

EXPERIMENTAL STUDY

Ascorbic acid modulates monosodium glutamate induced cytotoxicity in rat thymus

Pavlovic V¹, Pavlovic D², Kocic G², Sokolovic D², Sarac M³, Jovic Z⁴

Institute of Physiology, Medical Faculty University of Nis, Nis, Serbia. vojapav@yahoo.com

Abstract: *Background:* Monosodium glutamate (MSG) is a commonly used flavor enhancer in modern nutrition. It has been shown that administration of MSG induces toxic effects in various regions of brain, thymus, liver and kidney. Also, it is well-documented that Vitamin C (ascorbic acid) has a protective role in MSG-induced cytotoxicity in rat liver, kidney and various brain regions, but has not been studied in thymus.

Objectives: In the present study, we examined the possible protective role of Vitamin C in MSG-induced cytotoxicity in adult (Kindly indicate the strain of rat) rat thymus.

Material and methods: MSG was administrated intraperitoneally (4 mg/g of body weight), with or without Vitamin C (500 mg/kg of body weight), for six consecutive days. Animals were sacrificed at 1st, 7th and 14th day of last MSG dose.

Results: This study demonstrates that MSG administration in animals significantly decreases cell viability with significant down-regulation of Bcl-2 protein, while Bax protein expression was not significantly changed in rat thymocytes. Vitamin C was effective in ameliorating the 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.

Conclusion: These results suggest that the treatment with Vitamin C may prevent the MSG-induced cytotoxicity in rat thymocytes by up-regulating Bcl-2 protein expression resulting in a change in Bcl-2/Bax protein ratio (*Tab. 1, Fig. 1, Ref. 32*). Full Text (Free, PDF) www.bmj.sk.

Key words: vitamin C, monosodium glutamate, thymus, Bcl-2, Bax.

Monosodium glutamate (MSG), the sodium salt of glutamic acid (GA), is one of the most frequently applied food additive in the developed world. Modern nutrition enables a continuous intake of this flavor enhancer, with resulting rise and accumulation of GA in blood (1). This amino acid acts at multiple receptor types, divided into two main groups: ionotropic glutamate receptors (iGluR) and metabotropic (mGluR) glutamate receptors (2). In addition to the CNS, glutamate receptors (GluRs) are also found on various non-neuronal cells. GluRs were found in human lymphocytes (3), mouse (4), and rat thymocytes (5), suggesting their role in immune system. However, several studies showed toxic effects of MSG in various regions of central nervous system (6), thymus (7), liver and kidney (8), mainly by

generation of ROS and resulting oxidative stress. Toxic effect of MSG in human adults, as manifested by the Chinese restaurant syndrome, is also documented (9).

The immune system is highly dependent on adequate cell-cell communication, and any damage to the signaling systems (oxidative stress), will lead to an impairment in immune responsiveness. Vitamin C (ascorbic acid) is an essential water-soluble nutrient that primarily exerts its effect on host defense mechanisms and immune homeostasis by being the most important physiological antioxidant (10). It is present in extracellular fluid and the cytosolic compartment of cells and exerts several diverse effects on the immune system. Vitamin C increases the neutrophilic motility (11) and phagocytic functions (12) in human. Such macrophage functions as chemotaxis, phagocytosis, and superoxide anion production in mice are enhanced by several antioxidants, including Vitamin C (13). The administration of Vitamin C normalized the monocyte function in strong smokers, as was reviewed earlier (14). Increased proliferation of T cells (15), and inhibition of various forms of T cell death (16) and Fas-induced apoptosis of monocytes (17) by Vitamin C have also been reported. The increased cytotoxic activity of natural killer cells in humans is another example of Vitamin C supplementation effects (18). In line with previous results, the recent study showed a protective role of Vitamin C on MSG-induced cytotoxicity in rat liver, brain and kidney (8), but it has not been studied in thymus.

