International Journal of Pharmacology

ISSN 1811-7775

science alert

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Asian Network for Scientific Information

International Journal of Pharmacology 3 (3): 254-259, 2007
ISSN 1811-7775

Phytonutrient: Effects on Lipid Peroxidation in Experimental Gastritis Induced by Restraint Stress

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Abstract: In this research we studied the effect of free radical scavengers on restraint-induced lesions in rats. Forty rats were divided into 2 equal sized groups; a control group that was given a vitamin E deficient diet and a treatment group that was given the same diet but with oral supplementation of palm-based phytonutrient complex (PPC) at 60 mg kg^{-1} body weight for 28 days. At the end of the treatment period, half the numbers of rats in each group were sacrificed while the remaining half were subjected to daily restraint-stress for 2 h on 4 consecutive days. The rats were sacrificed after the fourth exposure, their stomach isolated and examined for lesions, gastric PGE_2 content, gastric malondialdehyde level and the gastric reduced glutathione level were measured as an index to reflect the scavenging abilities of PPC. Present findings showed hemorrhagic gastric lesions in rat exposed to stress. Rats that received PPC had less gastric lesions compared to the non-stressed control. The malondialdehyde level was also significantly lower in rats given PPC supplementation compared to the control while the reduced glutathione levels were preserved. We conclude that it is indeed probable that oxygen radicals are involved in the pathogenesis of restraint stress-induced lesions thus supplementation with antioxidant such as PPC may be able to reduce or inhibit the formation of these lesions.

Key words: Palm-based phytonutrient complex (PPC), restraint-stress, lipid peroxidation, gastric lesions

INTRODUCTION

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 (Salim, 1990; Nafeeza *et al*., 1999; Naito *et al*., 1993). Damage to gastric mucosa caused by restraint stress has been attributed to impaired blood flow that resulted in ischemia followed by reperfusion, a process known to generates free radicals. Oxygen-derived free radicals are cytotoxic and mediate tissue damage by injuring cellular membranes and releasing intracellular components (Salim, 1990). Although it is widely accepted that the pathogenesis of gastric mucosal lesions involves oxygen-derived free radicals the role of lipid peroxidation induced by stress remains uncertain. Among various stressor used in animals, one of the most reproducible results can be obtained by restraint stress (Ainsah *et al*., 1999; Al-Moutairy and Tariq, 1996).

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 (Nafeeza *et al*., 2002; Izgut-Uysal *et al*., 2001; Hirota *et al*., 1990). There are, however others who have shown antioxidant to be ineffective in inhibiting such damage, giving rise to the question of whether a combination of antioxidants is better than a single antioxidant against such injuries. Palm oil is a known source of phytonutrients. Palm oil derived vitamin E has been shown to accelerate repair of ethanol-induced gastric injury (Nafeeza *et al*., 1999; Kamsiah *et al*., 1999), aspirin (Nafeeza *et al*., 2002; Kamsiah *et al*., 2002) and indomethacin (Qodriyah *et al*., 2002) induced gastric injury. A Palm-based Phytonutrient Complex (PPC) an extract from palm oil is shown to have a mixture of antioxidant such as vitamin E (tocotrienols: 39% and tocopherol: 11.5%) and other nutrients such as phytosterol, phytocarotenoid complex and co-enzyme Q10. The effect of PPC on oxidative stress could account for the beneficial effect of this phytonutrient in model of stress injury. To confirm the hypothesis of the involvement of lipid peroxidation and PPC in stress, rats were subjected to restraint stress and stomach was examined for lesions and oxidative damage.

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MATERIALS AND METHODS

Male *Sprague-Dawley* rats (n = 40) were divided into two equally sized groups. The control group was fed with vitamin E deficient diet (VED) while the treatment group received a vitamin E deficient diet (VED) with oral supplementation of a PPC at 60 mg kg^{-1} body weight for 28 days. At the end of the treatment period, ten rats from each group were sacrificed. The remaining rats were exposed to restraint-stress. Stress-induced gastric lesions, gastric malondialdehyde level, gastric glutathione level and gastric prostaglandin E_2 (PGE_2) content were measured in all rats. The measurement was done immediately after the rats were sacrificed.

