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·Original Article·

Excitotoxicity effects of glutamate on human neuroblastoma SH-SY5Y cells via oxidative damage

Zhong-Wei SUN*, Lan ZHANG*, Shu-Jia ZHU, Wen-Chun CHEN, Bing MEI

Shanghai Institute of Brain Functional Genomics, and Key Laboratory of Brain Functional Genomics, MOE & STCSM, East China Normal University, Shanghai 200062, China

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Abstract: Objective To investigate the mechanisms of excitotoxic effects of glutamate on human neuroblastoma SH-SY5Y cells. **Methods** SH-SY5Y cell viability was measured by MTT assay. Other damaged profile was detected by lactate dehydrogenase (LDH) release and by 4', 6-diamidino-2-phenylindole (DAPI) staining. The cytosolic calcium concentration was tested by calcium influx assay. The glutamate-induced oxidative stress was analyzed by cytosolic glutathione assay, superoxide dismutase (SOD) assay and extracellular malondialdehyde (MDA) assay. **Results** Glutamate treatment caused damage in SH-SY5Y cells, including the decrease of cell viability, the increase of LDH release and the alterations of morphological structures. Furthermore, the concentration of cytoplasmic calcium in SH-SY5Y cells was not changed within 20 min following glutamate treatment, while cytosolic calcium concentration significantly increased within 24 h after glutamate treatment, which could not be inhibited by MK801, an antagonist of NMDA receptors, or by LY341495, an antagonist of metabotropic glutamate receptors. On the other hand, oxidative damage was observed in SH-SY5Y cells treated with glutamate, including decreases in glutathione content and SOD activity, and elevation of MDA level, all of which could be alleviated by an antioxidant Tanshinone IIA (Tan IIA, a major active ingredient from a Chinese plant *Salvia Miltiorrhiza Bge*). **Conclusion** Glutamate exerts toxicity in human neuroblastoma SH-SY5Y cells possibly through oxidative damage, not through calcium homeostasis destruction mediated by NMDA receptors.

Keywords: glutamate; excitotoxicity; cytosolic calcium; oxidative damage

1 Introduction

As an excitatory neurotransmitter, glutamate can accumulate in the brain when its concentration is abnormally high, which could be related to the etiology of some neurodegenerative diseases, such as amyotrophic lateral sclerosis[1-3], stroke or trauma[4], Alzheimer's disease[5,6], Parkinson's disease[7], and Huntington's disease[8]. Many *in vitro* experiments indicate that at high concentrations, glutamate acts as a neurotoxin inducing cell death. Further research has elucidated that glutamate induces excitotoxicity in primary cultured neurons through both oxidative damage and over-stimulation of *N*-methyl-*D*-aspartate (NMDA) receptors, the latter of which would lead to calcium homeostasis destruction[9-11]. In cell lines, it is shown that glutamate produces toxicity in PC12 cells through oxidative stress, while vitamin E, idebenone and selegiline could protect PC12 cells from this toxicity[12]. However, whether this excitotoxicity is dependent on NMDA receptors or not is still unclear[13-16]. Glutamate can also in-

*These authors contributed equally to this work.
Corresponding author: Bing MEI
Tel: 86-21-62233967; Fax: 86-21-62601953
E-mail: bmei@brain.ecnu.edu.cn
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duce apoptosis of human neuroblastoma SH-SY5Y cells, although the underlying mechanisms have not yet been clearly revealed. Besides, the application concentrations of glutamate in SH-SY5Y cells vary from 2 mmol/L to 100 mmol/L[17-19]. The present study thus aims to investigate whether glutamate toxicity in SH-SY5Y cells is dependent on NMDA receptors or through oxidative stress.

2 Materials and methods

2.1 Cell culture SH-SY5Y cells (ATCC, USA) were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Inc., USA) supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin and 100 μg/mL streptomycin at 37 ºC with 5% CO_2.

