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Folate Deprivation and Copper Exposure Potentiate Reactive Oxygen Species Generation and Chromosomal DNA Loss but not Mitochondrial DNA Deletions in Rat Hepatocytes

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Abstract: Both increased copper and reduced folate levels are commonly found in patients with liver diseases. To better understand the mechanisms by which folate deprivation interacts with copper to contribute to hepatocellular toxicity, rat primary hepatocytes were isolated, cultured in folate-deprived (FD) RPMI medium, and assayed for cytotoxicity after copper sulfate ($CuSO_4$) exposure. MTT measurement and trypan blue assay showed that elevated $CuSO_4$ levels aggravated cell death of folate-deprived but not folate-sufficient hepatocytes. $CuSO_4$ treatment increased the levels of intracellular reactive oxygen species (ROS) by 3 times in FD hepatocytes and tripled the proportion of FD hepatocytes with hypodiploid DNA contents. Measurement of membrane phosphatidylserine exposure indicated that the $CuSO_4$-mediated toxicity in FD hepatocytes was not mediated by the apoptotic pathway. Real-time polymerase chain reaction (PCR) analysis revealed that $CuSO_4$ treatment did not increase the occurrence of a 4834-bp mtDNA ($mtDNA^{4834}$) deletion in FD hepatocytes. Preincubation of FD hepatocytes with various concentrations of folate prior to $CuSO_4$ treatment did not modulate the $mtDNA^{4834}$ deletion. Taken together, the data suggest that elevated copper levels potentiate cell death of folate-deprived hepatocytes, which is primarily associated with increased ROS generation and chromosomal DNA loss. The cytotoxicity exerted by folate depletion and elevated copper levels, however, is not due to apoptosis or accumulated $mtDNA^{4834}$ deletions in primary hepatocytes.

Key words: Folate depletion, copper toxicity, reactive oxygen species, hypoploid DNA content, mtDNA deletion, primary hepatocytes

Abbreviations: DCFH-DA, 2',7'-dichlorofluorescein diacetate; FBS, fetal bovine serum; folate, pteroylmonoglutamic acid; FD, folate-deficient; $mtDNA^{4834}$ deletion, a 4834-bp deletion in mitochondrial DNA; PBS, phosphate-buffered saline; PI, propidium iodide; ROS, reactive oxygen species.

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Introduction

Toxicological levels of copper occur in Wilson's disease, an autosomal recessive disorder of copper disposition in the liver and extrahepatic organs [1]. In cirrhotic patients with liver disorders, plasma copper levels are abnormally elevated [2]. Excess copper, a pro-oxidant, facilitates the formation of hydroxyl radicals via the Fenton reaction and through redox alteration of antioxidant molecules to cause cellular damage [3,4]. In addition to elevated copper levels, reduced folate levels are commonly found in patients with alcoholic and liver diseases such as hepatitis C [5–7]. Growing evidence suggests that folate acts as an antioxidant, which is capable of scavenging peroxyl, azide, hydroxyl radicals and peroxynitrite [8,9]. Folate deprivation promotes reactive oxygen species (ROS) generation and lipid peroxidation in human hepatoma HepG2 cells [10]. Although either folate deprivation or elevated copper levels could independently elicit intracellular oxidative stress, the question as to whether these two factors interact to cause hepatocellular injury remains elusive.

Oxidative stress can promote apoptosis, a programmed cell death [11] characterized by chromosomal DNA damage such as chromatin condensation and DNA fragmentation, and membrane phosphatidylserine translocation [12,13]. Mitochondria DNA (mtDNA) is more susceptible to oxidative injury than chromosomal DNA partially due to lack of histone protection [14]. Among various types of mtDNA damage, large-scale deletions of mtDNA (a 4977 bp deletion in human mtDNA and a 4834 bp deletion in rodent mtDNA) are commonly found in aging tissues [15–17] and in livers of patients with liver diseases [18]. Accumulation of large mtDNA deletions beyond a certain threshold may involve the altered synthesis of mitochondrial proteins, respiratory chain dysfunction, and the production of free radicals [19]. Although the exact causes of accumulating large mtDNA large deletions are not clear, the occurrence of this common mtDNA deletion has been correlated with increased oxidative damage of lipid and increased 8-OHdG levels [20].

