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•Research article•

Heme peroxidases are responsible for the dehydrogenation and oxidation metabolism of harmaline into harmine

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[ABSTRACT] Harmaline and harmine are β-carboline alkaloids with effective pharmacological effects. Harmaline can be transformed into harmine after oral administration. However, enzymes involved in the metabolic pathway remain unclear. In this study, harmaline was incubated with rat liver microsomes (RLM), rat brain microsomes (RBM), blood, plasma, broken blood cells, and heme peroxidases including horseradish peroxidase (HRP), lactoperoxidase (LPO), and myeloperoxidase (MPO). The production of harmine was determined by a validated UPLC-ESI-MS/MS method. Results showed that heme peroxidases catalyzed the oxidative dehydrogenation of harmaline. All the reactions were in accordance with the Hill equation. The reaction was inhibited by ascorbic acid and excess H_2O_2. The transformation of harmaline to harmine was confirmed after incubation with blood, plasma, and broken blood cells, rather than RLM and RBM. Harmaline was incubated with blood, plasma, and broken cells liquid for 3 h, and the formation of harmine became stable. Results indicated an integrated metabolic pathway of harmaline, which will lay foundation for the oxidation reaction of dihydro-β-carboline. Moreover, the metabolic stability of harmaline in blood should not be ignored when the pharmacokinetics study of harmaline is carried out.

[KEY WORDS] Harmaline; Harmine; Oxidative dehydrogenation; Heme peroxidases; Myeloperoxidase; Horseradish peroxidase; Lactoperoxidase

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Introduction

β-Carboline alkaloids, namely, harmaline and harmine, have attracted much attention because of their effective pharmacological effects. These compounds exhibit pharmacological functions, such as anti-tumor, anti-hypertension, anti-coagulation, anti-diabetes, anti-bacteria, anti-plasmodium, anti-pruritic, and anti-pyretic acvitiy in various fields [1]. The psychotropic properties of harmaline and harmine are important. Harmaline and harmine can effectively ameliorate scopolamine-induced memory impairment in mice by improving cholinergic system function, modulating vital neurotransmitters, and revealing their potential therapeutic effect on Alzheimer's disease (AD) [2]. Recent studies have confirmed that harmaline and harmine are potential candidates for the treatment of AD, Parkinson's disease, and depression due to its inhibitory effect against acetylcholinesterase and butyrylcholinesterase [3] as well as monoamine oxidase A (MAO-A) [4]. Harmaline and harmine also played a therapeutic role in the treatment of convulsion by interaction with γ aminobutyric acid receptor [5], withdrawal syndrome by interaction with imidazoline receptor [6], and central and peripheral analgesia by interaction with opioid receptor [7]. However, harmaline and harmine exert toxic effects. An acute onset of tremor occurred in rats, mice, cats, rabbits, monkeys, and cattle following administration of harmaline and harmine [1, 4, 8-9].

Harmaline and harmine are widely distributed in nature, especially the hallucinogenic plant, *Ayahuasca*, which is used as an ingredient of the popular sacred and psychoactive drinks in South American Indian culture [10]. Harmaline and harmine are also the dominant compounds in *Peganum harmala* Linn., which has been used for the treatment of nervous, cardiovascular, gastrointestinal, respiratory, and endocrine diseases in America, Australia, Northwest China,

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These authors have no conflict of interest to declare.

Central Asia, Middle East, Northwest India, Europe, and North Africa [1]. These compounds are found in common plant-derived foodstuffs (e.g., wheat, rice, corn, barely, soy, beans, rye, grape, mushroom, and vinegar), well-cooked meat, plant-derived beverages (e.g., wine, beer, whisky, brandy, and sake), and plant-derived inhaled substances (e.g., tobacco) [11]. Thus, the *in vivo* processes of harmaline and harmine have attracted increasing attention given the widespread distribution and pharmacological effects of these β-carboline alkaloids.

The metabolic pathways of harmaline and harmine were investigated in different mammals. Numerous studies have revealed the *in vivo* metabolism of harmaline and harmine in rats, mice, dogs and humans; the underlying mechanisms include oxidative dehydrogenation, 7-*O*-demethylation, hydroxylation, *O*-glucuronization, and *O*-sulfonation [12-13]. Harmaline and harmine are mainly catalyzed to generate harmalol and harmol through 7-*O*-demethylation by CYP2D6, CYP1A1, and CYP1A2, where CYP2D6 contributes the most [12]. Interestingly, harmine was detected in the urine after oral administration of harmaline in rats [13]. In *in vitro* studies, harmine was formed after co-incubation of harmaline with the liver microsomes of rats, mice, pigs, rabbits, bulls, sheeps, and camels [14]. However, catalytic enzymes involved in the reaction remain unknown.

