

Activity of Tacrolimus: An Immunosuppressant, in Pyloric Ligation Induced Peptic Ulcer in Rat

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The effect of tacrolimus (FK506) on peptic ulcer was evaluated using pyloric ligation (PL) model in rats. Tacrolimus was administered orally at different doses (1, 2 and 3 mg/kg) and it showed a gastric ulcer healing effect in a dose dependent manner. Gastric volume, total and free acidity and ulcerative index parameters were reduced in the tacrolimus treated rats as compared to pyloric ligated rats. The higher dose (3 mg/kg) treated group produced significant results similar to that of the ranitidine (50 mg/kg) treated group. Pretreatment with tacrolimus also produced significant ($p < 0.05$) reduction in TBARS, total calcium, TNF- α , IL-8 and MPO whereas it showed an increase in GSH level at higher dose. The anti-secretory and anti-ulcerative effect of tacrolimus may be due to immunosuppressive actions by inhibition of calcineurin and the oxidative pathway. It can be concluded that tacrolimus can play an important role in the treatment of peptic ulcer disorder to improve the quality of life.

Key words—tacrolimus; pyloric ligation; calcium; myeloperoxidase; calcineurin

INTRODUCTION

Tacrolimus (FK506) is a potent immunosuppressive drug that has been widely used for organ transplantation and atopic dermatitis.^{1,2} Recent clinical studies have demonstrated the beneficial effects of this agent in the treatment of various autoimmune and inflammatory diseases, including rheumatoid arthritis and inflammatory bowel diseases.³ It has further been shown that tacrolimus inhibited the induction of iNOS by suppressing the activation of nuclear factor kappa-B (NF- κ B), which may be responsible for its protective effect.^{4,5} It was also reported earlier that the immunosuppressive agent tacrolimus prevented gastric mucosal lesions.^{6,7} In the peptic ulcer condition mast cells play an important key role. Activated mast cells release various biochemical mediators like cytokines (TNF, interleukin, interferon, leukotriene *etc.*), chemokines, histamine, serotonin, eicosanoids and myeloperoxidase enzymes.⁸ Mucosal infiltration by neutrophils and mast cells activation is well known in the pyloric ligation (PL) model.^{9,10}

Pyloric ligation is one of the major factors of mucosal damage because it interferes with gastric mucosal resistance and alters the level of cytoprotective prostaglandins, cytokines, membrane lipid peroxidation (TBARS) and endogenous glutathione.¹¹

Moreover, pyloric ligation causes an increase in calcium level which, in turn, is known to stimulate free radical generation.^{12,13} This increase in calcium and free radicals is documented to have induced tissue injury and peptic ulcer.¹⁴ Hence, as we know that tacrolimus binds to the FK506-binding protein (FKBP) and this tacrolimus-FKBP complex interacts with calcineurin which inhibits the catalytic activity of calcineurin, this activity of tacrolimus can thus be explored for its antiulcer potential. Although it has been speculated that the preventive effects of tacrolimus in gastric ulcers are derived from the suppression of cytokine synthesis and neutrophil infiltration,¹⁵ the exact mechanisms have not been explored yet. The present study was therefore designed to explore the mechanisms underlying the gastroprotective effect of tacrolimus in pyloric ligation induced peptic ulcer.

MATERIALS AND METHODS

Experimental Animals Male Wistar rats weighing between 200–250 g were used. They were kept on a standard laboratory diet with environmental temperature and humidity. A 12 hour light-dark cycle was maintained throughout the experimental protocol. This experimental protocol was duly approved by the Institutional Animal Ethics Committee (IAEC) and care of the animals was carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on

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Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg No:- 874/ac/05/CPCSEA).

Experimental Design Six groups, each comprised of six rats, were included in the antiulcer studies.

Group I Sham control group: normal saline (p.o.), Group II Pyloric ligation control group, Group III Ranitidine control group (50 mg/kg, p.o.), Group IV Tacrolimus treated group (1 mg/kg, p.o.), Group V Tacrolimus treated group (2 mg/kg, p.o.), Group VI Tacrolimus treated group (3 mg/kg, p.o.).

Surgical Procedure Ulcer study was performed by the pyloric ligation process in rats as described by Shey *et al.*¹⁶⁾ Animals were fasted for 24 h before pylorus ligation with water *ad libitum*. Under light ether anesthesia, the abdomen was opened by midline incision below the xiphoid process. The pyloric portion of the stomach was slightly lifted out and ligated, avoiding damage to its blood supply. On the day of the experiment on groups II to VI, the normal saline (0.9% w/v of sodium chloride), ranitidine and tacrolimus was administered orally 1 h before pylorus ligation, respectively, whereas in group II normal saline was administered orally 1 h before the surgical procedure but no pyloric ligation was performed. This group II was employed as the sham control group. The stomach was carefully placed back and the abdominal wall was closed with sutures. Animals were sacrificed 6 h after pylorus ligation and gastric content and isolated tissues were subjected to further studies.

