Studies on the protective effects of betaine against oxidative damage during experimentally induced restraint stress in Wistar albino rats

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Abstract Stress can be defined as physical and psychological modifications that disrupt the homeostasis and the balance of organisms. Stress is known as one of the most important reasons of several diseases. In the present study, the anti-stress effect of betaine was evaluated with reference to its antioxidant property. Wistar albino rats were divided into four groups such as control, betaine, restraint stress (6 h/day for 30 days), and betaine+restraint stress. The oxidative damage was assessed by measuring the protein and corticosterone in plasma, lipid peroxidation, non-enzymic (reduced glutathione), and enzymic antioxidants (glutathione peroxidase, glutathione-S-transferase, catalase, and superoxide dismutase) in the lymphoid organs of thymus and spleen. Followed by the induction of restraint stress, the non-enzymic and enzymic antioxidants were significantly decreased with concomitant increase observed in the levels of corticosterone and lipid peroxidation. Oral pretreatment with betaine (250 mg/kg body weight daily for a period of 30 days) significantly ($P<0.001$) prevented the restraint stress-induced alterations in the levels of protein and corticosterone in plasma of experimental groups of rats. It counteracted the restraint stress-induced lipid peroxidation and maintained the antioxidant defense system in the lymphoid tissues at near normal. The findings suggest that

betaine possesses significant anti-stress activity, which may be due to its antioxidant property.

Keywords Restraint stress · Antioxidant status · Betaine · Lipid peroxidation · Corticosterone

Introduction

Stress is characterized by physiological reorganization that occurs in response to novel or menacing stimuli. The maladaptive effects of repeated stress include susceptibility to depression, panic disorder, posttraumatic stress disorder, drug abuse, and cognitive impairment (Goel and Bhattacharya 1991). Rapid urbanization and the changing social fabric in our society lead to heightened levels of stress both mental and physical. Stress exerts detrimental effects on several cellular functions through impairment of antioxidant defenses, leading to oxidative damage, a process central to many diseases (Torres et al. 2004). A substantial literature has focused on anatomical, behavioural, and neuroendocrine changes associated to the exposure to chronic stress. For instance, it has been demonstrated that chronic exposure to stress decreases body weight and food intake, induces adrenal hypertrophy, involution of thymus, spleen and lymph nodes, and affects neuronal plasticity (Martí et al. 1994; Zivkovic et al. 2005).

Generation of free radicals is an integral feature of normal cellular functions, in contrast, excessive generation and/inadequate removal of free radical results in destructive and irreversible damage to the cells (Lopaczyski and Zeisel 2001). Cells of the immune system are particularly sensitive to changes in the antioxidant status because they carry out important functions through the generation of a high number of oxygen free radicals (Pieri et al. 1993).

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Antioxidants play an important protective role against the reactive oxygen species. This antioxidant–oxidant balance is an important determination of immune cell function, including maintenance of the integrity and functionality of membrane lipids, cellular proteins and nucleic acids in immune cells. Moreover, the cells of the immune system have a high percentage of polyunsaturated fatty acids (PUFA) in their plasma membrane, and therefore, it is not surprising that these cells usually contain higher concentrations of antioxidant nutrient than other cells (Meydani et al. 1995). Antioxidant administration has been shown to reduce restraint stress-induced oxidative damage (Liu et al. 1996). Reports indicate that there is an inverse relationship between the dietary intake of antioxidant-rich foods and the incidence of human diseases (Sies 1993). Hence, the search for natural antioxidants is essentially important.

In recent years, there has been a global trend towards the use of natural substances existing in fruits, vegetables, and herbs as antioxidants and functional nutrients. Betaine (trimethylglycine) is a natural component that is widely found in animals, plants, and microorganisms and rich dietary sources include seafood, especially marine invertebrates; wheat germ or bran; and spinach. Betaine has been used as a dietary feed supplement in animal nutrition for more than 50 years, and this use has provided useful insights into human nutrition. In the living body, it is an endogenous catabolite of choline. Choline, or its metabolites, is needed for the structural integrity and signaling functions of cell membranes; it is a major source of methyl groups in the diet, and it directly affects cholinergic neurotransmission, transmembrane signaling, and lipid transport/metabolism (Zeisel and Blusztajn 1994). Since three methyl groups are contained in its structure (Fig. 1), betaine is presumed to work as a methyl donor and to play an important role in homocysteine metabolism (Barak et al. 1996).

Betaine is catabolized via a series of enzyme reactions that occur mainly in the mitochondria of liver and kidney cells. These transmethylation reactions involve the transfer of methyl (one carbon) groups via the methionine cycle (Fig. 2) in vital biological processes. The conversion of homocysteine to methionine is important to conserve methionine, detoxify homocysteine, and produce *S*-adenosylmethionine (SAM). Elevated total homocysteine concentrations and low SAM concentrations have been associated with chronic disease. The formation of methionine from homocysteine can occur either via betaine or via 5-methyltetrahydrofolate (CH₃-THF).

