In vitro Evaluation of the Antiproliferative Effect, Cytotoxicity and Proinflammatory Activity of the Food Additive Monosodium Glutamate on RAW 264.7 Cell Line

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Monosodium glutamate (E-621, abbreviated MSG) is a food additive widely used in the food domain as a flavor and taste enhancer. The present study aims to evaluate in vitro the effects of increased concentrations of MSG, using the RAW 264.7 murine macrophage line. The study was conducted in 3 complementary directions: first, establishing the ability of monosodium glutamate to induce cell mortality, by using the MTT assay to determine cell viability; second, establishing by its oxidizing activity if MSG is toxic for cells inducing oxidative stress, measured by the Griess test for determination of extracellular NO; third, to observe whether MSG presence induces an immune response quantified by the secretion of proinflammatory cytokines, TNFa in this case, measured through the immunoenzymatic ELISA technique.

Keywords: monosodium glutamate, viability, cytotoxicity, proinflammatory effect, TNFa

The contemporary lifestyle involves long working days, unused vacation days, lack of time for adequate meals; this is the "fast food era" and despite alarming scientific reports, the tendency is to use attractive foods containing additives. For an appetizing appearance and a better taste, food products contain additional and not necessarily necessary agents like dyes, flavor enhancers, sweeteners, etc. Many natural occurring aliments, such as soy and tomatoes, as well as fish and meat contain high levels of natural glutamate.

Monosodium glutamate (MSG), commonly known as Ajinomotto, is the sodium salt of L-glutamic acid (glutamate) and it is used in the food industry as a **flavor enhancer** with an **"umami"** taste (the fifth taste) that intensifies the meaty, savory flavor of food, as naturally occurring glutamate does in foods such as stews and meat soups. The European Union classifies it as *generally recognized safe* food additive which is permitted in certain foods and subject to quantitative limits. MSG has the HS code 29224220 and the E number E-621 [1]. Recently, in 2017 the European Food Safety Authority (EFSA) concluded safety re-evaluation and established an acceptable daily intake level of 30 mg/kg body weight [1]. In actuality, the US Food and Drug Administration (FDA) permitted MSG intake as 0.55 g/day.

MSG as a flavor enhancer balance, blends, and rounds the perception of other tastes [2]. MSG is used in canned food, crackers, meat, salad dressings, frozen dinners and a myriad of other products. It is found in local supermarkets, restaurants and school cafeterias alike [3]. It is important to note that monosodium glutamate can also be found in vaccines (even anti-influenza), intravenous infusions (with maltodextrin) and in vitamin supplements. Monosodium glutamate is used in all vitamin or mineral supplements encapsulated in gelatinous form [4]. Scientists isolated MSG from plants in the early 1900s. However, MSG is the source of some controversy, because although MSG has proven its value as an enhancer of flavour, different studies have hinted at possible toxic effects related to this popular food-additive [5]. MSG has been linked with obesity, metabolic disorders, *Chinese restaurant syndrome*, neurotoxic effects and detrimental effects on the reproductive organs [2, 6].

In fact, numerous studies have been reported in the literature regarding potential antiproliferative, cytotoxic and genotoxic effects, as well as MSG proinflammatory activity, some examples being references [2, 7-12]. European Union limits MSG concentration in foods (10 mg/g of product), but actually it is typically found in high concentrations in food claiming to contain no added MSG [6, 12].

Glutamate is the excitatory neurotransmitter in the mammalian central nervous system (CNS) playing an important role in both physiological and pathological processes [13]. Several studies investigated the effect of monosodium glutamate on the CNS of rodents and recorded that MSG induces neuronal necrosis and Alzheimer disease [14, 15]. Administration of MSG in high doses, repetitive and systemic ways causes neuronal death through excitotoxicity mechanism in which glutamate receptors activation induced intracellular Ca²⁺ increment [15-18]. In addition to its key roles in fundamental neurological processes and neurological disorders, glutamate is important in protein synthesis, protein degradation and nitrogen metabolism.

