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## *Critical Review*

# Hydrogen Peroxide. Ubiquitous in Cell Culture and In vivo?

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***Summary***

**Hydrogen peroxide ($H_2O_2$) is widely regarded as a cytotoxic agent whose levels must be minimized by the action of antioxidant defence enzymes. In fact, $H_2O_2$ is poorly reactive in the absence of transition metal ions. Exposure of certain human tissues to $H_2O_2$ may be greater than is commonly supposed; levels of $H_2O_2$ in the human body may be controlled not only by catabolism but also by excretion, and $H_2O_2$ could play a role in the regulation of renal function and as an antibacterial agent in the urine.**

**Cell culture is a widely used method for the investigation of "physiological" processes such as signal transduction and regulation of gene expression, but chemical reactions involving cell culture media are rarely considered. Addition of reducing agents to commonly used cell-culture media can lead to generation of substantial amounts of $H_2O_2$. Some or all of the reported effects of ascorbic acid and polyphenolic compounds (e.g., quercetin, catechin, epigallocatechin, epigallocatechin gallate) on cells in culture may be due to $H_2O_2$ generation by interaction of these compounds with cell culture media.**

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**Keywords** Ascorbate; breath; catalase; catechins; cell culture; epigallocatechin; epigallocatechin gallate; FOX assay; hydrogen peroxide; hydroxyl radical; oxygen electrode; quercetin; urine.

## INTRODUCTION

Hydrogen peroxide ( $H_2O_2$) is a pale-blue covalently bonded liquid, freely miscible with water and apparently able to cross cell membranes readily (*1*). High (usually $\geq 50\,\mu M$) levels of $H_2O_2$ are cytotoxic to a wide range of animal, plant and bacterial cells, although $LD_{50}$ values and the mode of cell death depend on multiple parameters, including the cell type used, its iron content, length of exposure to $H_2O_2$, the $H_2O_2$ concentration used, and the cell culture media employed (*1–5*). It is therefore widely thought that $H_2O_2$ is toxic in vivo and must be rapidly eliminated, employing enzymes such as catalases, glutathione peroxidases and thioredoxin-linked systems (*1*, *6–8*). Paradoxically, however, acatalasaemia (*1*, *9*) or deletion of glutathione peroxidase appear to produce no significant phenotype, except perhaps under high oxidative stress conditions (*9–13*).

In chemical terms, $H_2O_2$ is poorly reactive: it does not oxidize most biological molecules including lipids, DNA, and proteins (unless the latter have exposed, or hyperreactive, thiol groups or methionine residues (*1*, *3*, *14*)). The danger of $H_2O_2$ largely relates to its ease of conversion to the indiscriminately reactive hydroxyl radical ($OH^\bullet$), either by exposure to ultraviolet light (*15*)

$$H_2O_2 \xrightarrow{uv} 2OH^\bullet$$

or by interaction with transition metal ions, of which the most important in vivo is probably iron (*1*, *16*)

$$Fe^{2+} + H_2O_2 \longrightarrow \text{intermediate complexes (ferryl??)} \longrightarrow Fe^{3+} + OH^\bullet + OH^-$$

Living organisms have evolved mechanisms to sequester transition metal ions into forms non-catalytic for $OH^\bullet$ generation or other free radical reactions in vivo (*1*, *16*, *17*). Nevertheless, millimolar levels of $H_2O_2$ can contribute to Fenton chemistry by not only providing one of the substrates (i.e., itself) but also by liberating the other, e.g., releasing iron from haem proteins (*1*, *16–19*). Release of iron by this and other mechanisms explains why addition of $H_2O_2$ to cells in culture can lead to

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$OH^{\bullet}$-mediated oxidative DNA damage (*1*), although such damage is rapidly repaired provided that the cells are not rendered nonviable by the $H_2O_2$ (20). However, levels of $H_2O_2$ at or below about 20 $\mu$M exert only limited damaging effects on most cells. Indeed, there is evidence that $H_2O_2$ is used as an inter- and intracellular signalling molecule (*21–26*), e.g., in the activation of NF$\kappa$B in some (*23*), but not all (*27*), cell types. Hence its use in physiological signalling mechanisms may be a good reason not to eliminate all the $H_2O_2$ generated in vivo (*3*, *4*, *21–26*, *28–33*). Indeed, we have recently argued that the exposure of human tissues to $H_2O_2$ may be greater than is commonly supposed, and that excretion of $H_2O_2$ in urine and exhaled air may contribute to regulation of its levels in vivo (*34*).

