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In vivo characterization of toxicity of norcocaethylene and norcocaine identified as the most toxic cocaine metabolites in male mice

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ABSTRACT

Background: Majority of cocaine users also consume alcohol, and concurrent use of cocaine and alcohol produces cocaethylene, norcocaine, norcocaethylene, and other non-toxic metabolites. It is essential to know their relative toxicity for development of a truly effective therapeutics for cocaine toxicity treatment.

Methods: Drug (norcocaethylene or norcocaine)-induced acute toxicity was characterized by the occurrence (and the timing) of prostration, seizure, and death after intraperitoneal administration of the drug (n = 15) using the same strain (Swiss Webster) of male mice reported in previous study by Hearn et al. to determine LD_{50} of cocaine and cocaethylene. In addition, drug (cocaine, cocaethylene, norcocaine, or norcocaethylene)-induced hyperactivity was determined by locomotor activity testing (n = 8).

Results: According to the animal data, norcocaethylene (LD_{50} = ~39.4 mg/kg) and norcocaine (LD_{50} = ~49.7 mg/kg) are the most toxic metabolites, but they do not induce significant hyperactivity. In addition, the relative toxicity of drugs correlates with the time to the occurrence of prostration/seizure/death after the drug administration.

Conclusions: The relative toxicity of these toxic drugs can be ranked in this order: norcocaethylene > norcocaine > cocaethylene > cocaine. The data suggest that norcocaethylene, norcocaine, and cocaethylene are all significant contributors to acute toxicity of cocaine in concurrent use of cocaine and alcohol. Hence, future therapeutic development for cocaine toxicity treatment must account for detoxification of these more toxic metabolites. In addition, the relative toxicity of different drugs correlates with the average time to the occurrence of death, seizure, or prostration after the drug administration with a same dose close to their LD_{50} values.

1. Introduction

Cocaine, known as one of the most reinforcing drugs of abuse (Ersche et al., 2012; Landry et al., 1993; Milton and Everitt, 2012), is highly toxic in animals and humans due to its multiple physiological effects on central nervous and cardiovascular systems etc. (Heard et al., 2008). The use of cocaine leads to neurological impairments because of its neurotoxic effects mediated by several dopaminergic and glutamatergic neurotransmitter systems (Cunha-Oliveira et al., 2008; Mohammad Ahmadi Soleimani et al., 2016). The acute toxicity in the cases of cocaine overdose could be life-threatening, reflected by events like seizure, cardiovascular failure, respiratory depression, and/or death (Heard et al., 2008; Zhang et al., 2017; Zheng et al., 2018).

It has been known that cocaine has three metabolic pathways (Zheng and Zhan, 2016) in the body, including cocaine benzoyl ester hydrolysis (catalyzed mainly by plasma butyrylcholinesterase) to produce metabolites ecgonine methyl ester (EME) and benzoic acid, cocaine methyl ester hydrolysis (catalyzed by liver carboxylesterases-1, denoted as hCE-1) to generate benzoylecgonine and methanol, and cocaine oxidation (catalyzed by liver microsomal cytochrome P450 3A4) to norcocaine. Of these metabolites, norcocaine is significantly more toxic than cocaine itself (Evans and Morarity, 1980; Zhan et al., 2014) and, thus, has been considered as a significant contributor of the cocaine toxicity (Evans and Morarity, 1980).

Notably, concurrent use of cocaine and alcohol produces additional metabolites, including cocaethylene (Hearn et al., 1991) in male and female Swiss Webster mice and norcocaethylene (Chen et al., 2017) in male Sprague-Dawley rats. In particular, alcohol (ethanol) can react

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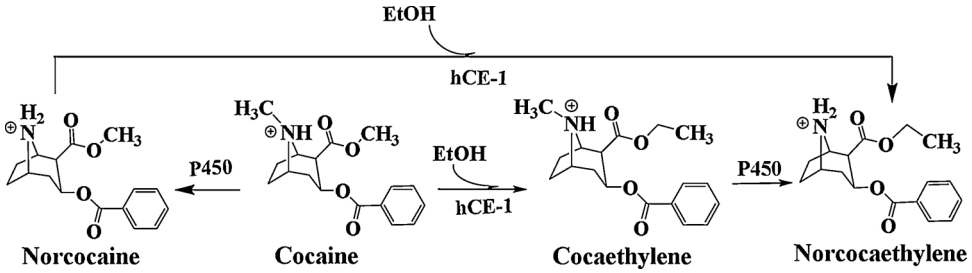

