

## Biochemical Approach to Investigate Cause of Wilson Disease via Functional Expression of Genes in Human Hepatoma Cells

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### ABSTRACT

**Background & Objective:** The basic abnormality of Wilson Disease (WD) is the improper excretion of copper through bile that results to copper accumulation and damage of tissue, firstly within the liver and then other organs including brain eventually. Excess dietary intake of copper cause disturbance in regulatory pathways. This excess copper enhances free radical production that results to oxidative stress and cellular damage. The study designed to identify the possible safe dose of copper in dietary intake for safeguard of human beings.

**Materials and methods:** The examination of Cu<sup>2+</sup> ion binding potential with CTR1, ATOX1 and ATP7B proteins shows their pairing affinity. On the basis of potential metal binding site, in the MIB server molecular docking performed. In this study we have performed MTT assay for cellular cytotoxicity and H2DCFDA method for intracellular ROS generation. Along with this study we have also performed JC-1 stain assay to check mitochondrial membrane potential. To check transcriptional gene expression, we also studied about Transcriptional gene expression by Real Time PCR.

**Results:** The study concludes that CuCl<sub>2</sub> at 1µg/ml, was not reduced cellular viability but no significant change observed at safe conc. of CuCl<sub>2</sub>. The CuCl<sub>2</sub> treated cells showed non-significant generation of ROS in a dose dependent manner. No diverse changes observed in control samples and no significant disturbed morphology was seen in CuCl<sub>2</sub> treated cells at its safe concentration. Cytotoxicity test and intracellular ROS generation support control supply of copper in dietary intake below the 5µg/ml. The *in-silico* docking result supports our view point about safe concentration and copper delivery in cellular system. The result showed non-significant change in treated cells compare to control cells. So, the study has proved the selected dose of copper is possibly safe for dietary intake.

**Conclusion:** In this study we concluded that excess dietary intake of copper can cause molecular and morphological disturbance in cells physiology. This study will help the researchers to uncover the critical areas of WD which is a problem of late detection due to one of the causes to heavy dietary intake of copper. Safe dose of copper will be a safeguard and its new subject to study for many researchers. Thus, a new theory on Wilson disease through cellular investigation at genes levels might have been arrived for future verification.

**Keywords:** Copper, Liver, Wilson disease, Acute liver failure, Mutation

### INTRODUCTION

The hepatic presentation can be divided into four main presentations, acute hepatitis, chronic active hepatitis, cirrhosis, and acute fulminant hepatic failure [1]. Wilson's disease (WD) also called hepatolenticular degeneration is an autosomal recessive inherited disorder caused due to ATP7B gene mutations. Mutations in the ATP7B gene present on chromosome 13 ireregulate the normal copper metabolism and that results to the toxic copper accumulation. ATP7B gene controls the protein transporter responsible for excreting excess copper into bile and out of the body. The liver is responsible for the major system of copper excretion around 95%. WD appears to be typical of rare autosomal recessive diseases in that it is present at a low frequency in all populations. WD is a rare inherited disorder that causes

copper to accumulate in your liver, brain and other vital organs WD is not just a disease of children and young adults, but may present at any age [2]. Most people with the disease

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are diagnosed between the ages of 5 and 35, but it can affect younger and older people, as well. The disease is not manifested clinically before 4 to 5 years of age because it takes time for copper to accumulate to toxic levels in the liver till such age. Unfortunately, WD is often misdiagnosed, and the average time from symptom onset to diagnostic treatment is long, about 12 months [3,4].

An estimate for the disease frequency in most populations is about 17 per million, which would lead to a carrier frequency of 1 in 122 [5]. The disease is recognized to be more common than previously thought, with a gene frequency of 1 in 90–150 and an incidence (based on adults presenting with neurologic symptoms [6] that may be as high as 1 in 30,000. More than 500 distinct mutations have been described in the Wilson gene, from which 380 have a confirmed role in the pathogenesis of the disease [7]. Copper encodes an ATP and membrane-bound copper-transporting ATPase. Deficiency in ATP7B protein damages excretion of biliary copper, resulting in hepatic copper accumulation, and oxidant damage copper toxicity [8]. High copper levels include hepatocytes necrosis, followed by increased copper release into the bloodstream, leading to haemolysis and accumulate in kidney, brain, and cornea. Copper is a transition metal, and excess iron levels lead to the formation of a toxic hydroxyl group and increase oxidative stress in the cells. This oxidative stress damages the cells and leads to the clinical manifestation, namely liver failure, behavioural problems, movement disorders, and Kayser-Fleisher rings in the cornea [9].

