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EXPERIMENTAL STUDY

Effect of ascorbic acid on the monosodium glutamate-induced neurobehavioral changes in periadolescent rats

Sareesh Naduvil Narayanan[1], Raju Suresh Kumar[1], Jaijesh Paval[2], Satheesha Nayak[2]

Melaka Manipal Medical College (Manipal Campus), Manipal University, Manipal, Karnataka, India.
sareeshnn@yahoo.co.in

Abstract: *Aim:* In the current study we evaluated adverse effects of monosodium glutamate (MSG) on memory formation and its retrieval as well as the role of ascorbic acid (Vitamin-C) in prevention of MSG-induced alteration of neurobehavioral performance in periadolescent rats.
Materials and methods: Healthy male albino Wistar rats (4–6 weeks old), were randomly allotted in four groups. Group I: normal control, who remained in their homecage throughout the experimental period. Group II: vehicle control, who were orally administered with normal saline for three weeks. Group III: MSG, who were orally administered with aqueous solution of MSG (2 mg/g b.w/day), for three weeks. Group IV: MSG+AA, who were administered with aqueous solution of MSG, and subsequently by ascorbic acid (100 mg/kg b.w/day) orally for three weeks. After the experimental period, all animals from all groups were first tested for anxiety followed by passive avoidance behavior.
Results: MSG significantly altered the neurobehavioral performance in rats. The alteration manifested as less time spent on the open arm during the EPM test and shorter entrance latency to the dark compartment during the passive avoidance task. All behavioral changes were significantly prevented by simultaneous administration of ascorbic acid with MSG.
Conclusion: The present data point to the neuroprotective role of ascorbic acid. The ascorbic acid can be used as a therapeutic agent in various cognitive deficits *(Fig. 5, Ref. 25).* Full Text (Free, PDF) *www.bmj.sk.*
Key words: periadolescent, monosodium glutamate (MSG), ascorbic acid, neurobehavioral performance, anxiety, passive avoidance behavior.

Glutamate, an excitatory amino acid, is a natural constituent of many foods, including meat, cheese, and vegetables. It is the main excitatory neurotransmitter in the brain and the spinal cord. The areas of its higher concentration are the cerebral cortex, hippocampus and cerebellum. Glutamate plays an important role in some physiologic processes. It helps in the differentiation, migration and survival of neurons in the developing brain largely through facilitating the entry of Ca^{2+}. It is also known to help in long-term potentiation (LTP), an electrochemical phenomenon involved in memory consolidation. Free glutamate also enhances the palatability of foodstuffs, and in modern nutrition it continues to be a flavor enhancer in the form of monosodium glutamate (MSG). There is considerable evidence suggesting the neurotoxic effect in high concentrations of MSG when given to neonatal animals. Administration of subneurotoxic doses of monosodium-L-glutamate (MSG) (2 mg/g, p.o., for 10 days) during the weaning stages caused behavioral abnormalities such as a decrease in the active avoidance learning performance in rats during the learning (acquisition) phase without any changes in the extinction and relearning phases (1). In a study, the rat pups were subcutaneously given MSG (3 mg/g) daily during the postnatal period from 5th to 12th day. When they were tested for behavioral analysis, on the 56th and 84th days, they showed significant deficits in the behavioral tests (2). Male and female Sprague-Dawley-derived CD strain pups exposed to L-glutamic acid (2–3.5 mg/g) on days 1–15 *post partum* showed measurable, long-term behavioral and somatic alterations in female rats, and to a lesser degree in male rats (3).

Even though the reports on the neurotoxic effect of MSG on neonates are numerous, the concerns regarding the latter effect on the nervous system in young children and prevention remain unanswered. Recent reports state that the treatment with vitamin C (500 mg/kg of body weight) prevents the MSG-induced (4 mg/g of body weight) cytotoxicity in rat thymocytes by up-regulating Bcl-2 protein expression resulting in a change in Bcl-2/Bax protein ratio (4). Farombi et al have reported that dietary antioxidants such as vitamin C, vitamin E, and quercetin have a protective potential against oxidative stress induced by MSG (5). In the present study, we investigated the neurobehavioral alterations of rats due to MSG administration during periadolescent period (age in range of 4–6-weeks) and its prevention by ascorbic acid (vitamin C).

