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# Baicalein Reverses the Methamphetamine-Induced Striatal Dopaminergic Neurotoxicity in Mice

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## ABSTRACT

The potential for neuroprotection by baicalein (5,6,7-trihydroxyflavone), a major constituent from the root of a widely used Chinese medicinal herb *Scutellaria baicalensis* Georgi, against methamphetamine-induced neurotoxicity was studied. All ICR mice were treated by 4-times repeated intraperitoneal administration, at 2 hr intervals, of either methamphetamine (5 mg/kg), saline, baicalein (1 mg/kg) or baicalein pretreatment followed by methamphetamine. In the striatum of mouse, the tissue level of dopamine was monitored on day 3 and nitric oxide was assayed after 1 hr, 24 hrs and 3 days of the above treatments. The results showed that striatal dopamine was significantly depleted by methamphetamine and elevated by baicalein. Pretreatment with baicalein prevented the methamphetamine-induced dopamine depletion. Nitric oxide was depressed by methamphetamine, elevated by baicalein, but remained suppressed with baicalein plus methamphetamine after 1 hr post-treatment. At 24 hrs nitric oxide concentration was unaffected by methamphetamine but was significantly elevated by baicalein or baicalein plus methamphetamine administration. On 3 days post-treatment nitric oxide was elevated by methamphetamine, baicalein and further markedly elevated by the administration of baicalein plus methamphetamine. These results suggest a potential neuroprotective role for baicalein with the possible involvement of nitric oxide.

Key words: Baicalein, Methamphetamine, Dopamine, Nitric oxide

## INTRODUCTION

The toxic effects of methamphetamine (METH) specified on monoamine systems are partly involved in the acute and long-term depletion on dopamine (DA) and serotonin in striatal nerve terminals<sup>(1-2)</sup>. Recent studies have demonstrated that chronic use of METH damages DA fibers and leads to a prolonged functional changes. These changes are including the decreases of DA transporter numbers and tyrosine hydroxylase activity as well as the releases of DA which in turn interacts with the previous two factors in striatum<sup>(3-8)</sup>. Neurotoxicity caused by repeated administration of high dose of METH (5 mg/kg, i.p., × 4, 2 hr intervals) has heavily reported. However, the pathways involved in the METH-induced neurodegeneration were not clear.

Current views indicated that the major mechanisms responsible for long-lasting toxic effects of METH are associated with DA and glutamate efflux<sup>(9-11)</sup>. Studies also support the hypothesis that METH-induced dopaminergic neurotoxicity is related with the formation of free radicals<sup>(12-13)</sup>. Following the auto-oxidation process, the concentrations of superoxide radical, hydrogen perox-

ide and hydroxyl radicals then heavily raised<sup>(10,14)</sup>. On the other hand, METH also promotes the generation of peroxynitrite, a neurotoxin produced from the interaction of nitric oxide (NO) and superoxide radical. Peroxynitrite has potentials for the lipid peroxidation which is linked to neuronal degeneration.

NO is a general free radical gas that exerts both neurotoxic and neuroprotective effects<sup>(15-18)</sup>. Previous studies have suggested that N-Methyl-D-aspartate (NMDA) glutamate receptor activation-induced elevation of nitric oxide synthase (NOS) activity is mainly responsible for the NMDA-dependent neurotoxicity as it is preventable by NOS inhibitors. For instance, high dose of N $\omega$ -nitro-L-arginine methyl ester (L-NAME, 30 mg/kg, i.p., × 2), significantly reduced the METH-induced DA depletion in the striatum<sup>(19)</sup>. However, NO-donor, 3-morpholino-sydnnonimine (SIN-1) has also been shown to be neuroprotective in a cerebral ischemia rat model<sup>(20)</sup>.

Baicalein, one of the major flavonoids in the dry root of *Scutellaria baicalensis* Georgi, is widely used in traditional Chinese medicine<sup>(21)</sup>. It has a variety of biological activities such as anti-bacterial, anti-inflammatory, antioxidant, antithrombotic and antitumor properties. In 1999, Chen *et al.* found that baicalein inhibited the endothelium/NO-mediated relaxation at low concentrations

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(0.3-10  $\mu\text{M}$ ) and relaxed the endothelium-denuded vessels at higher concentrations (30-300  $\mu\text{M}$ )<sup>(22)</sup>. Furthermore, Lee *et al.* reported that baicalein exhibited significant protective effects on central neurons against glutamate-induced and glucose deprivation-induced neuronal death in 2003<sup>(23)</sup>. Such findings prompted the present attempt to investigate the effects of baicalein on METH-induced neuronal toxicity and the possible role of NO.

