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Research article

Abuse potential and dopaminergic effect of alkyl nitrites

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HIGHLIGHTS

- The abuse of alkyl nitrites is common among adolescents and young adults worldwide.
- Mice treated with alkyl nitrites showed greater drug-paired place preference.
- Isobutyl nitrite induced greater dopamine release from striatal synaptosomes.
- Alkyl nitrites could lead to dependence and dopaminergic effects.
- This provides evidence for controlling alkyl nitrites as psychoactive substances.

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ABSTRACT

The abuse of alkyl nitrites is common among adolescents and young adults worldwide. However, the information regarding the effects of alkyl nitrites on the central nervous system and the associated psychological abuse potential is scarce. The abuse potential of 3 representative alkyl nitrites – isobutyl nitrite, isoamyl nitrite, and butyl nitrite – was evaluated in mice using conditioned place preference tests with an unbiased method. The dopamine levels released by synaptosomes extracted from the striatal region were measured using high performance liquid chromatography. Mice treated with the test substances (50 mg/kg, i.p.) exhibited a significantly increased drug-paired place preference. Moreover, greater levels of dopamine were released by striatal region synaptosomes in response to isobutyl nitrite treatment in mice. Thus, our findings suggest that alkyl nitrites could lead to psychological dependence and dopaminergic effects. Furthermore, these results provide scientific evidence to support the regulation of alkyl nitrites as psychoactive substances in the future.

1. Introduction

Alkyl nitrite – generally known as popper – is a flammable liquid chemical that is commonly sold as room air fresheners or deodorizers in some clubs, headshops, and online [1]. In addition, poppers are used for recreational purposes as they quickly induce euphoria and sexual arousal [2,3]. Amyl nitrite has also been previously used to manage medical conditions such as angina pectoris and cyanide poisoning [4], as it results in blood vessel dilation. However, nausea, disorientation, and maculopathy were found to be the adverse effects of this medication, and its use in the medical setting was consequently discontinued [5,6]. Nevertheless, certain alkyl nitrites are still being marketed as "rush," "poppers," "snappers," and other names, particularly for aphrodisiac purposes. This constitutes a major problem as they are easily available to young people [7], and their use is associated with attempted suicide [8].

The decision to control alkyl nitrite use as a psychoactive substance has been controversial in many countries. In France, alkyl nitrites were controlled under Decree No. 2007-1636 on November 20, 2007, "on products containing aliphatic alkyl nitrites, cyclic, heterocyclic or their isomers for their isomers for the consumer and not benefiting from a marketing authorization," which was abolished in 2009 due to trade-related problems. Nevertheless, certain articles have emphasized on the need to control these substances [9].

Abbreviations: CPP, conditioned place preference; CNS, central nervous system; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; ECD, electrochemical detector.

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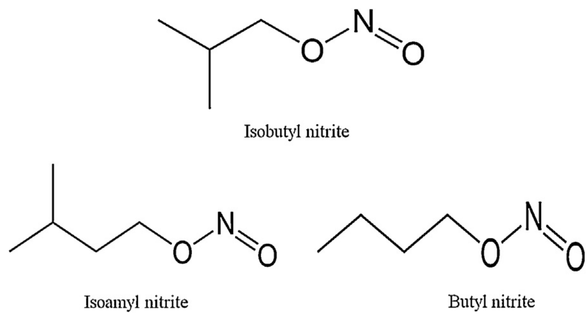

Fig. 1. Chemical structures of isobutyl nitrite, isoamyl nitrite, and butyl nitrite.

In Japan, 6 alkyl nitrites (isobutyl nitrite, isopropyl nitrite, isoamyl nitrite, tertiarybutyl nitrite, cyclohexyl nitrite, and butyl nitrite) are controlled as "designated substances" under the "Pharmaceutical Affairs Law." In some countries such as Korea, it is vital to first elucidate the action on the central nervous system (CNS) and the dependence potential of a drug before it can be controlled as a psychoactive substance. Thus, the findings of the present study could serve as a legal basis for controlling the tested substance as a psychoactive substance, under the "Act on Narcotics Control".

