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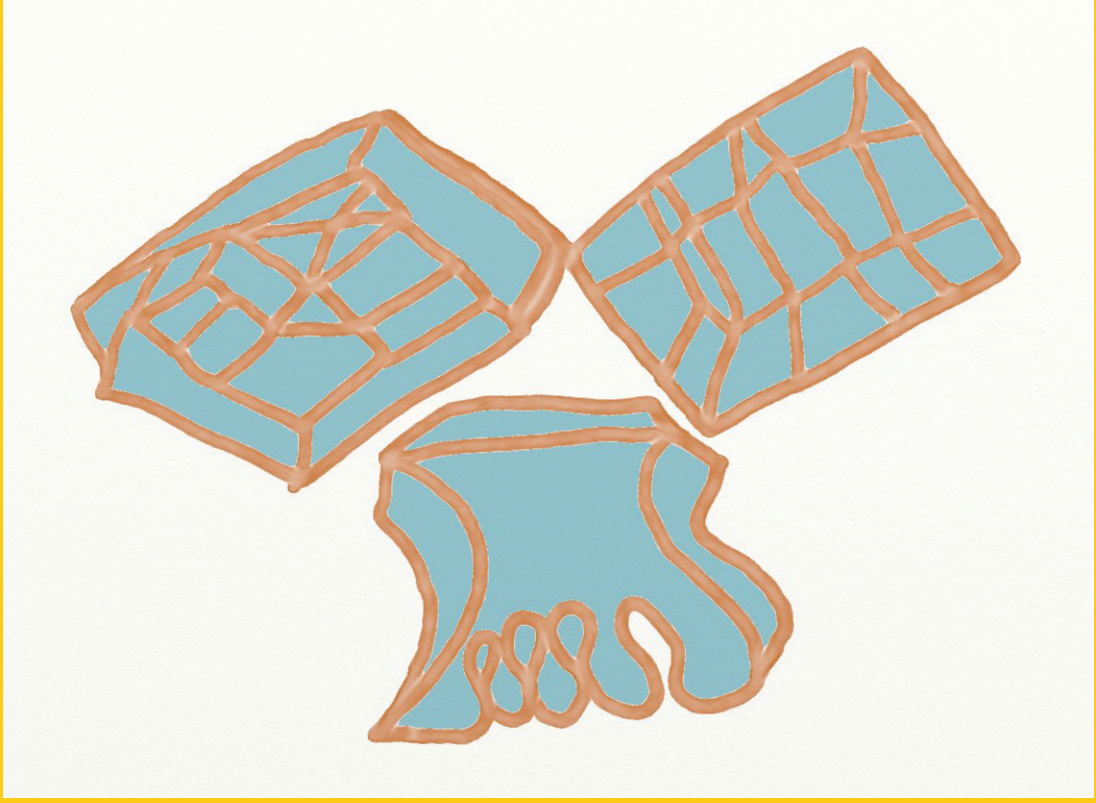

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Original Article

Ascorbic acid glucoside reduces neurotoxicity and glutathione depletion in mouse brain induced by nitrotriazole radiosensitazer

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ABSTRACT

Aim: To investigate the potential of the anti-oxidant ascorbic acid glucoside (AA-2G) to modulate neurotoxicity induced by high doses of nitrotriazole radiosensitizer.

Materials and Methods: Male and female C56Bl/6xCBA hybrid mice aged 8-14 weeks (weight 18-24 g) were used. Nitrotriazole drug radiosensitizer sanazole at a high dose of 2, 1 g/kg was per os administered to induce neurotoxicity at mice. Ascorbic acid glucoside was given 30 min before the sanazole administration. Serum ascorbic acid, brain glutathione level, as well as behavioral performance using open field apparatus were measured.

Results: Administration of high (non-therapeutic) doses of the nitrotriazole drug sanazole results in neurotoxicity in mice as evidenced from behavioral performance, emotional activity and depletion of the cellular antioxidant, glutathione, in the brain. The serum levels of ascorbic acid was also found reduced in high dose sanazole treated animals. Per os administration of ascorbic acid glucoside significantly reduced the neurotoxicity. This effect was associated with the prevention of glutathione depletion in mouse brain and restoring the ascorbic acid level in serum.