Animal studies have shown that both pathways are equally important (Barak et al. 1996; Finkelstein and Martin 1984) and that betaine is a vital methylating agent (Barak et al. 1982). Betaine transfers a methyl group via the enzyme betaine homocysteine methyl transferase (BHMT) to become dimethylglycine. The CH₃-THF reaction utilizes the enzyme methionine synthase to transfer a methyl group to the cofactor cobalamin (vitamin B₁₂), forming methylcobalamin. Then methylcobalamin transfers the methyl to homocysteine to form methionine. Methylenetetrahydrofolate reductase (MTHFR; EC 1.1.1.68) is involved in reforming CH₃-THF from methylene-THF. In addition, homocysteine can be metabolized via transsulfuration to form cystathionine. This is catalyzed by cystathionine β-synthase (CBS; EC 4.2.1.22), which utilizes vitamin B₆ (pyridoxine) as a cofactor. Thus, betaine supplementation is effective for reducing plasma homocysteine levels in humans (Steenge et al. 2003) and in homocystinuria patients with MTHFR deficiency (Smolin et al. 1981).

Betaine generally appears to be safe at a daily intake of 9–15 g (average of 12 g). Subacute and subchronic rat studies determined that betaine is nontoxic at all doses studied (0–5% of the diet) (Hayes et al. 2003). Studies by Erman et al. (2004) showed that betaine exerted cellular and subcellular membrane stabilization in the liver by restoring both non-enzymic and enzymic antioxidants. It has been shown that betaine is involved in the synthesis of methionine, which serves as a major supplier of cellular cysteine via trans-sulfuration pathway for the synthesis of reduced glutathione that protects the cell from reactive metabolites and reactive oxygen species (Craig 2004).

Earlier, we reported the protective effect of betaine on mitochondrial function (Ganesan et al. 2007a) protein and glycoprotein metabolism (Ganesan et al. 2007b) and lipid metabolism (Ganesan et al. 2008) and lysosomal function (Ganesan and Anandan 2009) against isoprenaline-induced myocardial infarction in rats. In the present study, an attempt was made to assess the protective effects of betaine against oxidative damage during experimentally induced restraint stress in Wistar albino rats with respect to changes in the levels of lipid peroxidation, antioxidant status (GST, GPx, CAT, SOD activities, and GSH level) in lymphoid organs of thymus and spleen, changes in the adrenal weight, plasma protein, and corticosterone levels.

Materials and methods

Chemicals

Epinephrine, bovine serum albumin, and betaine were obtained from Sigma Chemical Company, St. Louis, MO, USA. All the other chemicals used were of analytical grade.

Fig. 1 Chemical structure of betaine

Fig. 2 Betaine, choline, and methionine cycle (*B6*, vitamin B-6; *B12*, vitamin B-12 (cobalamin); *CBS*, cystathionine β -synthase; *MS*, methionine synthase; *MTHFR*, methylenetetrahydrofolate reductase)

Animals

Wistar strain male albino rats, weighing 150–180 g, were selected for the study. The animals were housed individually in polypropylene cages (with stainless steel grill top) under hygienic and standard environmental conditions ($28 \pm 2^\circ\text{C}$, humidity 60–70%, 12 h light/dark cycle). The animals were allowed a standard diet [Krish Feeds (Scientist's Choice Laboratory Animal Feeds), Bangalore, India] and water ad libitum. The diet contained carbohydrate (Nitrogen free) 56.2%, crude protein 22%, ash 7.5%, crude oil 4.2%, crude fibre 3%, glucose 2.5%, vitamin 1.8%, sand silica 1.4%, calcium 0.8%, phosphorus 0.6%, and provided metabolizable energy of 3600 kcal. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee of the Central Institute of Fisheries Technology, Cochin.

Experimental protocol

The experimental animals were divided into four groups of six rats each. Rats in Group I (normal control) received the standard diet. In Group II, received the standard diet (normal rats) and were orally administered with betaine

[250 mg (dissolved in distilled water)/kg body weight/day] by intragastric intubation for a period of 30 days. In Group III, received the standard diet and restraint stress was induced by keeping the animals in wire mesh restrainer for 6 h/day for 30 days. In Group IV, rats were provided with a standard diet and were orally administered with betaine [250 mg (dissolved in distilled water)/kg body weight/day] by intragastric intubation for a period of 30 days, and the restraint stress was induced as described in Group III animals.

Stress procedure

Rats were subjected to restraint stress in a wire mesh restrainer, 6 h/day for 30 days (Sunanda Rao and Raju 2000). The stress procedure was carried out in the institutional animal facility throughout the experimental period between 10 A.M. and 4 P.M. The wire mesh restrainer had a wooden base and a stainless steel wire mesh restrainer hinged to the base. A restrainer with dimensions of 8 cm (L) $\times$ 4 cm (B) $\times$ 4 cm (H) was used in this experiment.

