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Molecular basis of pathophysiology of Indian childhood cirrhosis: Role of nuclear copper accumulation in liver

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Abstract

Indian Childhood Cirrhosis (ICC) is a disease of abnormal copper metabolism commonly characterized by swelling and degeneration of liver cells along with the presence of orcein staining deposits of copper. Hepatic copper content of ICC patients was about 43 fold higher than those of control subjects. The data on sub-cellular distribution of copper revealed massive accumulation of Copper (73%) of total cell copper) in the nuclear fraction (455 μg Cu/g tissue nuclei). On further distribution of copper in nuclear fraction, the enrichment of copper in heterochromatin and euchromatin of ICC nuclei was found to be 48 and 15 fold higher over control fractions respectively. The ultra-violet spectra of heterochromatin and euchromatin isolated from ICC nuclear fraction showed a broad absorption maxima as compared to controls. Further, A260/A280 ratio was markedly lower in heterochromatin and euchromatin of ICC liver in comparison to controls. An antioxidant enzyme, catalase activity was also significantly reduced in ICC liver as compared to control. Further, DNA fragmentation studies indicated that there was significantly increased DNA fragmentation in ICC liver. Collectively, these findings suggest that massive accumulation of copper in nucleus and decrease in catalase activity was associated with DNA fragmentation in hepatocyte of ICC disease. (Mol Cell Biochem **156:** 25–30, 1996)

Key words: Indian childhood cirrhosis, hetero and euchromatin, copper, catalase, DNA fragmentation

Abbreviations: ICC – Indian Childhood Cirrhosis

Introduction

Previous studies have demonstrated the presence of orcein staining deposits in ICC liver cells. These were known to be associated with high copper content which often exceeds the level observed in Wilson's disease and resulted in swelling and degeneration of liver cells [1–6]. The etiology and pathophysiology of the disease is not completely understood. However, a role for excess dietary copper ingestion in the etiology and pathogenesis of ICC is being incriminated. However, the mechanism by which excess copper results in cellular toxicity has not been well defined despite *in vitro* studies that demonstrate alterations in membrane proteins, several hepatocellular enzymes and reduced stability of DNA through the formation of highly reactive hydroxide radicals [6, 7].

DNA is the primary site for all information regarding the metabolic and structural proteins which are responsible for the maintenance and regeneration of cell. High copper content and H_2O_2 have been shown to damage DNA include single and double strand breaks and chemical changes in purine and pyrimidine bases especially conversion of guanine into 8-hydroxy guanine *in vitro* [7, 8]. Pathophysiology of hereditary disorders of copper toxicosis like Wilson's and Menke's

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disease, where copper has been implicated in its pathogenesis, has been fairly worked out [9–11]. Preliminary reports from our laboratory [12] as well as other [13] have shown high accumulation of copper in nuclear fraction of ICC liver. The present study has been undertaken to find out the distribution of copper within subcellular fractions with special reference to nuclear fractions. In order to extend our understanding, the impact of massive accumulation of copper in nucleus was carried out on DNA damage by DNA fragmentation studies. We also examined catalase enzyme activity which protects DNA damage by catalysing H_2O_2.

Materials and methods

Liver samples of children suffering from ICC, who died during their admission at the Nehru Hospital, Postgraduate Institute of Medical Education and Research, Chandigarh, in the advanced stage of the disease, were obtained post-mortem immediately after death. Specimens were stored at –70°C till used, part of the specimen was fixed in 1% formal saline for histopathological features of ICC, which are as follows: (i) presence of Mallory's hyaline (ii) pericellular fibrosis (iii) presence of neutrophilic exudate (iv) ineffective nodulation (v) presence of copper binding proteins as demonstrated by Orcein stain. (Bhagwat *et al* [14]). Control liver samples were included in which copper content was in normal range (4–22 μg Cu/g wet tissue) liver structure was normal with normal histopathology. All the subjects in both the groups were male and below 5 years of age.

Sub-cellular fractionation was carried out by differential centrifugation. A 20% (w/v) homogenate of the liver tissue was prepared in a solution consisting of 0.25 M sucrose, TMKC buffer (0.05 M Tris, 5.0 mM $MgCl_2$, 3.0 mM KCl, 0.2 mM $CaCl_2$, pH 7.4) and 0.4 mM Phenylmethyl sulfonyl fluoride (PMSF). Tissue disruption was affected utilizing a motor-driven Potter-Elvehzem homogenizer with a teflon pestle for 20 strokes. After filtering through four layers of cheese cloth, the homogenate was centrifuged at 700 g for 15 min in a refrigerated centrifuge at 4°C. The pellet represented the crude nuclear fraction. The supernatant was centrifuged at 9000 x g for 10 min. The pellet represented the crude mitochondrial fraction. The supernatant was centrifuged at 30,000 x g for 15 min and then 105,000 x 9 for 1 h in Beckman L5-50B ultracentrifuge, USA, saving the pellet as crude lysosomal and microsomal fraction respectively. Post microsomal supernatant represented cytosolic fraction. The recovery of copper in all fractions in relation to the total homogenate was 95.6% ± 0.86 (mean ± S.E.).

