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Modulation of Restraint Induced Gastric Oxidative Changes in Rats by Tocotrienol and Tocopherol

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Abstract: The present study compares the effect of tocotrienol mixture and α-tocopherol on gastric malondialdehyde (MDA), glutathione peroxidase (GPx), superoxide dismutase (SOD), reduced and oxidized glutathione (GSH and GSSG) content and prostaglandin E_2 level in rats exposed to restraint stress. Twenty-four male *Sprague dawley* rats were randomly assigned into 4 equal sized groups; two control groups and two treated groups which were supplemented with either tocotrienol (TT) or α-tocopherol (TF) orally at a dose of 60 mg kg^{-1} body weight. After 28 days of treatment, one control group, the TT and TF groups were subjected to restraint stress, 2 h daily for 4 consecutive days. After the last exposure to stress, the stomach was excised for the evaluation of the parameters. Present findings showed that TT was better in preventing the formation of gastric lesion compared to TF while both TT and TF significantly reduces the gastric MDA content compared to stress control. We also found that both TT and TF have the ability to reduce prostaglandin E_2 loss which was apparent with stress exposure. The endogenous content of antioxidant enzyme GPx activity and GSH content was maintained towards the normal levels in rats receiving TT but not in the TF treated group. The SOD level however was not altered in stressed rats. As a conclusion, tocotrienol posses a better protective effect against stress-induced gastric lesions compared to α-tocopherol. The protective effect was associated with decreased lipid peroxidation, increased prostaglandin E_2 (PGE_2) and restoration of GPx activity and GSH content which was altered by stress.

Key words: Tocotrienol, α-tocopherol, restraint-stress, gastric lesions, malondialdehyde, glutathione peroxidase

INTRODUCTION

Various responses to stress exposure are important for human survival. However repeated activation of responses to stress as well as sustained activation will cause an overexposure to stress hormones, thereby increasing risk to various health problems (Lundberg, 2005). One common health problem related to stress is the formation of gastric lesions, more well known as stress ulcers. The mechanism of experimental stress ulceration appears to depend on an interaction between changes in gastric acid output, gastric barrier mucus, mucosal blood circulation, gastric motility, mitotic activity of the mucosal lining of the stomach and oxidative stress (Martinez-Augustin *et al.*, 2000).

Many studies have shown that despite the diverse causes of gastritis, a common factor implicated at the molecular level in the pathogenesis of this clinical entity are free radicals (Naito *et al.*, 1993; Kamsiah *et al.*, 1999) which overwhelm the endogenous antioxidant system. Agents with ability to catalytically reduced free radical or act as antioxidant had been shown to protect the gastric mucosa against a variety of noxious stimuli (Hirota *et al.*, 1990; Izgut-Uysal *et al.*, 2001; Izgüt-Uysal and Agac Deri, 2007). Oxygen-derived free radicals are cytotoxic and mediate tissue damage by injuring cellular membranes and releasing intracellular components thus resulting in oxidative stress.

Although it is widely accepted that the pathogenesis of gastric mucosal lesions involves oxygen-derived free radicals, the role of lipid peroxidation and the endogenous

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antioxidant enzymes in stress remains a question. Among various stressor used in animals, one of the most reproducible results can be obtained by restraint stress (Salim, 1990; Hayase and Takeuchi, 1986) which lead to the formation of gastric lesions.

The effect of tocotrienol and α-tocopherol on oxidative stress could account for the beneficial effect of this vitamin in model of stress induced gastric injury. Vitamin E is known to have a scavenging effect on reactive oxygen species and a stabilizing effect on damaged cell membrane. To confirm the hypothesis of the involvement of lipid peroxidation and compare the effect of tocotrienol and α-tocopherol in parameters involved in stress-induced gastric lesion, rats were subjected to restraint stress and stomach was examined for lesions and oxidative damage.