Harmaline and harmine have similar structures, except that the double bonds at positions 3 and 4 are different. The chemical structures of the two alkaloids are shown in Fig. 1. Tetrahydro-β-carboline alkaloids, such as 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid and 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, can generate aromatic β-carboline alkaloids, norharmane, and harmane under the catalysis of heme peroxidases [15]. 2-Methyl-1,2,3,4-tetrahydro-β-carboline and 2,9-dimethyl-1,2,3,4-tetrahydro-β-carboline can be oxidized by heme peroxidases [16]. Thus, we hypothesize that harmaline can presumably form harmine by the oxidative dehydrogenation of heme peroxidases. In the current study, enzyme incubation *in vitro* and mass spectrometry were established. Horseradish peroxidase (HRP), lactoperoxidase (LPO), and myeloperoxidase (MPO), the three heme peroxidases that were easily obtained, were used to verify the hypothesis. The blood and microsomes were also used to verify the occurrence of the reaction due to the existence of a large number of peroxidases in tissues and body fluids [17]. The results will clarify the reaction process of harmaline into harmine.

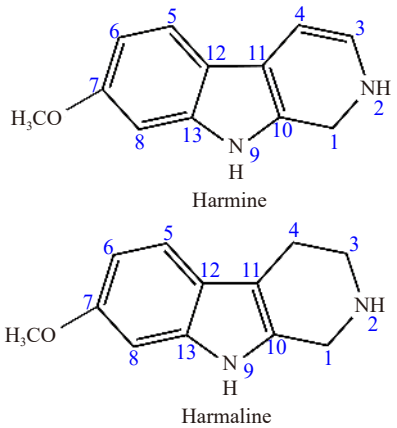

Fig. 1 Chemical structures of harmaline and harmine

Materials and Methods

Chemicals and regents

Harmaline and harmine with purity of more than 99.0% were isolated from the seeds of *P. harmala* in our laboratory [18]. 9-Aminoacridine hydrochloride was used as an internal standard (IS). HRP, LPO, MPO, H_2O_2, ascorbic acid, and heparin sodium were obtained from Sigma Aldrich Co. (St. Louis, MO, USA). Acetonitrile, methanol, and formic acid were of HPLC grade and purchased from Fisher Scientific Co. (Santa Clara, CA, USA). Deionized water (> 18 mΩ) was produced using a Milli-Q Academic System (Millipore Corp., Billerica, MA, USA). All other chemicals and reagents were of either analytical or HPLC grade.

Animals

Male Wistar rats weighing 180–220 g were obtained from the Drug Safety Evaluation and Research Center of Shanghai University of Traditional Chinese Medicine. All the animals were housed under standard environmental conditions (room temperature: 25 °C ± 1 °C, relative humidity: 60%–65%, 12 h light–dark cycles: lights on from 7:00 to 19:00) and given free access to rodent chow and water. Before experiments, the rats were allowed to acclimatize for at least seven days. The animal experiment was performed in compliance with the regulations for animal experimentation issued by the State Committee of Science and Technology of the People's Republic of China on 14 November 1988 and approved by the Experimental Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine (No. PZSHUTCM18122111; Approval date: 21, December, 2018).

Preparation of rat blood cells, liver, and brain microsomes

Ten male Wistar rats weighing 180–220 g were used in the experiment. All the rats were anesthetized with 10% chloral hydrate, and the blood, brain, and liver were collected.

Blood cells were incubated to determine whether the reaction may occur in the blood. All the rats were anesthetized, and their plasma was collected by the abdominal aortic method. Their fresh whole blood was added to a centrifuge tube containing heparin sodium for immediate anticoagulation and centrifuged at 3 000 × *g* at 4 °C for 10 min. Centrifugation was conducted at a low speed to ensure that the blood cells were not broken. Heparin sodium was added in excess to ensure anticoagulation in all experimental time periods. The upper plasma was removed, while 0.01 mol·L^{-1} PBS (pH 7.4) at the same volume as the plasma was added to the precipitate. The cells were crushed by ultrasond, completely broken, and then mixed to obtain broken cell liquid. The broken blood

cells were immediately used for incubation experiment without any cold storage.

Liver microsomes were incubated to determine whether the reaction may occur in the liver. The liver tissues of rats were collected and rinsed with cold normal saline for three times. The liver tissue was cut and added with three times the amount of phosphate (K_2HPO_4-KH_2PO_4, 100 mmol·L^{-1}, pH 7.4)-KCl (150 mmol·L^{-1}) buffer. The sample was homogenized with a handheld homogenizer in an ice bath. After centrifugation at 9000 × *g* at 4 °C for 20 min, the supernatant was taken to obtain S9 liquid. S9 liquid was centrifuged at 100 000 × *g* at 4 °C for 60 min, and the resultant supernatant was discarded. The same amount of phosphate buffer (K_2HPO_4-KH_2PO_4, 100 mmol·L^{-1}, pH 7.4) as the supernatant was added, and the microsome precipitate was repeatedly blown with a straw until the system was uniform. The centrifugation step was repeated. An appropriate amount of phosphate buffer solution (K_2HPO_4-KH_2PO_4, 100 mmol·L^{-1}, pH 7.4) was added again, and rat liver microsomes (RLM) were obtained after full blow.