Previous studies have shown that folate deficiency leads to oxidative stress-related apoptotic DNA damage in human hepatoma cells [21] and rat liver [22]. Folate supplementation has been found to modulate the accumulation of large mtDNA deletions in the liver of rats after chemotherapy [23], and in the liver of aging rats [24]. Whether elevated copper levels may potentiate the cytotoxicity of folate-deprived hepatocytes via increased oxidative stress is currently not known. We hypothesized that elevated copper levels may interact with folate deprivation to cause hepatic injury through apoptotic DNA damage and the accumulation of large mtDNA deletions. For this reason, a rat primary hepatocyte model was used to assess the individual and combined effects of folate deficit and increased copper levels on cellular viability, intracellular ROS generation, chromosomal DNA loss, apoptotic markers, and the large mtDNA deletions.

Material and Methods

Materials

Folate (pteroylmonoglutamic acid), 2',7'-dichlorofluorescein diacetate (DCFH-DA), insulin, transferrin, copper sulfate, MTT (3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyl-tetrazolium bromide), and propidium iodide (PI) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RPMI 1640 medium, RPMI 1640 medium without folate, penicillin, streptomycin, fungizone, trypsin, and trypan blue were obtained from Gibco BRL Life Technologies Inc. (Gaithersburg, MD, USA).

Primary hepatocyte cultures

Primary hepatocytes were isolated from young male Wistar rats (200–250 g; 6 weeks) by using a two-stage collagenase perfusion technique [25] with some modifications. The experimental protocols were approved by the Institutional Animal Care Committee of Fu-Jen University. Briefly, the liver was perfused via the portal vein at a flow rate of 25 mL/minute for 10 minutes with a buffer solution (25 mmol/L sodium phosphate buffer pH 7.6, 3.1 mmol/L KCl, 119 mmol/L NaCl, 3.5 mmol/L glucose, 1.0 g/L bovine serum albumin, and 5 mg/L phenol red). The perfusion was then continued for an additional 10 minutes at a flow rate of 18 mL/minute with the same buffer supplemented with 50 mg collagenase. The liver was then minced and suspended in Krebs–Henseleit buffer containing 10 g/L bovine serum albumin and 8 mmol/L HEPES (pH 7.4). After centrifugation at low speed (130 × g) for 10 minutes, the parenchymal cells in the pellet were resuspended and washed twice with buffer. Cells prepared in this manner had greater than 90% viability by trypan blue exclusion. The hepatocytes were seeded at a density of 1×10^5 cells/mL in RPMI 1640 medium supplemented with 2 g/L sodium bicarbonate, 100 units/mL penicillin, 100 units/mL streptomycin, 100 µg/mL fungizone, 10% fetal bovine serum (FBS), 5 mg/L transferrin, and 5 mg/mL insulin. The cell culture was incubated in a CO_2 incubator at 37 °C.

Folate deprivation and copper treatment

Primary hepatocytes (1×10^5/mL) were cultured in the RPMI complete medium (control) or folate-deprived medium (FD) for 6 hours, treated either with or without copper sulfate ($CuSO_4$) for 24 or 48 hours, and then harvested for various assays. The concentrations and the time of pro-oxidant treatment were previously tested [26]. The concentrations (4–16 μM) of copper selected for this study were in the range of plasma levels of patients with liver diseases [27]. For folate supplementation, hepatocytes were cultured in FD medium supplemented with various levels of folate. A stock solution of folate was prepared at 10 mmol/L in bicarbonate solution.

Measurement of cytotoxicity

The viability of hepatocytes was determined by MTT colorimetric assay [28]. After termination of copper treatment, 10 μL MTT solution (5 mg/mL in phosphate-buffered saline; PBS) was added to each plate. The absorbance of each well was determined with an automated plate reader at 550 nm (Bio-Tek Instrument, Inc., Vermont, USA). Survival was calculated as the percentage of the staining value of untreated cultures. The rate of cell death was also characterized by trypan blue assay.

Determination of intracellular reactive oxygen species

Intracellular ROS were labeled using an oxidation-sensitive probe, DCFH-DA, and assayed for by flow cytometry. The detailed procedure has been described elsewhere [29]. The excitation wavelength was 488 nm and the emission wavelength was 525 nm for the fluorescent 2', 7'-dichlorofluorescein (DCF). The fluorescence in control cells was designated as 100%, and the ratio of treated cells to control cells was calculated.