Conclusion: Administration of ascorbic acid glucoside, but not ascorbic acid, before sanazole administration protected from sanazole-induced neurotoxicity by preventing the decrease in the brain reduced glutathione level and providing high level of ascorbic acid in plasma.

KEY WORDS: Antioxidants, ascorbic acid glucoside, ascorbic acid, neurotoxicity, sanazole

INTRODUCTION

Chemotherapy and radiotherapy are currently the principal modalities of cancer treatment. These treatments affect normal cells causing certain side effects, which at times dominate and limit the treatment. One way to overcome this problem is the use the hypoxic cell-sensitizing drugs as adjuvant to enhance the medical effectiveness of the treatment. One of the most useful hypoxic cell-sensitizing drugs which have successfully completed the phase III clinical trials for use as an adjuvant in radiotherapy is sanazole, a nitrotriazole drug.[1,2] High doses or frequent administration of the sanazole bring about neurotoxicity as a side effect in the sensitization of cancer treatments.[3] Neurotoxicity could result from the increased production of reactive oxygen species (ROS) and/ or a decreased anti-oxidant capacity of the cells of the brain tissue, which is especially endangered with regard to the generation and insufficient ROS detoxification, resulting in the oxidative stress.[4,5] The disturbance of balance between ROS generation and anti-oxidant processes was found for several neurological disorders, and anti-tumor chemotherapy drugs such as cisplatin, paclitaxel, methotrexate,[6,7] as well as a number of widely used radiosensitizers misonidazole (MISO), metronidazole.[8-10] Because ROS are known to be involved in the neurotoxicity induction, it is expected that compounds with anti-oxidant activity could be used to neutralize free radicals and prevent the side effect. Ascorbic acid functions as anti-oxidant by directly reacting oxygen intermediates and has a vital role in defenses against oxidative stress.[11] It inhibits peroxidation of membrane phospholipids and act as scavengers of free radicals. Brain concentration of vitamin C is 10-fold higher than its plasma levels.[12] The decrease in neuronal cell viability by oxidative stress inducing agents was markedly attenuated by ascorbic acid, as well as other antioxidants such as glutathione, N-acetyl-L-cysteine and sodium metabisulphite.[13] Ascorbic acid is a potential cerebra-protective agent. Although, it does not penetrate the blood-brain barrier, its oxidized form, dehydroascorbic acid, enters the brain by means of facilitative transport and plays

an important role in preventing oxidative stress by trapping hydroxyl radical in the central nervous system.[14]

2-O- α-D-glucopyranosyl-L-ascorbic acid (AA-2G) as a glucoside derivative of ascorbic acid has been found to exhibit the higher stability in aqueous solution than ascorbic acid (AA) and can manifest the inherent vitamin C radical scavenging activity. Just after administration into the body it is hydrolyzed to release free AA by mammalian α-glucosidase.[15,16] According to a recent data of Takebayashi *et al*. (2007),[17] AA-2G is thought to exert long-lasting radical scavenging activity without pro-oxidant effect and has benefit to inactivate a larger amount of radicals than does AA that appeared to enhance lipid peroxidation associated with iron ions in extracellular fluids.[17,18] AA-2G produces anti-oxidant and radioprotective effects due to blocking the radiation-induced lipid peroxidation processes and strand breaks in DNA.[19-21] We have shown that AA-2G markedly protects from the decrease in blood leukocytes, thymocytes, splenocytes and bone marrow cells in healthy mice administered with high CPA dose (250 mg/kg).[22] The present study was undertaken to investigate the potential of the anti-oxidant ascorbic acid glucoside in comparison with ascorbic acid to modulate sanazole-induced neurotoxicity.

