Characterisation of oxidative injury to an intestinal cell line (HT-29) by hydrogen peroxide

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Abstract

Reactive oxygen metabolites have been implicated in causing epithelial cell injury in colonic inflammation. A model of oxidative injury in intestinal epithelial cells has been developed in which HT-29-18-C1 cells are injured with graded concentrations of hydrogen peroxide and characterised by the MTT test. The MTT test was validated as a cytotoxicity assay and has a similar sensitivity to hydrogen peroxide induced injury as the assay of intracellular adenosine triphosphate. Exposure to a range of hydrogen peroxide concentrations (0-05-20 mM) for varying duration (5-120 min) showed that injury was dependent on time and concentration. The median lethal dose (LD50) for one hour exposure to hydrogen peroxide was approximately 0.1 mM. Injury from hydrogen peroxide was only partially reversible as determined by the MTT test and assay of cellular proliferation by crystal violet staining. There was an exponential loss of hydrogen peroxide when incubated with HT-29-18-C1 cells (t1/2 35 min). Experiments with 0.5 mg/ml aminotriazole and 0.5-2 mM buthionine sulfoximine suggested hydrogen peroxide breakdown was predominantly caused by catalase rather than glutathione peroxidase. Injury resulting from 1.18-C1 hydrogen peroxide could be reduced by either coinoculation of cells with 1,10-phenanthroline, an Fe2+ chelator, or preincubation with deferoxamine, an Fe3+ chelator, suggesting the participation of Fe2+ and Fe3+ in hydrogen peroxide induced injury. In conclusion, hydrogen peroxide induces injury in HT-29-18-C1 cells both directly and by generation of the hydroxyl radical.

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Reactive oxygen metabolites (ROMs) are oxygen centred free radicals or their reduction products, which are released by stimulated neutrophils. Although ROMs kill microorganisms they may also injure the surrounding tissue. Evidence for an important role of ROMs in the pathogenesis of inflammatory bowel disease (IBD) is accumulating. Production of ROMs has been detected by chemiluminescence assays in colonic mucosal biopsy specimens of patients with IBD. Sulphasalazine and 5-aminosalicylic acid may owe their therapeutic effect partly to their powerful antioxidant properties. A range of ROMs including H2O2, O2-, hypochlorous acid, and monochloramine have been implicated in causing epithelial cell injury in IBD. Furthermore, transition metals such as iron can catalyse the conversion of H2O2 to the highly reactive hydroxyl radical (OH·), which can also injure intestinal cells.

Little is known about the effect of oxidant injury on intestinal epithelial cell function. The objective of this study was to develop a model of oxidant injury to intestinal epithelial cells for the future of novel therapeutic strategies for the treatment of IBD. Before such studies can be performed a model of intestinal epithelial cell injury needs to be established in which both the sensitivity to oxidant injury and the oxidants responsible are characterised. To ensure a source of uninjured intestinal epithelial cells that was free of other cell types (inflammatory or endothelial), which might contribute to oxidative epithelial cell injury, we used the HT-29-18-C1 cloned cell line derived from a human colonic carcinoma. This cell line has many of the phenotypic properties of human intestinal epithelial cells including regulated trans-epithelial ion transport. The MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), which measures mitochondrial dehydrogenase activity, was used to characterise the time course, concentration dependence, and reversibility of H2O2 induced injury. The role of iron in mediating H2O2 induced injury in HT-29-18-C1 cells was assessed with iron chelating agents.

Methods

CELL CULTURE

HT-29-18-C1 cells (passage 9-20) were cultured in a 5% CO2-95% air humidified incubator at 37°C in Dulbecco’s modified Eagle’s medium with 20 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid) (ICN Flow Ltd, Bucks) and supplemented with 10 μg/ml human transferrin, 50 IU/ml streptomycin, 50 μg/ml penicillin, 2 mM glutamine, 25 mM NaHCO3, and 10% fetal calf serum (Gibco BRL, Paisley). The HT-29-18-C1 cells were a gift of Dr M H Montrose, Johns Hopkins Medical School.

