulated \( \alpha \)-tocopherol in the liver of VESD-fed rats under WIRS exposure to induce oxidative stress, as described below, may result in further increase in the liver \( \alpha \)-tocopherol concentration. Ham and Liebler (34) have shown that when the liver of rats fed either a standard diet or a diet supplemented with vitamin E (2,000 IU \( \alpha \)-tocopherol acetate/kg diet) for 3.5–4.5 wk is perfused with a medium containing tert-butyldihydroperoxide, an agent to induce oxidative stress, for 10 min, a further increase in the increased \( \alpha \)-tocopherol content occurs with a reduction of enhanced lipid peroxidation in the perfused liver of rats with vitamin E supplementation, while no change in \( \alpha \)-tocopherol content occurs with enhanced lipid peroxidation in the perfused liver of rats without vitamin E supplementation.

In the present study, VESD-fed rats had lower serum and liver LPO concentrations than ND-fed rats under no stress-free conditions. Thus, dietary supplemental vitamin E was found to exert antioxidant action under stress-free conditions. Exposure to 6 h of WIRS caused a significant increase in serum LPO concentration in ND-fed rats, as reported previously (8–10, 17, 21). The stress exposure caused a significant increase in serum LPO concentration in VESD-fed rats but the increased serum LPO concentration was significantly less than that in ND-fed rats. Upon exposure to 6 h of WIRS, a significant increase in liver LPO concentration occurred in ND-fed rats, as reported previously (6–10, 17). However, the stress exposure caused no significant increase in liver LPO concentration in VESD-fed rats. Thus, dietary vitamin E supplementation was found to attenuate WIRS-induced increases in serum and liver LPO concentrations. Such a higher antioxidant effect of dietary vitamin E supplementation in rats with and without WIRS could be due to a large accumulation of supplemental vitamin E in the liver.

Ascorbic acid is known to support the chain-breaking antioxidant function of vitamin E for lipid peroxidation through regeneration of vitamin E from its radical form, resulting in the conversion of ascorbic acid to its oxidized form, dehydroascorbic acid (35). GSH is known to spare vitamin E by lowering the rate of the initiation of lipid peroxidation in the bilayer phase (36). GSH is also known to maintain the antioxidant action of ascorbic acid through regeneration of ascorbic acid from its oxidized form (37). It has been shown that the antioxidant role of ubiquinol-10, i.e., CoQ\(_{10}\)red, involves an interaction with \( \alpha \)-tocopherol (13). It has also been shown that ubiquinol-10, i.e., CoQ\(_{10}\)red, supports the chain-breaking antioxidant function of \( \alpha \)-tocopherol for lipid peroxidation by regeneration of \( \alpha \)-tocopherol from its oxidized form (38). In the present study, liver ascorbic acid and CoQ\(_{10}\)red concentrations in VESD-fed rats were significantly lower than those in ND-fed rats under stress-free conditions.
conditions. These results suggest that ascorbic acid and CoQ_{10,red} fed the liver of VESD-fed rats could contribute to maintenance of \( \alpha \)-tocopherol accumulated in the liver, resulting in consumption of ascorbic acid and CoQ_{10,red} in the tissue. Exposure to 6 h of WIRS decreased liver ascorbic acid, GSH, and CoQ_{10,red} concentrations in ND-fed rats, as shown in our previous report (17). Taking these results and the above-described changes in \( \alpha \)-tocopherol and LPO concentrations in the liver of ND-fed rats with WIRS into consideration, it can be thought that ascorbic acid, GSH, and CoQ_{10,red} in the liver of ND-fed rats are used to protect the liver tissue against WIRS-induced oxidative stress through maintenance of vitamin E in the liver tissue, resulting in decreases in ascorbic acid, GSH, and CoQ_{10,red} concentrations in the liver tissue. The WIRS exposure caused a decrease in liver GSH concentration and further decrease in liver ascorbic acid concentration but had no significant effect on decreased liver CoQ_{10,red} concentration in VESD-fed rats. Taking these results and the above-described changes in \( \alpha \)-tocopherol and LPO concentrations in the liver of VESD-fed rats with WIRS into consideration, it can be thought that ascorbic acid and GSH rather than CoQ_{10,red} in the liver of VESD-fed rats are used to protect the liver tissue against WIRS-induced oxidative stress through maintenance of vitamin E accumulated in the liver tissue, resulting in a further decrease in ascorbic acid concentration and a decrease in GSH concentration in the liver tissue.