Copper is a cofactor for some enzymes in the body such as cytochrome c oxidase, ceruloplasmin, dopamine beta-hydroxylase, tyrosinase and superoxide dismutase. Copper enters the body through the digestive tract with the help of a transporter protein in the cells of the small bowel, (CMT1) copper membrane transporter 1 (Ctr1; SLC31A1) [10]. CMT1, carries copper inside the cells, where some is bound to metallothionein and part is carried by ATOX1 to an organelle known as the trans-Golgi network [8]. Liver cells carry the metallothionein and CMT1 protein, and ATOX1 bind it inside the cell, but ATP7B present here that links copper to ceruloplasmin and then releases it into the bloodstream, along with eradicating excess copper by secreting it into bile. Both functions of ATP7B are impaired in WD [8]. Foods rich in copper must be avoided, in the early years after diagnosis at least. Drinking water usually contains less than 0.2 mg copper per litre but up to 10% of domestic drinking water has copper levels that may be too high for Wilson patients, so it should be tested [11]. Copper is conveyed to the liver *via* the portal circulation and excess removed by excretion into the bile at the apical aspect of hepatocytes, a process impaired by mutations in *ATP7B*. Copper plays an important role in the development of healthy nerves, collagen, bones, and the melanin (skin pigment). Generally, copper absorbed from your food, and excreted the excess through liver (bile). But in the condition,

when person with WD, copper isn't removed properly and starts accumulating instead, that possibly leads to a life-threatening level. When diagnosed early, Wilson's disease is treatable, and many people with the disorder live normal lives [12].

## MATERIALS & METHODS

### Culture wares and chemicals

Fetal bovine serum (FBS), Eagle's minimal essential medium (EMEM), antibiotic and antimycotic solution, trypsin, 2,7-dichlorodihydrofluorescein diacetate (DCFH2-DA), NAC (N-acetyl cysteine), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT), hank balanced salts solution (HBSS), JC-1 and phosphate buffers were procured from Sigma chemical company USA. Milli Q double-distilled deionised water was used throughout the study. All plastic wares including 96-well plates, 96-black well plates, 6/12/24 well plates, 90 mm plates and 25, 75 cm<sup>2</sup> (poly-L-lysine coated) culture flasks were purchased from Nunc (USA).

### Cell culture

HepG2 is a continuous cell line consisting of human liver carcinoma cells, derived from the liver tissue of a 15-year-old Caucasian male was grown in EMEM cell culture medium supplemented with 10% FBS and antibiotic-antimycotic solution (1.5%) at 5% CO<sub>2</sub> and 95% relative humidity at 37°C.

### Interaction study of Cu<sup>2+</sup> with CTR1, ATOX1 and ATP7B protein

The energy minimized structures obtained for each CTR1 (PDB ID: 2LS2), ATOX1 (PDB ID: 5F0W), and ATP7B (PDB ID: 2ROP) proteins were used for subsequent interaction study with Cu<sup>2+</sup> ion. Prior to docking, the CTR1, ATOX1 and ATP7B proteins were pre-processed in order to get correct conformation of the proteins by adding polar hydrogen and assigning charges using Kollman method using Autodock 4 (autodock.scripps.edu/). Metal Ion-Binding Site Prediction and Docking Server (MIB) was carried out for binding scores between Cu<sup>2+</sup> and CTR1, ATOX1 and ATP7B proteins [13]. The query protein structure should compare with each metal-binding template in the database to detect the metal-binding residues. Binding score is assigned to each residue of the query protein. With the principle of conserved structure and sequence-based information MIB server can give excellent predictions for Cu<sup>2+</sup> binding scores for amino acid residues than geometric subgraph and artificial neural network-based methods.

MIB server provides an accurate, integrated approach used to search for metal ion-binding residue sites using fragment transformation method. This server supports 12 metal ions for residue prediction. To locate metal-binding residues the query protein structure should compare with each metal-

binding template in the database and binding score is assigned to each residue of the query protein [14].

#### Cytotoxicity assay

Cells were seeded in 96 well plates after 70-80% confluency treated with  $\text{CuCl}_2$  at different concentration in complete medium. After treatment, they were incubated for overnight. After incubation absorbance was recorded at 540 nm at ELISA plate reader [15].

