

Original Article

A comparison between tocopherol and tocotrienol effects on gastric parameters in rats exposed to stress

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Rats exposed to stress developed various changes in the gastrointestinal tract and hormones. The present study was designed to compare the impact of tocopherol and tocotrienol on changes that influence gastric and hormonal parameters important in maintaining gastric mucosal integrity in rats exposed to restraint stress. These include gastric acidity, gastric tissue content of parameters such as malondialdehyde, prostaglandin E₂ (PGE₂), serum levels of gastrin and glucagon-like peptide-1 (GLP-1). Sixty male Sprague-Dawley rats (200-250g) were randomly divided into three equal sized groups, a control group which received a normal rat diet (RC) and two treatment groups each receiving a vitamin deficient diet with oral supplementation of either tocopherol (TF) or tocotrienol (TT) at 60mg/kg body weight. Blood samples were taken from half the number of rats (non-stressed group) after a treatment period of 28 days before they were killed. The remaining half was subjected to experimental restraint-stress, at 2 hours daily for 4 consecutive days (stressed groups), on the fourth day, blood samples were taken and the rats killed. The findings showed that the gastric acid concentration and serum gastrin level in stressed rats were significantly ($P<0.05$) reduced compared to the non-stressed rats in the control and TF groups. However, the gastric acidity and gastrin levels in the TT group were comparable in stressed and non-stressed rats. These findings suggest that tocotrienol is able to preserve the gastric acidity and serum gastrin level which are usually altered in stressed conditions. The PGE₂ content and the plasma GLP-1 level were, however, comparable in all stressed and non-stressed groups indicating that these parameters were not altered in stress and that supplementation with TF or TT had no effect on the gastric PGE₂ content or the GLP-1 level. The malondialdehyde, an indicator of lipid peroxidation was higher from gastric tissues in the stressed groups compared to the non-stressed groups. These findings implicated that free radicals may play a role in the development of gastric injury in stress and supplementation with either TF or TT was able to reduce the lipid peroxidation levels compared to the control rats. We conclude that both tocopherol and tocotrienol are comparable in their gastroprotective ability against damage by free radicals generated in stress conditions, but only tocotrienol has the ability to block the stress-induced changes in the gastric acidity and gastrin level.

Key Words: tocopherol, tocotrienol, stress, gastric mucosal integrity

Introduction

It has, for many centuries been known that all organisms survive by maintaining balance with its environment. Stress is a condition that can affect this balance and lead to various pathological changes. Stress in rats can be simply and reliably produced by the restraint model.¹⁻³ The effects of such a model on stress include changes in the gastrointestinal tract. In the gastric microenvironment, a crucial balance between the aggressive and defensive factors is required to maintain the integrity of the gastric mucosa.⁴⁻⁶ An imbalance of these factors lead to pathological changes or injury to the gastric mucosa.

Free radicals had been implicated as a common cause of injury to the gastric mucosa due to a variety of agents including alcohol,^{7,8} non-steroidal anti-inflammatory drugs (NSAIDs)⁹⁻¹¹ and stress.^{9,10,12,13} Agents with ability to catalytically reduce free radicals or act as an antioxidant had been shown to effectively protect the gastric mucosa against a variety of noxious stimuli.^{11,12,14,15}

Vitamin E, a naturally occurring antioxidant, is found in abundance in the environment. It is an essential fat-soluble vitamin and is considered a generic name describing bio-activities of two of its derivatives, tocopherol and tocotrienol.¹⁶ The two derivatives share a common general structure, an aromatic chromanol head and 16-carbon tail. The amount of methyl substitute in the chromanol nucleus varies among the isomers (alpha, beta, gamma and delta). Tocopherol has a saturated hydrocarbon side chain whereas tocotrienol possesses three unsaturated carbon chains.

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Accepted 30th June 2005

It was postulated that tocotrienols are more mobile and less restricted in their interactions with lipid radicals in the membrane than tocopherol.¹⁷ The biological activity of vitamin E is believed to be due to its antioxidant action to inhibit lipid peroxidation in biological membranes by scavenging the peroxy chain reaction. Studies have shown that tocotrienols are more potent antioxidants compared to tocopherols.¹⁷ The current study compares the effect of tocopherol and tocotrienol on parameters that are involved in maintaining gastric mucosal integrity in rats exposed to acute repetitive stress.

