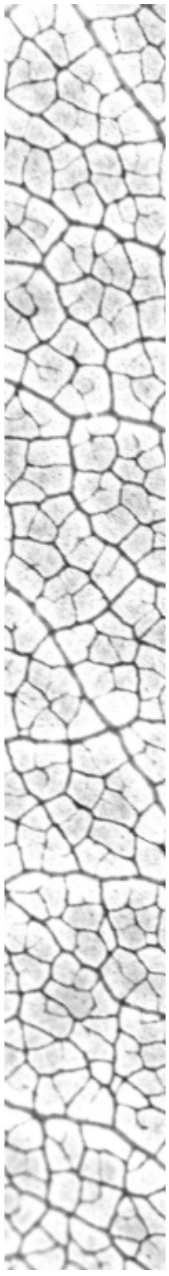

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Monosodium glutamate induced impairment in antioxidant defense system and genotoxicity in human neuronal cell line IMR-32

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Abstract
Monosodium glutamate (MSG) has been widely used as a flavor enhancer in many processed foods despite its toxic effects described in many reports. There is paucity of data regarding the mechanism of MSG induced toxicity on human neuronal cells. The present study was designed to investigate the alteration in antioxidant defense system and genotoxic effects after MSG exposure on human neuroblastoma cells IMR-32. The treatment of MSG was given for 24 h and cytotoxicity study was carried out by trypan blue dye exclusion assay. Apoptosis and necrosis were observed using Propidium iodide (PI) and Hoechst double staining method. Biochemical assays like total protein, protein carbonyl, lipid peroxidation and glutathione level were analyzed along with enzymatic activity of super oxide dismutase and catalase. Genotoxicity indices were measured by comet assay and DNA fragmentation assay. Result of cytotoxicity showed dose dependent decrease in percent viability and significant increase was observed in percent of apoptosis and necrosis. Moreover, exposure of MSG significantly increased lipid peroxidation and protein carbonyl formation along with the impairment in antioxidant defense mechanism. Comet assay and DNA fragmentation assay showed genotoxic effects of MSG on IMR-32 cells in dose dependent manner. Findings of these dose reliant toxicity study of MSG suggest that MSG might be responsible for oxidative stress as well as genotoxicity in human neuronal cells IMR-32 cells and consumption of MSG essential to be controlled.

Keywords: free radical toxicity, genotoxicity, IMR-32, monosodium glutamate, neurodegeneration

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INTRODUCTION

Neurodegenerative diseases are one of the major health challenges especially in elderly population. It includes hundreds of diseases categorized by progressive loss of neurons. Various genetic and environmental factors have been found to modulate the risk for neurodegeneration (Dugger and Dickson 2017). One of the important risk factors assigned to neurodegeneration is dilatory food additives like monosodium glutamate (MSG), a sodium salt of glutamate (Husarova and Ostatnikova 2013; Keservani et al. 2016). Monosodium glutamate gets absorbed in gastrointestinal tract and reaches brain through blood circulation. It gets highly accumulated in those regions of the brain that are essential in cognitive processes mediation such as cerebral cortex, hippocampal gyrus dentatus and striatum (Razali et al. 2017). It induces neurotoxicity, disorders of endocrine glands associated with neurological activities, learning difficulties, epileptic seizures and responsible for many more neurological impairments (Swamy et al. 2013).

Accumulation of MSG in certain regions of brain has upraised the attention for role of MSG intake in neurodegeneration. Considering the wide spread effect of MSG in neuro physiology, it becomes essential not only to understand dose dependent response in neuronal cell but also its effect on various cellular process. In this regard, studies have been conducted to understand mechanism of MSG induced toxicity using various model systems (Pelaez et al. 1999; Razali et al. 2017). MSG showed that high dose of MSG (4 mg/g b. w.) was able to induce neuronal necrosis in hypothalamic arcuate nuclei in neonatal rats treated orally for 4 days (Pelaez et al. 1999). In our previous

