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## Tocotrienol - Rich Fraction and its Effects on Parameters Affecting Gastric Mucosal Integrity after a Single Exposure to Indomethacin

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**Abstract:** The effect of treatment with a tocotrienol-rich fraction (TTRF) on acute single exposure to indomethacin was investigated. Forty-eight male rats of the *Sprague-Dawley* (200-250g) species were randomly assigned into two groups (N and T). The N group was fed with a commercially prepared normal rat diet and the T group was fed with an identical diet enriched with TTRF 150mg/kg diet. Each group was further subdivided into two subgroups that was either challenged (NI and TI) or not challenged with indomethacin (NX and TX). After eight weeks of treatment the NX and TX rats were killed and the stomachs isolated whereas the NI and TI rats were challenged with a single dose of indomethacin (80mg/kg body weight) orally and after six hours the rats were killed. Measurements for malondialdehyde (MDA), glutathione content, PGE<sub>2</sub>, gastric acid concentration and gastric adherent mucous (GAM) were done. Gastric PGE<sub>2</sub> content and acid concentration were comparable in the NI and TI groups compared to its corresponding group that was not challenged. The gastric MDA content and GAM concentration were increased in the NI and TI compared to its corresponding group that was not challenged. This indicated that indomethacin increased MDA and treatment with TTRF could not inhibit the rise of MDA whereas TTRF has no effect on GAM concentration. The glutathione ratio was however, only elevated in the TI group compared to the TX, which indicates that in acute mucosal injury by indomethacin, TTRF is able to preserve the ratio of the endogenous antioxidant. We conclude that TTRF has beneficial effects on gastric parameters.

**Keywords:** TTRF, indomethacin, MDA, glutathione, PGE<sub>2</sub>, gastric acid, gastric adherent mucous

### Introduction

The non-steroidal anti-inflammatory drugs (NSAIDs) have been among the most widely used drugs and although the non selective NSAIDs have been notorious in causing gastrointestinal lesions, the mechanism of how these lesions are generated have remained obscure. The emergence of the selective NSAIDs is indeed a breakthrough as it ameliorates the gastrointestinal side effects. The use of the non-selective NSAIDs is still popular due to its relatively cheap cost. Hence, studies exploring the possibilities of preserving gastric mucosal integrity during treatment with non-selective NSAID are still being conducted (Sontag *et al.*, 1994; Taha *et al.*, 1996; Hawkey *et al.*, 1998).

The therapeutic effects and major toxic side effects of NSAIDs have been attributed to the ability of these drugs to inhibit the synthesis of prostaglandin (PG), through a direct action on prostaglandin H synthetase, which serves both as a cyclooxygenase (COX) and as a peroxidase (Davies and Wallace, 1997). PGs increase both the synthesis and the release of gastric mucous while NSAIDs has the opposite effects. Free radicals production by NSAIDs is among the more probable mechanism suggested that disrupt the gastric mucosal integrity (Ali *et al.*, 1996; Granger *et al.*, 1986). The body has endogenous antioxidant, which under normal conditions is adequate to protect the organs. In situations that differ from normal such as exposure to noxious stimuli, vulnerable organs such as the lung, liver and stomach need a high level of nonprotein sulfhydryls (mainly reduced glutathione) to maintain integrity (Kosower and Kosower, 1978; Boyd *et al.*, 1979). In such situations, exogenous antioxidant may prove to be beneficial.

Alpha tocopherol (vitamin E) is a naturally occurring antioxidant in the biological systems and is present in the cell membrane of various tissues including intestines and stomach (Granger *et al.*, 1986). The biological activity of vitamin E is generally believed to be due to its antioxidant action rendering it capable to inhibit lipid peroxidation in biological membranes by scavenging the chain propagating peroxy radicals, thus blocking the free radical chain reaction.

Tocotrienol, the vitamin E that may be obtained from palm oil has been shown to be a better antioxidant (Serbinova and Packer, 1994; Afaf and Appleqvist, 1996). In our efforts to search for

avenues to minimise disturbances in the gastric environment due to NSAIDs, this study is carried out to determine the effects of TTRF on important gastric parameters after exposure to indomethacin. This study also investigated the effects of a single exposure of indomethacin on the same parameters.

