NR2B-DAPK1-P53 mediated hippocampal cell death following monosodium glutamate ingestion and interventions with luteolin, caffeic-acid and phoenix dactylifera

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Date of submission: 8th August 2022 Date of acceptance: 6th October 2022 Date of publication: 30th October 2022

Abstract

Introduction: Glutamate is the major excitatory neurotransmitter in the brain, but its accumulation potentiates excitotoxicity. In most food seasonings is the monosodium glutamate (MSG), whose over ingestion have been reported with glutamate-like neurotoxicity, thus, this study investigated the efficacy of *Phoenix dactylifera* and two of its phytochemicals MSG hippocampal toxicity.

Materials and Methods: Forty-eight male Wistar rats were randomly allocated to eight groups of six rats each (n=6). The control received normal saline, group 2 received 4 g/kg MSG, groups 3 to 5 received 4 g/kg MSG followed by 100 mg/kg caffeic-acid, 100 mg/kg luteolin and 500 mg/kg *Phoenix dactylifera*, while groups 6 to 8 received the above agents first followed by 4 g/kg MSG orally for 21 days. 24 hours after the last ingestion, the rats were euthanized and hippocamapal tissue was removed and processed for GluN2B, DAPK1 and p53 immuno histochemical staining.

Results: Repeated MSG ingestions caused high expressions of GluN2B, DAPK1 and p53 in the hippocampus of the exposed rats suggestive of an apoptotic cascades along the NR2B-DAPK1-P53 neuronal death pathway. Pre- or post-treatment with caffeic-acid, luteolin or *Phoenix dactylifera* markedly reduced the hippocampal expressions GluN2B, DAPK1 and p53.

Conclusion: *Phoenix dactylifera* and its flavonoids are capable of downplaying the activities GluN2B, DAPK1 and p53 in MSG toxicity, thereby preventing hippocampal cell death.

Key words: Apoptosis, Glutamate, Monosodium glutamate, Hippocampus, *Phoenix dactylifera,*

Access this article online

Website: https://www.nepjol.info/index.php/NJN

DOI: https://doi.org/10.3126/njn.v19i3.47346

HOW TO CITE

Ibiyeye R, Imam A, Adana M, Sulaimon F, Ajao M. NR2B-DAPK1-P53 mediated hippocampal cell death following monosodium glutamate ingestion and interventions with luteolin, caffeic-acid and phoenix dactylifera. NJNS. 2022;19(3):3-8.

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ISSN: 1813-1948 (Print), 1813-1956 (Online)

Introduction

Food seasonings are salts, herbs and spices used to draw out water or amplify the natural flavor of the food, and enhance edibility.[1] One of the commonly used food seasons is monosodium glutamate (MSG), used in canned foods, crackers, and lots of other products,[1] but which excessive use and accumulation are associated with wide range neurotoxicity.[2;3] This is because it is metabolized by glutamate in the body, which is the major excitatory amino acid in the mammalian central nervous system, known to mediate about 50% of all synaptic transmissions in the Central Nervous System (CNS).[4;5] More specifically, postsynaptic accumulation of glutamate over-excites glutamatergic receptors which may in turn cause excitotoxicity and possibly neurodegenerative like pathology such as cell death.

Considerable evidences indicate that an excitotoxic response arising from increased extracellular glutamate is likely to be important in determining the extent of damage to the brain, especially the hippocampus which is about the most vulnerable region of the brain.[6] Hippocampal

vulnerability to glutamate excitotoxicity is due to its abundant glutamate receptors, which may also be responsible for its sensitivity in to neuronal cell death after stroke, traumatic brain injury, and most neurodegenerative and neuroinflammatory diseases.[7;8]

Polyphenols are naturally occurring compounds found largely in fruits, vegetables, cereals and beverages and are secondary metabolites of plants, and they include but not limited to quercetin, luteolin, caffeic acid and ferulic acid.[9] One of the plants that contain a handful amount of these polyphenols, such as caffeic acid (dactyliferic acid), ferulic acid, luteolin and quercetin is the *Phoenix dactylifera* fruit, whose polyphenol content is said to have a linear relationship with its anti-oxidant and anti-inflammatory capacity among other biological therapeutic properties.[10] Depending on a well established hypothesis linking neuronal oxidative activities and glutamate functions, this study explored the therapeutic advantage of the antioxidant capacity of *Phoenix dactylifera* in MSG induced NR2B, DAPK1, and P53 cell death in the hippocampus of rats.