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Glutamate concentrations in the intracellular medium range from 2 to 20 mM [19], in plasma of approximately 150 μ M and in the cerebrospinal fluid 10 μ M [20]. The normal concentration of glutamate in the extracellular space varies between 1 and 80 μ M [21]. These values can increase significantly in relation to the ingestion of high amounts of MSG, and in this context, the present study is performed using variable concentrations of MSG.

The well-known murine macrophage cell line, RAW 264.7, is often used for initial screening of natural products for bioactivity and for predicting their potential effect in vivo or on primary cells. The response of this cell line is considered to reflect the potential de novo human response and is used to effectively evaluate the bioactivity of a compound [22]. Due to their nature as first barrier against potentially harmful agents to the body, these cells are often used to evaluate the immune response to various factors.

The MTT test is a colorimetric test for the evaluation of cellular metabolic activity [23]. NAD(P)H-dependent cellular oxidoreductases may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) to its insoluble formazan, which has a purple color. Treatment of cells with MTT allows the evaluation of oxidative metabolism and the response of a cell population to external factors that may have a positive or negative effect on cells in culture. Since for most cell populations the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the in vitro cytotoxic effects of drugs on cell lines or primary patient cells [24].

The murine macrophage cell line RAW 264.7 is able to express both tumor necrosis factor (TNF, a cell signaling protein involved in systemic inflammation) and the inducible NO synthase (an enzyme catalyzing the production of nitric oxide) [25]. Nitric oxide (NO) is an important physiological messenger and effector molecular in many biological systems, including immunological, neuronal and cardiovascular tissues [26]. Due to its involvement in these various systems, the interest in measuring NO in biological tissues and fluids remains a strong one. The Griess test is an indirect assay indicating the oxidation products of reacted nitrogen oxide NO. This analysis_detects the presence of nitrite ion and is based on a diazotization reaction that was initially described by Griess in 1879. Over the years, many changes to the original reaction have been described [27]. By this analysis only nitrite NO_2^- can be measured and nitrate NO_3^- must be reduced; the technique interferes with other nitrites, as well as with food and environmental nitrates.

Although NO and its metabolites (NO₂⁻ and NO₃⁻) are thought to be major mediators of tissue damage resulting from oxidant exposure, the cytokines, including TNF, are also involved. Tumor necrosis factor alpha (TNF α), a potent mediator of the immune system, is synthesized mainly by monocytes and macrophages upon stimulation, e.g. with lipopolysaccharide (LPS). Having a major role in regulating immune system cells, TNF α protein is mainly produced, but not only, by macrophages activated by the presence of stimuli, and its level is an indicator of the pro-inflammatory capacity of a substance.

These three corroborated tests provide a fairly comprehensive overview of the antiproliferative effects, oxidative stress-induced toxicity, and immune response-inducing ability of MSG.

Experimental part

Reagents and cells culture

The complete DMEM (Dulbecco's modified Eagle's medium) was purchased from Merck and was supplemented with 10% fetal bovine serum albumin and 1% antibiotics (penicillin/streptomycin, Lonza). A 1 M MSG stock solution was prepared by dissolving monosodium glutamate (purchased from Sigma-Aldrich) in bidistilled water and sterilized by filtering through 0.22 µm PVDF (Star Lab) filter. This solution was diluted 1:10 (1 mL sterile 1 M MSG + 9 mL sterile complete DMEM medium) for a working concentration of 100 mM. The MTT stock solution contained 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) in phosphate-buffered saline (PBS) solution. A cellular lysis solution containing 50% N-N-dimethylformamide (DMF), 4.8% sodium dodecyl sulphate (SDS), 8% acetic acid and 3 mM HCl was also prepared.

RAW 264.7 cells type ECACC (European Collection of Authenticated Cell Cultures, England) were cultured in DMEM Lonza (complete culture medium) on 75 cm² flasks in a humidified atmosphere at 37 ⁰C and 5% CO₂.

Cells stimulation

For the cytotoxicity assay, RAW cells were seeded in 96-well plates at a starting density of 5×10^4 cells/well and grown overnight to allow adherence (100 µl/well) in complete culture medium. After 24 h the medium was removed and the cells were washed with DMEM. The cells were then exposed in serial dilutions of MSG solution (starting with 200 mM MSG) in fresh complete culture medium for 24 h or 48 h. Cells grown in complete culture medium alone served as control. Cell viability was determined after 24 h and 48 h exposure, respectively.