All rats were kept on a regular night/day cycle, with natural light for a period of 10 h (07:00 to 17:00 h). Throughout the feeding period all rats were habituated to handling to reduce their stress-related disturbances. Food and water were given *ad libitum* throughout the experiment. The rats were housed in large cages with wide wire-mash bottoms to prevent coprophagy. This study was conducted in the Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia between May 2001 to March 2003. The Animal Care and Use Committee (ACUC) of the Faculty of Medicine, National University of Malaysia, had approved this study (approval number: FAR/2000/NAFEEZA/30-NOVEMBER/031).

Palm-based phytonutrient complex (PPC): Palm-Based Phytonutrient Complex is a reddish vegetable oil suspension of naturally occurring mixture of tocotrienols and tocopherol which were extracted from the fruit of oil palm tree (*Elaeis giineensis*). It contains 22% d-γ-tocotrienol, 5.5% d-δ-tokotrienol, 11.5% d-α-tocotrienol and 11.5% d-α-tocopherol. Additionally it also contains other phytonutrients also extracted from the palm fruit which includes 10% plant squalene, 3.5% phytosterol complex, 0.5% phyto-carotenoid which includes α-carotene and β-carotene and also 0.43% co-enzyme Q10.

Restraint-stress: Rats were restrained by placing them in individual plastic restrainer measuring approximately 17×5 cm (Ainsah *et al*., 1999), for 2 h daily for 4 consecutive days. Following the restraining procedure on the fourth day, the rats were sacrificed. The stomach was opened 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 was 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 (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 mn. The gastric tissue content was deter-mined by the Lowry *et al.* (1951) method and MDA was expressed in terms of gram protein.

Measurement of gastric GSH and GSSG content: Gastric glutathione content was measured using a well-established method previously described by Griffith (1979). The gastric tissue was homogenized in 4 volume of 5% TCA/0.01N HCl and centrifuged at 17000 x g for 15 min at 2°C. The supernatant was separated for GSH and GSSG assay. The ratio for reduced glutathione to oxidized glutathione was taken as data.

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).

Statistics: Results are expressed as mean±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

Rats exposed to restraint stress 2 h a day for 4 consecutive days showed presence of considerable ulcerogenicity in the form of hemorrhagic mucosal lesions

confined to the corpus (glandular part of the stomach). The gastric lesion index in the control group is 7.8 fold higher compared to the non-stressed rats in the same group (Fig. 1). Supplementation with PPC however was able to reduce the lesions in the gastric mucosa after stress insult. There was a significant reduction of the lesion index by 48.7% compared to the stressed control ($p = 0.0006$). Rats killed after the 28 days feeding period and not exposed to stress had no gastric mucosal lesion in the gastric mucosa.

Treatment of rats with PPC causes a 16.7% ($p = 0.007$) 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. The increased was 23.5% ($p = 0.0037$) in the stressed control compared to the non-stressed control. Supplementation with PPC although shown to reduced the MDA level compared to the stressed control, the level was still significantly higher compared to the non-stressed PPC group. This suggests that PPC can reduce lipid peroxidation even in normal, non-stressed rats (Fig. 2).

Reduced glutathione (GSH) is the major endogenous antioxidant in life organism (Inoeu *et al.*, 1987). 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 30.2% ($p = 0.026$) in the stressed control compared to the Non-Stress Control (NSC) group as shown in Fig. 3. Rats treated with either PPC showed no significant different in the gastric glutathione level compared to the non-stress

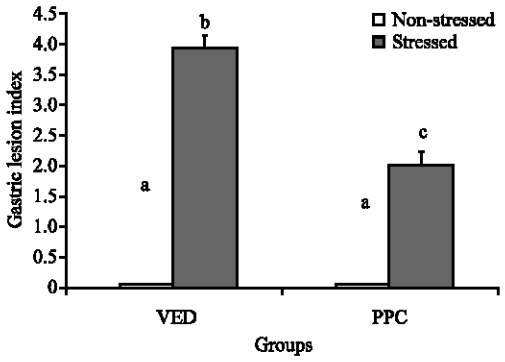

Fig. 1: Effects of PPC on lesion index in rats exposed to restraint-stress. Mean lesion index with or without exposure to restraint-stress in control rats and rats supplemented with PPC. Different letters between bars indicate significant difference ($p<0.05$)