MATERIALS AND METHODS

Male and female C56Bl/6xCBA hybrid mice aged 8-14 weeks (weight 18-24 g) from the vivarium of Pharmacology Research Institute (Tomsk, Russian) were housed under standard conditions of temperature and humidity. Mice were provided with food and water ad libitum. All experiments were conducted in accordance with the ethical guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

AA-2G and sanazole (AK 2123) were obtained from Dr. V.T. Kagiya (Health Research Foundation, Kyoto 606-8225, Japan). AA and glutathione (GSH) used in this study were from Sigma Chemical Company Inc., St Louis, MO, USA.

In preliminary experiment mice were treated with sanazole at different doses for searching suitable dose to induce neurotoxicity. A high non-therapeutic dose of 2, 1 g/kg per os appeared to be the mostly effective to induce neurotoxicity at mice. Neurotoxicity was evidenced from significant damage to behavioral features of mice on the first day 2 h after sanazole administration. Sanazole in this specified dose did not lead to death of animals, but significantly disturbed walking pattern, as well as emotional activity of mice.

The animals were randomly separated into 5 groups and treated as follows:

1. Group I, control, treated with 0.9% NaCl orally (“Saline”).
2. Group II, administered with Sanazole at a high non-therapeutic dose of 2, 1 g/kg in aqua solution orally + 0, 9% NaCl orally (“Sanazole”).
3. Group III, administered with AA at a dose of 50 mg/kg orally + Sanazole at a dose of 2,1 g/kg orally
4. Group IV, administered with AA-2G at a dose of 50 mg/kg orally + Sanazole at a dose of 2,1 g/kg orally
5. Group V, administered with GSH at a dose of 500 mg/kg orally + Sanazole at a dose of 2, 1 g/kg orally.

AA-2G, AA and GSH were administered 30 min before the sanazole administration. Six mice per group were used to measure ascorbic acid and glutathione level, as well as to monitor behavioral performance after treatment.

Neurotoxicity was diagnosed by the measure of behavior terms such as locomotor activity using open field apparatus and emotional status using special methods.[23] Behavioral performance was tested 2 h after sanazole administration. Open-field test was used to assess parameters of exploratory behavior of mice. All experiments were carried out from 10.00 to 14.00 h. Open field was a chamber measuring 40x40x20 cm with a square field and white-colored walls. The chamber’s floor divided into 16 squares had round holes of 3 cm in diameter in each of the squares. Electric lamp (100 watt) located 1 meter above the floor lighted the chamber. A mouse was placed into one of the chamber’s corners and the following parameters were registered over a 2-min period:

- Horizontal activity (from one square to another) was expressed as the average number of crossed squares per mouse in a group;
- Common motor activity (the number of hole explorations, the number of standings on back paws (vertical activity), the number of washings and the number of defecations counted by the number of fecal balls) was expressed as the average of the total number of locomotion per mouse in the group;
- Emotional activity was assessed in points considering such signs as resistance to capture by a hand, tonus, reaction to object approaching, reaction to push by pincers and vocalization.

The results were assessed 2 h after the AK 2123 administration. The “saline” groups of mice were examined for the waking pattern 2 h after saline per os administration. The same behavioral activity of these mice was evaluated the day before sanazole administration (background).

The animals were anaesthetized by ether and sacrificed by cervical dislocation. The brain tissue was extracted. Blood was collected by heart puncture.

The reduced glutathione (GSH) level in brain was examined by the Hissin P.J. and Hilf R. (1976) method.[24] Brain tissue (250 mg) was homogenized in Tris-phosphate buffer (1 ml), pH 8, 1 and centrifuged at 20000g, 2^{0}C and the GSH or GSSG content in the supernatant was measured. For GSH level assay the reaction was initiated by addition of 0.1 ml of o-phthaldialdehyde (1mg/ml in methanol).

The glutathione disulfide (GSSG) level was assayed by addition of 0.2 ml of the 0.04M NEM with following incubation at room temperature for 30 min. Then 2 ml 0, 1M NaOH and 0.1 ml of o-phthaldialdehyde was added. Initial and final readings were taken at λ excitation: 350 nm, λ emission: 420 nm. The differences in initial and final readings were compared with those in corresponding GSH standards (1-10 x 10^{-9} M) and GSSG standard processed in the same run. The GSH/GSSG ratio was calculated.