MATERIALS AND METHODS

Male *Sprague dawley* rats (n = 24) were divided into four equally sized groups. Two control groups were fed with normal Rat Diet (RC) while the treatment groups received the same diet but with oral supplement of tocotrienol (TT) or α-tocopherol (TF) at 60 mg kg^{-1} body weight for 28 days. The dose chosen was based on the earlier studies which had shown a protective effect of tocotrienol and tocopherol on stress-induced gastric lesions (Azlina *et al.*, 2005; Nur Azlina and Nafeeza, 2007). Tocotrienol and α-tocopherol were given in olive oil which acts as a vehicle and was administered by oral gavage using an 18 G gavage needle. The control groups were sham administered with olive oil. At the end of the treatment period, the rats from one control group (stressed control) and both of the treated groups were exposed to restraint-stress. After the last exposure to stress the rats were sacrificed. The dissected stomach was taken for evaluation of gastric lesion, malondialdehyde, reduced and oxidized glutathione, glutathione peroxidase, superoxide dismutase and prostaglandin E$_2$ levels. The measurement was done after the rats were sacrificed by overdose of anesthesia.

All rats were kept on a regular night/day cycle, with natural light for a period of 10 h (07:00-17:00 h). Throughout the feeding period all rats were habituated to handling to reduce their stress-related disturbances. The rats were housed in large cages with wide wire-mash bottoms to prevent coprophagy. Food and water were given ad libitum throughout the experiment. Prior ethical approval was obtained from University Kebangsaan Malaysia Animal Ethics Committee (UKMAEC). Approval number FAR/2004/AZLINA/12-JULY/086. This study was conducted from July 2004 until May 2005 in Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia.

Rats were restrained by placing them in individual plastic restrainer measuring approximately 17×5 cm for 2 h daily for 4 consecutive days, as previously described by Ainsah *et al.* (1999). Following the restraining procedure on the fourth day, blood was collected, after which the rats were sacrificed. The stomach was dissected along the greater curvature and examine for lesions.

Macroscopic assessment of stress-induced gastric lesions: The macroscopic assessment of stress-induced gastric lesions in the gastric mucosa was performed by two independent examiners who were blinded to the treatment that the rats received. The assessment of lesions were done according to a semi quantitative scale. The scale used was as followed 5 = generalized hemorrhage covering more than 90% of the gastric mucosa, 4 = hemorrhage covering 60-90% of the gastric mucosa, 3 = hemorrhage covering 30-60% of the gastric mucosa, 2 = hemorrhage covering 10-30% of the gastric mucosa, 1 = generalized erythema with present of hemorrhage and 0 = no visible lesion.

Measurement of gastric malondialdehyde content: The content of malondialdehyde (MDA) in the stomach was determined using the method described by Ledwozyw *et al.* (1986). A sample of 0.5 mL was acidified with 2.5 mL of 1.22 mol L^{-1} trichloroacetic acid in 0.6 mol L^{-1} HCl. The mixture was left to stand for 15 min. After this time, 1.5 mL of 0.6% thiobarbituric acid in 0.05 mol NaOH was added. The sample was then incubated in a 100°C water bath for 30 min. Subsequently it was cooled under running tap water and 4 mL of n-butanol was added. After thorough mixing, the mixture was centrifuged for 10 min at 1500 x g. The absorbency of the upper phase was read at 535 nm. The gastric tissue content was determined by the Lowry *et al.* (1951a, b) method and MDA was expressed in terms of gram protein.

Measurement of reduced glutathione and oxidize glutathione levels: Measurements of gastric glutathione (GSH and GSSG) content were done following method by Griffith and Meister (1979).

Measurement of glutathione peroxidase activity: Measurement of glutathione peroxidase (GPx) activities was done using RANSEL kit (RS504). The kit was acquired from RANDOX Laboratories Ltd., Ardmore, Diamond Road, Crumlin, United Kingdom.

Measurement of superoxide dismutase activity:
Superoxide dismutase (SOD) activity was measured by colorimetric assay. The RANSOD kit was acquired from RANDOX Laboratories Ltd., Ardmore, Diamond Road, Crumlin, United Kingdom.

Measurement of gastric prostaglandin E_2 content:
Sample preparation for prostaglandin E_2 (PGE_2) assay was done using the method previously described by Redfern *et al.* (1987). Prostaglandin E_2 was measured using Enzyme Immuno Assay (EIA) kit (RPA 530, IBL Hamburg).

Statistical analysis: Statistical analysis was carried out using the SPSS statistical package version 12 (SPSS Inc. USA). Normal distribution of all variables was examined by Kolmogrov-Smirnov test. The results showed that all variables were normally distributed. The results are expressed as Mean±SD. Statistical significance ($p<0.05$) was determined by ANOVA and Tukey's post-hoc test.

