RESEARCH PAPER

Accepted 30 October 2003
Revised 13 August 2003
Received 17 December 2001
Indian J. Pharm. Sci., 2004, 66(1): 36-43

Pharmacological Evaluations of the Possible Antiinflammatory, Antiischemic and Cytoprotective Action of Trimetazidine

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Trimetazidine is a cellular antiischemic agent with cytoprotective action and devoid of any hemodynamic activity. Extensive work has been carried out on this drug in cardiovascular therapeutics and in transplantation surgery. It was thus decided to investigate possible antiinflammatory activity it could exert by virtue of it being a calcium channel blocker. Trimetazidine being a cytoprotective agent could offer protection against experimentally induced ulcerations. Another objective was to extend the antiischemic activity of trimetazidine in the brain in the cryogenic cerebral ischemic injury model and finally to assess the free radical scavenging activity of the drug in the murine peritoneal macrophage model. Trimetazidine potentiated the antiinflammatory effect of ketoprofen and also significantly reduced the rate of edema formation. It was able to protect animals against the experimentally induced gastric ulcerations. Trimetazidine produced a significant antiischemic activity in the brain and also brought about a significant free radical scavenging effect.

Trimetazidine (TMZ) is a well known antiischemic agent in the cardiovascular therapy, which is also categorized as a member of the piperazine class of calcium channel blockers, devoid of hemodynamic activity[1,2]. TMZ is also used in transplantation surgery as a cytoprotective agent to preserve organ function of the graft against ischemia-reperfusion induced injury. Ischemia that results because of occlusion of cerebral artery, tissue hypoxia, energy depletion or severe head injury is succeeded by a cascade of pathophysiological events, which lead to neuronal death. One of the factors could be the disturbance of intracellular calcium homeostasis and damage to cell membranes[3].

Apart from TMZ's protective action on the cardiovascular system, not much work has been carried out on other potential biological systems, which can succumb to the deleterious effects of ischemia, such as the CNS. Thus it was decided to study the antiischemic effect of TMZ on the cryogenic cerebral ischemia model, which is a relatively simple but an excellent model of cerebral ischemic injury.

Just as cerebral ischemia is a form of a localized inflammatory process; it was decided to assess this activity at the peripheral level. The process of inflammation is one of the defense mechanisms of the body and when this process is altered or some disturbance in the regulatory mechanism occurs, it results in pathophysiological manifestations such as inflammatory diseases.

Calcium plays a key role in the inflammatory process, as it modulates the synthesis and release of various mediators of inflammation. Calcium channel blockers exert their antiinflammatory effect by modulating the phlogistic effect of bradykinin, PEG_1 and 5-HT[4]. Reports have shown the calcium channel blocker, verapamil to enhance the anti-inflammatory effect of diclofenac[4]. Therefore it was proposed to study TMZ alone and in combination with ketoprofen for any antiinflammatory activity it could exert by virtue of the

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fact that TMZ possesses a weak calcium channel blocking activity.

Calcium is also shown to stimulate acid secretion in the stomach and this could be one of the causes of ulceration[5]. Recently much attention has been focused on the role of free radicals causing tissue injury through lipid peroxidation and this injury is manifested in the form of damage to the gastric lumen as ulceration[6]. TMZ by virtue of its free radical scavenging activity[7] and also weak calcium channel blocking activity could offer protection against experimentally-induced ulcers. The murine peritoneal macrophage model has been used in the present investigation to assess free radical scavenging activity of TMZ in peritoneal macrophages, to provide an insight into the mechanism by which TMZ could prevent free radical-induced inflammatory response.

MATERIALS AND METHODS

TMZ was obtained as a gift sample from Cipla, Mumbai. Ketoprofen (KPF) was a gift sample from Rhone Poulenc, Mumbai. Omeprazole (OME) and famotidine (FAM) were gift samples from Kopran, Mumbai. Verapamil (VPL) was a gift sample from Nicholas Piramal India Ltd., Mumbai. 2,3,5-Triphenyltetrazolium chloride (TTC) was a generous gift sample from Quest Institute of LifeSciences, NPIL, Mumbai. Nitroblue tetrazolium chloride (NBT), phenazine methosulphate (PMS), 1,4-dioxan and dimethylsulphoxide (DMSO) were purchased from Himedia, Mumbai.