Several reports have provided evidence of the toxicity induced by alkyl nitrite inhalation. In particular, ophthalmological toxicity is one of the well-known adverse effects. Some reports have described cases of alkyl nitrite-induced maculopathy [10–12] or visual loss [13]. Other toxic effects such as immunotoxicity [14], hepatotoxicity [15], or cardiovascular toxicity [16] have also been reported in animals. Although the toxic effects of alkyl nitrites have been reported in the literature, the information on the abuse potential is scarce and is only briefly described in certain anecdotal reports [9].

Physical or psychological dependence is the state of tolerance to and withdrawal from a drug after a prolonged period of use [17]. Several experimental approaches have involved behavioral pharmacological techniques. One such method is the conditioned place preference (CPP) test, which specifically evaluates the rewarding effects of unknown substances [18–20]. Moreover, it is well known that substance dependence is related to the dopaminergic pathway of the CNS. Hence, the measurement of dopamine levels in the CNS can be useful for predicting the abuse potential of a substance. Several investigations of the CNS using brain slices or synaptosomes have indicated that neurotransmitters may be regulated via additional mechanisms [21,22].

In the present study, the CPP paradigm was adopted to evaluate the abuse potential of 3 representative alkyl nitrites – isobutyl nitrite, isoamyl nitrite, and butyl nitrite. Moreover, the changes in the dopamine levels induced by alkyl nitrites were measured via high performance liquid chromatography (HPLC) to elucidate the mechanism of action of alkyl nitrites.

2. Materials and methods

2.1. Animals and substances

Male ICR mice (age: 8–12 weeks; weight: 30–37 g) were obtained from Samtacobio Korea (Osan, Korea). The mice were maintained in a temperature- (23 ± 1 °C) and humidity-controlled (55% ± 5%) room with a 12-h light/dark cycle (lights on from 07:00 to 19:00); laboratory mouse chow and water were provided ad libitum. Handling occurred only during the light cycle. Alkyl nitrites (isobutyl nitrite, isoamyl nitrite, and butyl nitrite; Fig. 1), cocaine, and methamphetamine HCl were purchased from Sigma Aldrich (St. Louis, MO, USA). For the CPP test, animals received intraperitoneal (i.p.) injections of vehicle (saline) or cocaine (20 mg/kg) and 2 doses of the test substances (isobutyl nitrite [5 and 50 mg/kg], isoamyl nitrite [5 and 50 mg/kg], and butyl nitrite [5 and 50 mg/kg]). For HPLC analyses, 3 doses of methamphetamine (1, 10, and 100 μM) and 3 doses of isobutyl nitrite (0.3, 3, and 30 μM) were administered to the synaptosomes in the striatal regions of the brain in the mice. All the animal experiments in the present study were approved by the National Institute of Food and Drug Safety Evaluation/Ministry of Food and Drug Safety Animal Ethics Board (Approval number: 1501MFDS04).

2.2. CPP apparatus

The CPP test box and chamber is manufactured by Saeronbio Inc. (Korea) and consists of 2 distinct compartments (black and white) separated by guillotine doors. The dimensions of each compartment of the white and black box are 15 × 17 × 15.5 cm. The duration of time spent by the animals in each box for conditioning was recorded based on infrared detection via a sensor controller.

