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• ORIGINAL RESEARCH •

Copper transportion of WD protein in hepatocytes from Wilson disease patients *in vitro*

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Abstract

AIM: **To study the effect of copper transporting P-type ATPase in copper metabolism of hepatocyte and pathogenesis of Wilson disease (WD).**

METHODS: **WD copper trans porting properties in some organelles of the cultured hepatocytes were studied from WD patients and normal controls. These cultured hepatocytes were incubated in the media of copper 15 mg·L^{-1} only, copper 15 mg·L^{-1} with vincris tine (agonist of P-type ATPase) 0.5 mg·L^{-1}, or copper 15 mg·L^{-1} with vanadate (antagonist of P-type ATPase) 18.39 mg·L^{-1} separately. Microsome (endoplasmic reticulum and Golgi apparatus), lysosome, mitochondria, and cytosol were isolated by differential centrifugation. Copper contents in these organelles were measured with atomic absorption spectrophotometer, and the influence in copper transportion of these organelles by vanadate and vincristine were comparatively analyzed between WD patients and controls. WD copper transporting P-type ATPase was detected by SDS-PAGE in conj unction with Western blot in liver samples of WD patients and controls.**

RESULTS: **The specific WD proteins (Mr155 000 lanes) were expressed in human hepatocytes, including the control and WD patients. After incubation with medium containing copper for 2 h or 24 h, the microsome copper concentration in WD patients was obviously lower than that of controls, and the addtion of vanadate or vincristine would change the copper transporting of microsomes obviously. When incubated with vincri stine, levels of copper in microsome were significantly increased, while incubated with vanadate, the copper concentrations in microsome were obviously decreased. The results indicated that there were WD proteins, the copper transportion P-type ATPase in the microsome of hepatocytes. WD patients possessed abnormal copper transporting function of WD protein in the microsome, and the agonist might correct the defect of copper transportion by promoting the activity of copper transportion P-type ATPase.**

CONCLUSION: **Copper transportion P-type ATPase plays an important role in hepatocytic copper metabolism. Dysfunction of hepatocytic WD protein copper transportion might be one of the most important factors for WD.**

Subject headings glucuronosyltranferase/genetics; glucurono syltranferase/biosynthesis; DNA,complementary/ genetics; liver/cytology; hasters; lung/cytology; animal

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INTRODUCTION

Hepatolenticular degeneration (Wilson disease, WD) is an autosomal recessive disorder first described in detail by Wilson in 1912, which is characterized by excessive accumulation of copper in the liver, brain, cornea an d subsequently in kidneys and other organs. The disease has a world prevalence of 5-50 per million and a birth incidence from 17-29 per million[1-6]. In China, WD is one of the most common neurogenetic diseases. According to a survey reported in 1995, WD patients accouted for about 10.14% of the total 957 neurogenetic patients first visiting the Neurogenetic Clinic of the First Hospital affiliated to Sun Yat-Sen University of Medical Sciences, and ranked as the second on the list[7]. The principle of copper metabolic disturbance in WD includes low serum cerulopl a smin levels and low serum copper levels, as well as increased copper excretion in urine. By means of removing the excessive copper, the disease development will be inhibited, and if treatment started before the appearance of neurological manif estations, the latter can be prevented to a large extent[2-3,8]. However, why does the abnormal copper metabolism happen in WD? It has been shown that more than 95% of circulating plasma copper were bound to a blue-copper oxidize cerulopl smin (CP), while the levels of CP was magnificently reduced in the majority of WD patients[1,9]. But no relationship was found between the concentration of cellular copper and the CP gene expression or CP protein with rodent mod el of WD and patients' cultured fibroblasts[10-13]. Therefore, it was suggested that the genetic defect of copper transportation did not alter biosynthetic and secretary of CP. Seemingly, neither the theory of MT (metallothionein) nor lysoso me abnormality could well explain the pathogenesis of WD[14-16]. Recently more concerns were focused on ATP7B, the gene of WD, which was just found in 1993 and has been mapped to chromosome 13q14.3 by three different genetic techniques[17-19]. Many researchers are trying to search for clues to the copper metabolic abnormality from the mutations of this gene, and the latter was predicted to en cod e a putative protein product, the WD copper-transporting P-type ATPase (WD protein), which has 1411 amino acids and a calculated molecular mass of about 159ku[20-28]. But up to now, the cellular localization of WD protein apparently has not yet been documented. There were reports that canalicular membranes, mitochondri a, microsome, or Golgi apparatus had WD

