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Tetrahydroprotoberberines inhibit lipid peroxidation and scavenge hydroxyl free radicals[1]

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KEY WORDS stepholidine；lipid peroxidation；malondialdehyde；mitochondria；free radicals

ABSTRACT

AIM：To study the effects of tetrahydroprotoberberines (THPB) on rat liver and brain lipid peroxidation (LPO) and oxygen free radicals generation. **METHODS**：The malondialdehyde (MDA) levels in rat brain and liver homogenates，induction of MDA by Fe^{2+}-Vit C in mitochondria，$OH^{\cdot}$ generation by Fenton reaction，and $O_2^{\cdot-}$ generation by pyrogallol oxidation were observed *in vitro*. **RESULTS**：(1) THPB lowered the MDA contents in the liver homogenate and mitochondria，and the IC_{50} values of *l*-THPB-18 and *l*-stepholidine (SPD) in the liver mitochondria were 3.1 and 12.7 $\mu mol \cdot L^{-1}$ respectively. SPD decreased the MDA contents in the brain homogenate and mitochondria with IC_{50} values of 102 and 35.0 $\mu mol \cdot L^{-1}$ respectively. (2) THPB scavenged $OH^{\cdot}$，and the IC_{50} values of *l*-THPB-18 and SPD were 0.21 and 3.8 $\mu mol \cdot L^{-1}$ respectively，but no effect on $O_2^{\cdot-}$ was observed. **CONCLUSION**：THPB could reduce the MDA contents and scavenge $OH^{\cdot}$ and THPB-18 was the most potent amongst them.

INTRODUCTION

We have found that some dihydroxy-tetrahydroprotoberberine analogues (dihydroxy-THPB)，such as *l*-stepholidine (SPD)，THPB-18，and scoulerine，possess an intrinsic activity to D_1 receptors[1]，and have a D_1 agonistic action in the 6-OHDA-lesioned rats，a Parkinson disease (PD) animal model[2-4]. Furthermore，SPD has been used to treat tardive dyskinesia and alleviate PD syndrome in preliminary clinical trails[5,6].

According to the current insight，PD is a neurodegenerative disease of DA neurons in the substantia nigra based on the oxygen free radicals and lipid peroxidation (LPO) induced by the oxidative stress[7-9]. The new effective remedy that could stop or slow down this degeneration can be a strategical drug，which both directly activates DA receptors (particularly D_1) and protects the surviving DA neurons in the substantia nigra[10,11]. Although this viewpoint has been well understood，the perfect drug remains elusive. The present work studied the anti-oxidative effects of THPB，particularly SPD and THPB-18，on LPO and oxygen free radicals generation.

MATERIAL AND METHODS

Animals and Materials Sprague-Dawley rats (male，Grade Ⅱ，certificate № 005，weighing 200－300 g) were purchased from Shanghai Experimental Animal Center，Chinese Academy of Sciences. Eleven THPB and 2 protoberberines (PB) (Fig 1)，supplied by Shanghai Institute of Materia Medica，were dissolved in sulphuric acid 0.1 $mol \cdot L^{-1}$，and adjusted with NaOH 0.1 $mol \cdot L^{-1}$ to pH 4－5. Other reagents were AR.

Measurement of MDA in rat liver and brain homogenate The contents of MDA in the liver or brain homogenate were measured with the thiobarbituric acid (TBA) reaction[12]. In brief，the liver and brain were homogenized with Tris-KCl buffer (Tris 0.1 $mol \cdot L^{-1}$，KCl 1 $mol \cdot L^{-1}$，pH 7.4). The supernatants were incubated with THPB at 37 ℃，and then with TBA and kept in boiling water bath. After cooling，the optical density (OD) value was measured at 535 nm (752 C spectrophotometer). MDA content was expressed as $nmol \cdot g^{-1}$ wet tissue.

Measurement of MDA initiated by Fe^{2+}-Vit

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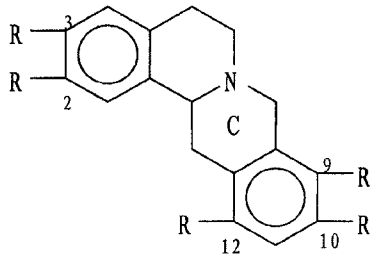
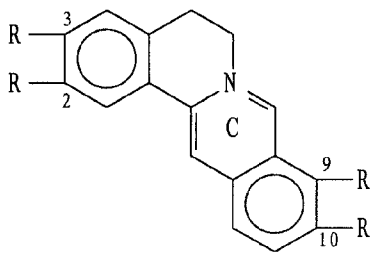


