Hydrogen peroxide commences copper induced DNA damage isolated from human blood: \textit{In vitro} study

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The present study revealed the damaging effects of copper and hydrogen peroxide on DNA isolated from human blood in \textit{in vitro}. Ultra violet spectral studies showed that copper and H$_2$O$_2$ alone (at 20 mM) caused destabilization of DNA structure. Notwithstanding, the effect was more prominent in combination of copper and H$_2$O$_2$. Further, agarose gel electrophoretic studies revealed that neither copper nor H$_2$O$_2$ alone had DNA fragmentation (up to 40 mM concentration), while copper and H$_2$O$_2$ together caused massive DNA fragmentation even at lower concentrations (4 mM copper + 4 mM H$_2$O$_2$). Therefore, it was concluded from the present study that the observed destabilization of DNA associated with alterations in configuration and subsequently massive DNA fragmentation was in response to copper and H$_2$O$_2$. Further, fluorescence spectroscopy and TUNNEL assay will address destabilization and fragmentation of naked DNA more precisely.

Keywords: Copper, DNA damage, DNA fragmentation, Hydrogen peroxide.

Copper is an important trace element that plays a fundamental role in the biochemistry of all living organisms. The unique electronic structure of copper permits the electronic interactions with the spin restricted dioxygen and serves as a cofactor for proteins in fundamental redox reactions in all living organisms. Copper affects enzymes activity both as a cofactor and as an allosteric component of cuproenzymes including cytochrome-c-oxidase, super-oxide dismutase, dopamine-$\beta$-hydroxylase, lysyl oxidase, tyrosinase and ceruloplasmin. Therefore, copper is essential for cellular respiration, free radical defense, neurotransmitter function, connective tissue biosynthesis, pigmentation and cellular iron metabolism. Excess copper can be lethal, which acts predominantly through formation of highly reactive hydroxyl radicals by Fenton type reaction, which damages DNA and other macromolecules.

Excess copper accumulation is toxic in all species. In human, copper toxicity often results from inherited abnormalities and leads to hepatic cirrhosis, hemolytic anemia and degeneration of basal ganglia. Wilson disease and Indian childhood cirrhosis (ICC) are the most common disorders of copper metabolism. Prasad \textit{et al.} have suggested that the nucleus is the principal site of copper cytotoxicity. In livers from ICC patients, copper content has been found higher (~43 folds) than control subjects. Copper is mainly present (73%) in the nuclear fraction and evidences are there about significant increase in DNA fragmentation. It is well established that both copper and H$_2$O$_2$ alone and together lead to DNA fragmentation due to oxidative stress from endonuclease activity (apoptosis) or direct attack by reactive oxygen species in presence of transition metal ions in \textit{ex vivo} condition. Further studies also documented chromatin destabilization and DNA fragmentation. In chromatin isolated from frozen calf thymus has been reported to contain 25 ng of tightly bound copper and approximately 10% of tightly bound copper has been found in normal nuclei. In view of this, copper in ICC nuclei is possible to bind naked DNA. Sagripanti \textit{et al.} have reported that there is an average of one copper atom bound for every two nucleotides equivalent to 1.5 µmole/mg of double strand DNA.

In view of the above facts, the present study was undertaken to study the effects of copper and hydrogen peroxide on DNA isolated from human blood in \textit{in vitro} and to study the direct interaction among copper and H$_2$O$_2$ with nucleotide bases.

