High cell density attenuates reactive oxygen species: Implications for in vitro assays

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ABSTRACT

In vitro cell-based assays are an essential and universally used step in elucidation of biological processes as well as in drug development. However, results obtained depend on the validity of protocols used. This statement certainly pertains to in vitro assays of oxidative stress. The holy grail of in vitro models is reliability and predictability of outcomes that relate to a single variable like addition of hydrogen peroxide or xanthine oxidase. Without such validated outcomes, comparison of results among different laboratories is not possible. Achieving this goal requires a thorough understanding of the complex interplay between the cells, their environment, and the experimental assays. Furthermore, as this knowledge is attained, it must be disseminated and used to update and standardize existing protocols. Here, we confirm and extend the effect of pyruvate and cell density on in vitro oxidative stress assays. Cell viability was assessed using a colorimetric assay measuring the reduction of a tetrazolium salt (XTT) into a colored formazan dye. Extracellular hydrogen peroxide concentrations were measured using the foxp3 assay. We confirmed a previously reported finding that pyruvate, a common ingredient in cell culture media, acts as an extracellular scavenger of reactive oxygen species. We also demonstrated that cell density directly correlates with resistance to oxidative stress in tissue culture. It is theorized that the protective effect due to cell density predominantly relates to intracellular factors such as reduced glutathione and extracellular factors such as catalase.

Oxidative stress is implicated in the pathogenesis of many acute and chronic disease processes including burn wound progression, atherosclerosis, neurodegenerative diseases, and aging. Sies defined oxidative stress as "a disturbance in the pro-oxidant–anti-oxidant balance in favour of the former, leading to potential damage."[1] In terms of chronic wounds, venous leg ulcers have been studied extensively, and reports for the last 15 years suggest that compromised healing may be related to high levels of oxidative stress.[2,3] Recently, Wall and colleagues demonstrated that chronic wound fibroblasts generate elevated levels of reactive oxygen species (ROS), and that this phenomenon may contribute to the persistence of chronic venous ulcers.[4] Studies also support the hypothesis that oxidative stress may promote injury progression after acute injuries such as burns.[5,6] Taken together, much evidence supports the hypothesis that therapies designed to inhibit free radical formation or to scavenge free radicals may be protective in these and many other disease processes.[7]

Not surprisingly, there has been a concomitant surge in research and development of antioxidant pharmaceuticals and nutraceuticals. Nutritional supplements such as vitamins C and E, omega-3 fatty acid, selenium, and polyphenol containing nutraceuticals are linked to a range of health benefits purportedly for their antioxidant properties. However, much of the data supporting these claims rely on in vitro experimentation.

In vitro assays are commonly used as a first step to investigate pathologic processes and assess the efficacy of potential treatment regimens. They are useful for screening potential therapeutic agents before initiating more costly and time-intensive animal studies and clinical trials. However, their validity rests on appropriate and correct use of experimental assays and culture environments that adequately model in vivo conditions.[8] The elements of in vitro systems can be divided into two categories that we refer to as intrinsic and extrinsic factors. Intrinsic factors are related to the cultured cells and include cell line, passage number, and density. Extrinsic factors are everything else within the culture environment, such as growth media, temperature and atmospheric conditions, and the experimental assay itself.

A critical extrinsic factor of in vitro systems is the medium. Characteristics of the medium and the frequency of

foxp3	PeroXOquant™ Quantitative Peroxide Assay Kit
XTT	sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate

replenishment can alter cell growth and function. Components within the media can also influence experimental assays. An important example, which relates directly to oxidative stress assays, was studied by Babich et al. His group demonstrated that pyruvate, a common ingredient in media, masks oxidative stress through a process of nonenzymatic decarboxylation. Although pyruvate's antioxidant effects have been known for decades, and even investigated as a therapeutic application in a number of organ systems, its effect on in vitro oxidative stress assays is only now being recognized.[9–13] In contrast, Halliwell's group demonstrated that antioxidants such as ascorbate, flavonoids, and other polyphenolic compounds can react with cell culture media to generate H_2O_2, and therefore act as pro-oxidants during in vitro experimentation.[14,15]