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study MSG was found to reduce 50 % cell viability of human neuronal cells IMR-32 at 7 mM (Shah et al. 2017). It also causes alterations in glucose utilization and antioxidant defenses in hepatic tissue of rat (Diniz et al. 2004). It also generates reactive oxygen species (ROS) in different cell types of body. Higher amount of ROS ultimately leading to DNA damage. It may also induce cell membrane damage by peroxidation of the polyunsaturated fatty acids (Ernst et al. 2004; Liao and Mani 2011). Studies showed that alteration in scavenging machinery that comprises superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione may lead to neurodegeneration (Uttara et al. 2004). Along with apoptosis, necrosis and alteration in antioxidant defense system MSG also caused genotoxicity in human lymphocytes culture (Ataseven et al. 2016). But there is a paucity of data regarding correlation between MSG induced free radical toxicity and genotoxicity which lead to neuronal cells damage. Hence, current study was designed with an intent to evaluate MSG induced dose dependent ROS modulated neurotoxicity on IMR-32 cells, which mimic cerebral cortex for better biological correlation.

MATERIALS AND METHODS

Chemicals

All chemicals were procured from HiMedia, Mumbai, India and Sigma-Aldrich, USA.

Cell Culture and Treatment

IMR-32 cell line was obtained from National Center for Cell Science (NCCS), Pune, India. Cell line were maintained in Minimal Essential Media (MEM) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids and 10% Fetal bovine serum (HiMedia, Mumbai, India) in CO_2 incubator at 37 ^{0}C with 5% CO_2.

Cytotoxicity Testing by Trypan Blue Dye Exclusion Assay

In our previous study, results of MTT assay showed that LC50 of MSG after 24 h of treatment to IMR-32 cells was 7 mM (Shah et al. 2017). Based on this data, three different doses (1.7 mM, 3.5 mM and 7 mM) of MSG were selected to evaluate the cytotoxic effects on human neuroblastoma cell line IMR-32 by trypan blue dye exclusion assay (Strober 2015).

Apoptosis and Necrosis Observation by Propidium Iodide (PI) and Hoechst 33342 Double Staining

Cultures of IMR-32 cells were set up on coverslip for different experimental groups (**Table 1**) and after 24 h of MSG treatment (1.7 mM, 3.5 mM and 7.0 mM doses) apoptosis and necrosis were observed using PI- 1 µL (10 mg/mL) and Hoechst 33342- 1 µL (10 mg/mL). Cells were examined under fluorescence microscope (Olympus B53F, Japan) and the percentage of apoptotic and necrotic cells were calculated (Gil-Gomez 2004; Zang et al. 2007).

Table 1. Experimental Groups

Groups	Doses
Group I- Control	-
Group II- Monosodium glutamate (MSG) low dose	1.7 mM
Group III- MSG mid dose	3.5 mM
Group IV- MSG high dose	7.0 mM

$$\text{Percentage of Apoptotic Cells} = \frac{\text{LA} + \text{DA}}{\text{LN} + \text{LA} + \text{DN} + \text{DA}} \times 100$$

$$\text{Percentage of Necrotic Cells} = \frac{\text{DN}}{\text{LN} + \text{LA} + \text{DN} + \text{DA}} \times 100$$

where,

LN= live cells with normal nuclei (PI/ Hoechst 33342: blue chromatin with organized structure)

LA= live cells with apoptotic nuclei (PI/ Hoechst 33342: bright blue chromatin that is highly condensed or fragmented)

DN= dead cells with normal nuclei (PI/ Hoechst 33342: pink chromatin with organized structure)

DA= dead cells with apoptotic nuclei (PI/ Hoechst 33342: bright pink chromatin that is highly condensed or fragmented).

Oxidative Stress Indices

IMR-32 cells were seeded in 12 well plates (10^5 cells/well) and cultured for 24 h. After 24 h of MSG treatment, these cells were used to make cell lysate. The cells were trypsinized and treated with lysis buffer (pH 7.5) containing 1% Triton X-100, 130 mM NaCl, 10 mM Tris-HCl and 10 mM NaH_2PO_4 (Shen et al. 2001). The mixture was incubated for 30 minutes at 4 °C. The supernatant was used for biochemical assays like total protein (TP) (Lowry et al. 1951), protein carbonyl (PC) (Levin et al. 1990), lipid peroxidation (LPO) (Ohkawa et al. 1979) and total glutathione (GSH) (Ellmann 1956) along with the activity of superoxide dismutase (SOD) (Kakkar et al. 1984) and catalase (CAT) (Sinha 1972).