Similar conditions of cell culture were used to determine the immune mediators secreted by RAW 264.7 cells after treatment with MSG solutions. The cells were exposed to serial diluted MSG solutions and the lipopolysaccharide (LPS from *E. coli* O111:B4, Invivogen) with 10 ng/mL concentration was used for TNF- α positive control. Cell Rev. Chim. \uparrow 71 \bullet no. 2 \bullet 2020 \bullet https://revistadechimie.ro 444 https://doi.org/10.37358/RC.20.2.7947

culture supernatants were collected after 3 h for TNF- α measurement and after 24 h for nitric oxide (NO) measurement.

MTT viability assay

Cell viability was determined as previously described [23]. Briefly, after 24 h exposure to MSG solutions, the culture medium was replaced by fresh culture medium containing 500 μ g/ mL MTT. The test is based on the ability of NADH mitochondrial dehydrogenases in living cells to reduce soluble tetrazolium salts from MTT (yellow) and to form insoluble crystals of formazan (purple). Cells were incubated for additional 3 h, then 100 μ L of lysis solution were added to dissolve the formazan crystals formed by reducing MTT.

The plate was incubated at room temperature in the dark until the cells were lysed and the purple crystals dissolved (about 48 h). The amount of water-insoluble blue formasan dye formed from MTT was proportional to the number of live cells and was determined by measuring the absorbance of all 96 wells using microplate reader spectrophotometer (ThermoScientific, USA) at 550 nm wavelength. The experiment was performed in triplicate and repeated three times.

Griess method for nitric oxide determination

The amount of NO was measured by the accumulation of nitrite in the culture supernatants, collected after 24 h of stimulation, using a colorimetric reaction with the Griess reagent (0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride and 1% (w/v) sulfanilamide containing 5% (w/v) H₃PO₄). Volumes of 80 μ L of cell culture supernatant were mixed with equal volumes of the Griess reagent and the cells plate incubated for 10 min in the dark. The standard curve (calibration curve) was created by using known concentrations of sodium nitrite, and the absorbance was measured within 30 min at 540 nm using the same spectrophotometer. The experiments were performed in triplicate and repeated three times.

TNF-\alpha determination

In order to evidence the proinflammatory effect of MSG, TNF- α concentrations were determined in cell supernatants collected after 3 h of stimulation using an ELISA kit (DuoSet, R&D Systems Inc., USA) according to the manufacturer's instructions. The experimental data were normalized to cell number determined by MTT assay using unstimulated cells as reference. The experiments were performed in triplicate and repeated three times.

Results and discussions

For all the experimental data, the t-test was performed and the p-values calculated. A *p*-value less than 0.05 (typically ≤ 0.05) is statistically significant. It indicates strong evidence against the null hypothesis, as there is less than a 5% probability the null is correct (and the results are random).

Anti-proliferative effect of MSG measured by determining cell viability using MTT assay

In order to examine the cytotoxicity of MSG, RAW 264.7 cells were incubated with MSG solutions of various concentrations ranging from 200 mM to 3.125 mM and cell viability was measured after 24 h or 48 h using MTT assay. As it can be seen both graphically (Fig. 1) and from the Table 1, there was a significantly statistical decrease in cell viability with increasingly concentrated MSG solutions, meaning that an increased cytotoxicity was recorded by gradual exposure of RAW cells to the concentration series in the culture medium. Except for the two very concentrated solutions, the viability decrease is more pronounced with the longer the contact time.



Fig. 1. Cell viability determined by MTT assay for RAW 264.7 murine macrophage cells treated with different MSG concentrations for 24 h (A) and 48 h (B) in comparison with control samples (Ctr). Results are representative of independent experiments performed in triplicate in same conditions and error bars expressed as mean \pm SD (standard deviation) of three replicates. * p<0.05 as compared to control (unstimulated cells)

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	Cell viability decrease %	
c MSG (mM)	24 h	48 h
200	56.23	78.28
100	38.61	40.25
50	30.12	25.28
25	19.82	12.81
12.5	13.42	7.26
6.25	10.89	2.12
3.125	11.05	5.82
Ctr	0	0