#### Intracellular ROS generation by $\text{H}_2\text{DCFDA}$

For the detection of intracellular ROS, cells were grown in 96 black well plates with 80% confluency. Cells were treated with or without  $\text{CuCl}_2$  and incubated for 30 min. After incubation images were recorded by fluorescent microscope (Nikon Eclipse, Germany) using  $\text{H}_2\text{DCFDA}$  followed by conc. 5  $\mu\text{M}/100\text{ml}$  DCFDA and quenching was done by NAC (N-acetyl cysteine) 50ppm [16]. Oxidative stress caused the depletion of reduced glutathione, while pre-treatment with antioxidant N-acetyl cysteine (NAC) was able to abrogate oxidative stress.

#### Cell morphology

Cells were seeded in 6 well plates at 80% confluency; cells were treated with or without  $\text{CuCl}_2$  and incubate for 3hr.

**Table 1.** The sequences (Sigma Aldrich) of forward and reverse primers.

S. No.	Gene Name	Forward Primer Sequence	Reverse Primer Sequence
1	B-Actin	5'-CCAACCGCGAGAAGATGA-3'	3'-CCAGAGGCGTACAGGGATAG-5'
2	Atox1	5'-GGCATGACTGCCAAGTCC-3'	3'-CCCACAGGATGGACCAAA-5'
3	ATP7B	5'-TGCTTCCGGGTTTCTTAGTG-3'	3'-AGTCCTGGAAGGCAATACCA-5'
4	CMT-1 (Ctr1; SLC31A1)	5'-TGGTGGGAATGATCCATTTT-3'	3'-TCGGAGAGAGAGGTGCTAGTG-5'

#### STATISTICS

Group means  $\pm$  SE were compared by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. All analysis was performed with Graph Pad Prism 5 (version.04). Simple linear regression and correlation analysis were used to assess the cell viability and ROS generation against different  $\text{CuCl}_2$  concentrations. A two-tailed ( $\alpha$ ) probability  $P < 0.05$ ,  $P < 0.01$  was considered statistically significant.

#### RESULTS

##### Interaction study of $\text{Cu}^{2+}$ with CTR1, ATOX1 and ATP7B protein

After incubation image captured by bright field microscope (Evos XL core, life technologies) [17].

#### Mitochondrial integrity detection by fluorescent microscopy

JC-1 is pH and potential dependent dye used for mitochondrial trans membrane potential (MTP) detection. After treatment, cells were incubated with 5 $\mu\text{M}$  JC-1 (Invitrogen) for 30 min at 37°C, washed and images were visualized by a fluorescence microscope (Nikon Eclipsed Made in Germany) [18].

#### Quantitative real-time PCR analysis

After treatment with  $\text{CuCl}_2$  total RNA was isolated using TRIzol reagent (Life Technologies) according to manufacturer protocol. For real-time PCR, cDNA was synthesized by ABI high capacity cDNA Reverse Transcription Kit. The sequences (Sigma Aldrich) of forward and reverse primers are as follows: Real-time PCR was carried out for 40 cycles on an Applied Biosystems 7900 HT Fast Real-Time PCR system [19] (Table 1).

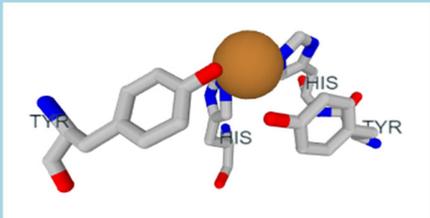
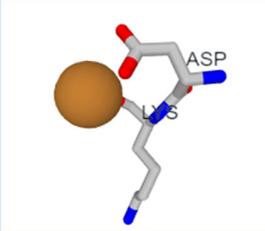
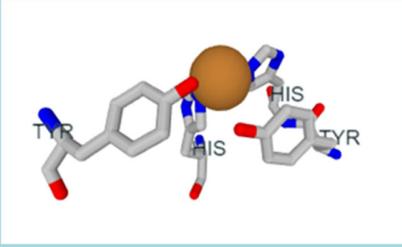
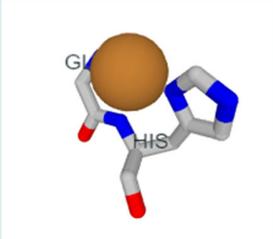
Metal ions help in stabilization of outer membrane protein structures and contribute in catalysis and transport. Scarcity and overdose of some metal ions both can lead to disease. A characteristic of metals is that they easily lose electrons from the familiar elemental or metallic state to form positively charged ions which tend to be soluble in biological fluids. Metal plays a critical role in function of proteins. With the underlying principle of conserved structure and sequence-based information MIB server provides predictions for  $\text{Cu}^{2+}$  binding scores for amino acid residues than geometric sub graph and artificial neural network-based methods. The examination of  $\text{Cu}^{2+}$  ion binding potential with CTR1, ATOX1 and ATP7B proteins shows their pairing affinity (Table 2). On the basis of potential metal binding site; in the

