GENDER-SPECIFIC DIFFERENCES OF OXIDATIVE PROCESSES IN THE POPULATION OF CIRCULATING NEUTROPHILS OF RATS IN A SETTING OF PROLONGED ADMINISTRATION OF MONOSODIUM GLUTAMATE

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Abstract

Background and aims: *Monosodium salt of glutamic acid (MSG) is one of the most common food additives. The aim of study was to assess, in gender-specific terms, how prolonged administration of MSG effects on reactive oxygen and nitrogen species formation and the apoptotic/necrotic processes in the population of rats circulating neutrophils.* ***Material and methods:*** *Experimental studies were conducted on 32 mature white rats. MSG was administered intragastrical at a dose of 30 mg/kg body weight for 30 days. The analysis of cell samples to determine neutrophils with overproduction of reactive oxygen species (ROS) and signs of apoptosis\necrosis was evaluated with flow laser cytometry method. The total nitric oxide synthase (NOS) activity was determined by monitoring the rate of conversion of L-arginine into citrulline. The total quantity of NO metabolites was assessed by evaluating of nitrite and nitrate ions.* ***Results:*** *We found a significant increase in generation of ROS, intensification of nitroxydergic processes, an increase in the percentage of apoptotic neutrophils and no changes in the percentage of necrotic neutrophils.* ***Conclusions:*** *We observed activation of oxidative and nitroxydergic processes in rats with prolonged administration of MSG, which initiate apoptosis. In gender-specific terms, a more pronounced changes were seen in male rats.*

key words: *monosodium glutamate, reactive oxygen species, nitric oxide, NO-synthase; apoptosis.*

Background and aims

The human diet contains thousands of structurally diverse chemical substances which may become components of food during processing and during food preparation that bring about chemical changes and introduce compounds not normally found in raw agricultural products. Further, chemicals are added to achieve certain technical effects such as preservation, color, consistency, flavoring, sweetening, and other physical effects. However, food additives are not always safe for human use [1,2].

Monosodium salt of glutamic acid (MSG) is one of the most common food additives in Ukraine and in Europe with global production over 200 thousand tons annually [3,4]. Encoded

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E621, it is a food additive from a group of flavor enhancers, used in a wide range of foods, such as soups, sauces, mixed condiments, chips, meat products, and puddings. The expanding use of MSG (including its use as a constituent in baby formulas and in certain vaccines) is raising concerns of its potential impact on human health [5], since its ingested amounts are virtually out of control. At present, there is no reliable data that demonstrate specific dose levels and situations when MSG continuously taken with food as an E621 additive would be a health hazard.

Glutamic acid and glutamates are normal metabolites in our bodies. However, any synthetic product differs from its natural counterpart, mostly in spatial configuration of the molecule. Concerning glutamates, there are studies (conceivably to the order of food industry corporations) that fully justify the use of this substance as a food additive. At the same time, US epidemiological studies show that up to 25% to 30% of the population cannot tolerate MSG and prove that its use is associated with excessive weight and obesity [3].

The literature reports on the toxic effects of MSG are scarce and mostly address the issues of glutamate-induced obesity [6,7] and neurotoxicity [8-10]. Moreover, it should be pointed out that the majority of toxicity studies have been performed with large doses of sodium glutamate. Today, it is expedient not only to assess the established hazardous doses of E621, but also to look into the molecular mechanisms of how the allowed (reportedly safe) doses of sodium glutamate impact the living organism.

A key universal mechanism through which most toxic agents work is activation of free-radical processes and overproduction of reactive oxygen species (ROS) [11]. In recent years, apart from reactive oxygen species, the researchers have progressively more interest in

reactive nitrogen species (RNS) [12]. The molecules altered as a result of impact by the reactive oxygen species and the reactive nitrogen species can be viewed as signals that carry the biological information required for regulation of various cellular functions, in part, for initiation of apoptosis.

Therefore, the objective of our study was to assess, in gender-specific terms, how prolonged administration of monosodium glutamate effects on reactive oxygen and nitrogen species formation and the apoptotic/necrotic processes in the population of circulating neutrophils of rats.

Material and methods

Experimental tests were conducted on 32 inbred, mature white rats weighing 180-200 g that were housed at 25 ± 3 °C and humidity of $55\pm2\%$, under a constant 12 h light and dark cycle. Water was available *ad libitum*.

Animals were divided into four subgroups: Group I, intact male rats (n=8); Group II, male rats injected with monosodium glutamate (n=8); Group III, intact female rats (n=8) and Group IV, male rats injected with monosodium glutamate (n=8).

The investigations were conducted under the general rules and regulations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986) [13].

Monosodium glutamate (MSG) was administered intragastrically at a dose of 30 mg/kg body weight (1\500 of LD_{50}) for 30 days [14]. MSG was purchased from Sigma-Aldrich (USA).