Materials and Methods

Ethical approval:

Ethical approval for the study was obtained from the Ethical Committee of the University of Ilorin following the Faculty of Basic Medical Sciences ethical committee recommendation (UERC\ASN\2018\1258).

Chemicals:

Luteolin (CAS No 491-70-3) and caffeic acid (CAS No 331-39-5) were procured from the Henan Kaixiang Biological Technology Ltd., China. Fruits of *Phoenix dactylifera* (identified as date fruit) were obtained from a local market in Ilorin, Kwara State. Analytical grade of methanol (CAS 67-56-1) was procured for the methanolic crude extract of *Phoenix dactylifera* fruits.

Experimental Animals

Forty-eight male Wistar rats weighing between 120 - 150 g were used in this study. They were housed in the animal holding of the Faculty of Basic Medical Sciences, College of Health Sciences, University of Ilorin. They were allowed free access to food and water, in line with the Guide for the Care and Use of Laboratory Animals of University of Ilorin, Ilorin Kwara State in accordance with the NIH guidelines for the use and care of laboratory animals (NIH, 2011).

Experimental Design

The 48 rats were randomly allocated to eight groups of six rats each, receiving normal saline (Group A - Control), 4 g/kg of MSG (Group B), 4 g/kg MSG followed by 100 mg/kg caffeic-acid, 100 mg/kg luteolin and 500 mg/kg *Phoenix dactylifera* respectively (Group C,D,E), or 100 mg/kg caffeic-acid, 100 mg/kg luteolin and 500 mg/kg *Phoenix dactylifera* followed by 4 g/kg MSG (Groups F,G,H). The exposure was through oral gavage for a consecutive 21 days in alla groups.

Immunohistochemical Protocol

24 hours after the last ingestions of the agents, the rats were euthanized with intramuscular injection of ketamine, and transcardiaclly perfused with normal saline followed by 4% paraformaldehyde. The brains were removed and fixed in 4% paraformaldehyde for at least 24 hours before the histochemistry procedures. Antigen unmasking of the paraffin embedded hippocampal sections was done by incubating in antigen retrieval solution at 70˚C FOR 50 min. This was followed by endogenous peroxidase inhibition, and then incubated in the primary antibodies (anti- NR2B, DAPK1 or P53) overnight at 10˚C. After rinses, the sections were incubated in the secondary antibody (goat anti-rabbit \ mouse) for 30 min and then reactivity was revealed with DAP + hydrogen peroxide. The immunopositive cells were counted and expressed graphically as percentage fold increase as compared to the control group

Statistical Analysis

Data was subject to one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, using Graph pad prism (version 5). Data was presented as mean ± SEM

Results

GluN2B Immunoreactivity in the hippocampus of MSG, caffeic acid, luteolin and *Phoenix dactylifera* exposed rats

There was 14 folds increase of the GluN2B immunopositive cells in the hippocampal dentate gyrus of rats that received oral MSG only when compared to the control. However, pretreatment and concurrent treatment with luteolin reduced the increased immunoreactive cells to about 0.3 fold, *Phoenix dactylifera* reduced it to about 2.6 and 2.7 folds, while caffeic acid reduced it to about 1.9 and 2.0 folds increase respectively when compared with the MSG only treated rats. (Fig. i and Fig. ii)

Effects of monosodium glutamate, caffeic acid, luteolin and *Phoenix dactylifera* on the population of DAPK1 Immunoreactive cells in the hippocampus

There was about 11 folds increase in the DAPK1 immunoreactive cells in the hippocampus of the MSG treated rats when compared to the normal saline control. However, pretreatment and concurrent treatment with

luteolin reduced the increased immunopositive cells to about 2.4 and 0.1 fold, *Phoenix dactylifera* reduced it to about 3.0 and 2.9 folds and caffeic acid reduced it to about 3.6 folds and increased it to 3.8 folds when compared with the MSG only treated rats (Fig. iii and iv).

