Cell Culture, Oxidative Stress, and Antioxidants: Avoiding Pitfalls

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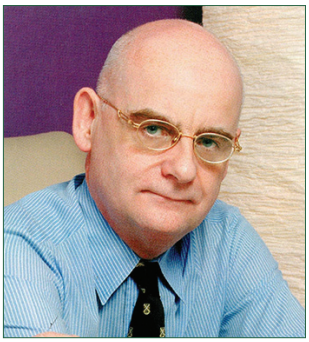
Cell culture is widely used by biochemists and cell/molecular biologists, but the fluctuating (and often elevated) levels of O_2 to which cells in culture are exposed can affect many of their properties. So can the low level of antioxidants found in some cell culture media. Reagents, especially "antioxidants," added to cell culture media can react with the constituents of the media to produce H_2O_2 and degradation products that can influence cell behavior. Several published papers describing the cellular effects of ascorbate, polyphenols, and carotenoids have, in fact, reported artifacts due to the actions of the degradation products of these "antioxidants." A greater awareness of the potential artifacts in cell culture studies is needed among the free radical/antioxidant community. *(Biomed J 2014;37:99-105)*



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This review is an update and expansion of my article[1] in 2003, entitled "Oxidative stress in cell culture: An under-appreciated problem." Over 10 years later, the problems are still under-appreciated and artifactual results are still being published, hence the need for another commentary on the topic.

Cell culture is used by researchers worldwide to elucidate metabolic pathways and to discover the mechanisms involved in cell signaling, regulation of gene expression and protein synthesis, cell proliferation, senescence, and cell death. Cell culture studies have provided a huge amount of valuable information, including helping to elucidate important physiological roles of mitogen-activated protein (MAP) kinases, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), activator protein-1 (AP-1), nuclear transcription factor erythroid 2p45-related factor-2 (Nrf2), nitric oxide, and caspases, among many other agents. There is also growing interest in using reprogrammed stem cells from patients to study the mechanisms of disease, e.g. in amyotrophic lateral sclerosis.[2]

However, it must be realized that cells in culture are different from those *in vivo* in many ways. The normal cell matrix (which has important influences on cell morphology and function) is absent, as are other cell types that normally surround the cells in question and communicate with them. For example, isolated neurons are very vulnerable to damage, but *in vivo* glia help to regulate their metabolism and protect them against damage.[3] A huge range of agents, from cytotoxic drugs to dietary antioxidants, show remarkable effects when added to cells in culture, yet many are a disappointment when tried *in vivo*.[1] Rapid metabolism and limited bioavailability of agents tested *in vivo* are obvious explanations for this discrepancy. However, cell culture imposes an oxidative stress that may, under certain circumstances, lead to misleading conclusions.[1] Another important factor is misidentification of cell lines (the cell is not what it is supposed to be).[4-9] Even when the cell line is correct, it may have a genotype (e.g. *apoE*) not reflective of the *in vivo* status.[10] Genetic drift is frequent; mutations occur rapidly in cell culture,[11] as exemplified by studies on HeLa cells.[12] Indeed, many cell culture studies are carried out using malignant cell lines such as HeLa because such cells are robust and grow and divide easily in culture. This makes sense when looking for chemotherapeutic agents, but perhaps less sense when trying to elucidate normal metabolic or signaling pathways.

An alternative is primary culture, where cells are harvested from a tissue and plated. Some of them will survive, but many die because of the stress of the isolation procedure and the "foreign environment" of the culture conditions. This is also true of many cancer cells; they often need support from the surrounding stroma *in vivo*

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to survive and only those that can adapt to the loss of this, and to other aspects of cell culture conditions, will proliferate.[13] Values of 1-10% have been quoted for the number of originally harvested cells from a tissue that survive.[14] The words "culture shock" have been used to describe this phenomenon.[1,14] To take examples, isolation of rat hepatocytes can lead to activation of nitric oxide synthase within them, generating levels of nitric oxide ($NO^{\bullet}$) sufficient to alter cell metabolism.[15] Treatment of cells with trypsin can cause a decrease in the cellular levels of reduced glutathione (GSH).[16,17] The cells which survive the culture shock appear to be those that have adapted rapidly, with multiple changes in gene expression, metabolic activity, and the levels of many enzymes.[13,17-19] Some enzyme levels are increased, other swiftly decreased.[19] Thus, only a fraction, and probably an unrepresentative fraction, of the initially plated cells survives. As an example, p53 is not expressed in 14-day mouse embryos, but in culture of fibroblasts from them, the cells that survived were those that had begun to express it.[20] Indeed, cell cultures often show abnormal p53 activity.[21] Cells may change their properties, e.g. glial cells can be activated during the processing of brain tissue, which is required for their isolation.[22]

The oxidative stress of cell culture

Culture shock affects cells in multiple ways, one key action being to impose oxidative stress. The term "oxidative stress" refers to a serious imbalance between the levels of reactive oxygen species (ROS) in a cell and its antioxidant defenses in favor of the ROS.[23,24] Cell culture causes oxidative stress for two reasons: (A) It leads to more ROS generation and (B) it can impair cellular antioxidant defenses. Let us examine how these events happen.