All the animal protocols were approved by the Institutional Animal Ethics Committee and conducted according to CPCSEA guidelines. The CPCSEA registration number of the institute is 242/CPCSEA.

Antiinflammatory activity in the carrageenan paw edema model in rats:

Wistar rats of either sex in the weight range of 200-250 g were randomly divided into 10 groups of 5 animals each, consisting of the following groups. Group C received the vehicle and served as a control group. Five standard treatment groups were as follows, ketoprofen administered orally at doses 25 mg/kg (group K1), 50 mg/kg (group K2) and 100 mg/kg (group K3). Group V received VPL 50 mg/kg i.p., whereas Group T received TMZ 200 mg/kg orally. Of the 4 combination groups, group TK1 received a combination of TMZ 200 mg/kg and KPF 25 mg/kg orally; group TK2 received TMZ 200 mg/kg and KPF 50 mg/kg orally. Group TK3 received TMZ 200 mg/kg and KPF 100 mg/kg orally and group VK2 received VPL 40 mg/kg i.p. and KPF 50 mg/kg orally. Animals in the combination groups received both drugs simultaneously.

All the groups received intraplantar injection of 0.1 ml of 1% λ-carrageenan into left hind paw 60 min after drug treatment[4]. The volume of the injected paw was measured on a plethysmometer (UGO Basile, Model 7140) before the carrageenan injection and then every half an hour up to 4 h after injection of carrageenan. The percent inhibition of edema was calculated as % Inhibition=1-(Vd-Vp/Vc-V'p)x100 where, Vd=volume of paw after carrageenan injection of drug treated animal. Vp= volume of preinjected paw of drug treated animals. Vc=volume of preinjected paw after carrageenan injection of control animals. V'p=volume of preinjected paw of control animals.

The data was analyzed using Student's t-test for % inhibition of edema of combination groups as compared to the respective standard treatment groups. A value of $p<0.05$ was considered to be statistically significant as compared to the control. A graph of percent edema formation was plotted against time and various groups were compared with control, with a value of $p<0.05$ considered to be statistically significant to control.

Effect of TMZ on gastric ulceration induced by ketoprofen in rats:

Wistar rats of either sex in the weight range of 200-250 g were randomly divided into 5 groups of 5 animals each consisting of control and 4 treatment groups. All animals were fasted for 36 h prior to study with free access to water. Group I, which served as the control received vehicle. Group II received 200 mg/kg TMZ orally. Group III received VPL 40 mg/kg intraperitoneally. Group IV received 100 mg/kg OME orally and Group V received 10 mg/kg, FAM intraperitoneally. Animals in each of the above groups were dosed with KPF (100 mg/kg p.o.) one hour following oral drug treatment and 0.5 h after VPL and FAM injection in order to induce the ulcers.

Six hours after KPF treatment the animals were sacrificed by cervical dislocation and the stomachs were removed and cut along the greater curvature and washed with saline. Degree of injury to stomachs was evaluated according to the method of Nambu[8]. Data was analyzed using Dunnett's test for ulcer score and compared to the control. A value of $P<0.05$ was considered to be statistically significant.

Antiischemic activity of TMZ in the cryogenic cerebral ischemia model in rats:

Wistar rats of either sex and weight range of 250-300 g were randomly divided into 3 groups of 5 animals each. Normal group which did not undergo any surgery, while the control group received only vehicle 0.5 h before surgery and the treatment group received intraperitoneal injection of TMZ (250 mg/kg) 0.5 h before the surgery.

Animals in the control and treatment groups were anesthetized using pentobarbitone Na (40 mg/kg, i.p.) and a small hole was drilled into the skull 5 mm above the left eye using a hand drill. A stick of dry ice was placed on the exposed dura for a period of 120 s in order to induce the cryogenic injury. The skull was bandaged, and animals were allowed to recover under light treatment.