Plasma samples (200 μl) were mixed with 100 μl of ice cold 5% metaphosphoric acid and proteins were removed by centrifugation at 12,000g for 5 min. Aliquots (50 μl) of deproteinized plasma were mixed with 930 μl of 2 M Na-acetate buffer, pH 6,2, and 10 U of ascorbate oxidase to convert free AA to DHAA (dehydroascorbic acid). The reaction was completed in 30 min. Then, 0.2 ml 92.5 mM o-phenylenediamine was added, and the fluorophore formation was completed after 30 min. The initial and final readings are taken at λ excitation: 350 nm, λ emission: 430 nm.[25]

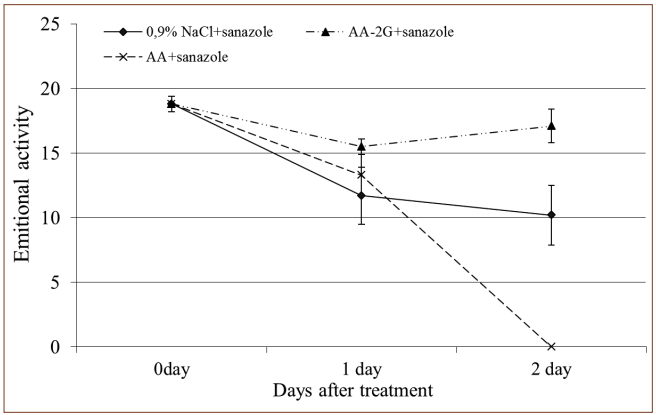

Figure 1: Effect of ascorbic acid glucoside and ascorbic acid on emotional activity in mice when administered at a single dose of 50 mg/kg orally 30 min before sanazole treatment at a high (non-therapeutic) dose 2, 1 g\kg orally. Each value represents the mean ± SD for 6 mice.

The values given in the figures represent the mean values. Drug effects were assessed by comparing drug-treated animals with control animals using non-parametric Wilcoxon Mann-Witney test. A value of $P < 0.05$ was considered as statistically significant difference.

RESULTS

On the first step, we studied the effect of ascorbic acid and ascorbic acid glucoside on the behavioral activities of mice administered with Sanazole at neurotoxic dose. In order to find if AA and AA-2G are able to attenuate sanazole induced neurotoxicity we used these drugs at different doses and schedules. Behavioral performance of mice was evaluated after their exposure to AA and AA-2G with the following doses: 10, 50, 100 and 300 mg/kg of body weight, and anti-oxidants were orally administered either 30 min before or just after or 1 h after sanazole administration at a high non-therapeutic dose of 2,1 g/kg per os. To attenuate sanazole induced damage to mice locomotor and emotional activity, AA-2G at the most effective dose 50 mg/kg orally administered 30 min before sanazole administration. So we have chosen "working" dose of 50 mg/kg in further studies. Per os administration of ascorbic acid glucoside significantly reduced the neurotoxicity while AA did not attenuate neurotoxicity at this regimen [Figures 1 and 2].

GSH level in the brain of mice after sanazole administration at a high non-therapeutic dose was decreased with concomitant increase in the level of GSSG as can be evidenced from the data presented in Table 1. AA-2G, when administered 30 min before sanazole, appeared to prevent the decrease in the GSH level and increase in the GSSG level. Thus, GSH/GSSG ratio came to 133, which was close to the parameters of control untreated mice. In the case of AA, these parameters did not essentially differ from those observed in the sanazole treated group. So AA-2G was found to be more effective in restoring the endogenous GSH level in brain than vitamin C.