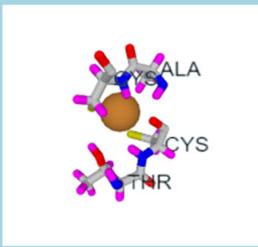
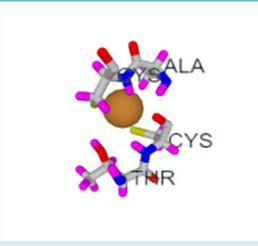
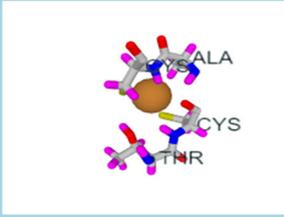
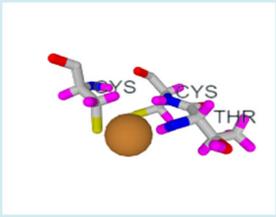
same server i.e. MIB molecular docking was performed. These results are similar to the findings of in vitro functional studies. Our findings can also explain the molecular reasons for subtle changes in structure, flexibility, and stability aspects of copper related safety.

Based on structural information of proteins, we had applied a fragment transformation method to predict the metal-ion binding sites of the closed conformation with the ion potentially with their nucleation sites for Cu<sup>2+</sup> during the

internalization and mineralization. **Table 2** highlights the metal-binding residues according to a scoring criterion that selects those amino acids with normalised scores higher than 2.2; these values are higher than the method thresholds and assure prediction accuracies higher than 95% for the Cu<sup>2+</sup>. This criterion guarantees that all the predicted residues are true positive since the false positive rate was <5% in all cases.

**Table 2.** Metal Ion Binding (MIB) Server results showing the different binding affinities and the different interacting residues per cofactor-bound CTR1, ATOX1 and ATP7B proteins with the cofactor represented as spherical ball.

Cofactor-Bond	Binding residues	Binding scores	3D model of the best binding model
Cu <sup>2+</sup> - CTR1	21Y, 22E	4.29	
	22E, 25K	4.08	
	20F, 21Y	3.21	
	23G, 24L	2.68	

Cu <sup>2+</sup> - ATP7B	132T, 133C, 135S, 136C	2.37	
	30H, 31C, 33S, 34C	3	
Cu <sup>2+</sup> - ATOX1	11T, 12C, 14G, 15C	2.92	
	12C, 15C	3.39	

### Cytotoxicity assay

CuCl<sub>2</sub> induced dose dependent decrease in cell viability of cells. There was no significant reduction in cell viability under control cells. Further, we have performed cell viability test with different conc. of CuCl<sub>2</sub> to find LD<sub>50</sub> of CuCl<sub>2</sub> i.e. 1 µg/ml. We find that, at 1µg/ml conc. CuCl<sub>2</sub>, not reduced cellular viability and no significant change observed in HepG2 cells at this safe conc. of CuCl<sub>2</sub> (Figure 1).

### Intracellular ROS generation by H<sub>2</sub>DCFDA

CuCl<sub>2</sub> has a strong potential to produce ROS at higher concentration but safe dose is exhibit it. We observed that there was no significant change in control and treated samples. CuCl<sub>2</sub> treated cells showed non-significant generation of ROS in a dose dependent manner. NAC (50 µg/ml) is a specific quencher of ROS which used with highest conc. Of CuCl<sub>2</sub> (2 µg/ml) followed by control cells.

CuCl<sub>2</sub> at 2 µg/ml was not quenched by NAC at safe concentration (Figure 2).

### Intracellular ROS generation through Fluorescent microscope

Fluorescent image of intracellular ROS generation showed there was no significant change was observed in cells. Result showed no change in control and treated cells. Suggested safe concentration of CuCl<sub>2</sub> exhibit ROS generation in cellular system (Figure 3A).

### Cell morphology

Toxic effect of CuCl<sub>2</sub> was observed in HepG2 cell line at lower to higher concentration. CuCl<sub>2</sub> not induced any significant morphological change in dose dependent manner in HepG2 cells. There was no drastic change observed in control samples and no significant disturbed morphology

was observed in CuCl<sub>2</sub> treated cells at its suggested safe concentration (Figure 3B).