Fig. 1. Cocaine and its three toxic metabolites produced in the concurrent use of cocaine and alcohol.

with cocaine to form cocaethylene through transesterification under the hCE-1 catalysis. With alcohol co-administration, ~24% (intravenous), ~34% (oral), or ~18% (smoked) of cocaine is converted to cocaethylene through transesterification in humans (Herbst et al., 2011). It was demonstrated that cocaethylene is more potent than cocaine in terms of lethality in both (male and female) Swiss Webster mice and male Sprague-Dawley rats (Hearn et al., 1991; Hou et al., 2014). Through testing the dose-dependent toxicity of cocaine and cocaethylene in Swiss Webster mice, Hearn et al. (Hearn et al., 1991) determined the LD_{50} of cocaine and cocaethylene when they were administered intraperitoneally (IP), revealing a significantly lower LD_{50} of cocaethylene (LD_{50} = 63.8 ± 1.0 mg/kg for male mice or 60.7 ± 1.0 mg/kg for female mice) compared to cocaine (LD_{50} = 93.0 ± 1.1 mg/kg for male mice or 92.4 ± 1.1 mg/kg for female mice). The LD_{50} values for male mice were very close to the corresponding LD_{50} values for female mice. Further, both cocaethylene oxidation under the P450 catalysis and norcocaine transesterification under the hCE-1 catalysis produce norcocaethylene, as depicted in Fig. 1. In fact, we were able to simultaneously detect cocaine and all cocaine metabolites, including cocaethylene, norcocaine, and norcocaethylene along with other non-toxic metabolites (Chen et al., 2017, 2016), in the blood (of male Sprague-Dawley rats) after co-administration of cocaine and alcohol by using a LC–MS/MS protocol. However, the toxicity of norcocaethylene has not been determined in previous studies.

This study was first aimed to determine the LD_{50} of norcocaethylene and norcocaine when they are administered IP to the same strain (Swiss Webster) of male mice used by Hearn et al. (Hearn et al., 1991) to determine the LD_{50} of cocaine and cocaethylene in order to compare the toxicity of all these toxic drugs/metabolites under the same conditions. In addition, their toxic and physiological effects were also characterized in terms of other behaviors of the mice. It has been demonstrated that norcocaethylene is the most toxic cocaine metabolite, and norcocaine is the second most toxic metabolite. Our animal data also suggest that both norcocaethylene and norcocaine are significant contributors of the acute cocaine toxicity associated with concurrent use of cocaine and alcohol. However, unlike cocaine and cocaethylene, both norcocaethylene and norcocaine do not produce hyperactivity at all.

2. Materials and methods

Materials. Cocaine, cocaethylene, norcocaine, and norcocaethylene were provided by the National Institute on Drug Abuse (NIDA) Drug Supply Program (Bethesda, MD). Calculations of the drug doses were all based on the free base weight. All other supplies were purchased from Thermo Fisher Scientific (Waltham, MA).

Animals. Male Swiss Webster mice (30–35 g) used in our studies were ordered from Charles River Laboratories (Wilmington, MA). All mice were allowed ad libitum access to food and water and maintained on a 12 h light/12 h dark cycle, with the lights on at 8:00 a.m. at room temperature of 21–22 °C. Experiments were performed in a same colony room in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The animal protocol was approved by the IACUC (Institutional Animal Care and Use Committee) at the University of Kentucky.

Acute toxicity testing. Drug (norcocaethylene or norcocaine)-induced acute toxicity was characterized in this study by the occurrence (and the time to the occurrence) of prostration, seizure, and/or death after IP administration of the drug (n = 15 for each group associated with each dose condition). Following the drug administration, mice were immediately placed in plexiglas containers (19.37 × 38.13 × 13.03 cm high) for visual observation of the presence or absence of prostration/seizure/death during the first 6 h (with a final check of the survived mice after 24 h) following cocaine administration (Zheng et al., 2008).