2.2 Treatment of SH-SY5Y cells with glutamate, MK801, LY341495 and Tan IIA Cells were divided into 8 groups: (1) control group; (2) glutamate group: cells were treated with 20, 40, 60, and 80 mmol/L glutamate (Sigma, USA), respectively; (3) Tan IIA group: cells were treated with 0.001, 0.01, 0.1, and 1μmol/L Tan IIA (National Institute for the Control of Pharmaceutical and Biological Products, China), respectively; (4) glutamate+Tan IIA group: cells were treated with 60 mmol/L glutamate and 1 μmol/L Tan IIA; (5) MK801 group: cells were treated with 5 μmol/L MK801 (Sigma, USA); (6) glutamate+MK801 group: cells were treated with 60 mmol/L glutamate and 5 μmol/L MK801; (7) LY341495 group: cells were treated with 10 μmol/L LY341495 (Tocris, UK); (8) glutamate+ LY341495 group: cells were treated with 60 mmol/L glutamate and 10 μmol/L LY341495. All these agents were added in the medium 24-48 h after cells were seeded. The following analyses were performed 24 h after the treatment.

2.3 MTT assay Cell viability was measured using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the MTT solution (5 g/L) was added into each well and incubated at 37 ºC for 4 h. After the removal of culture medium, 100 μL dimethyl sulfoxide was added into each well to dissolve the formazan. The optical density was measured at 532 nm using a microplate reader (Tecan, Austria). The absorbance of the control group was considered as 100% of the cell viability.

2.4 LDH release assay The CytoTox-96 assay (Promega, USA) was employed to evaluate the total release of cytoplasmic lactate dehydrogenase (LDH) into the medium, which is a consequence of cellular integrity damage. The assay is based upon a coupled enzymatic conversion from 2-*p*-(iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT, a tetrazolium salt) into a formazan product, and the enzymatic reaction is catalyzed by LDH released from cells and diaphorase in the assay substrate mixture. Absorbance was read at 450 nm by the microplate reader. The mean absorbance of each group was normalized to the percentage of the control value.

2.5 4', 6-Diamidino-2-phenylindole (DAPI) staining Briefly, after the treatment with glutamate and/or Tan IIA for 24 h, the cells were fixed with methanol:acetic acid (3:1) for 10 min at room temperature, and then were washed with PBS. Cells were stained with DAPI staining solution (Beyotime Ins. Bio, China) for 5 min, followed by examination with fluorescence microscopy (Leica, Germany). Cells exhibiting size-reduced nuclear, chromatin condensation, intense fluorescence and nuclear fragmentation were considered as apoptotic cells.

2.6 Measurement of intracellular calcium concentration For short-time (20 min) treatment of glutamate, cells were then incubated with 10 μmol/L Fluo-4 AM (Invitrogen Inc., USA) at 37 ºC for 40 min, and then were washed twice with Hanks balanced salt solution containing 20 mmol/L HEPES (pH 7.5), to remove the free Fluo-4 AM. Cells were further incubated at 37 ºC for 40 min to ensure complete de-esterification of Fluo-4 AM. Fluorescent changes in response to intracellular Ca^{2+} levels were recorded at an excitation wavelength of 488 nm and an emission wavelength of 525 nm at 37 ºC on Flex station imaging plate reader (Molecular Devices Corporation, USA). 100-s basal line recordings were first taken at 10-s intervals. For long-time (24 h) treatment of glutamate and other agents, cells were then incubated with 10 μmol/L Fluo-4 AM at 37 ºC for 40 min, and washed twice with D-Hanks balanced salt solution containing 20 mmol/L HEPES (pH 7.5) to remove any free Fluo-4 AM. After that, cells were further incubated at 37 ºC for 20 min to ensure complete de-esterification of Fluo-4 AM. Then endpoint data were collected using Flex station imaging plate reader at an excitation wavelength of 488 nm and an emission wavelength of

525 nm at 37 ℃. The drug application here included 60 mmol/L glutamate, 1 μmol/L Tan IIA, 5 μmol/L MK801, 10 μmol/L LY341495, 60 mmol/L glutamate plus 1 μmol/L Tan IIA, 60 mmol/L glutamate plus 5 μmol/L MK801, and 60 mmol/L glutamate plus 10 μmol/L LY341495. Data were expressed as Relative Fluorescence Units (RFU).