CHEMICALS AND SOLUTIONS

For most experiments cells were placed in a solution (Na+ medium) containing (in mM) 130 NaCl, 5 KCl, 2 CaCl2, 1 MgSO4, 0.83 NaH2PO4, 0.17 NaHPO4, 25 glucose, 20 HEPES, titrated to pH 7.40 at 37°C with

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**Figure 1:** (A) Comparison of the measurement of cell survival by the MTT test with measurement of cell number by the crystal violet assay. HT-29-18-C1 cells (5×10^6 cells/ml) were incubated for one hour with H_2O_2 (0-10 mM). Cell survival was measured by the MTT test (closed circles). Controls were cells incubated in Na^+ medium without H_2O_2. Percentage cell survival was calculated as the percentage of control optical density. Cell number was measured by the crystal violet assay (open circles) and the results expressed as the percentage of the initial total cell number. Each data point is the mean (SD) of eight replicates. (B) Comparison of sensitivity to H_2O_2 induced injury of the MTT test and cellular ATP content. HT-29-18-C1 cells were incubated for five minutes in Na^+ medium with H_2O_2 at the concentrations shown and percentage of control cellular ATP content measured (closed circles) or the percentage of cells surviving measured by the MTT test (open circles). Data points are the mean (SD) of three replicates for the ATP assay or eight replicates for the MTT test.

MTT assay

This assay was performed as described previously. Cells were seeded onto 96 well plates at a density of 5×10^4 cells/ml and cultured for 24 hours. After exposure to H_2O_2 in Na^+ medium for one hour at 37°C the medium was replaced with culture medium containing 0.56 mg/ml MTT (3-[4,5-dimethylthiazol-2-y]2,5-diphenyl tetrazolium bromide) for two hours at 37°C. The cells were then lysed by replacement of medium with lysing buffer containing 50% (vol/vol) N,N-dimethylformamide, 20% sodium dodecyl sulphate (wt/vol) titrated to pH 4-7 with equal parts of 80% acetic acid and 1 M HCl. After incubation overnight, the plate was read at 540 nm in a Titer-Tech Multiskan plate reader using the lysing buffer as blank. The surviving fraction was calculated as the fraction of uptake of control. Preliminary experiments showed that the optical density was proportional to the number of cells seeded up to a cell density of 1-5×10^6 cells/ml. Intra-assay reproducibility had a coefficient of variation of 6-2%. Inter-assay variation was greater with a coefficient of variation of 23-6%. Comparisons were only made between cells grown on the same plate.

H_2O_2 assay

H_2O_2 was measured fluorometrically according to the method of Hyslop and Sklar. Cells were seeded onto 96 well plates at a density of 5×10^4 cells/ml and cultured for 24 hours. After incubation of the cells with H_2O_2 in culture medium, aliquots of the culture medium were removed for assay. Samples were added to a fluorometer cuvette solution containing phosphate buffered saline, 1 mM PHPA (p-hydroxophenylacetate) and 53 mg/ml horseradish peroxidase type VI (Sigma), pH 7-40. Sample blanks were obtained before addition of the sample. The reaction was monitored in a Perkin Elmer LS-50 fluorometer (excitation 323±2.5 nm, emission 400±2.5 nm). Calibration was performed by addition of H_2O_2 of known concentration.

ATP assay

Cellular ATP content was measured fluorometrically as described previously. Cells were seeded onto 33 mm culture dishes at a density of 1×10^6 cells per dish and cultured for three days. After exposure to H_2O_2 in Na^+ medium at 37°C, the cells were extracted with ice cold 0.4 M perchloric acid. After neutralisation with 2.5 M KHCO_3, cellular debris was removed by centrifugation and the supernatant assayed for ATP in a buffered medium containing 100 mM TRIS (TRIS-(hydroxymethyl)aminomethane), 5 mM MgCl_2, 5 mM glucose, 10 μM NADP (nicotinamide adenine dinucleotide phosphate), 1.75 U/ml glucose-6-phosphate dehydrogenase, and 1 U/ml hexokinase (Sigma), titrated to pH 7-40. The reaction was carried out at 37°C in a Perkin Elmer LS-50 fluorometer (excitation 340±4 nm, emission 450±20 nm). Sample blanks were obtained before addition of cellular supernatant. Calibration was obtained by addition of ATP of known concentration. Results were normalised to cellular protein measured with the Bradford reagent (Bio-Rad Labs, Herts) using globulin as standard.