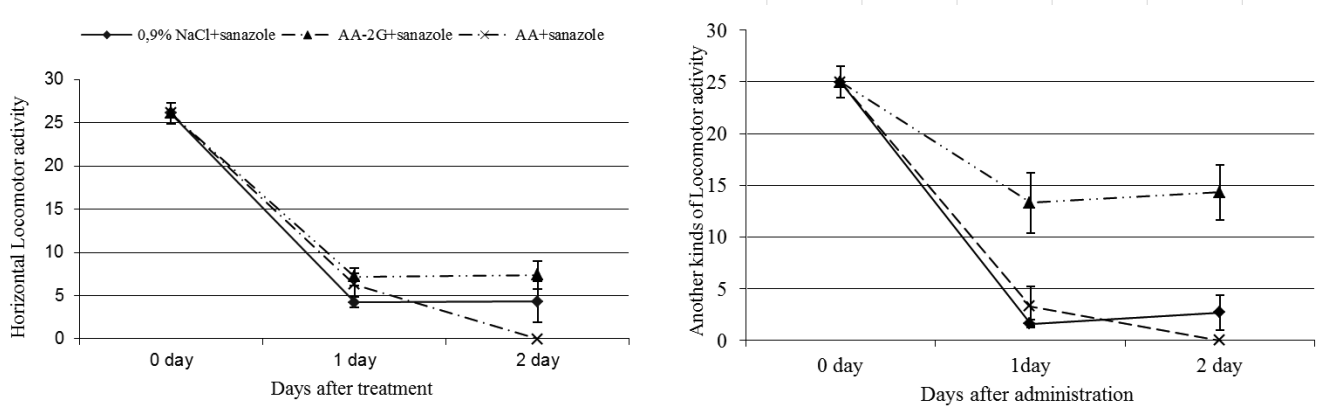

Figure 2: Effect of ascorbic acid glucoside and ascorbic acid on locomotor activity in mice when administered at a single dose of 50 mg/kg 30 min before sanazole treatment at a high (non-therapeutic) dose 2, 1 g\kg orally. Each value represents the mean ± SD for 6 mice.

Oxidative stress resulting from enhanced free-radical formation and/or a defect in anti-oxidant defense was shown to decrease the levels of tocopherol and ascorbic acid.[26,27] In order to make clear, the differences between AA-2G and AA ability to protect the GSH level, we compared the level of ascorbic acid in plasma after sanazole, AA-2G and AA administrations [Figure 3]. Administration of AA-2G prior to sanazole prevented the decrease in the ascorbic acid level in the plasma while similar administration of AA had no effect on the plasma ascorbic acid level [Figure 3].

As can be seen in Figure 4 administration of AA-2G and AA to mice resulted in time dependent increase in ascorbic acid levels in plasma. As it is seen from the figure, that the AA-2G administration resulted in the increase in ascorbic acid level in plasma in a shorter time than the AA administration; also, the high level of ascorbic acid remained for a longer time.

The greater stability and better bio-comprehensibility of ascorbic acid glucoside can explain these differences. It is well known, that ascorbic acid is transported to blood cells in oxidized form (dehydroascorbic acid) with transporter Glut-1.[28,29] The presence of glucose moiety in AA-2G can promote the greater affinity to conveyors, though it is known that some amount of AA-2G is subjected to hydrolysis by alpha - glucosidase of the intestine.[15]

Under conditions of oxidative stress ascorbic acid is known to maintain GSH level via its contribution to free radical trapping. Another possible mechanism for rising GSH level under the influence of ascorbic acid was also proposed by Puskas F. *et al.*, (2000).[30] It is known that dehydroascorbic acid (DHA) is the major transport form of ascorbic acid in blood and brain cells. DHA enters cells through the glucose transporters and is reduced to ascorbic acid intra-cellular.[14] DHA stimulates the pentose phosphate pathway providing the increase in NADPH2 generation that results in the GSSG regeneration through glutathione-reductase (GR) reaction. It is possible to assume, that oxidation of ascorbic acid in blood plasma after introduction of sanazole promotes an activation of the transport of the oxidized form of ascorbic acid in brain cells, the activation of the pentose phosphate pathway and enhancement in reduction of oxidized glutathione in glutathione-reductase reaction.

In order to confirm GSH contribution to AA-2G effect in attenuation of sanazole induced neurotoxicity we examined if exogenic GSH affects neurotoxicity and brain GSH level in mice treated with sanazole.