RESULTS

Rats exposed to restraint stress for 2 h a day for 4 consecutive days shows presence of considerable ulcerogenicity in the form of hemorrhagic mucosal lesions confined to the corpus (glandular part of the stomach). As shown in Fig. 1, the gastric lesions index in the Stressed Control (SC) group was higher by 46% ($p = 0.0068$) compared to the TT group and 31% ($p = 0.049$) compared

to TF group, this findings indicates that vitamin E both tocotrienol and tocopherol are able to reduce the formation of stress-induced gastric lesions. Rats killed after the 28 days feeding period and not exposed to stress had no gastric mucosal lesion. The gastric lesion index in the control group is 8.4 fold higher compared to the non-stressed rats in the same group.

As shown in Fig. 2, treatment of rats with both TT ($p = 0.046$) and TF ($p = 0.0042$) individually causes a significant reduction in gastric MDA level compared to stressed control. Stress causes an increased in gastric Thiobarbituric Reactive Substance (TBARS) as indicated by the increased of gastric MDA content.

Reduced glutathione (GSH) is the major endogenous antioxidant in life organism. The results are expressed by the ratio of GSH to the oxidized form of glutathione (GSSG). Exposure to restraint stress for 2 h a day for 4 consecutive days resulted in a significant reduction of gastric glutathione level by 26.3% ($p = 0.027$) in the stressed control compared to the Non-Stress Control (NSC) group as shown in Fig. 3. Rats treated with either TT or TF showed no significant different in the gastric glutathione level compared to the non-stressed control. The finding suggest that vitamin E can restore a normal gastric glutathione level which was altered by stress.

Exposure to restraint stress for 2 h a day for 4 consecutive days resulted in a significant reduction of gastric GPx activity by 21.2% ($p = 0.001$) in the stressed control compared to the NSC group as shown in Fig. 4. Rats treated with TT showed no significant different in the gastric GPx activity compared
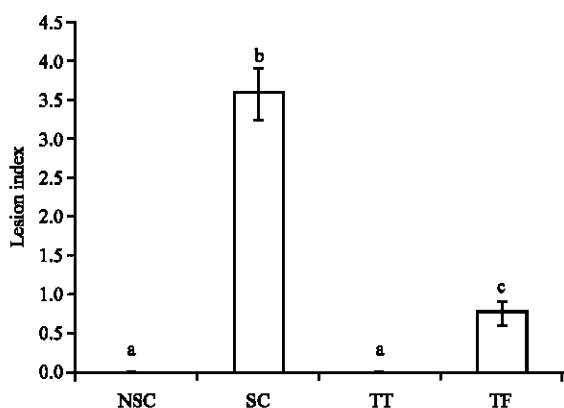

Fig. 1: Effects of TT and TF on lesion index in rats exposed to restraint-stress. Mean lesion index with or without exposure to restraint-stress in control rats and rats supplemented with TT or TF. Different letter(s) between bars indicate significant difference (p<0.05)
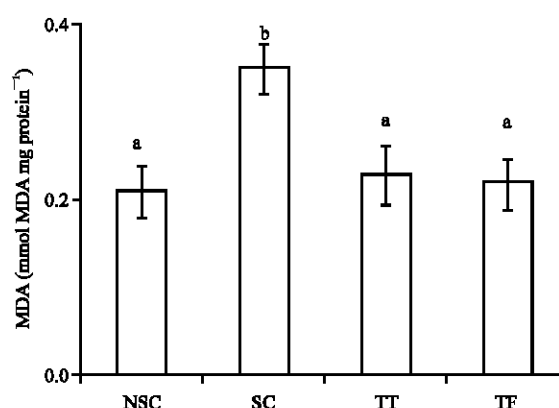