¹Institute of Physiology, Medical Faculty University of Nis, Serbia, ²Institute of Biochemistry, Medical Faculty University of Nis, Serbia, ³Medical Faculty University of Nis, Serbia, and ⁴Institute of Pharmacology, Medical Faculty University of Nis, Serbia

Address for correspondence: V. Pavlovic, MD, PhD, Inst of Physiology, Medical Faculty University of Nis, Bulevar dr Zorana Djindjica, 18000, Nis, Serbia.
Phone: +381.18276736

Temporally address: Institute for Microbiology and Immunology, Sahlgrenska Academy, University of Goteborg, Medicinaregatan 7A, Box 435 S-40530, Gothenburg, Sweden.

Acknowledgements: This work was supported by the Ministry of Science and Environmental Protection of Serbia (Project 145081).

Since Vitamin C may be present in diets and meals commonly consumed by humans, the next study was design to evaluate the possible protective role of this antioxidant on MSG-induced cytotoxicity in rat thymus.

Materials and methods

Animals

Experiments were performed on adult male Wistar rats (120–140 g), 8–10 weeks old, bred at the Vivarium of the Institute of Biomedical Research, Medical Faculty, Nis, under conventional laboratory conditions. The experimental animals were treated in accordance with national animal protection guidelines.

Materials

Culture medium (CM) was prepared using RPMI 1640 (Sigma, St Louis, Mo., USA), according to the manufacturer instructions. CM containing 25 mM HEPES, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 ug/ml) and 10% fetal calf serum (FCS).

Monosodium glutamate (MSG) was obtained from Fluka Chemika AG, Buchs, Switzerland.

Vitamin C (L-ascorbic acid) was purchased from Galenika a.d., Belgrade, Serbia.

Saponin-based permeabilization reagent, IntraPrept, was obtained from Immunotech, Marseille, France.

The following monoclonal antibodies were purchased from Immunotech (Marseille, France): Mouse anti-rat Bcl-2 (clone 5D4) and Goat F (ab)₂ phycoerythrin (PE)-conjugated anti-mouse IgG (H+L). Mouse anti-rat Bax (clone 6A7) monoclonal antibody was obtained from Sigma, St Louis, Mo., USA.

Monosodium glutamate animal treatment

Experimental animals were treated intraperitoneally, with 500 mg/kg of body weight Vitamin C (19), 4 mg/g of body weight MSG (20) in 1 ml physiologic saline and MSG only (4 mg/g of body weight) in 1 ml physiologic saline, for 6 consecutive days, regarding to our previous studies and continuous evaluation of MSG effects (7). Their respective controls (control animals) were treated with only 1ml of physiologic saline, for 6 consecutive days. Animals (experimental and control) were sacrificed (using ether anesthesia) at 1st, 7th and 14th day after last MSG dose.

Preparation of thymocytes

Thymocytes were prepared as described previously (7). Briefly, each thymus was extirpated using sterile technique and placed in cold CM containing 10 % FCS. The thymocytes were released by sliding the thymus along a steel-mesh. Cell suspensions were filtered through a sterile nylon-filter to remove stroma and then the cells were washed twice with cold CM containing 10 % FCS.

Determination of cell viability

At various time points (1st, 7th and 14th day after last MSG dose), after thymocyte isolation, cell viability was determined

Tab. 1. The effect of MSG and vitamin C on rat thymocytes viability.

Group	Viability (trypan blue exclusion test)		
	MSG treatment	MSG+VitC treatment	Control
D1	55.2±5.65*	72.3±4.56••	
D7	43.4±7.37**	56.23±5.61•	65.76±5.21
D14	36.23±4.81***	51.43±5.17•	

Rat thymocytes were isolated at 1st, and 14th day after MSG administration and cell viability was determined by using the trypan blue dye exclusion method, as described in Material and methods. Results are given as mean percentage ± SD of triplicate samples of one representative experiment (out of three with similar results). Abbreviations: D1 – animals sacrificed at 1st day after glutamate administration, D7 – animals sacrificed at 7th day after glutamate administration, D14 – animals sacrificed at 14th day after glutamate administration. MSG – animals treated only with MSG. MSG+VitC – animals treated simultaneously with MSG and vitamin C. *** p<0.001 compared to control (non-treated) animals. • p<0.05 compared to MSG treated animals.

by using the trypan blue dye exclusion method. Results were presented as the percentages of viable cells.