Material and methods

Rats used for this study were kept on a regular night/day cycle, with natural light for a period of 10 hours (0700 to 1700h). Throughout the feeding period all rats were habituated to handling to reduce stress-related disturbances. The rats were housed in large cages with wire-mesh bottoms to prevent coprophagy. Food and water were given ad libitum throughout the experiment. All the experiment protocols were approved by the Animal Care and Use Committee of the Faculty of Medicine, National University of Malaysia (*approval number: FAR/2000/NAFEEZA/30-NOVEMBER/031*).

Sixty male *Sprague-Dawley* rats weighing approximately 200–250g were purchased from the University Breeding Center (UKM, Kuala Lumpur) and divided into three equally sized groups. The control rats were given normal chow while the treated group was fed a vitamin E deficient diet (VED) with oral supplementation of either α -tocopherol or tocotrienol at 60mg/kg body weight for 28 days. At the end of the treatment period, ten rats from each group were killed. The remaining rats were exposed to restraint-stress. Stress-induced changes in the gastric acid concentration, serum gastrin level, plasma glucagons-like peptide 1, gastric malondialdehyde and prostaglandin E₂ content were measured in all rats with or without exposure to stress. All measurements were done immediately after the rats were killed.

Restraint-stress

Rats were restrained by placing them in individual plastic restrainers measuring approximately 12 x 5 cm for 2 hours daily for 4 consecutive days. Following the restraining procedure on the fourth day, blood was taken and the rats were killed. The gastric acid was collected and the acid concentration was measured immediately.

Measurement of gastric acid concentration

The junctions between the stomach–esophagus and duodenum-pylorus were secured before the stomach was isolated. Then 3 ml of distilled water was introduced into the stomach and the organ was carefully shaken. The gastric juice was then collected and centrifuged for 10 minutes at 3000rpm. The supernatant was then taken and diluted 10 times. Following this, a few drops of phenolphthalein was added to the solution. Titration was done using 0.01M solution until the color of the test solution changed to light pink indicating pH 7.0. The volume of sodium hydroxide (NaOH) needed for titration was used in the calculation to derive the hydrogen ion concentration according to the method described by Shay *et al.*¹⁸

Measurement of serum gastrin level

Blood taken immediately after the last exposure to restraint stress was cooled in an ice-bath and allowed to clot. Serum was separated by centrifugation at 1500xg at 4°C. Gastrin level was measured using an RIA kit (M13101, IBL Hamburg).

Measurement of plasma glucagon-like peptide 1 level

Plasma was collected immediately before the rats were killed. Dipeptidyl peptidase inhibitor (DPP-IV: EC 3.4.14.5 LINCO Research, Inc USA) was immediately added to the collected sample to prevent degradation of Glucagon-like peptide-1 (GLP-1) by dipeptidyl peptidase enzyme. Glucagon-like peptide 1 was then extracted from the plasma samples and assay using a RIA kit (GLP1T-36HK LINCO Research, Inc USA).

Measurement of gastric malondialdehyde content

The content of malondialdehyde (MDA) in the stomach was determined using the method described by Ledwozyz *et al.*¹⁹ A sample of 0.5ml was acidified with 2.5ml of 1.22mol/L trichloroacetic acid in 0.6mol/L HCl. The mixture was left to stand for 15 minutes. After this time, 1.5ml 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 minutes. Subsequently it was cooled under running tap water and 4ml of *n*-butanol was added. After thorough mixing, the mixture was centrifuged for 10 minutes at 1500xg. The absorbency of the upper phase was read at 535nm. The gastric tissue content was determined by the Lowry *et al.*,(1951) method and MDA was expressed in terms of gram protein.¹⁹

Measurement of gastric prostaglandin E₂ content

Sample preparation for prostaglandin E₂ (PGE₂) assay was done using the method previously described by Redfern *et al.*²¹ Prostaglandin E₂ was measured using Enzyme Immuno Assay (EIA) kit (RPA 530, IBL Hamburg).

Statistics

Results are expressed as mean \pm SEM. Statistical significance ($P < 0.05$) was determined by ANOVA or student's t-test for parametric analysis and Kruskal Wallis or Wilcoxon Signed Test for non-parametric analysis where appropriate.