Five hours from the time of surgery, animals in all the groups were sacrificed by cervical dislocation, brains were removed, washed with saline, sliced in 2 mm broad coronal sections and placed into vials containing 0.5% solution of TTC in phosphate buffered saline (PBS, pH 7.4). Half an hour later the sections were removed and dropped into preweighed vial containing 10 g of a 50:50 mixture of DMSO and ethanol. Fresh uninjured brain tissue samples on incubation with colourless TTC acquire a red coloured deposit of Formozan compound as a result of reduction of dye by mitochondrial enzymes. This coloured component is then extracted using DMSO- ethanol mixture. Vials were capped and placed in the dark. After 24 h, 100 μl of extract was diluted with 900 μl of fresh DMSO-ethanol mixture and absorbance of this solution was determined spectrophotometrically at 485 nm and absorbance per g was calculated for each tissue. % Loss of staining was calculated according to the method of Preston[9]. The % loss of staining of control and treated group were compared with the normal group in order to assess antiischemic activity and the absorbance/g values were compared using student's t test and $P< 0.05$ was considered as significant as compared to control.

Effect of TMZ on the free radical scavenging activity in the murine peritoneal macrophage model in mice:

Swiss mice of either sex in the weight range of 20-25 g were employed for the study. The animals were divided into two groups of five each. All animals were injected with 0.2 ml of 5% sodium caseinate i.p, in order to elicit peritoneal macrophages and on the day 5, PMS was injected intraperitoneally at a dose 100 μg/animal. This was an attempt to activate macrophages *in vivo*. The drug (TMZ 200 mg/kg, i.p.) or vehicle was administered half an hour before PMS injection. Two hours after PMS injection the animals were sacrificed by cervical dislocation. Two millilitres of PBS (pH 7.4) was injected intraperitoneally, 1 ml on either side prior to sacrifice. The abdomen was massaged for 3-4 min.

The abdomen was cut open and peritoneal fluid withdrawn. This was centrifuged at 2000 rpm on a Remi refrigerated centrifuge. The cell pellet was resuspended in 1 ml PBS by vortexing. The cell count was adjusted according to method of Anto[10]. One millilitre of cell suspension was then mixed with 1 ml of a mixture of 0.2% NBT in Phosphate buffered saline, 5% dextrose and Hanks balanced salt solution (6:2:4) and incubated for 2 h, in dark at room temperature. The mixture was then centrifuged and 3 ml of 1,4–dioxan was added to cell pellet. This was then boiled in a water bath for 10 min. After boiling it was cooled and again centrifuged and optical density of supernatant was read at 530 nm, this is a measure of superoxide produced by activated peritoneal macrophages. The data were analyzed using Student's t test, by comparison of the treated group with control. $P<0.05$ was considered statistically significant.

RESULTS

Group T produced a 13.0±6.71% inhibition of edema at 3 h. Among the three doses of KPF tested, group K1 showed maximum inhibition of 66.1±6.67% at 3 h as compared to 57.5±7.11% and 53.2±11.2% for groups K2 and K3, respectively. The percent inhibition of edema of TMZ and KPF combinations namely, group TK2 (66.0±6.16%) and group TK3 (62.0±7.23%) is significant in comparison to that of group T. However, these combination groups could not exhibit significant percent inhibition of edema in comparison to that of groups K2 and K3, respectively. TMZ in combination with 25 mg/kg KPF (Group TK1) did not show any improvement in the inhibition of edema as offered by Group K1 alone. Combination group VK2 inhibited edema formation by 64.0±4.9 percent, which was comparable to that of combination group TK2 as shown in Table 1.