Table 1PERCENTAGES OF DECREASE INCELL VIABILITY AFTER 24 HOURSAND 48 HOURS, RESPECTIVELY

MSG-induced cytotoxicity measured by testing the degree of oxidative stress induced by NO formation determined by Griess method

Nitric oxide (NO) is an important physiological messenger in many biological systems, including immunological, neuronal and cardiovascular tissues and plays a major role in the pathogenesis of acute and chronic inflammation [18]. The effect of MSG on NO secretion by macrophages was investigated by incubating RAW 264.7 cells with a series of MSG concentrations decreasing from 200 to 3.125 mM. The results of measurements were illustrated in Fig. 2.

Experimental results obtained during both exposure time intervals (24 h, 48 h) showed clearly a very high cytotoxicity (the highest NO concentration) at the highest concentration of MSG (200 mM). In each series of determinations NO concentration released in the extracellular space remains at a high level at 100, respectively 50 mM MSG solutions, after which the cytotoxicity is diminished, reaching values close to the control. Comparing the results for time of 24 hours and 48 hours, a substantial increase in NO concentration was observed for each solution concentrated in MSG (200, 100 and 50 mM).



Fig. 2. NO concentration determined by Griess method for nontreated (control) RAW 264.7 murine macrophage cells and for cells treated with different concentrations of MSG solution for 24 h (A) and 48 h (B). The calibration curve drawn with absorbance read using spectrophotometer is included. Each bar represents mean value of three replicates ± SD (standard deviation). Results are representative of three independent experiments performed in triplicate in same conditions;
* p<0.05 as compared to control (unstimulated cells). Data were normalized to cell number determined by MTT assay using unstimulated cells as reference

The potential proinflammatory effect of MSG measured by determining TNFa cytokine secretion

It has been reported that TNF α is the most important proinflammatory cytokine, which is released early after an inflammatory stimulus [28]. Having a major role in regulating immune system cells, TNF- α is considered as an indicator of the pro-inflammatory capacity of a substance. Figure 3 presents the obtained results of determining TNF α cytokine secretion.



Fig. 3. Results of TNF α secretion determined by ELISA method for murine macrophage cells stimulated with LPS and treated with different concentrations of MSG solution for 3 h: (a) the calibration curve drawn with absorbance read using spectrophotometer; (b) the determined values of TNF α concentration; data normalized with viability. Each error bar represents mean value of three replicates \pm SD (standard deviation). Results are representative of three independent experiments performed in triplicate in same conditions; * p<0.05 as compared to control (unstimulated cells). Data were normalized to cell number determined by MTT assay using unstimulated cells as reference

It can be observed a uniform decrease of $TNF\alpha$ secretion for exposure of RAW cells in more concentrated MSG solutions (higher than 25 mM MSG), a fact which is apparently contrary with some literature data [29-36] that indicated an increases of this cytokine levels following MSG intake. Our explanation consists in the particularity that nitric oxide downregulates tumor necrosis factor mRNA in RAW 264.7 cells, acting mainly by reducing its half-life time [37] and it was thus proven the existence of a negative feedback by endogenous NO oxide on TNF *in vitro* synthesis [25].

Conclusions

We demonstrated that concentrations of 200, 100, 50 and even 25 mM solutions of monosodium glutamate exerted multiple effects on the RAW 264.7 murine macrophage tumor cell line. The viability decreased by 30-60%, reaching even up to 80% in the case of prolonged exposure (48 h) to high concentrations of MSG. The viability increase was also noticed at other MSG concentrations but at a lower level, which suggests that MSG toxicity is due, in part, to the increase in oxidative stress.

TNF α level decreases with the MSG concentration for the three higher concentrations, reaching a plateau very close to the control level at of 25 mM MSG. The low levels of TNF α suggest that MSG does not act by inducing inflammation, but causes cellular damage through NO's production. However, we suppose that MSG *in vivo* is present in concentrations hundreds of times lower than those needed exerted experimental effects, *i.e.* concentrations at which, *in vitro*, no significant changes were observed. From the data obtained so far, no conclusion can be drawn regarding the cytotoxic effects induced by MSG on murine macrophages at concentrations similarly to native biological conditions.

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