Results

Comparison between tocopherol and tocotrienol on gastric acid concentration in rats exposed restraint stress

The effect of restraint-stress on gastric acid concentration in rats was obtained by the comparison of the concentration of H⁺ between the stressed and non-stressed rats in the study group. As shown in Figure 1, there was a 30% reduction ($P = 0.012$) in the concentration of H⁺ in stressed rats in the tocopherol group compared to the non-stressed rats in the same group, these findings were similar to the control group. Stressed rats that received supplementation of tocotrienol, however, did not show any change in the gastric acid concentration compared to its non-stressed group. The findings suggest that supplementation with tocotrienol and not tocopherol can block the

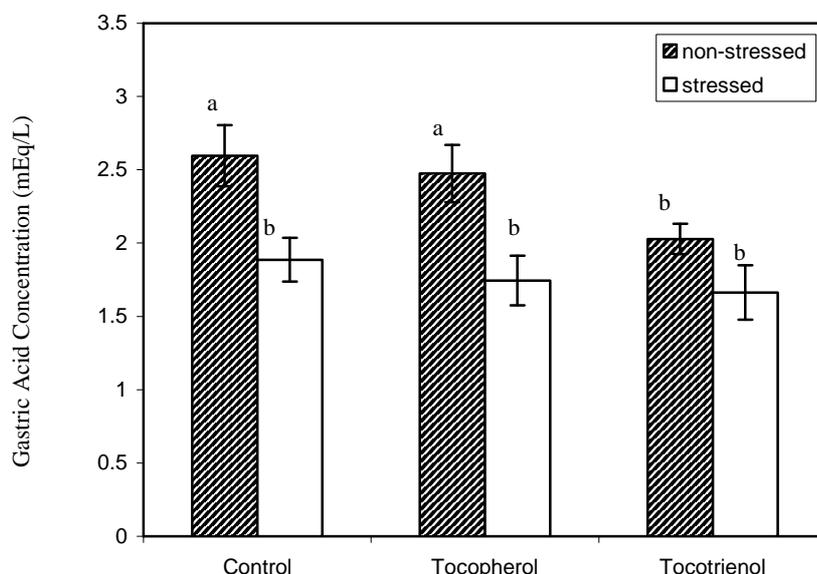


Figure 1. Comparison between tocopherol and tocotrienol on gastric acid concentration in rats exposed to restraint-stress. Mean gastric acid concentration with or without exposure to restraint-stress in control, tocopherol and tocotrienol supplemented rats. Different letters between bars indicate significant difference ($P < 0.05$).

changes in the gastric acidity induced by stress. The gastric acid concentration in non-stressed rats with supplemental tocotrienol was significantly ($P = 0.02$) lower compared to the non-stressed tocopherol group. This suggests the possible ability of tocotrienol to reduce gastric acidity under normal conditions.

Comparison between tocopherol and tocotrienol on serum gastrin levels in rats exposed restraint stress

Exposure to restraint stress two hours a day for four consecutive days resulted in a significant reduction in serum gastrin level by 40% ($P = 0.02$) in the control group and 38.1% ($P = 0.009$) in the tocopherol supplemented group, as shown in Figure 2. Similar to results of the acid concentration, the serum gastrin level in rats supplemented with tocotrienol had comparable gastrin level with or without exposure to restraint stress. The gastrin level was 38% ($P = 0.021$) and 28.6% ($P = 0.005$) higher in the tocotrienol group compared to the control rats and rats supplemented with tocopherol respectively with exposure to stress. The findings suggest that tocotrienol supplementation and not tocopherol can maintain a normal gastrin level in stress conditions.

Comparison between tocopherol and tocotrienol on plasma GLP-1 levels in rats exposed restraint stress

Another hormone that maybe altered during stress is GLP-1. It has been shown that GLP-1 plays a part in food intake regulation and gastric emptying.^{22,23} Our study showed that GLP-1 level was not altered in stress, where there was no significant difference in the plasma GLP-1 level between the non-stressed and stressed groups in rats given supplementation of tocopherol or tocotrienol. There was also no differences in levels between the two vitamin E supplemented groups, as shown in Figure 3.