TMZ showed a significant reduction in rate of formation of edema as compared to control animals. The combination of KPF in doses of 50 and 100 mg/kg along with 200 mg/kg TMZ showed significant reduction in the rate of formation of edema as compared to control animals. Thus, TMZ brought about a synergistic effect in reducing the rate of edema formation of KPF. Combination group VK2 consisting of VPL 40 mg/kg and KPF 50 mg/kg was equipotent as group TK2 which consisted of KPF 50 mg/kg and TMZ 200 mg/kg

TABLE 1: EFFECT OF VARIOUS TREATMENTS ON THE ANTIINFLAMMATORY ACTIVITY IN RATS

Group	Treatment	Dose (mg/kg p.o.)	% Inhibition of edema
T	TMZ	200	13.0±6.71
V	VPL^	40	27.9±7.0
K1	KPF	25	66.1±3.33
K2	KPF	50	57.5±7.11
K3	KPF	100	53.3±11.2
TK1	TMZ + KPF	25 + 200	61.3±6.88*
TK2	TMZ + KPF	50 + 200	66.0±6.16*
TK3	TMZ + KPF	100 + 200	62.0±7.23*
VK2	VPL^ + KPF	50 + 40	64.0±4.89#

Each value represents mean±s.e.m (n=5) of percent inhibition of edema offered by different treatments on the carrageenan-induced rat paw edema. TMZ-trimetazidine, KPF-ketoprofen, VPL-verapamil. ^indicates that VPL was administered intraperitoneally. *indicates statistical significance at $p<0.05$ in comparison with TMZ treatment alone and #indicates statistical significance at $p<0.05$ in comparison with VPL by the Student's t test.

combination as depicted in figs. 1, 2 and 3.

Control group showed an ulcer score of 2.9±0.25 while combination of KPF and TMZ in Group II reduced the ulcer score to 1.0±0.52, as shown in Table 2. TMZ when administered alone, did not induce any ulcers. The combination of TMZ and KPF significantly lowered the ulcer score as compared to Control. Group V was comparable with TMZ as it reduced ulcer score to 1.0±0.27 whereas best protection was offered by group IV (100 mg/kg OME p.o.) with a score of 0.15±0.1. Group III however did not show any significant antiulcer activity at the dose tested as is evident with an ulcer score of 1.9±0.68. Results were compared with KPF treatment at $p<0.05$ by Dunnett's test.

The results for antiischemic activity were analyzed in terms of percent loss of staining of brain tissues by TTC, which could be co-related to the ischemic foci. Normal animals showed 0.0% loss of staining while control animals showed 32.8% loss of staining. Animals treated with 250 mg/kg i.p. TMZ showed significant protection with only 4.52% loss in staining. The results were compared using student's t-test. Results have been depicted in Table 3.

TMZ has been reported to show free radical scavenging activity. It was decided to reproduce this activity in the murine peritoneal macrophage model. Results were analyzed in terms of the absorbance of the coloured reaction product formed, which gave an indication of the amount of free radicals produced. TMZ, 200 mg/kg, i.p., was able to significantly reduce the absorbance values as compared to the control. The percentage inhibition of free radical induced coloured radical product was 44.2±16.0%. The results were tabulated in Table 4.

DISCUSSION

The early phase of inflammation is reported to be due to the release of bradykinin, histamine and leukotrienes. This is later followed by prostaglandin activity. The delayed phase has been linked to PMN leukocyte infiltration and production of neutrophils and free radicals[11]. Calcium ions play an important role in the synthesis and release of chemical mediators of inflammation. Influx of calcium is an essential step in the release of histamine and 5-HT from mast cells and in the synthesis and release of prostaglandins[4]. Calcium channel blockers exert antiinflammatory effect by modulation of phlogistic effect of PG-E, bradykinin and 5-HT.

TMZ by virtue of its calcium channel blocking activity could show its action on the early phase of inflammatory response. It is also known to inhibit histamine and serotonin release[12]. It prevents the breakdown of membrane phospholipids, associated with free radicals[13]. TMZ inhibits neutrophil infiltration and also prevents against the deleterious effects of superoxide radicals[1] known to possess proinflammatory roles. Thus TMZ exhibits its weak antiinflammatory effect in the early and late phases of the

inflammatory response as evident in figs. 1, 2 and 3 because of its action on histamine release and on free radicals. Although it does not possess any significant action on the inflammatory response, it potentiaties the action of the NSAID, KPF.