2.3. The CPP test

The test consists of 5 phases: (1) Habituation: For 2 days (day 1 and 2), the mice were allowed free access to both compartments of the apparatus for 15 min on each day. (2) Pre-conditioning: The mice were drug-free and were allowed unrestricted access to both compartments of the apparatus for 15 min. The time spent by the mice in each compartment was recorded, and these values served as a baseline. Accordingly, the mice showed a preference for either the black or white compartment, and the preference was recorded within the scope of the mean (15–20%); based on these results, the mice were selected for further experiments and were assigned to 8 groups. (3) Conditioning: On day 4, one group of the selected mice was treated with drugs, and was maintained in the white compartment for 40 min. On the next day (day 5), the group of mice was treated with saline, and was maintained in the black compartment for 40 min. This protocol was repeated for 4 cycles (8 days). During the drug- and saline-paired sessions, the compartments were closed using a guillotine door. (4) Post-conditioning: On day 12, the mice were drug-free and were allowed free access to both compartments of the apparatus for 15 min. The time spent by the mice in each compartment was recorded, and these results were used as test values. (5) Scoring: The results were calculated based on the difference in post-conditioning (test values) and pre-conditioning (baseline values). During the 40-min pairing sessions, the mice were intraperitoneally injected with 5 or 50 mg/kg of alkyl nitrites in either the black or white compartment; the sessions were conducted on alternate days and alternated with saline administration, thus achieving a total of 4 pairings for each treatment.

2.4. Preparation of synaptosomal fractions

Synaptosomes were prepared as previously described [23–26], with slight modifications. Untreated male ICR mice were killed by cervical dislocation and decapitation, and the striatum region of their brain was quickly removed (n = 3). The striatum was homogenized in 10 volumes of ice-cold 0.32 M sucrose by using a Dounce tissue grinder (Kontes, USA). The lysates were centrifuged for 10 min/3000*g* at 4 °C. The supernatant (S1), containing the crude synaptosomal fraction, was transferred to a new tube. The S1 lysate was added and diluted in a 1:1 fashion with Krebe-Hepes buffer pH 7.4 (117 mM NaCl, 4.8 mM KCl, 2.5 mM $MgCl_2$, and 25 mM Hepes), and then centrifuged for 20 min/10,000*g* at 4 °C to obtain the pellet (P1).