The nuclei were purified by the method of Blobel and Potter [15]. Purified nuclei were then washed, swollen and fractionated into condensed (heterochromatin) and extended (euchromatin) fractions by the method as described by Yasmineh and Yunis [16]. Purified nuclei fractions were suspended in 0.25 M sucrose and allowed to swell by standing for 10 min at 4°C. Nuclei were disrupted by sonication for 10–15 sec in a sonicator (Sonifier cell disrupter model W1 85 heat systems ultrasonics, Inc., Plain Vew N.Y. USA) at 6 amps and 20,000 cycles per sec. Heterochromatin fraction was separated by sedimentation at 3500 x g for 25 min; 3500 g supernatant recentrifuged at 7800 x g for 1 h, and euchromatin fraction was separated by precipitation with the addition of alcohol to 7800 g supernatant. Heterochromatin and euchromatin fractions were then suspended in TMCK buffer pH 7.4. Preparation of nuclei, heterochromatin and euchromatin were monitored by phase contrast microscopy using Wright's stain. Absorption spectra of the purified heterochromatin and euchromatin fractions was recorded on a spectrophotometer (Model U-3200, Hitachi, Japan) in ultra violet range (200–400 nm}. Copper levels in tissue subcellular fractions and nuclear fractions were estimated by wet digestion method. Specimens were digested in 2 ml of digestion mixture (Conc Nitric acid: Perchloric acid = 5:1 in a digester (Model Buchi-445, Switzerland) under vaccum upto dryness and the residues reconstituted in 10 mM nitric acid. Copper was monitored on atomic absorption spectrophotometer (Model Perkin Elmer-4000, USA) using hollow cathode lamp of copper at 324.8 nm. The instrument was calibrated with Sigma Copper standard and standard reference material SRM – 1577 {Bovine liver) obtained from National Bureau of Standards (NBS), Washington (USA).

DNA analysis in nuclei, heterochromatin and euchromatin fractions was carried out by diluting these fractions into 1.0% sodium dodecyl sulfate, 8.0 M Urea and 0.05 M Tris pH 8.0 and absorbance was measured at 260 nm. DNA content was calculated assuming 50 μg DNA = 1.0 O.D. at 260 nm.

DNA fragmentation was determined spectrophotometrically after sedimentation of DNA from hepatocytes as described by Shen *et al* [17]. One gram of liver tissue was homogenized in lysis buffer consisting of 5 mM Tris-HCl, 20 mM EDTA and 0.5% Triton X-100 (pH 8.0) by hand homogenizer for 5 strokes. Homogenate was kept on ice for 15 min. The samples were then centrifuged at 27,000 x g for 20 min to separate intact chromatin pellet from DNA cleavage products that remained in the supernatant fraction [18]. Pellets were resuspended in 5 ml of 0.5 M perchloric acid. Concentrated perchloric acid (5.5 M) was also added to supernatant sample (100 μl/ml) . Supernatant and resuspended pellet samples were heated at 90°C for 15 min then centrifuged at 1000 x g for 10 min. Finally samples were assayed for DNA content using diphenylamine, a reagent that excludes RNA by reacting preferentially with 2-deoxysugars [19]. The amount of DNA fragmentation was expressed as the percentage of total DNA which appeared in the supernatant fraction.

Catalase activity was measured in the liver homogenate by the method of Luck [20]. A 10% tissue homogenates (w/v) were prepared in ice cold 0.15 KCI at 4°C. Activity of catalase was measured in the post nuclear fraction obtained by centrifuging homogenate at 1 000 g for 20 min at 4°C. 3 ml of H_2O_2 phosphate buffer was placed into the cuvette. 100 µl of tissue supernatant was added and mixed thoroughly. The decrease in absorbance at 240 nm was recorded after every 30 sec for 3 min. The enzyme activity was expressed as µmol H_2O_2 decomposed/mg protein/min. Protein was measured by the method of Lowry *et al* [21] using bovine serum albumin as standard. Results have been reported as means ± S.E., and compared by unpaired *t* test, with significance defined as $p < 0.05$.