DNA Fragmentation Assay

DNA fragmentation assay is performed to analyze DNA damage due to MSG induced toxicity following standard method (Garyantes et al. 2010). DNA isolation was carried out using Proteinase K method (Goldenberger et al. 1995). The DNA samples were visualized under 1.2 % Agarose gel electrophoresis containing ethidium bromide (0.03%). Gel image was captured in UV transilluminator (UVP Bio Doc-IT imaging system, USA) and the shearing pattern of DNA was compared to know the level of DNA damage induced by different doses of MSG.

Comet Assay

Comet assay was performed according to method of Singh and co-workers (Singh et al. 2988). The slides were observed under fluorescence microscope and 100 comets were scored from each group. Percentage DNA in tail and percentage DNA in head were analyzed with the help of Comet score software.

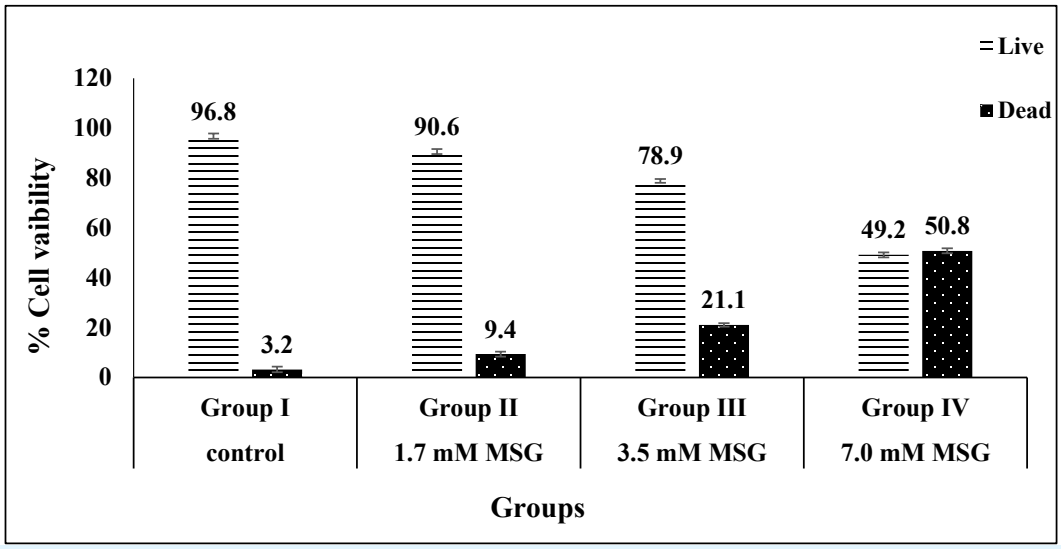

Fig. 1. Percentage of live and dead cells after trypan blue dye exclusion assay in control and all MSG treated groups

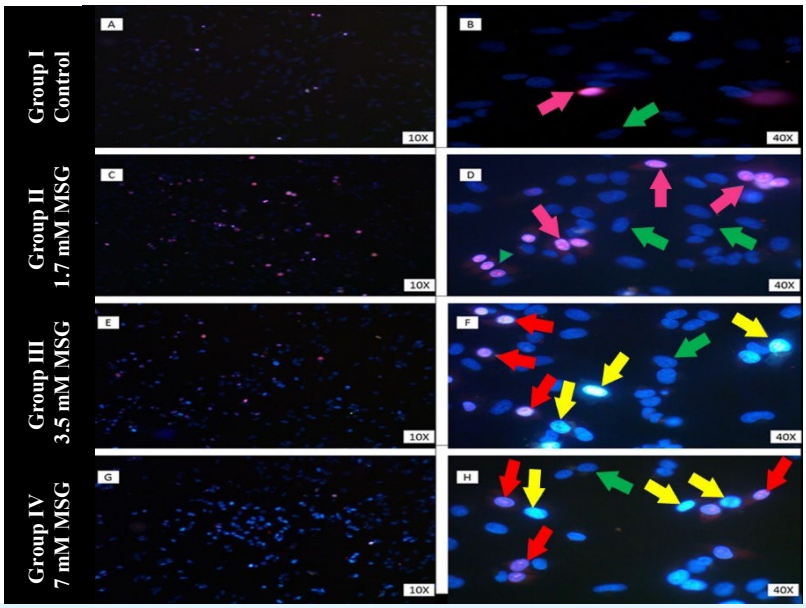

Fig. 2. Propidium Iodide (PI) and Hoechst double Staining. MSG induced toxicity on IMR-32 cells were studied by PI-Hoechst double staining method. Live cells with normal nuclei (LN) showed with green arrow. Live cells with apoptotic nuclei (LA) showed with yellow arrow. Dead cells with normal nuclei (DN) showed with pink arrow. Dead cells with apoptotic nuclei (DA) showed with red arrow