Immediately after the last session of the experiment, animals were killed by using chloroform anaesthesia, and blood was collected with anticoagulant for the separation of plasma. Plasma separated was used for the determination of protein and corticosterone (stress hormone) levels. Adrenal, thymus, and spleen tissues were dissected out immediately and washed with

chilled physiological saline. Ten percent homogenate was prepared using ice-cold Tris-HCl (10 mM, pH 7.4) buffer by motor-driven Teflon-glass tissue homogenizer. The homogenate was centrifuged at 2,000 rpm for 15 min at 4°C, and the supernatant was used for the determination of lipid peroxides (LPO), reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione-S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD).

Biochemical assays

Plasma corticosterone level

Whole blood, removed under ether anaesthesia, was collected to heparin-coated test tubes and centrifuged at 1,500×g for 10 min at 4°C to separate plasma from erythrocytes. The ImmChem™ ¹²⁵I Corticosterone RIA kit (ICN Biomedicals, Inc., Costa Mesa, CA, USA) that is designed for use in laboratory mice and rats were used for measuring plasma corticosterone levels. Plasma and standard samples, diluted with steroid diluent, were incubated with ¹²⁵I-corticosterone and corticosterone antiserum for 2 h. After the precipitation step, the samples were centrifuged at 1,000×g and 4°C for 15 min. The supernatant was removed. The radioactivity of the remaining pellet was counted by using Gamma-counter GC-20. The resulting concentration of plasma corticosterone was expressed as ng/mL using corticosterone standards prepared.

Plasma protein content

The protein content of the samples was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Lipid peroxidation (LPO)

Lipid peroxidation (LPO) was assayed by the method of Ohkawa et al. (1979), in which the malondialdehyde (MDA) released served as the index of LPO. 1,1,3,3-Tetraethoxypropane malondialdehyde bis (diethyl acetal) was used as standard. To 0.2 ml of tissue homogenate, 0.2 ml of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% thiobarbituric acid (TBA) were added. The mixture was made up to 4.0 ml with water and then heated in a water bath at 95.8°C for 60 min using glass ball as a condenser. After cooling, 1.0 ml of water and 5 ml of *n*-butanol/pyridine mixture were added and shaken vigorously. After centrifugation at 4,000 rpm for 10 min, the organic layer was taken, and its absorbance was measured at 532 nm. The level of lipid peroxides was expressed as nanomoles of MDA formed per milligram of protein.

Reduced glutathione (GSH)

Reduced glutathione (GSH) was estimated by the method of Ellman (1959). 0.1 ml of tissue homogenate was precipitated with 5% trichloroacetic acid (TCA). The contents were mixed well for complete precipitation of proteins and centrifuged. To 0.1 ml of supernatant, 2.0 ml of 0.6 mM DTNB [5,5 dithiobis (2-nitrobenzoic acid)] reagent and 0.2 M phosphate buffer (pH 8.0) were added to make up to a final volume of 4.0 ml. The absorbance was read at 412 nm against a blank containing TCA instead of sample. A series of standards treated in a similar way were also run to determine the glutathione content. The amount of glutathione is expressed as nanomoles per gram tissue. 5-Sulphosalicylic acid was used to prevent the oxidation of glutathione.

Glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) was assayed by the method of Paglia and Valentine (1967). The reaction mixture consisted of 0.2 ml of 0.8 mM ethylenediaminetetraacetic acid (EDTA), 0.1 ml of 10 mM sodium azide, 0.1 ml of 2.5 mM H₂O₂, 0.2 ml of reduced glutathione, 0.4 ml of 0.4 M phosphate buffer pH 7.0, and 0.2 ml of tissue homogenate and was incubated at 37.8°C for 10 min. The reaction was arrested by the addition of 0.5 ml of 10% TCA, and the tubes were centrifuged at 2,000 rpm. To the supernatant, 3.0 ml of 0.3 mM disodium hydrogen phosphate and 1.0 ml of 0.04% DTNB were added, and the color developed was read photometric mode at 420 nm immediately. The activity of GPx was expressed as nanomoles of glutathione oxidized per minute per milligram protein.

Glutathione-S-transferase (GST)

Glutathione-S-transferase (GST) was assayed by the method of Habig et al. (1974). To 0.1 ml of tissue homogenate, 1.0 ml of 0.3 M phosphate buffer pH 6.5, 1.7 ml of water, and 0.1 ml of 30 mM 1-chloro-2,4-dinitrobenzene (CDNB) were added. After incubation at 37.8°C for 15 min, 0.1 ml of GSH was added and change in OD was read at 340 nm for 3 min at an interval of 30 s. Reaction mixture without the enzyme was used as blank. The glutathione-S-transferase activity was expressed as μmoles of 1-chloro-2,4-dinitrobenzene (CDNB) conjugate formed per minute per milligram protein.

