Effects of alcohol on metabolism and toxicity of cocaine in rats

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**ABSTRACT**

As most cocaine users drink alcohol, it is interesting to understand how a non-lethal dose of alcohol affects the metabolism and toxicity of cocaine. In this study, we examined the correlation between dose-dependent toxicity and the metabolism/pharmacokinetic (PK) profile of cocaine with or without alcohol (ethanol, 1 g/kg) co-administration in rats. The cocaine toxicity in rats with or without alcohol co-administration is characterized by not only the commonly used LD_{50}, but also the average times for the appearance of convulsion and death as well as total toxicity level (TTL) in the blood. All these data have consistently demonstrated that co-administration of alcohol enhances cocaine toxicity, and that the alcohol-enhanced toxicity of cocaine is mainly attributed to the observed two additional metabolites (cocaethylene and norcocaethylene – products of chemical reactions of cocaine with alcohol catalyzed by metabolic enzymes carboxylesterase-1 and liver microsomal cytochrome P450 3A4) that are more toxic than cocaine itself. So, evaluation of the substance TTL should account for the blood levels of not only cocaine itself, but also its all toxic metabolites. In addition, for rats died of a lethal dose of cocaine (60 or 100 mg/kg) combined with 1 g/kg alcohol, we also determined the TTL at the time of death, demonstrating that death would occur once the TTL reached a threshold (~16 μM).

1. Introduction

As one of the most reinforcing drugs of abuse [1–3], cocaine is highly toxic [4]. When overdosed, cocaine may cause cardiovascular failure, respiratory depression, seizure, and death [4–6]. The toxicity of cocaine increases when it is used in combination with alcohol (which always refers to ethyl alcohol or ethanol in this report) [7–9]. Notably, concurrent use of cocaine and alcohol is very popular among drug users. Particularly, ~92 % of cocaine users drink alcohol [10]. Both cocaine and alcohol are toxic when the doses are high. It is also known [11] that concurrent use of cocaine and alcohol produces additional metabolites, and that multiple metabolites of cocaine are even more toxic than cocaine itself. Hence, it is reasonable to assume that the enhanced cocaine toxicity in the presence of alcohol is relevant to the alcohol-induced change in cocaine metabolism in the body.

Cocaine is metabolized via multiple pathways in the body [12–15]. First of all, cocaine is metabolized through hydrolysis of the benzoyl ester moiety (Fig. 1). The benzoyl ester hydrolysis produces metabolites ecgonine methyl ester (EME) and benzoic acid. This metabolic pathway is catalyzed mainly by plasma enzyme butyrylcholinesterase (BChE), although carboxylesterase-2 (hCE-2) also catalyzes benzoyl ester hydrolysis [16]. Second, cocaine is metabolized by liver carboxylesterase-1 (hCE-1) through cocaine methyl ester hydrolysis. This metabolic pathway generates metabolites benzoylecgonine and methanol (Fig. 1). In addition to the hydrolysis reactions, cocaine is also oxidized by liver microsomal cytochrome P450 3A4 (CYP3A4) to produce metabolite norcocaine [17] (Fig. 1). Further, in the presence of alcohol, cocaine metabolism also generates additional metabolites cocaethylene [18] and norcocaethylene [19] (Fig. 1). It has been demonstrated that cocaethylene, norcocaine, and norcocaethylene are all more toxic than cocaine itself, with the relative acute toxicity in the order of norcocaethylene > norcocaine > cocaethylene > cocaine [11]. These toxic metabolites (cocaethylene, norcocaine, and norcocaethylene) should be considered as significant contributors to cocaine toxicity in the presence of alcohol.

In the present study, we aimed to determine the detailed effects of alcohol on cocaine toxicity (including not only the LD_{50}, but also the timing of the substance-induced convulsion and death) and the
pharmacokinetic (PK) profile of cocaine (including the time courses of cocaine and its metabolites) in rats. To determine the effects of alcohol on the metabolism and PK profile of cocaine, an LC-MS/MS method was used to simultaneously determine the concentrations of cocaine and its metabolites in the blood samples collected at various time points after cocaine administration in combination with alcohol to compare with the corresponding PK profile without alcohol. The obtained data have revealed how alcohol affects the PK profile and metabolism of cocaine, as well as the total toxicity of cocaine and its toxic metabolites.