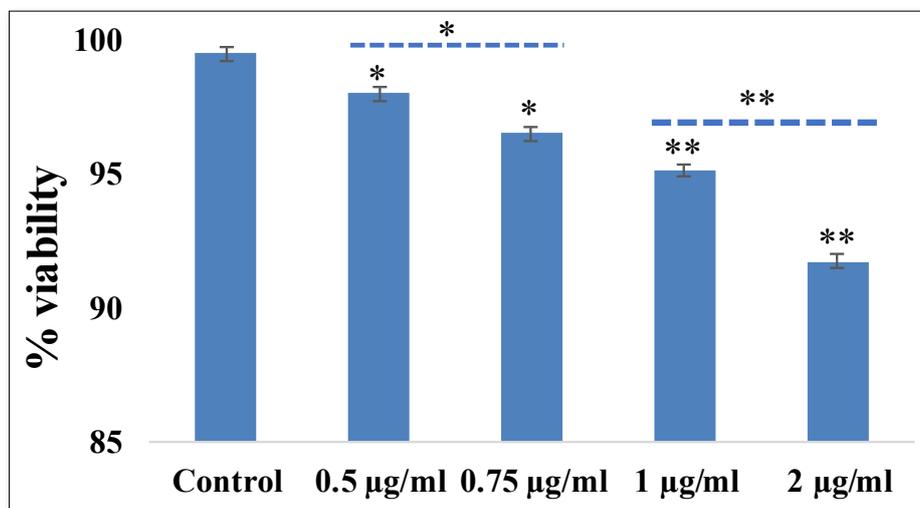


Figure 1. Cellular cytotoxicity through mitochondrial dehydrogenase assay with different concentration of CuCl<sub>2</sub> treatment lines in HepG2 cell

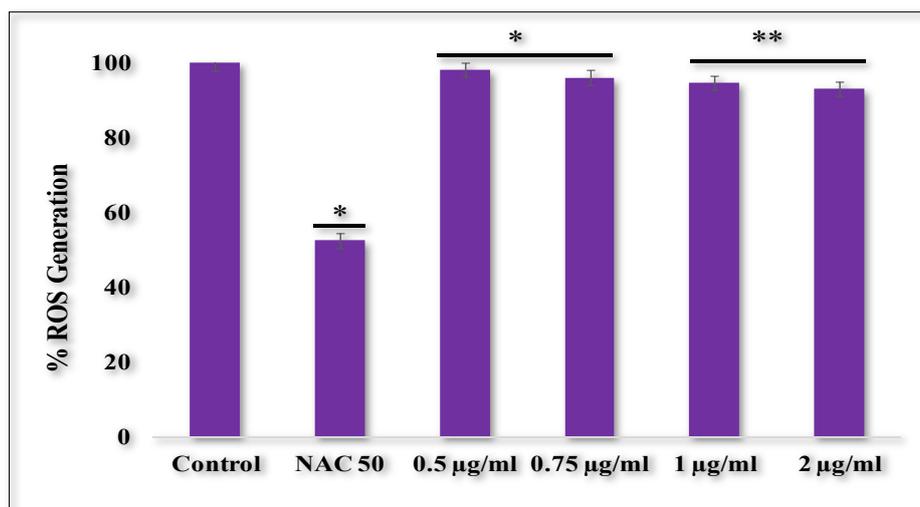


Figure 2. Intracellular ROS generation by H2DCFDA assay with different conc. of CuCl<sub>2</sub> in HepG2 cell line.