Calcium influx seems to play an essential role in the stimulation-secretion coupling in mammalian oxyntic cells, an effect that can be inhibited by calcium channel blockers. They also possess inhibitory effect on histamine, gastrin, carbachol and cAMP induced stimulation of gastric acid secretion[14].

Oxygen derived free radicals also play an important role in the pathogenesis of acute experimental gastric lesions induced by ischemia- reperfusion, stress, ethanol and NSAIDs. Oxygen derived free radicals cause tissue injury through lipid peroxidation. If the generation of free radicals exceeds the ability of free radical scavenging enzymes to dismute the radicals, gastric mucosa may be injured by free radicals[6]. Neutrophil adherence to vascular endothelium has been suggested to be a crucial event in the pathogenesis of NSAID induced gastric damage[15].

TMZ by being a calcium channel blocker, could protect the gastric mucosa against all the gastric secretory effects and histamine release. However, VPL a calcium channel blocker could not offer any protection and thus it could signify that calcium channel blocking activity by itself is not sufficient to render protection against NSAID induced ulceration.

Endothelial cells and leukocytes adhere to gastric vascular endothelium and are the main source for the production of oxygen free radicals. These in turn are capable of reacting with proteins, lipids and nucleic acids leading to lipid peroxidation of biological membranes[16] and in this case could lead to ulceration. TMZ has been shown to reduce the incidence and severity of peritoneal adhesion induced by ileal ischemia-reperfusion in the isolated ileal preparation[17].

TMZ is also a free radical scavenger and is also very effective against lipid peroxidation[16]. Thus it could exert a cytoprotective effect against all the factors having a damaging consequence on gastric mucosa and thus bring about an antiulcerogenic effect.

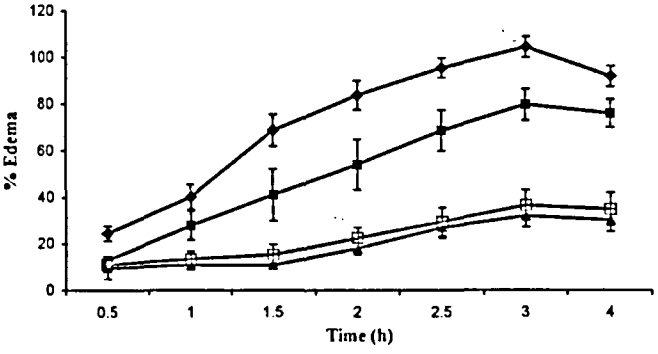

Fig 1: Effect of trimetazidine and ketoprofen alone and in combination on carageenan- induced paw edema in rats.

Each value is expressed as mean±s.e.m. (n=5) of percent edema formation versus time. (-◆-) Group C, (-■-) Group T* (200 mg/kg trimetazidine p.o.), (-▲-) Group K1* (25 mg/kg ketoprofen p.o.), (-□-) Group TK1* (200 mg/kg trimetazidine p.o. and 25 mg/kg ketoprofen p.o.). *P<0.05 is statistically significant as compared to control using Student's t test.

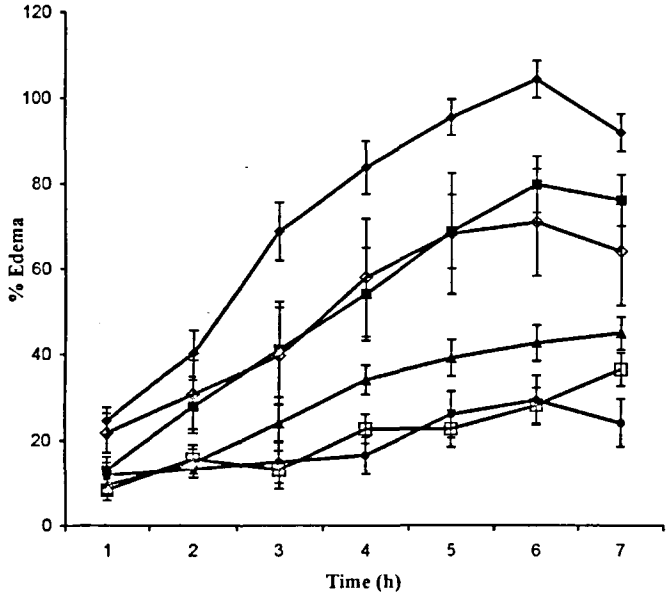

Fig 2: Effect of drug treatment on carrageenan- induced paw edema in rats.