2. Materials and methods

2.1. Materials

(-)-Cocaine was provided by the National Institute on Drug Abuse (NIDA) Drug Supply Program (Bethesda, MD). [3H](-)-cocaine (50 Ci/mmol) was ordered from PerkinElmer (Waltham, Massachusetts). Wild-type BChE protein was prepared in the same way as reported earlier [20]. Human hCE-2 protein was purchased from Sigma-Aldrich (CAS # 9016–18–6). All other chemicals were purchased from Thermo Fisher Scientific (Waltham, MA), Sigma-Aldrich (St. Louis, MO), or VWR International (Radnor, PA). Cocaine-HCl solution (1 mL/kg) and alcohol (20 % w/v for 1 g/kg dose, 30% w/v for 3 g/kg dose and 60 % w/v for 6 g/kg dose) was prepared using sterile saline and injected i.p. at different concentrations.

2.2. Animals

Rats were chosen so that blood sampling at multiple time points within a day for PK studies was feasible. Male Sprague-Dawley rats (250–275 g) used in this study were ordered from Harlan/Envigo (Indianapolis, IN), and housed initially as one or two rats per cage, including the following groups:

![Fig. 1. Metabolic pathways of cocaine in the presence of alcohol.](image-url)
Eight groups of rats (n = 8 per group) were used to determine LD_{50} of cocaine + 1 g/kg alcohol with a cocaine dose of 10, 20, 40, 50, 60, 75, 100, or 180 mg/kg (i.p.);

Eight groups of rats (n = 8 per group) were used to determine LD_{50} of cocaine (without alcohol) with a cocaine dose of 10, 20, 40, 50, 60, 75, 85, 100, or 180 mg/kg (i.p.);

A group of 4 rats were used to determine time-dependent blood concentrations of cocaine and cocaine metabolites with i.p. administration of 20 mg/kg cocaine and 1 g/kg alcohol;

A group of 4 rats were used to determine time-dependent blood concentrations of cocaine and cocaine metabolites with i.p. administration of 20 mg/kg cocaine only;

Additional rats were used for i.p. administration of 60 mg/kg cocaine + 1 g/kg alcohol until the number of survived rats reached 4 and the number of dead rats reached 4 required for determining time-dependent blood concentrations of cocaine and cocaine metabolites;

Additional rats were used for i.p. administration of 60 mg/kg cocaine only until the number of survived rats reached 4 and the number of dead rats reached 4 required for determining time-dependent blood concentrations of cocaine and cocaine metabolites.

All rats 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 a room temperature of 21–22 °C. Experiments were performed in the 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.

2.3. Acute toxicity testing

Substance (cocaine only, alcohol only, or combined use of cocaine and alcohol)-induced acute toxicity was characterized in this study by the occurrence (and the time to the occurrence) of convulsion and death. Rats were immediately placed in chambers for observation of the presence or absence of convulsion/death during the first 6 h (with a final check of the survived rats after 24 h) [21] after administration of the substance.

2.4. Metabolism of cocaine

After the intraperitoneal (i.p.) injection of cocaine (or both cocaine and alcohol), blood samples (75 μL/sample) were collected from saphenous veins into heparin-treated capillary tubes at various time points and mixed with 100 μL paraoxon solution (250 μM paraoxon with 10 U/mL heparin in 0.1 % formic acid). Blood samples were stored at −80 °C until analysis by using our previously developed LC-MS/MS method [22] for simultaneously detecting the concentrations of cocaine and metabolites in blood samples.

2.5. Enzyme activity assay against cocaine

To examine whether alcohol affects the catalytic activity of enzyme BChE or hCE-2 against (-)-cocaine, the catalytic activity of enzyme (BChE or hCE-2) was tested in the absence and presence of alcohol (at a concentration of 80 mg/dL or 17.6 mM, equivalent to the reported concentration of 80 mg/dL or 17.6 mM, equivalent to the reported catalytic parameters (K_{M} = 202 μM and k_{cat} = 5.3 min^{-1}) [25] of hCE-2 against (-)-cocaine. Concerning the enzyme concentration used in the assays, 10 μL of 1.85 mg/mL BChE was added to the reaction system (200 μL), with a final BChE concentration of 0.0925 mg/mL in the reaction system. The enzymatic reaction was allowed to continue for 8 min such that only ~20 % of cocaine was converted to metabolites before it was stopped by 0.02 M HCl. The enzymatic reactions catalyzed by hCE-2 were also carried out in the similar way, but with 10 μL of the original hCE-2 solution (received from Sigma-Aldrich) added to the reaction system (200 μL), and also with no more than ~20 % of cocaine converted to metabolites.

2.6. Data analysis

All animal data were analyzed by using the GraphPad Prism 7 software (GraphPad Software, La Jolla, CA). Data are presented as mean ± SEM. The PK parameters for cocaine and its metabolites were calculated by using the MATLAB software and the noncompartmental model coded in the Phoenix WinNonlin software (Certara, Princeton, NJ). The elimination half-life (t_{1/2}) was estimated by the elimination rate constant based on log-linear regression of the terminal portion of the plasma concentration-time curve. For the enzymatic reaction data, the enzyme activity of a given enzyme (BChE or hCE-2) against (-)-cocaine in the absence of alcohol was used as the standard reference (100% activity) to compare with the corresponding enzyme activity in the presence of alcohol (with t-test).