## MATERIALS AND METHODS

### I. Animals

Male ICR mice 4-5 weeks old, were obtained from Laboratory Animal Center, National Taiwan University College of Medicine. Mice were housed in the environmentally controlled quarters with a 12-hour light/dark lighting cycle and constant room temperature at  $23 \pm 1^\circ\text{C}$ . Food and water were given *ad libitum*.

### II. Drugs

Methamphetamine hydrochloride was purchased from National Bureau of Controlled Drugs, Department of Health, Taiwan, R.O.C. Baicalein was purchased from Sigma Chemical Co. (98%, Aldrich, US).

### III. Animal Models of METH-induced Neurotoxicity

Animals were divided into four groups ( $n = 9$  per group). Animals in Groups I & II received four intraperitoneal injections of saline or baicalein (1 mg/kg) at 2 hr intervals. Groups III & IV animals were pretreated with saline or baicalein (1 mg/kg, *i.p.*) 30 mins before each METH (5 mg/kg, *i.p.*,  $\times 4$ , 2 hr intervals). The mice were sacrificed 3 days after the last injection, and the striatal tissues were sliced from both hemispheres for the determination of DA levels. In addition, NO concentrations were determined one hr, 24 hrs and 3 days post treatment.

### IV. Dopamine Levels

The concentrations of DA in striatal tissues were quantified by a modified high performance liquid chromatography (HPLC) method combined with electrochemical (EC) detection<sup>(24)</sup>. Striatal tissue was homogenized in 600  $\mu\text{L}$  of DA mobile phase solution consisting of 75 nM sodium dihydrogen phosphate, 1.5 nM sodium dodecyl sulfate, 0.72 mM triethylamine, 200  $\mu\text{M}$  EDTA, 12% methanol and 13.5% acetonitrile (pH 4.0), and centrifuged at  $4^\circ\text{C}$  ( $1 \times 10^4$  rpm, 4 min). Three hundred microlitres of the supernatant was then removed and filtered through a 0.2  $\mu\text{m}$  Nylon-66 microfilter. Following a 10-fold dilution, aliquot of 20  $\mu\text{L}$  from each sample was injected into the HPLC/EC system for DA assay. The amount of DA was determined using standard curves that were generated by

determining in triplicates the responses of three known standards. Protein determinations were performed by the Lowry method<sup>(25)</sup>.

### V. Determination of Nitric Oxide

Brains of mice were rapidly removed and placed on ice. The striatums were quickly removed and homogenized at  $0^\circ\text{C}$  in 300  $\mu\text{L}$  of 3D water that had been degassed 15 min before. The homogenate was then centrifuged at  $1 \times 10^4$  rpm for 10 min at  $4^\circ\text{C}$ . The supernatants were divided into two parts for NO assay and for protein determination. For NO determination, the samples were deproteinized by adding 100% ethanol (volume 1:1) and then centrifuged at  $1.4 \times 10^4$  rpm for 10 min at  $4^\circ\text{C}$ . To avoid possible changes due to postmortem or storage, a 50  $\mu\text{L}$  aliquot of the supernatant was immediately detected by the NO/ozone chemiluminescence technique using NO-Analyzer (Model 280, Sievers Co. Ltd.). This method measures the oxidation form of NO (nitrate) with a powerful reaction agent (0.1 M vanadium chloride, Merck, Darmstadt, Germany) dissolved in 8% HCl solution. Sample was purged by a stream of helium (99%) from the reaction vessel to the chemiluminescence chamber. The reactions were measured and transformed into electrical signals by the photomultiplier tube. Fresh  $\text{NaNO}_3$  solution (100 mM in 3D water) was used for the standard curve, which was made by triple injections of each concentration (10, 100, 500, or 1000 nM NO).

### VI. Statistical Analysis

All data in these experiments are presented as mean  $\pm$  SEM. The SPSS (Statistical Package for the Social Science), version 11.0 was used for the statistical analysis. One-way ANOVA was performed to determine the differences between groups, and furthermore *post-hoc* comparisons between means were made by Tukey test. The significant levels were defined at  $p < 0.05$ .