### Materials and Methods

In this study, forty-eight male rats of the *Sprague-Dawley* (200-250g) species were randomly assigned into two groups (N and T). The N group was fed with a commercially prepared normal rat diet and the T group was fed with an identical diet enriched with TTRF 150mg/kg diet. The TTRF enriched diet was prepared by dissolving 150mg of palm oil in a sufficient amount of acetone, pouring it over 1 kg of rat pellet and allowing the acetone to evaporate. The normal rat pellets were treated with acetone only. Each group was further subdivided into two subgroups that was either challenged (NI and TI, each n = 12) or not challenged with indomethacin (NX and TX, each n = 12). After an eight-week study period the NX and TX rats were killed and the stomachs isolated. Whilst the NI and TI rats were first challenged with a single dose of indomethacin (80mg/kg body weight) orally and were killed only after six hours post challenged. Of the twelve rats in each group, measurements for MDA, glutathione content, PGE<sub>2</sub>, and gastric acid concentration were done in six rats while the remaining stomachs (n=6) were used for the analysis of gastric adherent mucous.

The lower end oesophagus and pylorus were clamped and the stomach was removed. Gastric tissue MDA content was measured using a modified method described by Ledwozyw *et al.*, 1988. The gastric tissue was homogenise in distilled water, centrifuged and the diluted supernatant was added with trichloroacetic acid. After 15 minutes at room temperature, thiobarbituric acid was added and the samples were incubated in 100°C water bath for 30 minutes. After cooling, n-butanol was added and the absorbency of the upper phase was read.

Gastric glutathione content was measured using a well-established method (Griffith, 1980). The gastric tissue was homogenise in 4 volume of 5% TCA/0.01N HCL and centrifuged at 17000 × g for 15mins at 2°C. The supernatant was separated for GSH and GSSG assay. The ratio for reduced glutathione to oxidised glutathione

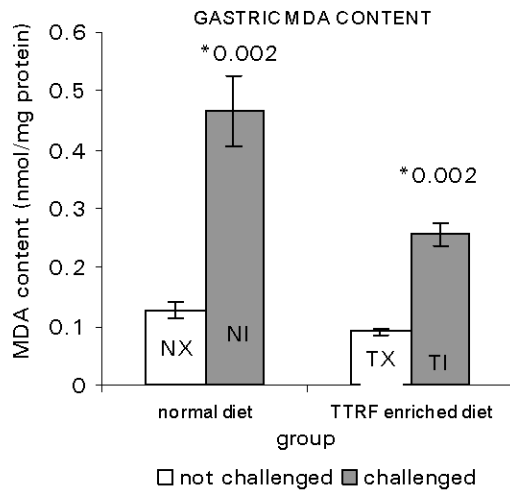


Fig. 1: Effects of TTRF and indomethacin on gastric MDA content. There was an increase in the gastric MDA content after challenged with indomethacin for both groups (NI and TI) compared to their respective controls (NX and TX) ( $P=0.002$ ).

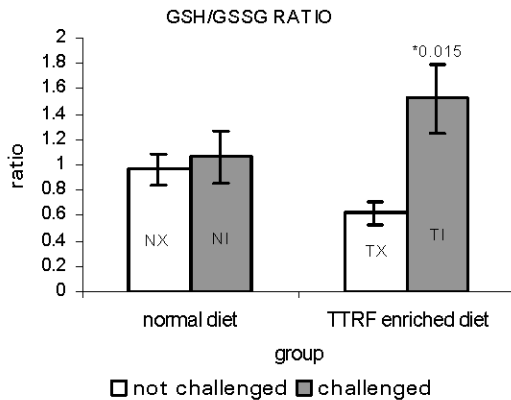


Fig. 2: Effects of TTRF and indomethacin on glutathione ratio. There was an increase in the glutathione ratio in the treated group that was challenged with indomethacin (TI) ( $P=0.015$ ) compared to the corresponding control (TX). The glutathione ratio in the normal challenged group (NI) did not differ from the normal unchallenged group (NX).

was calculated.

Samples of gastric mucosal tissue were prepared for prostaglandin analysis according to the method described by Redfern *et al.*, 1987. The extraction of  $PGE_2$  was performed using an Amprep C18 cartridge (Amersham International, UK) and the content was analysed using a kit ( $PGE_2[125]$  assay system, code RPA 530; Amersham International).

Samples of gastric juice were collected and centrifuged at 3000 r.p.m. for 10 min. Aliquots of each sample were titrated with 0.01N NaOH to a pH of 7.0. The concentration of hydrogen ion was calculated as described by Shay *et al.*, 1954.