## EXPOSURE OF HUMAN TISSUES TO $H_2O_2$

Hydrogen peroxide is generated in vivo by the dismutation of superoxide radical ($O_2^{\bullet-}$), both nonenzymically and catalysed by superoxide dismutase enzymes. Hydrogen peroxide is also directly produced by a range of oxidase enzymes including glycollate and monoamine oxidases as well as by the peroxisomal pathway for $\beta$-oxidation of fatty acids (*1*, *6*, *35*). It thus seems likely that most or all human cells are exposed to some level of $H_2O_2$. However, some tissues may be exposed to higher $H_2O_2$ concentrations (*34*).

### *The Gastrointestinal Tract*

Many beverages commonly drunk by humans contain $H_2O_2$ at concentrations above 100 $\mu$M, including teas and instant coffee (*36–38*). For example, Figure 1 shows the high levels of $H_2O_2$ that can be present within beverages prepared as one would normally prepare them for drinking, and kept at 90 °C. When such beverages are ingested, this $H_2O_2$ presumably rapidly diffuses into the cells of the oral cavity and upper part of the gastrointestinal tract (39). Oral bacteria are also capable of producing $H_2O_2$ (*40*, *41*), although the impact of this on oral tissues is uncertain.

### *The Respiratory Tract*

The cells lining the respiratory system, like the oral and oesophageal epithelia, are exposed to 21% $O_2$, hyperoxia as compared with most other body tissues (*1*). Hydrogen peroxide is present in exhaled air even in healthy subjects, although amounts are greater in patients with inflammatory lung diseases and in cigarette smokers (*42–50*). Exhalation of $H_2O_2$ might represent an excretory mechanism (*34*).

### *The Urogenital System*

Substantial concentrations of $H_2O_2$, sometimes exceeding 100 $\mu$M, can be detected in freshly voided human urine (Table 1) (*51–53*), even in babies (*54*). The simplest way of demonstrating its presence is to place urine into an oxygen electrode, and inject catalase through the cap. A "spike" of $O_2$ release results as the $H_2O_2$ present is decomposed by catalase (Fig. 2).

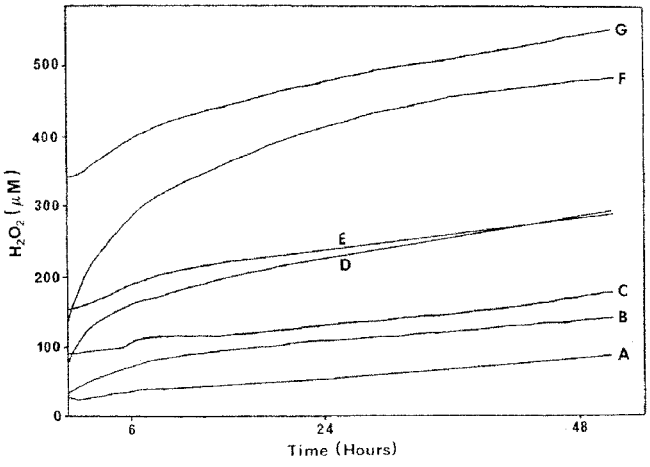


**Figure 1.** Generation of $H_2O_2$ within commonly drunk beverages. (A) Cocoa powder; (B) Oolong tea; (C) Green tea; (D) Black tea; (E) Ground coffee; (F) Tea granules; (G) Instant coffee. 0.25 g of the dried powders/leaves were weighed and 25 ml of deionised water (90 °C) was added, followed by stirring on a hot-plate to maintain the temperature for 15 min. Samples were then taken for analysis: this was taken as zero time. At every 30 min (up to 6 h), and at 24 and 48 h, 330 $\mu$l samples were taken and made up to 1.0 ml with deionised water. Ninety $\mu$l was taken to determine the amount of $H_2O_2$ generated. Data selected from (*36*).