3. Results and discussion

3.1. Acute toxicity of alcohol

In this study, we first wanted to determine the effects of a non-lethal dose of alcohol on the toxicity of cocaine in rats. Hence, with the acute toxicity testing of alcohol, we tried to identify an appropriate alcohol dose which is not lethal for rats. For this purpose, three different alcohol dose levels (1, 3, and 6 g/kg, i.p.) were tested in rats. All three dose levels should give a blood alcohol concentration much higher than the blood concentration of cocaine and its metabolites. Eight rats were used for each dose level (n = 8/group), and the observations were recorded over 6 h on day 0 and then 24 h after the alcohol injection. The first alcohol dose level tested in our study was 6 g/kg, because 6 g/kg of alcohol was used in previous studies on the acute toxicity of alcohol-cocaine combination in rodents [26]. All rats died within 5 min to 1 h after i.p., injection of 6 g/kg alcohol. So, the alcohol dose of 6 g/kg was too high for rats. When the alcohol dose was reduced to 3 g/kg, rats presented coma 1 min after the injection and the coma lasted for over 4 h although no rats died. However, after intraperitoneal injection of 1 g/kg alcohol, no toxic signs were observed in any rats, with all rats showing normal standing and reflection. Based on the dose-dependent toxicity, 1 g/kg (or ~22 mmol/kg) alcohol was determined as the appropriate dose of alcohol for further tests in this study. It has been known [23] that the average blood alcohol concentration in rats was ~80 mg/dL (or ~17.6 mM) after i.p. administration of 1 g/kg alcohol, which was far excess for reaction with cocaine (≤180 mg/kg, i.p. with a plasma concentration ≤150 μM [6]) used in this study.

3.2. Acute toxicity of the cocaine and alcohol combination in comparison with cocaine alone

The acute toxicity of a substance (or a combination) may be characterized by LD_{50} and the average times for the occurrence of convulsion and death after the substance administration. We tested eight different cocaine dose levels between 10 mg/kg and 180 mg/kg with or without co-administration of 1 g/kg alcohol in order to determine the LD_{50}, with eight rats for each group (n = 8/group). The eight cocaine dose levels are 10, 20, 40, 60, 75, 85, 100, and 180 mg/kg without alcohol or 10, 20, 40, 50, 60, 75, 100, and 180 mg/kg with 1 g/kg alcohol. The
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3.3. Effects of alcohol on metabolic profile of cocaine

To investigate how alcohol changes the metabolism and PK profile of cocaine, a cocaine pharmacokinetic (PK) study was performed to measure the blood concentrations of cocaine and its metabolites after the administration of cocaine and alcohol in additional rats. In this study, we determined the PK profile of 20 mg/kg and 60 mg/kg cocaine administered via i.p. injection with or without co-administration of 1 g/kg alcohol (i.p.). Specially for the i.p. injection of 60 mg/kg cocaine, the PK was determined in survived rats (n = 4), due to the death of some rats (25% without 1 g/kg alcohol co-administration or 75% with 1 g/kg alcohol co-administration). Depicted in Fig. 4 are the PK data from four rats injected with 20 mg/kg cocaine and four rats survived from 60 mg/kg cocaine. Notably, in the presence of alcohol, two additional metabolites, i.e., cocaethylene and norcocacethylene, were detected in the blood. Time-dependent blood concentrations of cocaine and its metabolites are all shown in Fig. 4.

In addition, we have also examined whether alcohol affects the catalytic activities of human BChE and hCE-2 against cocaine. The obtained activity data are depicted in Fig. 5, indicating that of alcohol (at a concentration of 80 mg/dL or 17.6 mM) has no significant effect on the activity of any of these enzymes.

Summarized in Table 1 are the key PK parameters from the mean concentrations for cocaine and its metabolites. As seen in Table 1, 20 mg/kg cocaine had an elimination half-life (t1/2) of ~30 min and 60 mg/kg cocaine had an elimination half-life of ~65 min. The observed change in cocaine elimination half-life due to the increase of cocaine dose is consistent with the previous observation that the elimination half-life of cocaine in the blood is dependent on the dose used in the experiment, as all endogenous enzymes responsible for cocaine metabolism are saturated when cocaine concentration is sufficiently high in plasma [29,30]. In comparison, 20 mg/kg cocaine with 1 g/kg alcohol had an elimination half-life of ~33 min, and 60 mg/kg cocaine with 1 g/kg alcohol had an elimination half-life of ~74 min. Alcohol slightly increased the elimination half-life of cocaine, particularly for the higher dose (60 mg/kg).