proteins[29-34]. However, all these researches were carried out in animal livers or in abnormal/immortal cell lines, which h ad much more different cell structures and biochemical metabolisms from human beings. We now set up a cultured hepatocyte model for studying WD copper transporting properties in such suborganelles as microsomes, lysosomes, cytosol, and mitochondria of the cultured hepatocytes from WD patients and normal controls under different incubative conditions with copper, ATP or the adjusting agents of WD proteins, and analyzed the cellular localization of WD proteins in hepatocytes.

MATERIALS AND METHODS

Subjects

Five (male 3, female 2) patients, aged 13-31 years, were diagnosed as having Wilson disease patients according to clinical symptoms, signs and copper biochemical laboratory assay by our Neurohereditary Clinic from 1998 to 1999. They had lower levels of serum ceruloplasmin and high levels of urinary copper. They all had liver cirrhosis accompanied by splenomegaly, and intended to receive splenectomy and liver biopsy. Five (male 4, female 1) controls, aged 28-49 years, were patients with hepatolith, cholith, or liver a ngioma, or healthy liver grantors, with normal neurological examinations and nor mal copper chemical tests, and were to receive hepatolobectomy. Immediately after operation, liver samples were rinsed and preserved in 4℃ F12/DMEM culture medium.

Hepatocyte culture and protein blotting

Hepatocytes were separated by 0. 5 g·L^{-1} type IV collagenase digested and cultured according to the methods introd uced by literatures[35-40]. The isolated hepatocytes were seeded and cultured in flasks pre-coated with rat tail collagen at 37℃ 50 mM·L^{-1} CO_2 with F12/DMEM supplemented with 200 mM·L^{-1} fetal bovine serum, 10 mmol·L^{-1} nicotinamide, 5 mg·L^{-1} a mphotericin B, 0.5 mg·L^{-1} glucagon,10 ìg·L^{-1} EGF(epidermal growth factor), 10 μg·L^{-1} insulin-transferrin-sodium selenite media supplement, and other growth factors. The cultured cells were observed and photographed and the media were partly changed every day. When they grew to contact with each other, discard the culture media, and cultured the hepatocytes further for 2 or 24 h in all WD patients and controls with culture media containing: copper 15 mg·L^{-1} only; copper 15 mg·L^{-1}, ATP 30 mmol·L^{-1} and vanadate 18.39 mg·L^{-1}; copper 15 mg·L^{-1}, ATP 30 mmol·L^{-1} and vincristihe 0.5 mg·L^{-1}; and copper 15 mg·L^{-1} and ATP 30 mmol·L^{-1}, respectively. After reincubation, cells were rinsed with D-Hanks solution atroom temperature, harvested by rubber policeman after adding 1 mL of 0.05 mol·L^{-1} Tris-HCl (2.5 g·L^{-1} Nonidet P-40, 0.5 mmol·L^{-1} PMSF, 0.1 g·L^{-1} aprotinin, 1 mg·L^{-1} leupeptin, 1 μmol·L^{-1} pepstantin, pH8.6), dissolved for 15 min at 4℃ and disintegrated under ultrasonic (80 W×90 s), and then centrifuged at 16 000× *g* for 20 min. Finally, the protein rich supernatants were transferred to separate vials for testing or preservation at-700℃.

Fifty μg proteins of the supernatants were first separated by SDS-PAGE on 200 volts in 60 g·L^{-1} gels for 45 min, followed by 90 min of electrophoretic transfer to nitrocellulose membranes on 120 volts[31,41] according to the instructions of the Bio-Lab kit (New England Bio-Lab Co.). Transferred membranes were incubated with the primary antibodies and goat anti -rabbit antibodies with HRP (Bio-Lab Co.), respectively. After rinsed with the buffer thoroughly, the membranes were reacted with enhanced chemiluminescence. The primary antibody was a rabbit anti-human WD protein antibody (anti-WD), a gift from Dr. Gitlin and Dr. Lutsenko. The band number, density and molecular weight of specific bands were observed and analyzed by Bio-Rad Gel 2000 Imaging System.

