Trace Amounts of Copper in Drinking Water Aggravate Cerebral Ischemic Injury via Impairing Endothelial Progenitor Cells in Mice

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Copper plays a crucial role in nutrition and normal brain function [1,2]. In daily life, we come into contact copper easily in our drinking water, food, and so on. The Environmental Protection Agency's (USA) maximum allowable contaminant level for copper in municipal drinking water is 1.3 mg/L [1]. But in some areas, especially in heavy metal pollution regions, copper content in the water exceeds the maximal level [1,2]. Moreover, copper has been implicated as an important factor in Alzheimer's disease (AD) and Huntington's disease (HD) [1,2]. However, there is limited information about the link between copper contamination of drinking water and stroke (a devastating disease and the major cause of mortality and morbidity worldwide) [3] to date.

Endothelial progenitor cells (EPCs) are a circulating, bone marrow-derived cell population that participate in vasculogenesis by differentiating into endothelial cells and play an important role in tissue repairing and regeneration in ischemic brain [4,5]. Long-term studies indicate that the number and function of EPCs in peripheral blood may be a new marker for stroke outcomes and cumulative cardiovascular risk [4,5]. Thus, EPC dysfunction and the consequent abnormality of endothelial regeneration may influence the susceptibility to cerebral ischemic injury. In addition, the involvement of copper in the pathogenesis of vascular dysfunction has been proposed [6]. Therefore, it can be logically speculated that copper contamination of drinking water might pose as a risk factor for stroke via impairing EPC function. To address this issue, this study sought to determine the effect of trace amounts of copper in drinking water on cerebral ischemic injury and EPC functions in mice.

Male C57BL/6 mice (10–12 week, 20–25 g) used in these experiments were obtained from Sino-British SIPPR/BK Lab Animal Ltd (Shanghai, China). All animals received humane care,

and the experimental procedures were in compliance with the institutional animal care guidelines. Mice were given trace amounts of copper (0.13 mg/L, about one-tenth of the Environmental Protection Agency's maximum allowable contaminant level in municipal drinking water) [1] as copper sulfate (469130; Sigma, St. Louis, USA) and water (Control) via drinking water for 14 weeks. The body weights were monitored every week. After long-term treatment, mice were subjected to permanent left middle cerebral artery occlusion by electrocoagulation, and the infarct volumes and neurobehavioral outcomes were determined according to the published protocols [7]. It was found that there was no significant difference in body weights between copper-treated and control mice (data not shown). However, the infarct volumes were significantly increased and the corresponding neurobehavioral outcomes were markedly impaired in copper-treated mice compared with control (Figure 1A). These results indicate that chronic exposure to trace amounts of copper could aggravate cerebral ischemic injury in mice.

To determine the potential mechanisms underlying trace amounts of copper aggravating cerebral ischemic injury in mice, EPC functions and local angiogenesis in ischemic brain were assessed according to previously described technique [3,4,8]. It was found that EPC functions (Figure 1B) and capillary density in ischemic brain (Figure 1C) were significantly reduced in the copper-treated mice compared with control. EPCs can secrete various angiogenic growth factors to promote angiogenesis and have been used to successfully restore endothelial function and enhance angiogenesis in ischemic brain tissue [4,5]. Therefore, the increase of cerebral ischemic injury produced by chronic copper intake may be partly attributed to the EPC dysfunction and consequent reduction of local angiogenesis in ischemic brain.

Figure 1 Chronic copper intake aggravated cerebral ischemic injury, impaired EPC functions, and reduced angiogenesis in ischemic brain in mice. **A**, The representative images of TTC-stained brain sections (a), cerebral infarct volumes (b), and neurobehavioral outcomes (c, body asymmetry test; d, beam test) at 3 days after the left middle cerebral artery occlusion. ** $P < 0.01$ vs. Control. $n = 9-11$. **B**, EPC functions were impaired in copper-treated mice compared with control. The migration (a, b, g), tube formation (c, d, h), and adhesion (e, f, i) assays of EPCs. $n = 7-8$, ** $P < 0.01$ vs. Control. Scale bar: 100 μm . **C**, CD31 immunostaining shows microvessels in ischemic brain of mice. The bar graph shows that the number of microvessels in copper-treated mice was decreased compared with that in control animals. * $P < 0.05$ vs. Control. $n = 12$. Scale bar: 100 μm (up); 35 μm (below). Control, vehicle-treated mice; Copper, copper-treated mice.

Next, we sought to investigate the potential mechanisms underlying the EPC impairment in copper-treated mice. It was found that, compared with control, p-eNOS and MnSOD expression levels (Figure 2A,B) and intracellular NO levels (Figure 2D) were significantly reduced, and TSP-1 expression levels (Figure 2C) were markedly increased in EPCs from copper-treated mice. It has been

shown that eNOS and MnSOD critically regulate EPC function [5,8], and decreased NO production can induce TSP-1 (a key inhibitor of endothelial cell and EPC function) expression in cultured endothelial cells and EPCs [8,9]. Thus, the decreased MnSOD and phosphorylated eNOS levels and consequent reduction in intracellular NO levels, together with the increase in TSP-1

Figure 2 Effects of chronic copper intake on eNOS, p-eNOS, MnSOD, and TSP-1 expression and intracellular NO levels of EPCs in mice.

A, eNOS and p-eNOS expression levels in EPCs. ** $P < 0.01$ vs. Control. $n = 3$. **B**, MnSOD expression levels in EPCs. ** $P < 0.01$ vs. Control. $n = 7$. **C**, The secreted TSP-1 levels of EPCs. ** $P < 0.01$ vs. Control. $n = 4$. **D**, intracellular NO levels (DAF fluorescence intensity) in EPCs. * $P < 0.05$ vs. Control. $n = 12$ –13. p-eNOS, phosphorylated eNOS; TSP-1, thrombospondin-1; Control, vehicle-treated mice; Copper, copper-treated mice.

levels, might represent a major mechanism underlying the EPC dysfunction in copper-treated mice.

In conclusion, chronic intake of trace amounts of copper aggravated cerebral ischemic injury in mice, which might be partly attributed to the impairment of EPCs and consequent reduction of angiogenesis in ischemic brain. This result implies that copper contamination of drinking water might be a risk factor for stroke, and the risk of cardiovascular diseases (especially stroke) might warrant further attention in heavy metal pollution regions.

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Conflict of Interest

The authors declare no conflict of interest.

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