According to the data in Table 1, when injected 20 mg/kg cocaine i.p. only, the peak blood concentration (Cmax) was ~1.5 μM for cocaine (COC), ~0.18 μM for norcocaine (NORCOC), ~7.3 μM for benzoylecgonine (BZE), and ~0.52 μM for ecegonine methyl ester (EME). For i.p. injection of 20 mg/kg cocaine and 1 g/kg alcohol, the peak blood concentration was ~2.0 μM for cocaine, ~0.17 μM for norcocaine, ~5.4 μM for benzoylecgonine, and ~0.68 μM for EME. In addition, co-administration of 20 mg/kg cocaine and 1 g/kg alcohol produced two additional metabolites, i.e., cocaethylene (CE) with Cmax = ~0.16 μM (or ~160 nM) and norcocacethylene (NORCE) with Cmax = ~0.035 μM (or ~35 nM), in the blood. All cocaine metabolites (in the presence of alcohol) summarized in Table 1 had longer elimination half-lives than that (t1/2 = ~33 min) of cocaine when the cocaine dose was 20 mg/kg. In addition, alcohol increased the area under the curve (AUC) values of cocaine and EME, and decreased the AUC values of norcocaine and benzoylecgonine, as seen in Table 1.

Injected with 60 mg/kg cocaine (i.p.) only, the peak blood concentration was ~7.0 μM for cocaine, ~0.96 μM for norcocaine, ~21.8 μM for benzoylecgonine, and ~1.4 μM for EME. For i.p. injection of 60 mg/kg cocaine and 1 g/kg alcohol, the peak blood concentration was ~8.1 μM for cocaine, ~0.75 μM for norcocaine, ~16.2 μM for...
benzoylecgonine, and \(~1.4 \mu M\) for EME. The observed alcohol-induced decrease in the peak blood concentration of norcocaine was unexpected, as it has been known that chronic alcohol consumption increases the expression of CYP3A4 (the enzyme responsible for metabolism of cocaine to norcocaine) \([31]\). However, the overall effects of alcohol on CYP3A4 might be complicated. Interestingly, while chronic alcohol use results in the increase of CYP3A4 expression and, thus, indirectly increases the CYP3A4 activity, alcohol itself also binds with CYP3A4 in the active site to directly affect the CYP3A4 activity \([32]\). The CYP3A4-alcohol binding affinity \((K_d)\) was determined as 5.9 mM \([33]\). The binding of alcohol with CYP3A4 in the active site may significantly impact the binding of CYP3A4 with its inhibitor or substrate \([32]\). For example, 20 mM alcohol significantly improves the binding affinity \((K_d)\) of nelfinavir as an inhibitor with CYP3A4, but significantly decreases the catalytic efficiency of CYP3A4 for nelfinavir metabolism by mainly changing the \(K_M\) from 1.3 \(\mu M\) to 4.4 \(\mu M\) \([33]\). In our current study, the peak blood concentration \((C_{\text{max}})\) of cocaine or norcocaine was reached quickly, at \(~10\) or \(~15\) min after the cocaine and alcohol co-administration. The actual effect of alcohol in the CYP3A4 expression within 10–15 min might not be significant. Hence, our data implies that alcohol might mainly affect the CYP3A4 activity through its direct binding interaction with CYP3A4. Further extensive studies on this

**Fig. 4.** Time-dependent blood concentrations of cocaine and cocaine metabolites in male rats after i.p. administration of 20 mg/kg cocaine or 60 mg/kg cocaine with or without co-administration of 1 g/kg alcohol \((n = 4\) per group). Data are plotted as mean ± SEM.
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Fig. 5. Effects of alcohol (17.6 mM) on the catalytic activities of enzymes (BChE and hCE-2) against cocaine (100 μM). The enzymatic reactions (n = 5 per reaction) were carried out in the presence of alcohol at a concentration of 80 mg/dL (17.6 mM) in comparison with the enzymatic reactions (n = 5 per reaction) without alcohol. Statistical analysis (t-test): ns, p > 0.05.