NaOH. All chemicals were obtained from Sigma or Fluka.
staining as described previously. Cells were cultured in 96 well plates in identical conditions to those described for the MTT assay. After exposure to H₂O₂, cells were fixed by addition of 1% glutaraldehyde (final concentration). The plate was shaken for 15 minutes, then washed three times in deionised water and air dried overnight. Crystal violet (0.1%) in 200 mM boric acid titrated to pH 9.0 was added to each well and the plate shaken for 20 minutes. After the plate was washed three times with deionised water and air dried overnight, bound dye was solubilised by addition of 10% acetic acid to each well and the optical density read at 540 nm on a Titer-Tech Multiskan plate reader. Preliminary experiments showed this assay was linear up to at least 10⁵ cells/well.

LACTATE DEHYDROGENASE RELEASE
Lactate dehydrogenase release into the medium was measured as described before. Lactate dehydrogenase release into the medium after exposure to 1 mg/ml digitonin was taken as 100% of total activity.

STATISTICAL ANALYSIS
Results are presented as the mean (SD). Comparisons of two means were performed by Student’s unpaired two tailed t test. A probability of <0.05 was considered significant.

Results
USE OF THE MTT TEST AS AN ASSAY OF H₂O₂ INDUCED CYTOTOXICITY
As the MTT test is an assay of both cell integrity and cell number, it was important to find out if it could be used as an assay of cytotoxicity of HT-29 cells exposed to H₂O₂. Therefore the MTT test was compared with the crystal violet assay, which only measures cell number. After exposure to graded concentrations of H₂O₂ (0-05–10 mM) for one hour, there was a greater decrease in the percentage of surviving cells as measured by the MTT test than cell number as measured by crystal violet staining (Fig 1A). This shows that under these conditions the MTT test is predominately an index of cellular integrity.

COMPARISON OF SENSITIVITY OF THE MTT TEST AND CELLULAR ATP CONTENT WITH H₂O₂ INDUCED INJURY
Previous work has shown that a fall in ATP concentration is one of the earliest changes in oxidative injury. As the MTT test is an assay of mitochondrial function, the sensitivity to oxidative injury of cellular ATP content was compared with that of the MTT test. Exposure to H₂O₂ for five minutes at concentrations of less than 2.5 mM caused little change in cellular ATP content while a fall in the surviving fraction could be detected by the MTT test (Fig 1B). We conclude that the MTT test is a very sensitive assay of cellular injury.

INDUCTION OF CELLULAR INJURY WITH H₂O₂
The concentration dependence of H₂O₂ induced injury after 5, 10, 30, 60, and 120 minutes exposure to H₂O₂ was assessed by the MTT test (Fig 2). After exposure for five minutes, the minimum H₂O₂ concentration causing detectable injury was found to be 0.5 mM. The LD₅₀ for one hour exposure was about 0.1 mM. Exposure to H₂O₂ for one hour produced the widest range of injury and was chosen for subsequent experiments. We also assessed cell injury by measurement of lactate dehydrogenase release. No release was detectable after exposure of cells to 1 ml H₂O₂ for one hour. As the MTT test was more sensitive than lactate dehydrogenase release to oxidant induced injury in this cell type it was used for all subsequent experiments.

REVERSIBILITY OF CELLULAR INJURY INDUCED BY H₂O₂
To determine the reversibility of injury induced by H₂O₂, the MTT test was performed at intervals (0–6 hours) after exposure of cells to 0.1 or 0.5 mM H₂O₂ for one hour. As Figure 3A shows, only partial reversal of injury was found over the six hour period. To confirm this result cell numbers were measured by the crystal violet assay over a 72 hour period after one hour exposure to 0.1, 1.0, or 10 mM H₂O₂ (Fig 3B). Cellular proliferation was seen in control cells, but was abolished after exposure to H₂O₂. We conclude that even mild degrees of injury induced by H₂O₂ are not fully reversible.
was occurring, the decay of 1 mM H$_2$O$_2$ in the extracellular medium of cells at a density of 5x10$^4$/ml was measured over time and found to fall exponentially with a t$_{1/2}$ of about 35 minutes (Fig 4B). Breakdown of H$_2$O$_2$ was significantly faster in the presence of 1-5x10$^6$ cells than 5x10$^4$ cells. Thus the dependence of cellular injury on cell number is caused by increased H$_2$O$_2$ breakdown by cellular enzyme systems.

