Original Article

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The therapeutic potential of trifluoperazine against ethanol and cold water stress-induced gastric lesions in rat

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Abstract

Gastric erosion is a multifactorial etiological disorder. The study aimed to evaluate the therapeutic effect of trifluoperazine (TFP) on gastric lesioninduced by cold-water stress and ethanol in rats. TFP 5, 10 and 20 mg/kg as well as pantoprazole (20 mg/kg), as the standard treatment, was administered via oral gavage, 1 h before gastric lesion-induced by 50% ethanol (5 ml/kg per oral) or immersion in cold water (20-22 °C) in rats. Animals were anesthetized 1 h after ethanol gavage or 4 h after immersion in cold water. The stomach tissue was removed and allocated for histopathological and biochemical parameters including malondialdehyde (MDA)level, superoxide dismutase (SOD) and catalase (CAT) activity assay. Both models were induced significant increase in MDA level and decrease in SOD and CAT activity. Pre-treatment with TFP at doses of 10 and 20 mg/kg significantly increased the activity of SOD and CAT enzymes and decreased the concentration of MDA in both models. Also, pre-treatment with TFP ameliorated ethanol and cold-water stress-induced damage. TFP demonstrated gastric mucosal protection against oxidative injuries caused by ethanol and cold-water stress because of its antioxidant properties and inhibition of toxic oxidant in the stomach tissues.

Keywords: Cold-water stress, Ethanol, Oxidative Stress, Stomach Ulcer, Trifluoperazine

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1. Introduction

Amongst the serious digestive system disorders, gastric ulcer is the most common chronic disease which affects 5-10 % of the world population. Gastric lesionis a complex and multifactorial disorder which has been shown to be associated with several factors like alcohol consumption, smoking, stress, chronic usage of non-steroidal anti-inflammatory drugs (NSAIDs), and *Helicobacter pylori* infection (1, 2). Experimental studies have demonstrated that oxygen-generated free radicals and lipid peroxidation are involved in the pathogenesis of acute gastric lesions induced by

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ethanol, nonsteroidal anti-inflammatory drugs, Helicobacter pylori, and stress (3). These agents mediate their effects in part, through the generation of reactive oxygen species (ROS). Various types of ROS such as hydroxyl radical plays a critical role in causing oxidative damage of mucosa in all types of lesions. Free radicals, involved in gastrointestinal injuries, may inactivate the synthesis of mucosal prostaglandin which alleviates the generation of ROS by accumulating H_2O_2, as an inhibitor of the prostaglandin synthesis (4). Moreover, the interaction between hydroxyl radicals and the cell membrane causes oxidative stress chain reactions to produce lipid-derived free radicals such as lipid hydroperoxides (4). Prolonged use of proton pump inhibitors and H_2 receptor blockers causes adverse effects and drug interactions (2, 5). Therefore, there is a strong need for new effective drugs for peptic lesions amelioration.

Previous studies revealed that patients with gastrointestinal tract diseases suffer from affective disorders such as depression and psychotic symptoms (6). On the other hand, recent reports showed a significant gastroprotective effect of antidepressant and antipsychotic drugs in some models of gastric lesions. Risperidone, an atypical antipsychotic, has been reported to have significant gastroprotective effects in cold restraint stress-induced gastric lesions (6). Many antidepressant drugs like maprotiline, mianserin, trimipramine, fluoxetine, etc. have been found to exhibit antiulcer activity. It is also reported that serotonin reuptake inhibitors prevent duodenal ulcer (7).

The antipsychotic effect of TFP is believed to be mediated by blocking dopamine D2-receptors in the brain (8). Furthermore, TFP showed anti-proliferative and pro-apoptotic activity and also acted as a calmodulin antagonist (9). Previously, studies have shown that TFP can inhibit intracellular reactive oxygen species (ROS) generation and prevent loss of mitochondrial membrane potential (MMP) followed by reducing apoptosis in the cells (9). The TFP is an inducer of hypoxia-inducible factor-one alpha (HIF-1α), an upregulator of angiogenesis (including Vascular endothelial growth factor (VEGF), gluconeogenesis, cell proliferation and survival, and metabolic adaptation genes, induced under hypoxia and stress oxidative conditions. TFP also reduced mitochondrial swelling induced by Ca^{2+} followed by the generation of reactive oxygen species and inhibited the mitochondrial permeability transition (10) that is an important mechanism of compounds toxicity (11, 12). However, so far TFP has not been explored for its gastroprotective activity. Therefore, the present study evaluates the gastroprotective effect of TFP against the ethanol and cold water stress-induced gastric erosions. In addition to its histological examination, the present study evaluated some biochemical parameters such as superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) levels.

