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Effects of tetrathiomolybdate and penicillamine on brain hydroxyl radical and free copper levels: A microdialysis study in vivo

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ABSTRACT

Wilson disease is an inherited disorder of excessive copper accumulation. The commonly used drug D-penicillamine (PA) or trientine both cause a high incidence (10–50%) of neurological worsening, which rarely occurs with tetrathiomolybdate (TM) treatment. To investigate the mechanisms of neurologic deterioration after the initiation of chelation therapy, brain hydroxyl radical and free copper were assessed in vivo in this study. On days 3, 7, 14, and 21 after PA or TM administration, striatal hydroxyl radical levels of both TX mice and controls were assessed by terephthalic acid (TA) combined with microdialysis and high-performance liquid chromatography (HPLC). Within the same microdialysis samples, free copper was measured by inductively coupled plasma mass spectrometry (ICP-MS). The results showed that both hydroxyl radical and free copper markedly increased in the striatum of TX mice during PA administration but were not elevated when administering TM. These results suggested that the further increased free copper in the brain and oxidative stress caused by some chelators might contribute to the neurological deterioration.

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1. Introduction

Wilson disease (WD), an inherited metabolic disorder, is characterized by copper accumulation and subsequent damage to organs, especially the brain and liver. Chelators such as D-penicillamine (PA) and trientine have been recommended as the first-line therapy for decades, but they carry the risk of neurological deterioration in 10–50% of patients with WD [1–3]. In contrast, neurologic worsening is rare when treated with tetrathiomolybdate (TM) [4,5]. Thus, the mechanisms of neurological deterioration with chelation therapy are still not fully understood.

Prior studies found that free copper levels and oxidative stress increased in the plasma or cerebrospinal fluid of patients with WD and in animal models [6–9]. During the administration of PA or trientine, enhanced oxidative stress and increased free copper levels were found and were associated with neurological disorders and deterioration [4,7,10,11]. When treated with TM, there were no increases in serum free copper, and neurological worsening was rare [4].

In vivo, free copper catalyzes the generation of hydroxyl radical, which is the most active reactive oxygen species (ROS) [12–14]. It is likely that the mechanism of penicillamine- or trientine-induced neurologic worsening is that they caused serum free copper to increase further. This additional free copper is transported to the brain where it leads to enhanced oxidative stress and induces neurologic damage [10,11,15]. However, there has been no direct evidence supporting this hypothesis yet. To investigate mechanism of chelator-induced neurologic worsening, we compared the free copper and hydroxyl radical levels in striatal extracellular fluid during PA or TM administration in toxic milk (TX) mice, an animal model of WD.

Abbreviations: OH-TA, 2-hydroterephthtate; PA, D-penicillamine; ROS, reactive oxygen species; TA, terephthalic acid; TM, tetrathiomolybdate; TX mice, toxicity milk mice.

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2. Materials and methods

2.1. Animals

The TX mice mutation, which first appeared in the DL strain, carries a methionine to valine (Met1386Val) mutation in the gene for ATP7B. In this model, elevated concentrations of copper are found in the cortex, striatum, thalamus plus hypothalamus, and brain stem [16–18]. The TX mice used in this study were originally provided by Professor Julian Mercer (School of Life and Environmental Sciences, Deakin University, Australia). They were outcrossed several times to C57BL/6 mice and maintained in the C57BL/6 background [16]. C57BL/6 mice were purchased from the Laboratory Animal Center of Sun Yat-sen University, Guangzhou, China. They were bred in the Laboratory Animal Center of Sun Yat-sen University and were housed in a temperature-controlled, 12:12 light/dark room. Procedures involving animals and their care were conducted in conformity with the animal ethics guidelines of the Institutional Animal Care and Use Committee of Sun Yat-sen University (ethics approval number 20100603005). All efforts were made to minimize the number of animals used and their suffering. All mice used in this study were 16 weeks old (peak time of copper deposition in TX mice).

2.2. Experiment design

PA and TM were dissolved in deionized water and given to mice (TX and C57BL/6 mice) via intragastric administration for 3, 7, 14, and 21 days. PA was given (200 mg/kg/d) in two divided dosages after 3 h without food; TM was given (12 mg/kg/d) in two divided dosage: one after 3 h without food and the other with food. TX mice with PA or TM were assigned to the model intervention groups (TX–PA or TX-TM group), and C57BL/6 mice with PA or TM comprised the normal intervention groups (C57-PA or C57-TM group) with $n = 5$ for each time point and group. Additional mice (TX and C57BL/6 mice, $n = 3$ for each time point and group, resulting in a total of 12 mice for each TX and C57BL/6 group respectively) were given deionized water via intragastric administration and served as blank control groups (TX-Con or C57-Con group). Every mouse underwent microdialysis for detection of free copper and hydroxyl radical only one time.