Brain microsomes were incubated to determine whether the reaction may occur in the brain. Brain tissue was collected and rinsed three times with cold saline. The tissue was crushed and added with five times the amount of Tris-HCl (50 mmol·L^{-1}, pH 7.4)-sucrose (250 mmol·L^{-1})-KCl (100 mmol·L^{-1}) buffer. The tissue was homogenized in an ice bath and centrifuged at 1000 × *g* at 4 °C for 10 min. The supernatant was taken and centrifuged at 17 000 × *g* at 4 °C for 45 min. The supernatant was collected again, centrifuged at 100 000 × *g* at 4 °C for 60 min, and then the resultant supernatant was discarded. An appropriate amount of the buffer was added, and the precipitation were blown repeatedly and rat brain microsomes (RBM) were obtained. The protein concentrations of RLM and RBM were measured by BCA protein concentration method.

Incubation in vitro by single enzyme

The following *in vitro* enzyme incubation systems were established to determine the enzyme that catalyzed the dehydrogenation of harmaline to harmine: 20 μL of enzyme solution (HRP, LPO, and MPO), 20 μL of 10 μmol·L^{-1} substrate solution, 140 μL of 0.01 mol·L^{-1} PBS (pH 7.4), and 20 μL of 250 μmol·L^{-1} H_2O_2. The total volume of the incubation system was 200 μL, where the final concentration of the substrate was 1 μmol·L^{-1}, the final concentration of hydrogen peroxide (H_2O_2) was 25 μmol·L^{-1}, the incubation temperature was 37 °C, and the incubation time was 40 min.

The *in vitro* incubation system with enzyme inhibitors was described as follows: 20 μL of enzyme solution, 20 μL of 10 μmol·L^{-1} substrate solution, 20 μL of enzyme inhibitor (10 mmol·L^{-1} ascorbic acid or 20 mmol·L^{-1} H_2O_2), 120 μL of 0.01 mol·L^{-1} PBS (pH 7.4), and 20 μL of 250 μmol·L^{-1} H_2O_2. The incubation condition was the same as above, and the incubation time was 40 min.

The enzyme that catalyzed the reaction was used to further explore enzymatic kinetics. The enzyme incubation systems were the same as the above single-enzyme incubation system. The substrate solution was diluted with 0.01 mol·L^{-1} PBS to achieve a various of concentrations: 2, 5, 10, 20, 50, 100, and 200 μmol·L^{-1}. The incubation temperature was 37 °C, and the incubation time was 20 min.

Incubation in vitro by blood as well as brain and liver microsomes

The brain, liver, and blood were used to identify the metabolic tissues for the oxidative dehydrogenation of harmaline into harmine. RLM and RBM were incubated at 37 °C, and the system was the same as the above single-enzyme incubation system. The incubation time was 40 min. The *in vitro* incubation system with broken cell liquid (plasma or blood) was described as follows: 5 mL of broken cell liquid (plasma or blood), 100 μL of 10 μmol·L^{-1} harmaline, and 100 μL of 250 μmol·L^{-1} H_2O_2. The incubation temperature was 37 °C, and the incubation time was 24 h.

The change in product content with incubation time was investigated by co-incubation with broken cell liquid, plasma, or blood. The anticoagulant blood from a rat was divided into two parts. One part for incubation of blood, and the other part was centrifuged to prepare plasma and broken cell liquid. The ratio of plasma to cells in each volume of blood was almost 1 ∶ 1. The *in vitro* incubation system with broken cell liquid (plasma or blood) was described as follows: 4 mL of broken cell liquid (plasma or blood), 80 μL of 10 μmol·L^{-1} harmaline, and 80 μL of 250 μmol·L^{-1} H_2O_2. The incubation temperature was 37 °C. About 200 μL of the liquid was collected after incubation for 1, 2, 3, 4, 6, 9, 12, and 24 h. The concentration of harmine was taken as abscissa *Y*, and the incubation time was taken as ordinate *X*.

Sample preparation

After enzyme incubation *in vitro*, 600 μL of IS solution (acetonitrile solution containing 16.67 ng·mL^{-1} tacrine) was added and vortex-mixed for 1 min. Following the centrifugation at 12 000 × *g* at 4 °C for 10 min, up to 720 μL of the supernatant was transferred to another clean tube and evaporated under slight stream of nitrogen to dryness at 37 °C. The dried residue was reconstituted with 90 μL of the initial mobile phase and vortex-mixed for 1 min. The mixture was centrifuged at 12 000 × *g* at 4 °C for 10 min, and the supernatant was immediately analyzed.