Analysis of chromosomal DNA loss

Cells were fixed in ice-cold 100% ethanol. RNase A (500 mg/L) and 0.5% Triton were added to the samples, which were further incubated at 37 °C for 60 minutes. Cells were then incubated with PI (50 mg/L) for 20 minutes at 37 °C. After centrifugation (300 × g, 5 minutes), 10,000 cells were assayed for cellular DNA content (red fluorescence) on the flow cytometer. Cells with DNA contents < 2N in the M1 region were defined as hypodiploid cells.

Analysis of membrane phosphatidylserine exposure

The Annexin-V-Fluos kit was used to measure apoptosis of cells with membrane phosphatidylserine (PS) exposure. The detailed procedure has been described elsewhere [29]. The green (Annexin-V stain: apoptotic cells) and red (PI stain: necrotic cells) fluorescence intensities were analyzed on the flow cytometer at 488 nm excitation with a 515-nm bandpass filter and a > 560-nm filter for PI detection. Cells with Annexin-V-positive and PI-negative fluorescence were defined as apoptotic.

Analysis of large mtDNA deletions

Whole DNAs were extracted as described by Huang *et al* [29]. Primers and probes for the mtDNA D-loop and a 4834-bp large deletion in rodent mtDNA ($mtDNA^{4834}$ deletion), and PCR conditions were modified from the method described by Branda *et al* [23]. The degree of $mtDNA^{4834}$ deletion was quantified with a DYXL-5' reporter and a 3'-BHQ1 quencher dye, and the amount of D-loop expression was quantified with a 6FAM-5' reporter and a 3'-BHQ1-labeled quencher dye. PCR amplification was carried out in a 50-μL reaction consisting of 1× TaqMan Universal Master Mix, 200 nM each $mtDNA^{4834}$ deletion primer, 100 nM each D-loop primer, and 100 nM each $mtDNA^{4834}$ deletion and D-loop probe primer. The cycling condition included an initial phase of 2 minutes at 50 °C, 10 minutes at 95 °C, then 40 cycles of 15 seconds at 95 °C and 0.5 minutes at 72 °C (LightCycler, Roche Diagnostics, Mannheim GmbH, Mannheim, Germany). Each sample was assayed in duplicate, and the fluorescence spectra were monitored by the LightCycler Sequence Detection System with Sequence Detection Software version 4.

Statistical analysis

Results are expressed as means ± SEM. Differences between groups were analyzed by one-way ANOVA and Dunnett's multiple-range test with the General Linear Model of SAS (SAS Institute, Cary, NC, USA). $P < 0.05$ is considered significant.

Results

Folate deprivation and copper treatment potentiated cell death

Figure 1 presents the data of cellular viability measured by MTT and trypan blue assays. Treatment with $CuSO_4$ levels of 4, 8, and 16 μM for 48 hours did not exert cytotoxicity in folate-sufficient cells (controls) as shown in Figure 1A. In contrast, treatment of folate-deprived hepatocytes with 16 μM for 24 hours significantly decreased cellular viability. At 48 hours of $CuSO_4$ treatment, the inhibitory effect of copper treatment on cellular viability reached a plateau at 16 μM $CuSO_4$ (Figure 1A). Trypan blue assay showed that the death rate of FD cells treated with 16 μM $CuSO_4$ for 48 hours was 28 ± 5%, which was significantly higher than death rates of control cells without $CuSO_4$ treatment (Figure 1B).

Copper treatment aggravated ROS generation in folate-deprived hepatocytes

Figure 2A showed that $CuSO_4$ treatment for 48 hours increased DCF fluorescence intensity in both control and FD cells, as reflected by the shift of mean DCF intensity to the right. The intracellular ROS levels in FD cells were higher than those in controls, regardless of the presence or absence of $CuSO_4$ treatment (16 μM). FD hepatocytes receiving $CuSO_4$ treatment (8 and 16 μM) for 48 hours displayed 2- to 3-fold increases in ROS production when compared to untreated FD hepatocytes (Figure 2B). Conversely, hepatocytes with sufficient folate produced less ROS (40–60% increases) after receiving the same $CuSO_4$ treatment as FD hepatocytes did.