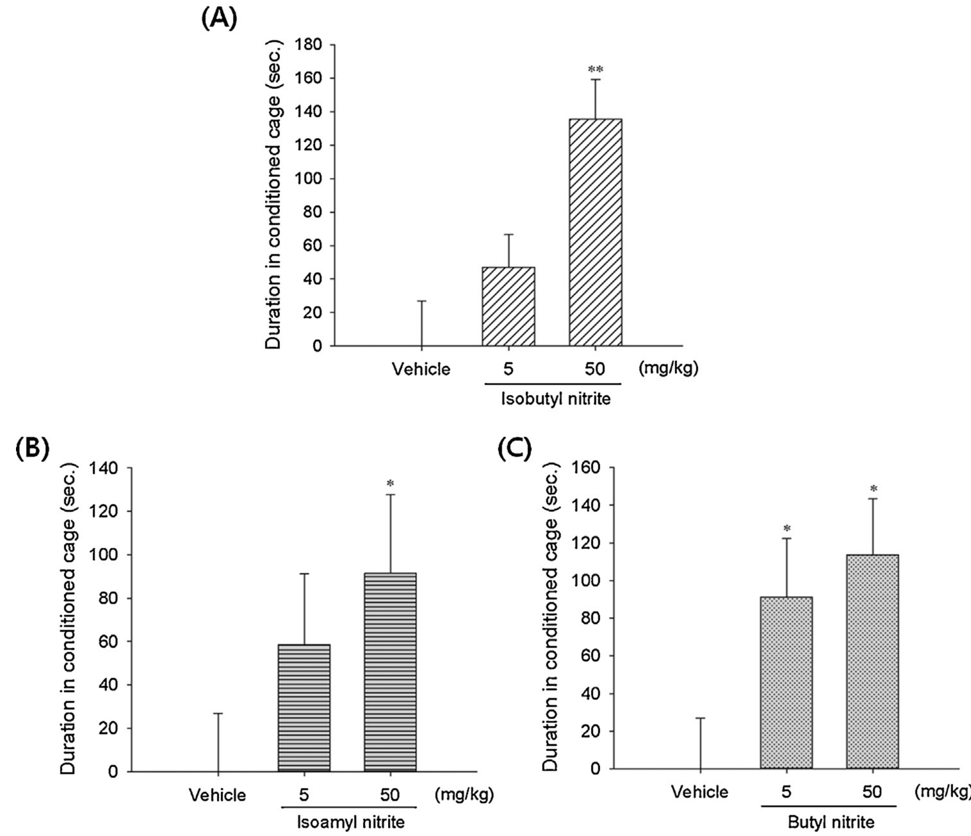

Fig. 2. Effects of intraperitoneally injected isobutyl nitrite, isoamyl nitrite, and butyl nitrite, as determined by the conditioned place preference (CPP) test. (A) isobutyl nitrite, (B) isoamyl nitrite, and (C) butyl nitrite induced CPP at 5 and 50 mg/kg doses in mice. Data are expressed as mean ± standard error of the mean of 8 animals per group. *P < 0.05, **P < 0.01 indicates statistical significance, when compared with the vehicle-treated group (one-way analysis of variance, followed by a Holm-Sidak post-hoc test).

2.5. Western blot

The protein concentration of the synaptosome was determined by the Smart BCA Protein Assay Kit (iNtRON Biotechnology, Seongnam-si, South Korea). The protein (10 μg protein per lane) was run on 10% SDS-PAGE gels and then transferred electrophoretically to a polyvinylidene difluoride membrane (Invitrogen, Carlsbad, CA, USA) for 2 h at 200 mA. The membranes were blocked with 5% bovine serum albumin prior to incubation with the primary antibody overnight at 4 °C. It was then treated with a horseradish peroxidase (HRP)-conjugated antibody (secondary antibody) for 1 h at room temperature. We used the following primary and secondary antibodies: rabbit. anti-*N*-methyl-D-aspartic acid receptor 2A (NMDAR2A; G9038, Sigma, St. Louis, MO, USA; 1:3000 dilution), anti-β-actin (PA1-183, Thermo Fisher Scientific, Waltham, MA, USA; 1:000 dilution), and anti-rabbit IgG HRP-linked antibody (7074S, Cell Signaling Technology, MA, USA; 1:2000 dilution). The blots were visualized using the ECL Chemiluminescense detection kit (#32132, Thermo Fisher Scientific, Waltham, MA, USA). Moreover, the blots were analyzed via digital image analysis with a chemidoc (Bio-rad, Hercules, CA, USA) and lab program (version 4.1, Bio-rad, Hercules, CA, USA).

2.6. Treatment of substances

The pellet (P1) was mixed with 80 volumes of 20 nM dopamine, and then incubated at 37 °C for 15 min. The pellet (P2) was obtained after 10 min of centrifugation at 10,000*g*. Thereafter, several doses of 300 μl methamphetamine (1, 10, and 100 μM) and isobutyl nitrite (0.3, 3, 30 μM) were added to the same amount of P2 prior to incubation at 37 °C for 15 min. The supernatant was obtained after the addition of 1 ml of 0.1 M perchloric acid and centrifugation for 3 min at 20,000*g*. After filtration, the supernatant was measured by using a HPLC-electrochemical detector (ECD) instrument (DIONEX UltiMate 3000, Thermo Fisher Scientific, Waltham, MA, USA).

2.7. Measurement of dopamine levels

Dopamine levels in the synaptosome were detected by using an Acclaim™ RSLC120 C18 column (2.2 μm 120 Å 2.1 × 50 mm; Thermo Fisher Scientific, Waltham, MA, USA) and an oven temperature of 35 °C. The flow rate was 0.5 ml/min, and the injection volume was 40 μl. The voltage of the ECD was maintained at 250 mV, and sample detection was conducted for 3.5 min. Dopamine was separated by using a mobile phase consisting of 6.9 g NaH_2PO_4, 250 mg 1-heptanesulfonic acid sodium salt, 80 mg EDTA (pH 3.2), and 10% HPLC-grade methanol. The dopamine levels were analyzed by using Chromeleon™ 7 (Thermo Fisher Scientific, Waltham, MA, USA).