UPLC-ESI-MS/MS conditions

Chromatographic separation was performed on an Agilent Triple Quadrupole LC/MS 6410 (Agilent Technologies, Germany) by an ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm particle size). The gradient mobile phase consisted of an aqueous solution of 0.1% formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.3 mL·min^{-1} and the equivalent elution program was performed as follows: 0–3.00 min, 22%–22% B. The injection volume was 5 μL, and the temperatures of the column and autosampler were maintained at 40 °C and 10 °C, respectively. Tandem mass spectrometry was performed using a triple-quadrupole mass spectrometer (Agilent Technologies, Ger-

many) equipped with electrospray ionization (ESI) in the positive ionization mode. The multiple reactions monitoring (MRM) was chosen for the quantification of harmine and IS. The MS parameters were optimized as follows: capillary voltage, 4000 V; gas temperature, 350 °C; gas flow, 10 $L \cdot min^{-1}$, and the MS parameters of compounds were also optimized as shown in Table 1. The results of method validation are shown in supplementary material Table S1.

Statistical analysis

Experimental data were presented as mean ± standard deviation (SD). Statistical analysis was carried out using one-way analysis of variance by SPSS version 21.0 software. *Post-hoc* comparisons were performed using LSD (homogeneous variance) or Dunnett T3 (nonhomogeneous variance) test to determine statistical significance between groups. $P < 0.05$ was considered statistically significant in all cases. The enzyme kinetics curve was fitted by GraphPad Prism 5.

Results

Determination of protein concentration

The protein concentrations of RLM, RBM, HRP, LPO, and MPO were measured by the BCA protein concentration method. The absorbance of BSA serial standard protein solution and the sample solution to be tested was detected at the wavelength of 562 nm. The protein concentrations of BSA serial standard protein solution was taken as abscissa *X*, and the corresponding absorbance value was taken as ordinate *Y*. The linear regression was carried out to obtain the following standard curve equation: $Y = 0.0008733X + 0.01333$ ($r^2 = 0.9992$), and the linear range was 20.00–1000.0 $\mu g \cdot mL^{-1}$. According to the standard curve equation and the calculation of the dilution ratio of the sample, the protein concentrations of RLM, RBM, HRP, LPO, and MPO were 699.9, 931.4, 56.88, 62.61, and 3.00 $\mu g \cdot mL^{-1}$, respectively.

Identification of metabolic enzymes for the oxidative dehydrogenation of harmaline into harmine

The enzyme that catalyzed the oxidative dehydrogenation of harmaline to harmine was determined through enzyme incubation *in vitro*. As shown in Fig. 2, harmaline was transformed into harmine through the metabolism of HRP, LPO, and MPO. Harmine can only be detected when the substrate, enzyme, and H_2O_2 are all present. The activity of the three enzymes was detected and calculated. Approximately 0.017 $\mu mol \cdot L^{-1}$ harmine was generated after incubation of 3.00 $\mu g \cdot mL^{-1}$ MPO, 10 $\mu mol \cdot L^{-1}$ harmaline and appropriate H_2O_2. After catalysis by 62.61 $\mu g \cdot mL^{-1}$ LPO and 56.88 $\mu g \cdot mL^{-1}$ HRP, the concentration of harmine was 0.018 $\mu mol \cdot L^{-1}$ and 0.23 $\mu mol \cdot L^{-1}$, respectively. The enzyme activity of HRP, LPO, and MPO was defined as the amount of harmaline (μmol) per milligram of enzyme per minute, and the unit was U/mg prot. The sequence of the enzyme activity of HRP, LPO, and MPO in the oxidative dehydrogenation of harmaline to harmine was followed the order of MPO, HRP, and LPO, in which MPO was 1.46 times higher than HRP and 20.48 times higher than LPO.

Table 1 Optimized parameters of MS

Compds	Precursor ion	Product ion	Fragmentor/V	Collision energy/V	Cell accelerator voltage/V
Harmine	213.1	170.1	130	34	1
Tacrine	199.1	171.1	165	36	1

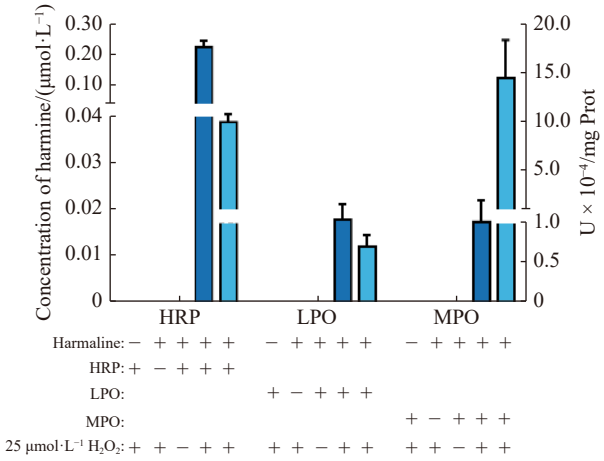

Fig. 2 Identification of metabolic enzymes for the oxidative dehydrogenation of harmaline into harmine ($n = 5$). The left ordinate (histogram with point) displays the production of harmine and the right ordinate (histogram with line) displays the activity of enzyme.