Administration of GSH, 30 min before sanazole prevented the behavioral defects induced by sanazole as can be seen from the data presented in Table 2. It is known, that GSH can cross the blood-brain barrier and its administration increases its level in plasma more effectively than administration of the individual amino acids, from which GSH is synthesized.[27]

The ratio GSH/GSSG may be considered as a marker of oxidative stress and a reflector of the activity of the anti-oxidant system. Sanazole treatment resulted in oxidative stress and increased the GSSG level due to binding of GSH to free radicals and its transformation to GSSG [Table 2]. The GSH administration at

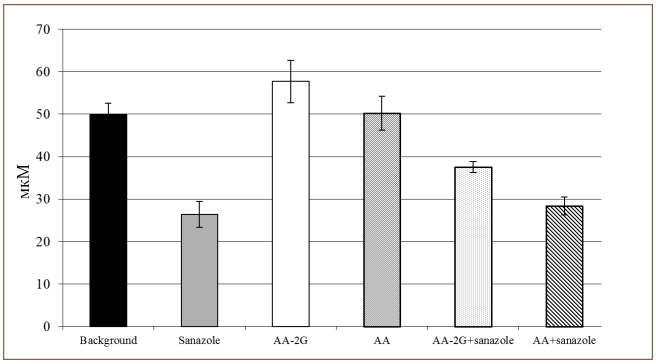

Figure 3: Concentration of ascorbic acid in plasma of mice 2 h after sanazole administration at a high (non-therapeutic) dose 2, 1 g\kg orally. Each value represents the mean ± SD for 6 mice.

Table 1: The levels of GSH, GSSG and the GSH/GSSH ratio in brain of mice treated with sanazole, ascorbic acid (AA) and ascorbic acid glucoside (AA-2G).

	GSH (imol/g of tissue)	GSSG (nmol/g of tissue)	GSH/GSSG
Saline	1.34 ± 0.08	8.48 ± 0.53	158
Sanazole, 2,1 g/kg	1.13 ± 0.08*	10.64 ± 0.64*	106*
Sanazole+ AA	1.06 ± 0.08*	10.08 ± 0.35	98*
Sanazole+ AA-2G	1.30 ± 0.05**	9.76 ± 0.3**	133**

Each value represents the mean ± SD for 6 mice* - differences are significant with the Saline group ($P < 0.05$); **: Differences are significant with the Sanazole group ($P < 0.05$)

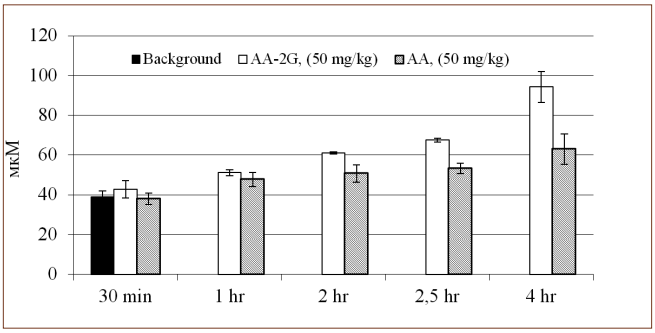

Figure 4: Kinetics of ascorbic acid levels in plasma after administration ascorbic acid glucoside and ascorbic acid at a single dose of 50 mg/kg orally. Each value represents the mean ± SD for 6 mice.

Table 2: The levels of GSH, GSSG and GSH/GSSH ratio in brain of intact mice and sanazole and sanazole + GSH-treated mice.

	GSH (imol/g of tissue)	GSSG (nmol/g of tissue)	GSH/GSSG
Saline	1,79± 0,02	9,6	186
Sanazole, 2,1 g/kg	1,5± 0,09*	10,8	138
Sanazole+GSH	1,78± 0,009**	9,58	186

Each value represents the mean ± SD for 6 mice * - differences are significant with the Saline group ($P < 0,05$);** - differences are significant with the Sanazole group ($P < 0,05$)

a dose of 50.0 mg/kg, 30 min before sanazole prevented the decrease in the GSH level and maintained the GSH/GSSG ratio and also, the GSH levels were found restored in the brain to the control levels.