2. Materials and methods

2.1. Chemicals

Trifluoperazine, pyrogallol, ammonium molybdateand, MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) were purchased from Sigma–Aldrich (St. Louis, MO). Thiobarbituric acid (TBA) and ethanol were obtained from Merck (Germany).

2.2. Animals

2.2.1. Animals grouping and experimental procedure

Male adult Wistar rats (n=50) weighting between 180-250 g were obtained from the Center of comparative and experimental medicine (Shiraz University of Medical Sciences, Shiraz, Iran) and randomly divided into 10 groups, each including 5 rats. Induction of lesions was carried out by ethanol and cold water stress models (13). For each experimental model, five groups (25 rats) were used. The animals were housed in suspended bracket cages in a climate controlled room with temperature of 23±2 °C in a cycle of 12 h light and 12 h darkness with free access to food and water. All procedures on animals were performed in accordance with Animal Rights Monitoring Committee Guide of Shiraz University of Medical Sciences (95-01-103-11946).

2.2.2. Ethanol induced-erosions

Animals were deprived of food for 24 h before the experiments but had free access to water. Then, Group 1 received 2. 5 ml/kg saline, as

vehicle control, followed by 5 ml/kg ethanol after 1 h, and Group 2 received 20 mg/kg pantoprazole, as a standard control, followed by 5 ml/kg ethanol (% 50) after 1 h. Groups3, 4, 5 respectively received 5, 10 and 20 mg/kg TFP followed by 5 ml/kg ethanol (% 50) after 1 h. All treatment were carried out by intragastric gavage. One hour after the experimental period, all animals were sacrificed by ether anesthesia.

2.2.3. Stress induced erosions (cold water stress)

Animals were fasting for 24 h prior to the experiment. Then, Group 1 received 2.5 ml/kg saline as the vehicle control; Group 2 received 20 mg/kg pantoprazole as the standard control; Groups 3, 4 and 5 respectively received 5, 10 and 20 mg/kg TFP. One hour after receiving corresponding treatment in the experimental groups, animals were allowed to swim in cold water for 4 h. Thus, rats were put into water (water depth 10 cm to avoid drowning and water temperature set to 20-22 °C) and allowed free movement in the water. All treatment procedures were carried out by intragastric gavage. After the experimental period, all animals were sacrificed by ether anesthesia. Each stomach was opened along the greater curvature and examined macroscopically for gastric erosions under a dissecting microscope (10×).

2.3. Histopathology

At the end of the experiment, the whole stomach was dissected. The stomach tissues were fixed in formalin 10 % and paraffin embedded for microscopic examination in accordance with routine laboratory procedures. Sections of 5 µm thickness were cut and mounted on the glass microscope slides, using standard techniques. After staining with hematoxylin-eosin, the sections were examined under light microscope and photographed (14).

2.4. Biochemical examination of gastric mucosa

The method described by Madesh and Balasubramanium (15) was used for the determination of superoxide dismutase (SOD) activityin the stomach homogenates. A colorimetric assay involving generation of superoxide by pyrogallol auto-oxidation and the inhibition of superoxide-dependent reduction of the tetrazolium dye, MTT into formazan by SOD was measured at 570 nm. The amount of MTT-formazan assay was calculated by using molar extinction coefficient E570 of 17,000 M^{-1} cm^{-1}. One unit of SOD was defined as the amount of protein required to inhibit the MTT reduction by 50%.

Catalase (CAT) activity was measured in homogenates using Goth method(16). Briefly, 0.2 ml of the samples was incubated in 1 ml substrate (65 µmol/ml hydrogen peroxide in 60 mmol/l sodium-potassium phosphate buffer, pH 7.4) at 37 °C for 60 s. One unit CAT decomposes 1 µmol of hydrogen peroxide per minute, under these conditions. The enzymatic reaction was terminated with 1 ml of 32.4 mmol/l ammonium molybdate, and the yellow complex of molybdate and hydrogen peroxide were measured at 405 nm using a spectrophotometer. Values of CAT activity were expressed as kU/l, where k is the first-order rate constant.