[1]Department of Physiology, Melaka Manipal Medical College (Manipal Campus), Manipal University, Manipal, Karnataka, and [2]Department of Anatomy, Melaka Manipal Medical College (Manipal Campus), Manipal University, Manipal, Karnataka, India

Address for correspondence: Sareesh Naduvil Narayanan, Dept of Physiology, Melaka Manipal Medical College (Manipal Campus), Manipal University, Manipal – 576 104, Karnataka, India.

Materials and methods

Animals: Healthy male albino Wistar rats (age in range of 4–6 weeks) with weight in range of 120–140 g were used in the present study. These were obtained from the litters of eight normal female rats. The animals were housed in plastic cages of size 14"x9"x8" (3 rats in each cage). All animals had free access to food and water. They were maintained under standard laboratory conditions, i.e. in an air-conditioned room with alternating light and dark cycles of 12 hours each. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC). Care was taken to handle the animals in humane manner.

Drugs: Monosodium glutamate and ascorbic acid (Vitamin C) were obtained from *Merck, India* and dissolved in isotonic sodium chloride (*Merck, India)* solution to get the desired concentrations. Both solutions (MSG – 2 mg/g of b.w; Ascorbic acid – 100 mg/kg of b.w.) were administered orally using a gastric gavage needle. The selection of the above doses of drugs was based on previous reports (1, 6).

Experimental design: The animals were randomly allotted into four groups.

The Group I (n=10) was the *normal control group* and the animals of this group remained in their homecage throughout the experimental period. The Group II (n=10) was the *vehicle control group* and the animals of this group were orally administered with normal saline for three weeks. The Group III (n=10) was *the MSG-treated group* and the animals of this group were orally administered with aqueous solution of MSG (2 mg/g body weight/day) for three weeks. The Group IV (n=10) was the *MSG+AA group* and the animals of this group were orally administered with aqueous solution of MSG (2 mg/g of body weight/day), followed by ascorbic acid (100 mg/kg of body weight/day) for three weeks.

The administration of the drugs was done between 7.00 pm and 8.00 pm everyday. After the experimental period (three weeks), all animals from all groups were first tested for anxiety using elevated plus maze (EPM), followed by passive avoidance behavior using passive avoidance apparatus. The path of each rat during the EPM test was recorded by a video camera (*Sony color camera and F1.2 Lens, 0.7 Lux*) fixed at the ceiling of the room where the experiment was conducted. The data were analyzed by using a computerized system (*Panlab Smart Version 2.5 Software, Barcelona, Spain*).

Behavioral tests: Behavioral tests were conducted in a separate large room between 7.00 pm and 11.00 pm. Two behavioral paradigms, such as elevated plus maze (EPM) and passive avoidance were studied.

Elevated plus maze test:

Apparatus: Elevated plus maze (EPM) apparatus was made of black plexiglass with two closed and two open arms (50 x 10 cm) from the open center (5 x 5 cm) in plus shape. Closed arms were surrounded by high walls (40 x 10 cm) and the whole apparatus was raised to a height of 50 cm above the floor.

Elevated plus maze test: EPM test was carried out in a sound-attenuated, temperature-controlled room, illuminated by a 40 W white light bulb. The observer stayed in the same room, one meter away from the maze. At the start of each trial, each animal was placed on the central platform facing the closed arm. Over a period of 5 min, the time spent in the open arm was recorded. The criterion for an arm visit was considered only when the rat decisively moved all its four limbs into the arms. The maze was cleaned using 70 % ethanol after each trial. The number of defecation pellets was also noted. All events were recorded by a video camera.

Passive avoidance test: Passive avoidance test was conducted by the method of Bures et al (7) with modifications.