### Mitochondrial integrity detection by fluorescent microscopy

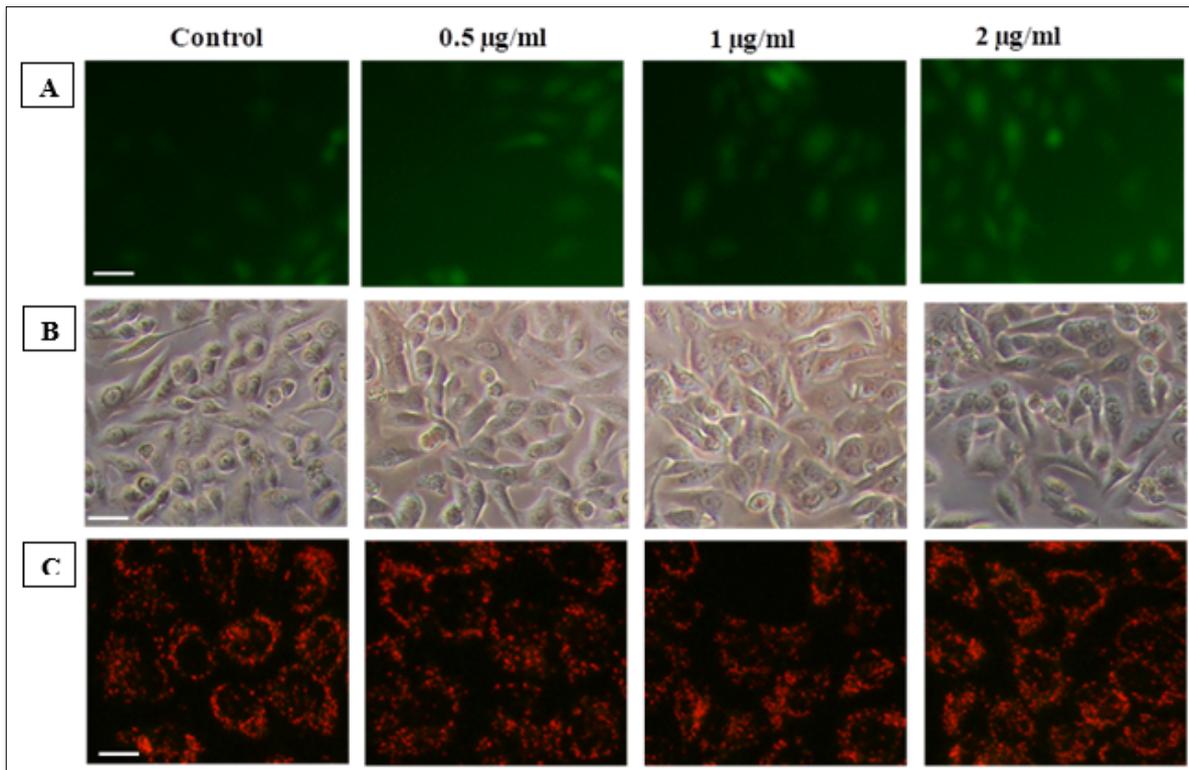
Result showed healthy mitochondria appeared in figure as dense red color in control cells along with treated cells showed stable mitochondrial membrane potential. Control cells showed normal mitochondrial membrane potential; thus, mitochondria were able to load with JC-1 dye and formed J-aggregates, which was represented by intense red fluorescence. Result observed through fluorescent microscope showed that normal cells in conc. dependent manner (Figure 3C).

### Transcriptional level expression by CuCl<sub>2</sub>

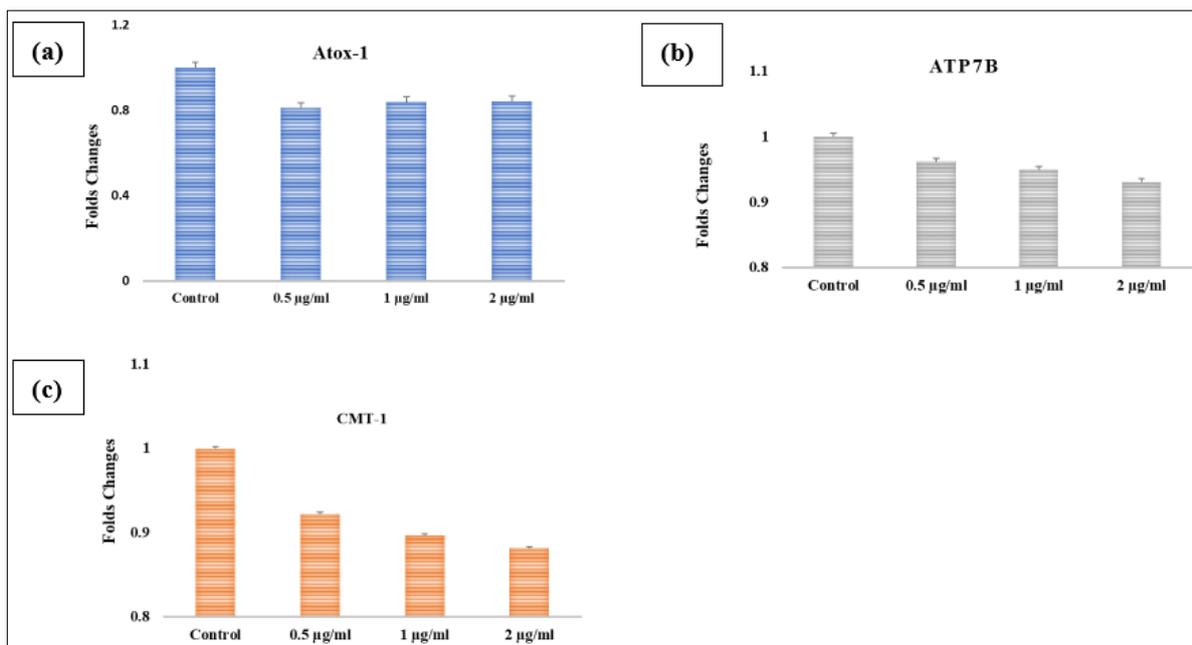
CuCl<sub>2</sub> induced changes and subsequent cytotoxic response and ultra-structural changes in cells exposed with different concentration. A non-significant (p < 0.05) regulation of pro-apoptotic genes, i.e. Atox-1 (~1.0 folds), ATP-7B (~0.9 folds) and CMT-1 (~0.97 folds) was observed in experimental cells than control cells (Figure 4).