Enzyme inhibition studies were performed to assess the comparative importance of catalase and glutathione peroxidase in this cellular system. Aminotriazole (0.5 mg/ml), an inhibitor of catalase, 18 partially inhibited the breakdown of H$_2$O$_2$ (Fig 4B). Incubation of cells at a density of 5x10$^4$ cells/ml with 0-5 mg/ml aminotriazole increased cellular injury from exposure to 1 mM H$_2$O$_2$ for one hour confirming that catalase contributes to the defence of cells (Fig 4C). Complete inhibition of catalase was not possible as aminotriazole at concentrations higher than 0.5 mg/ml was found to be toxic (Fig 4C). Buthionine sulfoximine (0-5-2 mM), a specific inhibitor of de novo glutathione synthesis, 17 did not increase cellular injury after exposure of cells at 5x10$^4$ cells/ml to 1 mM H$_2$O$_2$ for one hour (surviving fraction for 0-5, 1-0, and 2-0 mM buthionine sulfoximine 0-26 (0-03), 0-29 (0-03), 0-26 (0-04), and 0-28 (0-03) respectively, there were no significant differences between these values). These concentrations of buthionine sulfoximine have been shown to substantially inhibit intracellular glutathione concentrations in HT-29 cells. 18 This suggests that glutathione peroxidase does not play an important part in protection of HT-29-18-C1 cells against H$_2$O$_2$ when H$_2$O$_2$ was used at these concentrations.

Determination of the oxidant responsible for cellular injury

Work with other cell types has shown that Fe$^{2+}$, Fe$^{3+}$, HO$_2^-$ and O$_2^-$ can play an important part in mediating the toxic effects of H$_2$O$_2$. 6, 7, 19 Therefore the effect of iron chelating agents in protecting against injury from exposure to 1 mM H$_2$O$_2$ for one hour was tested. Coincubation with 1,10-phenanthroline (1-0-33 $\mu$M), a chelator of Fe$^{2+}$, protected the cells in a concentration dependent manner from injury caused by H$_2$O$_2$ (Fig 5A). Preincubation with 1,10-phenanthroline was not tested as prolonged exposure was found to be toxic. By contrast, coincubation of 1 mM deferoxamine, a specific chelator of Fe$^{3+}$, did not protect the cells against injury 8 (Fig 5B). Preincubation with deferoxamine for 18 hours before exposure to H$_2$O$_2$ however, resulted in a concentration dependent protection against H$_2$O$_2$ induced injury (Fig 5C). The superoxide radical did not seem to play an important part in H$_2$O$_2$ cytotoxicity as 100 U/ml superoxide dismutase did not protect against injury from 1 mM H$_2$O$_2$ (Fig 5D). Additional experiments using superoxide dismutase at a concentration of 250 U/ml failed to protect against H$_2$O$_2$ induced injury (data not shown). Neither did

**Figure 3:** (A) Reversibility of cellular injury induced by H$_2$O$_2$, HT-29-18-C1 cells at a density of 5x10$^4$/ml were incubated in Na$^+$ medium with either 0-1 mM (open circles) or 0-5 mM (closed circles) H$_2$O$_2$ for one hour. The percentage cell survival was determined by the MTT test performed 0, 1, 2, 4, and 6 hours after H$_2$O$_2$ exposure. Each data point is the mean (SD) of eight replicates. At each H$_2$O$_2$ concentration the surviving percentage was significantly higher at time points 1, 2, 4, and 6 hours than at time zero, p<0.02.

(B) Cell counts after exposure to H$_2$O$_2$. HT-29-18-C1 cells at a density of 5x10$^4$/ml were incubated in Na$^+$ medium alone (open circles) or with 0-1 mM (closed circles), 1 mM (closed triangles) or 10 mM H$_2$O$_2$ (open triangles) for one hour. Cell counts were performed with the crystal violet test at zero, 24, 48, and 72 hours after exposure. Data points are the mean (SD) of eight replicates.

**Effect of catalase and glutathione peroxidase on degree of H$_2$O$_2$ induced injury**

The degree of injury sustained by cells after exposure was found to be dependent on the number of cells incubated. Cellular injury, as determined by the MTT test, after one hour exposure to 1 mM H$_2$O$_2$ was greater when cells were seeded at a density of 5x10$^4$ cells/ml than 1-5x10$^6$ cells/ml (Fig 4A). To find out if breakdown of H$_2$O$_2$ in the external medium
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**Discussion**

While the effects of oxidant injury have been studied extensively in hepatocytes and endothelial cells, intestinal epithelial cells have received much less attention. The sensitivity to ROMs has been studied in cultured gastric cells and a small intestinal cell line but not in epithelial cells of colonic origin. Electrophysiological responses of colonic epithelium to ROMs has been reported but little attention was paid to the degree of cellular injury. The objective of this study was to establish a model of oxidant injury in intestinal epithelial cells as a preliminary to studying cellular protective mechanisms against oxidative stress. We have developed this model in HT-29-18-C1 cells, using H₂O₂ as the source of oxidative stress and evaluated the degree of injury using the MTT test.