More ROS generation

Most cells *in vivo* in animals are exposed to low O_2 concentrations, in the range of 1-10 mm Hg, although there are obvious exceptions (including skin epidermis, cornea of the eye, and the cells lining the respiratory tract).[1,25-27] Cell culture is commonly performed under 95% air/5% CO_2, an O_2 tension of approximately 150 mm Hg. Since the rates of production of ROS [superoxide $\left(O_2^{\bullet-}\right)$ and hydrogen peroxide (H_2O_2)] by cellular enzyme systems and by "leakage" of electrons from electron transport chains are limited at normal cellular levels, elevated O_2 will thus increase ROS production.[1,25-27] Therefore, more ROS will be produced in cells in culture. Depending on the magnitude and time course, increased ROS production might cause increased cell proliferation, halt proliferation, or lead to senescence, cell death, or adaptation.[24,28] Even in the cells that adapt and proliferate, their properties are likely to be altered (e.g. changes in mitochondrial morphology and function).[29] One important example of this was revealed by attempts to determine the "Hayflick limit," the number of times that cells can divide in culture before they undergo senescence.[14] For human fibroblasts, a value of 40-50 doublings was originally suggested. A study by Shay *et al*.[14] revealed that if cells are grown at low O_2, many more doublings are possible. The original Hayflick limits were artifactually low, due in part to culture-related oxidative stress causing accelerated telomere shortening.[14,30,31] Cells whose properties are very different when grown under physiological O_2 levels as opposed to 21% O_2 include chondrocytes,[32] hepatocytes,[33] and several malignant cell types, especially as many tumor cells experience hypoxia *in vivo*.[13,34,35] Another problem is that the O_2 levels in cell culture can fluctuate wildly, being high when the cells are first placed in air-saturated culture medium and dropping as the cells grow, proliferate, and consume O_2. Often an O_2 gradient is established between the surface of the medium and the cells beneath. When the medium is changed, cells can therefore switch from relative hypoxia to hyperoxia and suffer the equivalent of a hypoxia-reperfusion injury.[24] The density of the cells under study is also important to consider; the denser they are, the faster the O_2 will be consumed, and products such as H_2O_2 can more readily diffuse into adjacent cells for metabolism.

How can cells adapt to the oxidative stress of cell culture? This might involve, in principle, increased levels of antioxidant defenses, down-regulating the production of ROS, such as decreasing the levels of or adapting mitochondrial or endoplasmic reticulum electron transport chains to produce less $O_2^{\bullet-}$. An additional strategy is to change cellular targets of oxidative damage so that they become more resistant to damage by ROS.[24,25,36-38] All these adaptations can be facilitated by an ROS-dependent increased frequency of mutations. Further evolution might then take place; a cell that has adapted to cope with the oxidative stress of cell culture might further evolve to use ROS. For example, some malignant cells in culture appear to have "learned" to use ROS to promote proliferation and suppress apoptosis.[39,40] Non-malignant cells may evolve similarly; because more ROS are produced under the pro-oxidant environment of cell culture, cells might adapt to utilize ROS-dependent signal transduction pathways that rarely, if ever, operate *in vivo* in healthy tissues.[41,42] It is not impossible that some of the widely investigated ROS-dependent signaling pathways used by cells in culture to respond to cytokines, growth factors, and other stimuli are not physiological.[24,41,42]

The culture process also alters cell metabolism. For example, Chinese Hamster Ovary (CHO) cells in culture metabolize abnormally large amounts of glucose via the pentose phosphate pathway. They also increase oxaloacetate production, both events seemingly as an adaptation to the oxidative stress of cell culture.[43] Indeed, cellular production of pyruvate was suggested many years ago as a similar adaptation,[44] since pyruvate, oxaloacetate, and α-ketoglutarate can scavenge H_2O_2 readily in cell culture media.[44-49]

Hyperglycemia

In the human body, cells are normally exposed to a glucose level of around 4-5 mM, although of course this may rise shortly after a meal and then fall again. By contrast, the concentrations of glucose in various cell culture media are highly variable. Several media use 5.5 mM levels, which are approximately physiological. Yet others have levels of 11 to as high as 55 mM. High glucose for prolonged periods increases mitochondrial $O_2^{\bullet-}$ generation in cells, leading to severe perturbation of metabolism and alterations of cell function.[50,51] High glucose also causes glycation and glycoxidation of proteins, with the potential to impair cell metabolism (analogous to the sequelae of diabetes in humans).