Fig. 2: Effects of TT and TF on gastric MDA level in rats exposed to restraint-stress. Mean MDA level with or without exposure to restraint-stress in control rats and rats supplemented with TT or TF. Different letter(s) between bars indicate significant difference (p<0.05)
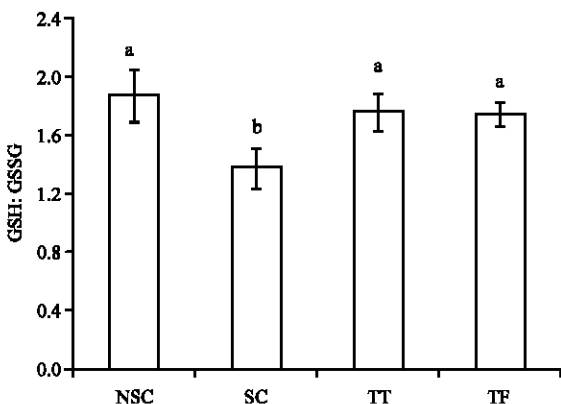
Fig. 3: Effects of TT and TF on gastric glutathione ratio in rats exposed to restraint-stress. Mean gastric glutathione ratio with or without exposure to restraint-stress in control rats and rats supplemented with TT or TF. Different letter(s) between bars indicate significant difference ($p < 0.05$)
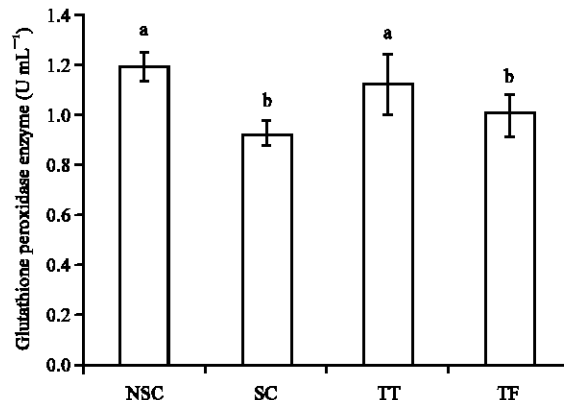

Fig. 5: Effects of TT and TF on gastric glutathione peroxidase activity in rats exposed to restraint-stress. Gastric glutathione peroxidase activity with exposure to restraint-stress in control rats and rats supplemented with TT or TF. Different letter(s) between bars indicate significant difference ($p < 0.05$)

Fig. 4: Effects of TT and TF on gastric prostaglandin E_2 content in rats exposed to restraint-stress. Mean gastric prostaglandin E_2 content with or without exposure to restraint-stress in control rats and rats supplemented with TT or TF. Different letters between bars indicate significant difference ($p < 0.05$)
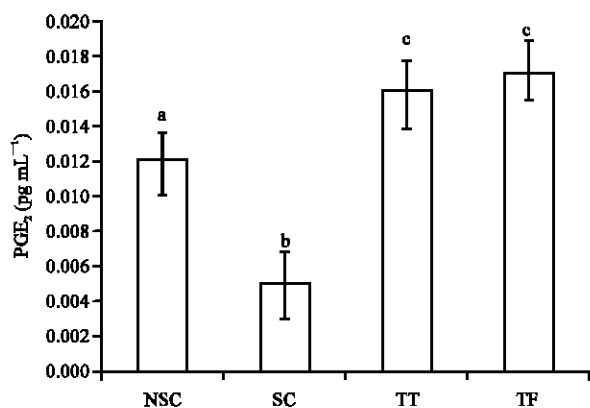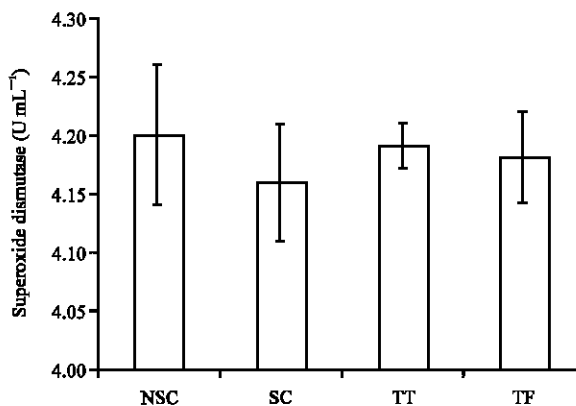

Fig. 6: Effects of TT and TF on gastric superoxide dismutase activity in rats exposed to restraint-stress. Gastric superoxide dismutase activity with exposure to restraint-stress in control rats and rats supplemented with TT or TF. No significant difference was observed

to the non-stressed control. However supplementation with TF did not have effect on maintaining GPx activity towards the non stressed level. The finding suggests that TT, not TF have the ability to restore a normal gastric GPx activity which was altered by stress.

Superoxide dismutase activity was slightly reduced in rats exposed to stress, but this reduction was not significant. Rats supplemented with TT and TF have a similar SOD values with the non-stressed control rats as shown in Fig. 5.