### DISCUSSION

The major route of copper exposure is oral for non-occupationally exposed healthy humans. Once the metal



**Figure 3.** Morphological changes were observed through different staining in HepG2 cells. (A) Intracellular ROS generation through H2DCFDA. (B) Morphological image of CuCl<sub>2</sub> treated HepG2 cells. (C) Mitochondrial depolarization in HepG2 Cells. Using scale bar 20 μm.



**Figure 4.** Alterations in the relative genes and their expressions studies of genes involved in (a) Atox-1 (b) ATP7B (c) CMT-1.

passes through the basolateral membrane it is transported to the liver bound to serum albumin [20]. Liver is the critical organ for copper homeostasis. In adults the mean dietary daily intake of copper is ranges between 0.9 and 2.2 mg. All other intakes of copper (inhalation and dermal) are insignificant in comparison to the oral route. Inhalation adds 0.3-2.0  $\mu\text{g/day}$  from dusts and smoke [21]. Every molecule has response machinery in cellular system through some signaling pathways or receptors. So, we have performed *in silico* study to prior check copper efficiency to get inside in cells with safe concentration. Some selected genes which are mainly responsible for copper introduction and transportation in cells by control regulation [14]. Our *in-silico* docking result has been supporting our viewpoint about suggested safe concentration and delivery of copper in cellular system. To locate metal-binding residues the query protein structure should compare with each metal-binding template in the database and binding score is assigned to each residue of the query protein [22]. Women using copper IUDs are exposed to only 80  $\mu\text{g}$  or less of copper per day from this source. The homeostasis of copper involves the dual essentiality and toxicity of the element [23]. In the study cellular cytotoxicity of copper chloride in hepatocyte carcinoma cell line at selected concentration was studied. There was no significant change observed in suggested safe concentration [14]. Its essentiality rises from its specific incorporation into a large number of proteins for structural and catalytic purposes. The cellular pathways of uptake, incorporation into protein and export of copper are conserved in mammals and modulated by the metal itself [24]. Cells have their own mechanism to maintain homeostasis. An optimum level of intracellular ROS was neutralized in cellular system through superoxide through superoxide dismutase, Nrf-2, Heme oxygenase and some amino acid. The intracellular ROS generation through H2DCFDA dye was performed. Non-significant intracellular ROS generation was observed in treated cells at selected concentration [21]. Copper is mainly absorbed through the gastrointestinal tract. From 20 to 60% of the dietary copper is absorbed, with the rest being excreted through the feces. The fluorescent image detection of H2DCFDA dye in treated HepG2 cell line was also performed. Fluorescent image results also support the quantitative analysis data of ROS generation. There was no substantial change was observed in figures. So, the study result showed a possible safe dose of copper which proves to be beneficial for daily dietary intake of copper. Research study got corroborated by the fact that, heavy intake of copper, generated ROS which alter the normal cellular physiology. For morphological detection, the bright field microscope image detection was performed. Images did not show any noticeable changes compare to control cells [21]. Hence, the study suggests that normal intake of copper acts as a safeguard for human being. The copper transport to the peripheral tissues is completed through the plasma that attached to serum albumin low-molecular weight complexes or ceruloplasmin [25]. The