Flow cytometric evaluation of Bcl-2 and Bax levels

The levels Bcl-2 and Bax were measured by flow cytometry, as described previously (7), by using retrospective monoclonal antibodies. Non-specific binding was detected by the control cells, which were incubated with the secondary antibody (PE-conjugated anti-mouse IgG) alone. Labeled cells were analyzed (5000 analyzed cells/per sample) using EpicserXL flow cytometer (Coulter, Krefeld, Germany).

Statistical analysis

The results are presented as the mean ± SD. Significant differences between groups were evaluated using Student's t-test.

Results

MSG enhances the viability of rat thymocytes

The cytotoxic effect of MSG on rat thymocytes, was evaluated at various time points (1st, 7th and 14th day after last MSG dose), by using trypan blue dye exclusion method. The obtained results, presented in Table 1, show that MSG administration to animals significantly decreased cell viability, during examination period, in a time-dependent manner. As shown in Table 1, the maximal increased cytotoxicity (p<0.01), during examination period, was detected at 14th day after MSG administration.

Effect of MSG on Bcl-2 and Bax protein expression

Taking into account the different intracellular signal transduction pathways which might be involved in T-cell death, in next experiments we studied the influence of MSG on Bcl-2 and Bax protein expression in rat thymocytes, by using flow cytometric method. As shown in Figure 1, MSG administration to animals induced significant down-regulation of Bcl-2 protein (p<0.05), during whole examination period, in rat thymocytes. On the other hand, no significant changes in the expression of Bax protein were detected during examination period (data not shown).

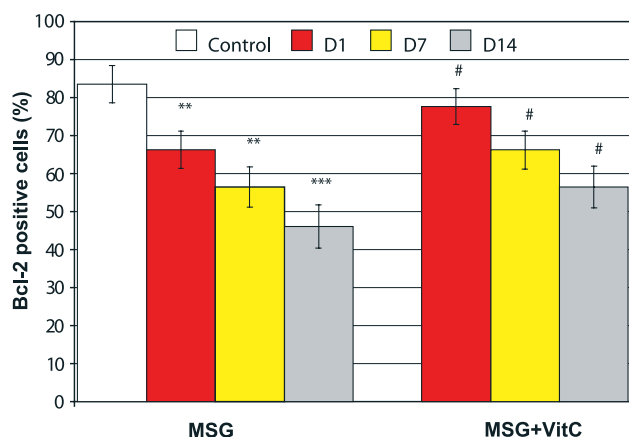


Fig. 1. The effect of monosodium glutamate (MSG) and vitamin C on Bcl-2 protein expression in rat thymocytes. Thymocytes were incubated with anti-Bcl-2 monoclonal antibody, as described in Material and methods. Percentages of positive cells were determined by flow cytometric analysis. Results are given as mean percentage \pm SD of triplicate samples of one representative experiment (out of two with similar results). Abbreviation as in Table 1.

Vitamin C decreases cytotoxicity and up-regulates the expression of Bcl-2 in rat thymocytes

Further, we investigated the possible protective role of Vitamin C on MSG-induced cytotoxicity in rat thymocytes. We showed that administration of Vitamin C to the animals significantly reduced the MSG-induced cytotoxicity of rat thymocytes, during whole examination period. The maximal increase in cell viability ($p < 0.01$) was observed at 1st day, after last MSG dose (Tab. 1).

To shed some light on molecular effects of ascorbic acid on T cells, we next studied the modulatory effect of Vitamin C on Bcl-2 and Bax expression in MSG treated rats. Treatment of rats with Vitamin C induced significant increase in Bcl-2 protein ($p < 0.05$), during the whole examination period, in rat thymocytes. The maximal up-regulation, of Bcl-2 protein, was observed at 1st day after the last MSG administration (Fig. 1). However, application of Vitamin C together with MSG was not able to modulate the expression of Bax protein in rat thymocytes, during the whole examination period (data not shown).