Statistical Analysis

The results were expressed as Mean ± S.E. The statistical significance was evaluated by Analysis of Variance (ANOVA) with the help of GraphPad Prism 7. The individual comparison was obtained by Tukey's multiple comparison test. Value of $p<0.05$ was considered to indicate significant difference.

RESULTS

Cell Viability Study

The trypan blue dye exclusion test showed cytotoxic effect of MSG on cell viability. When IMR-32 cells were exposed to 1.7 mM dose of MSG, it showed nearly 91 % cell viability. At 3.5 mM MSG dose, it exhibited approximately 79 % of live cells, while 7 mM MSG dose showed approximately 50 % cell mortality and thus dose dependent decrease in percentage of live cells was observed (**Fig. 1**).

Apoptosis and Necrosis Observation

MSG treatment of 1.7 mM showed non-significant increase in apoptosis, whereas 3.5 mM and 7 mM treatment showed highly significant increase ($p<0.001$) in apoptosis as compared to control. On the other hand, all three doses of MSG showed highly significant increase ($P<0.001$) in necrosis when compared to control. Whereas, steep increase in number of apoptotic cells was observed (**Fig. 2**; **Table 2**).

Table 2. Percentage of apoptosis and necrosis by Propidium iodide and Hoechst double staining method

Parameter	Group I Control	Group II 1.7 mM MSG	Group III 3.5 mM MSG	Group IV 7.0 mM MSG
Apoptosis	4.5±0.5	13.5±1.5***	29.0±2.0***	46.0±1.5***
Necrosis	8.5±1.0	28.5±0.5***	25.5±0.5***	22.3±1.0***

Table 3. Result of oxidative stress related parameters in control and all MSG treated groups

Groups	TP (mg protein/ 10^5 Cells)	PC (nM Pro Carbonyl /mg protein)	LPO (nM MDA formed /60min/mg protein)	GSH (mM GSH/mg protein)	SOD (U SOD/mg protein)	CAT (nM H_2O_2 consumed/min/mg protein)
I-Control	0.42±0.12	4.02±0.11	1.12±0.33	1.69±0.02	41.31±0.11	156.6±0.12
II-1.7 mM MSG	0.40±0.34^{ns}	5.61±0.05**	1.71±0.11**	1.46±0.12*	41.12±0.12^{ns}	141.7±0.14**
III-3.5 mM MSG	0.39±0.41*	6.32±0.11***	2.81±0.06***	1.16±0.05**	40.14±0.23**	130.6±0.07***
IV-7.0 mM MSG	0.37±0.39**	8.18±0.05***	3.46±0.15***	0.92±0.09***	34.61±0.12***	108.2±0.21***

Oxidative Stress Analysis

Treatment of MSG showed that value of total protein decreased significantly at 3.5 mM and 7 mM doses ($p<0.05$ and $p<0.01$ respectively) whereas non-significant alteration was observed at 1.7 mM treatment as compared to control (**Table 3**). Protein carbonyl can be formed inside the cell due to the breakage of protein backbone by generation of ROS or direct oxidation of amino acids and it showed significant increase ($p<0.01$) at 1.7 mM dose. A highly significant increase ($p<0.001$) was observed at both 3.5 and 7 mM doses of MSG. The level of malondialdehyde was measured to know the lipid peroxidation (LPO) and it increased significantly ($p<0.01$) at low doses (1.7 mM) of MSG treatment (Group II), while the mid and high doses of MSG (Group III and IV respectively) showed highly significant increase ($p<0.001$) in LPO as compared to control (**Table 3**).