THPBs	R_2	R_3	R_9	R_{10}	R_{12}
l-THPB-18	OH	OCH_3	OH	OCH_3	Cl
d-THPB-18	OH	OCH_3	OH	OCH_3	Cl
l-THPB-19	OH	OCH_3	OH	OCH_3	Br
scoulerine	OH	OCH_3	OH	OCH_3	H
SPD	OH	OCH_3	OCH_3	OH	H
THPB-1	OH	OH	OCH_3	OCH_3	H
H-143	OH	OCH_3	H	H	H
H-25	OH	OCH_3	H	Cl	H
H-149	OH	OCH_3	C_2H_3O	H	OCH_3
l-THP	OCH_3	OCH_3	OCH_3	OCH_3	H
THB	$-OCH_2O-$		OCH_3	OCH_3	H



PBs	R_2	R_3	R_9	R_{10}
palmatine	OCH_3	OCH_3	OCH_3	OCH_3
berberine	$-OCH_2O-$		OCH_3	OCH_3

Fig 1. The chemical structures of tetrahydro-protoberberines (THPBs) and proto-berberines (PBs).

C in liver and brain mitochondria Rat liver and brain were homogenized with 10 % (w/v) ice-cold Tris-KCl buffer, then the supernatant was centrifuged at 18 000 × g, 4 ℃ for 15 min (HITACHI 25-PR520). The mitochondrial suspension 0.5 mL (containing 1 mg protein) was incubated at 37 ℃ for 60 min, in the presence of $FeSO_4$ 5 $\mu mol \cdot L^{-1}$, Vit C 10 $\mu mol \cdot L^{-1}$, and the test compounds, then 2 mL 0.1 $mol \cdot L^{-1}$ HCl and 1 mL 0.67 % TBA were added, suspension mixture was then heated for 15 min in a boiling water bath, and extracted with 4 mL BuOH after cooling. The OD value of the BuOH phase was measured at 535 nm[13]. The MDA content was expressed as $\mu mol \cdot g^{-1}$ protein.

Measurement of hydroxyl radical ($OH^{\cdot}$) generated in Fe^{2+}-EDTA and H_2O_2 system *in vitro* The suspension mixture 3.5 mL containing benzoic acid 2.187 $mmol \cdot L^{-1}$, $FeSO_4$ 21 $\mu mol \cdot L^{-1}$, edetic acid-2Na 64 $\mu mol \cdot L^{-1}$, K_2HPO_4-KH_2PO_4 buffer 109 $mmol \cdot L^{-1}$ (pH 7.4), H_2O_2 32 $\mu mol \cdot L^{-1}$, and the test compound was incubated for 5 h at 25 ℃. The fluoresence intensity of $OH^{\cdot}$ radicals generated as a result was measured (300/408 nm, HITACHI 650-10S fluorospectrophotometer)[14].

Measurement of superoxide anion ($O_2^{\cdot-}$) resulting from pyrogallol autoxidation The effects of THPBs on scavenging $O_2^{\cdot-}$ was measured by determining of rate of pyrogallol autoxidation[15].

Statistical analysis The inhibitory concentration 50 % (IC_{50}) and its 95 % confidence limits were calculated by logit's method. Statistical analysis was performed using unpaired *t* test.

RESULTS

Effect of SPD on MDA content SPD decreased the MDA contents in rat liver and brain homogenate in a dose-dependent manner (Fig 2). At the concentrations from 1.875 – 300 $\mu mol \cdot L^{-1}$, the inhibitory rates (IR) in the liver and brain homogenate were (4 ± 7) % to (83 ± 4) % and (18 ± 5) % to (80 ± 6) % respectively, and its IC_{50} values (95 % confidence limits) were 18.5 (15.5 – 22.0) and 102 (94 – 110) $\mu mol \cdot L^{-1}$, respectively.

SPD could also inhibit the MDA induced by Fe^{2+}-Vit C in rat brain mitochondria (Fig 3). At the concentrations form 7.5 – 240 $\mu mol \cdot L^{-1}$, IR were (24 ± 5) % to (92 ± 14) % with IC_{50} value of 35.0 (21.7 – 56.7) $\mu mol \cdot L^{-1}$. These results indicate that SPD possesses anti-LPO activity.

Effect of THPB on MDA content of rat liver homogenate Among the tested 11 THPB and 2 PB, *l*-THPB-18, *d*-THPB-18, *l*-THPB-19, THPB-1, H-143, and SPD all lowered the MDA level at 5 $\mu mol \cdot L^{-1}$ ($n = 5$, $P < 0.05$ *vs* control) with IR of 68 %, 56 %, 56 %, 56 %, 32 %, and 28 % respectively. The anti-LPO effect of *l*-THPB-18 was more potent than that of SPD and other THPB. At 10 $\mu mol \cdot L^{-1}$, *l*-THP could lower the MDA content ($P < 0.01$ *vs* control) with an IR of 89 %, but palmatine (dehydrogenated *l*-THP) had no obvious effect on it.