Locomotor activity testing. Drug (cocaine, cocaethylene, norcocaine, or norcocaethylene)-induced hyperactivity was monitored by using a video-tracking system in our lab. The locomotor activity tests were performed in high-density, non-porous plastic chambers measuring 50 cm (L) × 50 cm (W) × 38 cm (H) in a light- and sound-attenuating behavioral test enclosure (San Diego Instruments, San Diego, CA). Using the system, we were able to test 8 mice at the same time. Cumulative distance traveled was recorded by ANY-maze video tracking system (San Diego Instruments, San Diego, CA) to represent the locomotor activity. Four groups (n = 8 for each group) of mice were used, with one group for each drug (cocaine, cocaethylene, norcocaine, or norcocaethylene). Prior to the drug test, each group was tested using saline to establish the control locomotor activity. Then, each drug group was tested further for three times with different doses of drug: 10 mg/kg (IP) on Day 1, 20 mg/kg (IP) on Day 3, and 30 mg/kg (IP) on Day 5. For each test, before drug (cocaine, cocaethylene, norcocaine, or norcocaethylene with a given dose) or saline administration (n = 8 for each group), mice were allowed to acclimate to the test chambers for 4 h in a day prior to the testing day, and then 2 h right before the test. For each drug administration, the distance traveled was collected in 5-min bins. After drug or saline administration, mice were immediately returned to the test chamber for activity monitoring for 2 h after the drug/saline administration.

Data analysis. All animal data were analyzed by using the GraphPad Prism 7 software (GraphPad Software, La Jolla, CA). The statistical significance was based on one-way analysis of variance (ANOVA).

3. Results

Acute toxicity in terms of LD_{50}. To determine the LD_{50}, we tested 7 different dose conditions from 30 mg/kg to 60 mg/kg for norcocaethylene and 8 different dose conditions from 35 mg/kg to 70 mg/kg for norcocaine (see Fig. 2); 15 mice were used for each dose condition (n = 15) for each drug. In general observation of the toxicity signs, for each drug with a lethal dose, most mice first shown prostration, followed by Straub tail reaction before seizure and eventual death. By using the data shown in Fig. 2, we obtained LD_{50} = ~39.4 mg/kg for norcocaethylene, and LD_{50} = ~49.7 mg/kg for norcocaine when they were administered IP. According to the LD_{50} values summarized in Table 1, cocaine metabolites cocaethylene, norcocaine, and norcocaethylene are all more toxic than cocaine, with norcocaethylene being

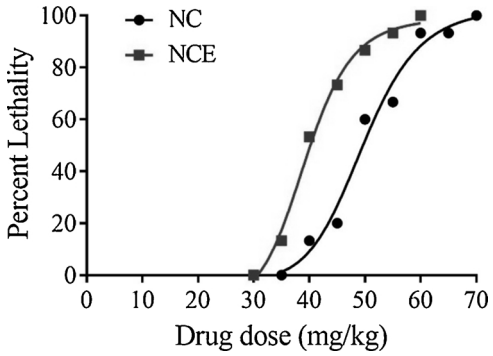

Fig. 2. Dose-dependent lethality of norcocaethylene (NCE) and norcocaine (NC). For each group of mice (n = 15), a dose of drug (NCE or NC) was administered IP. Following the drug administration, mice were immediately placed in plexiglas containers for observation.

Table 1
LD_{50} of cocaine and its toxic metabolites (given IP) in male Swiss Webster mice.

Compound	Cocaine[a]	Cocaethylene[a]	Norcocaine[b]	Norcocaethylene[b]
LD_{50} ± SD (mg/kg)	93.0 ± 1.1	63.8 ± 1.0	49.71 ± 1.64	39.39 ± 0.95

[a] Data from reference (Hearn et al., 1991).
[b] This study.

the most toxic and norcocaine being the second most toxic.