2.3. Microdialysis procedure

For technical reasons, free copper and hydroxyl radical were measured in anesthetized animals in this study. Mice were anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, USA). Microdialysis probes (CMA 7 Metal Free with 6000 molecular weight cut-off, CMA Microdialysis, Kista, Sweden) with 2-mm active lengths were used in the experiment. Each probe was reused no more than four times. The active area of the probe was implanted in striatum at the following coordinates: 0.7 mm anterior of bregma, 1.2 mm lateral of bregma, and 3.2 mm ventral of the dura [19]. The whole system was perfused with Ringer's solution (147 mM NaCl, 4 mM KCl, and 1.3 mM CaCl2) containing terephthalic acid (0.25 mM) at a flow rate of 2 μl/min by a BASi/100 microinjection pump (BASi, West Lafayette, IN, USA). The perfusate was freshly made every time just before the start of the experiment and was passed through a 0.22-μm filter before use. A 90-min stabilization period was permitted before initiating dialysate sampling. Dialysate samples were manually collected every 30 min and stored at −80 °C until assayed [20,21]. The experiments were performed over 3 h; after each experiment, the probe was perfused with Millipore water at 2 ml/min for 1 h and kept in Millipore water.

2.4. Determination of free copper concentrations

Microdialysis in the striatum was performed as described above. The recovery rate of the microdialysis probe was determined in vitro. Before and after each animal microdialysis, the probe was placed into an Eppendorf 50-ml centrifuge tube containing Ringer's solution with 2 μg/ml Cu^{2+}. The solution was incubated at 37 °C and collections made at 2 μl/min for 3 h. The microdialysis probes provided approximately 30–35% relative recovery for free copper. The concentrations of free copper in the dialysate were determined by ICP-MS. For ICP-MS analysis, 200 μl of the dialysate was diluted to 2 ml with Milli-Q water. An Agilent 7700x quadrupole ICP-MS equipped with a He Octapole reaction/collision cell was used. The instrument parameters were as follows: 1550 W RF power, 1.05 l/min carrier gas, 0.1 rps nebulizer pump, 2 °C spray chamber temperature. He flows were set at 4.3 ml/min when interference removal by the collision cell was required. Calibration solutions were obtained from a 100-ppm multielement stock solution (CPI International, Santa Rosa, CA). All samples and standards were analyzed in the quantitative mode.

2.5. Determination of hydroxyl radical formations

Samples were analyzed by reversed-phase high-performance liquid chromatography (HPLC) coupled with fluorescence detection (Agilent Technology, Vienna, Austria) using an excitation wavelength of 315 nm and an emission wavelength of 435 nm. The mobile phase was composed of 50 mM KH_2PO_4 and 30% methanol, adjusted to pH 3.2 with 1 M H_3PO_4. This mobile phase was delivered at 0.8 ml/min flow rate (Pump 420, Kontron Instruments, Milano, Italy) through a Hypersil column (C18, 4.6 × 150 mm, 5 μm; Sigma–Aldrich Chemicals, USA). A sample volume of 10 μl was injected into the HPLC system with a total elution time of 10 min [20,21]. The concentration of OH-TA in each sample was determined against a standard curve of OH-TA generated from the authentic standard. Before and after each microdialysis, probe recovery was determined by putting the probe into a 50 ml centrifuge tube with 1 μM OH-TA (dissolved in Ringer's solution) and perfused with Ringer's solution for 3 h. The results suggest that within the four times used, these probes provided approximately 28–35% relative recovery for OH-TA. Microdialysis data is presented without corrections for probe recovery in this article.

2.6. Chemicals

D-Penicillamine (D-PA), $CuSO_4$, terephthalic acid, and its hydroxylated metabolite OH-TA were purchased from Sigma–Aldrich (St. Louis, MO, USA). All standard solutions were freshly prepared using water from a Milli-Q system, (Millipore, Bedford, MA, USA). The pH of the standard solutions was maintained in the pH 6–9 range unless stated otherwise.

2.7. Statistical analysis

All values are presented as means ± standard errors of the mean (SEM). Tests for statistical significance (one-way ANOVA followed by post hoc Bonferroni) were performed using SPSS 20.0. Values were considered to differ significantly at the level of $p < 0.05$.

