

# The effect of monosodium glutamate on the apoptosis of rat thymocytes and Bcl-2 protein expression

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## Abstract

**Introduction:** Monosodium glutamate (MSG) is the sodium salt of glutamic acid widely spread in modern nutrition. Numerous recent studies have shown the existence of glutamic receptors on different non-neuronal cells, which among others also include lymphocytes and thymocytes. However, it has not yet been precisely established what modulatory effect is created by the activation of these receptors on the immune system cells.

**Material and methods:** We have evaluated the effect of different MSG doses on the intensity of apoptosis of rat thymocytes, as well as the expression of their antiapoptotic protein, Bcl-2. Apoptosis was detected using the Annexin V-FITC apoptosis detection kit and cells were analysed using a flow cytometer. Expression of Bcl-2 was determined with flow cytometry using respective monoclonal antibodies.

**Results:** The current study results demonstrate that different MSG doses significantly increase the intensity of thymocyte apoptosis in a culture. Also, the expression of Bcl-2 proteins in the thymocyte culture, after it has been cultivated with different MSG doses, is significantly reduced.

**Conclusions:** The results presented in our study indicate that different MSG doses significantly modulate the intensity of the apoptotic thymocyte process in a culture, and that one of the possible ways through which MSG induces apoptosis is the reduction of Bcl-2 protein expression.

**Key words:** monosodium glutamate, thymocytes, apoptosis, Bcl-2.

## Introduction

Monosodium glutamate (MSG) is one of the most frequently applied nutritive additives today, especially in Chinese and Japanese food [1]. According to its chemical structure, MSG is the sodium salt of glutamic acid (GA). The quantity of 32 grams of MSG, taken in with food, is equivalent to the quantity of 25 grams of GA [1]. The wide MSG distribution in modern nutrition enables a continuous intake of this substance into organism, which results in accumulation and rise of the GA concentration in blood.

GA represents one of the main excitatory amino acids in the central nervous system (CNS), where it exerts its effects by binding to ionotropic (iGluR) and metabotropic (mGluR) receptors, thus modulating the intracellular processes [2]. Ionotropic receptors function as ionic channels and they take part in Na and Ca ions transport over the cell membrane, while metabotropic receptors fully function by activating G proteins and making secondary messengers [3].

Over the past few years the researches have been directed towards examining the possible function of GA as a peripheral neurotransmitter and they indicated the existence of iGluR and/or mGluR on different non-neuronal cells [2, 4]. The initial researches in the field of human lymphocytes showed that peripheral lymphocytes bind glutamate with high affinity [5]. The researches carried out later showed that mGluR exists on mice thymocytes, as well as on the thymic stromal cells [6]. Further researches showed that ionotropic receptors exist on human lymphocytes and that their activation brings about the rise of the intracellular Ca [7], as well as the rise of ROS (reactive oxygen species) [8]. However, a precise function of the glutamic receptors on lymphocytes still remains unknown. Therefore, the current study was designed to evaluate the effect of MSG on rat thymocyte apoptosis and the expression of their antiapoptotic protein Bcl-2.

## Material and methods

### Animals

Adult Wistar rats (150-200g), both sexes, 8±12 weeks old, were bred at the Vivarium of the Institute of Biomedical Research, Medical Faculty, Nis, under conventional laboratory conditions. All animals used for experiments were healthy.

### Materials

Monosodium glutamate (MSG) was obtained from Fluka Chemika AG, Buchs, Switzerland. MSG was dissolved in RPMI medium at two different concentrations: 1mM and 10 mM.

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 µg/mL) and 10% fetal calf serum (FCS).

Anti-Bcl-2 monoclonal antibody and phycoerythrin (PE)-conjugated anti-rat IgG (H+L) monoclonal antibody were purchased from Immunotech, Marseille, France.

### Preparation of thymocytes

The animals were sacrificed using ether anesthesia. The thymus was extirpated using sterile technique and placed in RPMI medium/10% FCS. Thymocytes were released by teasing thymus through a steel-mesh. Cell suspensions were filtered through sterile nylon-filter to remove stroma and then the cells were washed twice with RPMI medium/10% FCS. The trypan blue exclusion test was performed to evaluate cell viability (always >95%) at the end of cell isolation.

### Cell culture

Thymocytes were counted and adjusted to a density of  $1 \times 10^7$  cells/mL. Cells were cultured in 96-well flat-bottom plates (Sarstedt, Newton, USA), containing a 100 µl of cells suspension ( $1 \times 10^6$  cells) in each well. The cells were treated with either RPMI/10%FCS (control) or MSG at two different concentrations: 1mM and 10 mM. All cultures were done in triplicates. The thymocytes were cultured for 24 hours in an incubator (Assab, Sweden) at 37°C in an atmosphere of 95% air and 5% carbon dioxide.