On Day 31, experimental animals were sacrificed under deep thiopental anesthesia and their whole blood, serum and lung homogenates were used for further tests.

The population of neutrophils was obtained by centrifugation of whole blood at double

density gradient 1.077 and 1.093 of Ficoll-Urografin.

The analysis of cell samples to determine neutrophils with overproduction of ROS (hydrogen peroxide) was evaluated with flow laser cytometry method on Epics XL flow cytometer (Beckman Coulter, USA) using 2.7-dichlorodihydrofluorescein diacetate.

The analysis of cell samples to determine neutrophils with signs of apoptosis was evaluated with flow laser cytometry method on Epics XL flow cytometer (Beckman Coulter, USA) using ANNEXIN V FITC reagent kit (by Beckman Coulter, USA).

Preparation of a 10% pulmonary homogenate: the samples of lungs taken immediately post-euthanasia were cooled down to 1...3 °C in normal saline, dried with filter paper and then chopped with scissors and homogenized in 0.05 M Tris-HCl buffer (pH 7.4) using a Silent Crusher S magnetic homogenizer (by Heidolp, Germany). The ratio of tissue weight to buffer volume was 1:9. The resulting homogenate was centrifuged at 3000 rpm for 30 minutes on a Hermle Z 32 HK centrifuge with cooling; supernatant liquid was used for the tests.

Nitric oxide synthase (NOS) activity assay in the lung tissue supernatant was performed by monitoring the rate of conversion of L-arginine into citrulline [15]. Total protein was measured with Lowry assay [16].

Quantitative assessment of total concentration of $NO_2^- + NO_3^-$ was performed by evaluation of their amount, which included nitrite ions that were previously present in the sample (NO_2^-) as well as nitrate ions reduced to nitrites (NO_3^-) [17]. The reduction was performed using zinc dust in acidic environment. Nitrites underwent a reaction of diazotisation with sulphanilic acid. The obtained diazotisation compound with N-1-naphthtylethylendiamin

formed the azo dye. Optical density of the obtained color solution was evaluated by spectrophotometry at 536 nm.

Statistical processing of digital data was carried out using the software Excel (Microsoft, USA) and STATISTICA 6.0 (Statsoft, USA). The distribution of data was analyzed according to assessment of normality by Kolmogorov-Smirnov criterion. The obtained values had a normal distribution, so the difference between the groups was analyzed using the Student's t-criterion. All data were presented as M (mean) ± m (standard error). A probability level (p value) of less than 0.05 was considered to be statistically significant. Gender-specific differences of test parameters were detected with the one factor dispersion analysis (ANOVA).

Results

As we compared the levels of ROS generation by circulating neutrophils of mature male and female rats in the control groups (Table 1), the males were reported to have 20.3 % (p<0.05) higher values; this suggests that ROS production in males more was intensive compared to females. Prolonged administration of MSG to mature male rats caused a 40.3 % increase in ROS generation by circulating neutrophils (p<0.001) compared to control animals; the respective increase in female rats was 25.1 % (p<0.001). In gender-specific terms, the intensity of changes of ROS generation in females was 15.2 % lower compared to mature males. The ANOVA factor analysis has demonstrated a significant impact of gender on ROS generation by circulating neutrophils of rats in a setting of prolonged administration of sodium glutamate.

When comparing serum levels of nitrogen (II) oxide metabolites (NO_x) in female and male mature controls, the female rats were found to have higher values, suggesting that NO

production is more intensive in females compared to males (Table 2). Thus, this parameter was 29.0 % higher in serum of females than in males (p<0.02); for lung homogenate, this difference was 21.6 % also in favor of females (p<0.05). That said, the total activity of NO-synthase (NOS) in the supernatant of lung homogenate in control female rats was 17.4 % higher than the respective finding in males (p<0.05).

Table 1. The influence exerted by sodium glutamate on generation of ROS by circulating neutrophils and indices of apoptosis/necrosis in rats (M±m, n=8).

Parameter	Groups of animals			
	Controls	Mature male rats	Controls	Mature female rats
ROS$^+$-cells, %	17.98±0.86	25.23±1.19 p<0.001	14.95±0.98	18.71±0.78 p<0.02
ANV$^+$-cells, %	4.43±0.17	5.32±0.27 p<0.05	3.84±0.15	4.32±0.12 p<0.05
PI$^+$-cells, %	1.86±0.09	2.01±0.08 p>0.05	1.78±0.11	1.91±0.10 p>0.05

Note: p is the significance of differences between the control group and the experimental group

In a setting of prolonged administration of MSG, there were substantially higher NO_x levels compared to the findings in the control group both in serum and in lung homogenate in mature male rats, a 2.3-fold and a 2.7-fold difference (p<0.001), respectively. That said, the overall NOS activity in lung homogenate was increased by 65.2 % (p<0.001).