Although CoQ_{9} and CoQ_{10} are mainly present in the liver of rats, CoQ_{9} is the predominant form in the liver (15). In the present study, CoQ_{9,red} concentration was approximately 6-fold higher than CoQ_{10,red} concentration in the liver of ND-fed rats. In addition, it has been demonstrated in isolated rat hepatocytes that CoQ_{9,red}, together with \( \alpha \)-tocopherol, constantly acts as an antioxidant when oxidative stress is induced with a hydrophilic radical initiator, 2,2′-azobis (2-aminopropane) dihydrochloride (39). Therefore, changes in total CoQ_{9}, CoQ_{9,red}, and CoQ_{9,ox} concentrations and the ratio of CoQ_{9,red} to total CoQ_{9} were further examined in the liver of ND-fed and VESD-fed rats with and without 6 h of WIRS. VESD-fed rats showed significant decreases in total CoQ_{9} and CoQ_{9,red} concentrations and the ratio of CoQ_{9,red} to total CoQ_{9} in the liver tissue as compared with those in ND-fed rats under stress-free conditions. Taking these results and the above-described changes in liver \( \alpha \)-tocopherol and LPO concentrations in VESD-fed rats into consideration, it can be thought that CoQ_{9,red} in the liver of VESD-fed rats is used to maintain \( \alpha \)-tocopherol accumulated in the liver tissue and to protect the accumulated \( \alpha \)-tocopherol against its oxidation in the liver tissue, resulting in decreases in total CoQ_{9} and CoQ_{9,red} concentrations and the ratio of CoQ_{9,red} to total CoQ_{9} in the liver tissue. In addition, it seems that the reduction of CoQ_{9,red} concentration in the liver is, at least in part, due to its metabolism after its conversion to CoQ_{9,ox}, because both total CoQ_{9} and CoQ_{9,red} concentrations were reduced in the liver of VESD-fed rats, as described above. In contrast, Galleano and Puntarulo (16) have reported that when rats are given a diet supplemented with vitamin E (200 IU \( \alpha \)-tocopherol acetate/kg diet) for 6 wk, about 2.7-fold increase in liver \( \alpha \)-tocopherol content occurs with about 1.5- and 1.9-fold increases in CoQ_{9,red} and CoQ_{10,red} contents, respectively, as compared with rats fed a standard diet containing vitamin E (50 IU \( \alpha \)-tocopherol acetate/kg diet) for the same period. In the present study, liver \( \alpha \)-tocopherol concentration was 5.9-fold higher in rats fed a diet supplemented with vitamin E (500 IU \( \alpha \)-tocopherol acetate/kg diet) for 4 wk than in rats fed the ND containing vitamin E (50 IU \( \alpha \)-tocopherol acetate/kg diet) for the same period. From this finding, there seems to be a possibility that the difference in the ratio of increased \( \alpha \)-tocopherol level in the liver of rats with dietary vitamin E supplementation between the present study and the study reported by Galleano and Puntarulo (16) causes a reverse effect on liver CoQ_{9,red} and CoQ_{9,ox} levels. Upon exposure of ND-fed rats to 6 h of WIRS, total CoQ_{9}, CoQ_{9,red}, and CoQ_{9,ox} concentration were significantly reduced without any change in the ratio of CoQ_{9,red} to total CoQ_{9} in the liver. These results suggest that CoQ_{9,red} could not sufficiently contribute to the antioxidant action of \( \alpha \)-tocopherol in the liver of ND-fed rats with WIRS, resulting in increased lipid peroxidation in the tissue. These results also suggest that CoQ_{9,red} may be degraded with its constant conversion to CoQ_{9,ox} in the liver of ND-fed rats with WIRS. It has been reported that ubiquinone (CoQ) is degraded in beef heart submitochondrial particles during lipid peroxidation, resulting in reduction of its content, although its degraded product is unknown (40). In contrast, upon exposure of VESD-fed rats to 6 h of WIRS, decreased total CoQ_{9} and CoQ_{9,red} concentrations in the liver recovered to the levels of unstressed ND-fed rats, while a significant increase in CoQ_{9,ox} concentration and further decrease in the ratio of CoQ_{9,red} to total CoQ_{9} in the liver. These results suggest that the recovered CoQ_{9,red} could sufficiently contribute to the antioxidant action of \( \alpha \)-tocopherol in the liver of VESD-fed rats with WIRS, resulting in no increase in lipid peroxidation in the tissue. This recovery of decreased CoQ_{9,red} concentration in VESD-fed rats with WIRS may be an adaptation to WIRS-induced oxidative stress, which may lead to protection of the liver tissue against enhanced lipid peroxidation through a further increase in increased \( \alpha \)-tocopherol level in the liver tissue. However, the mechanism by which decreased CoQ_{9,red} concentration in the liver of VESD-fed rats recovers upon exposure to WIRS is unknown at present. Therefore, further study is needed to clarify this mechanism.