Acute toxicity in terms of the average times to the occurrence of prostration, seizure, and death after the drug administration. In order to characterize the acute toxicity of the drug (norcocaethylene or norcocaine), we also analyzed the average times (in seconds) between the drug (norcocaethylene or norcocaine) administration and occurrence of the event (prostration, seizure, or death) under all dose conditions. Within each group of mice associated with a given dose condition, the average time was calculated as the harmonic mean, i.e. the reciprocal of the arithmetic mean of the reciprocals. Depicted in Fig. 3 are the obtained average times under all dose conditions tested. According to data in Fig. 3, for a given drug (norcocaethylene or norcocaine), each event (prostration, seizure, or death) occurred earlier when the drug dose became higher. In comparison of the two drugs, each event (prostration, seizure, or death) occurred earlier in the mice administered with a given dose of norcocaethylene compared to that with the same dose of

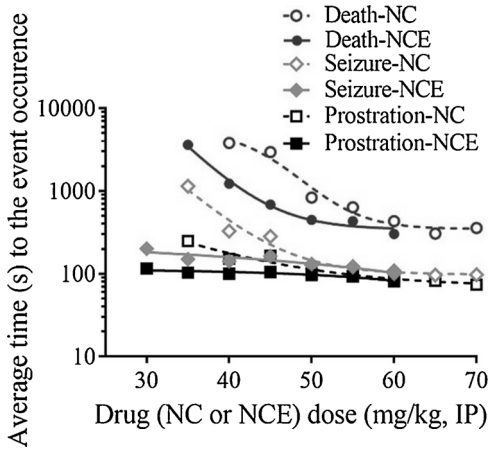

Fig. 3. Dose-dependent average times (in seconds) to the occurrence of prostration, seizure, and death of mice after IP administration of norcocaethylene (NCE) or norcocaine (NC). For each group of mice (n = 15), a dose of drug (NCE or NC) was administered IP. Following the drug administration, mice were immediately placed in plexiglas containers for observation.

norcocaine. The lower the dose, the larger the difference. The difference became smaller with increasing the dose. All these data also consistently indicate that norcocaethylene is more toxic than norcocaine.

Drug-induced hyperactivity. The stimulant effect of cocaine can be represented conveniently by the drug-inducted hyperactivity, i.e. the drug-induced increase in the locomotor activity. As seen in Fig. 4A, each dose of cocaine significantly induced hyperactivity in mice. Each dose of cocaethylene also significantly induced hyperactivity in mice (Fig. 4B). But the hyperactivity induced by a dose of cocaethylene is weaker than that by the same dose of cocaine (Figs. 4A-B and 5). In contrast, under all of the dose conditions tested, neither norcocaethylene nor norcocaine induced significant hyperactivity in mice (Figs. 4C-D and 5).

4. Discussion

Majority of cocaine users, e.g. 92% as of August 2013 (Gorelick et al., 2013), also consume alcohol. It has been known that cocaine metabolites include cocaethylene, norcocaine, norcocaethylene, and other non-toxic metabolites in concurrent use of cocaine and alcohol. But the toxicity, as well as the potential stimulant effect, of norcocaethylene are characterized for the first time in this study. It has also been known that norcocaine is more toxic than cocaine (Evans and Morarity, 1980; Zhan et al., 2014); but the dose-dependent toxicity data have never been presented in literature. So, this is the first report of the dose-dependent toxicity data for both norcocaethylene and norcocaine. For the doses used to test each drug (norcocaethylene or norcocaine), we started from 50 mg/kg for the first group. Then, the dose was decreased (or increased) by 5 mg/kg for the next group and continued until reaching 0% lethality (or 100% lethality). All of the tests were conducted in the absence of alcohol. If the tests were conducted in the presence of alcohol, norcocaine could react with alcohol to produce norcocaethylene (see Fig. 1), similar to the reaction of cocaine with alcohol. It has been known that with the alcohol co-administration, about 18% to 34% of cocaine was converted to cocaethylene, depending on the route of administration (Herbst et al., 2011). So, in the presence of alcohol, certain percent of norcocaine would be converted to norcocaethylene in the body. As a result, norcocaine would become a slightly more toxic mixture of norcocaine and norcocaethylene. Meanwhile, there should be no chemical reaction between alcohol and norcocaethylene. For a given total molar concentration, the pure norcocaethylene is still expected to be more toxic than a mixture of norcocaine and norcocaethylene. So, the presence of alcohol would not be expected to change the relative toxicity of these toxic metabolites.