Results

Biochemical parameters estimated on patients during admission for assessment of ICC included serum copper and ceruloplasmin which were slightly elevated but urinary copper was significantly higher as compared to normal value (20 µg/dl). The hepatic copper concentration of ICC patients was 620 + 73 µg Cu/g wet wt. or 2294 ± 353 µg Cu/g dry weight which was approximately 42 times higher than control livers (14.57 ± 4.23 µg Cu/g wet wt. or 52.50 ± 14.37 µg Cu/g dry weight) (Table 1). Subcellular distribution studies revealed that there was a marked shift of copper from cytosolic, microsomal and mitochondrial fraction to nucleus in ICC liver cells as compared to control (Table 2). Seventy three percent of total cell copper in the ICC hepatocytes was present in the nuclear fraction (455 ± 56 µg Cu/g tissue nuclei). On the other hand, only 28% of total cell copper was found in nuclear fraction (4 µg Cu/g tissue nuclei) of control liver. Wright stained preparations of heterochromatin revealed the light grey masses of nuclei which are characteristically associated with the dark masses of heterochromatin. By phase contrast, the preparation of euchromatin showed no heterochromatin. The results on intranuclear distribution of copper in different fractions of chromatin with special reference to heterochromatin and euchromatin are presented in Tabie 3. The copper content in ICC liver nuclei was 92 fold higher than control liver nuclei when copper levels were expressed as µg copper/mg DNA. The copper contents in heterochromatin and euchromatin isolated from ICC liver were significantly higher in comparison to controls. Copper content in heterochromatin was two fold higher than euchromatin in ICC liver nuclei. In contrast to this, copper content in euchromatin was higher than heterochromatin in control liver nuclei.

The ultra violet spectral studies of heterochromatin and euchromatin were carried out to elucidate the destabilizing effect of excess copper in ICC liver nuclei on heterochromatin and euchromatin. The U.V. spectra of heterochromatin and euchromatin from ICC nuclei showed a broad peak with a shoulder around 264 nm which indicated a change in their absorption towards higher wave-length as compared to control fraction spectra which showed a shoulder around 260 nm (Fig. 1). A260/A280 ratio was about 1.14 in heterochromatin and euchromatin isolated from ICC liver where as this ratio was about 1.6 in heterochromatin and euchromatin of control liver (Fig. 1A and B).

An antioxidant enzyme, catalase which catalyzes the decomposition of hydrogen peroxide, was significantly reduced in ICC liver (100 ± 8.0 µmol H_2O_2 decomposed/mg protein/min) as compared to control (160 ± 9.5 µmol H_2O_2 decomposed/mg protein/min.) This study suggests more accumulation of H_2O_2 in hepatocytes. DNA fragmentation experiments were conducted using liver samples from ICC and control liver samples (Fig. 2). DNA fragmentation in ICC liver (218 ± 12%) was about 8 fold higher than control liver (27 ± 2.53%).

Discussion

In the present study, elevated hepatic copper (2294 µg/g dry weight tissue) and Orcein stained hepatocyte granules which suggest the presence of copper binding proteins as well as cytosolic copper content. Liver of ICC disease and normal human liver differ primarily by having different percentages of total tissue copper in the nuclear fractions, about 73% in ICC disease and 28% in the normal human. These findings are fairly consistent with the observations of Adamson [13] who has reported that about 87% of hepatic copper sedimented at less than 10,000 × g in ICC liver. In adult human liver, about 20% of hepatic copper was observed in nuclear fraction [22]. The distribution of copper within liver cells is

Table 1. Serum, Urinary, Hepatic copper and Serum ceruloplasmin levels in Control and ICC disease

Group	Serum copper (µg/dl)	Serum ceruloplasmin (mg/dl)	Urinary copper (µg/24 h urine)	Hepatic copper (µg/g wet tissue)	Hepatic copper (µg/g dry tissue)
Control	ND	ND	ND	14.37 ± 11	36.42 ± 7.0
ICC (3)	130 ± 13.58	53.48 ± 5.66	460 ± 86	630 ± 73***	2294 ± 176***

Values are expressed as mean ± S.E. of three experiments. ND Not done; ***$p < 0.001$ as compared to control group; *$p < 0.05$ as compared to control group

Table 2. Distribution of Hepatic Copper in Sub-Cellular fractions (Percentage of homogenate content) in control and ICC disease