Apparatus: The apparatus had two compartments. The larger compartment was rectangular, with a 50 x 50 cm grid floor and wooden walls of 35 cm height. It had a roof, which could be opened or closed. One of the walls had a 6 x 6 cm opening connecting the larger compartment with a dark smaller compartment. The smaller compartment had a 15 x 15 cm electrifiable grid connected to a constant current stimulator, wooden walls of 15 cm in height and a ceiling, which could be opened or closed. The connection between the two compartments could be closed with a sliding door made of plexiglass. The larger compartment was illuminated with a 100 W bulb placed 150 cm above the center.

Passive avoidance test: The experiment had three parts (1) Exploration test (2), Aversive stimulation and learning test, and (3) Memory retention test.

Exploration test: This test had three trials. During the test, each rat was kept in the center of the larger compartment facing the dark compartment away from the entrance. The door between the two compartments was kept open. The rat was allowed to explore the apparatus (both larger and smaller compartments) for 3 minutes. In each trial, the total time taken by the animal to enter the dark compartment was noted using a stopwatch. At the end of the trial, the rat was replaced to its homecage, where it remained during the inter-trial interval, i.e. for five minutes.

An aversive stimulation and learning test: After the last exploration trial, the rat was again kept in the center of the larger compartment as in the trial sessions. When the animal entered the smaller compartment, the sliding door between the two compartments of the apparatus was closed and three-foot shocks (50 Hz, 1.5 mA, and 1 sec duration) were given at 5-second intervals. The ceiling was then opened and the rat was returned to its homecage.

Memory retention test: The retention test was carried out after 24 and 48 hours. The rat was kept in the center of the larger compartment facing the smaller compartment away from the entrance for a maximum period of three minutes. The sliding door was kept open during this period. The latency time required for the animal to enter the dark compartment was recorded. The latency time was recorded as 3 minutes for animals that did not enter the dark compartment within 3 minutes. The absence of entry into the dark compartment indicated positive memory retention.

Statistical analysis: All results were expressed as mean ± standard error of mean (S.E.M.). The significance of differences

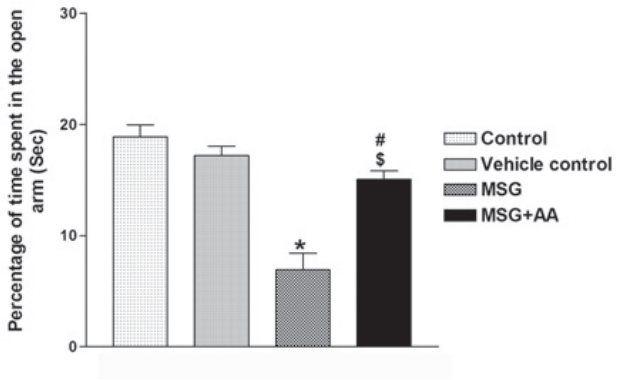

Fig. 1. Elevated plus-maze open arm exploration time: Rats treated with MSG (2 mg/g b.w) alone spent significantly less time in the open arm (Control/Vehicle-control vs MSG, * p<0.001, One-way ANOVA; Tukey's test). Simultaneous treatment with ascorbic acid (100 mg/kg b.w) and monosodium glutamate significantly increased the open arm exploration time in rats when compared with the group treated with MSG alone but not with control/vehicle-control (MSG vs MSG+AA; $ p<0.001, MSG vs Control/Vehicle-control; # p>0.05, One--way ANOVA, Tukey's test). The results are expressed as Mean±SEM.

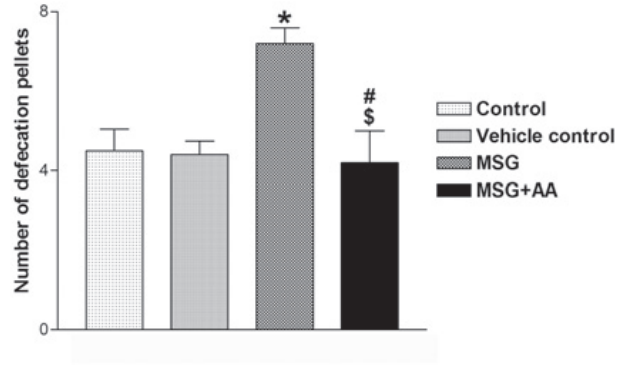

Fig. 2. Number of defecation pellets during EPM test: Greater number of defecation pellets was found with MSG-treated group (2 mg/g b.w) (Control/Vehicle-control vs MSG, * p<0.01 One-way ANOVA; Tukey's test) while the treatment with ascorbic acid along with MSG significantly decreased the anxiety-induced defecation in animals (MSG vs MSG+AA, $ p<0.01, MSG vs Control/Vehicle-control; # p>0.05, One--way ANOVA, Tukey's test). The results are expressed as Mean±SEM.