Glutathione is a major defense molecule against ROS. The level of GSH was observed to be reducing significantly ($p<0.05$, $p<0.01$ and $p<0.001$ respectively for 1.7 mM, 3.5 mM and 7 mM doses of MSG) in dose dependent manner as compared to control. These parameters showed an excellent linear dose response relationship in cultured IMR-32 cells after 24 h of MSG exposure (**Table 3**).

Superoxide dismutase is an enzyme that catalysis the dismutation of O_2^- into oxygen and H_2O_2 whereas, catalase converts H_2O_2 to non-toxic water molecule. The SOD activity was lower in 7 mM MSG treated group (Group IV) with high significant levels ($p<0.001$), while decrease was moderately significant ($P<0.01$) in 3.5 mM and non-significant in 1.7 mM MSG treatment (Group II and III respectively) in comparison to control (**Table 3**). The treatments of 3.5 mM and 7 mM MSG (Group III and IV respectively) to the IMR-32 cells showed highly significant decrease ($p<0.001$) in catalase activity as compared to untreated group. Whereas, CAT activity was noticed to moderately reduce ($p<0.01$) for the group of cells treated with 1.7 mM MSG (Group II) (**Table 3**).

DNA Fragmentation Assay

Fragmentation of DNA is a marker for late apoptosis. DNA extracted from untreated control cells (Group I) showed no shearing in agarose gel electrophoresis (**Fig. 3**; Lane 1), thus indicating no DNA damage. On the other hand, when the cultures were treated with different doses of MSG, a significant shearing was observed with concomitant increase in dose dependent manner. DNA extracted from 3.5 mM and 7 mM MSG treated IMR-32 cells (Groups III and IV respectively) showed pronounced increase in shearing of DNA (**Fig. 3**; Lane 3 and 2 respectively) due to its fragmentation. But there was not much difference in the shearing appearance in 1.7 mM MSG treatment (Group II) (**Fig. 3**; Lane 4) as compared to control group.

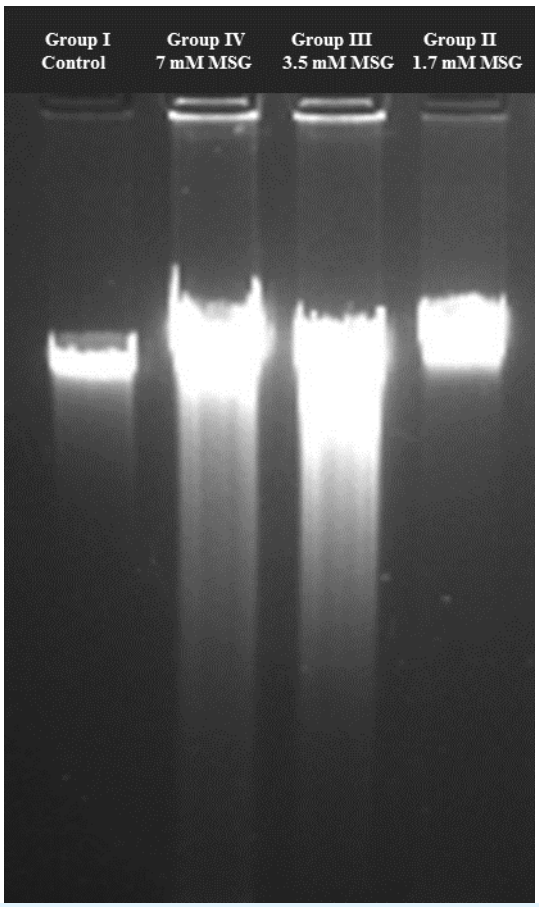

Fig. 3. Result of DNA fragmentation assay. Damaged and fragmented DNA was stretched out during agarose gel electrophoresis. Shearing of DNA increased in dose dependent manner