3.4. Contributions of toxic cocaine metabolites to the total toxicity

As norcocaine, cocaethylene, and norcocaethylene are all more toxic than cocaine itself [11], the total toxicity level (TTL) of cocaine and its metabolites in the blood may be estimated by using the following equation:

\[ TTL = \sum_{i=0}^{3} TL_i = \sum_{i=0}^{3} \left( \frac{LD_{50, i}}{LD_{50, cocaethylene}} \right) i \]

where \( i = 0 \) (cocaine), 1 (norcocaine), 2 (cocaethylene), and 3 (norcocaethylene). \( C_i \) represents the blood concentration of toxic compound \( i \). According to Eq.(1), the contribution from each toxic metabolite to the total toxicity is weighted by the ratio of cocaine LD\(_{50}\) to its own LD\(_{50}\). So, TTL represents the total toxicity of all these toxic compounds in terms of the cocaine toxicity-equivalent concentration. Using Eq.(1), one needs to use the LD\(_{50}\) values determined in a same animal species. In fact, the LD\(_{50}\) values have been known for all these four toxic entities (cocaine, norcocaine, cocaethylene, and norcocaethylene) in male mice: LD\(_{50}\)(cocaine) = 93.0 mg/kg; LD\(_{50}\)(norcocaine) = 49.71 mg/kg; LD\(_{50}\)(cocaethylene) = 63.8 mg/kg; and LD\(_{50}\)(norcocaethylene) = 39.39 mg/kg [11]. So, these LD\(_{50}\) values may be used to estimate TTL using Eq.(1). Correspondingly,

complex drug-drug interaction question will be needed in the future to fully understand the observed effects of alcohol on the metabolic profile of cocaine.

Further, co-administration of 60 mg/kg cocaine and 1 g/kg alcohol also produced cocaethylene with \( C_{max} = -0.58 \mu M \) (or ~580 nM) and norcocaine with \( C_{max} = -0.11 \mu M \) (or ~110 nM) in the blood. In the presence of alcohol, the higher the dose of cocaine, the higher concentrations of toxic metabolites (norcocaine, cocaethylene, and norcocaethylene) produced in the blood. Notably, the toxic metabolites norcocaine, cocaethylene, and norcocaethylene all had relatively shorter elimination half-lives than that \( t_{1/2} = ~74 \text{ min} \) of cocaine when the cocaine dose was as high as 60 mg/kg. Under this cocaine dose, alcohol also increased the cocaine AUC and decreased the AUC values of norcocaine and benzoylecgonine (see Table 1), due to the reason that alcohol can inhibit CE-1, the enzyme for catalyzing the hydrolysis of cocaine to benzoylecgonine [34].

In addition, it was reported that cocaine metabolites had longer half-lives than cocaine itself in humans [35]. According to our data summarized in Table 1, at the lower dose (20 mg/kg) of cocaine, cocaine did have a shorter elimination half-life in rats compared to the metabolites. However, the elimination half-life of cocaine is dose-dependent: the higher the dose, the longer the elimination half-life of cocaine, as noted above and observed previously [29,30].

When the cocaine dose was as high as 60 mg/kg, cocaine had an even longer elimination half-life than all its toxic metabolites (norcocaine, cocaethylene, and norcocaethylene).

Table 1

Pharmacokinetic parameters for cocaine and its metabolites in rats after i.p. injection of 20 or 60 mg/kg cocaine with or without 1 g/kg alcohol co-administration. For the lethal dose (60 mg/kg) of cocaine, we were able to collect blood samples from only survived rats for this analysis.

| Dose     | Parameter | COC | NOROCOC | CE | NORCE | RZE | EME |
|----------|-----------|-----|---------|----|-------|-----|-----|
| 20 mg/kg cocaine | \( T_{max} \) (min)\(^a\) | 10 ± 5 | N/A     | N/A | 45 ± 17 | 45 ± 11 |
|          | \( C_{max} \) (nM) | 1520 ± 279 | N/A | 7278 ± 872 | 558 ± 81 |
|          | AUC\(_{0-\infty}\) (nM min) | 83,537 ± 21,446 | N/A | 1216,085 ± 140,061 | 94,980 ± 7289 |
|          | AUC\(_{0-\infty}\) (nM min) | 84,939 ± 23,644 | N/A | 1649,319 ± 261,735 | 157,889 ± 9107 |
|          | \( t_{1/2} \) (min)\(^a\) | 10 ± 2 | N/A | 123 ± 22 | 154 ± 32 |
| 20 mg/kg cocaine + 1 g/kg alcohol | \( T_{max} \) (min)\(^a\) | 10 ± 5 | N/A | 45 ± 11 | 34 ± 10 |
|          | \( C_{max} \) (nM) | 1967 ± 285 | N/A | 19,970 ± 308 | 898,330 ± 160,183 |
|          | AUC\(_{0-\infty}\) (nM min) | 113,437 ± 28,855 | N/A | 2352 ± 308 | 126,010 ± 11,463 |
|          | AUC\(_{0-\infty}\) (nM min) | 113,956 ± 29,337 | N/A | 214,298 ± 298 | 220,714 ± 10,653 |
| 60 mg/kg cocaine | \( t_{1/2} \) (min)\(^a\) | 33 ± 3 | 41 ± 8 | 109 ± 9 | 193 ± 13 |
|          | \( T_{max} \) (min)\(^a\) | 10 ± 7 | 15 ± 1 | N/A | 45 ± 11 | 60 ± 8 |
|          | \( C_{max} \) (nM) | 6997 ± 918 | N/A | 21,781 ± 726 | 1399 ± 130 |
|          | AUC\(_{0-\infty}\) (nM min) | 782,035 ± 54,512 | N/A | 4011,650 ± 228,926 | 255,870 ± 13,323 |
|          | AUC\(_{0-\infty}\) (nM min) | 837,080 ± 50,917 | N/A | 6359,744 ± 379,083 | 534,753 ± 28,828 |
| 60 mg/kg cocaine + 1 g/kg alcohol | \( t_{1/2} \) (min)\(^a\) | 65 ± 15 | N/A | 167 ± 4 | 100 ± 15 |
|          | \( T_{max} \) (min)\(^a\) | 15 ± 5 | 15 ± 1 | 60 ± 14 | 120 ± 15 | 120 ± 0 |
|          | \( C_{max} \) (nM) | 8056 ± 720 | N/A | 16,220 ± 267 | 1363 ± 125 |
|          | AUC\(_{0-\infty}\) (nM min) | 865,453 ± 98,761 | N/A | 3157,382 ± 435,775 | 240,308 ± 1834 |
|          | AUC\(_{0-\infty}\) (nM min) | 971,732 ± 54,904 | N/A | 5467,289 ± 9778 | 503,646 ± 22,666 |
|          | \( t_{1/2} \) (min)\(^a\) | 74 ± 11 | 58 ± 8 | 67 ± 13 | 164 ± 19 | 234 ± 39 |