Inhibition rate of the conversion of harmaline into harmine by excess H_2O_2 and ascorbic acid

The inhibitors of HRP, LPO, and MPO were used in the experiment. The activity of the three enzymes was inhibited by heme peroxidase inhibitors, such as excessive H_2O_2 (2 $mmol \cdot L^{-1}$) or ascorbic acid (1 $mmol \cdot L^{-1}$). After inhibition by 2 $mmol \cdot L^{-1}$ H_2O_2, the concentration of harmine catalyzed by HRP decreased to 7.12 ± 0.53 $ng \cdot mL^{-1}$, which was significantly lower than that before inhibition (47.95 ± 3.91 $ng \cdot mL^{-1}$) ($P < 0.001$, Fig. 3). For the inhibitory effects of excessive H_2O_2 against LPO, the concentration of harmine was reduced to 2.56 ± 0.48 $ng \cdot mL^{-1}$, which was significantly lower than that before inhibition ($P < 0.05$). Moreover, 2 $mmol \cdot L^{-1}$ H_2O_2 inhibited the activity of 3.00 $\mu g \cdot mL^{-1}$ MPO. The inhibition rate of excessive H_2O_2 (2 $mmol \cdot L^{-1}$) against HRP, LPO, and MPO was 85.16%, 31.96%, and 100%, respectively. The inhibition rate of ascorbic acid (1 $mmol \cdot L^{-1}$) was higher than that of excessive H_2O_2. After the addition of ascorbic acid in the incubation system, the activity of HRP, LPO and MPO was inhibited by 100%.

Enzyme kinetics of HRP, LPO, and MPO for the oxidative dehydrogenation of harmaline into harmine

The enzymec kinetic curves of HRP, LPO, and MPO are shown in Fig. 4. After the detection of harmine in the product, the reaction rate (defined as the amount of harmaline per milligram of enzyme per minute, with a unit of pmol/min/mg) was calculated. Peroxidase-catalyzed oxida-

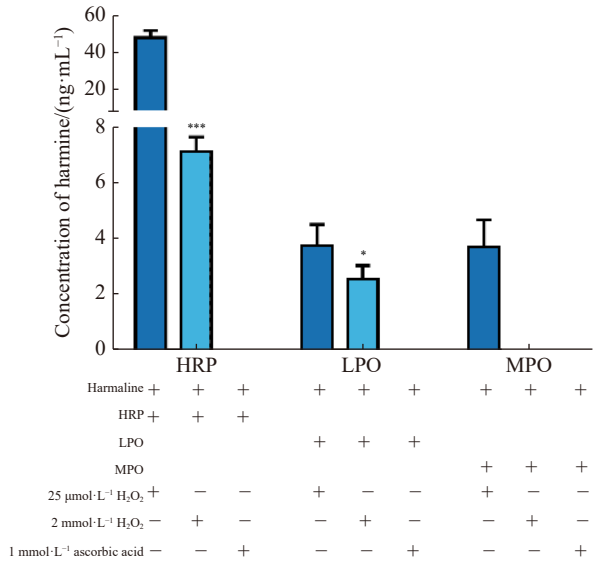

Fig. 3 Inhibition rate of the conversion of harmaline into harmine by excess H_2O_2 and ascorbic acid (n = 5)

tion was studied as a function of the concentration of substrates. k' and V_{max} were determined from non-linear regression fitting by GraphPad Prism 5. The enzymatic reactions were all in accordance with the Hill equation. The values of k' were 17.03 ± 0.90, 11.73 ± 3.57, and 11.95 ± 5.28 μmol·L^{-1}, the values of V_{max} were 20 053.00 ± 1119.00, 1367.00 ± 431.30, 28 308.00 ± 11 999.00 pmol·min^{-1}·mg^{-1}, and the values of n were 0.75 ± 0.01, 0.74 ± 0.09, and 0.58 ± 0.06 for HRP, LPO, and MPO, respectively.

Identification of metabolic tissues for the oxidative dehydrogenation of harmaline into harmine

As shown in Fig. 5, harmaline was not transformed into harmine through the metabolism of RLM and RBM under the incubation condition. The concentration of harmine was detected after incubation of harmaline with blood, plasma, and broken cell liquid. The harmine product in blood (20.27 ± 0.50 ng·mL^{-1}) was higher than that in broken cell liquid (13.10 ± 0.15 ng·mL^{-1}) and plasma (9.94 ± 0.11 ng·mL^{-1}) (P < 0.05).