The $H_2O_2$ detected in human urine appears to arise, at least in part, by $O_2^{\bullet-}$-dependent autoxidation of urinary molecules, some of which originate from diet (*38*, *53*, *55*). Traces of superoxide dismutase are present in urine (*56*): this enzyme, as well as the acidic pH of urine, should facilitate dismutation of $O_2^{\bullet-}$ to $H_2O_2$ (*1*). The $pO_2$ of urine within the bladder is below that of ambient air (*57*, *58*) and so the rate of $H_2O_2$ generation in urine may well increase upon voiding. Nevertheless, the high levels of $H_2O_2$ that are detected in some urine samples (Table 1) strongly suggest that at least some $H_2O_2$ generation occurs within the bladder. Indeed, $H_2O_2$ has been detected in urine sampled by catheterisation (*59*). Since $H_2O_2$ has an antibacterial effect (*1*, *2*, *60*), we have argued that its presence at high levels in urine could be advantageous in diminishing infections of the bladder and urinary tract (*34*). $H_2O_2$ might also modulate renal function (*34*, *61–63*). The urinary excretion of $H_2O_2$ may represent a mechanism for controlling its levels in the human body. If so, measurement of urinary $H_2O_2$ levels may represent a valuable tool for assessment of "oxidative stress," because $H_2O_2$ can be measured rapidly and simply (*53*, *55*).

### *Vascular Endothelial and Circulating Blood Cells*

Some studies have claimed substantial levels of $H_2O_2$ (up to ~35 $\mu$M) in human blood plasma (*64–66*), but others have claimed levels to be at or close to zero (*67*). The latter data are more consistent with the observation that $H_2O_2$ added to human plasma disappears rapidly (*68*). In part, it is degraded by the

**Table 1**
Levels of Hydrogen Peroxide in freshly voided Human Urine

| Gender of subject | Age (Years) | $H_2O_2$] in urine ($\mu$M) |
|---|---|---|
| Male | 20 | 26.5 |
| Male | 21 | 16.3 |
| Male | 23 | 5.2 |
| Male | 26 | 5.9 |
| Male | 28 | 18.9 |
| Male | 30 | 22.3 |
| Male | 34 | 11.0 |
| Male | 49 | 109.6 |
| Female | 18 | 5.0 |
| Female | 19 | 8.0 |
| Female | 19 | 0.4 |
| Female | 21 | 6.2 |
| Female | 22 | 7.7 |
| Female | 25 | 11.5 |
| Female | 27 | 13.0 |
| Female | 35 | 3.5 |

*Note*: Spot urine samples were collected from healthy human volunteers and assayed immediately. Subjects undertook no special dietary or other preparation before providing samples. Data are means of triplicate determinations on each sample; replicates varied by <5%. $H_2O_2$ was analysed by the ferrous ion oxidation-xylenol orange (FOX) assay. Data abstracted from (*34*, *53*).

traces of catalase present, but $H_2O_2$ can also react with ascorbate and protein-SH groups (68). In vivo, $H_2O_2$ generated in plasma could also diffuse into erythrocytes, platelets, white cells, and vascular endothelial cells for catabolism. However, the studies in (*64–66*) may be explained by proposing that human blood plasma is continuously generating $H_2O_2$ (*34*). In vivo, this $H_2O_2$ is rapidly removed but if plasma is assayed under conditions where the $H_2O_2$ is no longer removed, it will accumulate (*34*). One enzyme likely to be involved in plasma $H_2O_2$ generation, at least under pathological conditions, is xanthine oxidase (*69*). Levels of circulating and endothelium-bound xanthine oxidase are increased as a result of tissue injury (*70*, *71*).