2.7 Glutathione assay Contents of intracellular reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined using commercially available kits (Beyotime Ins. Bio, China), according to manufacturer's instructions. Absorbance was read at 450 nm by the microplate reader. Total glutathione (total GS) content is the sum of GSH content and GSSG content.

2.8 Superoxide dismutase (SOD) and malondialdehyde (MDA) assays After 24-h exposure to glutamate and/or Tan IIA, the cell medium was collected for MDA detection, using the MDA Assay Kit (Nanjing Jiancheng Corp, China). MDA is an intermediate product of lipid peroxidation, and its content is determined by the thiobarbituric acid (TBA) reaction. The absorbance was read at 532 nm with a spectrophotometer (Biochrom, UK). For SOD detection, the cells were lysed using cell lysis buffer (Beyotime Ins. Bio, China) and the lysates were centrifugated at 10 000 g at 4 ºC for 5 min. Supernatant was collected for SOD analysis by SOD kit (Dojindo Laboratories, Japan). Absorbance was read at 450 nm by the microplate reader. The mean value of each group was calculated as the percentage of the control value.

2.9 Statistical analysis Data were presented as mean±SEM, and analyzed with one-way ANOVA, followed by a Newman-Keuls test. $P < 0.05$ was considered as statistically significant.

3 Results

3.1 Glutamate induced a decrease of neuronal cell viability which could be ameliorated by Tan IIA As detected by MTT method, 20-80 mmol/L glutamate treatment in SH-SY5Y cells for 16, 24, and 32 h all caused a decrease of cell viability in a time- and dose-dependent way, except the 20 mmol/L glutamate treatment for 16 h (Fig. 1A). Besides, 0.001-10 μmol/L Tan IIA treatment alone did not exert any significant influence on the survival rate of SH-SY5Y cells, while 100 μmol/L Tan IIA dramatically decreased the cell viability (Fig. 1B). Therefore, 60 mmol/L glutamate and 0.001 μmol/L, 0.01 μmol/L, 0.1 μmol/L and 1 μmol/L Tan IIA were selected for cell co-treatment in this study. Results revealed that at all the 4 doses, Tan IIA could ameliorate the decrease of cell viability and inhibit glutamate-induced LDH release (Fig. 1C, D).

3.2 Glutamate induced alterations in nuclear morphology, which could be alleviated by Tan IIA Alterations in nuclear morphology after glutamate treatment were observed by DAPI staining. As shown in Fig. 2, cell nuclei in control group had a regular and oval shape. However, apoptotic symptoms appeared after 24-h treatment with 60 mmol/L glutamate, such as nuclear condensation and fragmentation. Moreover, co-treatment with 1 μmol/L Tan IIA could alleviate the glutamate-induced nuclear morphological alterations.

3.3 Effects of glutamate, Tan IIA, MK801 and LY341495 on intracellular calcium levels As shown in Fig. 3A, 20-min treatment of glutamate (< 80 mmol/L) failed to induce a significant increase of cytoplasmic calcium level in SH-SY5Y cells, as compared to the control group. However, glutamate treatment (60 mmol/L) for 24 h caused a great increase of calcium level, which could not be inhibited by MK801 (an antagonist of NMDA receptors), Tan IIA or LY341495 (an antagonist of metabotropic glutamate receptors) (Fig. 3B). Besides, co-treatment of MK801 or LY341495 did not affect the toxicity of glutamate in SH-SY5Y cells, as indicated by MTT assay (Fig. 3C).

3.4 Glutamate induced oxidative damage which could be ameliorated by Tan IIA In this study, glutamate-induced oxidative stress in SH-SY5Y cells was assessed by glutathione assay, SOD and MDA assays. Results showed that glutamate treatment induced a dramatic decrease of total GS (GSH plus GSSG) content to 34% of that in control group, while Tan IIA could obviously ameliorate this decrease (Fig. 4A).

The content of MDA represents the extent of lipid peroxidation. As shown in Fig. 4B, glutamate treatment (60 mmol/L) induced a significant increase in MDA content by 13% (P=0.007). Moreover, 1 μmol/L Tan IIA treatment could decrease MDA content by 7%, as compared to that in glutamate group (P=0.037).