Intrinsic factors are also critically important in experimental in vitro systems. Cell density has been studied extensively, particularly with respect to spontaneous cell death. Most studies report increased cell death in cultures grown to confluence and attribute the cause to contact inhibition and autocrine-mediated mechanisms.[16,17] However, one recent study illustrated that high cell density was critical for human T cell activation and subsequent proliferation, underscoring how different cell types can have diametrically opposite responses to similar conditions.[18] With respect to ROS studies, intrinsic factors such as increased cell age and passage number have also been shown to reduce viability in response to hydrogen peroxide exposure.[19]

In the present work, we confirmed the previously reported finding that pyruvate acts as an extracellular scavenger of ROS.[20] While Babich et al. used human gingival fibroblasts, we extended his work to apply to AHDFs. We also have demonstrated that cell density greatly affects oxidative stress outcomes. In 2002, Moran showed that mouse fibroblast L-cells were more resistant to oxidative stress at high density.[21] Their evidence highlighted several of the upstream signaling pathways leading to oxidative stress induced apoptosis. Here, we corroborate and extend Moran's work to AHDFs and suggest for the first time a cell density-dependent mechanism for the neutralization of ROS.

MATERIALS AND METHODS

Cell culture

Adult human dermal fibroblast (AHDF) strain (CF31) isolated from a healthy 31-year-old Caucasian female was purchased from BioWhittaker (Walkersville, MD). Cells were thawed, washed in phosphate-buffered saline, and resuspended in complete Dulbecco's modified Eagle's medium (DMEM) (GIBCO #11054, Carlsbad, CA) freshly supplemented with 10% fetal bovine serum (FBS) and 584 mg/L glutamine. Cells were of passage 7–10 and cultured at 5% CO_2, 100% humidity, and 37 °C for all experiments.

Effect of pyruvate and cell density

All assays were carried out on 96-well plates (plastic, uncoated) in either pyruvate-containing (GIBCO #11885) or pyruvate-free (GIBCO #11966) DMEM. Experimental media were amended with 10% FBS, 1% Pen/Strep, and

1 g/L D-glucose. AHDFs were seeded at a density of 1,000 cells/well for experiments comparing the presence or absence of pyruvate in the media. For the other experiments, cells were seeded at various densities (500, 1,000, 2,000, 4,000 cells/well). Cell densities were verified by visual counting under light microscopy, and all studies were conducted in quadruplicate.

Cells were incubated for 24 hours prior to ROS treatment. Two strategies were used to administer ROS, either a single bolus of H_2O_2 or the hypoxanthine/xanthine oxidase (HX/XO) system. In the latter, HX was brought to a working concentration of 2 mM per well prior to inclusion of XO. XO was then added to wells at various concentrations. After ROS treatment, the cells were incubated for 20 hours. Next, the media in all wells for both the single bolus H_2O_2 and HX/XO systems were replaced with 100 μL pyruvate-free media. The XTT cell proliferation assay was then used to assess cell viability following manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). Absorbance was recorded at 450 nm with a microtiter plate spectrophotometer.

Extracellular H_2O_2 quantification

Measurement of H_2O_2 was carried out by the ferrous ion oxidation xylenol orange (FOX) method using the PeroXOquant Quantitative Peroxide Assay Kit, lipid-soluble formulation (Pierce Biotechnology, Rockford, IL). AHDFs were seeded at various densities onto 96-well plates in pyruvate-free DMEM. Cells were incubated for 24 hours prior to ROS treatment. Both single bolus doses of H_2O_2 and HX/XO were used for this experiment. Following a 2-hour incubation, the FOX assay was used according to manufacturer protocol. All studies were conducted in quadruplicate.

Intracellular glutathione quantification

All assays were carried out on polystyrene, uncoated 100-mm cell culture dishes in pyruvate-free (GIBCO #11966) DMEM with 10% FBS, 1% Pen/Strep, and 1 g/L D-glucose. AHDFs were seeded at various densities (50,000, 25,000, 12,500, 6,250 cells/mL) equivalent to the seeding densities used in the 96-well plates (4,000, 2,000, 1,000, 500 cells/well, respectively). All studies were conducted in triplicate.