The female rats were also observed to have NOS activation and increased NO_x content vs control animals; these changes, however, were less pronounced. Thus, the increase in serum NO_x was 73.4 % (p<0.001), while the respective increase in lung homogenate was 89.6 % (p<0.001). That said, the overall NOS activity in lung homogenate was increased by 44.4 % (p<0.01).

When comparing the intensities of changes in NO_x levels in rats of either sex, it was found that mature females had lower intensities compared to mature males: serum levels were 58.7 % lower and the levels in lung homogenate were 85.0 % lower. As for the intensity of changes in overall NOS activity, it was 20.8 % lower in females than in males.

One-way ANOVA analysis has demonstrated a significant impact of gender on generation of nitrogen (II) oxide in rats (p<0.001) in a setting of prolonged administration of MSG.

When comparing the intensity of early apoptosis of circulating neutrophils in mature female controls vs. mature male controls, male rats were found to have higher values (Table 1). Thus, in males this variable was 15.4 % higher than in females (p<0.05).

Prolonged administration of MSG in mature male rats caused the counts of circulating neutrophils with signs of early apoptosis (ANV$^+$-cells) to increase by 20.0 % (p<0.05) compared to the findings in control rats; the respective increase in females was 12.5 % (p<0.05). In gender-specific terms, the intensity of changes in apoptosis initiation was 7.5 % lower in female rats compared to the findings in mature males. Using an ANOVA factor analysis, we did not find the gender of rats to have any statistically significant impact on apoptosis of neutrophils.

Table 2. The influence exerted by monosodium glutamate on the activity of NO-synthase (NOS) and on the content of nitrogen (II) oxide metabolites (NO_x) in rats (M±m, n=8).

Parameter	Groups of animals			
	Controls	Mature male rats	Controls	Mature female rats
Serum				
NO_x, μmol/L	30.61±2.20	71.06±2.29 p<0.001	39.50±2.04	68.50±2.58 p<0.001
The supernatant of pulmonary homogenate				
NO_x, μmol/kg	43.73±3.27	120.09±5.76 p<0.001	53.19±2.32	100.83±3.33 p<0.001
NOS, nmol/(min×mg of protein)	0.69±0.03	1.14±0.07 p<0.001	0.81±0.04	1.17±0.04 p<0.01

Note: p is the significance of differences between the control group and the experimental group.

No significant differences in test parameters were found in mature female and male animals in control groups during assessment of quantity of PI^+-neutrophils in blood, a parameter that describes the intensity of necrotic processes. Prolonged administration of MSG to rats did not cause a significant increase in the percentage of circulating PI^+-neutrophils either in mature male or in mature female rats.

Discussion

What comes under notice during review of the results obtained in the study, is the quite high percentage of circulating neutrophils with increased ROS generation in animals of control groups. Perhaps this can be explained by the regulatory influence of ROS, since these substances may act as secondary messengers in maintenance of physical and chemical properties of biological membranes and also in regulation of such cellular reactions as proliferation, differentiation and apoptosis [18].

As we compared the levels of ROS production by circulating neutrophils of mature male and female rats in control groups, the males were found to have higher values, which suggests that the production of ROS in males was more intensive compared to females. In a like manner, ROS production by human endothelial cells was found to be higher in men compared to women [19].

As for NO, our data concerning the impact of sex on the intensity of nitroxydergic processes in rats of control groups agree with the data by other authors [20], who have also found females to have higher NO levels. Estrogens activate endothelial NOS with a subsequent increase in nitrogen (II) oxide production by endothelial cells [21]. It appears likely that estrogens not only stimulate nitrogen (II) oxide production but also reduce its inactivation by reactive oxygen species.

Prolonged administration of MSG at the dose of 30 mg/kg to mature male rats caused an increase in ROS generation by circulating neutrophils compared to control animals. In gender-specific terms, the intensity of changes of ROS generation in female rats was lower compared to mature male rats.

In a setting of prolonged administration of MSG at the dose of 30 mg/kg, there were substantially higher NO_x levels compared to the findings in the control group. The female rats were also observed to have NOS activation and increased NO_x content vs control animals; these changes, however, were less pronounced.

In a setting of exposure to MSG, mitochondrial respiratory chain is the principal

source of ROS. Moreover, an increase in extracellular glutamate increases the production of hydroxyl radicals. A study by Sharma A. has demonstrated increased activity of α-ketoglutarate dehydrogenase in a setting of MSG use; this may activate oxygen and stimulate the production of superoxide anion and hydrogen peroxide [22].