Effects of Monosodium glutamate, Caffeic acid, Luteolin and *Phoenix dactylifera* on p53 Expression in the Hippocampus

There was a marked ($p<0.05$) increment in the p53 expression of rats exposed to MSG alone when cmpared with the saline treated rats, and other groups. The administrations of caffeic acid, luteolin and P. dactylifera before and/or concurrently exposed to MSG significantly ($p<0.05$) prevented as much increment in the p53 expression as observed in the MSG only exposed rats. The administration of caffeic acid, luteolin and P. dactylifera before exposure to MSG significantly prevented increment in the p53 expression as observed in the groups that were exposed to concurrent administration of caffeic acid, luteolin and *P. dactylifera*

Table i: The Hippocampal p53 concentration in rats exposed to MSG, Caffeic acid, Luteolin and Phoenix dactylifera

Experimental groups	P53 (ng\mL)
Control	0.43±0.13
MSG	8.30±0.24[a]
MSG+CAF	5.84±0.31[a]
MSG+PD	5.50±0.62[ab]
MSG+LUT	4.80±0.46[ab]
CAF – MSG	4.30±0.48[ab]
PD – MSG	3.6±0.63[ab]
LUT -- MSG	2.9±0.34[b]

Data was presented as mean±SEM. Saline (Control), 4 g/kg MSG (MSG), 4 g/kg MSG posttreated with 100 mg/kg caffeic-acid, 100 mg/kg of luteolin and 500 mg/kg of *Phoenix dactylifera* (MSG+CAF, MSG+LUT and MSG+PD), or concurrently treated with the above agents (CAF-MSG, LUT-MSG and PD-MSG) respectively. Superscripts (a and b) indicates significant ($P<0.05$) differences compared to the control and the MSG only groups.

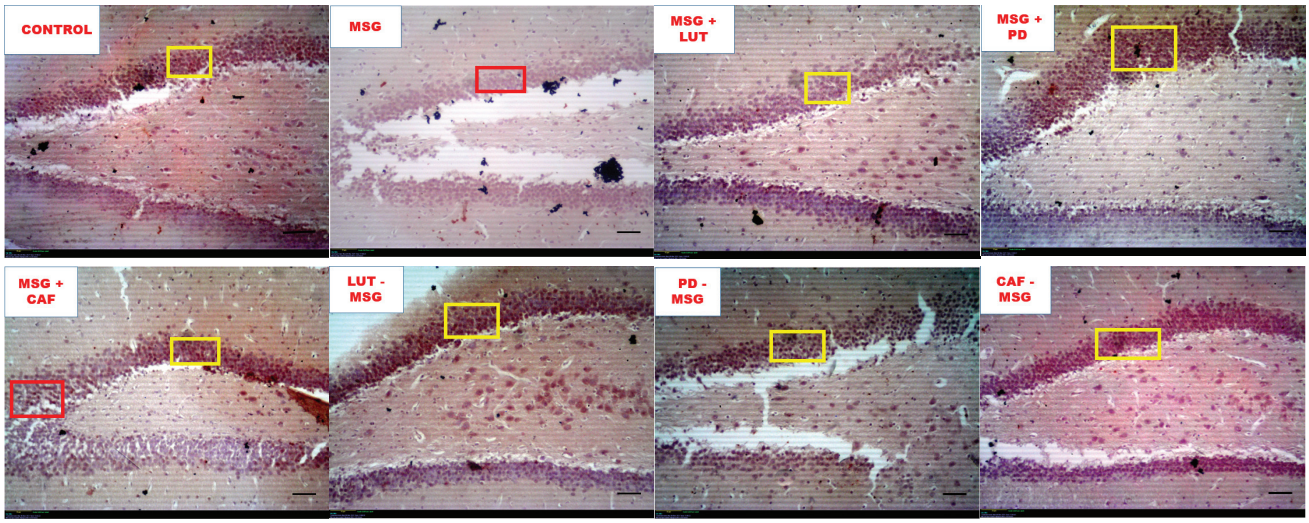

Figure i: Representative photomicrographs with GluNR2B expression in the hippocampal dentate gyrus of rats treated with saline (Control), 4 g/kg MSG (MSG), 4 g/kg MSG posttreated with 100 mg/kg caffeic-acid, 100 mg/kg luteolin and 500 mg/kg Phoenix dactylifera (MSG+CAF, MSG+LUT and MSG+PD), or concurrently treated with the above agents (CAF-MSG, LUT-MSG and PD-MSG) respectively. Anti- GluNR2B immunohistochemistry. Red rectangle encircles the used up GluN2B expression, Yellow rectangle covers the uncoupled GluN2B expression.