## RESULTS

The levels of DA formation in striatum of mice 3 days after the last injection were shown in Figure 1. In this experiment, it was found that baicalein reversed the neurotoxicity of METH. Repeated injections of METH (5 mg/kg, *i.p.*,  $\times 4$ , 2 hr intervals) significantly decreased the contents of DA in striatal tissues as compared with saline-treated mice ( $F = 38.657$ ,  $p < 0.05$ ). In addition, mice treated with baicalein (1 mg/kg, *i.p.*,  $\times 4$ ) showed an increase of DA concentration in striatum after treatment as compared with SS ( $F = 38.657$ ,  $p < 0.05$ ). In the group of baicalein + METH (BM), it significantly reversed DA level in striatal tissues ( $F = 38.657$ ,  $p < 0.05$ , compared with SM mice) and attenuated METH toxic effects on dopaminergic system.

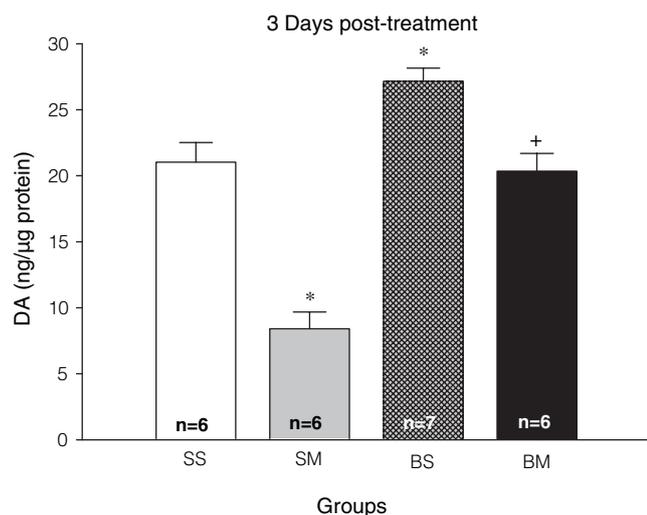
The tissue levels of NO collected one hour after mice exposed to a toxic dose regimen of METH (5 mg/kg, i.p.,  $\times 4$ , 2 hr intervals) were shown in Figure 2A. There was a significant decrease in striatal NO concentrations in the SM group as compared with SS ( $F = 46.372$ ,  $p < 0.001$ ). The mice that received baicalein before saline injection (BS) showed a significant increase in NO levels ( $F = 46.372$ ,  $p < 0.001$ , compared with SS). The NO concentration decreased dramatically in BM group. It was due to that the enhancing effect of baicalein was unable to overcome the suppressing effects of METH, resulting in an overall decrease in striatal NO.

The formation of NO in the mice striatum over a period of 24 hrs after the last injection was shown in Figure 2B. In SM mice, it was observed that NO, significantly reduced 1 hr after the last injection, had returned to a level similar to that of the control after 24 hrs. On the other hand, in mice treated with baicalein and saline (BS), NO formation was still enhanced at the similar level as compared to the 1hr data. Since the suppressing effect of METH was diminished and the enhancing effect of baicalein remained the same, the NO of BM group was therefore in a net increase. Both BS and BM groups were significantly different from control ( $F = 22.450$ ,  $p < 0.05$ , compared with SS). In addition, there were no significant difference in NO concentrations between BM and BS mice.

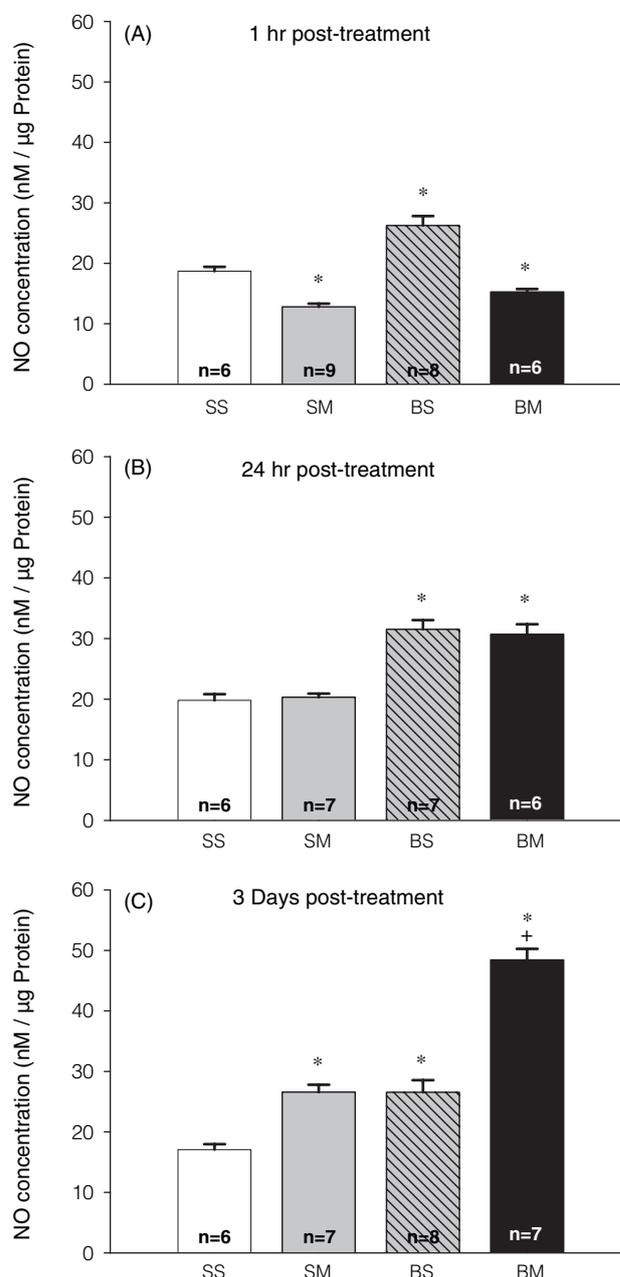
As depicted in Figure 2C, METH significantly increased NO productions in the striatum after 3 days ( $F = 60.893$ ,  $p < 0.001$ , SM compared with SS). Again, mice treated with baicalein and saline still exhibited an increased level of NO ( $F = 60.893$ ,  $p < 0.001$ , BS compared