Catalase (CAT)

Catalase (CAT) was assayed by the method of Takahara et al. (1960). To 1.2 ml of 50 mM phosphate buffer pH 7.0, 0.2 ml of the tissue homogenate was added, and reaction was started by the addition of 1.0 ml of 30 mM H₂O₂ solution.

The decrease in absorbance was measured at nm at 30 s intervals for 3 min. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide. The enzyme activity was expressed as nanomoles of H_2O_2 decomposed per minute per milligram protein.

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) was assayed by the method of Misra and Fridovich (1972). 0.1 ml of tissue homogenate was added to the tubes containing 0.75 ml ethanol and 0.15 ml chloroform (chilled in ice) and centrifuged. To 0.5 ml of supernatant, 0.5 ml of 0.6 mM EDTA solution and 1 ml of 0.1 M carbonate-bicarbonate (pH 10.2) buffer were added. The reaction was initiated by the addition of 0.5 ml of 1.8 mM epinephrine (freshly prepared), and the increase in absorbance at 480 nm was measured by kinetic mode using a Shimadzu UV-1601 visible spectrophotometer. One unit of the SOD activity was the amount of protein required to give 50% inhibition of epinephrine autoxidation.

Betaine content

Betaine content in the lymphoid tissues (thymus and spleen) was determined by the method of Mar et al. (1995) using a Shimadzu LC 10 ATvp HPLC-PDA system with Lichrosorb C8 RP Column (4.6 mm ID $\times$ 125 mm).

Statistical analysis

Results are expressed as mean $\pm$ SD. Multiple comparisons of the significant (ANOVA) were performed by

Tukey's multiple comparison tests. A P value <0.05 was considered as statistically significant. All data were analyzed with the aid of statistical package program SPSS 10.0 for Windows.

Results

Figures 3 and 4 depict the levels of initial body weight, accumulated weight gain, total feed consumption, and feed efficiency ratio in control and experimental groups of rats. There were significant ($P<0.001$) decreases observed in the levels of accumulated weight gain, total feed consumption, and feed efficiency ratio in Group III restraint stress-induced rats as compared to that of Group I control rats. Oral pretreatment with betaine (250 mg/kg body weight/day for a period of 30 days) significantly ($P<0.001$) maintained these levels in Group IV rats at values comparable to that of control animals. The Group II rats receiving betaine alone did not show any significant change when compared with control rats, indicating that it does not have any adverse effects.

Figure 5 shows the levels of protein and corticosterone in plasma of control and experimental groups of rats. There was a significant ($P<0.001$) increase noted in the levels of protein and corticosterone in plasma of Group III restraint stress-induced rats as compared to that of Group I control rats. Prior oral administration of betaine (250 mg/kg body weight/day for a period of 30 days) significantly ($P<0.001$) prevented the stress-induced release of protein and stress hormone (corticosterone) into plasma of Group IV animals as compared to that of Group III rats.

Fig. 3 Levels of initial body weight, accumulated weight gain and total feed consumption in control and experimental groups of rats (A): Betaine orally administered [250 mg (dissolved in distilled water)/kg body weight/day] by intragastric intubation for 30 days. (B): Restraint stress was induced by keeping the animals in a wire mesh restrainer,

for 6 h a day for 30 days. Results are mean $\pm$ SD ($n=6$); one-way ANOVA; Tukey's multiple comparison test. [#] $P<0.001$ significantly different compared with control rats. ^{*} $P<0.001$ significantly different compared with restraint stress-induced animals

Fig. 4 Level of feed efficiency ratio in control and experimental groups of rats (A): Betaine orally administered [250 mg (dissolved in distilled water)/kg body weight/day] by intragastric intubation for 30 days. (B): Restraint stress was induced by keeping the animals in a wire mesh restrainer, for 6 h a day for 30 days. Results are mean $\pm$ SD ($n=6$); one-way ANOVA; Tukey's multiple comparison test. [#] $P<0.001$ significantly different compared with control rats. ^{*} $P<0.001$ significantly different compared with restraint stress-induced animals

Figure 6 depicts the mean weight of adrenal gland (mg g bwt⁻¹) in control and experimental groups of rats. Restraint stress caused significant ($P<0.001$) alterations in the adrenal weight of Group III rats as compared to that of Group I control rats. Oral pretreatment with betaine (250 mg/kg body weight/day for a period of 30 days) significantly ($P<0.001$) counteracted the stress-induced alterations and maintained the adrenal gland in Group IV rats at a levels comparable to that of control animals.