In conclusion, the results obtained from the present study indicate that dietary vitamin E supplementation protects against oxidative liver damage in rats with WIRS through maintenance of the antioxidant defense system associated with vitamin E without affecting the stress response.

Conflict of interest

The authors have no conflict of interest to disclose.
REFERENCES

1) Landeira-Fernandez J. 2004. Analysis of the cold-water restraint procedure in gastric ulceration and body temperature. *Physiol Behav* **82**: 827–833.

2) Nishida K, Ohta Y, Kobayashi T, Ishiguro I. 1997. Involvement of the xanthine-xanthine oxidase system and neutrophils in the development of acute gastric mucosal lesions in rats with water immersion restraint stress. *Digestion* **58**: 340–351.

3) Nishida K, Ohta Y, Ishiguro I. 1997. Role of gastric mucosal constitutive and inducible nitric oxide synthases in the development of stress-induced gastric mucosal lesions in rats. *Biochem Biophys Res Commun* **236**: 275–279.

4) Hamaguchi M, Watanabe T, Higuchi K, Tominaga K, Fujitawa Y, Arakawa T. 2001. Mechanisms and role of neutrophil infiltration in stress-induced gastric injury in rats. *Dig Dis Sci* **46**: 2708–2715.

5) Yasukawa K, Kasazaki K, Hyodo F, Utsumi H. 2004. Non-invasive analysis of reactive oxygen species in rats with water immersion restraint-induced gastric mucosal lesions using in vivo electron spin resonance spectroscopy. *Free Radic Res* **38**: 147–155.

6) Iwai K, Onodera A, Matsue H. 2001. Antioxidant activity and inhibitory effect of Gamazumi (*Viburnum dilatatum* Thunb.) on oxidative damage induced by water immersion restraint stress in rats. *Int J Food Nutr* **52**: 443–451.

7) Iwai K, Onodera A, Matsue H. 2001. Mechanism of preventive action of *Viburnum dilatatum* Thunb. (Gamazumi) crude extract on oxidative damage induced in rats subjected to stress. *J Sci Food Agric* **83**: 1593–1599.

8) Ohta Y, Chiba S, Tada M, Imai Y, Kitagawa A. 2007. Development of oxidative stress and cell damage in the liver of rats with water-immersion restraint stress. *Redox Rep* **12**: 139–147.

9) Kaida S, Ohta Y, Imai Y, Kawanishi M. 2010. Protective effect of L-ascorbic acid against oxidative damage in the liver of rats with water-immersion restraint stress. *Redox Rep* **15**: 11–19.

10) Ohta Y, Kaida S, Chiba S, Tada M, Teruya A, Imai Y, Kawanishi M. 2009. Involvement of oxidative stress in increases in the serum levels of various enzymes and components in rats with water-immersion restraint stress. *J Clin Biochem Nutr* **45**: 347–354.

11) Liebler DC. 1993. The role of metabolism in the antioxidant function of vitamin E. *Crit Rev Toxicol* **23**: 147–169.

12) Maroz A, Anderson RF, Smith RAJ, Murphy MP. 2009. Reactivity of ubiquinone and ubiquinol with superoxide and the hydroperoxyl radical: implication for in vivo antioxidant activity. *Free Radic Biol Med* **46**: 105–109.

13) Niki E. 1997. Mechanisms and dynamics of antioxidant action of ubiquinol. *Mol Aspects Med* **18** (Suppl): S63–70.

14) Quinn PJ, Fabisiak JP, Kagan VE. 1999. Expansion of antioxidant function of vitamin E by coenzyme Q. *Biofactors* **9**: 149–154.

15) Thurner M, Olsson J, Dallner G. 2004. Metabolism and function of coenzyme Q. *Biochim Biophys Acta* **1660**: 171–199.

16) Galleano M, Puntarulo S. 1997. Dietary α-tocopherol supplementation on antioxidant defenses after in vivo iron overload in rats. *Toxicology* **124**: 73–81.

17) Ohta Y, Yashiro K, Ohashi K, Imai Y, Kusumoto C, Matsura T, Hidaka M, Fukuzawa K. 2013. Vitamin E depletion enhances liver oxidative damage in rats with water-immersion restraint stress. *J Nutr Sci Vitaminol* **59**: 79–86.

18) Aziz Ibrahim IA, Kamisukh Y, Nafeeza MI, Nur Adzina MF. 2012. The effects of palm vitamin E on stress hormone levels and gastric lesions in stress-induced rats. *Arch Med Sci* **8**: 22–29.

19) Mohd Fahami NA, Ibrahim IA, Kamisukh Y, Mohd Ismail N. 2012. Palm vitamin E reduces catecholamines, xanthine oxidase activity and gastric lesions in rats exposed to water-immersion restraint stress. *BMC Gastroenterol* **12**: 54.