The activity of SOD is one of the indicators of anti-

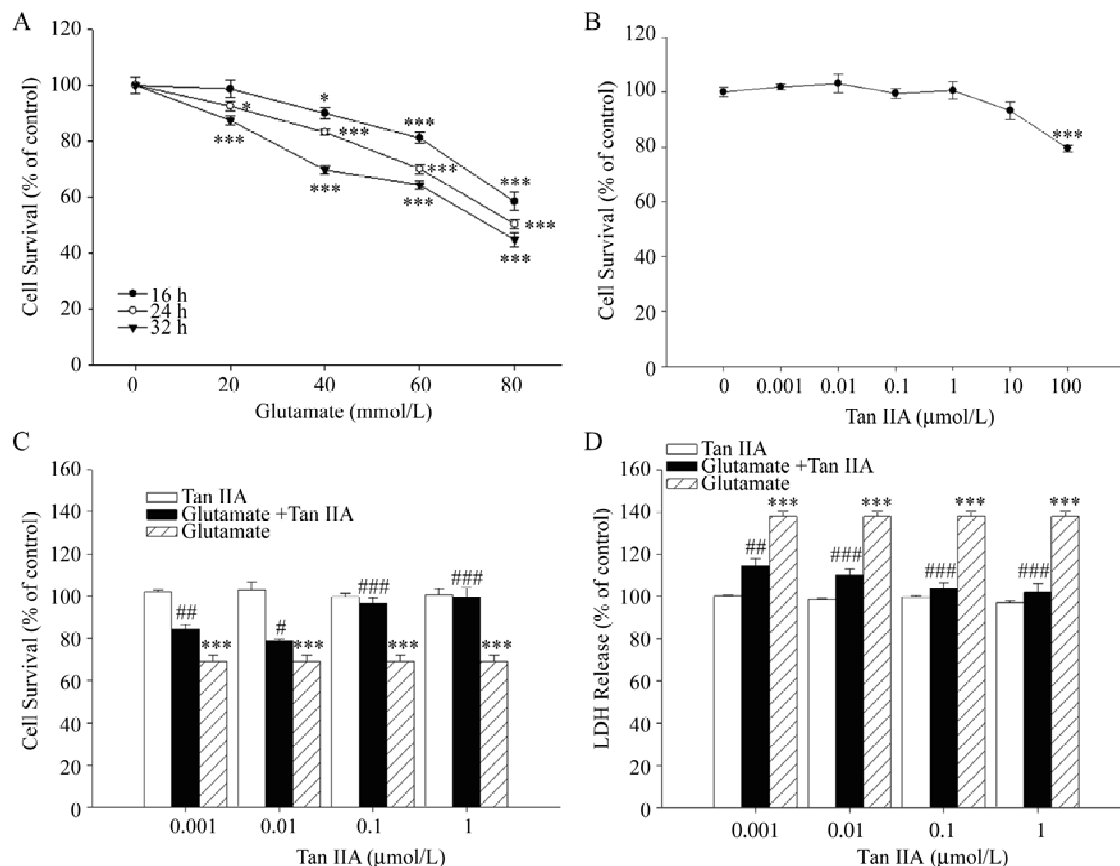

Fig. 1 Glutamate induced a decrease of cell viability and LDH release from SH-SY5Y cells, both of which could be ameliorated by Tan IIA. A: Treatment of glutamate at various doses for 16 h, 24 h, and 32 h. B: Cells treated with Tan IIA at indicated doses for 24 h. C: Changes in cell survival rate after the treatment with 60 mmol/L glutamate for 24 h in the absence or presence of Tan IIA at indicated doses. D: Changes in LDH release after the treatment with 60 mmol/L glutamate for 24 h in the absence or presence of Tan IIA at indicated doses. Data were the mean value of 3 separate experiments (*n* = 4 in each experiment). $^{*}P < 0.05$, $^{***}P < 0.001$ *vs* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* glutamate group.

oxidation ability of the cells. As shown in Fig. 4C, the activity of SOD decreased to 92% after glutamate treatment and Tan IIA rescued the activity of SOD to 96% of the control level.