Cells were incubated for 24 hours prior to ROS treatment. A single dose bolus of 100 μM H_2O_2 was used. Following a 2-hour incubation, cells were washed, harvested, and then lysed using an extraction buffer and freeze-thaw cycles. Glutathione was measured according to a modification of Tietze's recycling assay thoroughly described by Rahman et al.[22] Briefly, it is a spectrophotometric reader assay, relying on oxidation of reduced glutathione (GSH) by the sulfhydryl reagent 5,5′-dithio-bis(2-nitrobenzoic acid) to form the yellow derivative 5′-thio-2-nitrobenzoic acid, measurable at 412 nm. Glutathione disulfide (GSSG) is recycled to GSH by glutathione reductase in the presence of NADPH. This constant recycling of GSSG and GSH leads to a linear relationship between the change in absorbency of the solution and the total concentration of glutathione present. A value for the total intracellular glutathione content is assigned by comparing the rate of change of absorbency of a sample to a standard curve, which is generated by performing the assay with known concentrations of GSH.

Data analysis

Analysis of covariance (ANCOVA) was performed to identify differences in outcome (viability) by treatment (pyruvate) in combination with level of H_2O_2 or XO. ANCOVA was also used to detect differences in outcomes (viability, % remaining extracellular H_2O_2) by cell density in combination with level of H_2O_2 or HX/XO. Treatment effects were defined as pyruvate/pyruvate-free media or cell density, as appropriate. Levels of XO or H_2O_2 were included as covariates as appropriate. Interaction terms were included in the analysis in order to determine whether the outcome profile shapes across levels of H_2O_2 or HX/XO were similar. As outcomes were standardized to have a mean of 100% at the baseline of the covariates for all treatments, baseline values were excluded when conducting the analyses. Analyses were performed using SPSS 18 (SPSS Inc., IBM, Armonk, NY) using a significance level of 0.05.

Statistical significance between treatment and control groups for the GSH assay was assessed with a paired Student's t test. A p-value of less than 0.05 was defined as statistically significant.