Discussion

In the present study, we investigated the ability of MSG to induce toxicity in rat thymocytes. We also examined the modulatory effect of MSG on Bcl-2 and Bax protein expression in rat thymocytes. Furthermore, we tested the possibility of Vitamin C to prevent the MSG-induced cytotoxicity in rat thymocytes. The present study demonstrated the protective effect of Vitamin C in rat thymocytes treated with MSG by modulation the Bcl-2 protein expression in rat thymocytes.

Results presented in our study, demonstrated that MSG administration to animals significantly increased cytotoxicity during examination period, in a time dependent manner, as deter-

mined by trypan blue exclusion test. Further, flow cytometric results showed that MSG administration to animals induced significant down-regulation of Bcl-2 protein expression, during whole examination period, with resulting change of Bcl-2/Bax protein ratio in rat thymocytes. Recent results support previous findings that treatment with MSG induced thymocytes apoptosis and down regulation of Bcl-2 protein expression in vivo (7, 21), as well in vitro conditions (22) suggesting that the Bcl-2/Bax ratio rather than the Bax level is the important determinant for the induction of cell death in thymocytes by MSG.

To investigate the hypothesis that ascorbic acid modulates the MSG-induced toxicity in rat thymocytes, the animals were treated with ascorbic acid and MSG simultaneously during 6 consecutive days. The obtained data showed that the application of ascorbic acid significantly decreased the toxicity in rat thymocytes during the whole examination period. These findings confirm earlier investigations, which showed that after MSG treatment, ascorbic acid prevented liver, kidney and brain cytotoxicity (8), and here we demonstrated the protective role of ascorbic acid in thymocytes cytotoxicity. It is well-known that ascorbic acid is a component of the first line of antioxidant defense against oxidative processes. Ascorbic acid is involved in the antioxidant defense of cells and participates in free radical scavenging as an antioxidant, as well as plays an important role in biosynthetic processes, such as the synthesis of collagen, catecholamines or leukotrienes (23). The antioxidant ascorbic acid has beneficial effects on the function of immune cells such as T lymphocytes (24), polymorphonuclear leukocytes and macrophages (13). Ascorbic acid has also been shown to enhance antioxidant defenses of T cells as well as to increase T-cell responsiveness to antigens, suggesting a role in regulating the immune function (25). Therefore, a decrease in the intracellular content of these antioxidants may result in local immunosuppression (26). It has been demonstrated that Vitamin C could modulate the immune system by inhibiting T-cell apoptosis signaling pathways (16). The intracellular antioxidant-oxidant balance is critical for immune cell functions because it maintains the integrity of cellular components and their function. Immune cells are particularly sensitive to oxidative stress because of the high content of polyunsaturated fatty acids in their plasma membranes and a high production of ROS, which is part of their normal function (27). These findings may suggest that an imbalance between pro-oxidant-antioxidant systems in thymus, after MSG treatment, with resulting impaired immune function, can be restored by ascorbic acid. Previous findings are supported by our recent report, which indicated that MSG administration to animals induced the significant oxidative stress and apoptosis in rat thymus (21) as well as by a study which indicated high sensitivity of thymocytes to oxidative stress (28).

In an attempt to gain insight into the molecular effects of ascorbic acid, we studied the influence of this antioxidant on the expression of Bcl-2 and Bax protein in rat thymocytes. Our results indicate that ascorbic acid significantly up-regulated the expression of Bcl-2 protein in rat thymocytes after MSG treatment with resulting changes in Bcl-2/Bax protein ratio. These

results correlate with increased cell viability in rat thymus after ascorbic acid treatment, suggesting that ascorbic acid prevents MSG-induced cytotoxicity by increasing the Bcl-2 protein expression in rat thymocytes. These observations are in accordance with the findings that ascorbic acid in various cell types, prevents apoptosis by upregulating the Bcl-2 protein expression (29). Intracellular located Bcl-2 is essential to provide antioxidant protection against apoptosis (30), and it has been demonstrated to prevent the release of the redox compound of cytochrome c from mitochondria (31). However, ascorbic acid can provide a way to protect by antioxidant function at the plasma membrane, independent of Bcl-2 that is not located in this membrane, and can act at a very early phase of apoptosis initiation (32). Thus, overexpression of Bcl-2 may allow cells to cope better with the effects of ROS, possibly by allowing increases in endogenous antioxidant enzymes. By detoxifying ROS, antioxidants may therefore reverse the ROS-induced decline in Bcl-2 and prevent the cellular death (30).