20) Arakawa H, Kodama H, Matsukawa N, Yamaguchi I. 1997. Stress increases plasma enzyme activity in rats: differential effects of adrenergic and cholinergic blockades. *J Pharmacol Exp Ther* **260**: 1296–1303.

21) Ohta Y, Yashiro K, Ohashi K, Imai Y. 2012. Disruption of non-enzymatic antioxidant defense system in the brain of rats with water-immersion restraint stress. *J Clin Biochem Nutr* **51**: 136–142.

22) Guillemin R, Clayton GW, Lipscomb HS, Smith JD. 1959. Fluorometric measurement of rat plasma and adrenal corticosterone concentration. A note on technical details. *J Lab Clin Med* **53**: 830–832.

23) Kamiya Y, Ohta Y, Imai Y, Arisawa T, Nakano H. 2005. A critical role of gastric mucosal ascorbic acid in the progression of acute gastric mucosal lesions induced by compound 48/80 in rats. *World J Gastroenterol* **11**: 1324–1332.

24) Zannoni V, Lynch M, Goldstein S, Sato PA. 1974. A rapid micromethod for the determination of ascorbic acid in plasma and tissues. *Biochem Med* **11**: 41–48.

25) Yagi K. 1976. A simple fluorometric assay for liperoxides in plasma sample. *Biochem Med* **15**: 212–216.

26) Sedlak J, Lindsay RH. 1968. Estimation of total, protein-bound, and nonprotein sulhydryl groups in tissues with Ellman’s reagent. *Anal Biochem* **25**: 192–205.

27) Matsura T, Yamada K, Kawasaki T. 1992. Antioxidant role of cellular reduced coenzyme Q homologs and α-tocopherol in free radical-induced injury of hepatocytes isolated from rats fed diets with different vitamin E contents. *Biochim Biophys Acta* **1127**: 277–283.

28) Kusumoto C, Kinugawa T, Morikawa H, Teraoka M, Nishida T, Murakawa Y, Yamada K, Matsura T. 2010. Protection by exogenous added coenzyme Q9 against free radical-induced injuries in human liver cells. *J Clin Biochem Nutr* **46**: 244–251.

29) Ohkawa H, Ohishi N, Yagi K. 1979. Assay for lipid peroxides in animal tissue by thiobarbituric acid reaction. *Anal Biochem* **95**: 351–358.

30) Ohta Y, Imai Y. 2011. Effect of vitamin E intake status on stress-induced gastric mucosal lesions. *Uler Res* **38**: 180–183 (in Japanese with abstract in English).

31) Nakano K, Suzuki S. 1984. Stress-induced changes in tissue levels of ascorbic acid and histamine in rats. *J Nutr* **114**: 1602–1608.

32) Nathani MG, Nath MC. 1972. Effect of corticotrophin on ascorbic acid metabolism in rats. *Metabolism* **21**: 137–142.

33) Padayatty SJ, Doppman JL, Chang R, Wang Y, Gill J, Papanicolaou DA. 2007. Human adrenal glands secrete vitamin C in response to adrenocorticotropic hormone. *Am J Clin Nutr* **86**: 145–149.

34) Ham AJL, Liebler DC. 1997. Antioxidant reactions of
vitamin E in the perfused rat liver: product distribution and effect of dietary vitamin E supplementation. Arch Biochem Biophys 339: 157–164.

35) Beyer RE. 1994. The role of ascorbate in antioxidant protection of biomembranes: interaction with vitamin E and coenzyme Q. J Bioenerg Biomembr 26: 349–358.

36) Barelay LR. 1988. The cooperative antioxidant role of glutathione with a lipid-soluble and a water-soluble antioxidant during peroxidation of liposomes initiated in the aqueous phase and in the lipid phase. J Biol Chem 263: 16138–16142.

37) Winkler BS, Orselli SM, Rex TS. 1994. The redox couple between glutathione and ascorbic acid: a chemical and physiological prospective. Free Radic Biol Med 17: 333–349.

38) Yamamoto Y, Komuro E, Niki E. 1990. Antioxidant activity of ubiquinol in solution and phosphatidylcholine liposome. J Nutr Sci Vitaminol 36: 505–511.

39) Matsura T, Yamada K, Kawasaki T. 1992. Difference in antioxidant activity between reduced coenzyme Q9 and reduced coenzyme Q10 in the cell: studies with isolated rat and guinea pig hepatocytes treated with a water-soluble radical initiator. Biochim Biophys Acta 1123: 309–315.

40) Forsmark-Andrée P, Lee C-P, Dallner CV, Ernster L. 1997. Lipid peroxidation and changes in the ubiquinone content and the respiratory chain enzymes of mitochondrial particles. Free Radic Biol Med 22: 391–400.