2.8. Statistical analysis

All the results are expressed as means and standard error of the mean (±S.E.M.). CPP test and HPLC analysis data were analyzed via one-way analysis of variance tests using a sigma plot (13.0). Holm-Sidak post-hoc tests were used to identify the drug doses that induced significant changes in comparison to those noted in the vehicle-treated animals. *P < 0.05, **P < 0.01 compared to the vehicle-treated group.

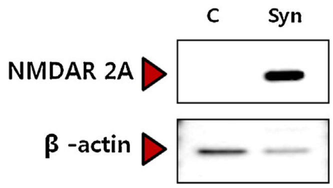

Fig. 3. Protein expression from the striatal synaptosomes of mice. A total of 10 μg of protein obtained from mouse brain tissue cytosol (C) and synaptosomes (Syn) were analyzed via western blotting. NMDA receptor 2A was used as a synaptic marker, whereas β-actin was used as an internal control for western blotting. An analysis of the blots was performed via digital image analysis with chemidoc (Bio-rad).

3. Results

3.1. Effects of alkyl nitrites, as determined via the CPP test

Place preference, followed by 4 pairs of conditioning with the tested alkyl nitrites at 2 doses (5 and 50 mg/kg), was measured. Cocaine (20 mg/kg) and saline (vehicle) were used as a positive control (data not shown) and negative control, respectively. The experimental mice who were treated with 50 mg/kg of isobutyl nitrite, isoamyl nitrite, and butyl nitrite showed significantly higher preference scores than the vehicle group in the substance-paired compartment ($P < 0.05$). Although isobutyl nitrite and isoamyl nitrite induced a significant preference only at the 50 mg/kg dose, butyl nitrite showed place preference at both 5 and 50 mg/kg doses ($P < 0.05$, Fig. 2).

3.2. Identification of isolated synaptosomes

To identify whether the synaptosomes were isolated from the striatal region of experimental animals, the NMDA 2A receptor levels were assessed via western blotting. The expression levels of the NMDA 2A receptors from the extracted synaptosomes were greater than those in the cytosol (Fig. 3).

3.3. Effects of isobutyl nitrite on the synaptosomal release of dopamine

The extracted synaptosomes were treated with test substances, and the changes in the dopamine levels were measured using HPLC-ECD. Methamphetamine was used as a positive control (data not shown) and vehicle (saline) was used as a negative control. We found that isobutyl nitrite and methamphetamine significantly increased the dopamine levels at methamphetamine doses of 1, 10, and 100 μM, and isobutyl nitrite doses of 0.3, 3 and 30 μM ($P < 0.05$, Fig. 4).

4. Discussion

Alkyl nitrites are commonly used, particularly by young individuals, due to their easy availability. These individuals are often unaware about the adverse effects of alkyl nitrites, including the related toxicity. However, several previous reports have described the toxicity of alkyl nitrites. Nitrite inhalation induces changes in the immune system by initially suppressing immune function, followed by a period of non-specific immune stimulation [27]. Nitrites are also known to lead to vision loss and reduced visual acuity [28,29]. Nevertheless, no studies have focused on the action of alkyl nitrites on the CNS or the potential for their abuse.

In the present study, we assessed the abuse potential in terms of the behavioral pharmacological aspect by using experimental animals. Since alkyl nitrites – sold as poppers on the street – are increasingly being used for recreational purposes [30,31], the need for its control as a psychoactive substance is becoming a major