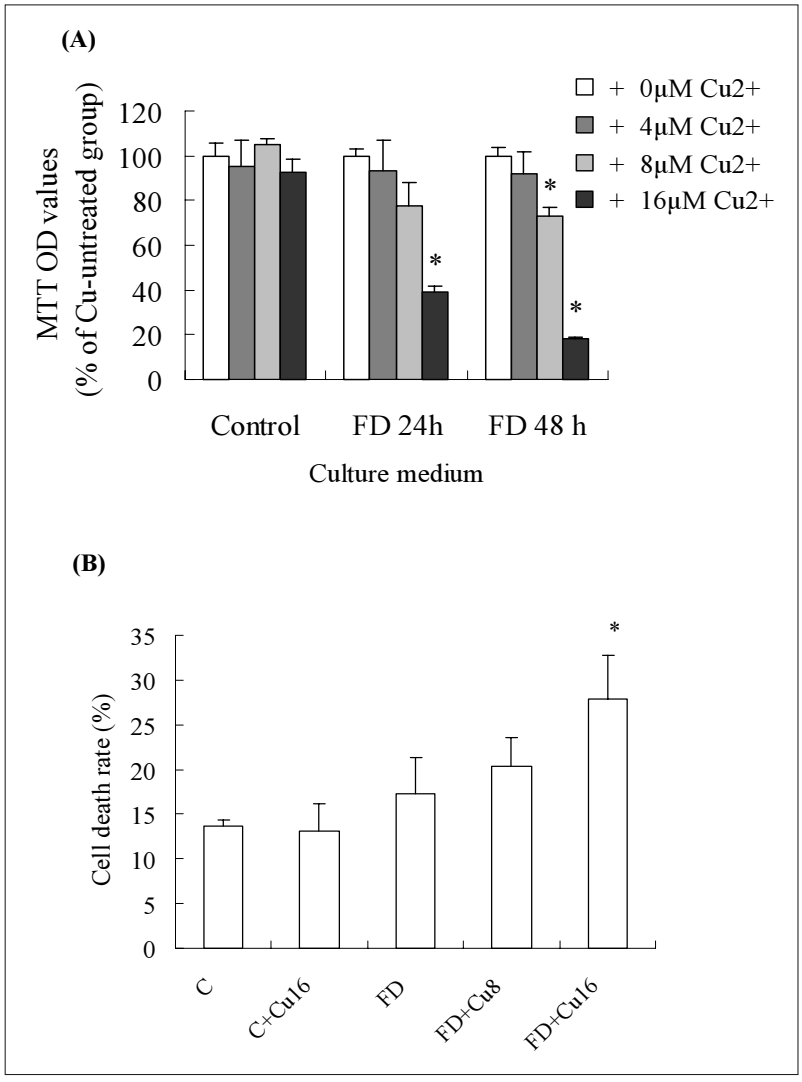

Figure 1: Effects of folate depletion and copper treatment on cellular viability. (A) Freshly isolated hepatocytes were cultured in folate-deprived (FD) and complete medium (control) for 6 hours, and treated with various concentrations of copper sulfate ($CuSO_4$) for 24 or 48 hours. Viability was measured by MTT assay, and expressed as a percentage of Cu-untreated values in the respective group. ANOVA was performed for each culture group. *: Significant at $P < 0.05$ compared with values of $CuSO_4$-untreated respective groups. (B) Death rates of primary hepatocytes incubated in control (C) and FD culture medium with or without 8μM (Cu8) or 16 μM (Cu16) $CuSO_4$ treatment for 48 hours. Death rate is expressed as percentage of dead cells (trypan blue-stained) to total cells (both live and dead). Means ± SD were obtained from triplicate cultures of three independent experiments. *: Significant at $P < 0.05$ compared with the control group.

Copper treatment aggravated chromosomal DNA loss in folate-deprived hepatocytes with no apoptotic induction

As indicated in Figure 3A, $CuSO_4$-treatment (16 μM) did not induce significant nuclear DNA loss in control hepatocytes. However, it is interesting to note that $CuSO_4$ treatment caused a 3-fold increase in hypoploid DNA content in FD hepatocytes compared to their untreated counterparts. To investigate whether $CuSO_4$-treated cells underwent apoptosis, cells with membrane disruption due to phosphatidylserine (PS) exposure were analyzed by fluorescent labeling with Annexin-V-Fluos. PI labeling is an index of necrotic cells with impaired membrane for dye penetration. Apoptotic cells were characterized by Annexin-positive and PI-negative fluorescence. In the bivariate scatter plots shown in Figure 3B, neither folate deprivation alone nor $CuSO_4$ treatment of FD hepatocytes induced apoptosis (no cells with Annexin+ and PI- fluorescence were found). Oligonucleosomal DNA fragmentation and DNA ladders were not detected in FD, $CuSO_4$-treated FD, and control hepatocytes (data not shown).