biochemical toxicity of copper, when it exceeds homeostatic control, was derived from its effects on the structure and function of biomolecules, such as DNA, membranes and proteins directly or through oxygen-radical mechanisms. The toxicity of a single oral dose of copper varies widely among different species. The major soluble salts like copper (II) chloride and copper (II) sulphate are usually more toxic than the less soluble salts like copper (II) oxide and copper (II) hydroxide). Long-term exposure in rats and mice showed no overt signs of toxicity other than a dose-related reduction in growth after ingestion [22]. The effects included inflammation of the liver and degeneration of kidney tubule epithelium. Mitochondrion is most important cellular organelle which regulates most of cellular processes. Apoptosis is considered to be a crucial phenomenon of cellular system for programmed cell death which occurs inside the mitochondria [26]. So, role of mitochondria in the study was accessed through mitochondrial depolarization using JC-1 dye [14]. Our result showed non-significant change in treated cells compare to control cells. So, this study has been proved selected dose of copper is possibly safe for dietary intake. Some testicular degeneration and reduced neonatal body and organ weights were seen in dye. Neurochemical changes have been reported after oral administration [26]. A limited number of immunotoxicity studies showed humoral and cell-mediated immune function impairment in mice after oral intakes in drinking-water. Copper is an essential element and adverse health effects in humans are related to deficiency as well as excess [27]. Copper deficiency is associated with anemia, neutropenia and bone abnormalities but clinically evident deficiency is relatively infrequent in humans. Effects of exposure have been reported as epigastric pain, metallic taste, nausea, headache, dizziness, diarrhea and vomiting, respiratory difficulty, hemolytic anemia, haemolytic anaemia, massive gastrointestinal bleeding, haematuria, liver and kidney failure, and death [21]. Copper ( $\text{Cu}^{2+}$ ) is an essential transition metal ion required as cofactor in many key enzymes. After  $\text{Cu}^{2+}$  uptake of cell, it gets transported by the cytoplasmic  $\text{Cu}^{2+}$  Atox1 chaperone to PIB-type ATPases in the Golgi network for its incorporation into Cu-dependent enzymes in the secretory path [14]. The selected copper concentration in the study did not induce any significant up or down regulation in hepatic carcinoma cell lines. The accumulation of copper in these areas can present a wide spectrum of symptoms, including liver cirrhosis; neuronal degeneration of the brain, particularly in the basal ganglia; Kayser-Fleischer (K-F) rings at the corneal limbus; and kidney damage [28]. In the study there was no any change was observed. The protein (CMT-1: SLC31A1) encoded by this gene (CTR1; COPT1) is a high-affinity copper transporter found in the cell membrane [21]. The encoded protein functions as a homotrimer to affects the uptake of dietary copper. Gastrointestinal effects could also have resulted from single and repeated incorporation of high copper concentrations containing drinking-water, and liver

failure has been reported following chronic ingestion of copper. Dermal exposure has not been associated with systemic toxicity, but copper may induce allergic responses in sensitive individuals [13].

## CONCLUSION

In addition to the study the cytoplasmic Cu<sup>2+</sup> transport and nuclear transcription factor roles, Atox-1, ATP7B and CMT-1 genes appears to have a new role in cell migration that entails positioning at the cell border. However, excess dietary intake of copper induced disturbance in normal cells physiology. Every molecule has response machinery in cellular system through some signaling pathways or receptors. In silico techniques are often used to predict the effects on some known target. So, the *in-silico* study has been performed to prior check copper efficiency to get inside in cells with safe concentration. Some selected genes which are mainly responsible for copper introduction and transportation in cells by control regulation. The study concludes the possible safe dose of copper through cell viability assay that can be beneficial for users and researcher and drug designer and will be healthy for normal dietary intake [29].

## Significance Statements

This study will help the researchers to uncover the critical areas of WD which is problem of late detection that many researchers were not able to explore. Thus, a new theory on WD through cellular investigation at genes and proteins levels need to be study and justify.

## ACKNOWLEDGMENT

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## AUTHORS CONTRIBUTION

- 1) Conceived and designed the experiments- Dr. Budhayash Gautam and Shikha Agnihotry
- 2) Performed the experiments- Shikha Agnihotry, Ajeet kumar Srivastav
- 3) Analyzed and interpreted the data- Shikha Agnihotry
- 4) Contributed reagents, materials, analysis tools or data- Pradeep K. Shukla.
- 5) Wrote the paper\*- Shikha Agnihotry and Budhayash Gautam

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