As shown in Fig. 6, the production of harmine initially showed an increasing trend and then stabilized. The production of harmine increased in the first 3 h. The concentrations of harmine were almost constant from 3 h to 24 h. The same variation was observed in broken cell liquid, plasma, and blood. The ratio of plasma to cells in each volume of blood was almost 1 ∶ 1. The enzyme concentration in the cell liquid group was the same as that in the blood group, while the enzyme concentration in the plasma group was almost twice as high as that in the blood group of each analysis unit. Thus, the amount of the harmine products after incubation with plasma and broken cell liquid was lower than that in blood.

Discussion

Enzyme incubation *in vitro* was used to determine the enzymes that catalyze the oxidative dehydrogenation of harmaline into harmine. The high protein concentrations of self-made RLM and RBM were satisfactory for *in vitro* incubation experiments. UPLC-ESI-MS/MS method for the determination of harmine in *in vitro* incubation systems was simple, rapid, specific, and repeatable, which is consistent with the requirements of the guiding principle of quantitative analysis method validation of biological samples, and thus could be used to determine harmine in biological samples.

HRP, LPO, and MPO catalyzed the oxidative dehydrogenation of harmaline into harmine, where MPO exhibited the highest catalytic activity, followed by HRP and LPO. HRP is a plant enzyme, while the other two are mammalian enzymes [19]. The presence of HRP may lead to alarm of biocatalysis in complex extraction. MPO is mainly distributed in neutrophil granulocytes, macrophages, and peritoneal B lymphocytes, while LPO is mainly distributed in exocrine glands and mucosal surfaces [17]. In oral drug delivery system of harmine and harmaline, MPO may play a more important role. The positive result of broken cell liquid, plasma, and blood incubation may be partly related to the presence of MPO in the blood. The two enzymes (MPO and LPO) do not exist in the brain and liver and may account for the uselessness of RLM and RBM. Other heme peroxidases, such as eosinophil peroxidase, thyroid peroxidase, etc., are seldom found in the brain and liver, and their catalytic activity should be further confirmed [17]. The property of drug metabolism was determined by the enzyme kinetic characteristics of the drug metabolism enzyme. The product generation method was used to accurately determine enzyme kinetic parameters, because the substrate elimination method might result in a large deviation when the drug metabolism pathway is complex [20]. In the classic Michaelis–Menten model, V_{max} and K_m, as the important parameters of drug enzyme kinetics, reflect the maximum reaction speed of the enzymatic reaction and the affinity between the enzyme and the substrate [21]. In the Hill model, the Hill constant k', V_{max}, and the Hill coefficient n are used to describe the catalytic properties of coenzymes. k' is related to the dissociation constant of the enzyme substrate and the index of enzyme affinity to substrate; n is the synergy index of substrate binding process, and the larger the value is, the better the synergy is [22]. In the present study, the reactions catalyzed by HRP, LPO, and MPO were all in accordance with the Hill equation. The values of n were all less than 1, indicating that there were negative synergistic effects in the reactions for HRP, LPO and MPO; in other words, the affinities of the enzyme to other substrates would decrease after the combination of substrates and the enzyme.

Peroxidases play an important role in a number of physiologically important processes. Excessive peroxidase activity is associated with oxidative damage to cells and tissues, leading to a variety of human diseases [23]. The MPO level was found to be associated with neurologic diseases, thereby corroborating experimental and clinic evidence of the possible deleterious role of MPO in various neurodegenerative diseases, such as AD, Parkinson's disease, and so on. Fur-