Effect of folate status on levels of the mtDNA^{4834} deletion in FD hepatocytes during copper treatment

The levels of the mtDNA^{4834} deletion were evaluated by quantitative real-time PCR. The cycle at which a statistically significant increase in normalized fluorescence was first detected was designated as the threshold cycle number (C_t) (Figure 4A). The relative amount *(R)* of deleted mtDNA to total mtDNA was calculated as $R = 2^{-\Delta Ct}$, where ΔCt is $Ct_{\text{deleted mtDNA}} - \Delta Ct_{\text{D-loop value}}$. We checked the specificity of the amplification products by 2% agarose gel electrophoresis, which showed unique PCR fragments of the expected size (Figure 4B). Figure 4C shows that copper treatment did not significantly increase the levels of mtDNA^{4834} deletion in FD hepatocytes. Preincubation of hepatocytes with 10, 100, and 1000 μM folate for 6 hours did not modulate levels of mtDNA^{4834} deletion.

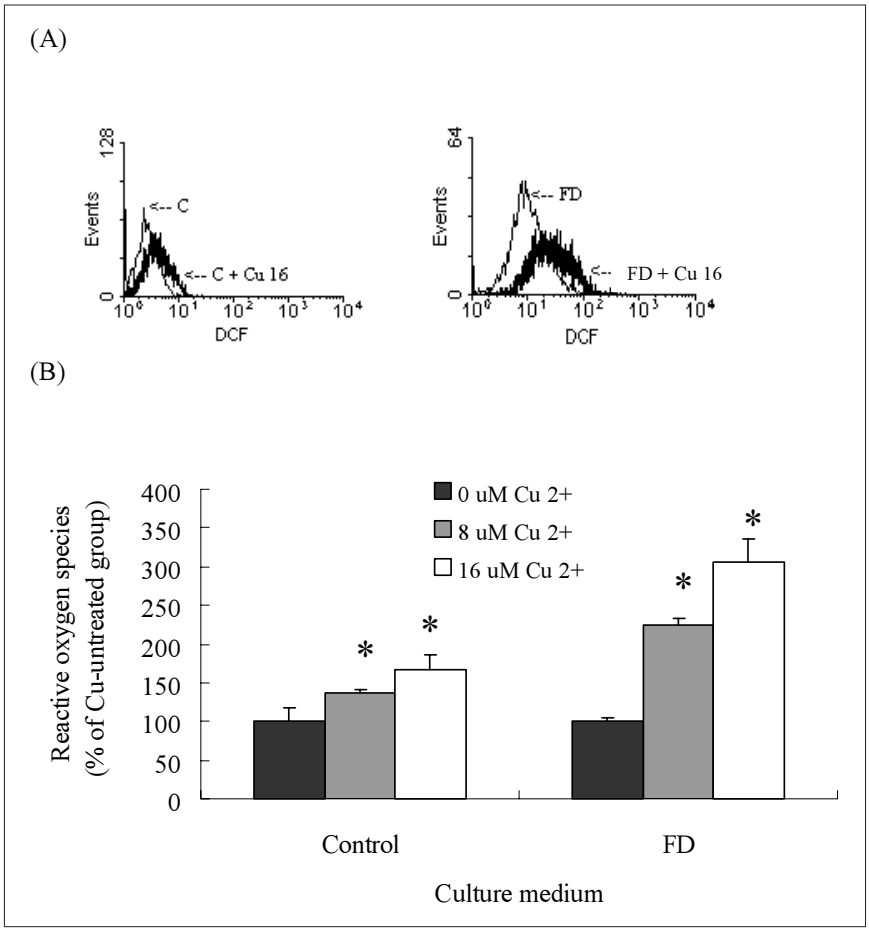

Figure 2: Effects of folate depletion and copper treatment on reactive oxygen species generation. Culture conditions and $CuSO_4$ treatment are as described in Figure 1. Intracellular ROS in hepatocyte cultures were measured by DCF fluorescence and flow cytometry. (A) DCF fluorescence distribution in control and FD hepatocyte cultures treated with or without 16 μM $CuSO_4$ for 48 hours. Histograms represent three independent experiments. (B) Dose effects of $CuSO_4$ treatments on intracellular ROS generation in control and FD hepatocytes. The relative ROS levels are expressed as percentages of the $CuSO_4$-untreated values from the respective group. Means ± SD were obtained from triplicate cultures of three independent experiments. *: Significant at $P < 0.05$ compared with values of $CuSO_4$-untreated group.