Concentration of malondialdehyde was measured by the thiobarbituric acid test (17). Briefly, 0.5 mL of homogenates was added to 2 ml of TBA reagent containing 0.375 % TBA, 15 % trichloroacetic acid and 0.25 mol/L HCl. The mixture was boiled for 15 min, cooled and centrifuged at 1700×g for 15 min at 4 °C. The absorbance of the supernatant was measured at 532 nm. Results were expressed as nmoles/mg protein.

The protein concentration of the tissue samples was determined by Bradford assay using bovine serum albumin as a standard, as previously described (18).

2.5. Gastric mucosa image processing

To perform a metric analysis on the wound area based on image processing techniques, an in house toolbox was developed using graphical user interface (GUI) in Matlab. The toolbox made it possible to define different wound types including slight, moderate and severe wounds as well as normal tissue based on RGB component of each pixel. The values were then used to calculate the percentage of the wound area for each image. Validation of the software was done with circles subdivided into four quarters and filled with different color types. The software was able to calculate the color

types for each quarter using defined RGB values.

2.6. Statistical analysis

All values are presented as mean ± standard error. Statistical analyses were done using Graph pad prism version 6. The significance of the differences between the mean values was determined by analysis of variance (ANOVA) and then Tukey post test, and a P value of less than 0.05 was considered statistically significant. Matlab, version 2012, was used for image processing.

3. Results

3.1. Effect of TFP pretreatment on lipid peroxidation and antioxidant enzymes activity in ethanol induced-erosions

The pH changes measured at the end of the experiment and after removing the stomach were not significant. Therefore, its data were not shown.

As shown in Figure 1A, TFP increased the SOD activity in a dose dependent manner in ethanol induced-erosions rat model. There was a significant increase in SOD activity in doses of 10 and 20 mg/kg TFP, as compared to the controls, whereas no significant difference was found in the SOD activity in TFP 5 mg/kg. There was also a significant difference in SOD activity between TFP in doses of 5, 10 and 20 mg/kg, compared to the positive control group($p<0.05$)

As shown in Figure 1B, TFP also increased the CAT activity in ethanol induced-erosions rat model. A significant difference in CAT activity was just observed in a dose of 20 mg/kg of TFP, compared with the controls. No significant increase was found in the CAT activity in doses of 5 and 10 mg/kg TFP,when compared to the controls. There was also a significant difference in the CAT activity between TFP in doses of 5, 10 and 20 mg/kg, compared with the positive control group.

The results, as shown in Figure 1C, indicated that TFP could decrease the MDA level in ethanol induced-erosions rat model. There was a significant decrease in MDA concentration in doses 5, 10 and 20 mg/kg TFP, as compared to the controls. Further analysis showed that there was a significant difference in MDA concentration between TFP in doses of 5, 10 and 20 mg/kg, when compared to the positive control group.

3.2. Effect of TFP pretreatment on lipid peroxidation and TFP enzymes activity in cold water immersion induced-erosions

As shown in figure 2A, TFP increased the SOD activity in a dose-dependent manner in cold water immersion induced-erosions rat model. There was a significant increase in the SOD activity in doses of 10 and 20 mg/kg TFP, when compared to the control group, whereas no significant difference was found in the SOD activity between TFP in a dose of 5 mg/kg and control. Also, a significant difference was observed in the SOD activity between TFP in a dose of 5 and 10 mg/kg, when compared to the positive control group.