3. Results

3.1. Free copper levels in striatum during PA or TM administration

The means and SEM of free copper in striatal extracellular fluid are shown in Fig. 1 and Table 1. All the time points refer to the

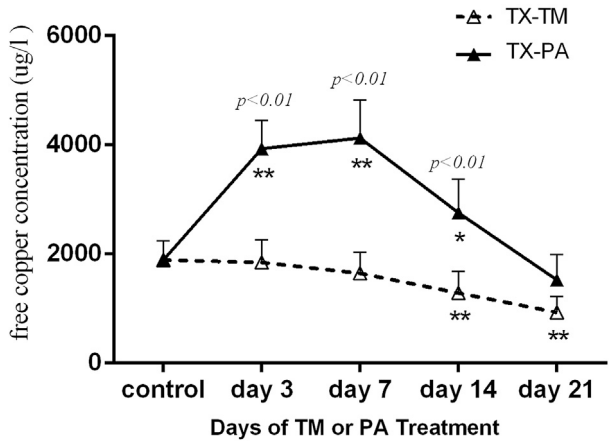

Fig. 1. Free copper levels in striatal extracellular fluids of TX mice. Means and SE of the free copper values during TM and PA administration. The *p*-values ($p < 0.01$) shown in the figure indicate significant differences between the TM group and PA group. The mean concentrations of the PA group were significantly higher than those of the control group at days 3, 7, and 14; while in the TM group, the mean concentrations were significantly lower than those of the control group at days 14 and 21 are shown by asterisks ($^{*}p < 0.5$, $^{**}p < 0.01$).

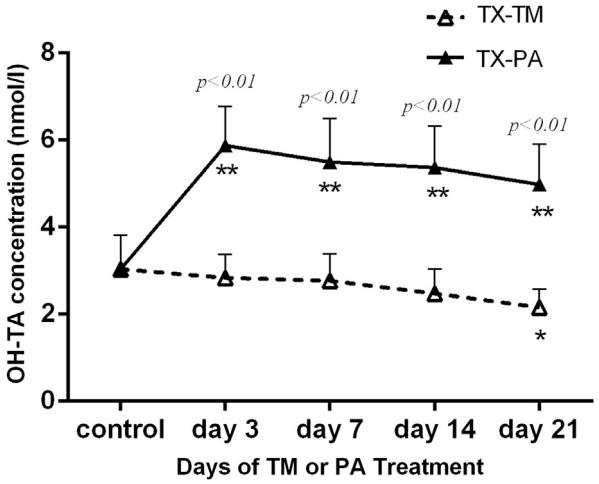

Fig. 2. OH-TA levels in the striatal dialysate of TX mice. Means and SE of the OH-TA values during TM and PA administration. The p-values ($p < 0.01$) shown in the figure indicate significant differences between the TM and PA groups. The mean concentrations of the PA group were significantly higher than those of the control group at days 3, 7, 14, and 21; while in the TM group, the mean concentrations were significantly lower than those in the control group at day 21, are shown by asterisks. ($^{*}p < 0.5$, $^{**}p < 0.01$).

number of days after the start of PA or TX treatment. The mean level of free copper in the TX–PA group increased on days 3, 7, and 14, and was significantly higher than that of the TX-TM group ($p < 0.05$, Fig. 1). From day 7, it progressively declined and fell to the level of the controls by day 21 ($p > 0.05$, Fig. 1). In the TX-TM group, the mean level of free copper continued to decline over the 21 days, and by day 14 it was significantly lower than that of the TX-Con group ($p < 0.01$, Fig. 1). The mean level of free copper in the C57-PA group also increased slightly within 3 days after PA administration but without significant difference compared to the C57-Con group ($p > 0.05$, Table 1). In the C57-TM group, the mean level of free copper progressively declined within the 21 days and reached significantly different levels compared to the C57-Con group by day 14 ($p < 0.01$, Table 1). In the control groups (TX-Con and C57-Con, mice without PA or TM), the free copper levels in the TX-Con group were slightly higher than those of the C57-Con group, but this difference was not significant ($p > 0.05$). Furthermore, no significant differences were observed between different time points in the TX-Con and C57-Con group.

3.2. Hydroxyl radical levels in the striatal dialysate during PA or TM administration

The means and SEM of OH-TA levels in the striatal dialysate are shown in Fig. 2 and Table 1. During the 21 days, the mean level of OH-TA in the TX–PA group was significantly higher than that of the TX-TM and control group ($p < 0.01$, Fig. 2). The mean level of OH-TA in the TX-TM group progressively decreased and became significantly lower than that of the TX-Con group at day 21 ($p < 0.05$, Fig. 2). The mean level of OH-TA in the C57-PA group also slightly increased 3 days after PA administration but without a significant difference compared to the C57-Con group ($p > 0.05$, Table 1). Over the course of 21 days, the mean level of OH-TA progressively declined in the C57-TM group and reached a significant difference compared to controls at day 21 ($p < 0.05$, Table 1). In the control groups (mice without PA or TM), similar to the free copper concentration, the OH-TA levels in the TX-Con group were slightly higher than those of the C57-Con group ($p > 0.05$). In addition, no significant differences were observed between various time points in the TX-Con and C57-Con group.