Comparison between tocopherol and tocotrienol on gastric MDA content in rats exposed restraint stress

When the effect of tocopherol and tocotrienol on gastric MDA content are compared, no significant difference ($P > 0.05$) was observed between the two vitamin E supplemented groups in both non-stressed and stressed rats. However, stress does cause an increase in the gastric MDA level, suggesting that lipid peroxidation mediated by free radicals may play a part in stress induced gastric injuries. The gastric MDA content increased 26.3% ($P = 0.0007$) in the control stressed group, 15.1% ($P = 0.0177$) in the tocopherol stressed group and 20.4% ($P = 0.0245$) in tocotrienol stressed group compared to their respective non-stressed groups. However, supplementation of both tocopherol and tocotrienol was able to reduce the increased MDA level compared to the control by 13.1% ($P = 0.031$) and 13% ($P = 0.042$) respectively.

Comparison between tocopherol and tocotrienol on gastric PGE₂ content in rats exposed restraint stress

The mean gastric PGE₂ content in all groups studied was not significantly different ($P > 0.05$), as shown in Figure 4. The findings suggests that stress does not alter the gastric PGE₂ content and supplementation with tocopherol or tocotrienol does not increase the gastric PGE₂ content in normal non-stressed rats.

Discussion

The current study showed that tocotrienol caused a reduction in the gastric acid concentration in rats not exposed to stress, which suggests that it may have an antisecretory effect. The study by Moutairy and Tariq⁴ also showed that a single large dose (300mg/kg) of α -tocopherol caused a reduction in gastric acid secretion in nonstressed rats. Based on these findings, they proposed

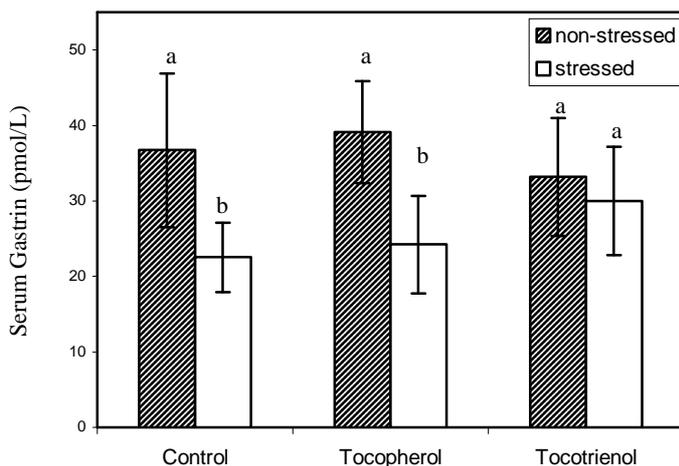


Figure 2. Comparison between tocopherol and tocotrienol on serum gastrin levels in rats exposed to restraint-stress. Mean gastrin level with or without exposure to restraint-stress stress in control, tocopherol and tocotrienol supplemented rats. Different letters between bars indicate significant difference ($P < 0.05$).

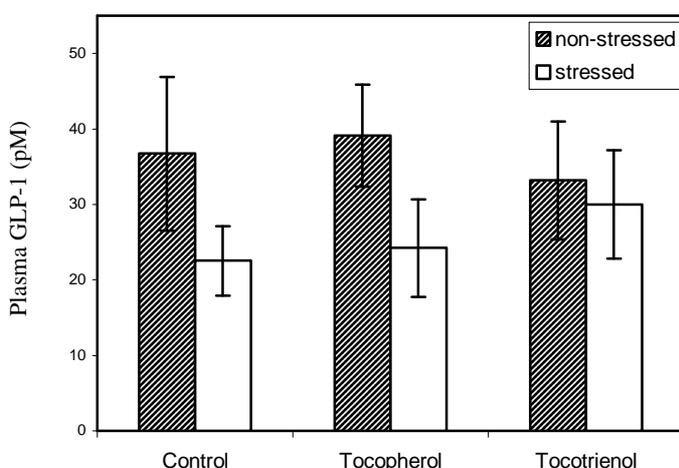


Figure 3. Comparison between tocopherol and tocotrienol plasma GLP-1 levels in rats exposed to restraint-stress. Mean GLP-1 levels with or without exposure to restraint-stress stress in control, tocopherol and tocotrienol supplemented rats. No significant differences were observed.