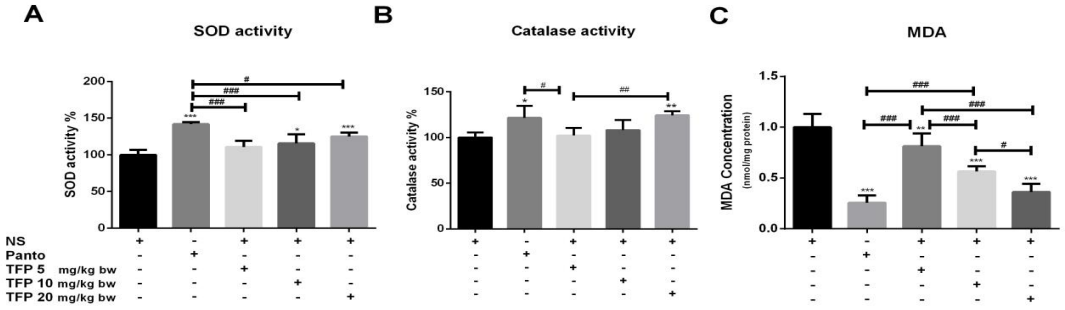

Figure 1. Effect of different doses of TFP and pantoprazole on SOD, CAT activity and MDA concentration in ethanol induced-ulcer. Animals were exposed to NS (black), 20 mg/kg pantoprazole and 5, 10, 20 mg/kg TFP (light and dark gray respectively for three consentration) and stomachs were removed and A) SOD, B) catalase activity, and C) MDA consentration were measured according to the materials and methods. * indicate significant difference(*p <0. 05,**p <0. 01, ***p <0. 001) between pretreatment groups and control, # indicate significant difference(#p < 0. 05, ##p <0. 01,###p <0. 001) between pretreatment groups.
NS: Normal saline, Panto:pantoprazole 20mg/kg, TFP: trifluoperazine.

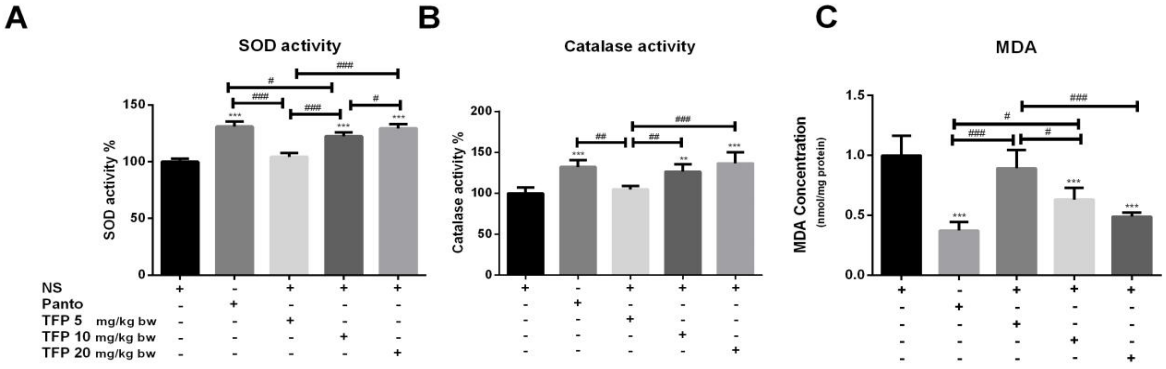

Figure 2. Effect of different doses of TFP and pantoprazole on SOD, CAT activity and MDA concentration in CWS induced-ulcer. Animals were exposed to NS (black), 20 mg/kg pantoprazole and 5, 10, 20 mg/kg TFP (light and dark gray respectively for three consentration) and stomachs were removed and A) SOD, B) catalase activity, and C) MDA consentration were measured according to the materials and methods. * indicate significant difference(*p<0.05,**p<0.01, ***p<0.001) between pretreatment groups and control, # indicate significant difference (#p<0.05, ##p<0. 01,###p<0.001) between pretreatment.
NS: Normal saline, Panto:pantoprazole 20mg/kg, TFP: trifluoperazine.

As shown in Figure 2B, TFP increased the CAT activity in a dose-dependent manner in cold water immersion induced-erosions rat model. A significant difference was observed in the CAT activity just in a dose of 10 and 20 mg/kg TFP, when compared to the controls. No significant increase was found in the CAT activity in a dose of 5 mg/kg TFP, when compared to the control group. A significant difference in the CAT activity was found between the dose of 5 mg/kg, when compared to the positive control group. Moreover, there was no significant difference between TFP in the dose of 10 and 20 mg/kg and the controls.