among the groups was assessed using one-way analysis of variance (ANOVA) test followed by Post-hoc Tukey test. *P* values <0.05 were considered as significant.

Results

Elevated plus maze test: Monosodium glutamate (2 mg/g b.w.) produced a substantial decrease in the time spent in the open arms, compared to rats in the control and vehicle-control groups (Fig. 1). The significance of these differences was borne out by One-way ANOVA and Tukey tests. This showed that the percentage of open-arm time displayed by the MSG group was significantly smaller (~ 9 % of the total time) than by either the control or the vehicle-control groups (Control/Vehicle-control

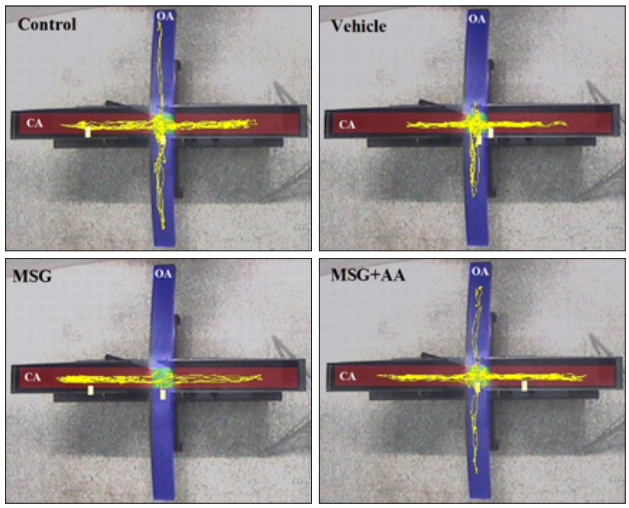

Fig. 3. Video tracking of the path taken by representative animals from each group during the EPM test. When compared with controls/vehicle-controls, the open arm exploration time was shorter in monosodium glutamate (MSG)-administered animals. Rats simultaneously treated with ascorbic acid and MSG (MSG+AA) spent more time in the open arm like control animals, ie open arm exploration time increased in these animals. CA; closed arm, OA; open arm.

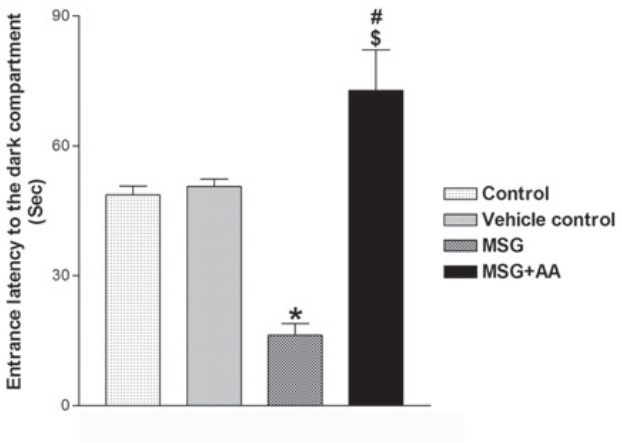

Fig. 4. Mean entrance latency to the dark compartment of the passive avoidance apparatus of animals tested 24 hours after the shock trial. Mean latency to enter the dark compartment was significantly less in MSG administered group (Control/vehicle-control vs MSG, 24 hrs; * p<0.001, One-way ANOVA; Tukey's test), while it was significantly higher in the group treated with ascorbic acid along with MSG in comparison to the group treated with MSG alone and controls/vehicle controls (MSG vs MSG+AA; $ p<0.001, Control/Vehicle-controls Vs MSG+AA; # p<0.05, One-way ANOVA; Tukey's test). Results are expressed as Mean±SEM.