The mean gastric PGE_2 content in rats exposed to restraint stress was significantly lower ($p < 0.05$) compared to the non-stressed control, as shown in Fig. 6. The findings suggest that stress alters the gastric PGE_2 content. Supplementation with either TT or TF increases PGE_2 level even higher than the non-stressed control values. The finding suggests that the protective effect of TT and TF could partly be due their abilities to increase the gastric PGE_2 content.

DISCUSSION

The finding showed that rats exposed to restraint stress for 2 h daily for 4 consecutive days developed gastric mucosal lesion at the glandular part of the stomach. The lesion was in the form of hemorrhage and generalized erythema. The results of this study demonstrates that pretreatment of rats with vitamin E either tocotrienol or α-tocopherol individually markedly reduce gastric mucosal damage induced by stress. It was also found that there was a difference between these two agents where a few rats in the α-tocopherol group developed lesions, while all the rats in the tocotrienol treatment group were completely protected against stress-induced gastric injury.

It is well known that gastric mucosa is continuously exposed to harmful factors. Destruction and protective capacity should be in balance to maintain functional integrity of the gastric mucosa. Among the various hazardous effects on biological system are oxidative destruction of membrane polyunsaturated fatty acid or more commonly known as lipid peroxidation has been observed in numerous tissue (Kujubu *et al.*, 1991).

As in the earlier studies done by Azlina *et al.* (2005) and Nur Azlina and Nafeeza (2007), in this study, the high gastric MDA content in the stressed control stomach supports the hypothesis that stress-induced injury is mediated by lipid peroxidation process. This indicates that reactive oxygen species and lipid peroxidation is important in the pathogenesis of gastric mucosal injury induced by stress. The study also showed that vitamin E decreases the breakdown of gastric mucosal barrier by reducing the product of lipid peroxidation (MDA). The reduced MDA levels accompanied by the improved gastric lesions in these groups suggests that vitamin E probably reduced injury by retarding the lipid peroxidation process. The findings also showed no difference in the ability to reduced gastric MDA content between tocotrienol and tocopherol, suggesting the similar radicals scavenging ability.

There was also the observation of significant depletion of reduced glutathione content in the gastric mucosa following exposure to stress. The treatment of rats with either tocotrienol or α-tocopherol significantly attenuated stress-induced depletion of gastric mucosal reduced glutathione. Studies had shown that reduced glutathione, a major endogenous non-protein sulfhydryl (NP-SH) compound in the stomach, plays an important role in the formation of gastrointestinal mucosa mucus, which protect the underlying gastric mucosa against acid secretion, pepsin and exogenous necrotizing agents (Stein *et al.*, 1990; Szabo and Brown, 1987). Hirota *et al.*

(1989) found that intraperitoneal injection of reduced glutathione significantly increases plasma level of glutathione and inhibit the occurrence of gastric injury induced by stress.

The decrease in gastric mucosal glutathione content with the development of stress-induced gastric mucosal lesions shows the important of free radicals in causing gastric injury in stress, thus prevention using exogenously administration of vitamin E seems to be a logical alternative to the prevention of such injuries. A study by Wan and Wang (2000) found that rats subjected to water-imerssion restrain stress develops gastric lesions and this was correlated with a reduction in the gastric GSH content. Treatment with exogenous GSH (100 and 200 mg kg^{-1}) intraperitoneally protected against stress gastric mucosal lesion (p<0.001 and p<0.001). In their study GSH (100 mg kg^{-1}) significantly increased secretion of gastric barrier mucus, but had no effect on secretion of gastric acid in restraint water immersed rats.

Tissues contains various endogenous antioxidant enzymes like superoxide dismutase, catalase (CAT) and glutathione peroxidase, which scavenge Reactive Oxygen Species (ROS) and therefore preventing lipid peroxidation and tissue damage. However in pathological conditions like gastric ulcerations, ROS maybe produced in excess and the delicate balance between ROS and endogenous antioxidant enzymes shifts towards increment of ROS production (Xie *et al.*, 1991). In such situations, agents which can augment the activity of ROS scavenging enzymes and prevent lipid peroxidation may prove beneficial.