4 Discussion

Previous research has demonstrated that at abnormally high concentrations, glutamate may cause neuron death, namely excitotoxicity[20], mainly in 2 ways. One is associated with an exaggerated and prolonged rise in intracellular Ca^{2+} concentration following the over-stimulation of NMDA receptors[21,22]. Another involves the oxidative injury pathway which is independent of NMDA receptors[11] and can be specifically inhibited by vitamin E, the caspase inhibitor, extracellular cystine in an elevated level, and the removal of extracellular glutamate[23,24]. However, contradictory findings concerning glutamate toxicity in PC12 cells have also been reported. Some groups find that glutamate toxicity in PC12 cells is not mediated by NMDA receptors[13-15], while another group has reported that application of MK-801 (an antagonist of NMDA receptors) could markedly attenuate glutamate toxicity in PC12 cells[16]. In addition, although mRNAs of NMDA receptor subunits are expressed in PC12 cells[14,25,26], and the receptor proteins and their functional activities are also detected in PC12 cells[26], other studies indicate that no functional NMDA-operated channels are found in the same cell line, and only trace amounts of the receptor proteins are present[27]. In the SH-SY5Y cell line, earlier studies have detected the expressions of NMDA receptors[28,29]. In our pre-

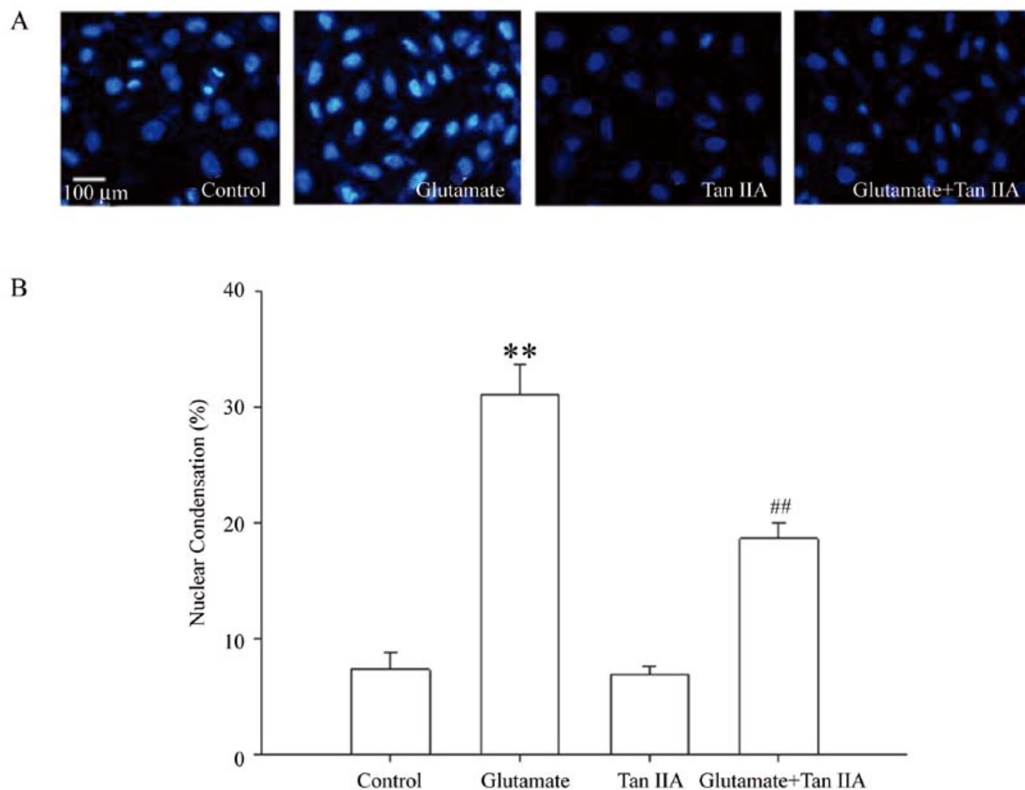

Fig. 2 A: Nuclear morphological assessment of SH-SY5Y cells by fluorescence microscopy. The neuclei were stained by DAPI. Each photograph was representative of 3 independent observations. B: The percentage of condensed nuclei of DAPI-stained cells. The dosages of glutamate and Tan IIA were 60 mmol/L and 1 μmol/L, respectively. $^{**}P$ < 0.01 *vs* control group, $^{\#\#}P$ < 0.01 *vs* glutamate group.