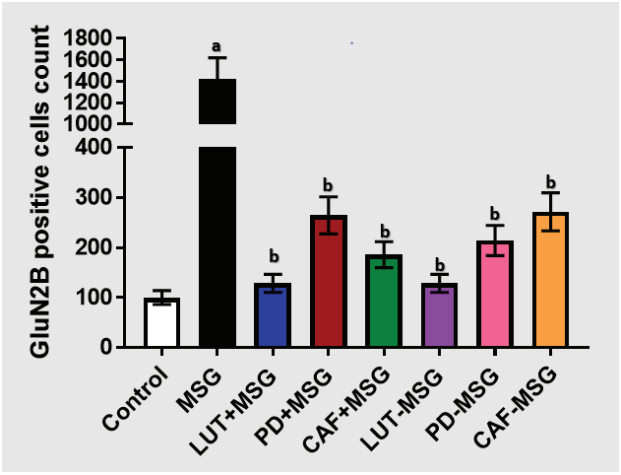

Figure ii: Showing percentage GluNR2B expression in the hippocampal dentate gyrus of rats treated with saline (Control), 4 g/kg MSG (MSG), 4 g/kg MSG posttreated with 100 mg/kg caffeic-acid, 100 mg/kg luteolin and 500 mg/kg Phoenix dactylifera (MSG+CAF, MSG+LUT and MSG+PD), or concurrently treated with the above agents (CAF-MSG, LUT-MSG and PD-MSG) respectively. Data in mean ± SEM. Superscripts (a and b) indicates significant (P<0.05) differences compared to the control and the MSG only groups

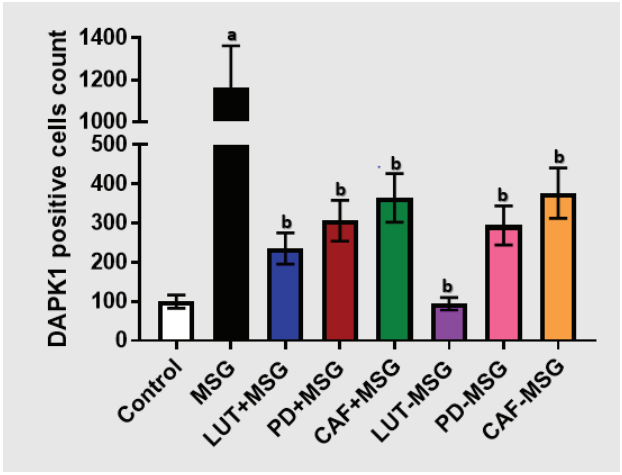

Figure iv: Showing percentage DAPK1 expression in the hippocampal dentate gyrus of rats treated with saline (Control), 4 g/kg MSG (MSG), 4 g/kg MSG posttreated with 100 mg/kg caffeic-acid, 100 mg/kg luteolin and 500 mg/kg Phoenix dactylifera (MSG+CAF, MSG+LUT and MSG+PD), or concurrently treated with the above agents (CAF-MSG, LUT-MSG and PD-MSG) respectively. Data in mean ± SEM. Superscripts (a and b) indicates significant (P<0.05) differences compared to the control and the MSG only groups.