\(^a\) \( T_{max} \) refers to the time when the peak concentration \( (C_{max}) \) was reached.
injection of 100 mg/kg cocaine following the alcohol injection.

Table 3

| Dose condition | TTL (μM) | COC (%) | NORCOC (%) | CE (%) | NORCE (%) |
|----------------|---------|---------|------------|--------|-----------|
| 20 mg/kg       | 1859    | 82      | 18         | 0      | 0         |
| 60 mg/kg       | 8789    | 80      | 20         | 0      | 0         |
| 20 mg/kg + alcohol | 2618    | 75      | 13         | 9      | 3         |
| 60 mg/kg + alcohol | 10,550  | 76      | 13         | 8      | 2         |

* Calculated by using Eq. (1) with the Cmax values listed in Table 1.

the relative contribution (TL in percentage) from each compound (j) to TTL can be evaluated as

\[ \text{TTL}(\%) = 100 \times \frac{(L_{D50})_b C_i}{(L_{D50})_a} \sum_{i=1}^{3} \frac{(L_{D50})_b C_i}{(L_{D50})_a} \]  (2)

As seen in Table 2, with i.p. injection of 20 mg/kg cocaine only, cocaine itself accounted for ~82 % of the total toxicity, with norcocaine accounting for ~18 % of the total toxicity. When the cocaine dose increased from 20 mg/kg to 60 mg/kg, the contribution of norcocaine to the total toxicity slightly increased from ~18 % to ~20 %. In the presence of alcohol, due to additional contributions from the more toxic cocaine metabolites (cocaethylene and norcocaethylene), cocaine itself only accounted for ~75 % (for 20 mg/kg cocaine) or ~76 % (for 60 mg/kg cocaine in survived rats) of the total toxicity, with the metabolites contributing to the remaining ~25 % or ~24 % of the total toxicity. So, the alcohol increased the contributions of cocaine metabolites to the total toxicity. As a result, alcohol increased the total toxicity (TTL), as seen in Table 2.

3.5. Blood concentrations of cocaine and its metabolites at the time when rats died of the co-administration of cocaine and alcohol

In order to examine the correlation between the lethality of cocaine and its metabolic profile in the presence of alcohol, blood samples were also collected from additional (un-survived) rats (n = 4 per group) immediately after death following intraperitoneal administration of 1 g/kg alcohol and cocaine (60 mg/kg with 75 % rats died or 100 mg/kg with 100 % rats died). The collected blood samples were also analyzed for the concentrations of cocaine and its metabolites (see Table 3). With the blood concentrations of cocaine and its metabolites at the time of death, instead of Cmax, using Eq. (1), we calculated the TTL and the individual contributions from all toxic compounds (see Table 3) at the time of death.