Fraction	Control		ICC	
	Copper content (μg/g tissue)	Percent distribution	Copper content (μg/g tissue)	Percent distribution
Homogenate	14.37 ± 4.23	100	620 ± 73	100
Crude nuclei (800 × g for 10 min)	4.00 ± 1.56	28.00 ± 0.65	455.00 ± 56***	73 ± 1.55***
Crude mitochondria (7500 × g for 10 min)	1.02 ± 0.23	7.10 ± 0.58	14.23 ± 4.0**	2.3 ± 0.10**
Crude lysosome (30,000 x g for 15 min)	0.60 ± 0.18	4.20 ± 0.46	12.83 ± 2.47**	2.07 ± 0.12
Crude micyosomes (105,000 × g for 60 min)	0.43 ± 0.20	3.00 ± 0.42	7.00 ± 1.82*	1.20 ± 0.11
Supernatant	7.50 ± 1.28	52.20 ± 0.95	105.00 ± 9.0***	17.28 ± 0.65***
Total copper recovered		94.50 ± 1.51		95.60 ± 0.86

Values are expressed as mean ± S.E of three experiments carried out in duplicate with different liver samples; ***$p < 0.001$ as compared to control group; **$p < 0.01$ as compared to control group; $p < 0.05$ as compared to control group

Table 3. Copper content in chromatin fractions from control and ICC liver

Group	Chromatin (μg/mg DNA)	Copper Heterochromatin (μg/mg DNA)	Euchromatin (μg/mg DNA)
Control	0.88 ± 0.25	0.20 ± 0.07	0.42 ± 0.20
ICC	81.30 ± 9.0***	19.22 ± 2.9***	9.5 ± 1.6**

Values are expressed as mean ± SD of three experiments carried out in duplicate with different preparations of purified nuclei (6 observations): ***$p < 0.001$ as compared to control group; **$p < 0.01$ as compared to control group.

altered to some extent in the neonate and in the case of deficiency or excess of copper within the body, induced by dietary restriction or excess administration. In the liver of copper loaded adult rats, the proportion of copper in the nuclei is also higher but the absolute amounts in all fractions are increased to some extent [23]. However in Wilson's disease which is also characterized by the toxic accumulation of copper in liver, about 73% of subcellular Cu was present in the cytoplasmic fraction and most of it was in association with metallothionein [24].

In the present study, largest proportion of the excess copper is deposited in ICC hepatic nuclei with a substantial portion bound to heterochromatin and euchromatin fraction of the DNA. Copper at micromolar concentration has been shown to enhance DNA damage in several biological systems, a property which has been exploited in cancer therapy [25]. In the present study, copper content in the purified nuclei from ICC liver was 81.30 μg Cu/mg DNA which is equivalent to 90% saturation limit of copper to bind to DNA. Chromatin isolated from frozen calf thymus was reported to contain 25 ng of tightly bound copper and would approximate perhaps 10% of the Cu found in the normal nuclei [26]. The saturation studies conducted by Sagripanti *et al* [27] indicate that there is an average of one Cu (II) molecule bound for every two nucleotides equivalent to 1.5 μmol copper per milligram of double strand DNA. Taken together, these results indicate that only 1 in 3700 copper sites are actually occupied in DNA *in vivo*. The copper content in heterochromatin and euchromatin from ICC liver nuclei was about 96 and 23 fold higher as compared to the corresponding fractions of normal liver nuclei respectively.

The ultra-violet spectra of heterochromatin and euchromatin showed a more pronounced broad of absorption maxima which suggests destabilization of DNA which could be due to excess copper. It was predicted by Coates *et al* [28] that the occurrence of this type of change in absorption maxima can be attributed to the perturbation of the electronic state of the bases by the interacting Cu^{2+} ions. A marked decrease in A260/A280 ratio in heterochromatin and euchromatin of ICC liver could be associated with decrease in GC content in DNA. The appearance of a very marked difference in the different spectra of DNA Cu^{2+} complexes between 260 and 280 nm depends upon the GC content of DNA [29]. This suggests perturbation of electronic system due to Cu2+ in GC base pairs, which could result in destabilization of DNA.

Copper was seen to enter into the nucleus and excess accumulation of copper in ICC nucleus exhibited a massive DNA fragmentation. Interestingly, the catalase activity in ICC liver was also significantly lower as compared to control which could result in more formation of H_2O_2. Thus, DNA damage in ICC liver cells could be hypothesized due to elevated level of copper and reduced catalase activity by a Fenton type mechanism in which transition metal ions are cycled by being reduced by superoxide radical then oxidized H_2O_2. Copper in the presence of peroxide produces DNA lesions located specifically in the polyguanosine sequences through a mechanism that involves .OH radicals *in vitro* [7]. This damage inactivates transforming ability that include single and double strand breaks and alterations of sites of guanosine. DNA damage due to copper and H_2O_2 was pre-