### *The Eye*

The presence of $H_2O_2$, at widely varying levels (in some cases, 100 $\mu$M or more), has been reported in aqueous and vitreous humors (*72–75*). The explanation might be essentially the same as that advanced to account for the conflicting data reported for blood plasma, i.e., that ocular fluids constantly generate $H_2O_2$, which is usually rapidly removed (*72*). Any impairment in the capacity of the lens epithelium, retina or other ocular tissues to dispose of $H_2O_2$ would then result in its accumulation. The origin of ocular $H_2O_2$ is uncertain, but oxidation of ascorbate may contribute (*73*).

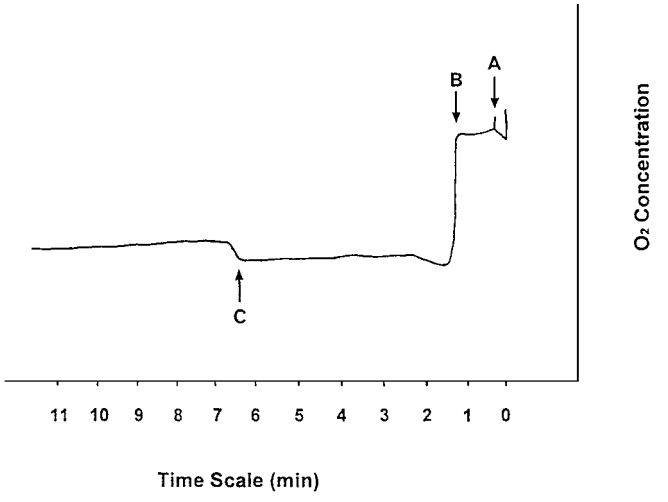


**Figure 2.** A sample of freshly-voided human urine was placed in the chamber of an oxygen electrode. At point A, buffer (phosphate-buffered saline PBS] pH 7.4) was added and at point B, urine was added. At point C, 10 $\mu$l of catalase dissolved in PBS ($10^3$ units of catalase) was injected through the cap. Note the immediate burst of $O_2$ evolution. Adapted from (*53*).

## ARTEFACTS OF CELL CULTURE: GENERATION OF $H_2O_2$ IN CELL CULTURE MEDIA

Cell culture is a universally used technique for examination of "physiological" and pathological phenomena and elucidation of signal transduction, regulation of gene expression, cell proliferation, and cell death mechanisms. Yet, most cells in culture exposed to 95% air/5% $CO_2$ (or even to pure $O_2$ in some studies) are essentially in a hyperoxic environment compared to the in vivo situation (*1*). Cell culture media are frequently deficient in antioxidants (e.g., ascorbate and $\alpha$-tocopherol) or antioxidant precursors (e.g., selenium) (*76*, *77*). The cells that can tolerate, or can adapt to, such conditions to allow them to grow and proliferate may behave very differently from cells in vivo.

Another important point is that agents added to cells in culture can undergo chemical reactions with constituents of cell culture media, some of which lead to $H_2O_2$ generation. For example, several phenolic compounds, including L-DOPA, dopamine, epigallocatechin, catechin, quercetin, and epigallocatechin gallate, can interact with commonly used cell culture media (e.g., Dulbecco's modified Eagles' medium DMEM], RPMI, and McCoy's 5A medium) to generate significant levels of $H_2O_2$ (*78 and our unpublished data*). Levels of over 50 $\mu$M can be achieved with some of these compounds (Fig. 3). Such $H_2O_2$ generation could account for some (or perhaps even all) of the reported effects of these phenolic compounds on cells in culture (*78*).