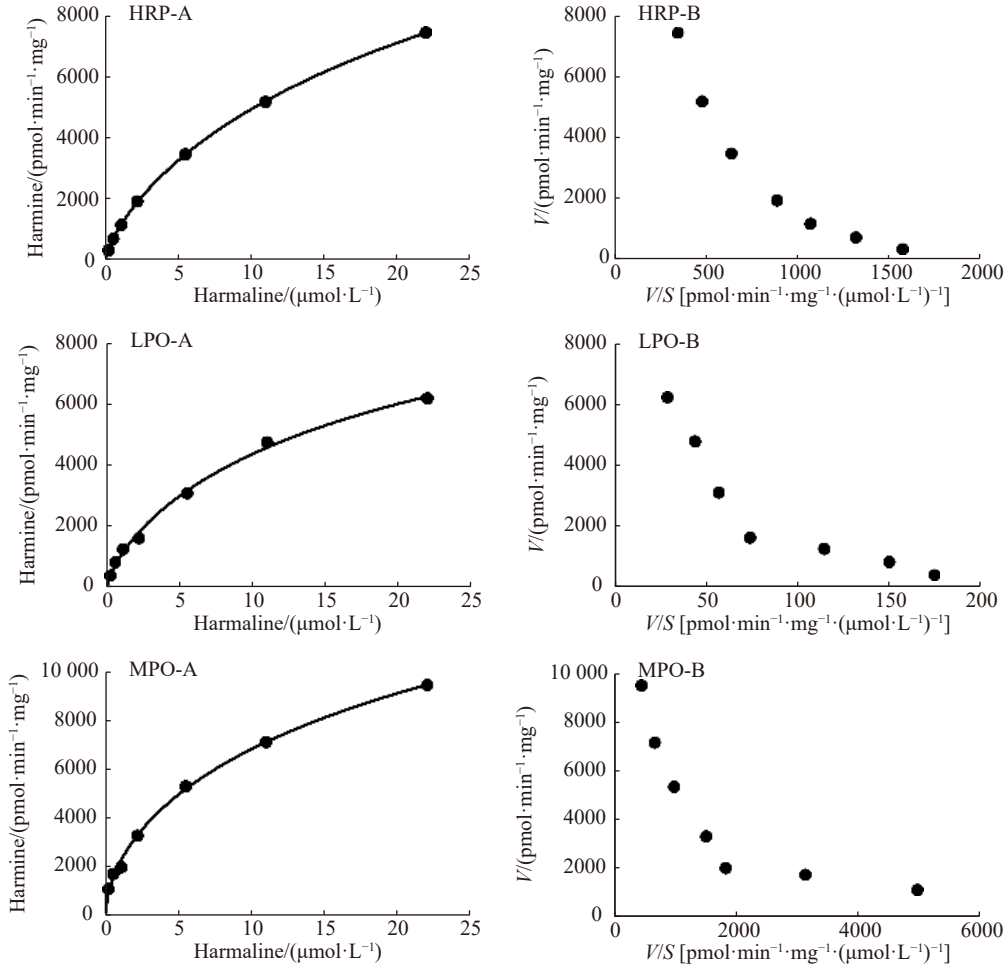

Fig. 4 Enzyme kinetic curves of HRP, LPO, and MPO (n = 5)

thermore, MPO is associated with with renal diseases, pulmonary inflammation, rheumatoid arthritis, skin inflammation, metabolic syndrome, and atherosclerosis/cardiovascular diseases [24-25]. Based on the above results, the binding of harmaline and MPO may affect the process of disease. The presence of LPO in bovine milk had a certain inhibitory effect against dextran sulfate sodium-induced colitis in mice [26]. Thus, the presence of harmaline in exocrine gland secretions may reduce the anti-inflammation properties of LPO, which provided guidance for the development of exocrine drugs. In addition, LPO is a major contributor to airway defenses and to the antimicrobial properties of exocrine gland secretions [23]. A variety of studies reported the antibacterial activities of harmaline and harmine [1]. Thus, the existence of harmaline and harmine has little effect on antibacterial function.

H_2O_2 plays an indispensable role in the reaction of the oxidative dehydrogenation of harmaline into harmine. Harmine can only be detected in the incubation system in the presence of H_2O_2, harmaline, and catalytic enzyme. Heme peroxidases show different specificity for different oxidizable substrates, including indoles, phenols, aromatic amines, lignin, and other proteins [27], but they share the same catalytic cycle. According to previous studies about heme peroxidases *in vivo*, H_2O_2 is essential in the catalytic process and the catalysis based on three consecutive redox steps [28]. Ferric is the central reaction part of an enzyme, so marking heme peroxidases as compound 1 ($ProFe^{3+}$). H_2O_2 is reduced into H_2O and reacts with compound 1 to convert into compound 2 ($\cdot$+$ProFe^{\text{IV}}$ = O). Then, compound 2 oxidizes substrate. Compound 2 can catalyze the two-electron oxidation of substrate, thereby completing the peroxidase cycle [23]. The reaction mechanism is shown in Fig. 7, in which AH_2 is the peroxidase substrate and oxidized to form A.

Blood, not RLM and RBM, can catalyze the metabolism of harmaline to produce harmine. Researches confirmed that harmaline was converted into harmine *in vivo* [13]. The formation of harmine in the incubation system of harmaline and broken cell liquid *in vitro* could partly explain the phenomenon. In this experiment, harmaline was not transformed into harmine under the incubation of RBM or RLM. This finding is in accordance with the report by Yu *et al*. [12]. Hence, CYP1A2, CYP2C9, CYP2D6, CYP3A4, CYP19, pooled recombinant CYP450s, and human liver microsomes did not catalyze harmaline to harmine upon the co-incubation of NADPH [12]. However, another research reported that harmine was formed after co-incubation of harmaline with the liver

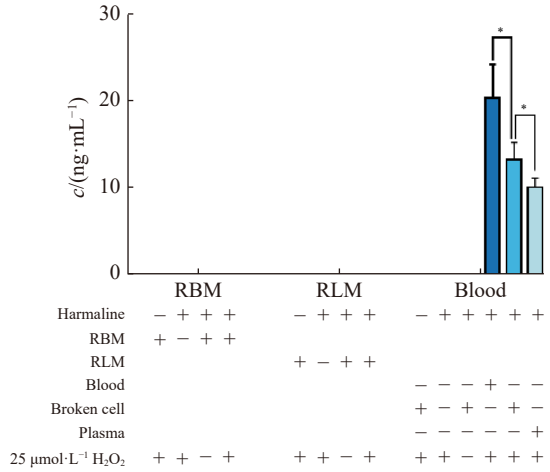

Fig. 5 Identification of metabolic tissues for the oxidative dehydrogenation of harmaline into harmine (n = 5)