vious experiment, we also found NMDA receptor expressions in SH-SY5Y cells by Genechip analysis method (data not shown). Moreover, if glutamate exerts its toxic effect in SH-SY5Y cells by NMDA receptor over-stimulation, there would be an exaggerated rise in intracellular Ca^{2+} concentration within a short time[30]. However, from our cytoplasmic-calcium assay, 20-min glutamate treatment did not lead to an increase of cytoplasmic calcium. Thus, we speculate that glutamate exerts its toxic effects in SH-SY5Y cells may not by NMDA receptors. Although long-time treatment (24 h) of glutamate increased the cytoplasmic calcium level, which is consistent with the result of another group[19], it could not be lessened by MK801, which again indicates that calcium increase may be independent of NMDA receptor over-stimulation. Based on our studies on SH-SY5Y cells, we infer that SH-SY5Y cells are possibly deficient in NMDA receptors, or perhaps the NMDA receptors have no functions, which leads to the difficulty of calcium flow into cytoplasm. It is possibly due to this deficiency that the SH-SY5Y cells become insensitive to low concentrations of glutamate. Therefore, we applied high concentrations of glutamate in the present study, which is consistent with that in other studies[17,18]. According to previous reports, metabotropic glutamate receptors could regulate intracellular calcium pump by inositol 1,4,5-trisphosphate receptor or ryanodine-sensitive Ca^{2+}-release channels[31,32]. However, LY341495, an antagonist of metabotropic glutamate receptors, also failed to inhibit glutamate-induced cytoplasmic calcium increase in this study. This also suggests that intracellular calcium increase induced by long-time treatment of glutamate may be independent of glutamate receptors. Moreover, whether the increased calcium is from the outside of the cells or from intracellular calcium pump is still unclear.

In this study, glutathione assay showed that glutamate treatment decreased the total GS content. Since the cystine/