### Flow-cytometric analysis

At the end of the culture, thymocytes were collected and washed with PBS containing 5% FCS. Permeabilization of thymocytes was done using Intra Prep, permeabilization reagent (Immunotech, Marseille, France), according to the manufacturer instructions. Incubation with anti-Bcl-2 and anti-rat IgG monoclonal antibodies was performed following the monoclonal antibodies manufacturer recommendations. Labeled cells were fixed in 4% formalin. The samples were analyzed on a flow cytometer (Coulter XL-MCL, Krefeld, Germany). Results were presented as the mean percent of positive cells ±SD for four different experiments. Non-specific binding was detected by the control cells, which were incubated with the secondary antibody alone.

### Detection of apoptosis (flow cytometry)

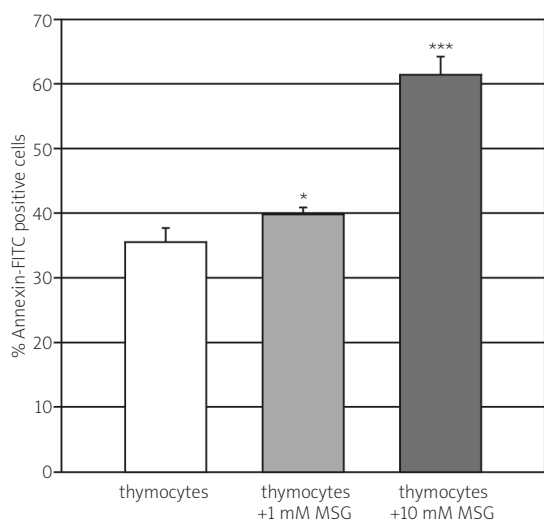
Detection of apoptosis by flow cytometry was performed using the Annexin V-FITC apoptosis detection kit (Immunotech, Marseille, France), according to instructions of the manufacturer. Total percentage of Annexin-FITC+ cells are considered as total percentage of apoptotic cells. Results are given as the mean percent apoptosis ±SD (standard deviation) for four different experiments. Hydrogen peroxide (10 µmol/L) treated cells were used as a positive control for flow cytometry.

### Statistical analysis

Results from fluorescence assays are expressed as mean ±SD. Significant differences between the groups were analysed with Student's t-test.

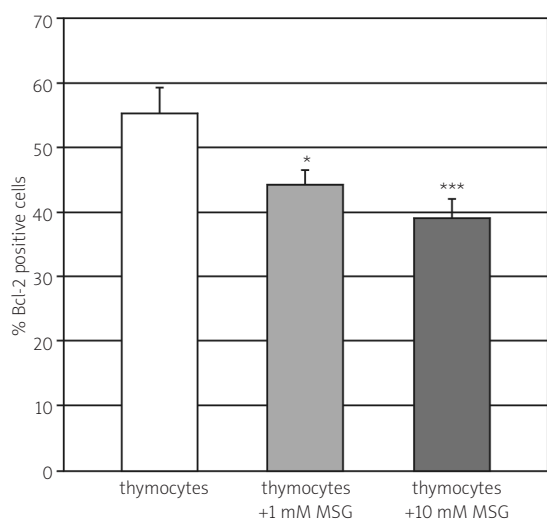
## Results

The first part of our research was related to the examination of the MSG effect on the thymocytes apoptosis in in vitro conditions, in the presence of different MSG concentrations. The results shown in Figure 1 indicate that different concentrations of MSG (1 mM and 10 mM) significantly increase thymocyte apoptosis. The most significant apoptosis increase was detected in the cultures with the highest MSG concentration (10 mM).



Thymocytes were cultivated in culture medium alone (thymocytes), with 1 mM MSG (thymocytes +1 mM MSG), or with 10 mM MSG (thymocytes +10 mM MSG), for 24 h and then stained with Annexin V-FITC apoptosis detection kit. Cell death was determined after 24 h and presented as percent apoptosis as previously described. Values are given as the mean percent apoptosis  $\pm$  SD for four different experiments. \* $p < 0.05$ ; \*\*\* $p < 0.001$  compared to medium controls (thymocytes).