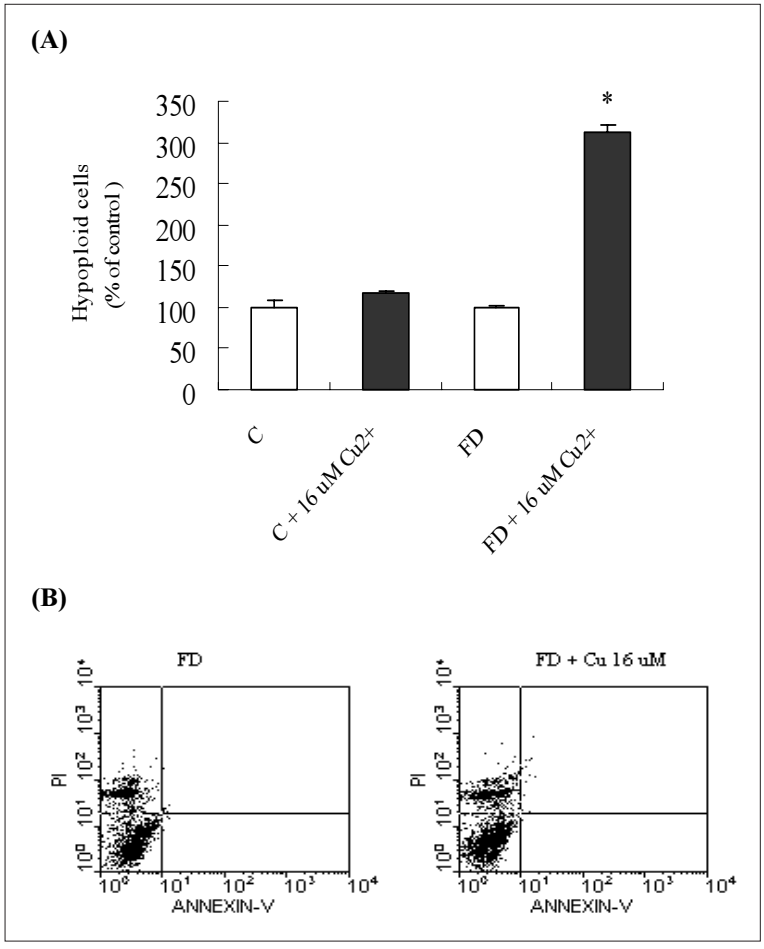

Figure 3: Effects of folate depletion and copper treatment on chromosomal DNA loss and apoptosis induction. Hepatocytes treated with or without 16 μM $CuSO_4$ for 48 hours were harvested, fixed, stained with DNA-interacting dye, and analyzed by flow cytometry. (A) Hypoploid cells were quantified and expressed as percentages of $CuSO_4$-untreated values from the respective group. Data are means ± SD from triplicate cultures of three independent experiments. *: Significant at $P < 0.05$ compared with values of $CuSO_4$-untreated group. (B) Representative scatter plots of apoptosis in FD hepatocytes treated with or without 16 μM copper for 48 hours. Apoptotic cells binding to Annexin V (AV fluorescence-positive) and necrotic cells with PI staining (PI-positive) were analyzed by flow cytometry. Data were obtained from triplicate cultures of three independent experiments.