with SS) measured 3 days after the last injection. Therefore, the synergistic enhancing effects of METH and baicalein resulted in a large increase of NO in the BM group.

Together, these findings indicate that baicalein persists



**Figure 1.** Mice striatal dopamine levels at 3 days post-treatment. Animals were injected intraperitoneally 4 times at 2 hrs intervals with saline (SS), saline + 4  $\times$  METH (SM), baicalein + 4  $\times$  saline (BS) and baicalein + 4  $\times$  METH (BM). Results are shown as mean  $\pm$  S.E.M. DA level was significantly depressed by 5 mg/kg METH (in SM), and elevated by 1 mg/kg baicalein (in BS) (\* $P < 0.05$ , vs. SS). Baicalein pretreatment prevented the METH-induced DA depression ( $^+P < 0.05$  vs. SM).



**Figure 2.** The Nitric oxide levels in mice striatum after drug treatments. Mice were injected intraperitoneally 4 times at 2 hrs intervals with saline (SS), saline + 4  $\times$  METH (SM), baicalein + 4  $\times$  saline (BS) and baicalein + 4  $\times$  METH (BM). Results are shown as mean  $\pm$  S.E.M. NO level was significantly depressed by METH (5 mg/kg) which elevated by baicalein (1 mg/kg) and depressed by baicalein + METH at 1 hr post-treatment [A]; NO concentrations in both BS and BM groups were significantly elevated at 24 hrs post-treatment [B]; and it was significantly elevated either by METH or baicalein, furthermore it increased more higher by baicalein + METH in 3 days post-treatment [C]. \* $P < 0.0001$  vs. SS and  $^+P < 0.0001$  vs. SM and BS.

tently kept elevated striatal NO concentrations up to 3 days after treatment. However, administration of four injections of METH caused a short-term decrease in NO formation in one hour; NO level returned to the normal at 24 hrs after last injection, and then reached a significant peak that will last to the end of the experiment (3 days).

## DISCUSSION

The major aim of this present study was to examine whether baicalein could protect dopamine neurons from METH toxic effects. Consistent with previous studies<sup>(19)</sup>, it was shown that the multiple-dose regiment of METH significantly decreased the striatal DA concentrations. We found that baicalein could prevent the METH-induced depletion of DA in the striatum (Figure 1). Previous research has shown that METH-induced neurotoxicity to the DA terminals is caused by the over releasing of DA and glutamate, which in turn result in enhanced free radicals formation. Blocking NO formation has been reported to attenuate METH-induced toxic effects<sup>(26-27)</sup>.

We observed that METH caused a significant decrease in striatal NO concentrations 1 hr after the last injection and NO levels returned to near control values at 24 hrs. This pattern was similar to the observation of NOS by Halasz *et al.* after one high dose of METH (7.5 mg/kg, i.p.)<sup>(28)</sup>. They reported that 0-2 hrs after METH administration, nNOS activity gradually decreased in the striatum, reaching a maximum decline at 2 hrs but returned to the control level after 12-24 hrs of the toxic insult. Based on these findings, we suggest that METH in the initial stage may diminish NOS enzyme activity, leading to lower NO formation. The main cause of this outcome was possibly related to the ATP-depleting effects of METH. Early in 1994, Chan *et al.* found that a four-dose regimen of METH caused ATP loss in the striatum<sup>(29)</sup>, and the pattern of ATP changes were quite similar to our NO results. In addition, we also observed a delayed increase of NO formation after 3 days of last METH injection which was consistent with previous studies by Lin *et al.*<sup>(30)</sup>.