Isolation of organelles

Total homogenates of cytosol, lysosome, microsome and mitochondria were isolated at 4℃ by differential centrifugation (8 000×g, 10 min; 9 000×g, 10 min; 30 000×g, 15 min; 108 000×g, 60 min) using super-high speed centrifuge (Beckman L8-55M, USA). Degree of contamination of cytosol, lysosome and microsome were estimated by measuring the lactate dehydrogenase a ctivities, acid-phosphatase activity and glucose-6-phosphatase activityies, respectively.

Content of copper and protein assay

All samples were assayed for protein concentration by the methods described by Bradford[4], using the bovine serum albumin as a standard. Copper contents were measured with atomic absorption spectrophot ometer, and expressed as copper/protein ratios (Cu/Pr):

$$\text{Cu/Pr } (\mu\text{g}\cdot\text{g}^{-1}) = \frac{\text{Copper contents } (\mu\text{g}\cdot\text{L}^{-1})}{\text{Protein contents } (\text{g}\cdot\text{L}^{-1})}$$

Statistical analysis

Results were given as the mean with the corresponding standard deviation ($\bar{x}\pm s$). Statistical analysis was performed with SPSS/8.0. F test and Student's t test were used to determine the differences between the means of different groups. Statistical significance was considered at the level of $P<0.05$.

RESULTS

Hepatocyte morphology

After the first 24 h culture, viable hepatocytes changed their shape from spherical to flat on the substrate and displayed one or two long cytoplasmic projections onto the substrate, and a ppeared three to six sided in shape. After 4 d, a widespread and a monolayer of hepatocytes could be found. After 7 d, they became smaller and with more granules. After 21 d culture, hepatocytes began to fall from the flasks and died.

Protein blotting

Western blotting analysis of WD protein separated from cultured human hepatocytes two main lanes with molecular mass of M_r 155 000, 90 000 and 80 000 were found in normal human and WD patient hepatocytes, but non could be seen in the blood vessel endotheliocytes of human liver (Figure 1).

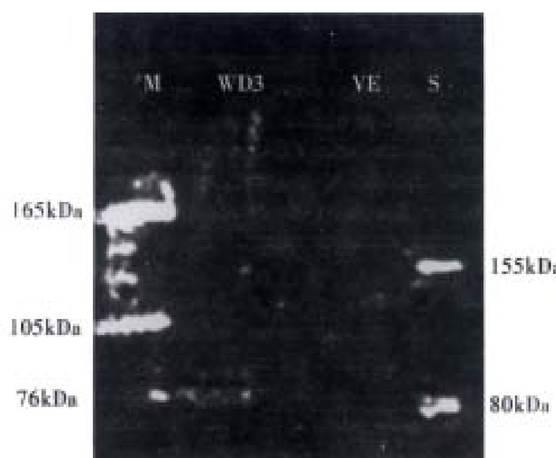

Figure 1 Western blotting analysis of WD protein separated from cultured human hepatocytes. M: Protein molecular mass markers; WD3: The hepatocytes of one WD patient; VE: Blood vessel endotheliocyte; S: Normal human hepatocytes.

Copper transportion of hepatocytes

Normal subjects After 2 h incubation with 15 mg·L^{-1} copper, the copper levels of all organelles increased significantly. When adding 30 mmol ·L^{-1} ATP to the culture media, there were different changes of copper concentrati ons in different organelles. Lysosome and microsome copper contents were much higher with ATP than without ATP, and the cytoplasmic copper level with ATP was lower than that of without ATP, and the differences between each group were not significant by Student's *t* test. The copper level of mitochondria showed no significant change. After 24 h copper incubation, the copper contents of microsome, mitochondria and cytoplasm with ATP became much lower than that without ATP, while the copper contents in lysosome showed no changes (Table 1).