Figures 7 and 10 depict the levels of lipid peroxides (LPO), reduced glutathione (GSH), and activity of glutathione peroxidase (GPx) in the lymphoid tissues (thymus and spleen) of control and experimental groups of rats. Chronic stress caused significant ($P<0.001$) elevation in the level of lipid peroxidation in the thymus and the spleen tissues of Group III rats as compared to that of Group I

Fig. 5 Levels of protein and corticosterone in plasma of control and experimental groups of rats (A): Betaine orally administered [250 mg (dissolved in distilled water)/kg body weight/day] by intragastric intubation for 30 days. (B): Restraint stress was induced by keeping the animals in a wire mesh restrainer, for 6 h a day for 30 days. Results are mean $\pm$ SD ($n=6$); one-way ANOVA; Tukey's multiple comparison test. [#] $P<0.001$ significantly different compared with control rats. ^{*} $P<0.001$ significantly different compared with restraint stress-induced animals. Concentration of plasma corticosterone was expressed as ng/mL using corticosterone standards prepared

Fig. 6 Mean weight of adrenal gland (mg g bwt⁻¹) in control and experimental groups of rats (A): Betaine orally administered [250 mg (dissolved in distilled water)/kg body weight/day] by intragastric intubation for 30 days. (B): Restraint stress was induced by keeping the animals in a wire mesh restrainer, for 6 h a day for 30 days. Results are mean $\pm$ SD ($n=6$); one-way ANOVA; Tukey's multiple comparison test. [#] $P<0.001$ significantly different compared with control rats. ^{*} $P<0.001$ significantly different compared with restraint stress-induced animals. Values expressed: Mean weight of adrenal gland mg g bwt⁻¹

control rats. This was paralleled by a decline in the level of reduced glutathione and the activity of glutathione peroxidase. In the present study, prior oral administration of betaine (250 mg/kg body weight/day for a period of 30 days) significantly ($P<0.001$) counteracted all these stress-induced adverse effects and maintained the lymphoid tissue antioxidants in Group IV rats at a level comparable to that of control animals.

Figures 8, 9 and 11 show the activities of glutathione-S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD) in the lymphoid tissues (thymus and spleen) of control and experimental groups of rats. There were significant ($P<0.001$) reductions observed in the activities of GST, catalase, and SOD in the lymphoid tissues of Group III restraint stress-induced rats as compared to Group I control animals. In the present investigation, prior oral administration of betaine (250 mg/kg body weight/day for a period of 30 days) significantly ($P<0.001$) prevented the stress-induced alterations in the activities of GST, catalase, and SOD in the thymus and spleen tissues of Group IV rats.

Figure 12 depicts the level of betaine content in the lymphoid tissues (thymus and spleen) of control and experimental groups of rats. There was a significant ($P<0.001$) decline observed in the level of betaine in the lymphoid tissues (thymus and spleen) of Group III restraint stress-induced rats as compared with Group I control rats. In Group II rats, there was a significant ($P<0.001$) elevation noted in the betaine content in the lymphoid tissues as compared with that of Group I control rats. Oral pretreatment with betaine (250 mg/kg body weight/day for a period of 30 days) significantly ($P<0.001$) prevented the stress-induced betaine depletion in the lymphoid tissues of Group IV rats as compared with that of Group III rats.

Fig. 7 Levels of lipid peroxides (LPO), reduced glutathione (GSH) and activity of glutathione peroxidase (GPx) in thymus of control and experimental groups of rats (A): Betaine orally administered [250 mg (dissolved in distilled water)/kg body weight/day] by intragastric intubation for 30 days. (B): Restraint stress was induced by keeping the animals in a wire mesh restrainer, for 6 h a day for 30 days.

Results are mean $\pm$ SD ($n=6$); one-way ANOVA; Tukey's multiple comparison test. [#] $P<0.001$ significantly different compared with control rats. ^{*} $P<0.001$ significantly different compared with restraint stress-induced animals. Values expressed: LPO, nmol malondialdehyde released/mg protein; GSH, nmol g^{-1} wet tissue; GPx, nmol GSH oxidized $min^{-1} mg^{-1}$ protein

Discussion

Immobilization/restraint stress is a well-known method for the production of chronic stress (Kvetnansky and Mikulaj 1970). This study was of interest as human beings are exposed to both emotional and physical stress daily in their life (Pylman et al. 2002). Chronic stress may also impair antioxidant defenses, leading to oxidative damage, by changing the balance between oxidant and antioxidant factors (Liu et al 1994; Sosnovsky and Kozlov 1992).

Rats were fed with standard commercial diet. Significant ($P<0.001$) decreases observed in the levels of accumulated weight gain, total feed consumption, and feed efficiency ratio in Group III restraint stress-induced rats as compared to that of Group I control rats (Figs. 3 and 4). The possibility that a reduced food intake, due to their inability to feed while in the restrainer, is most unlikely to have affected their bodyweight because the rats were restrained for 6 h daily, and it is very likely that they would have compensated for this after their release. The bodyweight loss was therefore more likely to have been due to a release of hormones such as thyroid-stimulating hormone, which could have acted by increasing the basal metabolic rate and led to a decrease in bodyweight. Although no detailed behavioural studies were made, it was noticed that the behaviour of the rats while they were being put into the restrainer during the first 7 days was different from their behaviour between 7 and 30 days. During the first 7 days, the rats resisted entering the restrainer, whereas during the second week they resisted less, appearing to show some habituation, and after 14 days they no longer showed any resistance. The rats also defecated and urinated more during

the first 7 days than subsequently. However, the behavior of the animals orally administered with betaine was comparable to that of the normal controls.