Gastric adherent mucous level was determined by Alcian blue dye binding method as described by Corne *et al.*, 1974. The gaster was isolated and immersed in the Alcian blue solution. After two hours the unbound Alcian blue was removed by washing it twice in 0.25M sucrose solution. The mucous bound dye was eluted by using 0.5M magnesium chloride solution. The samples were added with diethylether and centrifuged. The absorbency of the

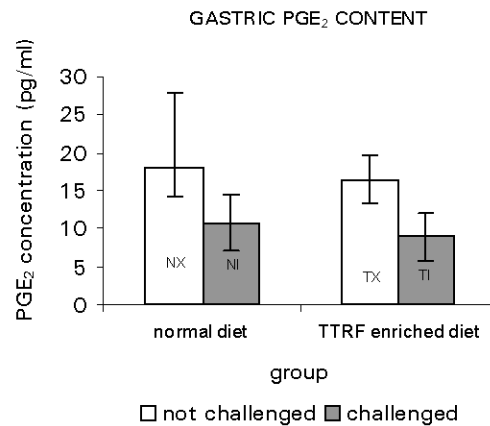


Fig. 3: Effects of TTRF and indomethacin on gastric tissue content of  $PGE_2$ . There were no difference in the gastric content of  $PGE_2$  in the normal and TTRF group whether or not the rats were challenged with indomethacin.

aqueous phase was measured using spectrophotometer. All results were expressed as mean  $\pm$  SEM. Statistical analysis was performed using t test and a P value of  $< 0.05$  was considered statistically significant for all parameters.

## Results

**Effect on gastric malondialdehyde (MDA) content:** The effects of TTRF and indomethacin on gastric MDA content are shown in Fig. 1. There was an increase in the gastric MDA content after challenged with indomethacin for both groups (NI and TI) compared to their respective controls ( $P=0.002$ ). There was a 3.5 fold increment in MDA content for the NI group and 3 fold increment for the TI group compared to the treated, unchallenged group. On a percent basis, the increment in MDA is smaller in the treated group (TI).

**Effect on glutathione ratio:** The effects of TTRF and indomethacin on glutathione ratio are shown in Fig. 2. There was an increase in the glutathione ratio in the treated group that was challenged with indomethacin (TI) ( $P=0.015$ ) by 2.5 fold compared to the corresponding control (TX). The glutathione ratio in the normal challenged group (NI) did not differ from the normal unchallenged group (NX).

**Effect on gastric tissue content of  $PGE_2$ :** The effects of TTRF and indomethacin on gastric tissue content of  $PGE_2$  are shown in Fig. 3. The gastric tissue content of  $PGE_2$  in the NI group did not differ from the unchallenged group (NX). There was also no difference in the gastric tissue content of  $PGE_2$  in the treated group whether or not the rats were challenged with indomethacin. This also demonstrated that indomethacin did not have an effect on gastric tissue content of  $PGE_2$  as the content remain unchanged in the untreated group (NI), compared to whether or not challenged (NI Vs NX).

**Effect on gastric acid concentration:** The effects of TTRF and indomethacin on gastric acid concentration are shown in Fig. 4. The gastric acid concentration in the NI group did not differ from the unchallenged group (NX). Similar observations were made in the groups treated with TTRF that is there was no difference in the gastric acid concentration in the TTRF group whether or not the rats were challenged with indomethacin. This also demonstrated that indomethacin did not have an effect on gastric acid concentration as the concentration remain unchanged in the untreated group (NI), compared to whether or not challenged (NI Vs NX).

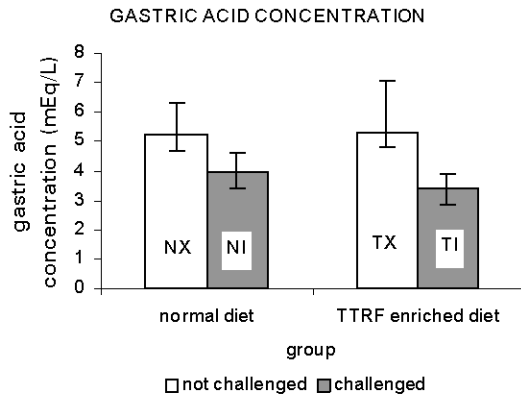


Fig. 4: Effects of TTRF and indomethacin on gastric acid concentration. There were no difference in the gastric acid concentration in the normal and TTRF group whether or not the rats were challenged with indomethacin.

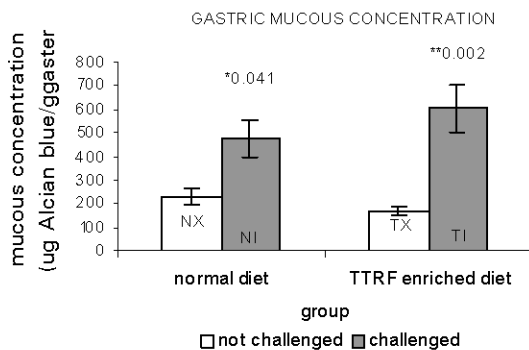


Fig. 5: Effects of TTRF and indomethacin on gastric adherent mucous quantity. There was an increase in gastric mucous quantity after challenged with indomethacin for both normal (NI) and the TTRF groups (TI) ( $P=0.041$  and  $P=0.002$  respectively) compared to their corresponding controls.