Materials and Methods

\textit{Isolation of DNA from human blood}—Venous blood was collected from healthy subjects and DNA...
was isolated as described by Daly et al.\textsuperscript{13}. Whole blood (5 ml) was withdrawn in a vial containing 975 μl of ACD (as an anticoagulant) and 25 ml of lysis buffer [320 mM, sucrose; 5M, MgCl\textsubscript{2}; 1%, Triton X-100; and 100 mM, Tris-HCl (pH 7.4)] was added followed by thorough mixing. Then, tube was centrifuged at 2000 g at 4°C for 15 min and pellet was collected. Pellet was resuspended in 2 ml of suspension buffer [150 mM, NaCl; 60 mM, EDTA; 1%, SDS; and 400 mM, Tris-HCl (pH 7.4)] and 500 μl of sodium perchlorate (5 M). The suspension was mixed gently for 15 min at room temperature and incubated at 65°C for 30 min. Two ml pre-cooled chloroform was added and tube was centrifuged at 400 g for 10 min. DNA was precipitated from upper aqueous phase by adding 2 volume of ice cooled ethanol. DNA was spooled out with an autoclaved tip and washed with ethanol (70%). DNA was resuspended in 200 μl of TE [10 mM, Tris; and 1mM, EDTA (pH 7.4)] buffer and incubated at 60°C for 6-8 h. DNA was stored at 4°C till further use. The quantity and quality of DNA was measured at 260 and 280 nm in UV spectrophotometer (Model-DU 640, Beckman Coulter, USA) in the ultra violet range (200-400 nm) at various concentrations of copper (20, 30 and 40 mM) and H\textsubscript{2}O\textsubscript{2} (20, 30 and 40 mM).

**DNA fragmentation studies**—DNA fragmentation study was carried out by the method of Sagripanti et al.\textsuperscript{14}. DNA (15 μg) was added to incubation buffer, [0.145M, NaCl; 0.02M, EDTA; 0.01M, sodium phosphate], pH 7.0 (12 μl) and TE buffer (3 μl) in the presence of copper (10-40 mM) or H\textsubscript{2}O\textsubscript{2} (10-40 mM) and in combination (4-8 mM). The samples were incubated at 37°C for 30 min. After incubation samples were electrophoresed on agarose gel (0.8%). The gels were placed on transilluminator for visualization of DNA fragments.

**Results and Discussion**

In the present study, ratio of absorbance at 260 and 280nm for DNA was 1.7, indicating a good quality of DNA as well as agarose gel electrophoresis showed no shearing of DNA, which was further subjected for UV spectral and fragmentation studies.

The ultra violet spectral studies of DNA was carried out to elucidate the destabilizing effect of copper and H\textsubscript{2}O\textsubscript{2} on DNA. In the blanks (at various concentrations of copper and H\textsubscript{2}O\textsubscript{2} and in combination) no peak was observed at the UV range (200-400 nm). The different UV spectra of DNA under various conditions have been shown in Fig. 1.

![Fig. 1](image_url)—Ultra violet spectral studies of DNA. (A)—The ultraviolet spectra of native DNA: Control native DNA showing a broad peak with a shoulder around 260 nm; (B)—In the presence of 20 mM copper. The distortion of UV spectra indicates destabilization of configuration of DNA; (C)—In the presence of H\textsubscript{2}O\textsubscript{2} (20 mM). The distortion of UV spectra was more prominent; (D)—In the presence of copper (20 mM)+H\textsubscript{2}O\textsubscript{2} (20 mM). A flat spectra indicates destabilization of configuration of native DNA.
The ultraviolet spectra of native DNA showed a broad peak with a shoulder around 260 nm (Fig. 1A). When 20 mM of copper as cupric chloride was added to native DNA, the distortion of UV spectra was observed indicating destabilization of configuration of DNA in the presence of copper (Fig. 1B). This effect was more prominent in the presence of 20 mM of H\textsubscript{2}O\textsubscript{2} (Fig. 1C). In combination (20 mM, copper and 20 mM, H\textsubscript{2}O\textsubscript{2}), a flat spectra of DNA was observed (Fig. 1D). Further, the spectral studies of DNA were carried out in the presence of copper and H\textsubscript{2}O\textsubscript{2} (30 mM, copper+30 mM, H\textsubscript{2}O\textsubscript{2} and 40 mM, copper+40 mM, H\textsubscript{2}O\textsubscript{2}). In both cases, there was a distortion of spectra of DNA as well as change in maxima absorbance. The occurrence of this type of change in absorption maxima can be attributed to the perturbation of electronic state of the basis by interactive Cu\textsuperscript{2+} ions in G-C base pairs, indicating destabilization of DNA\textsuperscript{15}. Excess copper in the presence of H\textsubscript{2}O\textsubscript{2} can produce more hydroxyl free radicals which can cause massive DNA fragmentation as reported in ICC liver\textsuperscript{3}. Copper and H\textsubscript{2}O\textsubscript{2} are required for DNA damage which can be explained by the Fenton type of mechanism in which transition metal ions are cycled by first being reduced by superoxide and then oxidized by H\textsubscript{2}O\textsubscript{2}. Production of hydroxyl free radicals would then damage the DNA. In \textit{in vivo}, DNA fragmentation during oxidative stress results from endonuclease activity (apoptosis). The findings of present study confirmed the DNA destabilization.