Besides NO production, could ATP-depleting effects of METH kill DA neurons? When DA neurons lacked energy support, glutamate may become a toxicant that facilitated  $Ca^{2+}$  influxes through NMDA receptor into the presynaptic DA neurons to cause cell death as described in 1994<sup>(31)</sup>. Also, Tung *et al.* mentioned that D-amphetamine could deplete both DA and energy in the striatum of rats<sup>(32)</sup>. This could be another reason of DA neuronal death.

In 2003, Moncada S. *et al.* mentioned that NO might be a physiological regulator of cell mitochondrial respiration<sup>(33)</sup>. This finding could somewhat link NO production with energy depletion together. Since baicalein could also cause vessel relaxation through inhibiting the protein kinase C-mediated cellular pathway in vascular smooth muscle<sup>(22,34)</sup>, it is reasonable to infer that the neuroprotective effect of baicalein against the ATP-depleting effects

of METH can simply come from the increasing of brain blood flow.

Studies of autoimmune inflammatory diseases of the central nervous system have identified both astrocytes and microglia as iNOS-expressing cells. According to these studies, the sources of NO were more important than the quantity of NO<sup>(35)</sup>. It has point out that METH-induced inflammatory reactions are involved in activating both microglia and astrocytes. The experiments by Thomas *et al.* demonstrated that the NO expressions by microglia were significantly increased at 24, 48, and 72hr after four times injections of 10 mg/kg METH. On the other hand, they also observed the depletion of DA after METH administration was in an inverse ratio with microglial activation<sup>(36)</sup>. A recently research by Kawasaki *et al.* shown that the activations of microglia and astrocytes were respectively noted on 1 to 3 days and 3 days after repeated METH treatment (4 mg/kg x 4)<sup>(37)</sup>. And further, inflammatory reactions in activated microglia may be involved in the molecular pathways of METH-induced neurotoxicity<sup>(38)</sup>. Nevertheless, mechanism for microglial activation as part of the METH neurotoxic cascade is still unclear.

Our results indicated that the BS groups constantly maintained NO at a high level from 1hr to 3 days (Figure 2). The over stimulation of NO formation after four-injections of baicalein is an interesting point because *in vitro* studies had indicated that baicalein could suppress lipopolysaccharide-induced increase of NO production and the expression of iNOS in microglia<sup>(39-40)</sup>. The great amount of NO may play a role in dopaminergic neuroprotection. Of course, further studies need to be done to differentiate the source of NO either from astrocytes or microglia cells.

DA levels in baicalein treated mice were slightly higher than those in the saline control group; we speculate that baicalein could indirectly increase DA level based on the report by Hanbauer *et al.*<sup>(41)</sup>. They demonstrated that exogenously applied NO evoked [<sup>3</sup>H]-dopamine release from cultured synaptosomes. Besides, NO synthesized by iNOS from astrocyte end-feet improves local blood flow<sup>(42-43)</sup>. As the study of mitochondria impairment in dopaminergic neurons showed, raising the concentration of glucose or mannose could enhance intracellular ATP synthesis via the citric acid cycle and thus prevent neuronal cell death. Furthermore, sufficient amount of glucose can also increase DA release and uptake<sup>(44-45)</sup>. Taken together, baicalein might increase DA level through, 1) the elevation of NO concentration by increasing iNOS activity, that leading to vasodilation, and in turn raises blood flow, glucose concentration, and promoting ATP synthesis, 2) extra NO production (either from astrocyte or microglia) might stimulate DA release. These two factors may contribute to the enhancement of DA level of striatal tissue in BS group.

In summary, in the present study we demonstrated that pretreatment with baicalein before METH, could prevent the suppression of DA levels in the striatum with the probable involvement of NO and vessel relaxation.

Baicalein might first stimulate iNOS activity in the glial cells and finally causing a long-lasting NO production (at least 3 days). The enhancement of vasodilation in the striatum by large amount of NO and whether baicalein helps to maintain sufficient ATP synthesis to support normal neuronal functions will be the subjects for our future work.

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