In summary, we have confirmed the previous data that prolonged the effect of MSG administration to animals resulted in increased cytotoxicity in rat thymus with significant down-regulation of Bcl-2 protein in rat thymocytes. Food substances rich in antioxidants, such as ascorbic acid, may significantly modulate the cytotoxicity induced by MSG administration, namely by increasing the expression of Bcl-2 protein in rat thymocytes resulting in a change in Bcl-2/Bax protein ratio.

References

1. Walker R, Lupien JR. The safety evaluation of monosodium glutamate. *J Nutr* 2000; 130: 1049S–1052S.
2. Hinoi E, Takarada T, Ueshima T, Tsuchihashi Y, Yoneda Y. Glutamate signaling in peripheral tissues. *Eur J Biochem* 2004; 271: 1–13.
3. Lombardi G, Miglio G, Dianzani C et al. Characterization of ionotropic glutamate receptors in human lymphocytes. *Brit J Pharmacol* 2001; 133: 936–944.
4. Storto M, de Grazia U, Battaglia G et al. Expression of metabotropic glutamate receptors in murine thymocytes and thymic stromal cells. *J Neuroimmunol* 2000; 109: 112–120.
5. Rezzani R, Corsetti G, Rodella L, Angoscini P, Lonati C, Bianchi R. Cyclosporine-A treatment inhibits the expression of metabotropic glutamate receptors in rat thymus. *Acta Histochem* 2003; 105: 81–87.
6. Park CH, Choi SH, Piao Y et al. Glutamate and aspartate impair memory retention and damage hypothalamic neurons in adult mice. *Toxicol Lett* 2000; 115: 117–125.
7. Pavlovic V, Cekic S, Sokolovic D, Djindjic B. Modulatory effect of monosodium glutamate on rat thymocyte proliferation and apoptosis. *Bratisl Lek Listy* 2006; 107: 185–191.
8. Farombi EO, Onyema OO. Monosodium glutamate-induced oxidative damage and genotoxicity in the rat: modulatory role of vitamin C, vitamin E and quercetin. *Hum Exp Toxicol* 2006; 25: 251–259.
9. Geha RS, Beiser A, Ren C et al. Review of allergic reaction to monosodium glutamate and outcome of a multicenter double blind placebo-controlled study. *J Nutr* 2000; 130: 1032–1038.
10. Hartel C, Strunk T, Bucsky P, Schultz C. Effects of vitamin C on intracytoplasmic cytokine production in human whole blood monocytes and lymphocytes. *Cytokine* 2004; 24: 101–106.
11. De la Fuente M, Ferrández MD, Burgos MS, Soler A, Prieto A, Miquel J. Immune function in aged women is improved by ingestion of vitamins C and E. *Can J Physiol Pharmacol* 1998; 76: 373–380.
12. Bergman M, Salman H, Djaldetti M, Fish L, Punskey I, Bessler H. In vitro immune response of human peripheral blood cells to vitamins C and E. *J Nutr Biochem* 2004; 15: 45–50.
13. Del Rio M, Ruedas G, Medina S, Victor VM, De la Fuente M. Improvement by several antioxidants of macrophage function *in vitro*. *Life Sci* 1998; 63: 871–881.
14. Ginter E. Chronic vitamin C deficiency increases the risk of cardiovascular diseases. *Bratisl Lek Listy* 2007; 108: 417–421.
15. Noh K, Lim H, Moon S et al. Mega-dose Vitamin C modulates T cell functions in Balb/c mice only when administered during T cell activation. *Immunol Lett* 2005; 98: 63–72.
16. Campbell JD, Cole M, Bunditrutavorn B, Vella AT. Ascorbic acid is a potent inhibitor of various forms of T cell apoptosis. *Cell Immunol* 1999; 194: 1–5.
17. Perez-Cruz I, Carcamo JM, Golde DW. Vitamin C inhibits FAS-induced apoptosis in monocytes and U937 cells. *Blood* 2003; 102: 336–343.
18. Vojdani A, Bazargan M, Vojdani E, Wright J. New evidence for antioxidant properties of Vitamin C. *Cancer Detect Prev* 2000; 24: 508–523.
19. Mahfouz MM, Kummerow FA. Vitamin C or Vitamin B6 supplementation prevent the oxidative stress and decrease of prostacyclin generation in homocysteinemic rats. *Int J Biochem Cell Biol* 2004; 35: 1919–1932.
20. Ortiz GG, Bitzer-Quintero OK, Beas Zárate C et al. Monosodium glutamate-induced damage in liver and kidney: a morphological and biochemical approach. *Biomed Pharmacother* 2006; 60: 86–91.
21. Pavlovic V, Pavlovic D, Kocic G et al. Effect of monosodium glutamate on oxidative stress and apoptosis in rat thymus. *Mol Cell Biochem* 2007; 303: 161–166.
22. Pavlovic V, Cekic S, Kocic G, Sokolovic D, Zivkovic V. Effect of Monosodium glutamate on apoptosis and Bcl-2/Bax protein level in rat thymocyte culture. *Physiol Res* 2007; 56: 619–626.
23. Linster CL, Van Schaftingen E. Vitamin C, biosynthesis, recycling and degradation in mammals. *FEBS J* 2007; 274: 1–22.
24. Mortola E, Okuda M, Ohno K, Watari T, Tsujimoto H, Hasegawa A. Inhibition of apoptosis and virus replication in feline immunodeficiency virus-infected cells by N-acetylcysteine and ascorbic acid. *J Vet Med Sci* 1998; 60: 1187–1193.
25. Wu CC, Doriarajan T, Lin TL. Effect of ascorbic acid supplementation on the immune response of chickens vaccinated and challenged with infectious bursal disease virus. *Vet Immunol Immunopathol* 2000; 74: 145–152.
26. Carbonell LF, Nadal JA, Llanos C, Hernindez I, Nava E, Diaz J. Depletion of liver glutathione potentiates the oxidative stress and decreases nitric oxide synthesis in a rat endotoxin shock model. *Crit Care Med* 2000; 28: 2002–2006.