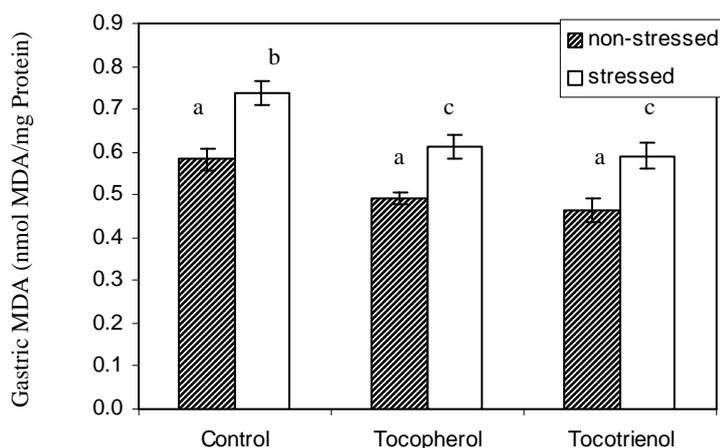


Figure 4. Comparison between tocopherol and tocotrienol on gastric MDA content in rats exposed to restraint-stress. Mean MDA with or without exposure to restraint-stress stress in control, tocopherol and tocotrienol supplemented rats. Different letters between bars indicate significant difference ($P < 0.05$).

that one of the protective effects by vitamin E against gastric injury could be through its antisecretory function. The main difference in the findings from Moutairy and Tariq⁴ and this study is the dose of tocopherol used. In their study a single large acute dose of α -tocopherol can exert antisecretory effects compared to the low dose of 60mg/kg bodyweight in our study. It is possible that a larger dose of α -tocopherol is needed to produce its

antisecretory effect; however a similar dose of 60mg/kg body weight of tocotrienol exerts a significant antisecretory effect at normal conditions. The findings suggest a better or a more potent antisecretory effect of tocotrienol compared to tocopherol.

The mechanism by which vitamin E exerts this effect remains unclear and alludes further exploration. It is known that gastric acid secretion is a calcium dependent

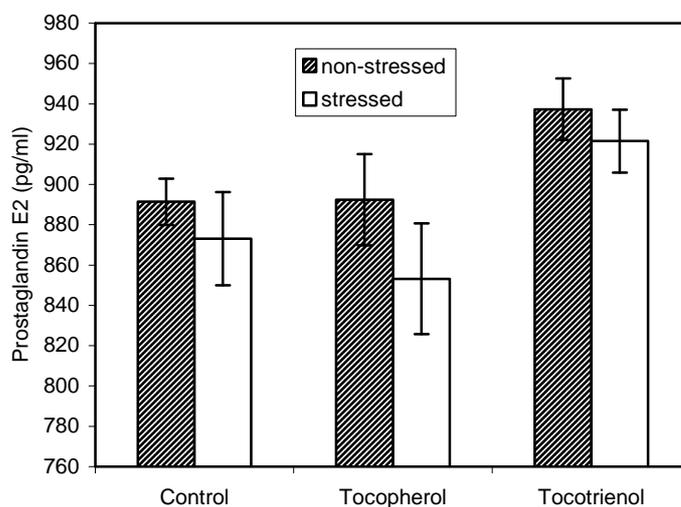


Figure 5. Comparison between tocopherol and tocotrienol gastric PGE₂ content in rats exposed to restraint-stress. Mean PGE₂ with or without exposure to restraint-stress stress in control, tocopherol and tocotrienol supplemented rats. No significant differences were observed.

process. Intravenous or intragastric calcium administration causes a significant increase in acid secretion²⁴, while verapamil, a calcium-channel blocker, had been shown to reduce calcium stimulated gastric acid secretion.²⁵ Vitamin E had been shown to reduced calcium influx across cell membranes²⁶, thus the antisecretory effect of vitamin E could be attributed to its effect on the influx of calcium, which overall effect causes a calcium dependent lower acid secretion.