It is reasonable to compare the LD_{50} values obtained from these dose-dependent toxicity data for both norcocaethylene and norcocaine with those (Hearn et al., 1991) for cocaine and cocaethylene, because they were obtained using the same strain (Swiss Webster) of male mice with the same route (IP) of drug administration; no female mice were used in the study. In fact, Hearn et al. (Hearn et al., 1991) demonstrated that the LD_{50} values of cocaine and cocaethylene in male mice were very close to the corresponding LD_{50} values in female mice. In comparison of these LD_{50} values in male mice (Table 1), we know the relative toxicity of these toxic drugs in this order: norcocaethylene > norcocaine > cocaethylene > cocaine. The toxicity data suggest that metabolites norcocaethylene, norcocaine, and cocaethylene are all significant contributors to the acute toxicity of cocaine in concurrent use of cocaine and alcohol. Hence, future therapeutic development for cocaine toxicity treatment should also account for detoxification of these more toxic metabolites including norcocaethylene, norcocaine, and cocaethylene. For example, in development of monoclonal antibodies or vaccines for cocaine, truly effective antibodies for the toxicity treatment should be capable of binding with all these toxic metabolites in addition to cocaine itself. In development of an enzyme therapy for cocaine toxicity treatment, the most effective enzyme should be capable of degrading not only cocaine itself, but also these toxic metabolites