DISCUSSION

It is known that nitroimidazole MISO is a severe neurotoxic radiosensitizer. The nitrotriazole derivative, sanazole has low penetration to brain tissue [31] and elicits mild neurotoxic effect. However, the frequent high dose administration of sanazole to enhance the efficacy of radiotherapy leads to numbness and peripheral neuropathy in some patients. In order to characterize the nature of sanazole-induced neurotoxicity in an adult mice model, we have administered sanazole at a high (non-therapeutic) dose of 2, 1 g/kg body weight orally in C57Bl/6 or F1 (CBAx C57Bl/6) mice. Locomotor and rearing activities in mice were determined in an "open field" apparatus, as well as emotional reaction to different actions. Sanazole treated mice displayed significant decrease in the parameters of horizontal and other kinds of locomotor activity in comparison to control groups [Figures 1, 2]. The summary index defining the emotional status was also decreased in the sanazole treated group, compared to the untreated group of mice. These findings provide evidence of high dose sanazole neurotoxicity, because the measurement of walking pattern and spatial orientation performance are sensitive indicators to monitor behavioral changes in relation to neurotoxicity in different animal models.[32]

The formation of nitrofurantoin radical, semiquinone radicals of catecholamines, and oxygen-derived radicals by chromaffin granules is proposed to cause damage to adrenal medulla, and this process may lead to neurotoxicity.[33] Glutathione is known to play a major role in protecting cells against oxidative stress.[34,35] Reduced glutathione has important functions as cellular anti-oxidant, for transport and storage of cysteine, for the detoxification of xenobiotics, apart from being a cofactor in isomerization reactions.[36,37] The glutathione system is especially important for cellular defense against ROS. GSH reacts directly with radicals in non-enzymatic reactions and is the electron donor in the reduction of peroxides catalyzed by glutathione peroxidase (GPx). The product of the GSH oxidation is GSSG. GSH is regenerated from GSSG within cells in a reaction catalyzed by the flavoenzyme glutathione reductase. This enzyme regenerates GSH by transferring reduction equivalent from NADFH to GSSG. GSH depletion was shown to result in the neurodegeneration indicating its crucial role in prevention of neurotoxicity.[38] Chemotherapeutic agent- induced neurotoxicity was shown to correlate with a significant decrease in activity of brain GSH.[5] Oral administration of high non-therapeutic dose of sanazole to mice leads to significant decrease in GSH levels in brain tissues compared to the controls [Table 1]. The decrease of the reduced glutathione levels in the brain of mice after sanazole treatment was concomitant with increased levels of oxidized glutathione. The decreasing GSH/GSSG ratio (138 in "Sanazole" vs. 186 in "Saline" group) could be a sensitive indicator of oxidative stress.

Our *in vivo* model of the sanazole-induced behavioral alterations indicated that sanazole-neurotoxicity is related to damage to anti-oxidant mechanisms. These data provide the first direct evidence of sanazole toxicity in the mammalian brain. Despite the limited ability of sanazole to pass the blood-brain barrier, its neurotoxicity may stem from the mechanism related to induction of tumor necrosis factor (TNF)- alpha, as TNF-alpha has been shown to be an important mediator by which some chemotherapeutic agents induce central nervous system (CNS) injury.[39]

Oxidative stress followed by the decreasing GSH level in brain is appeared to be involved in the neurotoxic effect of sanazole. Administration of ascorbic acid glucoside, but not ascorbic acid, before sanazole administration protected from sanazole-induced neurotoxicity by preventing the decrease in the GSH level.

In contrast to ascorbic acid, ascorbic acid glucoside is characterized by the high stability toward thermal and oxidative degradation in aqueous solutions and non-reducibility.[15,40] Indeed, we showed that per os AA-2G administration resulted in the higher level of ascorbic acid in plasma within up to 4 h in health mice as compared to AA treated mice.

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