vs MSG, p<0.001). The rats administered with ascorbic acid (100 mg/kg b.w) along with MSG showed a substantially longer open arm exploration time (~ 16 % of the total time) when compared with MSG group but not with the control/vehicle-controls (MSG vs MSG +AA; p<0.001, MSG vs Control/Vehicle-control; p>0.05, One-way ANOVA, Tukey's test). Furthermore, when compared with controls and vehicle-controls, the number of defecation pellets (Fig. 2) was found to be higher with MSG-ad-

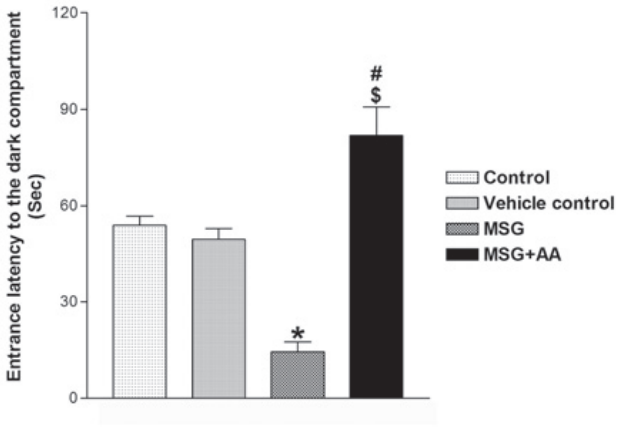

Fig. 5. Mean entrance latency to the dark compartment of animals tested 48 hours after the shock trial. Mean latency to enter the dark compartment was significantly lower in MSG-administered group 48 hrs after the shock trial (Control/Vehicle-control vs MSG, 48 hrs; * p<0.001, One-way ANOVA; Tukey's test). The memory retention was significantly higher in the group treated with ascorbic acid along with MSG in comparison to the group treated with MSG alone and controls/vehicle controls (MSG vs MSG+AA, $ p<0.001, Control/ Vehicle-control Vs MSG+AA; # p<0.01, One-way ANOVA; Tukey's test). Results are expressed as Mean±SEM.

ministered (2 mg/g b.w.) group (Control/Vehicle-control vs MSG, p<0.01 One-way ANOVA, Tukey's test). The treatment with ascorbic acid along with MSG significantly decreased the anxiety-induced defecation in animals (MSG vs MSG+AA, p<0.01, MSG vs Control/Vehicle-control; p>0.05, One-way ANOVA, Tukey's test) (Fig. 3).

Passive avoidance test: Performance of MSG-administered rats (2 mg/g b.w) in the memory retention test (both 24 and 48 hours after the shock trial) was impaired when compared to the controls and vehicle-controls (Figs 4 and 5). There was a significant difference between the groups (Control/vehicle-control vs MSG, 24 hrs; p<0.001, 48 hrs; p<0.001, One-way ANOVA; Tukey's test). The rats treated with ascorbic acid (100 mg/kg b.w) along with MSG showed an increase in the retention (both 24 and 48 hours after the shock trial) of the passive avoidance response than MSG group (MSG vs MSG+AA, 24 hrs; p<0.001, 48 hrs; p<0.001, One-way ANOVA; Tukey's test). Furthermore, the memory retention of the MSG+AA group was higher than in controls and vehicle-controls, and this difference was statistically significant (Control/vehicle-control vs MSG+AA, 24 hrs; p<0.05, 48 hrs; p<0.01, One-way ANOVA; Tukey's test).

Discussion

Glutamate is known to be neurotoxic in high concentrations and contributes to the development of certain neurodegenerative diseases. The excitotoxic effect of glutamate is mediated by an interaction with NMDA receptors. It leads to an uncontrollable rise in intracellular calcium concentrations and this raised intracellular Ca^{2+} activates various enzymes contributing to cell death by various mechanisms. Recent reports suggest that necrosis and apoptosis in cultured HT22 cells (an immortalized mouse hippocampal cell line) induced by glutamate are time-dependent. The glutamate-induced apoptosis in these cells is AIF-dependent but caspase-independent, accompanied by DNA ladder formation but not by chromatin condensation (8).