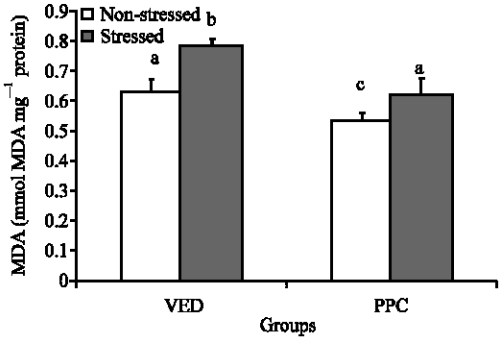

Fig. 2: Effects of PPC on gastric MDA level in rats exposed to restraint-stress. Mean MDA level with or without exposure to restraint-stress in control rats and rats supplemented with PPC. Different letters between bars indicate significant difference ($p<0.05$)

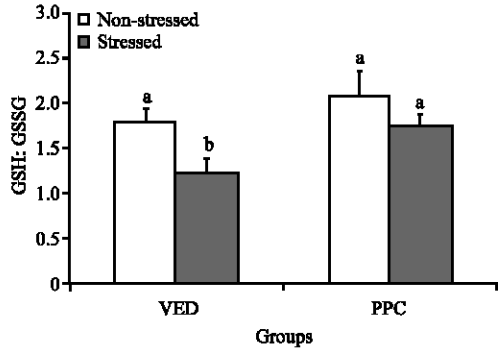

Fig. 3: Effects of PPC on gastric glutathione level in rats exposed to restraint-stress. Mean gastric glutathione level with or without exposure to restraint-stress in control rats and rats supplemented with PPC. Different letters between bars indicate significant difference ($p<0.05$)

control A significant increased of 43.2% ($p = 0.019$) was observed when comparing the PPC group with the stressed control. The finding suggests that PPC can restore a normal gastric glutathione level which was altered by stress.

The mean gastric PGE_2 content in rats exposed to restraint stress was significantly lower ($p<0.05$) compared to the non-stress control, as shown in Fig. 4. The findings suggest that stress alters the gastric PGE_2 content and the gastric mucosal injuries in rats exposed to stress could partly be due to disruption in these protective prostaglandin. Supplementation with PPC increases PGE_2 level even higher than the non-stress control values. This suggests a protective effect of PPC could be due to its ability to increase the gastric PGE_2 content.

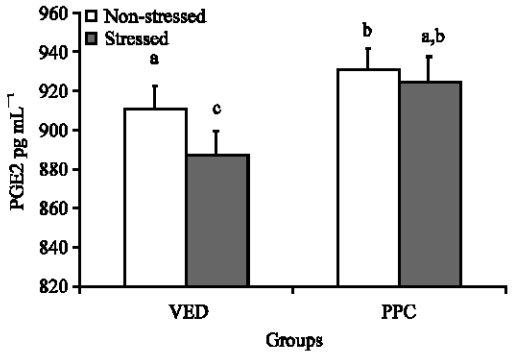

Fig. 4: Effects of PPC on gastric prostaglandin E_2 content in rats exposed to restraint-stress. Mean gastric prostaglandin E_2 content with or without exposure to restraint-stress in control rats and rats supplemented with PPC. Different letter(s) between bars indicate significant difference ($p<0.05$)

DISCUSSION

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. Amongst the various hazardous effects on biological system is oxidative destruction of membrane polyunsaturated fatty acid or more commonly known as lipid peroxidation which had been observed in numerous tissues (Izgut-Uysal *et al*., 2001). Present findings showed that rats exposed to restraint stress for 2 h daily for 4 consecutive days developed considerable amount of lesions in the form of mucosal hemorrhage and erythema confined to the corpus (glandular part of the stomach). As hypothesized, rats pre-supplemented with PPC for 28 days prior to stress exposure, had less severe gastric lesions compared to the control. However, PPC does not completely inhibit the formation of lesions but could effectively reduce the lesion formation which shows a protective effect against stress-induced lesions.