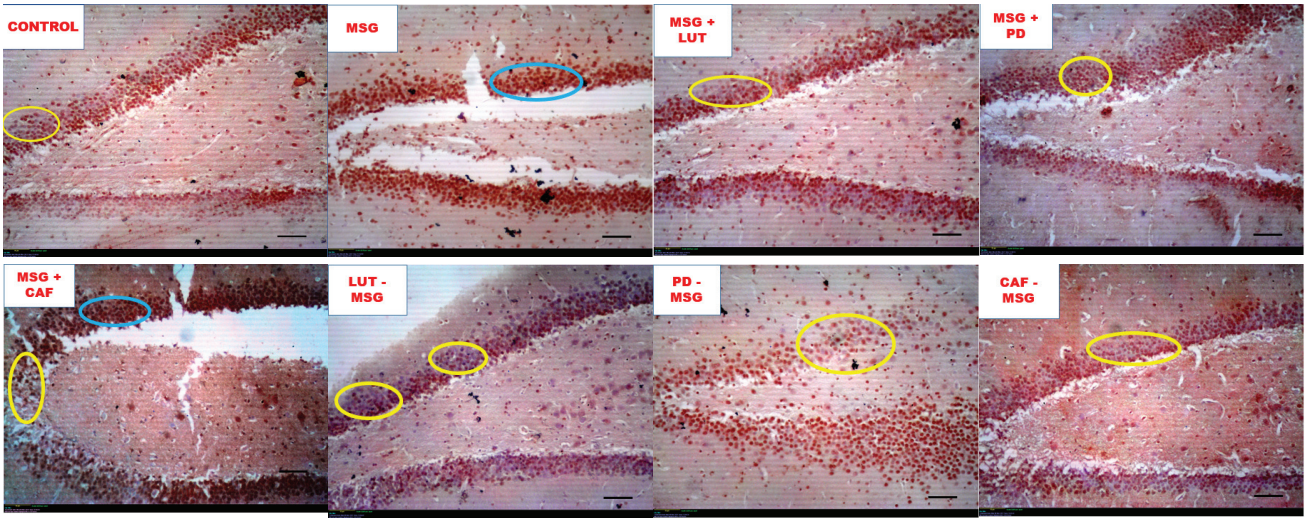

Figure iii: Representative photomicrographs with DAPK1 expression in the hippocampal dentate gyrus of rats treated with saline (Control), 4 g/kg MSG (MSG), 4 g/kg MSG posttreated with 100 mg/kg caffeic-acid, 100 mg/kg luteolin and 500 mg/kg Phoenix dactylifera (MSG+CAF, MSG+LUT and MSG+PD), or concurrently treated with the above agents (CAF-MSG, LUT-MSG and PD-MSG) respectively. Anti- DAPK1 immunohistochemistry. Yellow oval encircles the non\low DAPK1 expression, blue oval encircles the high DAPK1 expression

Discussion

In this study, repeated ingestions of monosodium glutamate led to a considerable increase in the expression of NR2B which indicates high activation of extrasynaptic NMDA. This was moderate in the control indicating a shift from the traditional physiological functioning of the NMDA receptor and balanced synaptic activities to an irregular and excitotoxic cascade in the MSG. These activities appeared to be normalized with luteolin and caffeic acid, but not *Phoenix dactylifera* interventions where moderate expressions of NR2B was recorded, suggestive of the potent activities of these agents in blocking the coupling of NR2B, and progressive outturns

to neuronal cell death pathways (11;12;13). It is note worthy that as promising as this sounds, a reduced expression or activities of the NR2B at this pont, may not be conclusive of inhibition of cell death (14), due to the multi modal pathways of cell death.

The death-associated protein kinase 1 (DAPK1) is a calcium/camodulin protein which is dependent on serine/threonine protein kinase and express abundantly in the brain. It plays diverse roles in apoptosis such as tumor suppression as well as neuronal cell death, thus making it a potent therapeutic target for neurological diseases, possible DAPK1-NR2B and/or DAPK1-p53 couplings (14). These tempt us to investigate its involvement in the MSG induced cell death-like pathologies and in the dissociative mechanisms of the intervention agents. More so, DAPK1 is reported to have a direct link with NR2B subunits and implicated in the initiation of cell death signaling, specifically by binding to the carboxyl tail region of NR2B, phosphorylates the NR2B and enhances its channel conductance. Therefore, uncoupling of activated DAPK1 from NMDA receptor complex protects the brain against damage. Thus, the inhibition of both NR2B and DAPK1 by the agents, evidenced with the reduced expression is an indication that the therapeutic agents may be blocking or disrupting these pathways to effect their neuroprotections against MSG hippocampal excitotoxicity (15;14). These may as well contribute to mechanistic roles of MSG induced cell death and the effectiveness of the polyphenols in reducing neuronal loss (11;12;15;14;7;8;13).

Another indicator of apoptotic cell death is the p53. P53 is a tumour suppressor gene, a translational dependent transcriptional regulator that controls apoptotic and necrotic pathways. It is normally low in concentration but increases with signal of DNA damage to initiate protective activities (16). At its death domain, it interacts with DAPK1 to intimate apoptotic processes, making the uncoupling of DAPK1-p53 bond a potent neuroprotective target in neurodegeneration (16;8). In this study, oral MSG resulted in relatively high concentration of p53 protein in the hippocampal tissue, indicative of severe DNA damage or possibly the coupling of the reported activated DAPK1 above. Luteolin, caffeic acid and *Phoenix dactylifera* treatments pre- or concurrent to MSG was however effective in downplaying these cascade by the depleting the expressions and concentrations of NR2B, DAPK1 and p53, preventing their interactions and consequently cell death in the hippocampus.

Conclusion

MSG induced NR2B-DAPK1-p53 mediated excitotoxicty and cell death in the hippocampus, effectively downplayed by the potent anti-apoptotic properties of the tree anti-oxidant and anti-inflammatory phenols used, including luteolin, caffeic acid and *Phoenix dactylifera.*

Acknowledgements

All the technical staffs of the Department of Anatomy, Afe Babalola University, Ekiti State and the Central Research Laboratory, Tanke, Ilorin, Kwara State, Nigeria are appreciated for their supports.

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