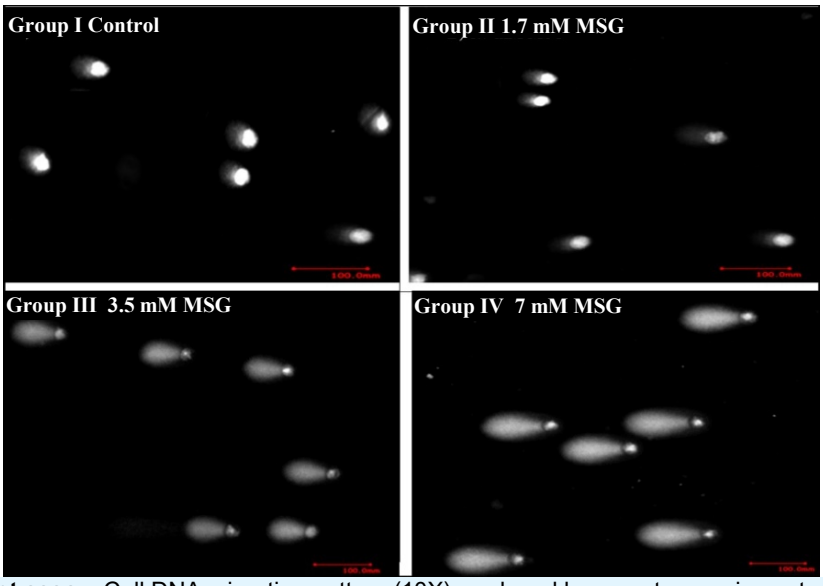

Fig. 4. Result of comet assay. Cell DNA migration pattern (10X) produced by comet assay in control and all MSG treated groups of cells

Table 4. Measurements of DNA damage by comet assay in control and all MSG treated groups

Parameters	Group I Control	Group II 1.7 mM MSG	Group III 3.5 mM MSG	Group IV 7.0 mM MSG
% DNA in tail	0.84±0.18	1.88±0.27^{ns}	2.44±0.28**	4.49±0.48***
% DNA in head	99.75±0.39	96.81±1.03^{ns}	95.67±1.17***	89.81±1.01***

Values are Mean ± S.E. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ and ^{ns} = nonsignificant when groups II, III and IV compared with group I

Comet Assay

Comet measurements were referred to be the excellent indicator for DNA damage study. Comet assay results were analyzed by finding percentage of DNA in the tail and percentage of DNA in head in control and all MSG treated groups (**Fig. 4**; **Table 4**). In 1.7 mM MSG treated group (II), there was no significant increase in percentage of DNA in tail as compared to control (Group I), while at 3.5 mM and 7 mM MSG treated groups (III and IV) there was significant increase ($p<0.01$ and $p<0.001$) in the percentage of DNA in tail. As concentration of MSG increases, a subsequent reduction in the percentage of head DNA head was observed. At 1.7 mM MSG treatment (Group II), non-significant results were obtained as compared to control (Group I), but there was highly significant ($p<0.001$) reduction in the percentage of head DNA in other two groups (III and IV) after MSG treatment (**Fig. 4**; **Table 4**).

DISCUSSION

Neurodegenerative disorders are heterogeneous group of diseases of the nervous system, including the brain, spinal cord and peripheral nerves that have different etiologies (Przeedborski et al. 2003). Due to the prevalence, morbidity and mortality of the neurodegenerative diseases, they represent significant medical, social, and financial burden to the society (Honda et al. 2004). Many genomic and environmental factors are associated with neurodegeneration. The environmental constituents including foods, metal, pollutants and lifestyle, play a direct and indirect role on brain health (Modggil et al. 2014). Food additives like monosodium glutamate (MSG) is known to impair brain functions and health. Yi and Hazell (2006) showed that the neuronal death is linked closely to glutamate-evoked excitotoxicity. Monosodium glutamate alters lipid peroxidation and antioxidant status in cerebral hemispheres, cerebellum, brain stem, diencephalon and causes damage to hypothalamic neurons as reported in study on rat brain (Farombi and Onyema 2006). It also induced elevation of glutamate level in the brain that end in destruction of the neurons (Husarova and Ostatnikova 2013). In a previous study, 7 mM MSG was found to cause cytotoxicity in cultured human neuroblastoma cell line IMR-32 studied using MTT assay (Shah et al. 2018). In this study also, similar dose of MSG confirmed its toxicity by trypan blue dye exclusion assay and decreased 50 % of cell viability of IMR-32 after 24 h exposure. Fukui and coworkers (2009) studied HT22 mouse hippocampal cells and after 30 min of 50 µM/mL glutamate treatment, they also recorded similar results. The ratio of live and dead cells clearly showed that treatment of MSG reduced cell viability. Bai and coworkers (2016) also showed similar results in rat spiral ganglion neurons upon the treatment of glutamate. Moreover, Propidium iodide and Hoechst 33342 double staining results revealed that MSG induces apoptosis as well as necrosis in dose dependent manner. Study carried out other scientists also support this finding by

showing similar effects on HT22 mouse hippocampal cells (Fukui et al. 2009)