The results, as shown in Figure 2C, indicated that TFP could decrease the MDA concentration in a dose-dependent manner in cold water immersion induced-erosions rat model. There was a significant decrease in the MDA concentration

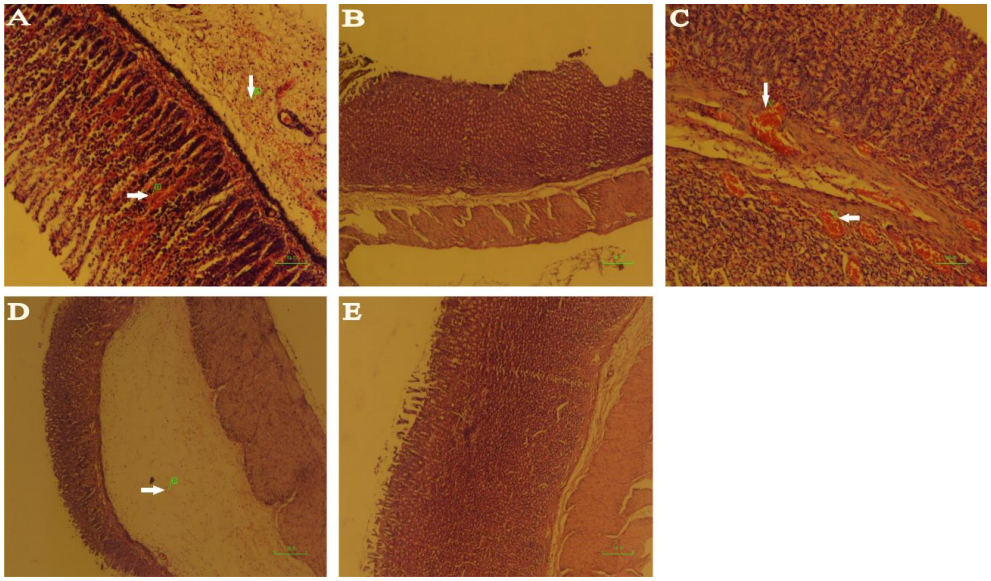

Figure 3. Histological examination of stomach of control and experimental groups of rats in ethanol induced-ulcer (X100, hematoxylin and eosin staining). (A) Control rats showed mucosal ulceration and edema; (B, E) pretreated rats with pantoprazole (standard control) and 20 mg/kg TFP showed normal gastric mucosa; (C) pretreated rat with 5 mg/kg TFP showed high level of hyperemia and edema; (D) pretreated rat with 10 mg/kg TFP showed few level of edema. arrows point to hyperemia and edema.

in dosesof 10 and 20 mg/kg TFP, when compared to control. Further analysis showed that there was a significant difference in the MDA concentration between TFP in doses of 5, 10 mg/kg, when compared to the positive control group ($p<0.05$). Further, no significant difference were observed between TFP in a dose of 20 mg/kg and the controls.

3.3. Effect of TFP pretreatment on histopathological changes in ethanol induced-erosions

Histological examination of the gastric mucosa showed ethanol induced extensive damage of the surface epithelium, with lesions extending up to the submucosa in some cases. Various histopathological changes including hemorrhage, submucosal edema, erosions and lesions were seen (Figure 3A). All these changes were significantly less expressed in the rats pretreated with both TFP 20 mg/kg (Figure 3C, 3D). Figures 3B and 3E show the normal gastric mucosa.

3.4. Effect of TFP pretreatment on histopathological changes in CWE induced-erosion

Histological examination of the gastric mucosa showed ethanol induced extensive damage of the surface epithelium, with lesions extending up to the submucosa in some cases. Various histopathological changes including submucosal edema and erosions were seen (Figure 4A). All these changes were significantly less expressed in the rats pretreated with both TFP 20 mg/kg (Figure 4E) and pantoprazole (Figure 4B).

3.5. Image processing of gastric mucosa in ethanol induced-erosions model and CWS

The percentage of the wound areas in gastric mucosa in ethanol induced-erosions and CWS models are shown in Tables 1 and 2, respectively.

4. Discussion

Gastric ulcer is the most common gastrointestinal disorder, which occurs due to an imbalance between the offensive (gastric acid secretion) and defensive (gastric mucosal integrity) factors (1). The current treatment of peptic ulcer is mainly performed with H_2 receptor antagonists, antacids, and proton pump inhibitors (2, 5). However, prolonged use of these treatments produces adverse reactions, like hypersensitivity, arrhythmia, impotence, and hematopoietic disorders. Therefore, there is a strong need to new effective drugs for peptic lesion therapy.