To ascertain DNA fragmentation in response to copper and H\textsubscript{2}O\textsubscript{2} alone, as well as in combination, isolated DNA was incubated with different concentrations of copper and H\textsubscript{2}O\textsubscript{2} in a shaking water bath at 37°C for 30 min. Then, the samples were electrophoresed. The control native DNA was run simultaneously in each experiment. DNA fragmentation was not observed in presence of various concentrations of copper (10-40 mM; Fig. 2A). Similarly, no fragmentation of DNA was observed at various concentrations of H\textsubscript{2}O\textsubscript{2} up to 40 mM alone (Fig. 2B). However, massive DNA fragmentation was observed in the presence of both copper and H\textsubscript{2}O\textsubscript{2} even at lower concentrations (4 and 8 mM; Fig. 2C). Therefore, these observations strongly indicated that H\textsubscript{2}O\textsubscript{2} in presence of copper could induce DNA fragmentation. The sensitizing effect of copper has been previously reported in phage inactivation after ionizing radiation\textsuperscript{16} and in reduction of sedimentation speed of T\textsubscript{7} bacteriophage DNA after exposure to reduced oxygen\textsuperscript{17}. High concentrations of H\textsubscript{2}O\textsubscript{2} have been known for many years to produce single and double strand breaks\textsuperscript{17}. Recently, we have demonstrated the effect of copper toxicity in peripheral blood mononuclear cells in \textit{ex vivo} conditions\textsuperscript{19}. Human DNA damage involving copper would be relatively favoured in diseases where copper concentration is elevated such as Wilson disease\textsuperscript{19} and Indian childhood cirrhosis\textsuperscript{3,7}.

In conclusion, direct interaction of transition metal (copper) to DNA in the presence of H\textsubscript{2}O\textsubscript{2} caused

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Fig. 2—DNA fragmentation pattern in presence of copper/ H\textsubscript{2}O\textsubscript{2} /copper+H\textsubscript{2}O\textsubscript{2}. (A)—Native DNA incubated with copper alone [lane C-control; lane 1-10 mM; lane 2-20 mM; lane 3-30 mM; lane 4-40 mM]; (B)—H\textsubscript{2}O\textsubscript{2} alone [lane C-control; lane 1-10 mM; lane 2-20 mM; lane 3-30 mM; lane 4-40 mM]; and (C)—In combination of copper and H\textsubscript{2}O\textsubscript{2}[lane C-control; lane 1-copper (4 mM)+ H\textsubscript{2}O\textsubscript{2} (4 mM); lane 2-copper (8 mM)+ H\textsubscript{2}O\textsubscript{2} (8 mM)].
destabilization and fragmentation of naked DNA. It suggested that after destabilization of chromatin structure i.e when DNA was not protected within a nuclear cellular milieu, nucleotides bases were more prone to interact with transition metal and caused massive DNA fragmentation. Further, fluorescence spectroscopy and TUNNEL assay will address destabilization and fragmentation of naked DNA more precisely.

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