As summarized in Table 3, for the rats died of the co-administration of 60 mg/kg cocaine and 1 g/kg alcohol, the average time at the occurrence of death was ~8.9 min, and the average blood concentration at the time of death was ~9.1 μM for cocaine, ~0.57 μM for EME, ~6.0 μM for benzoylcegonine, ~1.8 μM for norcocaine, ~1.8 μM for cocaethylene, and ~0.32 μM for norcocaethylene. Notably, cocaine itself only accounted for ~57 % of the total toxicity, with the metabolites (norcocaine, cocaethylene, and norcocaethylene) contributing to the remaining ~43 % of the total toxicity. The ~43 % contribution from the cocaine metabolites to the total toxicity in these un-survived rats was much higher than that (~24 %; see Table 2) in the survived rats discussed above. This is because the blood samples collected from these un-survived rats had higher levels of the toxic metabolites (Table 3) compared to those from the survived rats (see Table 1).

In comparison with the 60 mg/kg cocaine and 1 g/kg alcohol combination, for the rats died of the co-administration of 100 mg/kg cocaine and 1 g/kg alcohol, the average time (~3.9 min) at the occurrence of death was shorter, the blood concentration (~12 μM) of cocaine was higher, and the corresponding blood concentrations of cocaine metabolites were all lower because there was a shorter period of time (~3.9 min vs ~8.9 min) for converting cocaine to its metabolites before the occurrence of death. As a result, cocaine itself accounted for ~71 % of the total toxicity, with the metabolites contributing to the remaining 29 % of the total toxicity at ~3.9 min (the time of death).

Concerning the total toxicity (TTL), as seen in Table 3, TTL = 15,982 ± 601 nM (or 15,982 ± 601 μM) when the rats died (at ~8.9 min) from the combined 60 mg/kg cocaine and 1 g/kg alcohol, and TTL = 16,649 ± 1965 nM (or 16,649 ± 1965 μM) when the rats died (at ~3.9 min) from the combined 100 mg/kg cocaine and 1 g/kg alcohol. There was no significant difference between these two dose conditions in TTL at the time of death, suggesting that death may occur once the TTL reaches a threshold of ~16 μM considering the TTL data of 15,982 ± 601 μM or 16,649 ± 1965 μM after the alcohol and cocaine co-administration.

4. Conclusion

In this study, we aimed to understand how a non-lethal dose of alcohol (1 g/kg) affects the toxicity and metabolism/PK profile of cocaine in rats. For this purpose, the toxicity of cocaine, or the cocaine and alcohol combination, is characterized by multiple parameters, including not only the commonly used LD50, but also the harmonic mean of time for the appearance of convulsion and harmonic mean of time for the appearance of death. The animal data revealed that co-administration of alcohol markedly decreased the LD50 of cocaine

| Table 2 |
|----------|
| The total toxicity level (TTL) of cocaine and metabolites and the percentage contributions from individual toxic compounds with and without co-administration of 1 g/kg alcohol in rats. |
| Dose condition | TTL (μM) | COC (%) | NORCOC (%) | CE (%) | NORCE (%) |
|----------------|---------|---------|------------|--------|-----------|
| 20 mg/kg       | 1859    | 82      | 18         | 0      | 0         |
| 60 mg/kg       | 8789    | 80      | 20         | 0      | 0         |
| 20 mg/kg + alcohol | 2618    | 75      | 13         | 9      | 3         |
| 60 mg/kg + alcohol | 10,550  | 76      | 13         | 8      | 2         |

* Calculated by using Eq. (1) with the Cmax values listed in Table 1.

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| Table 3 |
|----------|
| Blood concentrations (nM ± SEM) of cocaine and its metabolites at the time of death (in blood samples collected immediately after death) following i.p. administration of 1 g/kg alcohol (ethanol) and cocaine (60 or 100 mg/kg) in rats. Death occurred at 8.9 ± 2.8 min after injection of 60 mg/kg cocaine or at 3.9 ± 1.2 min after injection of 100 mg/kg cocaine following the alcohol injection. |
| Cocaine or Metabolite | 1 g/kg alcohol + 60 mg/kg cocaine Blood Level (nM) | Toxicity Level (TL in nM) [%] | 1 g/kg alcohol + 100 mg/kg cocaine Blood Level (nM) | Toxicity Level (TL in nM) [%] |
|-----------------------|-----------------|-----------------|-----------------|-----------------|
| Cocaine               | 9094 ± 151      | 9094 ± 151 [57%]| 11,791 ± 957    | 11,791 ± 957 [71%]| |
| Egonnine Methyl Ester | 573 ± 37        | N/A             | 349 ± 78        | N/A             | |
| Benzoylcegonine        | 6006 ± 372      | N/A             | 1977 ± 465      | N/A             | |
| Norcocaine            | 1846 ± 89       | 3454 ± 167 [22%]| 1543 ± 261      | 2887 ± 488 [17%]| |
| Cocaethylene          | 1846 ± 149      | 2691 ± 217 [17%]| 1174 ± 306      | 1711 ± 446 [10%]| |
| Norcocaethylene       | 315 ± 28        | 744 ± 66 [55%]  | 110 ± 31        | 260 ± 73 [24%]  | |
| TTL (Total Toxicity Level in nM) | 15,982 ± 601 | 16,649 ± 1965 |

* TL = (blood level of the metabolite) × (ratio of the cocaine LD50 to the metabolite LD50 for mouse). Mouse LD50 data used: LD50(cocaine) = 93.0 mg/kg; LD50(norcocaine) = 49.71 mg/kg; LD50(cocaethylene) = 63.8 mg/kg; and LD50(norcocaethylene) = 39.39 mg/kg [11]. The number in bracket refers to the relative contribution of the molecular entity to the Total Toxicity Level (TTL).