Effect of THPB on Fe^{2+}-Vit C initiated MDA content in rat liver mitochondria Fe^{2+}-Vit C

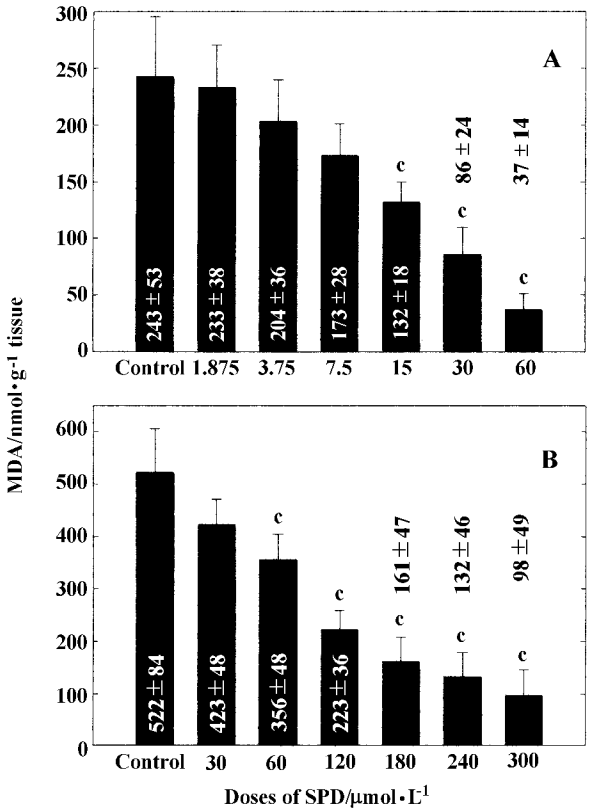


Fig 2. Effect of SPD on the MDA levels in rat liver (A) and brain (B) homogenate. $n = 4$ experiments in triplicate. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

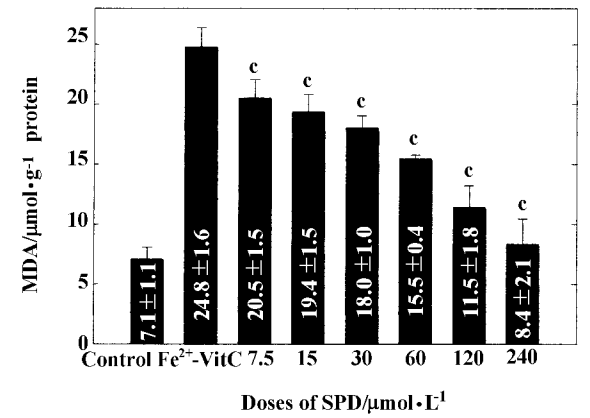


Fig 3. Effect of SPD on Fe^{2+}-Vit C induced increase in MDA levels in rat brain mitochondria. $n = 4$ experiments in duplicate. $\bar{x} \pm s$. $^cP < 0.01$ *vs* Fe^{2+}-Vit C group.

significantly increased the MDA levels in rat liver mitochondria ($P < 0.01$ *vs* control). *l*-THPB-18 (0.5 – 10 μmol·L^{-1}), *d*-THPB-18 (0.5 – 10 μmol·L^{-1}), THPB-1 (1 – 50 μmol·L^{-1}), H-143 (0.3 – 30 μmol·L^{-1}), H-25 (1 – 10 μmol·L^{-1}), scoulerine (1 – 45 μmol·L^{-1}), SPD (3.75 – 60 μmol·L^{-1}), and tea polyphenols (0.625 – 5 μg·mL^{-1}) all inhibited the MDA levels dose-dependently ($r = 0.9186 - 0.9973$, $P < 0.01$ or $P < 0.05$). Among them, *l*-THPB-18 was most potent, while SPD was the least (Tab 1). The IC_{50} of tea polyphenols was 1.49 ± 0.20 μg·mL^{-1} ($n = 4$).

Tab 1. IC_{50} values of THPB on MDA induced with Fe^{2+}-Vit C in rat liver mitochondria and fluorescence intensity of the hydroxyl radical ($OH^·$). $\bar{x} \pm s$. $n = 4$ experiments in duplicate. $^cP < 0.01$ *vs* *l*-THPB-18.