**Effect on gastric adherent mucous quantity:** The effects of TTRF and indomethacin on gastric adherent mucous quantity are shown in Fig. 5. There was an increase in gastric mucous quantity after challenged with indomethacin for both normal (NI) and the TTRF groups (TI) ( $P=0.041$  and  $P=0.002$  respectively) compared to their corresponding controls. The GAM quantity double in the NI group whilst a 3.5 fold increment was observed in the TI group compared to before challenged with indomethacin.

## Discussion

Just as many factors are involved in the maintenance of gastric mucosal integrity, factors causing the injury are also many and diverse. Some common identified aggressive factors include smoking, drugs such as NSAIDs and steroids, *Helicobacter pylori*, acid and pepsin. Although the gastric mucosa has protective factors such as adherent mucous layer, bicarbonate, phospholipids, prostaglandin and antioxidants, there are situations whereby these protection is breached. Given the widespread use of NSAIDs and their adverse effects on the gastric injury, studies such as the current one is indeed required.

Studies have shown that free radicals are involved in the development of mucosal damage by NSAIDs (Pihan *et al.*, 1987; Naito *et al.*, 1995). These excessive free radicals induce lipid peroxidations which is believed to be an important cause of destruction and damage to the gastric cellular membrane. In the

current study, we found that MDA was increased after challenged with indomethacin. Elevated gastric MDA reflects an intensification of lipid peroxidation process. Antioxidants such as TTRF used in this study is expected to retard lipid peroxidation process but this was not the case in our study. Even though TTRF is unable to inhibit the rise of MDA, on a percent basis the increment in MDA is smaller in the treated group (TI) compared to the untreated group (NI). Amongst the factors causing the lack of antioxidant effects of TTRF is the dose of indomethacin used. If, in fact the dose of indomethacin used is high and indomethacin increases the production of free radical, it is highly possible that the amount of TTRF used is insufficient to scavenge the excessive free radical. Hence, it is possible to increase the dose of TTRF in future studies. In contrast to the findings on MDA, interestingly we found that another indicator of antioxidant status that is the glutathione ratio, increased in the TTRF group that was exposed to indomethacin. Similar changes were not seen in the TTRF group that was not challenged to indomethacin. These observations suggest that TTRF on its own does not increase glutathione synthesis or its production. The ratio is enhance, however only after exposure to indomethacin which indicates that TTRF is able to scavenge the free radical and this reduce the consumption of reduced glutathione (GSH).

Studies have shown that chronic exposure to indomethacin suppressed the gastric prostaglandin synthesis (Redfern *et al.*, 1987; Shorrock and Rees, 1992). In this current study, there was no difference in gastric  $PGE_2$  content. It is therefore evident that a single dose of indomethacin does not inhibit prostaglandin synthesis after a single exposure. Complete inhibition of COX leading to reduce in  $PGE_2$  content will consume a much longer duration and will probably not be seen in a single dose only after 6 hours. The  $PGE_2$  measured is most probably the pre-formed  $PGE_2$ . Chronic treatment with indomethacin may lead to irreversible inhibition of  $PGE_2$ , in which case the reduced in  $PGE_2$  coupled with lipid peroxidation may caused sustained gastric injury.

Acid an aggressive factor that will ultimately leads to gastrointestinal lesions. Current treatment of the GI lesions employs anti secretory agents that is the  $H_2$  receptor antagonist, proton pump inhibitor and antimuscarinic agent. This study showed that there was no significant changes in acid concentration after exposure to a single dose of indomethacin either in the normal or the TTRF treated group. Previous studies done (Feldman and Colturi, 1984; Wagner *et al.*, 1995) showed that long term exposure to indomethacin led to a significant increase in mean gastric acid concentration. Exogenous prostaglandin has been shown to inhibit basal and stimulated acid secretion in man and animals (Levine and Schwartzel, 1984). As mentioned above, our study showed no changes in the gastric  $PGE_2$  content. This might explained why there were also no changes in the acid secretion after exposure to indomethacin.

After indomethacin exposure, there was increased in gastric adherent mucous content whether or not groups were treated with TTRF. A previous study done also showed that TTRF does not stimulate gastric mucous production (Nafeeza *et al.*, 2000). This showed that the increased in mucous content is probably not due to TTRF but may be the response of a protective mechanism in the gastric environment so as to minimise injury caused by indomethacin. Mucous released in response to topical application of an irritant, played an important role in the repair of epithelial damage through the process of restitution.

We found from this study that TTRF has no effect on  $PGE_2$  content, gastric acid and GAM concentration. TTRF was not able to inhibit the rise of MDA content by indomethacin but able to preserve the ratio of the endogenous antioxidant that is the glutathione. We conclude that TTRF has beneficial effects on gastric parameters.

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