N/A (not applicable) because this metabolite is not toxic and, hence, does not contribute to the toxicity level.

TTL represents the Total Toxicity Level in terms of cocaine toxicity-equivalent concentration for the overall toxicity.
from 73 mg/kg to 56 mg/kg (~23 % decrease) and shortened the average times for the appearance of death and/or convulsion after cocaine administration. All these toxicity data have consistently demonstrated that co-administration of alcohol makes cocaine more toxic.

Further, we also determined the detailed PK profiles of cocaine at doses of 20 and 60 mg/kg with co-administration of 1 g/kg alcohol in rats, including the time courses of the blood concentrations of cocaine and its metabolites. According to the obtained PK data, the alcohol-enhanced toxicity of cocaine is mainly attributed to two additional metabolites (cocaethylene and norcococaine) that are more toxic than cocaine itself. Cocaethylene and norcococaine can be produced only in the presence of alcohol. Co-administration of 1 g/kg alcohol remarkably changed the metabolic profile of cocaine. So, the total toxicity level (TTL) of the substance should account for blood concentrations of cocaine and its toxic metabolites including not only norcocaine, but also cocaethylene and norcocaethylene. In general, TTL should account for contributions from all the four molecular entities, including cocaine itself and three toxic metabolites (norcocaine, cocaethylene, and norcocaethylene). Accordingly, Eqs. (1) and (2) were established. Based on the obtained PK data, co-administration of alcohol markedly increased the TTL due to the production of additional toxic metabolites (cocaethylene and norcocaethylene). As a result, in the co-administration of alcohol (1 g/kg) and cocaine (20 or 60 mg/kg), only ~75 % (for 20 mg/kg cocaine) or ~76 % (for 60 mg/kg cocaine in the survived rats) of the total toxicity was attributed to cocaine itself, with the remaining ~25 % or ~24 % contributions from the toxic metabolites. With this TTL distribution in mind, to develop a truly effective treatment for concurrent use of cocaine and alcohol, rational design of the possible treatment must account for the physiological and toxic effects of not only cocaine itself, but also norcocaine, cocaethylene, and norcocaethylene.

In addition, we also determined the blood concentrations of cocaine and its metabolites in rats died of co-administration of alcohol (1 g/kg) and cocaine (60 or 100 mg/kg) at the time of death. In this way, we were able to determine the TTL at the time of death in the un-survived rats. Notably, co-administration of 1 g/kg alcohol and 60 mg/kg cocaine had a 75 % lethality. Under this dose condition, compared to the TTL distribution (76 % from cocaine, 13 % from norcocaine, 8 % from cocaethylene, and 2 % from norcocaethylene) in the survived rats, the un-survived rats had larger percentage contributions from the metabolites – norcocaine (22 %), cocaethylene (17 %), and norcocaethylene (5 %) – to the TTL, and cocaine itself accounted for only 57 % of the TTL. As a result, TTL = ~16 μM in the un-survived rats, and TTL = ~11 in the survived rats, which is consistent with the observation that some rats died (because they had a larger TTL, ~16 μM) and some rats did not die (because they had a TTL lower than the threshold of ~16 μM) of co-administration of 1 g/kg alcohol and 60 mg/kg cocaine.

Further, the 1 g/kg alcohol and 100 mg/kg cocaine combination (with 100 % lethality) should be more toxic than the 1 g/kg alcohol and 60 mg/kg cocaine combination (with 75 % lethality). Hence, rats with co-administration of 1 g/kg alcohol and 100 mg/kg cocaine died faster (at ~3.9 min) than the rats died (at ~8.9 min) of co-administration of 1 g/kg alcohol and 60 mg/kg cocaine. However, the rats died of co-administration of 1 g/kg alcohol and 100 mg/kg cocaine had an essentially the same TTL value (~16.6 ± 2.0 μM) at the time of death compared to that (TTL ~16.0 ± 0.6 μM) in the rate died of co-administration of 1 g/kg alcohol and 60 mg/kg cocaine. So, the TTL value of ~16 μM may be used as an approximate predictor (a threshold) for the occurrence of death for rats.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Data availability**

Data will be made available on request.

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