Discussion

Our data demonstrated that copper exposure (16 μM) within the range of the reported copper concentrations in patients with liver diseases [2, 27, 30] promoted cell death of folate-deprived hepatocytes. Furthermore, we found that folate sufficiency could prevent these hepatocytes from being injured while receiving the same copper treatment. These findings are consistent with a report elsewhere which demonstrated that the threshold concentrations of copper-induced hepatocellular toxicity *in vitro* were substantially higher (50–100 μM) under the condition of normal folate status [31]. Lack of folate, a proposed antioxidant vitamin, resulted in the depletion of antioxidant molecules and weakened the cellular oxidative defense system [10]. Folate-deficient rats have compromised antioxidant enzymatic activities and elevated lipid peroxidation in their livers [32], which may potentiate copper-induced oxidative stress in folate-deprived hepatocytes. Indeed, copper-mediated toxicity coincided with a 3-fold elevation in intracellular ROS generation in folate-deprived hepatocytes. These results are consistent with those from human hepatoma cells. Folate deficiency sensitized HepG2 cells to pro-oxidant-elicited cell death by promoting ROS generation and oxidative damage [33].

In the present study, copper-mediated toxicity of folate-deprived hepatocytes was correlated with chromosomal DNA loss. These hypodiploid cells were not apoptotic, because no membrane PS exposure, a hallmark of apoptosis, was detectable. Instead, the cell death mode appeared to be necrosis, supported by the evidence of PI-positive staining and trypan-blue assay. Acute oxidative damage can elicit necrosis with chromosomal DNA loss, whereas chronic and moderate oxidative stress promotes apoptosis [34]. Folate depletion combined with elevated copper levels apparently promoted acute oxidative stress, since ROS generation tripled in only 48 hours (Figure 2). In contrast, folate deficiency for 2 to 4 weeks without any pro-oxidant stimulation essentially promoted chronic oxidative stress, which was associated with apoptotic induction in HepG2 cells [21, 33].