Table 1
Striatal free copper and OH-TA levels in C57 mice during PA and TM administration.

Days of PA or TM administration	PA administration		TM administration	
	Free copper	OH-TA	Free copper	OH-TA
Control	1647.35 ± 368.73	2.61 ± 0.74	1647.35 ± 368.73	2.61 ± 0.74
Day 3	1737.62 ± 385.06	3.31 ± 1.77	1579.62 ± 344.99	2.23 ± 0.45
Day 7	2036.08 ± 395.69	3.20 ± 1.16	1278.3 ± 216.45	2.28 ± 0.47
Day 14	1804.18 ± 459.30	2.14 ± 0.47	990.66 ± 188.61**	2.03 ± 0.33
Day 21	1484.46 ± 329.68	2.55 ± 0.90	861.42 ± 78.59**	1.87 ± 0.40

The units of free copper levels are expressed as μg/L, and OH-TA levels are expressed as nmol/L. All values are means ± SEM. Intervention group, n = 5 for each time point and group; control group, n = 12. Values marked with asterisks indicate significant differences between intervention group and control group. $^{*}p < 0.05$; $^{**}p < 0.01$.

4. Discussion

The neurological deterioration of patients with WD that occurs during chelation therapy has puzzled neurologists for decades. We hypothesize that PA or trientine cause serum free copper further increase, and the additional free copper is transported into the brain where it leads to enhanced oxidative stress and induces neurologic damage. However, few studies monitored the changes of free copper and oxidative stress in the brain during chelation therapy in vivo; thus, there was no direct evidence to support this hypothesis.

Traditional and the direct method cannot be used to assay the free copper levels in the extracellular fluid of brain in vivo [9,22]. In this study, the free copper levels in the brain are been assayed by microdialysis in vivo [23]. Several methods were developed to measure the brain's oxidative stress in WD and other processes [24,25]. However, few studies have monitored the oxidative stress in the brain in vivo during administration of chelators. Furthermore, hydroxyl radical, the most reactive and damaging ROS that could be enhanced by free copper, has not been assessed during chelation therapy. In this study, striatal hydroxyl radical is measured by TA-trapping method combined with microdialysis [21]. The detection of hydroxyl radicals is based on the conversion

of TA into the strongly fluorescent 2-hydroxyterephthalate (OH-TA). This is a new valid and sensitive method to detect hydroxyl radical [21,26].

In our study, the striatal free copper and hydroxyl radical levels in the TX–PA group were much higher than those of the TX-TM group during the 21 days of study. Compared to the control group (administration of water), free copper and hydroxyl radical showed significant spikes in the TX–PA group; while in the TX-TM group, free copper and hydroxyl radical gradually declined and became significantly lower than control at day 14 and 21. We suggest that this may be the reason why neurological worsening is rare with TM treatment compared to other chelating agents.

A previous study found that when C57 mice were treated with PA, they did not show significant spikes in serum free copper [11]. Our results showed that although the mean levels of free copper and hydroxyl radical in the C57-PA group slightly increased 3 days after PA administration, there was no significant difference compared to control. In the C57-TX group on the other hand, levels gradually declined and free copper even became significantly lower than control by day 14.

Our findings suggest that the large stores of copper in the TX mice could be mobilized by penicillamine, which caused further elevation of free copper in the serum and brain extracellular fluid and thus enhanced brain oxidative stress. As the C57 mice had no large stores of copper, the increased free copper and enhanced oxidative stress were not significantly higher than the control. We also found that when treated with TM, the mean level of striatal free copper and hydroxyl radical gradually declined in both TX and C57 mice.

5. Conclusion

D-Penicillamine might mobilize stored copper and further result in increased free copper and enhanced oxidative stress in the brain, while TM administration does not lead to spikes of hydroxyl radical and free copper levels in the brain. The likely mechanism of neurologic worsening induced by penicillamine and trientine is that these chelators cause further elevation of brain free copper. Therefore, we suggest that the control of free copper levels represents a promising strategy in the treatment of neurological WD patients.

Conflicts of interest

The authors declare no conflict of interest.

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Transparency document

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