Table 1 Concentrations of copper in organelles of nor mal hepatocytes after 2 h or 24 h culture ($\bar{x}\pm s$, μg·g^{-1})

t (culture) /h	ATP/ (30 mmol·L^{-1})	Microsomes	Lysosomes	Cytoplasm	Mitochondria
0		74±13	70±10	526±63	85±11
2	absence	231±31	306±19	1571±115	420±43
2	presence	269±43[a]	342±26[a]	1488±129[a]	395±35
24	absence	346±52	322±40	1589±137	458±68
24	presence	288±39[a]	369±46	1464±110[b]	417±73[a]

[a]$P<0.05$, *vs* ATP absence; [b]$P<0.01$, *vs* ATP absence.

WD patients

Copper contents of cytoplasm after incubation with medium containing 15 mg·L^{-1} copper for 2 h, cytoplasmic copper concentration in WD patients became obviously higher than that of controls under all incubative conditions ($P<0.05$ *vs* control). When co-incubated with 0.5 mg·L^{-1} vincristine, there was no significant change of copper concentration in WD patients, while it decreased in the controls; when adding 18.39 mg·L^{-1} vanadate, there was no significant change of copper concentration in WD patients, while it inicreased in the controls ($P<0.05$). After 24 h culture with copper, cytoplasm copper levels of WD patients were higher than the controls ($P<0.01$ *vs* control). The adding of 0.5 mg·L^{-1} vincristine decreased its cop per level ($P<0.01$ *vs* no vincrinstine), but there was no difference between the WD group and the controls. When adding 18.39 mg·L^{-1} vanadate, the copper level in WD group increased ($P<0.05$ *vs* no vanadate), while that of the controls did not change, and that of WD group was higher than the controls ($P<0.01$ *vs* control, Figure 2).

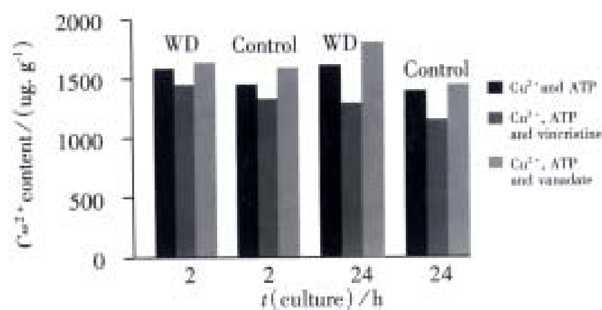

Figure 2 Cu^{2+} levels in hepatic cytosol at different culturing conditions.

Copper contents of microsome Copper concentrations of microsome in WD patients were obviously lower than that of controls after incubation for 2 h under each incubative condition ($P<0.05$ *vs* control). After incubation of copper and 0.5mg·L^{-1} vincristine, copper levels in the microsome were significantly increased ($P<0.05$ *vs* no vincrinstine). And after incubation with copper and 18.39 mg·L^{-1} vanadate, the copper concentrations of microsome in WD patients were obviously decreased ($P<0.05$ *vs* 2 h of no vanadate).

After 24 h incubation with 15 mg·L^{-1} copper and 30 mmol·L^{-1} ATP, copper contents in WD group were lower than the controls ($P<0.05$ *vs* control). When adding 0.5 mg·L^{-1} vincristine, there was no change in the WD group, and when adding 18.39 mg·L^{-1} vanadate, the copper contents increased ($P<0.05$ *vs* 24 h of no vanadate, Figure 3).

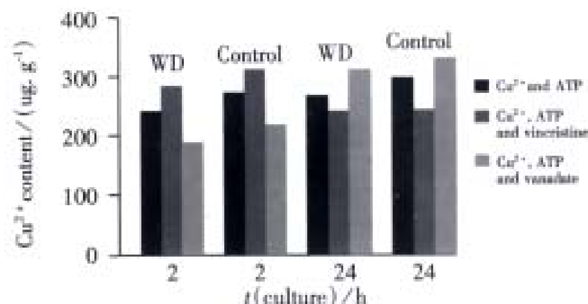

Figure 3 Cu^{2+} levels in hepatic microsome under different culturing conditions

Copper contents of lysosome After incubation with medium containing 15mg·L^{-1} copper and ATP for 2 h, lysosomal copper concentrations in WD patients were lower than that of controls ($P<0.05$ *vs* control), and when adding vanadate, there was significant decrease of copper concentration in the controls ($P<0.05$ *vs* no vanadate). With incubation up to 24 h, the WD patients' lysosome copper concentrations rose to the same level as the controls. When co-incubated with vincristine or vanadate, there was no change in the copper concentration of WD patients (Figure 4) .