Similarly earlier studies (Nur Azlina *et al*., 2005a, b) had showed that supplementation with tocotrienol or tocopherol individually at the dose of 60 mg kg^{-1} bodyweight for 28 days significantly reduces lesion formation although unable to fully protect the gastric mucosa against stress-induced lesion. The combination of tocotrienols and tocopherol as well as the other phytosterol complex in PPC was still insufficient for total prevention of stress-induced gastric lesion. Thus it is possible that the effect of PPC may be dose dependent, this however would require a further research on the dose-dependent response.

Damage to gastric mucosa caused by restraint stress has been attributed to impaired blood flow that resulted in ischemia followed by reperfusion, a process that generates free radicals. We confirmed that the stress-induced gastric lesions could partly be due to an excessive amount of free radicals formation due to stress with the observation of an elevation of gastric MDA content, which reflects an intensification of lipid peroxidation process. The finding indicates that reactive oxygen species and lipid peroxidation is important in the pathogenesis of gastric mucosal injury induced by stress. We also showed that PPC decreases the breakdown of gastric mucosal barrier by reducing the end product of lipid peroxidation (MDA). The reduced MDA level accompanying the improved gastric lesions in this supplemented group suggests that PPC probably reduces gastric injury by retarding the lipid peroxidation process.

The failure of the endogenous antioxidant defense system during stress, could be attributed to an excessive generation of free radicals. We observed a significant depletion of glutathione content in the gastric mucosa following exposure to stress. The treatment of rats with PPC however, significantly attenuated stress-induced depletion of gastric mucosal glutathione. PPC supplementation was able to increase the glutathione ratio towards the non-stressed value. Similar changes were not seen in the PPC group that was not exposed to stress. These observations suggest that PPC on its own does not increased the glutathione synthesis or its production. The ratio was however enhanced only after the exposure to repeated stress which indicates that PPC is able to scavenge free radicals and this reduces the consumption of reduced glutathione (GSH).

Studies had shown that reduced glutathione, a major endogenous non-protein sulfhydryl 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 strengthen the importance of free radicals in causing gastric injury in stress, thus prevention using exogenously administration of PPC seems to be a logical alternative to the prevention of such injuries.

Prostaglandins are generated in the gastric mucosa via the activity of an enzyme cyclooxygenase (COX), which exist in two genetically different isoforms,

constitutive of COX-1 forms and inducible COX-2 (Feng *et al*., 1995; Hla and Nieilson, 1992). 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 (Kujubu *et al*., 1991; Xie *et al*., 1991).

Study by Bregonzio *et al*. (2003) found that stress-induced mucosal ulcerations were 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 the rats exposed to stress compared to the non-stressed control. We also found that PPC supplementation has 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 PPC supplemented groups plus maintenance of gastric PGE_2 levels, we can also associate the decreased in the gastric lesions to the levels of PGE_2.

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 (Takeuchi *et al*., 1999). Present 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 PPC proved to be a good alternative towards reducing stress induced gastric lesions.

In conclusion, present data suggest that stress induced gastric lesion is multifactorial and from this study two factors had been determined, which is elevation of free radicals formation and depletion of the protective prostaglandin. We also found a possible solution to decrease the gastric injury due to stress, where supplementation with a palm-based phytonutrient complex which contains mainly tocotrienols and tocopherol was shown to reduce the occurrence of gastric mucosal lesions by strengthening the gastric mucosal barrier against stress-induced elevation of lipid peroxidation and restoring the gastric prostaglandin content. However, the effect of a larger dose of PPC for complete prevention against lipid peroxidation due to stress requires further investigation.

ACKNOWLEDGMENTS

This study was made possible by the research grant (code number: F/9/2000) from the Faculty of Medicine, UKM. We would like to acknowledge Carotech Sdn. Bhd. for the supply of PPC (Tocomin) used for this research. Thank you to the members of the Pharmacology Department, Faculty of Medicine, UKM, particularly Mrs. Azizah Osman for the technical support extended in this research.

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