**Figure 1.** The effect of MSG on apoptosis of rat thymocytes



Thymocytes were cultivated in culture medium alone (thymocytes), with 1 mM MSG (thymocytes +1 mM MSG), or with 10 mM MSG (thymocytes +10 mM MSG), for 24 h and then stained with anti-Bcl-2 and anti-rat IgG monoclonal antibodies as previously described. Values (percentage positive cells) are given as the mean  $\pm$ SD for four different experiments. \* $p < 0.05$ ; \*\* $p < 0.01$  compared to medium controls (thymocytes).

**Figure 2.** The effect of MSG on the expression of Bcl-2 protein on rat thymocytes

Our further study was designed to examine the level of expression of Bcl-2 molecules, the antiapoptotic protein in thymocyte culture. The obtained results, shown in Figure 2, indicate that the MSG presence in the cultures significantly reduces the expression of Bcl-2 protein. The maximum expression reduction of this protein was obtained in the cultures with the highest MSG concentration ( $p < 0.01$  in relation to the thymocyte cultures without MSG).

### Discussion

Apoptosis represents a controlled form cell death, which selectively eliminates individual cells without damaging the surrounding tissue. This process plays a very significant role in removing autoreactive or non-functional T-cells, during their development within thymus [9]. Programmed cell death is an active process and it is regulated by Bcl-2 protein family, which has the ability to inhibit or catalyse the apoptosis. There are two basic classes of Bcl-2 protein family. One class includes Bcl-2 and Bcl-x proteins which tend to inhibit apoptosis, while the other class is made up of Bax, Bak and Bim proteins, which promote apoptosis [10, 11]. Besides these naturally occurring processes, apoptosis can be experimentally induced by a variety of exogenous stimuli. Recent studies have demonstrated that ROS and the resulting oxidative stress play a pivotal role in apoptosis [12, 13]. GA is just one of many factors which may induce oxidative stress [14]. It is now established that mitochondria play a prominent role in apoptosis. During mitochondrial dysfunction, several essential players of apoptosis, including procaspases, cytochrome C, apoptosis inducing factor and apoptosis protease-activating factor 1 (APAF-1) are released in the cytosol. The complex forming of cytochrome C, APAF-1 and caspase 9 leads up to a chain activation of other caspases, which results in apoptosis [15, 16].

The results obtained in our study show that after 24-hour culture about 35% of the thymocytes undergo spontaneous apoptosis. It is believed today that such a spontaneous thymocyte apoptosis in a culture develops out of the lack of various surviving factors which are normally present under in vivo conditions, and are provided by thymocyte microenvironment [17]. Similar results have been issued out by Campbell and associates' researches [18]. Further researches were directed towards the examination of the MSG effect on the thymocyte apoptosis. Different MSG concentrations (1 and 10 mM) significantly modulate thymocyte apoptosis, as shown in Figure 1. This kind of thymocyte apoptosis modulation shows a dose-dependent effect. The results obtained in this way indicate that different MSG concentrations strongly induce thymocyte apoptosis. One of the reasons of such an apoptosis

induction probably lies in the fact that MSG [19, 20] and GA [14] are the substances which may strongly induce oxidative stress, lead to the increase of the ROS concentration and the activity of caspase 3 and start the apoptotic process [21]. Schelman and associates' researches [22] have also showed that different GA concentrations significantly induce the nerve cell apoptosis. Tunev and associates' research results also indicate that activation of glutamate receptors lead to the increase of the ROS concentration in human lymphocytes [8].

The final part of our researches was related to the examination of the expression degree of Bcl-2 proteins in thymocyte culture. By using cytofluorometry method, we have showed that MSG significantly modulates the expression of Bcl-2 proteins in thymocyte culture. The modulation of Bcl-2 protein expression shows a dose-dependent effect (Figure 2). The results obtained in this way indicate that one of the possible ways in which MSG induces apoptosis is by reducing the expression of Bcl-2 proteins. However, that does not exclude the possible modulatory effect of other members of the Bcl-2 protein family on the thymocyte apoptosis. Regular Bcl-2 protein expression is necessary for the normal T-cell development, during the primary, as well as during the secondary T-cell differentiation [23]. The decrease in Bcl-2 protein concentration induces the release of cytochrome C from mitochondria, by which the apoptotic process is activated [17]. The results obtained in this way are in accordance with the Esposti and associates' researches [24]. The earlier Perovic and associates' researches [25] also indicate that the increased expression of Bcl-2 proteins significantly reduces the apoptosis in the nerve cell cultures.

## Conclusions

The results obtained in our study indicate that different MSG doses significantly induce thymocyte apoptosis in a culture. One of the possible mechanisms through which MSG completes its apoptotic effect is the reduction of the expression level of Bcl-2 proteins in thymocyte cultures.

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