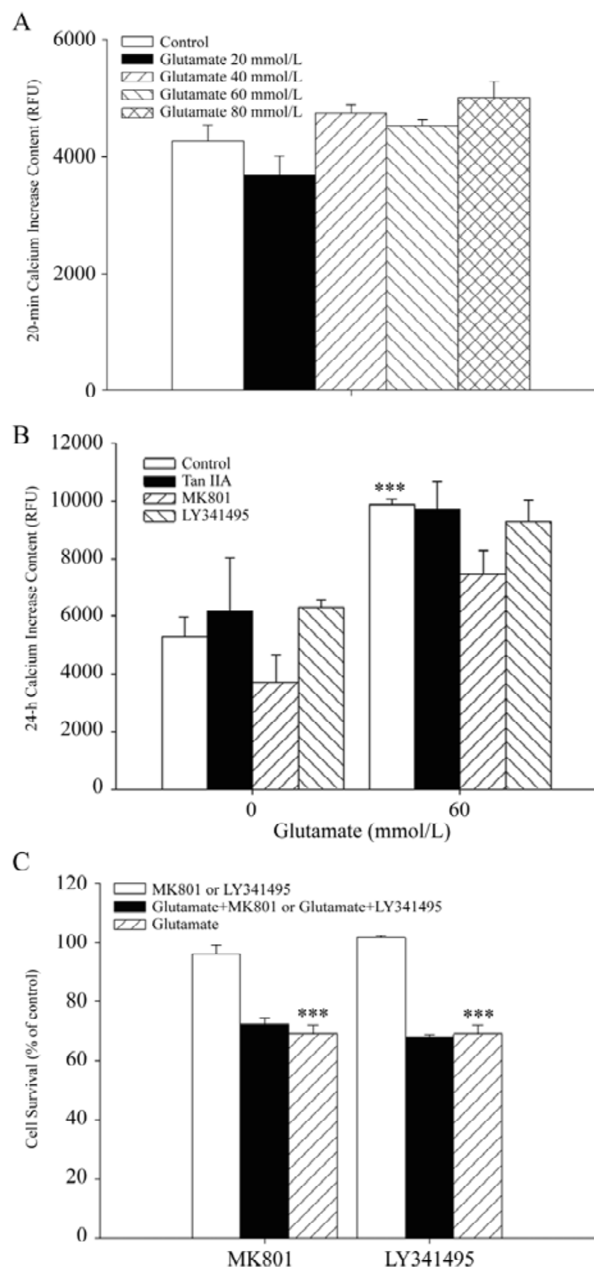

Fig. 3 Effects of glutamate, Tan IIA, MK801 and LY341495 on cytoplasmic calcium level. A: Glutamate treatment for 20 min did not induce any significant increase in level of cytoplasmic calcium. Data were calculated by the last 5-min mean value minus the first 100-s mean value. B: Glutamate treatment (60 mmol/L) for 24 h led to an increase in calcium level (* $P <$ 0.001), compared to that in control group. And MK801 (5 μmol/L), Tan IIA (1 μmol/L), and LY341495 (10 μmol/L) all had no significant effect on this calcium increase. Data were calculated by subtraction of the end-point data before reagent treatment from the endpoint data at 24 h of reagent treatment. C: MK801 (5 μmol/L) and LY341495 (10 μmol/L) had no significant effects on glutamate (60 mmol/L) toxicity. Data were from 3 separate experiments (n = 4 in each individual experiment). *** $P <$ 0.001 *vs* control group.**

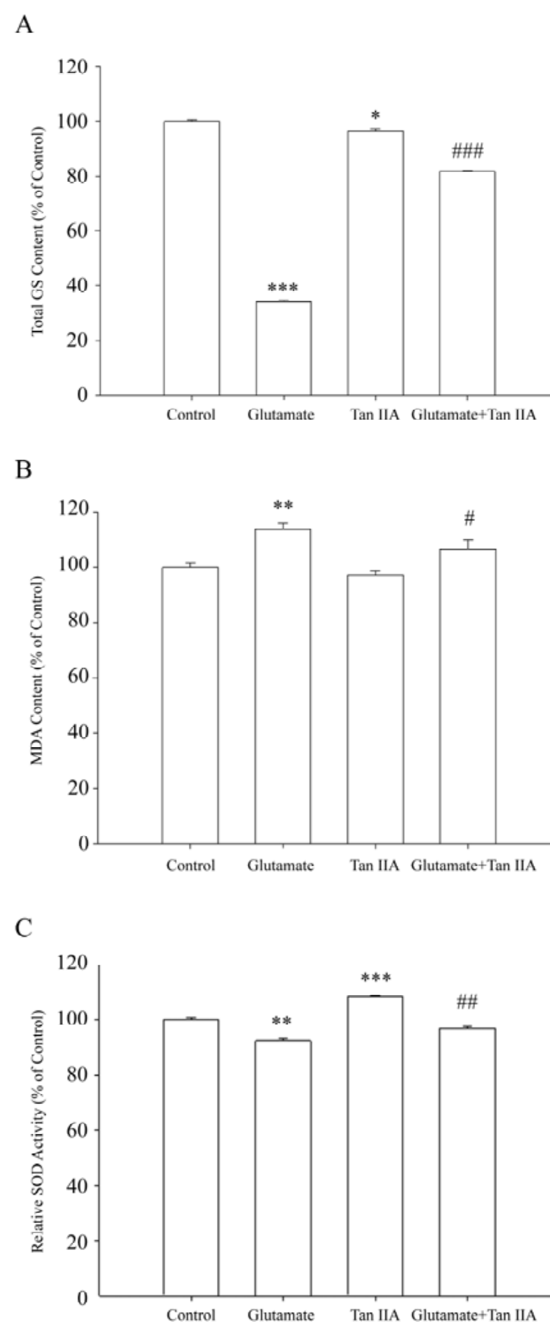

Fig. 4 Glutamate induced oxidative stress in SH-SY5Y cells, and this stress could be ameliorated by Tan IIA. A: Total GS (GSSH plus GSSG) content was measured after 24-h treatment of glutamate (60 mmol/L) and/or Tan IIA (1 μmol/L) treatment. B: MDA content was measured after 24-h treatment of glutamate (60 mmol/L) and/or Tan IIA (1 μmol/L). C: SOD activity was measured after 24-h treatment of glutamate (60 mmol/L) and/or Tan IIA (1 μmol/L) treatment. Data were from 3 separate experiments (n = 4 in each individual experiment). * $P <$ 0.05, ** $P <$ 0.01, *** $P <$ 0.001 *vs* control group; # $P <$ 0.05, ## $P <$ 0.01, ### $P <$ 0.001 *vs* glutamate group.