The MTT test measures cell integrity and cell number. It differs from many other assays of cell viability such as ⁵¹Cr release from prelabelled cells, lactate dehydrogenase release, and cellular trypan blue staining because it is not an assay of plasma membrane integrity. Instead, the MTT test depends on dehydrogenase activity in intact mitochondria, which converts a tetrachloride salt to a coloured formazan product. It is convenient for use with cultured cells as the coloured formazan product can be measured by spectrophotometric scanning of large samples of multwells. Tests of mitochondrial function are sensitive indicators of oxidant injury, as loss of mitochondrial function is an early event in oxidant and hypoxic injury whereas loss of plasma membrane integrity occurs later. The sensitivity of the MTT test was confirmed by the finding that it has a similar sensitivity to H₂O₂ induced injury as loss of intracellular ATP (Fig 1B). This is to be expected as one of the mechanisms of decrease of intracellular ATP during oxidative injury is de-energisation of mitochondria by peroxidation of their lipid membranes. Loss of ATP may also occur by other mechanisms, including activation of poly-ADP-ribose polymerase by DNA single strand breaks and inhibition of glyceroldehyde-3-phosphate dehydrogenase, which may explain why ATP loss does not correlate precisely with the MTT test through the range of H₂O₂ concentrations used. In our hands, lactate dehydrogenase release proved to be an insensitive assay of injury to HT-29-18-C1 cells. This may partly be because the sensitivity of HT-29-18-C1 plasma membrane to oxidant induced lysis is low compared with reduction of mitochondrial dehydrogenase activity.

As noted with other cell types, H₂O₂ is degraded by HT-29-18-C1 cells. This could reflect the presence of catalase (which catalyses the disproportionation of H₂O₂ to water) or of glutathione peroxidase (which catalyses the reduction of H₂O₂ by glutathione). Both enzymes have been shown previously in HT-29 cells. The protective effect of catalase against H₂O₂ induced injury of HT-29-18-C1 cells was shown by the increase in H₂O₂ induced injury that occurred when intracellular catalase was partially inhibited by aminotriazole. Aminotriazole decreases catalase activity by forming a high affinity complex with compound I (catalase plus H₂O₂). Aminotriazole also reacts with tryptophan moieties, however, which may explain its toxicity in concentrations above 0.5 mg/ml. By contrast, reduction of...
in intracellular glutathione with buthionine sulphoximine at concentrations (0.5–2 mM) known to cause greater than 90% depletion of glutathione in HT-29 cells did not increase the sensitivity of the cells to H₂O₂ induced injury. This is consistent with the previous finding that supplementation of HT-29 cells with glutathione does not protect them against H₂O₂ injury. The dominance of catalase over glutathione peroxidases as the protective enzyme against injury from H₂O₂ at these concentrations may reflect the different kinetic properties of the two enzymes. Glutathione peroxidase approaches its \( V_{\text{max}} \) at 100 \( \mu \)M H₂O₂, while catalase activity, being a first order process, will reduce the same fraction of H₂O₂ regardless of concentration. Enzyme mediated breakdown of H₂O₂ makes comparison of the sensitivity of different cell types to oxidant injury difficult, as the degree of injury will not only depend on the concentration of oxidant added but also on its rate of consumption. Nevertheless the sensitivity of HT-29-18-C1 cells to H₂O₂ is of the same order as that reported previously for HT-29 cells and other cell systems. The H₂O₂ concentration in inflamed tissue is of the order 0.1–0.5 mM. In a variety of cell types H₂O₂ not only damages cells directly but can also act as a substrate for the generation of the highly toxic \( \text{OH}^{-} \) by via the Fenton reaction as follows:

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^{-} + \text{OH}^-
\]