Stress causes an increased in lipid peroxidation as shown with the increased in the MDA levels. The gastric GPx level was found to be lower in the control stressed group as compared to the non-stressed control groups studied. This proved that there was depletion in the endogenous antioxidant enzymes activity and that ROS plays a significant role in stress induced damages. As predicted supplementation with tocotrienol was able to block the reduction of the circulating enzyme levels, maintaining it towards the normal non-stressed values. Thus prevention using exogenously administration of tocotrienol seems to be a logical alternative to the prevention of such injuries. However supplementation with α-tocopherol failed to enhance GPx activities but was still able to decreased lipid peroxidation as was found in this study. The different mechanism in the protection between tocotrienol and tocopherol as seen in this study open the door for further research.

The finding of the current study also found that SOD activities was unchanged in stress rats. Similarly, Ohta and Nishida (2003) found that rats exposed to water immersion restraint stress had an increased TBARS

concentration and reduced non-protein sulfhydryl (NP-SH) concentration but the SOD activity did not change. It was also showed that pre-treatment with exogenous SOD and CAT increases the level of NP-SH concentration but have no effect on the SOD activities. Thus, based on their study and the current study, it can be concluded that SOD does not play a role in stress induced gastric injury. Tocotrienol and α-tocopherol supplementation prior to exposure to stress had no effect on SOD activities, where the activity remains unchanged compared to the non-stress control.

A different scenario in the serum SOD activity was previously reported (23, 24) where the SOD activity was increased in the stress rats. This was followed by the formation of gastric lesions. Kashif *et al.* (2003) found that treatment with tocopherol prior or after stress was able to increased the activities of both CAT and SOD activities with the decreased lipid peroxidation in the serum samples. A probable explanation for the inability of tocopherol to reduced gastric GPx level would be that it decreases lipid peroxidation by enhancing the activities of other antioxidant enzymes such as SOD or CAT.

Prostaglandins are generated in the gastric mucosa via the activity of the enzyme cyclooxygenase (COX). It exist in two genetically different isoforms, constitutive of COX-1 forms and inducible COX-2 (Konturek *et al.*, 2001). COX-1 had been shown to exhibit cytoprotective effects on gastric mucosa where as COX-2 had been implicated in the inflammatory reactions and tissue damage involving various cytokines, endotoxins and growth factors (Xie *et al.*, 1991; Takeuchi *et al.*, 1999).

Study by Bregonzio *et al.* (2003) found that stress-induced mucosal ulcerations were also associated with a significant decrease in the gastric mucosal levels of PGE_2. In this study, a similar findings was observed, where the PGE_2 levels were lower in rats exposed to stress compared to the non-stressed control. It was also found that vitamin E supplementation in both forms, tocotrienol or α-tocopherol; have the ability to block the changes in PGE_2, where the level was not significantly different to the non-stressed control. With a lower lesions occurrence in the vitamin E supplemented groups plus maintenance of gastric PGE_2 levels, we can also associate the decreased in the gastric lesions to the maintenance of the levels of PGE_2. Other study by this group had also shown the beneficial effect of vitamin E treatment in enhancing the PGE_2 level in NSAIDs induced gastric ulcers in rats (Nafeeza and Kang, 2005).

Konturek *et al.* (2001) showed that the healing of stress lesions results in the restoration of mucosal prostaglandin generation and this effect are accompanied by overexpression of EGF and TNF alpha as well as COX-1 and COX-2 mRNA and by the increased biosynthesis of gastroprotective prostaglandins. A study had also found that treatment with 16, 16-dimethyl PGE_2, was able to decrease the mucosal ulcer in rats exposed to stress (Bregonzio *et al.*, 2003). This finding as well as others suggests that PGE_2 seems to be an important determinant in the pathogenesis of stress induced gastric mucosal lesions. If this is true then supplementation with vitamin E proved to be a good supplementation alternative towards reducing stress induced gastric lesions.

In conclusion, the data from the current study suggests that stress induced gastric injuries involves free radical formation and lipid peroxidation. Supplementation with vitamin E, both tocotrienol and α-tocopherol maybe beneficial where their intake prevented the occurrence of gastric mucosal lesions. The protective effect of vitamin E was related to a decreased lipid peroxidation, increased PGE_2 and the preservation of gastric reduced glutathione content and glutathione peroxidase enzyme activity. There was also significant difference between the two forms of vitamin E, where tocotrienol posses a better protective effect compared to α-tocopherol against stress-induced gastric lesions and their abilities was related to a decreased lipid peroxidation, increased PGE_2 and restoration of the GPx level which was altered by stress.

ACKNOWLEDGMENTS

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