Fewer antioxidants

Cell culture media are frequently deficient in the antioxidants that are normally obtained in the human diet and delivered to cells by the bloodstream. This is especially true of tocopherols and ascorbate. Vitamin E is rarely added to cell culture media because it is insoluble in water (some amount will be provided in calf serum, although the exact levels are seldom measured) and vitamin C is not added because it is unstable. Hence, cells in culture can be deficient in important antioxidants, a situation which can lead to overinterpretation of the actions of added antioxidants.[1,24,52,53] Antioxidants often appear to have beneficial effects when added to cultured cells, but this can be because a severe deficiency is being corrected, rather than being a real beneficial effect of "extra antioxidants." Culture media can be deficient in selenium;[52,54,55] this deficiency may decrease (or at least prevent oxidative stress–triggered increases in) the activities of selenium-dependent antioxidant systems. These include thioredoxin reductase, certain methionine sulfoxide reductases, and most members of the glutathione peroxidase enzyme family.[24,52] If thioredoxin reductase activity is decreased, cell metabolism and antioxidant defenses will be seriously impaired, particularly of the peroxiredoxins, the major scavengers of H_2O_2 in most cell types.[24,56]

Cell culture media can be pro-oxidant[1,24]

Cells require transition metal ions, especially iron and copper, in order to grow. Indeed, all laboratory solutions and cell culture media are contaminated with such ions.[24,57,58] In some cell culture media, inorganic metal ions are also added deliberately. Indeed, the widely used Dulbecco's modified Eagle's medium (DMEM) contains added iron(III) nitrate, $Fe(NO_3)_3$. "Free" iron is also present in Ham's FIO medium, often used to study the oxidation of low density lipoprotein (LDL) *in vitro*.[58] In other media, iron is mostly supplied in transferrin-bound form, usually in the widely used calf serum. Whereas transferrin-bound iron will not normally catalyze free radical reactions,[24,59] contaminating (or added) "free" iron ions can be pro-oxidant, as can be copper and many other transition metal ions.[24]

Ascorbate, flavonoids, many other polyphenolic compounds, and thiols are unstable in the commonly used cell culture media, undergoing rapid oxidation to generate H_2O_2 and often other ROS such as superoxide.[1,45-47,60-67] Studies in my laboratory have revealed that many of the apparent effects of these molecules on cells in culture are artifacts due to their oxidation in the cell culture medium. Examples to illustrate this are accumulating in the literature; Table 1 summarizes some of them. Indeed, the problem may be more widespread. Many substances, including agents used as allegedly specific inhibitors of signal transduction systems and metabolic pathways, are polyphenolic compounds, and thus likely to be highly oxidizable in cell culture media and prone to generate artifacts if added at high concentrations [Table 1]. Yet another mechanism of pro-oxidant effects is photochemical. If light intensity is sufficiently high, riboflavin in culture media can cause photochemical ROS formation.[68,69]

The extent of the artifacts depends on several factors, including the batch of cell culture medium, the cell density, and the medium content of keto acids such as pyruvate. Of course, if the keto acids present scavenge H_2O_2, they will then be depleted, causing further changes in cell metabolism. Leakage of catalase from cells in culture can increase the medium levels of this enzyme in some cases, and a human T-cell line was able to grow in culture at high, but not low cell densities for this reason.[70] A "protective factor" isolated from medium previously used to grow hepatocytes was able to protect other cells against the cytotoxicity of gallic acid; the factor was identified as catalase.[71] This is not surprising because the toxicity of gallic acid is due to its oxidation to produce H_2O_2 in the cell culture medium.[72] Extracellular superoxide dismutase may also be secreted into the surrounding medium by some cell types and can slow down the autoxidation rate of polyphenols, which often involves $O_2^{\bullet-}$.[73,74] Catalase in the medium can protect against H_2O_2 generated both intracellularly and extracellularly, since H_2O_2 readily crosses the cell membranes and so external catalase can "drain" H_2O_2 out of the cell.[24]

Sometimes compounds oxidize in cell culture media but do not generate H_2O_2. Compounds that break down rapidly in cell culture media without substantial H_2O_2 generation include lycopene and some other carotenoids, resveratrol, and curcumin; the reported effects of these compounds on cells in culture may be due to their oxidation products.[75,76] Thus, if a compound does not generate H_2O_2 in a cell culture medium, or if its cellular effects are not prevented by adding catalase, do not assume that it is stable in that medium.[75] The toxicity of dopamine to cells in culture was found to be due to its rapid oxidation to form not only H_2O_2 but also

Table 1: Examples of artifacts caused by oxidation of compounds added to cell culture media*