Anabolism and catabolism of proteins are very much synchronized cellular processes important for cell viability. Variation in stable state protein metabolism is an essential factor in regulating cellular homeostasis in response to oxidative damage in restraint stress condition. In the present investigation, there was a significant ($P<0.001$) increase observed in the levels of protein and corticosterone in plasma of Group III restraint stress-induced rats compared to Group I control rats (Fig. 5). This is in accordance with the earlier reported study (Sahin and Gümüslü 2007), which indicated that the increase observed in the protein content in

Fig. 8 Activity of glutathione-S-transferase (GST) in thymus and spleen of control and experimental groups of rats (A): Betaine orally administered [250 mg (dissolved in distilled water)/kg body weight/day] by intragastric intubation for 30 days. (B): Restraint stress was induced by keeping the animals in a wire mesh restrainer, for 6 h a day for 30 days. Results are mean $\pm$ SD ($n=6$); one-way ANOVA; Tukey's multiple comparison test. [#] $P<0.001$ significantly different compared with control rats. ^{*} $P<0.001$ significantly different compared with restraint stress-induced animals. Values expressed: GST, μmol 1-chloro-2,4-dinitrobenzene conjugate formed $min^{-1} mg^{-1}$ protein

Fig. 9 Activities of catalase (CAT) and superoxide dismutase (SOD) in Thymus of control and experimental groups of rats (A): Betaine orally administered [250 mg (dissolved in distilled water)/kg body weight/day] by intragastric intubation for 30 days. (B): Restraint stress was induced by keeping the animals in a wire mesh restrainer, for 6 h a day for 30 days. Results are mean $\pm$ SD ($n=6$); one-way ANOVA; Tukey's multiple comparison test. [#] $P<0.001$ significantly different compared with control rats. ^{*} $P<0.001$ significantly different compared with restraint stress-induced animals. Values expressed: CAT, nmol H_2O_2 decomposed $\text{min}^{-1} \text{mg}^{-1}$ protein; SOD, one unit of the SOD activity is the amount of protein required to give 50% inhibition of epinephrine autoxidation

plasma of restraint stress-induced rats is, at least in part, due to leakage of protein and stress hormone corticosterone from the damaged lymphoid tissues and adrenal cortex respectively into the systemic circulation.

In the present study, oral pretreatment with betaine significantly ($P<0.001$) prevented the restraint stress-induced alterations in the levels of protein content and corticosterone in plasma of Group IV rats compared to Group III rats (Fig. 5), possibly by inhibiting the disaggregation of polyribosomes or by attenuating the restraint stress-induced oxidation of lymphoid tissue proteins. Balkan et al. (2004) reported that supplementation of betaine protected the structural and functional integrity of the cell membranes by counteracting reactive oxygen

species-mediated lipid peroxidation and protein carbonyl formation. Reports by Ali et al. (2006) indicated that pretreatment with betaine significantly ameliorated the endocrinological effects induced by the stressful stimulus in desert sheep and goats.

Chronic stress caused hypertrophy of the glands. The weight of adrenal gland was increased significantly ($P<0.001$) in Group III restraint stress-induced rats as compared to Group I control rats (Fig. 6). This is in accordance with the earlier reported study (Tuli et al. 1995), which indicated that prolonged stress may result in weight gain and hyper-activity of adrenal glands. In the present study, prior oral administration of betaine significantly ($P<0.001$) prevented the stress-induced alterations in the weight of adrenal gland in Group IV rats as compared to that of Group III rats. It probably did so by its antioxidant property. Experimental evidence suggests that betaine is nonperturbing to cellular metabolism, highly compatible with enzyme function, and stabilizes cellular metabolic function under different kinds of stress in various organisms and animal tissues (Chambers 1995).

Oxidative stress is known to play an important role in the pathogenesis of restraint-stress injury. Lipid peroxidation is a free radical-mediated process, which has been implicated in a variety of disease states. It involves the formation and propagation of lipid radicals, the uptake of oxygen and rearrangement of double bonds in unsaturated lipids, which eventually results in destruction of membrane lipids. The oxidation of polyunsaturated fatty acids in biological membranes may cause impairment of membrane function, decrease in membrane fluidity, inactivation of membrane receptors and enzymes, and increase of non-specific permeability to ions and disruption of membrane structure (Hernández-Muñoz et al. 2000). In the present

Fig. 10 Levels of lipid peroxides (LPO), reduced glutathione (GSH) and activity of glutathione peroxidase (GPx) in spleen of control and experimental groups of rats (A): Betaine orally administered [250 mg (dissolved in distilled water)/kg body weight/day] by intragastric intubation for 30 days. (B): Restraint stress was induced by keeping the animals in a wire mesh restrainer, for 6 h a day for 30 days.