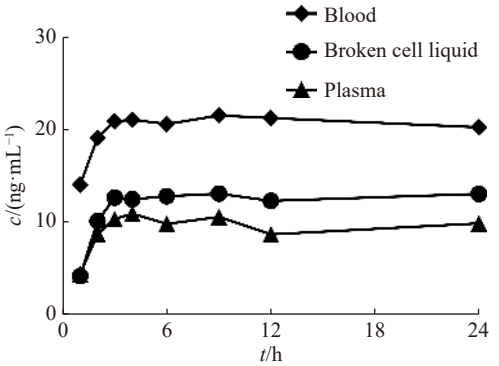

Fig. 6 Changes in the yield of harmine after incubation of harmaline with broken cell liquid, plasma or blood (n = 3)

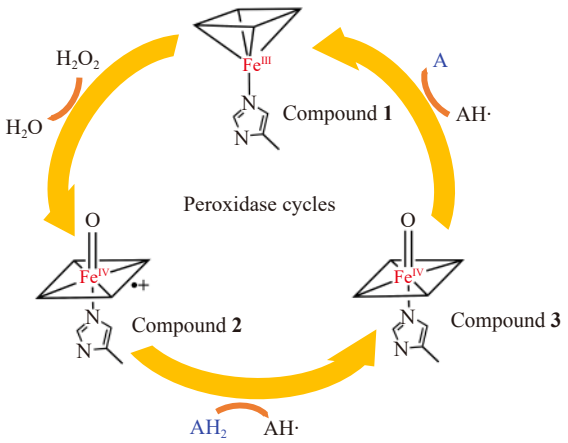

Fig. 7 Catalytic cycle of heme peroxidases. AH_2 is the peroxidase substrate [23, 27-28].

microsome of various mammals [14]. The purity of harmaline used was not sufficiently high and may contain a small amount of harmine, thereby indicating that a certain amount of harmine was produced. Different incubation conditions may account for differences in the results. The experiment was conducted without glucose 6-phosphate, glucose-6phosphate dehydrogenase, NADP+, and UDPGA, which are important in Li's research[14]. H_2O_2 is indispensable in the current research but not reported in the studies of Yu and Li [12,14].

Blood, plasma, and broken cell liquid have different types of activity; among which, blood has the highest catalytic activity. The emergence of harmine during harmaline co-incubation with broken cell liquid, plasma, and blood may be partly related to the presence of MPO in blood. Blood includes various cells and plasma. Thus, the product of blood incubation was significantly more than that of plasma and broken cells. MPO is mainly stored in the matrix of azurophilic granules of neutrophil granulocytes in a mature, dimeric form, and dimerization did not affect enzymatic activity [17]. When neutrophils in the peripheral blood and tissues were activated, MPO was released into phago-lysosomal compartment and the extra-cellular environment [25]. Thus, both plasma and broken cell liquid showed catalytic activity, where the catalytic activity of broken cell was stronger. The total harmine products of plasma and broken cell liquid incubation are less than those of blood, which could be attributed to enzyme activity loss during the process of broken.

Harmaline and harmine are β-carboline alkaloids that have similar structure but differ in double bond. Harmaline and harmine should always be studied together due to their similar structure and pharmacology. The transformation of harmine and harmaline should be paid more attention in *in vivo* study. Harmine can be detected after co-incubation of harmaline with broken blood cells, indicating that the pharmacokinetics of plasma harmaline and harmine may be affected if the supernatant of blood (plasma or serum) was not timely collected.

Conclusion

The oxidative dehydrogenation of harmaline into harmine requires the catalysis of HRP, LPO, or MPO in the presence of hydrogen peroxide, and MPO has the highest catalytic activity. The reactions catalyzed by HRP, LPO, and MPO are all in accordance with the Hill equation. Harmaline can be converted into harmine in the blood but not in the brain and liver. This study is the first to report an enzyme *in vitro* to oxidize harmaline and identify the specific catalytic enzyme. Furthermore, these findings reveal the metabolic pathway of harmaline and lay foundation for the oxidation reaction of tetrahydro-β-carboline and dihydro-β-carboline *in vitro*. The results further suggest that metabolism in blood should not be ignored when investigating the blood pharmacokinetics of compounds structurally similar to harmaline.

Abbreviations:

AD: Alzheimer's disease; HRP: Horseradish peroxidase; LPO: Lactoperoxidase; MAO: Monoamine oxidase; MPO: Myeloperoxidase; RLM: Rat liver microsomes; RBM: Rat brain microsomes

Supplementary Material

Supplementray information can be acquired by e-mail to

corresponding author.

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