27. **Victor VM, Guayerbas N, De la Fuente M.** Changes in the antioxidant content of mononuclear leukocytes from mice with endotoxin-induced oxidative stress. *Mol Cell Biochem* 2002; 229: 107—111.
28. **Aulwurm UR, Brand KA.** Increased formation of reactive oxygen species due to glucose depletion in primary cultures of rat thymocytes inhibits proliferation. *Eur J Biochem* 2000; 267: 5693—5698.
29. **Saitoh Y, Ouchida R, Kayasuga A, Miwa N.** Anti-Apoptotic Defense of bcl-2 Gene Against Hydroperoxide-Induced Cytotoxicity Together With Suppressed Lipid Peroxidation, Enhanced Ascorbate Uptake, and Upregulated Bcl-2 Protein. *J Cell Biochem* 2003; 89: 321—334.
30. **Hildeman DA, Mitchell T, Aronow B, Wojciechowski S, Kappler J, Marrack P.** Control of Bcl-2 expression by reactive oxygen species. *Proc Natl Acad Sci USA* 2003; 100: 15035—15040.
31. **Kannak K, Jain SK.** Oxidative stress and apoptosis. *Pathophysiology* 2000; 7: 153—163.
32. **Barroso MP, Gomez-Diaz C, Lopez-Lluch G, Malagon MM, Crane FL, Navas P.** Ascorbate and α -Tocopherol Prevent Apoptosis Induced by Serum Removal Independent of Bcl-2. *Arch. Biochem Biophys* 1997; 343: 243—248.

Received November 27, 2008.

Accepted January 23, 2009.