Results are mean $\pm$ SD ($n=6$); one-way ANOVA; Tukey's multiple comparison test. [#] $P<0.001$ significantly different compared with control rats. ^{*} $P<0.001$ significantly different compared with restraint stress-induced animals. Values expressed: LPO, nmol malondialdehyde released/mg protein; GSH, nmol g^{-1} wet tissue; GPx, nmol GSH oxidized $\text{min}^{-1} \text{mg}^{-1}$ protein

Fig. 11 Activities of catalase (CAT) and superoxide dismutase (SOD) in Spleen of control and experimental groups of rats (A): Betaine orally administered [250 mg (dissolved in distilled water)/kg body weight/day] by intragastric intubation for 30 days. (B): Restraint stress was induced by keeping the animals in a wire mesh restrainer, for 6 h a day for 30 days. Results are mean $\pm$ SD ($n=6$); one-way ANOVA; Tukey's multiple comparison test. [#] $P<0.001$ significantly different compared with control rats. ^{*} $P<0.001$ significantly different compared with restraint stress-induced animals. Values expressed: CAT, nmol H₂O₂ decomposed min⁻¹ mg⁻¹ protein; SOD, one unit of the SOD activity is the amount of protein required to give 50% inhibition of epinephrine autoxidation

study, the significant ($P<0.001$) increase observed in the level of lipid peroxides in the lymphoid tissues of Group III restraint stress-induced rats as compared to that of Group I control animals reflected the oxidative deterioration of lymphoid cells (Figs. 7 and 10). This is in corroboration with an earlier investigation (Zafir and Banu 2009), which suggested that the high vulnerability of lymphoid tissues to peroxidative damage is mainly due to a decline in the level of free radicals for scavengers.

In the present investigation, prior oral administration of betaine resulted in a significant ($P<0.001$) reduction in the level of lipid peroxidation in the lymphoid tissues of Group IV rats as compared to that of Group III rats (Figs. 7 and 10). It probably did so by counteracting the restraint stress-generated free radicals by its antioxidant property. Antioxidants are necessary for preventing the information of free radicals, and they inhibit some of the deleterious actions of reactive oxygen species that damage lipids, DNA and proteins. Betaine is highly lipotropic and, when administered exogenously, it can readily pass across the membrane lipid bilayer (Kanbak et al. 2001). The ability of betaine to diffuse into intracellular compartments aids the capabilities of this natural product as an antioxidant. Reports by Balkan et al. (2004) indicated that betaine supplementation was effective in the prevention of lipopolysaccharide-induced necrotic damage in liver by inhibiting Kupffer cell activation and behaving as an antioxidant. Betaine is also involved in the synthesis of methionine, which serves as a major supplier of cellular cysteine via trans-sulfuration pathway for the synthesis of reduced glutathione.

Tissue antioxidant system plays a fundamental role in cellular defense against reactive free radicals and other oxidant species. Glutathione functions by reaction with

superoxide radicals, peroxy radicals and singlet oxygen, followed by the formation of oxidized glutathione and other disulphides (Aweda et al. 2003). Depletion of GSH results in enhanced lipid peroxidation, and excessive lipid peroxidation can cause increased GSH consumption (Ganesan et al. 2007a), as observed in the present study (Figs. 7 and 10). This is in accordance with a previous reported study (Zafir et al. 2009), which indicated that the depletion of tissue GSH by restraint stress-induced oxidative stress. This reduction might have resulted from the oxidation of GSH to restraint stress-induced generation of free radicals. The depletion of GSH further enhances the susceptibility of the lymphoid tissues to oxygen metabolites and acid-mediated cell damage.

In the present study, prior oral administration of betaine significantly ($P<0.001$) maintained the level of GSH, which protects against oxidative damage by regulating the redox status of proteins in the lymphoid tissues at near normalcy in Group IV rats as compared with that of Group III restraint stress-induced rats (Figs. 7 and 10). This indicates that normal maintenance of lymphoid tissue GSH levels by betaine in restraint stress-induced rats may be the result, but not the cause, of the cytoprotection by betaine. The ability of betaine to maintain the tissue antioxidant status at higher rate demonstrates its possible preventive efficacy in inhibiting free radical mediated restraint stress-induced alterations. It may be possible that by blocking oxidative damage through lipid peroxidation, betaine prevents loss of membrane permeability and dysfunction of cellular proteins, leading to the survival of the functionally active cells. A previous report also reveals that betaine supplementation could protect the structural and functional integrity of the cell membrane by counteracting reactive oxygen species-mediated lipid peroxidation and protein carbonyl function (Balkan et al. 2004).