It has been manifested that gastrointestinal tract patients suffer from some depression and psychotic symptoms (6). Interestingly, clinical application of mental illness drugs in these patients have protective effects on the gastrointestinal tract. In this regard, beneficial effects of many antide-

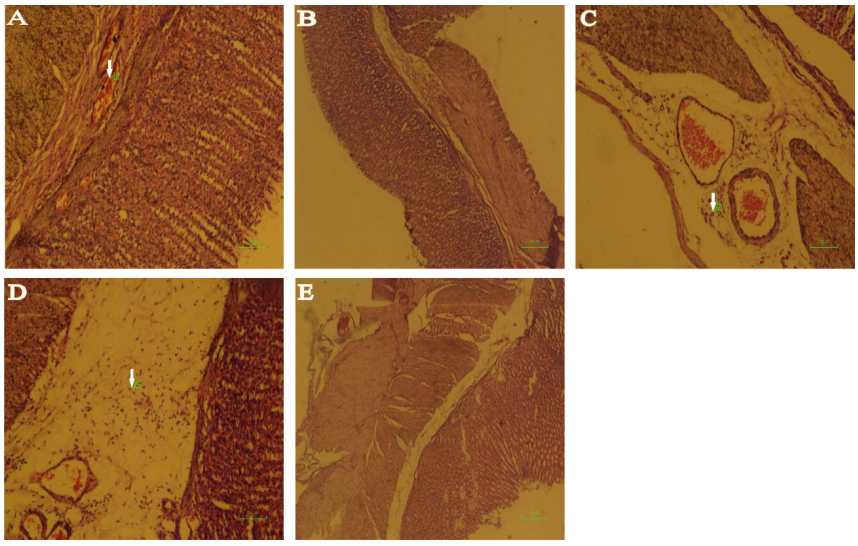

Figure 4. Histological examination of stomach of control and experimental group of rats in cold water stress (CWS) induced-ulcer (X100, hematoxylin and eosin staining). (A) Control rats showed mucosal ulceration and edema; (B, E) pretreated rats with pantoprazole (standard control) and 20 mg/kg TFP showed normal gastric mucosa; (C, D) pretreated rats with 5,10 mg/kg TFP showed high level of edema. arrows point to hyperemia and edema.

pressant and antipsychotic drugs have been proved in experimental studies. For instance, antidepressant drugs like maprotilin, mianserin, trimipramine, fluoxetine, etc. have been found to exhibit antiulcer activity (19, 20). Furthermore, similar effects have been observed in antipsychotic drugs such as clozapine, risperidone. To explain the gastro-protective activity of antidepressant drugs, we propose that they are able to inhibit histamine and acid secretion and blocking leukotriene receptors. Meanwhile, its interaction with some antioxidant pathwaysis suggested as another possible mechanism that exhibits antiulcer effect (21). We use pantoprazole (a proton pump inhibitor) as a positive control because of having gastro-protective as well as antioxidant properties performed through mechanisms such as restoring the levels of GSH and the diminished levels of SOD and catalase, and decreasing MDA concentration (22-24).

TFP is an atypical antipsychotic drug widely used for schizophrenia remedy(8). There is no report regarding the gastro-protective effect of TFP on gastric lesion, yet. To confirm the gastro-protective effect of TFP,we employed the ethanol and cold water immersion-induced gastric lesion models.

The formation of gastric mucosal lesions following ethanol administration involves several mechanisms, such as a reduction in the gastric blood flow, induction of oxidative stress, an increase in xanthine oxidase activity, increase in MDA levels, and a decrease in total glutathione content (25-28). Ethanol has also been reported to cause gastric lesions through vasoconstriction (25, 27).

Our results showed that SOD and CATactivity were decreased in the gastric tissue of ethanol induced-erosion animals. Inconsistent with our results, previous studies showed that the activity of SOD and CATdecreased after ethanol exposure as a result of increase in lipid peroxidation in animal-model (29, 30). Moreover, it has been suggested that ethanol cuases superoxide anion and hydroxyl radical production by neutrophils which increase the gastric lesions and tissuedamage (31).

SOD is able to convert superoxide to hydrogen peroxide, and subsequently, the catalase converts hydrogen peroxide to water (32). We observed that pre-treatment of animals by TFP restored, in a dose dependent manner, the oxidative stress and gastric mucosal damage induced by ethanol. Reactive oxygen species cuases oxidative stress, and lipid peroxidation has an important role in cell membrane damage. Our results showed that pretreatment of rats with the TFP significantly decreased the MDA level in the gastric tissues, as compared to the untreated control rates.