Each value is expressed as mean±s.e.m. (n=5) of percent edema formation versus time.(-◆-) Group C, (-■-) Group T* (200 mg/kg trimetazidine p.o.), (-▲-) Group K2* (50 mg/kg ketoprofen p.o.), (-◇-) Group V* (40 mg/kg verapamil i.p.), (-□-(Group TK2* (200 mg/kg trimetazidine p.o. and 50 mg/kg ketoprofen p.o.), and (-●-) Group VK2* (40 mg/kg verapamil i.p. and 50 mg/kg ketoprofen p.o.). *P<0.05 is statistically significant as compared to control using Student's t test.

Cryogenic injury uses dry ice to freeze viable tissues. Freezing can result in crystallization of electrolytes and denaturation of lipoprotein complexes, which are lethal to the cell. It can induce cell death through mechanical shock, rupture of cell membrane, osmotic shock and cellular hypoxia/anoxia[18]. The brain is susceptible to the effects of ischemia because it has high energy requirements and limited capacity to store oxygen, glucose and ATP[20]. Therefore, the brain tissue lesion induced by cryogenic injury is an excellent model for studying ischemic brain injury[19].

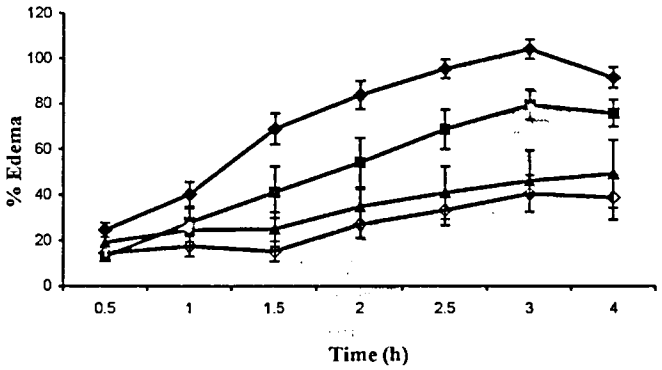

Fig 3: Effect of trimetazidine and ketoprofen alone and in combination on carrageenan- induced paw edema in rats.

Each value is expressed as mean±s.e.m. (n = 5) of percent edema formation versus time. (-◆-) Group C, (-■-) Group T* (200 mg/kg trimetazidine p.o.), (-▲-) Group K3* (100 mg/kg ketoprofen p.o.), (-◇-) Group TK3* (200 mg/kg trimetazidine p.o. and 100 mg/kg ketoprofen p.o.). *P<0.05 is statistically significant as compared to control using Student's t test.

Loss of ionic homeostasis following ischemic brain injury aggravates damage. Cellular influx of sodium and calcium, efflux of potassium and magnesium results. Calcium influx initiates secondary neuronal injury by mitochondrial dysfunction, stimulation of cyclooxygenases and 5-lipoxygenases, thereby yielding prostaglandins and leukotrienes causing lipid peroxidation, vasoconstriction and free radical generation[20].

Factors responsible for ischemic injury are increase in cellular free calcium concentration or overproduction of oxygen free radicals. Free radical formation results in structural membrane damage and neuronal loss[21]. As TMZ can counteract both these actions it behaves as a cytoprotective agent and offers protection against the deleterious effect of ischemia.

Moreover during ischemia, oxygen is slightly available or not available. Metabolic alterations that occur during ischemia facilitate the formation of free radicals from residual molecular oxygen. Upon reperfusion, the restored availability of oxygen enhances the formation of these toxic metabolites. Neutrophils, when activated, generate several types of free radicals, which can cause cellular injury to glia or neurons by membrane disruption. Thus neutrophils recruited to the infarct site may damage the neuronal tissue, producing oxygen free radicals[22]. During ischemia, activation of phospholipases increases the release of arachidonate. Lipid peroxidation is an especially damaging event as the formation of oxygen free radicals can propagate itself resulting in further cellular membrane damage[12].