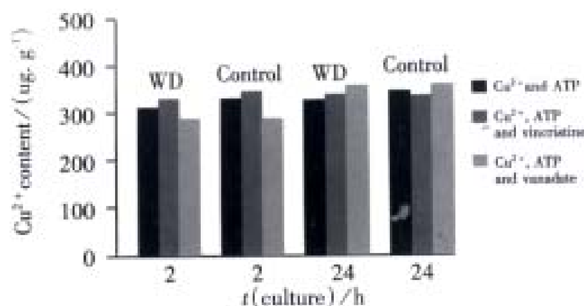

Figure 4 Cu^{2+} levels in hepatic lysosome under different culturing conditions.

Copper contents of mitochondria The copper content in mitochond ria was significantly lower than the controls when cultured 2 h or 24 h with 15 mg ·L^{-1} copper and 30 mmol·L^{-1} ATP. But when adding 0.5 mg·L^{-1} vincristine or 18.39 mg·L^{-1} vanadate, there was no significant change in the copper level of mi tochondria (Figure 5).

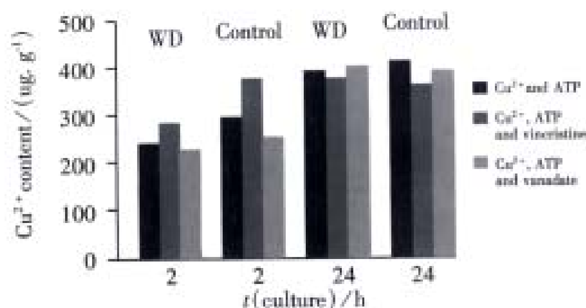

Figure 5 Cu^{2+} levels in hepatic mitochondria under different culturing conditions.

DISCUSSION

Copper is a trace element required by most organisms and is indispensable as a cofactor in a number of proteins, including cytochrome-c-oxidase, superoxide dismutase (SOD), dopamine o-hydroxylase, lysyl oxidase and ceruloplasmin. Both of lack and accumulation of copper may cause diseases[13,20]. One of the most important copper pumps in human body is considered as the copper transporting P-type ATPase, which is considered to play an essential role in cellular copper homeostasis[17-19,42]. In order to study the copper metabolism disorder of WD, Chan *et al* [43] ha d first applied the technique of skin fibroblast culture *in vitro* 20 years a go. Because it is easy to get the skin specimen, skin fibroblasts can be cultured and pass generations successfully, and its culture *in vitro* can be controlled and repeated steadily, this model had been widely used by researchers from all over the world[4,12,44]. In China, Liang and Chen *et al*[4,44] improved the fib roblast culturing model to study the copper metabolism of WD in 1992, and found that incubation with high contents of copper could promote the expression of hereditary abnormalities of copper metabolism in cultured skin fibroblasts of WD patients. They had studied the characteristics of copper uptake and excretion, analyzed the copper transporting manner of suborganelles, probed the actions of zinc on WD, and investigated the possible mechanisms of vanadate and vincristine to regulate the copper metabolism of WD cultured cells. However, as the copper metabolism disorders originated from the liver, and the main lesions were also localized in the liver, the study on the hepatocytes of WD patients can more directly reveal the possible mechanism of abnormal copper metabolism in WD.

Hepatocyte is one of the high-differentiated cells in human body[36-39,45]. Under normal biological conditions, hepatocytes of adult human body remain still and do not divide until stimulated by lesion, inflammation or other pathological factors. Cultured with common medium *in vitro*, hepatocytes can not multiply and divide. The rough endoplasmic recticulums disappear rapidly, cell appearance changed early, and its biochemical functions attenuated simultaneously. The cultured hepatocytes lost their tissue specific functions within 3 d to 5 d[35,36]. So it is important to improve the skills of hepatocyte separation and culture.