The current study also shows that normal rats and rats supplemented with tocopherol and exposed to acute repetitive stress had a lower gastric acid concentration compared to their non-stressed groups. The gastric acid reduced 31% and 30% respectively in the rats exposed to stress compared to the non-stressed control and TF groups. Similarly the same effect of stress in rats had been shown by Hayase and Takeuchi² where they reported a gradual reduction in the gastric acidity which fell 60% below the normal level after exposure to 2 hours of restraint stress in rats fed a normal diet. There are also other reports of a reduced gastric acid concentration and secretion in rats on exposure to stress.²⁷⁻³⁰ It is also possible that the reduction in the gastric acidity in stress is a result of dysfunction of gastric acid secreting cells. The dysfunction could be due to ischemia to the parietal cells as a result of the compromised gastric microcirculation under stress conditions. The parallel relationship between the gastric acidity and the gastric blood flow had been shown previously by Hayase and Takeuchi.²

The changes in the gastric microcirculation during stress has been extensively studied and one of the proposed mechanisms is the reduction in splanchnic blood flow as a result of the increase in adrenaline and noradrenaline secretions during stress.³¹ Alternatively it could be due to the increase of gastric motility which is a common feature of stress. The latter is supported by studies that found increased gastric contractions during stress.^{32,33} These gastric contractions may temporarily restrict blood flow to the gastric mucosa.

Our study also found that tocotrienol was able to retard changes in the gastric acid concentration due to stress. Although it may not be possible to block or eliminate

stress completely it is definitely advantageous to have drugs or more favorably a food supplement such as tocotrienol that can block undesirable responses to stress. It is still unclear how tocotrienol can block the changes in the gastric acidity due to stress. It is however possible that it prevents damage to the acid secreting cells by trapping free radicals or possibly by maintaining a normal blood flow to the gastric mucosa.

Previous studies had shown that the mechanisms involved in gastric protection are induced by the same factors that increase the gastric acidity.^{34,35} For example, gastrin which is known to increase the gastric acid secretion, at the same time promotes gastric protection by increasing gastric blood flow and thickening of gastric mucus.³⁵ The end effect is the maintenance of the balance between the aggressive and the defensive factors in the gastric mucosa. Administration of gastrin analog had been reported to protect rats against ethanol-induced gastric injuries.^{36,37} Takeuchi and Johnson³⁸ found that rats given a liquid diet to reduce gastrin secretion, had increased stress-induced lesions while administration of pentagastrin, a gastrin analog, was able to reduce the development of lesions due to stress. They also showed that lesion development had a high correlation with the reduction of DNA and RNA synthesis in the gastric mucosa, which caused the inability for the epithelial cell renewal and repair which lead to gastric erosion during stress. Gastrin trophic effects are known to stimulate DNA and RNA synthesis thus bringing about its protective function. Thus the importance of preserving gastrin levels in conditions susceptible to gastric mucosal damage.

This study found that gastrin levels reduced significantly in response to stress in rats receiving normal diet and tocopherol supplementation. The reduction in the gastrin level may not only cause the reduction in the acid secretion as discussed above but may also cause a reduction in the protective effects of gastrin on the gastric mucosa, which can eventually lead to formation of lesions in an impaired mucosa. This study also showed that the effect of stress on gastrin can be blocked by supplementation of tocotrienol, where it was found that the serum

gastrin level was comparable in both stressed and non-stressed rats. Treatment with gastrin had been shown to significantly increase the rate of repair of ulcers and to increase blood flow to the ulcer margins which leads to upregulation of cyclooxygenase (COX)-2 mRNA and COX-2 proteins in the mucosa.³⁹ These protective effects of gastrin may be preserved in the condition of stress by supplementation with the tocotrienol, but not tocopherol.

Glucagon-like peptide 1 is known to be released in response to food ingestion and function in inhibiting gastric emptying, gastric acid secretion as well as glucagon secretion.⁴⁰ Studies had suggested that GLP-1 may be involved in the modulation of hypothalamus-pituitary axis activity.⁴¹⁻⁴³ Glucagon-like peptide 1 signals had been linked to behavior and neuroendocrine response to stress. When we look at the effect of stress on GLP-1 by measuring plasma GLP-1 levels we found that this parameter was not altered in stress. Vitamin E, both tocopherol and tocotrienol, had no effect on GLP-1 levels in normal non-stressed rats as well as rats exposed to stress, which suggests that GLP-1 is not altered by stress and vitamin E had no direct effect on this parameter.

Since free radicals had been implicated in the injuries to the gastric mucosa by noxious agents, we studied the effect of stress on lipid peroxidation levels by measuring its by product malondialdehyde (MDA). Measurement of MDA is one of the oldest methods and most widely used to measure lipid peroxidation.⁹ This study confirms the involvement of free radicals in the pathogenesis of gastric injuries caused by stress - there was an increase in the MDA level of all stressed groups studied compared to the non-stressed rats. This finding was in agreement with other studies showing the important role of lipid peroxidation in causing injuries to the gastric mucosa.^{9,10,12,13} As mentioned previously, an impaired gastric microcirculation, which is a common finding during stress, could lead to anoxic damage or ischemia to the gastric mucosa and ultimately cause an increase in free radical formation in the gastric tissue.