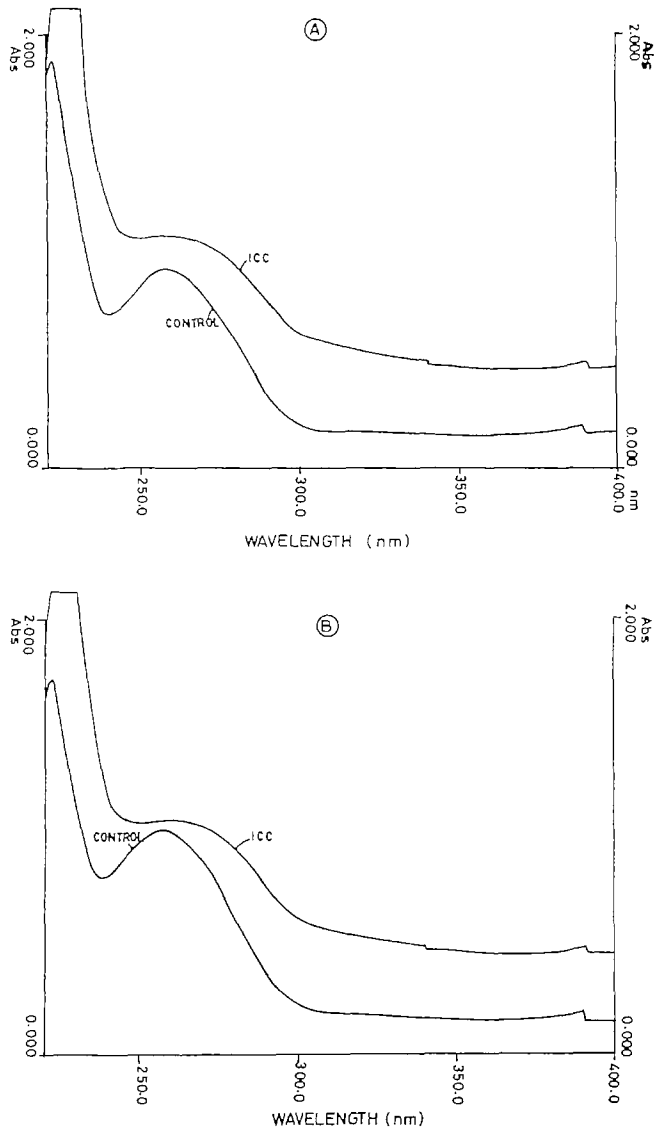

Fig. 1. Ultra violet absorption spectra of heterochromatin (A) and euchromatin (B) from control and ICC disease liver.

vented in the presence of EDTA and catalase [7]. Thus, the ability of copper chelator and .OH scavengers to protect DNA from damage, indicate that the copper and .OH free radicals participates in the mechanism of strand break formation produced by copper and H_2O_2.

Taken together, these data provide evidence that massive accumulation of copper in nuclei and reduced catalase activity in ICC liver are the prominent determinants for DNA destabilization and DNA fragmentation which may be critical to irreversible cell injury leading to cell death.

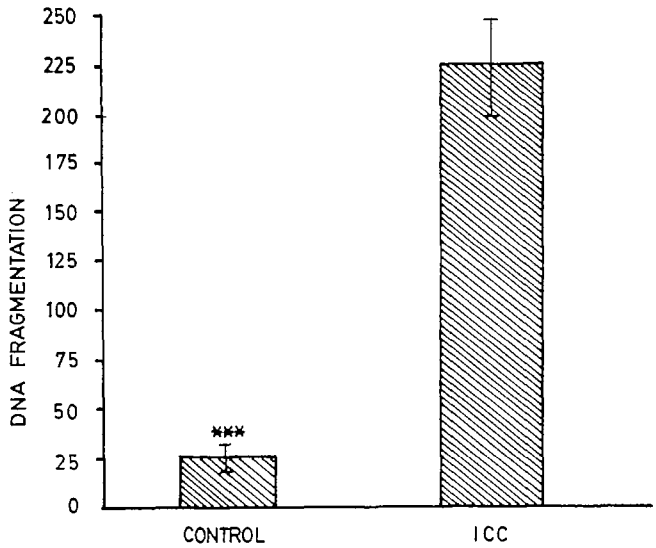

Fig. 2. Quantitation of DNA fragmentation in liver of Control and ICC disease. DNA fragmentation was expressed as the percentage of total DNA which appeared in the supernatant fraction. Values are mean ± S.E.M of observations made in duplicate in three independent experiments (n = 3). Asterisks indicate that values are significantly different ($p < 0.001$) between the two groups.

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