We had used thin biopsy liver pieces to culture rat hepatocytes by the method for skin fibroblasts culture[43,45,46]. In the first 3 days culture, there were round cells removing from the liver slices, and the cell number increased gradually. One week later, most of the cells grew to triangles or multipleangles in shape which were hepatocytes, and less cells showed shuttle-shaped, which were fibroblasts or other fibroblastoid. Up to 2-3 weeks, cells spread over the bottom of the flasks, but most of them were fusiform shaped and were fibroblastoid, and the hepatocytes were very rare then. This indicated that hepatocytes needed much higher culture condition than fibroblasts. When we cultured hepatocytes from embryo rats, the hepatocytes could be divided rapidly even if common culture media PMP L 1640 were used. And when various growth factors and mineral metals were added into the media, the embryo hepatocytes could maintain strong capacities of albumin synthesis and secretion up to one month or more. Most researchers agreed that embryo hepatocytes remained immature, so they could divide and be cultured easily[36].

WD patients we studied in this series, all had liver cirrhosis, and their hepatocytes were more difficult to culture. We mimicked the normal natural growth conditions of human liver and supplemented the culture media with fetal bovine serum, nicotinamide, amphotericin B, glucagon, EGF (epidermal growth factor), insulin-transferrin-sodium selenite media supplement. The hepatocytes in WD were then growing as the normal ones except that fewer fibroblasts speckled. We could erase the fibroblasts easily with rubble policeman under microscope or only by prolonging the collagenase digesting period to 60-90 minutes. We observed that copper uptake by normal hepatocytes, and found that the copper contents in microsomes (endoplasmic reticulum and Go lgi apparatus) and lysosome all increased significantly, while the copper contents in cytoplasm decreased markedly after 2 h of culture with 15 mg·L^{-1} copper and 30 mmol·L^{-1} ATP in the culture media. This indicated that there existed ATP de pendent copper uptake in these organelles of hepatocyte. We could not determine w here the concrete copper absorption took place at that time, because there is a variety of types of ATP-dependent copper transporters in hepatocytes, such as A TP-dependent glutathion coupling copper transporter, canalicular CMOT transporter and copper transporting P-type ATPase. When the culture medium was absent of magnesium, which was a necessary catalyzer to ATPase for the hydrolysis of its terminal phosphate, the copper accumulation in these organelles could not happen. And more importantly, after adding sodium vanadate, the specific antagonist sensitive to copper transporting P-type ATPase, the copper transporting in micro some and lysosome were inhibited markedly (Figures 3, 4). This proved that the A T P-dependent copper transporter was right the copper transporting P-type ATPase and suggested that it is located in both of the above two organelles, and our immunologic al blotting results with specific antibody against WD protein also proved that W D proteins were existing in hepatocytes. These were identical to the results of that of Bingham[48,49], Dijkstra[29], Shah[24] *et al* .

Liver plasma membranes in canalicular and basolateral fractions from Wistar rats were fractionated on discontinuous sucrose gradients by Dijkstra *et al*[29] and Usta *et al*[32], and it was found that there was ATP-stimulated uptake of radiolabeled copper in canalicular membranes, which was consistent with Adachi *et al*' studies[47] about the biochemistry of copper transporting in LEC rat, an animal model of Wilson disease. If this copper transporter functioned abnormally, it would lead to copper acc umulation in the liver as a result of deficient biliary copper excretion. This w ell explained the mechanism of copper excretion disorder of WD, but it could not answer the question of the deficiency of ceruloplasmin (CP), since CP is formed in endoplasmic reticulum where copper was transported to apo-ceruloplasmin. The research of Bingham *et al* (1995)[49] on rat hepatocytes indicated that copper transporting P-type ATPase might exist in endoplasmic reticulum. Furthermore, Shah[24] and other researchers did immunohistochemical studies using antibodies against the cation combining sites or other function domains and also found the specific reaction in endoplasmic reticulum in the transferred cell strains[33,41]. These results all suggested that copper transporting P-type ATPase should be in endoplasmic reticulum.