These finding suggested that gastro-protective effect of TFP might be associated with antioxidant properties, as demonstrated by decreased levels of MDA and increase in the antioxidant defenses such as SOD and CAT. Dalla Libera *et al.* previously described that clozapine, chlorpromazine and TFP, as very good antioxidants, markedly inhibited lipid peroxidation and protein carbonyl formation in aqueous and non-aqueous environments (33). Our results were in line with those of the studies that mentioned antioxidant mechanisms of TFP, such as scavenges of free radical, inhibit intracellular reactive oxygen species, preventing loss of MMP and protecting Ca^{2+}-ATPase (33-36).

We also observed the protective effect of TFP in CWS-induced erosion. The CWS is commonly used model for evaluating the drugs having a gastro-protective activity by virtue of its anti-stress effect. Increase in gastric motility, elevation of gastric acid secretion, vagal overactivity, mast cell degranulation, decreased gastric mucosal blood flowand decreased prostaglandin synthesis are involved in the genesis of stress-induced lesions (37).

Compared to the standard animals subjected to the stress experience, the stressed untreated control animals showed a significant decrease in the CAT and SOD activity. TFP administration before stress resulted in an increase in the stomach tissue CAT and SOD activity also decreased the MDA level, dose-dependently. Our finding was in agreement with other published results; SOD and CAT activity in the rat stomach tissue was decreased by indomethacin- and HCl/ethanol induced oxidative gastric mucosal damage (38), and CAT activity decreased in CWS (39); SOD activity was significantly decreased in 3.5 h of water immersion and restraint stress (40). Also, previous

Table 1. Percentages of the wound area in gastric mucosa in ethanol induced-ulcer model.

Groups	normal	mild	moderate	Sever
Control	9.76±3.2	32.25±2.13	44.18±6.04	13.65±7.93
TFP 5 mg/kg	20.35±5.93	36.96±9.17	38.25±11.21	4.43±2.73
TFP 10 mg/kg	31.45±2.36	32.76±5.25	35.12±2.65	0.66±0.33
TFP 20 mg/kg	59.37±17.1	29.96±11.69	10.66±10.44	0
Panto	75.37±11.91	19.01±7.83	5.60±4.17	0

Panto: pantoprazole 20 mg/kg, TFP: trifluoperazine.

Table 2. Percentages of the wound area in gastric mucosa in CWS model.

Groups	Normal	Mild	moderate	Sever
Control	25.64±10.90	22.96±18.38	49.83±16.03	1.56±1.11
TFP 5 mg/kg	59.97±0.71	26.12±4.3	10.74±2.61	1.5±1.1
TFP 10 mg/kg	49.26±2.9	45.69±0.98	5.02±3.34	0.01±0.009
TFP 20 mg/kg	48.88±6.4	48.12±5.86	2.91±2.8	0.08±0.08
Panto	70.87±2.82	29.12±2.82	0	0

Panto:pantoprazole 20 mg/kg, TFP: trifluoperazine.

study reported that TFP depressed the secretion of gastric acid, and pepsin inhibited the gastric H+, K(+)-ATPase activity in stress and indomethacin lesions model in rats (41). The results suggested that these mechanisms in addition to antioxidant properties might be related to the TFP antiulcer effects *in vivo*.

Citalopram that was used as an antidepressant drug showed gastro-protective effect in cold water stress. A reduction in the MDA level was observed in the treated animals. It revealed that citalopram mediates its protective effect by endogenous NO, prostaglandins, and KATP channel opening (7). Another atypical antipsychotic drug, Risperidone, also showed gastro-protective effect in cold water stress. Risperidone mediated gastro-protective activity through antagonist activity on 5-HT2 receptor (6).

Histologic examination revealed similar results in ethanol and CWS induced lesions; this indicated that animals that have received 20 mg/kg TFP exert significantly reduced gastric lesion formation and submucosal edema similar to the pantoprazole treated animals. The results confirmed the present biochemical findings of the tissue.

5. Conclusion

The study showed that, orally administration of TFP demonstrated gastric mucosal protection against oxidative injuries caused by ethanol and CWS. This protection is most likely due to direct antioxidant properties of TFP and inhibition of toxic oxidant in the stomach tissues. according to previous researches TFP have antioxidant properties *in vitro* environment (22, 33).

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Conflict of Interest

The authors have no conflict of interest.

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