RESULTS

Effect of pyruvate-containing media on ROS-induced cytotoxicity

Pyruvate masked the effects of ROS-induced cytotoxicity. In the single bolus dose hydrogen-peroxide ROS model, AHDFs expressed greater cell metabolism levels in pyruvate-containing (P+) media compared to pyruvate-free (P−) media. There was a significant difference in percent viability between pyruvate and pyruvate-free media ($p < 0.001$), as well as differences across levels of H_2O_2 and XO ($p < 0.001$ for both, Figure 1). The hydrogen peroxide dose–response curve showed a leftward shift of the median lethal dose (LD_{50}) from 550 μM in P+ media to 60 μM in P− media (Figure 1A). A similar protective effect was observed in the HX/XO system of ROS delivery. However, while the profiles across all levels of H_2O_2 were also significantly different ($p = 0.003$), the profiles across levels of HX/XO were not significantly different (A paired t test would probably have been significant for the HX/XO experiment, but, unfortunately, the same doses
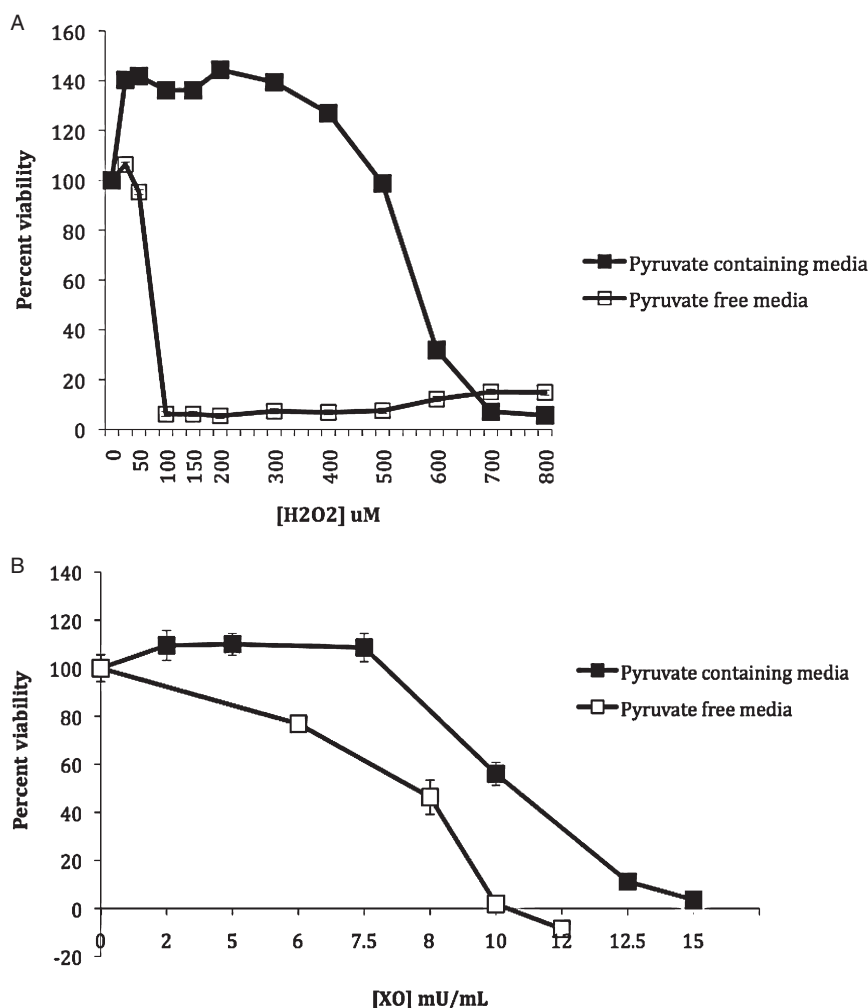
Figure 1. (A) Pyruvate in cell culture media masks cytotoxic effects of H_2O_2 ($p < 0.001$). Data points are means of quadruplicate wells. Cells seeded at 1,000 cells/well. Note errors bars are included, but often obscured due to their small size. (B) Pyruvate in cell culture media masks cytotoxic effects of ROS induced by the HX/XO system ($p < 0.001$). Cells seeded at 1,000 cells/well.
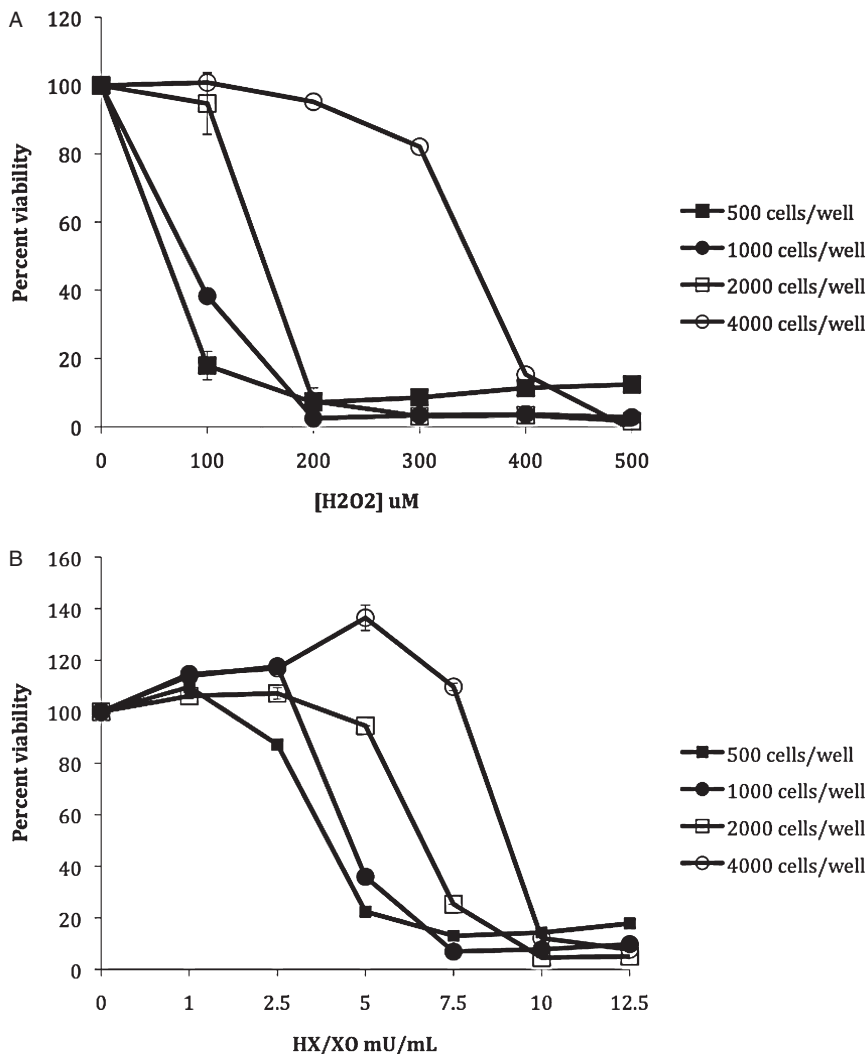
Figure 2. (A) Increasing cell densities facilitates increased resistance to H_2O_2-induced oxidative stress ($p < 0.001$). Data points are means of quadruplicate wells. Cells seeded at various cell densities in pyruvate-free media and incubated for 20 hours after treatment. (B) Increasing cell densities lead to increased resistance to oxidative stress via HX/XO ROS generation ($p < 0.001$). Data points are means of quadruplicate wells. Cells seeded at various densities in pyruvate-free media and incubated for 20 hours after treatment.