We observed a decrease in the open arm exploration time in MSG-treated rats during the EPM test. The open arm entries were also fewer in these animals. The MSG-treated rats showed an anxiety-like behavior when compared with controls/vehicle control rats during EPM test. Elevated plus maze (EPM) is used as a behavioral assay to study the brain sites such as limbic regions, hippocampus, amygdala, dorsal raphe nucleus, etc (9, 10). It is also used to study the role of various mechanisms such as effects of GABA and glutamate on hypothalamic-pituitary-adrenal axis neuromodulators in underlying anxiety behavior (11, 12). The altered neurobehavioral performance of rats on EPM could be addressed with respect to the modulatory role of MSG on hypothalamic areas. Reports suggest that the activation of the rostrocaudal axis of the arcuate nucleus in animals leads to their behavioral reactions such as anxiety-like behavior in EPM test (13). In one of the previous studies, the Fos protein immunohistochemistry technique was used to map the brain areas activated by a 15-minute exposure of rats to the elevated plus maze. This study revealed the involvement of piriform and entorhinal cortices, amygdala, midline thalamic nuclei, several medial hypothalamic nuclei, periaqueductal gray matter, superior and inferior colliculi, cuneiform nucleus, dorsal raphe nucleus and locus coeruleus in anxiety-like disorders (10).

Furthermore, passive avoidance behavior was also affected in MSG-treated rats. The passive avoidance tests or conditioned avoidance tests have been used in several studies to assess memory or retention as well as retrieval after or during various treatments (14, 15). In the present study, the rats trained in the passive avoidance apparatus for three times could not remember the noxious event 24 hours after the shock trial. The memory retention further reduced during 48 hours after the shock trial. The emotional memory developed over a number of trials was significantly altered by the administration of MSG (2 mg/g b.w) in rats.

The adverse effect of MSG on memory formation and its retrieval has been well documented in neonatal animals (1, 2, 3) but reports on young animals (those comparable with human young children) are scanty. There is considerable evidence to suggest that the altered passive avoidance behavior of MSG-treated rats is due to the effect of the excess of MSG on hypothalamic neurons (16). The role of hypothalamic neurons in emotional and memory functions of the hippocampus has been documented. Theta rhythm of hippocampus is important for learning and memory, and medial supramammillary nucleus (mSuM) of hypothalamus is involved in the control of the frequency of this rhythm. According to Shahidi et al, the supramammillary nucleus (SuM) contributes to passive avoidance (PA) consolidation at least 5 min after the acquisition trial, and this effect may be accomplished through SuM projections to the septal and/or hippocampal areas participating in PA memorization processes (17).

The role of MSG in modulating the normal functioning of these brain structures needs to be addressed. Excitotoxins act on brain in different ways. Excitotoxic lesions of the lateral hypothalamus have shown to produce neuronal loss, blood-brain barrier disruption, triggering of reactive gliosis, demyelination over an area coexistent with but not exceeding the area of neuronal loss, and remyelination (18, 19). Hence, we cannot exclude the possibility of high concentrations of glutamate on disruption of the blood-brain barrier (BBB), and there by causing the altered brain functions in the current study. Monosodium glutamate also affects the brain by increasing the free radical generation in the body. Reports suggest that high dietary intake of MSG induces oxidative stress in the brain, kidney, and liver of experimental animals (20, 21). In the current study also this oxidative stress might have contributed to the poor memory performance of MSG-treated rats during the passive avoidance task.