glutamate antiporter couples the export of glutamate to the import of cystine[33] and cystine is involved in the synthesis of potential intracellular antioxidant GSH, cells would die of apoptosis when GSH is depleted by extracellular glutamate[23,24,34]. In addition, from the results of SOD and MDA assays, we infer that the decrease of total GS content and down-regulation of SOD activity may result in a lower level of endogenous antioxidant, further inducing lipid peroxidation. MDA, a product of the breakdown of polyunsaturated fatty acid, serves as a convenient indicator of lipid peroxidation. Therefore, glutamate-induced cytotoxicity in SH-SY5Y cells could be mediated by oxidative damage.

Tanshinone (Tan) is a major active ingredient of the dried root or rhizome of *Salvia Miltiorrhiza Bge*. Tan IIA is the most abundant component and is structurally representative of Tan. Furthermore, it is well-known as an effective antioxidant whose protective function may be through enhancing antioxidant defense system, thereby decreasing cytotoxicity[35,36]. So we employed Tan IIA as an antioxidant to treat SH-SY5Y cells. Our data demonstrate that at concentrations between 0.001–1 μmol/L, Tan IIA could ameliorate glutamate-induced decrease of neuronal cell viability. In addition, Tan IIA could eventually lower the level of MDA, and rescue the total GS content and SOD activity at the concentration of 1 μmol/L. However, Tan IIA could not inhibit the calcium increase within 24 h of treatment. Consequently, our experiments suggest that through enhancing total GS content and SOD antioxidant defense system, Tan IIA could be promising in alleviating the damage induced mainly by oxidative stress, such as acute ischemia (hypoxia) and reperfusion[16,37]. Furthermore, our experiments also suggest that SH-SY5Y cell line is possibly not a suitable tool for studying NMDA receptor-mediated excitotoxicity.

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谷氨酸致人神经母细胞瘤细胞兴奋性毒损伤的机制

孙中伟*，张蓝*，竺淑佳，陈温纯，梅兵
华东师范大学脑功能基因组学研究所，教育部及上海市脑功能基因组学重点实验室，上海 200062

摘要 目的 探讨谷氨酸导致人神经母细胞瘤细胞(SH-SY5Y cells)兴奋性毒损伤的机制。**方法** MTT法检测SH-SY5Y细胞存活率；测定乳酸脱氢酶释放量观察细胞损伤程度；DAPI染色法观察细胞凋亡形态学特点；钙流法检测胞浆钙离子浓度变化；以胞内谷胱甘肽、超氧化物歧化酶活性和胞外丙二醛含量检测谷氨酸引发SH-SY5Y细胞的氧化应激状态。**结果** 谷氨酸导致SH-SY5Y细胞受损，包括存活率下降、乳酸脱氢酶释放量增多及形态结构发生改变；谷氨酸处理20 min后，胞浆钙离子浓度无显著改变，而处理24 h后，胞浆钙离子大量增加，且MK801 (NMDA受体拮抗剂)及LY341495 (代谢型谷氨酸受体拮抗剂)均不能抑制钙离子内流的增多；谷氨酸可导致SH-SY5Y氧化损伤，包括胞内谷胱甘肽含量减少、超氧化物歧化酶活性降低、胞外脂质过氧化产物丙二醛水平升高等，而丹参酮IIA (一种抗氧化剂)可减轻这些氧化损伤。**结论** 谷氨酸导致SH-SY5Y细胞兴奋性毒损伤可能是通过氧化损伤产生的，而不依赖于NMDA受体介导的钙稳态的破坏。

关键词：谷氨酸；兴奋性毒；胞浆钙离子；氧化损伤