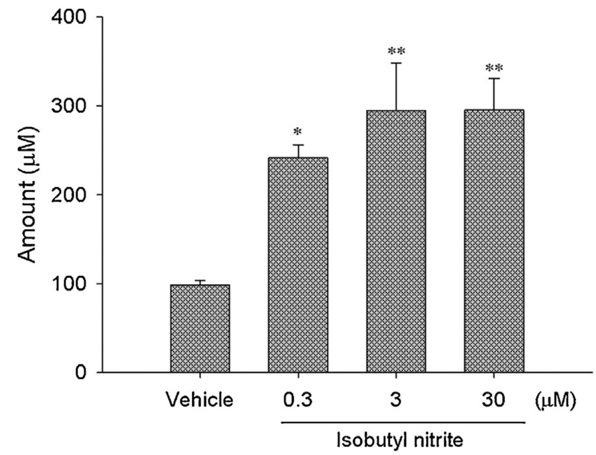

Fig. 4. Changes in the dopamine levels induced by the administration of isobutyl nitrite. The dopamine levels increased in a dose-dependent manner in the striatal synaptosomes of mice, as determined by the high performance liquid chromatography-electrochemical detector (ECD) method. (flow rate: 500 μl/min, ECD voltage: 250 mV, sample injection: 40 μl). $^{*}P < 0.05$, $^{**}P < 0.01$ indicates statistical significance, when compared with the vehicle-treated group (one-way analysis of variance, followed by a Holm-Sidak post-hoc test).

problem. In order to enact legislation for controlling alkyl nitrite use, its action on the CNS and its potential for abuse need to be determined. With regard to its action on the CNS, almost all the previous reports have focused on its effects on the peripheral nervous system, including smooth muscle relaxation and vasodilation [32]. Even though some reports on the CNS have been published, most of them have not provided scientific evidence. For instance, Klonne et al. indicated that individuals who used alkyl nitrites reported dizziness, nausea, and headache, thus indirectly suggesting their effects on the CNS [33]. Moreover, the neurotoxicity of alkyl nitrites has been mentioned in another recent report, although it primarily focused on learning and memory deficits [34]. The finding of increased dopamine levels from the synaptosomes in the present study could represent novel evidence regarding the effects of alkyl nitrites on the CNS.

Furthermore, we used the CPP test with experimental rodents to assess the potential for abuse of alkyl nitrites. CPP has been used to evaluate psychological dependence, particularly in terms of the rewarding aspect, in the behavioral pharmacology field [35]. Thus far, no scientific evidence of physical or psychological dependence caused by alkyl nitrite use has been reported; in fact, only a few anecdotal reports have suggested the abuse potential of alkyl nitrites. One such report states that a man developed alkyl nitrite-induced tolerance [9], and hence reasoned that this substance hence has a potential for abuse. Another report indicated that individuals who inhaled alkyl nitrites during their youth were more likely to use other drugs [36]. Thus, we believe that the use of the CPP on mice in the present study to determine the abuse potential of alkyl nitrites was the first such scientific assessment on this topic.

The relationship between CPP and dopamine has been assessed in several articles in the literature. Changes in dopamine levels are one of the factors influencing drug addiction; in fact, this is a relatively well-defined theory [37,38]. The CPP paradigm is also well-known for evaluating abuse potential, particularly the rewarding effects [39]. Hence, the changes in CPP could be evaluated for the dependence of a specific substance; once the substance has been confirmed to have dependence potential, the dopamine levels can then be assessed. Thus, we believe that the HPLC system established in the present study can be used as a prediction method for abuse potential of unknown substances.

There are several limitations of the present study. First, there is a lack of data regarding the proper concentration of alkyl nitrites used in the CPP test. Second, the experiment investigating the changes in the dopamine levels in synaptosomes usually involves only a single administration of isobutyl nitrite; however, repeated treatments are generally required to assess the dependence potential. Third, the dopamine levels released following the administration of other nitrite substances, such as isoamyl nitrite and butyl nitrite, should also be analyzed, although similar effects can be expected due to the similarity in the chemical structure of the three alkyl nitrites.

In conclusion, isobutyl nitrite, isoamyl nitrite, and butyl nitrite have the potential for psychological dependence. Moreover, the changes in the dopamine level induced by these substances were tested ex vivo by using synaptosomes at the striatal region in the mouse brain via HPLC; these findings provided clear evidence of the effect of alkyl nitrites on the CNS. The evidence of the CNS action and abuse potential of the tested substances could be used to ensure their control as psychoactive substances. Moreover, the method for measuring dopamine levels from synaptosomes can also be used to predict dopamine level changes in the CNS when screening for numerous new psychoactive substances, after some modifications.

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