Estimation of Gastric Volume, Total and Free Acidity The gastric juice was collected and its volume was measured. The gastric juice was then centrifuged and the clear supernatant was analysed for total and free acidity using the method of Hawk *et al.*¹⁷⁾ Briefly, 1 ml of supernatant liquid was pipetted out and diluted to 10 ml with distilled water. The solution was titrated against 0.01N sodium hydroxide using topfer's reagent as indicator. The end point was titrated when the solution turned orange in colour. The volume of NaOH was noted, which corresponded to free acidity and the solution was further titrated until it regained its pink colour. The total volume of NaOH was noted, which corresponded to the total acidity.

Measurement of Ulcerative Index Ulcerative index was measured by the method of Takagi *et al.*¹⁸⁾

Briefly, the stomach was opened and washed with running tap water, then was placed on a flat glass plate to measure the ulcerated area. Standardization was made with a 10×10 cm square glass plate. The opened stomach was laid on the glass plate and the mucous was exposed, allowing the counting of injuries per square mm. The ulcer index was determined by using the formula, Ulcer Index=10/X where, X= Total mucosal area/Total ulcerated area.

Biochemical Estimation Tissue homogenate was prepared with 10 volumes of 0.1M tris-HCL buffer (pH 7.4) and a supernatant of homogenate was employed to estimate the thiobarbituric acid reactive substance (TBARS), reduced glutathione, total calcium, TNF- α , IL-8 and myeloperoxidase activity.

Estimation of TBARS Estimation of lipid peroxidation was done by measuring the level of malondialdehyde (TBARS).¹⁹⁾ The concentration of TBARS in tissue homogenate was expressed in terms of nmol malondialdehyde/g of protein, 1,1,3,3-Tetramethoxypropane (1–10 nmol) was used as standard. Protein estimation was carried out as described by the method of Lowry.²⁰⁾

Estimation of Reduced Glutathione Reduced glutathione levels were estimated according to the method of Ellman.²¹⁾ An equal quantity of tissue homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate protein. To 0.01 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5,5'-dithio, bis (2-nitrobenzoic acid) and 0.4 ml of double distilled water was added. The mixture was vortexed and the absorbance was taken at 412 nm within 15 min. The concentration of reduced glutathione was expressed as $\mu\text{mol/g}$ of protein.

Estimation of Total Calcium Content Total calcium level was estimated in stomach tissues as described by Severinghaus and Ferrebee.²²⁾ Briefly, tissue homogenate was mixed with 1 ml of trichloroacetic acid (4%) in an ice-cold condition and centrifuged at 2500 rpm for 10 min. The clear supernatant was used for the estimation of total calcium by atomic emission spectroscopy at 556 nm.

Estimation of TNF- α and IL-8 Tissue samples were utilized for determination of the level of pro-inflammatory cytokines (TNF- α and IL-8). These levels were determined using an ELISA kit (SD Bio Standard Diagnostic, Gurgaon, India).

Estimation of Myeloperoxidase (MPO) Activity MPO enzyme activity measurement is an indication

of neutrophil infiltration. MPO activity was measured using a procedure described by Hillegass *et al.*²³⁾ Briefly, gastric tissue samples were homogenized in 50 mM potassium phosphate buffer (pH 6.0), and centrifuged at 2500 rpm (10 min); pellets were suspended in 50 mM phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (HETAB). After three freeze and thaw cycles, with sonication between cycles, the samples were centrifuged at 2500 rpm for 10 min. Aliquots (0.3 ml) were added to 2.3 ml of reaction mixture containing 50 mmol phosphate buffer, o-dianisidine, and 20 mmol H₂O₂ solution. The presence of MPO was measured at 460 nm for 3 min and MPO activity was expressed as U/g tissue. One unit of this activity was defined as that degrading 1 μ mol peroxide/min at 25°C.

Statistical Analysis All the results were expressed as mean \pm standard error of means (S.E.M.). The data was statistically analyzed by one way analysis of variance (ANOVA) followed by Tukey's multiple range tests using Sigmasat Version-2.0 Software. The p -value < 0.05 was considered to be statistically significant.