of XO were not used in the two conditions; hence, a paired t test is not possible). Here, the LD_{50} shifted from 10 mU/mL in P+ media to 7 mU/mL in P− media (Figure 1B). Interestingly, increased cell metabolism was observed at sublethal concentrations of either hydrogen peroxide or HX/XO in the presence of pyruvate. Growth-promoting effects of sublethal H_2O_2 concentrations have been noted in previous reports.[23,24]

### Effect of cell density on ROS-induced cytotoxicity

Cell density directly correlated with cell viability when exposed to oxidative stress in both the single bolus hydrogen peroxide and the HX/XO ROS models (Figure 2). There was a significant difference in viability by cell density ($p < 0.001$, Figure 2). Levels of H_2O_2 (Figure 2A) and HX/XO (Figure 2B) were also significantly related to viability; in both cases, there were significant ($p < 0.001$) differences in the profiles among cell density.

Notably in the hydrogen peroxide model, as the cell density doubled, the LD_{50} approximately doubled as well. A direct

correlation between LD_{50} and cell density was also seen in the HX/XO model, although the pattern was not as well defined. In these experiments, increased cell metabolism was observed at sublethal concentrations of HX/XO even in the absence of pyruvate. This was especially notable at higher cell densities, i.e., 2,000 or 4,000 cells per well. The twofold differences in cell densities were confirmed in these experiments by visual counts under light microscopy (Figure 3).