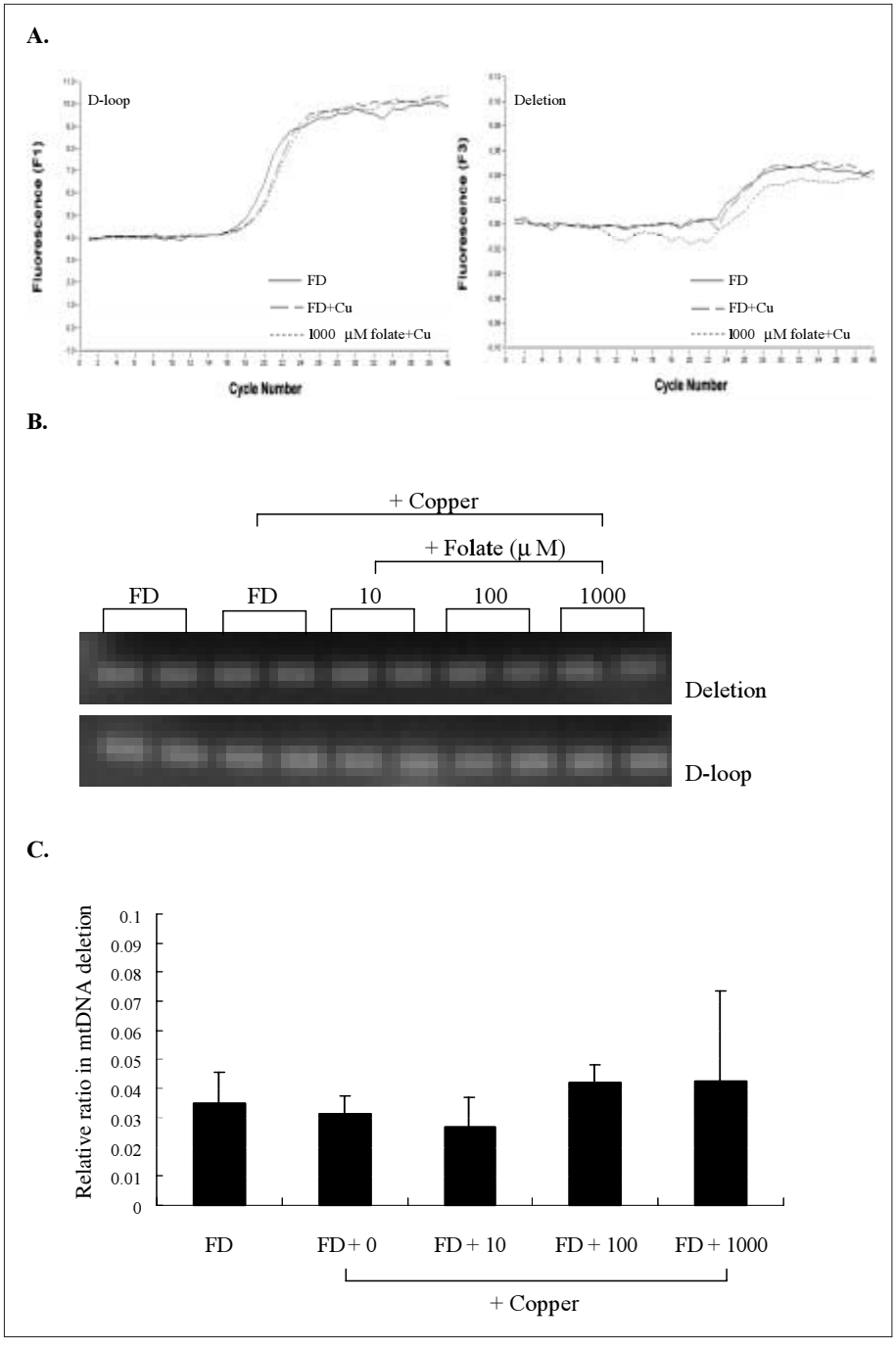

Figure 4: Effects of supplemental folate and copper treatment on mtDNA deletion of FD hepatocytes. FD hepatocytes were treated with or without 16 μM $CuSO_4$ for 48 hours. For folate supplementation, FD hepatocytes were preincubated with various concentrations of folate (0, 10, 100, and 1000 μM) for 6 hours prior to copper treatment. DNA was extracted from each group and analyzed by real-time PCR for the 4834-bp mtDNA deletion. (A) Representative plots of real-time PCR analysis. (B) Agarose gel electrophoresis of PCR products amplified from the primer pair detecting the 4834-bp mtDNA deletion and the primer pair for the D-loop region. (C) Quantification of the 4834-bp mtDNA deletion in FD hepatocytes supplemented with various levels of folate (0, 10, 100, and 1000 μM). Data are means ± SEM (n = 4).

Studies have reported that mitochondria are a potential target of copper-mediated toxicity in hepatocytes [31]. Our data provide several notable findings relative to mtDNA large deletions upon copper exposure: (1) $mtDNA^{4834}$ accumulation at 3–5% was not lethal in copper-untreated FD hepatocytes; (2) these levels of mtDNA deletion did not mediate necrotic cell death in copper-treated FD hepatocytes and; (3) folate deprivation and copper-induced ROS generation, an acute oxidative condition, seemed not to affect $mtDNA^{4834}$ accumulation. It is possible that oxidative stress elicited by copper in folate-deprived hepatocytes may be more pronounced in the cytosol than in the mitochondrial matrix, where the mtDNA is located. Elevated levels of copper have been documented to decrease cellular glutathione, cytosolic metallothionein levels, and glutathione peroxidase activity, and thus increased hydrogen peroxide

flux can be anticipated [35]. Consequently, acute oxidative stress may preferentially disrupt plasma membranes (Figure 3), impair mitochondrial membrane potential [36], and disturb mitochondrial function [37] without having an impact on the levels of mtDNA^{4834} accumulation.

Despite reports elsewhere indicating that increased folate levels could modulate the mtDNA^{4834} deletion in the rat liver after chemotherapy or in the liver of aging rats [23, 24], we could not find any protective effect by folate in modulating this deletion in copper-treated primary hepatocytes. Madsen *et al* proposed that mtDNA deletion may be due to slipped mispairing between repeated sequences during DNA replication or by erroneous RNA splicing [38]. It has been suggested that the favorable selection for and propagation of mutated mtDNA through tissue proliferation results in the accumulation of mutated mtDNA in individual cells [39]. However, this possibility can be excluded, since primary hepatocytes do not divide. Collectively, the evidence implies that folate may only modulate the common mtDNA deletion through mitochondrial biogenesis and mtDNA replication during cellular proliferation [23, 24].

In summary, our data demonstrate that folate deprivation and elevated copper levels potentiate necrotic cell death associated with elevated ROS generation and impaired chromosomal DNA integrity, but have no impact on accumulation of the common mtDNA deletion. Our observations from an *ex vivo* primary hepatocyte model may therefore provide mechanistic insights into the effect of folate insufficiency and increased copper toxicity on liver damage.

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