Other studies indicate that mitochondrial calcium load is a critical step of MSG toxicity. The generation of ROS by Ca^{2+}-loaded polarized mitochondria depletes the antioxidant potential of cells leading to ultimate impairment of cytoplasmic homeostasis of calcium. This in turn causes a release of cytochrome C, a change in oxidation-reduction potential and an increase in generation of superoxide anion radical [23]. Moreover, the ROS may affect NO metabolism by oxidizing tetrahydrobiopterin (BH_4), an eNOS cofactor, which also leads to an increase in generation of superoxide anion radicals [24].

On the other hand, increased concentrations of extracellular glutamate prevent cellular absorption of cysteine through the functioning of the cysteine/glutamate system; this leads to depletion of intracellular reserves of cysteine and glutathione. Reduced levels of glutathione also contribute to excessive accumulation of ROS, which adversely affects mitochondrial structure and function. A study by S. Wu has shown that oxidative stress could even lead to fragmentation of mitochondria [25].

However, most scientists associate the occurrence of oxidative stress in case of MSG injection with glutamate receptors. Glutamate receptors include three families of ionotropic receptors (iGluR) (NMDA - N-methyl-D-aspartate, AMPA - α-amino-3- hydroxy-5-methyl-4-isoxazolepropionic acid and kainate) and three groups of metabotropic receptors (mGluR) [26]. iGluR form ion channels permeable to particular cations, while mGluR

activate intracellular signaling mechanisms via several associated G proteins [27]. Activation of metabotropic receptors leads to an increase in the level of cAMP and releasing of calcium from intracellular stores. The level of calcium changes and this causes various cellular reactions, including activation of NO-synthase and protein kinase C. Glutamate induced Ca^{2+} influx and disruption of the inner transmembrane potential of the mitochondria, which resulted in opening the mitochondria permeability transition pore. When permeability transition pore is out of control, several essential players of apoptosis, including procaspases, cytochrome c, apoptosis-inducing factor and apoptosis protease-activating factor 1 are released into the cytosol. They have the ability to activate caspases, which results in apoptosis [27,28].

A plausible explanation for the pronounced increase in NO_x levels both in serum and in lung homogenate (in a setting of increased NOS activity in mature rats with prolonged administration of MSG) is that the increase in NO production is proportional to the influx of Ca^{2+} ions into the cytoplasm. Glutamate is among the factors that stimulate the influx of calcium into the cell and thus enhance the calcium-dependent activity of the NOS enzyme (among other biologically active substances) [29].

The toxic effect of NO is related both to its direct impact on iron-containing cellular enzymes and to the formation of peroxynitrite (ONOO-), a strong oxidant and a highly reactive/toxic free-radical compound, which is formed when NO interacts with the superoxide anion radical [30]. The toxic effect of ONOO- primarily manifests by inhibition of mitochondrial enzymes, leading to mitochondrial dysfunction and a reduction in ATP production [31]. In addition to that, peroxynitrite induces DNA damage and mutations and inhibits the

activity of the enzymes involved in DNA replication and may damage the DNA directly, which is one of the causes of apoptosis [32]. The prolonged overproduction of NO is acting as a proapoptotic modulator by activating the caspase family proteases via discharge of mitochondrial cytochrome C into the cytosol, regulation of p53 expression and changes in expression of apoptosis-associated proteins, including those of the Bcl-2 family [33].

It is evident that it is oxidative stress that accelerates apoptosis in a setting of prolonged administration of monosodium glutamate. Taking into consideration the antioxidant activity of estrogens, the less pronounced oxidative stress in female rats can be attributed to a greater capacity of their antioxidant system. Being phenolic compounds, estrogens suppress free-radical oxidation of lipids in biological membranes and lipoproteins. Besides, some metabolites of estrogens are capable of reducing tocopheroxyl radicals; both estradiol and tocopherol are synergetic with vitamin C, enhancing antioxidant effect [34].

Conclusions

Prolonged exposure of rats to monosodium glutamate at the dose of 30 mg/kg is accompanied by an increase in generation of reactive oxygen species by circulating neutrophils and by intensification of nitroxydergic processes both in serum and in lung homogenate, manifested as a significant increase in nitrogen (II) oxide metabolites and total NO-synthase activity. The ANOVA factor analysis has demonstrated a statistically significant impact of gender on generation of ROS and NO under given conditions. In gender-specific terms, a more pronounced oxidative and nitrooxidative stress was seen in male rats.

Prolonged exposure of rats to monosodium glutamate at the dose of 30 mg/kg is accompanied by an increase in the percentage of circulating neutrophils with signs of apoptosis and has no impact on the percentage of circulating neutrophils with signs of necrosis. In gender-specific terms, a more pronounced apoptotic changes were seen in male rats.

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