Observation	Comment
Induction of cell death by ascorbate in HL-60 or acute myeloid leukemia cells or human fibroblasts	Due to generation of H_2O_2 by ascorbate oxidation in cell culture media. Other reported effects of ascorbate on cells in culture (e.g. promoting differentiation of stem cells) might also be due to H_2O_2 generation
Ascorbate observed to inhibit cell proliferation and fibronectin synthesis in human skin fibroblasts	Inhibition by catalase suggests that the observed effect is due to H_2O_2 generation in the culture medium
Induction of apoptosis by green tea in PC12 cells	Due to generation of H_2O_2 by oxidation of tea components in cell culture media
Induction of cell death by l-DOPA and dopamine in PC12 and M14 cells	Due to H_2O_2, quinones, and semiquinones generated by the oxidation of l-DOPA and dopamine in the culture medium[77]
Toxicity of apple phenolics to cancer cells	Due to oxidation to produce H_2O_2 in the culture medium
Cytotoxicity of gallic acid	Largely or entirely due to oxidation of gallic acid to produce H_2O_2 in the culture medium[72]
Addition of grape seed extract to Caco-2 cell culture medium generates H_2O_2 due to oxidation of phenolics in the medium	-
Effects of polyphenols on c-jun phosphorylation in bronchial epithelial cell lines	Shown to involve H_2O_2, although H_2O_2 was not specifically identified as coming from the culture medium
Epigallocatechin gallate induces apoptosis in human oral cell lines	Due to production of H_2O_2 in the culture medium
Toxicity of myricetin to Chinese hamster lung fibroblast V79 cells	Due to H_2O_2 production, although H_2O_2 was not specifically identified as coming from the culture medium
Cell culture media found to generate ROS as detected by spin traps and fluorescent dyes	Ref[82]
Stimulation of SIRT1 activity by polyphenols in HT29 cells	Results confounded by instability of polyphenols in the culture medium
Cyanidin-3-rutoside toxic to HL60 cells	Shown to involve peroxide, although H_2O_2 was not specifically identified as coming from the culture medium
EGCG and green tea extract cause oxidative stress responses in *Saccharomyces cerevisiae*	Involves H_2O_2 production in the medium
Cytotoxicity of EGCG to oral carcinoma cell lines	Involves both H_2O_2 and quinones, although these did not account for all the effects
Activation of NF-κB in macrophages by coffee	Due to H_2O_2; coffee contains substantial H_2O_2 levels[83]
Toxicity of catechols to PC12-AC cells	Involves H_2O_2, mainly generated in the extracellular medium
Toxicity of EGCG to Jurkat T cells	Involves H_2O_2 generation in the culture medium
Cytotoxicity and genotoxicity of green tea extract to H260 and RAW264.7 cells	Involves H_2O_2 generation, although H_2O_2 was not specifically identified as coming from the culture medium
Toxicity of extracts of the oriental fungus *Ganoderma lucidum* to human lymphocytes	Involves H_2O_2 generation[80]
Toxicity of quercetin, catechin, and ascorbate to pancreatic β-cells	Involves H_2O_2 generation in the cell culture medium
Toxicity of 4-methylcatechol to murine tumor cells	Involves oxidation to form H_2O_2 and quinones/semiquinones in the cell culture medium
Toxicity of EGCG to ovarian cancer calls in DMEM	Due to H_2O_2 formation, probably both intracellularly and in the culture medium
Stimulatory effects of garcinol on growth of intestinal cells	Involves ROS production in the culture medium;[81] low levels of H_2O_2 often stimulate cell proliferation[1,24]

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cytotoxic quinones/semiquinones that could be scavenged by adding GSH to the culture medium.[77] Sometimes they can lead to adaptations by activating the Nrf2 system.[67,78]

Conclusion

Cells that survive and grow in culture are often not representative of cells *in vivo*, in terms of metabolism, gene expression, and enzyme levels, and there is a need for caution in extrapolating data obtained in cell culture to the *in vivo* situation. This is widely recognized by researchers, although not always fully considered. Less widely recognized is the extent of the oxidative stress that can be caused by the cell culture process. Thus, ROS-dependent signal transduction pathways identified in cultured cells need to be validated in knockout or transgenic animals.[24,41,42] Similarly, in studies of cellular effects of autoxidizable biomolecules or plant extracts containing them [Table 1], it must be realized that ROS production can occur by chemical reactions in the culture media.[79,80] The stability of such compounds in the culture medium, and their propensity to produce ROS and other oxidation products, must be checked before beginning cell studies. Depending on the cell type and the amount of

ROS produced, these species might exert toxic effects. This explains many of the previously reported cellular effects of thiols, ascorbate, and phenols [Table 1]. The resistance of cells to damage by ROS varies widely, depending on the extent to which the cell has adapted to the oxidative stress of the cell culture milieu. Low levels of H_2O_2 can have the paradoxical effect of accelerating proliferation in some cell types.[24,28,81]

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