Excitotoxicity exerted by glutamate is also responsible for increase in the cytosolic Ca^{2+} level, which is due to either influx from the extracellular space or release from the intracellular stores (Kataria et al. 2012; Szydlowaska and Tyamiaski 2010; Weber 2012)). In this condition the survival of a cell depends largely on functioning of the mitochondria (Fulda et al. 2010). As mitochondria not only satisfy the cellular energy demands but it is also involved in ROS generation, which in turn are suspected to cause cell death if they get out of control (Nicholls 2004). Increased ROS level causes damage to cell in several manner and oxidation of macromolecules like lipid, proteins and DNA. Increase in lipid peroxidation level due to ROS production results in loss of function and integrity of neuronal cell membranes, which in turn results in increase in non-specific permeability to ions, leading to disruption of membrane structure and cell functions (Evangelisti et al. 2013). The malondialdehyde, a marker of lipid peroxidation was increased upon MSG treatment in this study and it also reflected the membrane disruption mediated alteration in cellular functions.

Oxidation of proteins and amino acids induced by ROS generation also resulted in increase of protein carbonyl level and these could be determined by carbonyl groups (aldehydes and ketones) that are produced on protein side chains when they are oxidized (Dalle-Donne et al. 2003). In this study, increase in protein carbonyl level with parallel decrease in total protein confirms the damage to neuronal protein content. Antioxidant defense system consist mainly glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) as they are protagonist in our body. MSG alters the antioxidant defense system and causes oxidative stress, involving neurotoxicity in rats as exhibited by Swamy and coworkers (2013). GSH protects the cells against free radical peroxides and other toxic compounds (Uttara et al. 2009). In current study, dose dependent depletion of GSH level was found after MSG treatment, which in turn may result into oxidative stress mediated cell damage. These findings were in accordance with the study of Onyema and coworkers (2006) who suggested that MSG induced lipid peroxidation contributed to the depletion of GSH level in brain tissue. Superoxide dismutase defends the cell against free radical injury by converting O_2^- radical to hydrogen peroxide (H_2O_2) and prevents the formation of OH^- radicals through O_2^- driven Fenton reactions (Biebrn et al. 2012). The H_2O_2 formed by SOD is removed by catalase. Hence, if the activity of CAT is not adequate to degrade H_2O_2 into H_2O and O_2, than more H_2O_2 is converted into toxic hydroxyl radicals and finally responsible for cellular damage (Bai and Cederbaum 2001). Here, MSG is found to reduce GSH levels as well as inhibit the SOD and CAT activities, ultimately leading to neuronal cell damage. Similar results were also recorded by Swamy and coworkers (2013) during their *in vivo* experiment on rat neuronal cells.

Dead or dying cells can undergo rapid DNA degradation, which can be assessed by DNA fragmentation assay. Result of DNA fragmentation assay in the present study confirmed DNA damage representing an increase in shearing of DNA, when IMR-32 cells were exposed to different doses of MSG. The level of DNA damage reflects more accurately in comet measurement outcomes (Kumarvel and Jha 2006). Increased damaged DNA can be noted in the form of comet formation have also been analyzed in the present study. During electrophoresis, single-stranded DNA fragments move away from damaged nuclei within gel and form a comet-like pattern (Hartmann et al. 2004). In this cohort study, MSG treatment showed dose dependent DNA damage either in terms of decreased percent DNA in head or increased percent DNA in tail of comet. Similar results were also observed by Yang and co-workers while studying on cerebral cortical cell cultures of Sprague-Dawley rats after the glutamate treatment (Ynag et al. 2010).

From this study, it can be concluded that higher concentration of MSG increases free radical generation, thus leading to DNA damage and fragmentation and ultimately to apoptosis and necrosis of neuronal cells. This neuronal impairment can be associated with neurodegenerative diseases.

RECOMMENDATION

It is suggested that intake of monosodium glutamate should be strictly regulated because high amount of MSG as a food additive, obtained from preserved and packed food can cause adverse effects on neuronal cells.

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