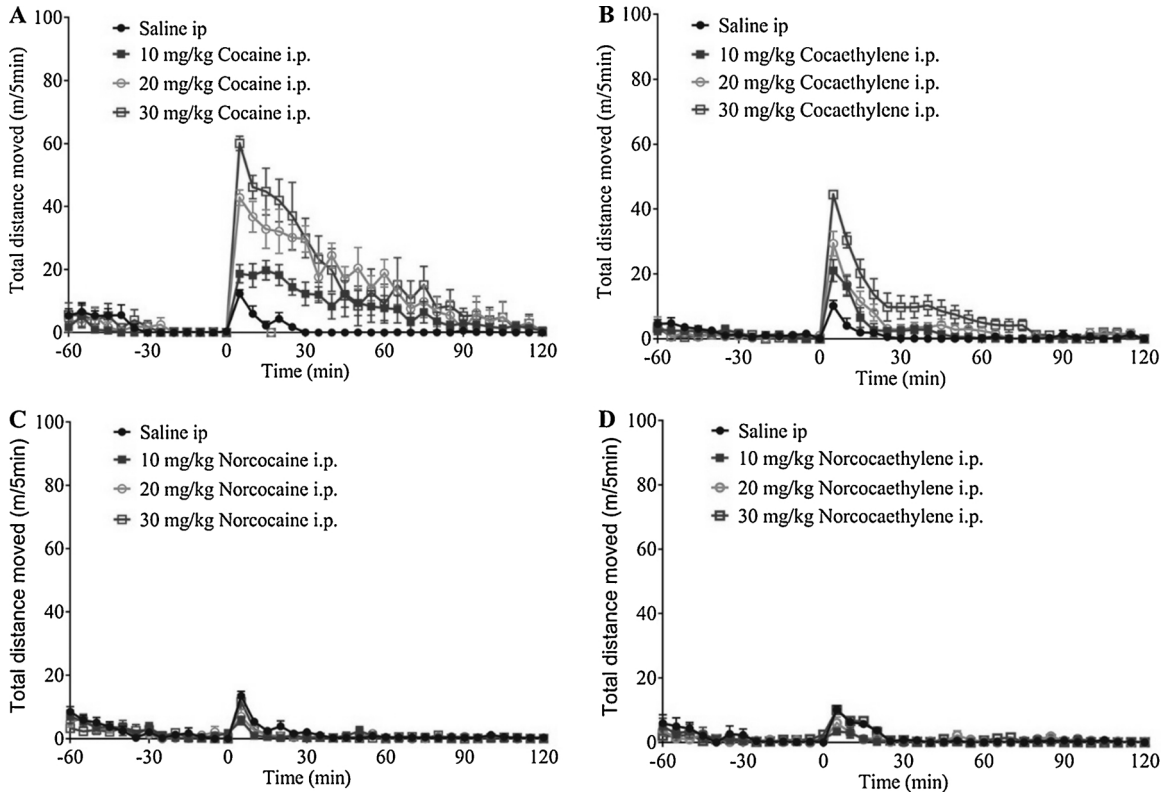

Fig. 4. Time-courses of locomotor activity (distance traveled per 5 min) produced by IP administration of cocaine (A), cocaethylene (B), norcocaine (C), or norcocaethylene (D) in comparison with the locomotor activity after the saline administration. Prior to the drug test, each group (n = 8) was tested using saline to establish the control locomotor activity. Then, each drug group was tested further for three times with different doses of drug: 10 mg/kg (IP) on Day 1, 20 mg/kg (IP) on Day 3, and 30 mg/kg (IP) on Day 5. For each test, before drug or saline administration, mice were allowed to acclimate to the test chambers for 4 h in a day prior to the testing day, and then 2 h right before the test. For each drug administration, the distance traveled was collected in 5-min bins. After drug or saline administration, mice were immediately returned to the test chamber for activity monitoring for 2 h after the drug/saline administration.

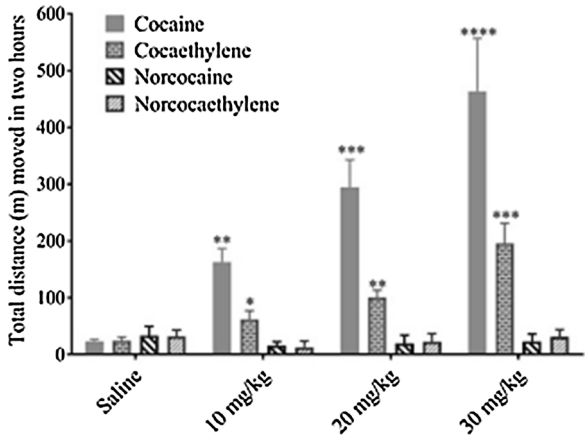

Fig. 5. Total distance (m) traveled in two hours after IP administration of saline, cocaine, cocaethylene, norcocaine, or norcocaethylene in four different groups (n = 8; see caption of Fig. 4 for the details). The statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$) was based on comparison with the saline in the same group.

(Hou et al., 2014; Pan et al., 2005; Xue et al., 2013, 2011; Zhan et al., 2014; Zheng et al., 2014, 2008). In development of a small-molecule therapeutic agent, one should account for not only the protein targets of cocaine, but also the protein targets of these toxic metabolites.

Further, based on the locomotor activity data shown in Figs. 4 and 5, within the four toxic drugs/metabolites (cocaine, cocaethylene, norcocaine, and norcocaethylene), the least toxic drug cocaine has the strongest stimulant effect, whereas the most toxic metabolites norcocaethylene and norcocaine have no stimulant effect at all. In addition, all of these four drugs are expected to have similar blood-brain barrier (BBB) permeability due to their similar structural features or according to the previously reported BBB permeability prediction method (Yuan et al., 2018). The obtained locomotor data together with the relative toxicity data further suggest that the primary targets for the most toxic metabolites norcocaethylene and norcocaine are likely different from those for cocaine and cocaethylene. This insight might make more complicated/difficult the development of a desirable small-molecule therapeutic agent for treatment of cocaine toxicity or addiction.

In addition, it is well-known that the relative toxicity of different drugs can be characterized by their LD_{50} values. So, to know the relative toxicity of different drugs, one may determine the LD_{50} for each drug by using a lot of animals associated with a series of doses. In this study, we have demonstrated that the relative toxicity of different drugs characterized by their LD_{50} values correlates with the average time (harmonic mean) to the occurrence of death, seizure, or prostration after the drug administration with a same dose, especially when the dose is close to or lower than their LD_{50} values (but high enough to have the event occurrence). For example, according to the data in Fig. 3, at the dose of 40 mg/kg, the average time to the occurrence of death was 1229s for norcocaethylene and 3822 s for norcocaine, the average time to the occurrence of seizure was 142 s for norcocaethylene and 328 s for norcocaine, and the average time to the occurrence of prostration was 100 s for norcocaethylene and 152 s for norcocaine. All these data are consistent with the relative LD_{50} values in terms of the relative toxicity.

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Contributors

FZ and CGZ conceptualized the present work. XZ and LS conducted the experiments. All authors analyzed the data. FZ and CGZ wrote the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors have no conflicts of interest.

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