Compounds	IC_{50}/μmol·L^{-1}	
	MDA	$OH^·$
l-THPB-18	3.1 ± 0.3	0.21 ± 0.04
d-THPB-18	3.9 ± 0.6	0.24 ± 0.19
THPB-1	4.1 ± 3.1	8.3 ± 2.9^c
H-25	4.2 ± 0.2^c	8.0 ± 1.9^c
H-143	4.9 ± 1.2^c	9.0 ± 0.9^c
Scoulerine	7.7 ± 2.0^c	0.59 ± 0.15^c
SPD	12.7 ± 2.5^c	3.8 ± 0.7^c

Effects of THPB on generation of hydroxyl radical ($OH^·$) and superoxide anion ($O_2^{·-}$)

l-THPB-18 (0.01 – 3 μmol·L^{-1}), *d*-THPB-18 (0.1 – 3 μmol·L^{-1}), *l*-THPB-19 (0.1 – 2 μmol·L^{-1}), THPB-1 (1 – 20 μmol·L^{-1}), scoulerine (0.03 – 30 μmol·L^{-1}), H-25 (0.1 – 30 μmol·L^{-1}), H-143 (0.3 – 60 μmol·L^{-1}), and SPD (0.3 – 60 μmol·L^{-1}) all could significantly scavenge $OH^·$ in a dose-dependent manner. The rank order of the inhibitory potency of THPB was *l*-THPB-18, *d*-THPB-18, *l*-THPB-19 > scoulerine > SPD > H-25, H-143, THPB-1 (Tab 1). The IC_{50} of *l*-THPB-19 was 0.23 ± 0.09 μmol·L^{-1} ($n = 3$). However, none of them had any significant effect on pyrogallol autoxidation rates even with the higher doses (100 – 640 μmol·L^{-1}), which indicated that THPB could not scavenge $O_2^{·-}$.

DISCUSSION

The MDA level, used as an autoxidation index of LPO in tissues, is used to screen the antioxidants. The present results show that some THPB can remarkably lower the MDA levels, which indicate that they possess the antioxidant effects. In addition, THPB can scavenge $OH^·$ radicals *in vitro*. Among them, the most potent one is THPB-18 which has a halogen substituted group at C_{12} position, suggesting that the halogen group is impor-

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tant for enhancing the pharmacological effects of THPB analogs[2,16].

THPB can scavenge $OH^{\cdot}$ radical, but has no effect on $O_2^{\cdot-}$. This property of THPB is to that of bromocriptine (D_2 agonist)[11]. Whether THPB has the scavenging effects in other $O_2^{\cdot-}$ generating systems such as the xanthine-xanthine oxidase system remains to be further studied.

The oxygen free radicals, especially $OH^{\cdot}$, induced in DA oxidative stress is very important in the neurodegeneration of DA neurons of the substantia nigra in PD[8,9]. The present results show that THPB, such as THPB-18 and SPD possess anti-oxidative pharmacological properties. Furthermore, recent *in vitro* and *in vivo* results have demonstrated that SPD and THPB-18 could protect DA neurons from the damage induced by selective DA neuron toxin MPTP and 1-methyl-4-phenyl pyridinium ion (MPP^+) (unpublished data). Recently, SPD has been preliminarily used to treat the PD patients in the later stage and has alleviated the syndrome by combination with the lowest dose of bromocriptine[6]. Thus it can be inferred that THPB can be potentially beneficial by preventing further DA neuro degeneration in PD.

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四氢原小檗碱同类物抑制脂质过氧化和清除羟自由基[1]

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关键词　千金藤立定；脂质过氧化；丙二醛；线粒体；自由基

目的：研究 THPB 对大鼠肝、脑脂质过氧化(LPO)和氧自由基的影响. **方法**：体外测定大鼠肝、脑匀浆 LPO 和 Fe^{2+}-Vit C 诱导肝、脑线粒体 LPO 产生的 MDA，Fenton 反应产生 $OH^{\cdot}$ 和邻苯三酚氧化产生 $O_2^{\cdot-}$. **结果**：(1) THPB 减少肝匀浆和线粒体 MDA，其中 *l*-THPB-18和 SPD 的 IC_{50}为 3.1 和 12.7 $\mu mol \cdot L^{-1}$. SPD 减少脑匀浆和脑线粒体 MDA，IC_{50}分别为 102 和 35.0 $\mu mol \cdot L^{-1}$. (2) THPB 能清除 $OH^{\cdot}$，其中 *l*-THPB-18和 SPD 的 IC_{50}为 0.21 和 3.8 $\mu mol \cdot L^{-1}$；但对 $O_2^{\cdot-}$ 无抑制作用. **结论**：THPB 能减少 MDA 和清除 $OH^{\cdot}$，其中 THPB-18 作用最强.

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