Effect of cell density on extracellular levels of ROS

Extracellular levels of H_2O_2 remaining in the media were inversely correlated with cell density. A control was determined as the amount of H_2O_2 remaining in a pyruvate-free and cell-free culture medium after 2 hours of incubation. Then levels of H_2O_2 after 2 hours in the presence of cells at different densities were determined and calculated as a percentage of control. Cell density had a significant effect ($p < 0.001$) on remaining extracellular H_2O_2 after accounting for either covariate (H_2O_2 [Figure 4A] or XO [Figure 4B]). Levels of H_2O_2 and XO also had significant effects on remaining extracellular
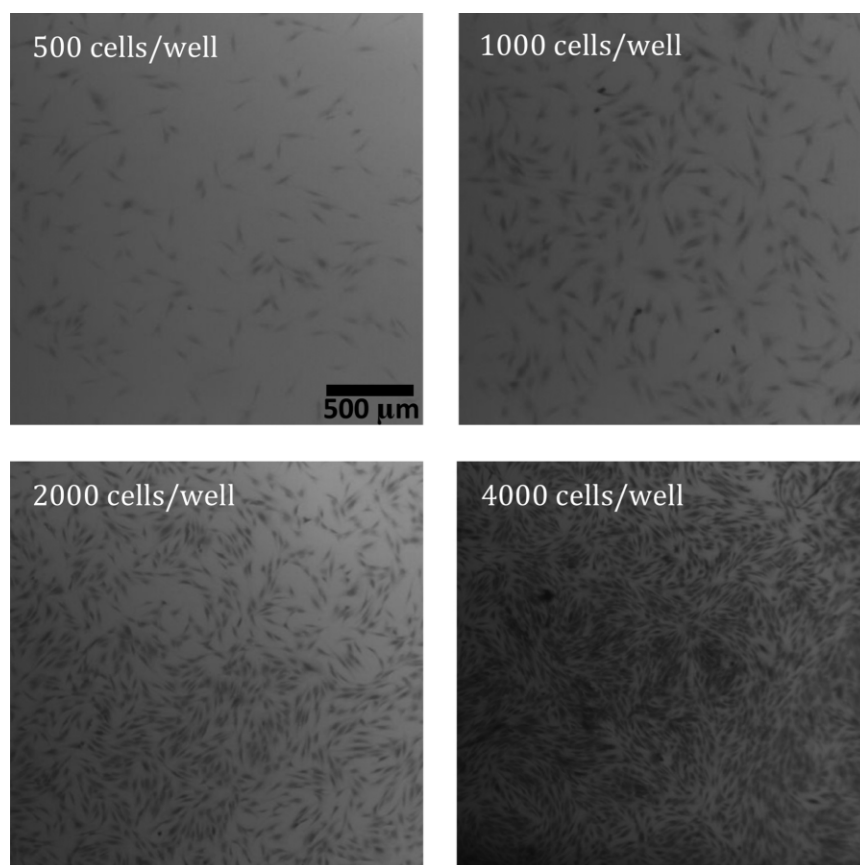
Figure 3. Images of AHDFs seeded at different densities. Cells were seeded at 500, 1,000, 2,000, and 4,000 cells/well, incubated overnight, and then counted via light microscopy. After incubation, the number of cells per well was calculated to be 1,033, 2,329, 4,227, and 10,014, respectively. Magnification bar represents 500 μm for all panels.

H_2O_2; however, no differences in profiles were detected. With a single bolus of H_2O_2, differences in remaining hydrogen peroxide were greatest at high dosages (Figure 4A). At 500 μM, H_2O_2 cells plated at 500 cells/well had ~65% H_2O_2 remaining in the media, while cells plated at 4,000 cells/well had only ~5% H_2O_2 remaining. The same pattern was seen in the HX/XO system of ROS delivery (Figure 4B). Here, the differences in residual H_2O_2 were also greatest at the highest XO dose, although overall the differences were not as substantial as a single bolus of H_2O_2. Subsequent analysis of cell viability with the XTT assay corroborated that cell density is directly correlated with cell viability after ROS treatment (data not shown).

### Effect of cell density on intracellular levels of GSH

Total intracellular GSH was directly correlated with cell density (Figure 5A). After normalization for cell number, intracellular GSH per cell was inversely correlated with cell density in experimental and control groups (Figure 5B). Cells seeded at the highest density (4,000 cells/well) showed a statistically significant ($p < 0.005$) reduction in total GSH after a 2-hour treatment with 100 μM H_2O_2 (Figure 5B). Cells seeded at 500, 1,000, and 2,000 cells/well all showed small, but nonstatistically significant, reductions in GSH.

DISCUSSION

Our data add to a growing body of evidence illustrating how intrinsic and extrinsic factors influence in vitro oxidative stress assays. Cell density is reported here as an intrinsic factor that is directly correlated with resistance to exogenously administered ROS. Specifically, we show that cells seeded at high density quench extracellular hydrogen peroxide in a density-dependent manner. We also confirm a previously reported finding that pyruvate in the media can mask the effects of exogenously introduced oxidative stress.

Cell density has been widely studied with respect to its relationship to spontaneous cell death. Contact inhibition, for example, is seen in vitro and refers to the process by which cells cease to proliferate when they reach confluence despite availability of extracellular nutrients and growth factors. Dysregulation of this process leads to hyperplasia in vivo and facilitates tumor progression by providing the incipient malignant cell with unrestrained proliferative capabilities.[25] Unregulated fibroblast proliferation leads to pathological states such as scleroderma, and myocardial and pulmonary fibrosis.[25–28] Therefore, cell density-dependent apoptosis provides a mechanism against overproliferation and subsequent pathology.