Ascorbic acid or vitamin C is a widely studied yet least understood of all vitamins. More than eighty years have passed since its discovery but till now, its precise biological function has remained an enigma. Ascorbic acid contributes to the synthesis of the amino acid carnitine and catecholamines regulating the nervous system. It is also necessary for the conversion of tryptophan to 5-hydroxy tryptophan and neurotransmitter serotonin as well as for the formation of neurotransmitter, namely nor-epinephrine from dopamine. Parle et al have reported that ascorbic acid (60, 120 mg/kg) injected for 3 and 8 consecutive days improved learning and memory of aged mice as indicated by decreased transfer-latency and increased step-down latency, and it also provided protection to the young animals from scopolamine and diazepam-induced impairment of memory (6). According to our observation, the ascorbic acid (100 mg/kg b.w.) significantly prevented the neurobehavioral changes induced by MSG (2 mg/g b.w). The underlying mechanism of action of ascorbic acid as a memory enhancer in our study might be attributed to its antioxidant property. Recent reports also suggest that MSG exposure induces oxidative damage in rat brain, kidney and liver, and this was completely ameliorated by the administration of dietary antioxidants such as vitamin C, vitamin E and quercetin. Moreover, vitamin C and quercetin have shown a greater ability to protect the brain from membrane damage than vitamin E (21). Recent data suggest that in the brain, ascorbic acid participates as a metabolic switch modulator in neuronal metabolism between rest and activation periods, helping the neuron to utilize lactate by stimulating the lactate transport instead of glucose (22). As an antioxidant, ascorbic acid can rejuvenate vitamin E, indirectly contributing to the fight against free radical damage in lipids, and these nutrients can be effective partners in reducing the destructive process of lipid peroxidation (23). Combined intake of vitamins C and E for at least ten years helps to maintain better cognitive functions in women in their 70‘s (24).

MSG, when administered intraperitoneally (4 mg/g of body weight), for six consecutive days significantly decreased the cell viability with significant down-regulation of Bcl-2 protein, while Bax protein expression was not significantly changed in rat thymocytes. The treatment with vitamin C was effective in ameliorating the above effect of MSG in rat thymocytes by increasing the proportion of viable cells and up-regulating the expression of Bcl-2 protein in rat thymocytes (4). Daily subcutaneous administration of monosodium glutamate (MSG) to adult male mice for 6 days at dose levels of 4 and 8 mg/g body weight induced oxidative stress in hepatic microsomes, and the attempt to maintain the redox state of the cells was increased by ascorbic acid content and the activities of glutathione-dependent enzymes (20). Another study also supports the vital role of ascorbic acid in brain functions. In knockout mice that cannot synthesize ascorbic acid, low levels of ascorbic acid significantly elevated the oxidative stress as well as produced motor deficits (25). These reports point to the fact that under oxidative stress, the ascorbic acid content in the cell itself is increased as an attempt to maintain the redox state of the cells. Therefore, in the current experiment, the additional administration of ascorbic acid would have further protected the brain tissue from the deleterious effect of oxidative damage due to the excessive concentration of glutamate.

There are many questions, which remain unanswered regarding the effect of monosodium glutamate on brain functions. Further studies are warranted to clearly conclude:

(1) The altered neurobehavioral performance (of rats 4–6 weeks old) is mediated via the excitotoxic effect of excessive concentration of glutamate on brain areas (hypothalamic areas) directly;

(2) Excitotoxicity disrupts the integrity of the blood-brain barrier and thereby alters the brain functions;

or (3) High concentration of MSG itself in animals causes oxidative stress, which in turn leads to the alterations in the functioning of various brain areas involved in anxiety, emotional learning and memory, and thereby causes neurobehavioral changes.

Whatever may be the underlying mechanism causing the behavioral changes in rats, it was significantly prevented by the administration of ascorbic acid.

Furthermore, during the passive avoidance task, the group treated simultaneously with ascorbic acid MSG performed better than the controls/vehicle controls, and this difference was statistically significant. The interaction between the ascorbic acid and glutamate in the brain is complex, and this effect could not be properly explained with our preliminary results.

Conclusion

The results of the current study clearly indicated that periadolescent (age in range of 4–6 weeks) treatment with monosodium glutamate significantly altered the neurobehavioral performance in rats. These behavioral changes were significantly prevented by simultaneous administration of ascorbic acid with MSG. This further proved the neuroprotective role of ascorbic acid and its potential role as a therapeutic agent in various cognitive deficits.

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