There were no differences in the MDA levels of both groups treated with either tocopherol or tocotrienol. There was a significant increase in the MDA level in both groups compared to their non-stressed groups. The findings suggest a similar potency of both preparations in controlling lipid peroxidation during stress. However, supplementation with tocotrienol or tocopherol significantly reduced the MDA content of gastric tissue compared to the stressed control, which suggests a protective effect against stress-induced injuries. Although this is true, it cannot completely inhibit the increased lipid peroxidation levels. It is possible that the dose given was not sufficient to completely prevent free radical induced lipid peroxidation in the gastric tissue during stress.

The level of PGE₂ did not differ significantly in the vitamin E supplemented groups compared to the non-stressed control. This shows that both tocopherol and tocotrienol in the dose used in this study, did not influence the gastric PGE₂ level in both normal and stressed rats. The findings also suggest that PGE₂ does not play a part in the stress induced injuries, where this parameter was not altered by stress.

We conclude that only tocotrienol has the ability to block the stress-induced changes in the gastric acidity and gastrin level but both tocopherol and tocotrienol are comparable in their gastroprotective ability against damage by free radicals in the stress condition. The effect of a larger dose of both tocopherol and tocotrienol on complete prevention against lipid peroxidation due to stress requires further investigation.

Acknowledgements

This study was made possible by the research grant (F/9/2000) from the Faculty of Medicine, Universiti Kebangsaan Malaysia. The authors would also like to acknowledge Carotech Sdn. Bhd. for the supply of tocotrienol for this research.

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A comparison between tocopherol and tocotrienol effects on gastric parameters in rats exposed to stress

生育酚和生育三烯酚影响应激小鼠胃参数的比较研究

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小鼠在应激状态下会引起胃肠道和激素的各种变化。本次研究比较了生育酚和生育三烯酚对一些影响小鼠胃和激素的参数所带来的变化，这些参数对小鼠在应激状态下保持胃粘膜完整性能中起重要作用。这些包括了胃酸度、胃组织中的一些成分如丙二醛、前列腺素 E₂ (PGE₂)，血清胃泌素和胰高血糖素样肽-1 (GLP-1)。60 只雄性 Sprague-Dawley 大鼠 (200-250g) 被随机分为三个组，对照组小鼠饲以正常的鼠饲料 (RC)，实验组两组均饲以维生素缺乏的饲料，并分别按体重计算口服 60mg/kg 量的生育酚 (TF) 或生育三烯酚 (TT)。经过 28 天饲喂后在宰杀前在非应激组中取一半小鼠的血清作为非应激组，另一半小鼠接受实验性的应激抑制反应，每天 2 小时，连续 4 天 (应激组)，第 4 天取小鼠血清并宰杀大鼠。实验结果显示，在对照组和 TF 组中，应激大鼠胃酸浓度和血清胃泌素水平相比非应激小鼠有显著降低 ($P < 0.05$)。然而，在 TT 组中，胃酸和胃泌素水平在应激和非应激小鼠中相当。这些结果表明，生育三烯酚能保持胃酸度和血清胃泌素水平，而这些指标在应激状态下经常改变。然而，PGE₂ 浓度和血浆 GLP-1 浓度在所有应激和非应激组中都相当，这表明这些指标在应激状态下未改变，添加 TF 或 TT 对胃 PGE₂ 浓度或血浆 GLP-1 浓度并没有影响。丙二醛作为脂类过氧化程度的指标，在应激组胃组织中其浓度相对非应激组要高。这些结果表明，自由基在应激状态下的胃损伤中发挥着作用，相比对照组大鼠，补充 TF 或 TT 能降低脂质过氧化程度。我们由此得出结论，生育酚和生育三烯酚对胃在应激状态下由自由基引起损伤时的保护能力相当，但生育三烯酚有阻止因应激导致的胃酸度和胃泌素水平发生改变的能力。

关键词：生育酚、生育三烯酚、应激、胃粘膜完整性