Our results suggest that such a system exists in HT-29-18-C1 cells, as the \( \text{Fe}^{2+} \) chelator 1,10-phenanthroline provided a significant degree of protection against H₂O₂ induced injury as previously described (Fig 5A). Protection by deferoxamine against H₂O₂ induced cell pulmonary artery endothelial cells has also been described. Iron is stored in cells as \( \text{Fe}^{3+} \) and must be reduced to provide \( \text{Fe}^{2+} \) for the Fenton reaction. Our finding that preincubation with deferoxamine protected the cells against H₂O₂ induced injury supports this notion and suggests that intracellular \( \text{Fe}^{3+} \) is important in mediating H₂O₂ toxicity (Fig 5C). Cellular uptake of deferoxamine and depletion of intracellular \( \text{Fe}^{3+} \) is slow, which may explain why simultaneous exposure of cells to deferoxamine and H₂O₂ did not prevent cell injury (Fig 5B). It is unlikely that 1,10-phenanthroline and deferoxamine acted by mechanisms other than chelation of iron. These agents cannot protect cells against cytotoxic agents, which act by mechanisms other than those entailing oxidants, and have no effect on cellular catalase or glutathione peroxidase.

Iron catalysed generation of \( \text{OH}^{-} \) by the Fenton reaction does not occur without previous reduction of \( \text{Fe}^{3+} \) to \( \text{Fe}^{2+} \). The identity of the reductant responsible is not clear. H₂O₂ is unlikely to be responsible because its reduction of \( \text{Fe}^{3+} \) is very slow at physiological pH. In a number of cell types \( O_2 \) has been shown to reduce \( \text{Fe}^{3+} \) by the following reaction and protect against H₂O₂-induced injury:

\[
\text{Fe}^{3+} + \text{O}_2^{-} \cdot \text{Fe}^{2+} + \text{O}_2
\]

This is unlikely to be occurring in our model as superoxide dismutase at high concentration did not protect HT-29-18-C1 cells against H₂O₂ induced injury. Of interest, superoxide dismutase (250 U/ml) also fails to protect cultured gastric cells, endothelial cells, fibroblasts, Staphylococcus aureus, and GPK cells against H₂O₂ induced injury. We cannot exclude the possibility, however, that the lack of effect of superoxide dismutase.
may reflect a failure of its endocytosis, which is required before it can protect against H$_2$O$_2$ induced injury.\textsuperscript{34} We assume there must be other reductants such as ascorbate or dithionate whose identity remains to be determined.\textsuperscript{29}

In view of the data presented in favour of iron catalysed generation of OH$,^\cdot$ it is of interest that a range of hydroxyl radical scavengers did not protect the cells against H$_2$O$_2$ mediated injury as reported in hepatocytes.\textsuperscript{19} Similar negative results of hydroxyl radical scavengers have also been obtained in endothelial cells, fibroblasts, and GPK cells.\textsuperscript{31 33} Although mannitol does not readily cross the plasma membrane, adequate entry to the cell interior would be expected of α-tocopherol, sodium benzoate, glucose, ethanol, and dimethylsulphoxide. It is known that OH$,^\cdot$ is so reactive that damage is restricted to Fe$^{2+}$ sites.\textsuperscript{6} The lack of effect of hydroxyl radical scavengers may reflect a failure to achieve an adequate concentration at the sites of Fe$^{2+}$ to compete effectively for OH$,^\cdot$ despite entry of the scavengers to intracellular compartments. It has also been suggested that cell injury may not be caused by OH$,^\cdot$ but by the highly reactive Fe$^{3+}$ or by ferryl species produced by Fenton reactions.\textsuperscript{35} The reason why 3-3 U/mL and 10 U/mL catalase increased cell survival compared with controls is not known. Although cellular generation of H$_2$O$_2$ de novo could not be detected, this possibility cannot be excluded as cellular catalase and glutathione peroxidase may have prevented its measurement in the incubating medium. Overall, these data suggest that both H$_2$O$_2$ and OH$,^\cdot$ may mediate H$_2$O$_2$ induced cytotoxicity in HT-29-18-C1 cells.

In summary, H$_2$O$_2$ causes injury to HT-29-18-C1 cells, which is essentially irreversible as judged by the MTT test and cellular proliferation. The injury is mediated by both the direct action of H$_2$O$_2$ and by the generation of OH$,^\cdot$ by the iron mediated Fenton reaction. This model should prove useful in the study of the mechanisms of oxidant injury in intestinal epithelial cells.

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