RESULTS

Effects of Tacrolimus on Gastric Volume, Total and Free Acidity Gastroprotective effect of tacrolimus was observed on the PL induced gastric damage in rats. The gastroprotective effects of 1, 2, and 3 mg/kg doses of tacrolimus on gastric volume, total and free acidity are shown in Table 1. The tacrolimus treated groups showed remarkable changes in the above parameters as compared to the PL animals; these changes produced by tacrolimus were comparable to the ranitidine treated group. These results showed that all doses of tacrolimus had a protective effect against the gastric damage caused by pyloric ligation but the higher dose had a significant ($p < 0.05$) gastroprotective effect since it decreased the gastric volume and reduced the total and free acidity similar to that of ranitidine.

Effects of Tacrolimus on Ulcerative Index Pyloric ligation caused severe gastric mucosal lesions in the rats. Oral administration of the tacrolimus (1, 2 and 3 mg/kg) reduced the severity of these gastric lesions in a dose dependent manner (Fig. 1), with a significant effect ($p < 0.05$) being observed at 3 mg/kg dose similar to that of ranitidine. Lower and medium doses however produced an insignificant gastroprotective

Table 1. Antisecretory Effect of Tacrolimus in Pyloric Ligated Rat

Group (mg/kg)	Gastric volume (ml/100 gm)	Total acidity (mEq/l)	Free acidity (mEq/l)
Pyloric ligation	2.78 \pm 0.74	98.84 \pm 1.93	61.78 \pm 2.35
Ranitidine (50)	1.10 \pm 0.16 ^a	58.39 \pm 2.14 ^a	21.06 \pm 2.92 ^a
Tacrolimus (1)	2.48 \pm 0.93 ^b	90.28 \pm 2.99 ^b	46.61 \pm 2.11 ^b
Tacrolimus (2)	2.09 \pm 0.36 ^b	80.54 \pm 2.36 ^b	33.82 \pm 1.54 ^b
Tacrolimus (3)	1.14 \pm 0.52 ^{ac}	61.61 \pm 3.15 ^{ac}	24.91 \pm 2.09 ^{ac}
F Value	4656.27	433.70	324.65

Values are mean \pm S.E.M. of 6 animals. a= $p < 0.05$, as compared to pyloric ligation control group; b= $p < 0.05$, as compared to ranitidine treated group; c= $p < 0.05$, as compared to tacrolimus 1 and 2 mg/kg pretreated groups.

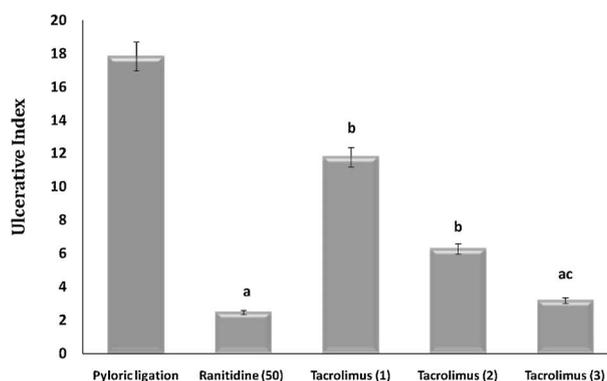


Fig. 1. Effect of Tacrolimus on Ulcerative Index

Values are mean \pm S.E.M. of 6 animals. a= $p < 0.05$, as compared to pyloric ligation control group; b= $p < 0.05$, as compared to ranitidine treated group; c= $p < 0.05$, as compared to tacrolimus 1 and 2 mg/kg pretreated groups.

effect as compared to ranitidine.

Effects of Tacrolimus on Biochemical Markers

The effects of tacrolimus on the ulceration process in gastric tissue were evaluated by the estimation of oxidative stress markers (TBARS and GSH), total calcium, TNF- α , IL-8 and MPO activity. These results are presented in Table 2 and showed that the GSH level for the PL group was lower whereas TBARS, total calcium, TNF- α , IL-8 and MPO levels were higher than those of group I. However, pretreatment with tacrolimus and ranitidine showed opposite results from PL. Tacrolimus has caused significant changes in the oxidative stress and proinflammatory marker levels and in the enzymatic system (MPO) in a dose dependent manner. A dose (3 mg/kg) has shown significant action ($p < 0.05$) similar to that of the ranitidine pretreated group.