Ascorbate (vitamin C) also oxidizes easily in certain cell culture media to generate $H_2O_2$ (*79–84*). For example, Figure 4 shows that ascorbate-induced apoptosis in HL-60 cells was significantly greater in DME medium than in RPMI medium, correlating with the increased rates of $H_2O_2$ generation on addition

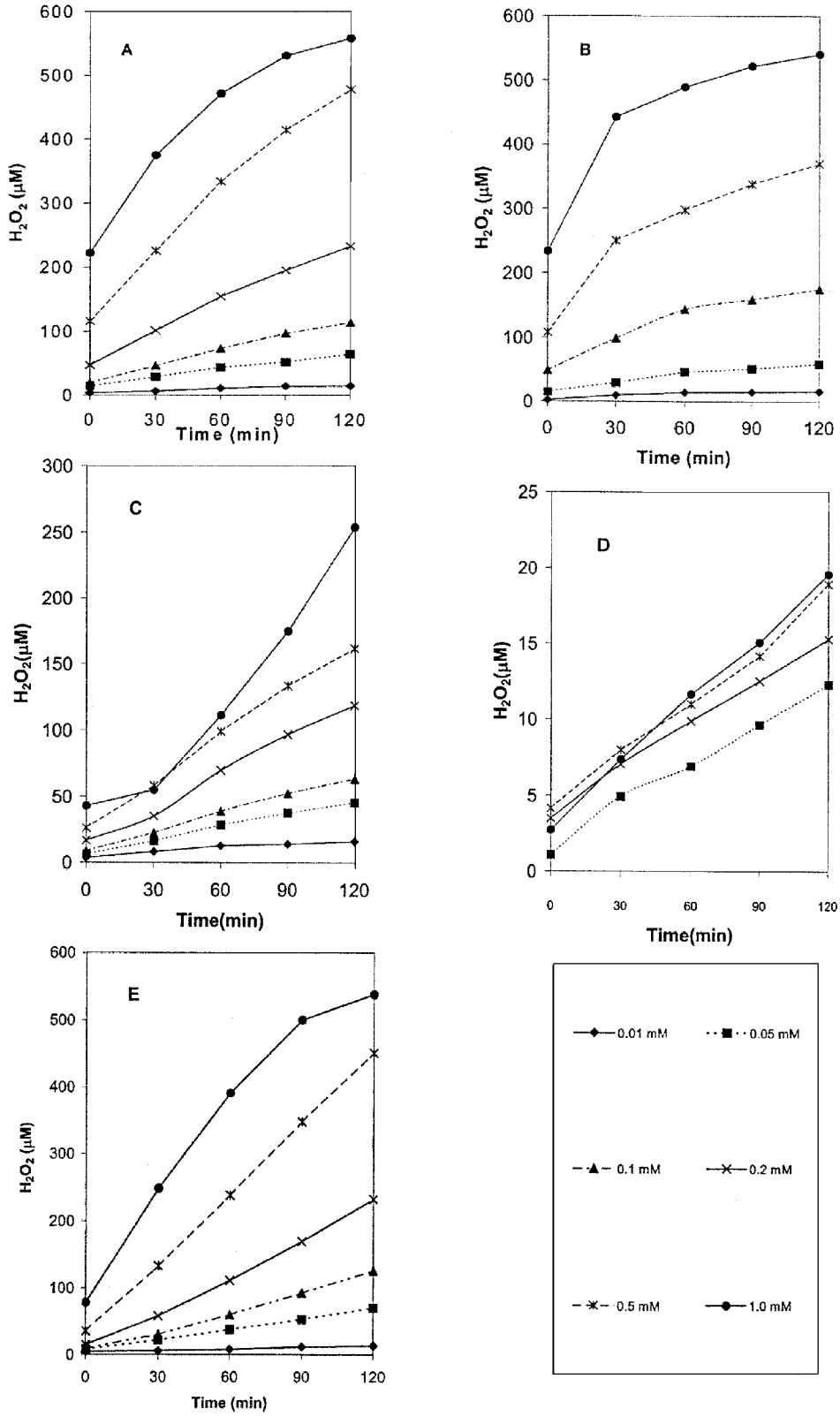


**Figure 3.** Generation of $H_2O_2$ on addition of (A) epigallocatechin gallate, (B) epigallocatechin, (C) quercetin, (D) catechin, or (E) gallic acid to Dulbecco's modified Eagles medium. Concentrations stated are the final concentrations in the medium. Data selected from (*78*).