Several groups had provided evidence which suggested that the copper transporting ATPase transport copper with the oxidation equivalence (I), that is Cu^{+} ion. One key evidence is the six conserved metal binding motifs Gly-Met-X-Cys-X-Ser-Cys in the amino terminal of each copper transporting P-type ATPase, where the cysteine residues only bind copper as Cu^{+}. Other reports an d our study did not support this hypothesis. It was shown that Cu^{+} transportion in microsomes was not dependent on ATP, but our data indicated that the copper accumulation in microsomes was ATP-dependent. Moreover, when we detected the concent rations of Cu^{+} in each organelles, we did not find any significant changes under different incubation conditions (data not shown), so all the copper contents provided in this paper were the results of Cu^{2+} detected by atomic absorption spectrophotometer. Finally, many papers have shown that the most common gene mutation of WD patients in Western Europe and Northern America is the His1069Glu in the conserved sequence Ser-Glu-His- Pro-Leu of copper transporting P-type ATPase[5,21,32], and we know histidyl residues bind copper as Cu^{2+}[48,49]. All this argued that copper transporting P-type AT Pase might transport copper as Cu^{2+}.

Microsome consists of endoplasmic reticulum and Golgi apparatus. The former mainly functions to synthesize proteins (including ceruloplasmin), and the latter works to process these proteins and make them glycosylated, while the function of lysosome is to digest the endogenous and exogenous fractions of cells, and to join the renewal of cells and tissues of the hepatocytes[48]. Harada *et al*[50] used colchicine to destroy microtubules in lysosome vesicles, and found that the secretion of bile copper was inhibited, therefore indicated that lysosome could work to transport cellular copper. We found that the copper levels in hepat ocyte lysosome of controls could be regulated by the antagonist of copper transporting P-type ATPase (vanadate) after incubation for 2 h, the agonist (vincristine) was not found in stimulating lysosome's copper transportion, and when incubated longer, vanadate did not show the inhibition to copper uptake. It remains unclear whether the copper transportion of lysosome is reached by WD proteins. Yin *et al*[46] regarded that copper transporting P-type ATPase was in both microsome and lysosomes of the cultured fibroblasts of WD patients. Our data showed that lysosome had copper transporting function too, but it did not belong to WD protein, because of its absence of the permanent inhibition by vanadate, the specific inhibitant to WD protein. This disagreement suggested that more evidences are needed to solve the problem.

In cultured hepatocytes of WD patients, the microsome copper contents after being co-cultured with copper and vanadate were significantly lower than that co-cultured with copper only, while being co-cultu re d with copper and vinscrtine, the copper contents in microsome and cytoplasm were significantly higher than that with copper only. This suggested that the agonists and antagonist of P-type ATPase affected the uptake and excretion of copper in microsome by inhibiting or increasing the activation of P-type ATPase. After 24 h of culture with copper and vanadate, the copper contents of microsome and cytoplasm were significantly higher than that with copper only, which indicated that the agonist and antagonist affected the secretion of copper significantly, which further suggested that there was copper transporting P-type ATPase in the microsome of human hepatocytes. Our data showed the significantly different copper levels of microsomes in WD patients' hepatocytes after co-cultured with copper as compared with the controls, which indicated that there were abnormalities of copper transportion in microsomes, that is the disturbance of WD proteins in view of the above results.

WD is one of the rare neurogenetic diseases that can be curable. Because of the frequent side effects (the most often applied medicine), there are about 10 to 30 percent of WD patients who could not tolerate the long-term use of the drug[2,4,51-53]. Much more adverse effects had also been found on the use of DMS, trientene or all other therapeutic drugs. These made it necessary to find new safe and effective alternation to D-Penicillamine to treat WD. When we added agonist (vincristine) to the culture media, the copper levels microsomes in WD patients' hepatocytes in c reased significantly, and showed no difference with the controls. This indicated that the function of the copper transporting ATPase in WD patients' hepatocytes could be promoted by vincristine's activation, and recover to the normal levels. Yin *et al*[46] also found that the agonist of copper transporting AT Pase could modify the impairment of copper excretion from the microsome of WD cultured cells . All the data suggested that it might be a new clinical approach for WD to useagonists of copper transporting ATPase by regulating this enzyme's activity of WD patients. Furthermore, cultured hepatocyte model using high content copper for copper transportion will provide a useful cytological tool for probing the mechanism and therapeutic methods of WD.

In conclusion, this paper used cultured hepatocyte model for WD copper studies, and the data indicated that there is copper transporting P-type ATPase in the microsomes; WD patients had abnormal functions of copper transportion P-type ATPase in the microsomes, and the agonist might correct the defect of copper transporting by increasing P-type ATPase activity.

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