Table 2. Effect of Tacrolimus on Biochemical Markers in Gastric Tissue

Group (mg/kg)	TBARS (nmol/g of protein)	GSH (μ mol/g of protein)	Total calcium (ppm/mg of protein)	TNF- α (ng/g of protein)	IL-8 (ng/mg of protein)	MPO (U/g of protein)
Normal	3.21 \pm 0.44	1.29 \pm 0.22	3.41 \pm 0.99	0.15 \pm 0.06	1.78 \pm 1.46	0.35 \pm 0.14
Pyloric ligation	4.61 \pm 0.31	0.72 \pm 0.29	13.70 \pm 1.54	0.63 \pm 0.13	32.18 \pm 2.77	1.08 \pm 0.13
Ranitidine (50)	3.27 \pm 0.25 ^a	1.27 \pm 0.56 ^a	3.83 \pm 1.36 ^a	0.18 \pm 0.09 ^a	2.48 \pm 0.96 ^a	0.38 \pm 0.06 ^a
Tacrolimus (1)	4.38 \pm 0.39 ^b	0.83 \pm 0.39 ^b	12.76 \pm 1.48 ^b	0.53 \pm 0.11 ^b	26.54 \pm 2.44 ^b	0.99 \pm 0.02 ^b
Tacrolimus (2)	4.11 \pm 0.72 ^b	0.93 \pm 0.21 ^b	9.89 \pm 1.97 ^b	0.45 \pm 0.05 ^b	18.28 \pm 1.79 ^b	0.92 \pm 0.01 ^b
Tacrolimus (3)	3.37 \pm 0.15 ^{ac}	1.24 \pm 0.49 ^{ac}	4.23 \pm 1.26 ^{ac}	0.21 \pm 0.07 ^{ac}	2.78 \pm 0.56 ^{ac}	0.40 \pm 0.11 ^{ac}
F Value	262.65	626.02	1258.47	426.65	3636.15	619.26

Values are mean \pm S.E.M. of 6 animals. a= p <0.05, as compared to pyloric ligation control group; b= p <0.05, as compared to ranitidine treated group; c= p <0.05, as compared to tacrolimus 1 and 2 mg/kg pretreated groups.

DISCUSSION

The present study showed that the immunosuppressing agent tacrolimus prevented PL induced gastric ulceration in rats and further demonstrated that this agent also potentially alters the level of gastric volume, total and free acidity, ulcerative index and various biochemical parameters (TBARS, GSH, calcium, TNF- α , IL-8 and MPO activity). Although various mechanisms are involved in the pathogenesis of pyloric ligation -induced peptic ulcer, gastric acid secretion and accumulation are thought to be the most important factors. In addition, it is well established that various antisecretory agents, such as H₂-receptor antagonists and proton pump inhibitors prevent gastric lesions.²⁴⁾ Moreover, ranitidine an H₂-receptor antagonist has often being reported to possess antioxidant and immunosuppressive actions, which may also be responsible for its antiulcerogenic activity.²⁵⁻²⁷⁾ Hence, in the present study ranitidine was taken as a reference drug with which to compare the antiulcer potential of tacrolimus. Since in this study tacrolimus significantly suppressed gastric acid secretion and acidic content which was indicated by a decrease in gastric volume and acidity, we considered that its protective effect may be partially mediated by its antisecretory effect. Results revealed that tacrolimus reduced the TBARS and calcium levels but increased the reduced glutathione levels, so this may be due to its effect on the decrease in free radical accumulation.^{12,13)} Moreover, it showed decrease in calcium levels which in turn led to a decrease in free radical generation.

Tacrolimus immunosuppressive effects are mediated by the suppression of T-cell activation through the

inhibition of a calcium/calmodulin activated protein serine/threonine phosphatase called protein phosphatase (calcineurin).^{28,29)} Many recent studies have reported that calcineurin may play an important role in intracellular signal transduction pathways in various secretory cells, pancreatic acinar cells, parotid gland, and chief gastric cells.^{30,31)} This inhibitory effect of tacrolimus on chief gastric cells through blockage of the calcineurin pathway leads to its antisecretory potential in pyloric ligation induced peptic ulcer. The tacrolimus-FKBP complex interacts with calcineurin and blocks the production of intermediate compounds which are involved with the expression of genes, regulating the production of cytokines. It is thus assumed that tacrolimus exerts a protective effect against PL induced peptic ulceration through alteration of the TNF- α and IL-8 levels. Moreover, the gastric mucosal damage is also caused by increased recruitment of neutrophils which is evidenced by elevated MPO activity in the PL model. Activated neutrophils are a potential source of oxygen metabolites which initiate the deactivation of antiproteases and activating cytotoxic enzymes including elastase, proteases, lactoferrin and MPO.^{32,33)} Result revealed that the tacrolimus treated group had decreased MPO activity, so this may be due to the drug's immunosuppressive action.

CONCLUSION

Our studies explored the ameliorative effect of tacrolimus on pyloric ligation induced peptic ulcer in rats. The ulcer protective activity of tacrolimus may be through its antisecretory, antioxidant and anti-inflammatory action and its inactivation of immune cells. Further, these actions may be produced by its

calcineurin blocking and immunosuppressive properties.

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