Fig. 12 Level of betaine content in lymphoid tissues (thymus and spleen) of control and experimental groups of rats (A): Betaine orally administered [250 mg (dissolved in distilled water)/kg body weight/day] by intragastric intubation for 30 days. (B): Restraint stress was induced by keeping the animals in a wire mesh restrainer, for 6 h a day for 30 days. Results are mean $\pm$ SD ($n=6$); $P<0.001$ significantly different compared with control rats. ^{**} $P<0.001$ significantly different compared with control and betaine administrated rats. ^{**} $P<0.001$ significantly different compared with restraint stress-induced rats. Values expressed: nmol/g wet tissue

Activities of glutathione-dependent antioxidant enzymes (GPx and GST) and antiperoxidative enzymes (catalase and SOD) were significantly ($P < 0.001$) lower in lymphoid tissues of Group III restraint stress-induced rats as compared with that of Group I control rats (Figs. 7, 8, 9, 10 and 11), which is in line with previous reports (Mannervick and Danielson 1998), suggested an enhanced oxidative stress in experimentally induced restraint stress. Glutathione protects the tissues against oxidative damage by way of regulating the redox status of proteins in the cell-surface membrane (Das et al. 1997). Reduction notified in the activities of GPx and GST in restraint stress-induced animals might be due to decreased availability of their substrate, reduced glutathione. This in turn may lead to increase the accumulation of oxidative-free radicals and also enhance the susceptibility of lymphoid cells to peroxidative damage. SOD and CAT, responsible for the destruction of peroxides have a specific role in protecting tissues against oxidative damage (Zafir et al. 2009). Reduction in the activities of these enzymes in restraint stress-induced oxidative stress may lead to the formation of O^{2-} and H_2O_2 , which in turn can form hydroxyl radical ($OH\cdot$) and bring about a number of reactions harmful to the cellular and subcellular membranes (Pryor 1988). The generation of free radical-scavenging enzymes in restraint stress-induced oxidative stress might have exceeded the ability of these free radical-scavenging enzymes to dismutate the radicals, resulting in peroxidative damage to lymphoid cells.

In the present study, preoral treatment with betaine significantly ($P < 0.001$) prevented the restraint stress-induced aberrations in the level of GSH and the activities of glutathione-dependent antioxidant enzymes (GPx and GST) and antiperoxidative enzymes (catalase and SOD) in lymphoid tissues of Group IV rats as compared to that of Group III rats (Figs. 7, 8, 9, 10 and 11). It probably did so by its antioxidant action (Go et al. 2007) against restraint stress-induced lipid peroxidation. Previous studies by Erman et al. (2004) indicated that lipotropes inhibit lipid peroxidation in cellular membranes as a result of distinct biophysical interactions with membrane lipid bilayer. Administration of betaine has already been reported to exert antioxidant action through forcing structural interactions with membrane lipids (Balkan et al. 2004). The unpaired electron present in the hydroxyl free radical, which is mainly responsible for the restraint stress-induced oxidative damage to the lymphoid tissues might have been trapped and subsequently dismutated by betaine. Earlier reports (Pourahmad et al. 2005; Kanbak et al. 2008) indicated that betaine had a potent reducing effect on the production free radicals in rats exposed to cytotoxicity. The protective effect of betaine against restraint stress-induced oxidative stress observed in this study may also be

associated with the restoration of SAM, which contributes to an increase in the supply of substrate needed for the synthesis of GSH that protects the cell from reactive metabolites and reactive oxygen species.

There was a significant ($P < 0.001$) reduction observed in the betaine content in lymphoid tissues (thymus and spleen) of Group III restraint stress-induced rats as compared with Group I control rats (Fig. 12), indicating alterations in normal functioning of the cellular membranes in the lymphoid tissues. A previous reported study showed that betaine supplementation in fish feed significantly protected fishes from the stress of moving from low to high salinity (Virtanen 1995). In the present study, oral administration of betaine resulted in increased accumulation of betaine in lymphoid tissues of Group II rats as compared with Group I control rats. Preoral treatment with betaine significantly ($P < 0.001$) attenuated the restraint stress-induced betaine depletion in lymphoid tissues of Group IV rats as compared with that of Group III animals, indicating its cytoprotective property (Ganesan et al. 2007b). This finding concurs with a previous reported study, which showed that an intravenous pretreatment with betaine significantly alleviated the effects induced by the stressful stimulus in sheep and goats (Ali et al. 2006).

In conclusion, the result of this study implies that lipid peroxidation and free radicals play an important role in restraint stress-induced oxidative damage. Moreover, elevated corticosterone levels were associated with a reduction in antioxidant status. Oral pretreatment with betaine significantly ($P < 0.001$) inhibits the level of lipid peroxidation, corticosterone and significantly protects the enzymatic antioxidant defense mechanisms in restraint stress-induced animals. The findings suggest that betaine ameliorates the tissue and cellular effect of stress. However, further studies have to be carried out for analyzing the thymus and the spleen versus other organs such as the liver, heart or brain to identify the precise mechanisms involved in the anti-stress effect of betaine.

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