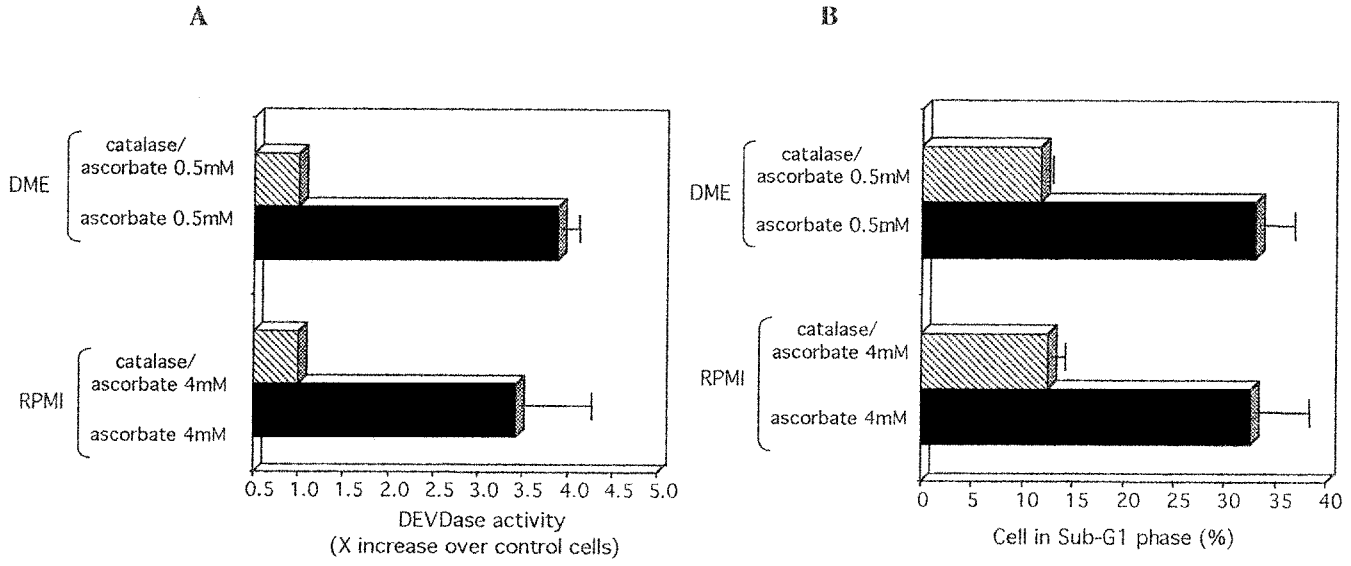


**Figure 4.** Induction of apoptosis measured by caspase 3 activation (A) or propidium iodide (B)] in HL-60 cells by ascorbate is $H_2O_2$-dependent. Ascorbate in Dulbecco's modified Eagle's medium induces apoptosis much more effectively than in RPMI medium (0.5 mM in DME but 4 mM in RPMI are required to give similar effects) correlating with rates of $H_2O_2$ production on addition of ascorbate to DMEM or RPMI media (*79*). Data are mean ± SD of ≥3 independent experiments and are selected from (*79*).

of ascorbate to DMEM. Apoptosis was suppressed by catalase, confirming a key role for $H_2O_2$. In other words, the cytotoxic effects of ascorbate on these cells in culture appeared entirely due to the generation of $H_2O_2$ in the culture media (*79*).

## CONCLUSIONS

Hydrogen peroxide appears to be a ubiquitous molecule. We exhale it, excrete it, and take it in from diet. The data reviewed in this paper re-emphasize the importance of metal ion sequestration in preventing the toxicity of $H_2O_2$ in vivo by decreasing the occurrence of Fenton chemistry, and help explain why a failure of such sequestration can cause severe tissue damage (*1*, *16*). They also underline the fact that increased attention must be paid to $H_2O_2$-generating chemical reactions in commonly used cell culture media.

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