On the other hand, dense cell cultures are more resistant to toxins, environmental stress, and extrinsic apoptotic signaling compared to sparse cultures.[29,30] The cytotoxicity of certain

A

B
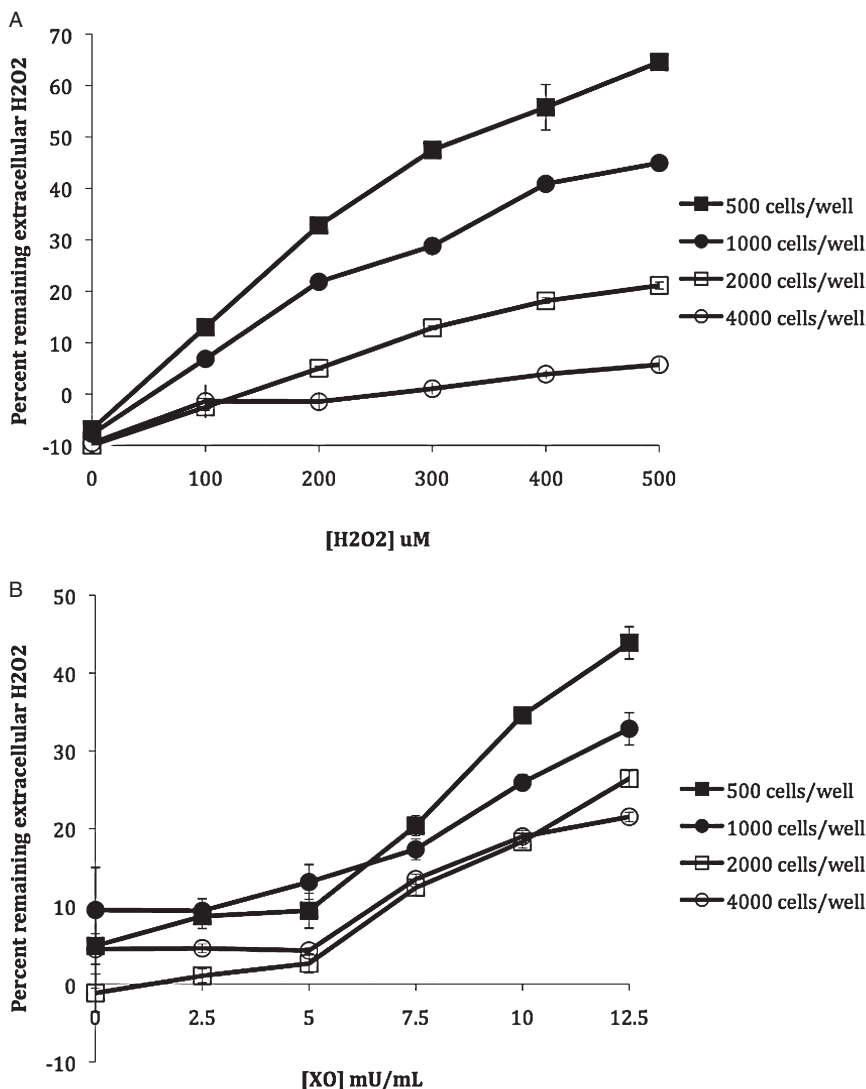
Figure 4. (A) Increase in cell density leads to decreased H_2O_2 within media when treated with single bolus dose of H_2O_2 ($p < 0.001$). Data points are means of quadruplicate wells. Cells seeded at various densities in pyruvate-free media. (B) Increase in cell density leads to decreased H_2O_2 within media when treated with ROS induced by the HX/XO system ($p < 0.001$). Data points are means of quadruplicate wells. Cells seeded at various densities in pyruvate-free media.

anticancer agents, such as doxorubicin and bleomycin, progressively decreases with increasing cell density.[31] Thus, cells at high density are simultaneously more likely to die spontaneously, although they are more resistant to toxins such as ROS. This protective effect from high cell density during exposure to toxins and environmental stressors may provide a possible advantage in pathologic situations induced by high oxidative stress such as burns, myocardial ischemia, or venous leg ulcers. High cell density as found in granulation tissue, it is speculated, may help to attenuate the progression of these diseases.