TABLE 2: ANTIULCEROGENIC EFFECT OF VARIOUS DRUG TREATMENTS ON KETOPROFEN INDUCED ULCERS

Groups	Treatment	Dose mg/kg (p.o.)	Mean scores of ulcers produced by KPF administration
I	Vehicle	----	2.9±0.25
II	TMZ	200	1.0±0.52*
III^	VPL	40	1.9±0.68
IV	OME	100	0.15±0.1*
V^	FAM	10	1.0±0.27*

Values are expressed as a mean±s.e.m (n=5) of ulcer scores induced by administration of ketoprofen. All groups received 100mg/kg KPF orally to induce ulcers. *indicates treatment groups which are considered to be statistically significant as compared to control by Dunnett's test at P<0.05. ^ indicates that test drug was administered intraperitoneally.

TABLE 3: ANTIISCHEMIC ACTIVITY OF TRIMETAZIDINE

Treatment	Mean absorbance/g of brain sections	% loss in staining
Normal [uninjured]	0.81±0.02	0.00
Control [vehicle treated, injured]	0.54±0.05	32.8
TMZ (250 mg/kg i.p)	0.77±0.04#	4.52#

Each value is expressed as mean±s.e.m (n=5) of absorbance/gm of brain sections exposed to TTC and % loss in staining of brain sections exposed to TTC in cryogenic cerebral ischemic injury model. #indicates that the treatment group is statistically significant as compared with the control at value of P<0.05 by the Student's t test.

TMZ is able to counteract all these following mechanisms and thus is a very effective anti-ischemic agent. It prevents changes in intracellular homeostasis, inhibits neutrophil infiltration at site of injury, and acts as a scavenger of free radicals[1]. It prevents the action of phospholipases and thus prevents lipid peroxidation[12] and by maintaining a balance between energy requirements and intracellular ionic homeostasis, TMZ acts as an effective anti ischemic agent in the cryogenic cerebral injury model in rats.

Oxygen free radicals are thought to produce cellular damage by oxidative modification of cellular macromolecules that lead to cellular and tissue dysfunction. Reactive oxygen species are implicated in a number of pathological processes including tissue injury, inflammatory disorders, cardiovascular diseases and neurodegenerative disorders[23].

Polymorphonuclear neutrophils and leukocytes are known to liberate free radicals in inflammatory response. The murine model is advantageous because free radicals are generated within the macrophages by the tumor inducing agent PMS and this could be a model that mimics the conditions generated in the inflammatory response. PMS produces superoxide anions and other active oxygen species capable of inducing lipid peroxidation[24]. The free radicals generated in macrophages reduce NBT to formazan (violet colored), which is estimated spectrophotometrically[10].

TABLE 4: EFFECT OF TRIMETAZIDINE ON FREE RADICAL SCAVENGING ACTIVITY IN RATS

Treatment	Dose (mg/kg i.p.)	Mean Absorbance value
Control	vehicle	0.48±0.04
Trimetazidine	200	0.27±0.08*

Each value is expressed as mean±s.e.m. (n=5) of the absorbance value obtained from the reduction of NBT by free radicals. *indicates that at P<0.05 treatment group is considered to be statistically significant as compared to control by Student's t test.

TMZ in the dose of 200 mg/kg showed free radical scavenging activity and since free radicals are reported to be involved in the pathology of ischemic injury and inflammation, the cytoprotective action of TMZ could be related to the free radical scavenging activity.

Therefore, although TMZ does not show any antiinflammatory activity, by itself the combination with an NSAID led to potentiation of the action of the NSAID and also the combination was able to reduce the ulcerogenic side effect of the NSAID. TMZ may prove to be very advantageous as a cerebral antiischemic agent. However, a long term study exploring the secondary growth kinetics of ischemic focus and effect of TMZ on the same can be studied.

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