The cell density-dependent resistance to ROS has been shown twice previously in transformed cells.[21,32] We hypothesized that increasing cell density increased the total intracellular antioxidant capacity of the cell culture, and thus increased viability when exposed to ROS. Our hypothesis would explain why dense cultures quenched hydrogen peroxide in the media more than sparse cultures. While we did find that dense cultures had greater total amounts of glu-

tathione in aggregate, we also found a surprising inverse relationship between cell density and intracellular glutathione concentration per cell. This novel finding supports the possibility of a cell density-mediated regulation of intracellular glutathione concentrations. Recent studies with quorum sensing have identified diffusible factors thought to be used by cells to sense their cell density and influence cell proliferation, motility, and signal transduction pathways.[33] It is certainly possible that a yet unidentified factor also modulates intracellular glutathione.

Our quantitative GSH studies also suggest that intracellular glutathione is only part of the story and support the likelihood that multiple mechanisms are at play for the neutralization of ROS. What our data show for the first time is the possibility that mechanisms for ROS neutralization may be cell density dependent and that GSH plays a larger role at high densities. While further studies clearly must be conducted to elucidate the nature of the differential effect highlighted here, this finding further underscores our aim which is to encourage the
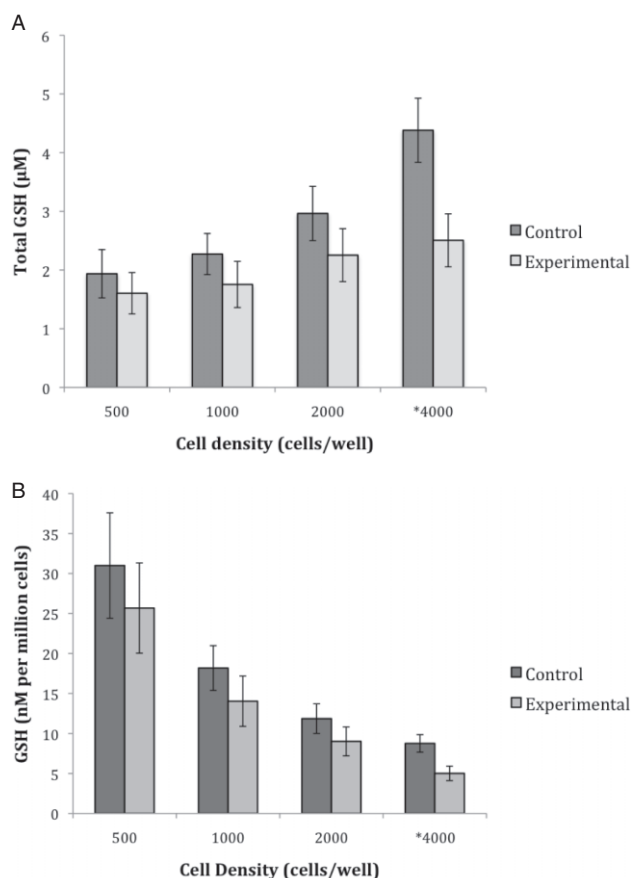
Figure 5. (A) Total reduced glutathione (GSH) is directly correlated with cell density when measured in aggregate. Total GSH is significantly decreased (*$p < 0.005$) in the highest density after 2-hour treatment with 100 μM H_2O_2 of various cell densities. (B) After normalization for cell number, intracellular GSH per cell is inversely correlated with cell density. High cell density (4,000 cells/well) shows a significant (*$p < 0.005$) reduction in total GSH as compared to lower cell densities vs. control.

intentional consideration of cell density when conducting in vitro oxidative stress assays to avoid inconsistency and artifactual results.

Extrinsic factors such as cell culture media, growth conditions, and experimental treatments have all been shown to influence oxidative stress experiments. Pyruvate was confirmed here as potent scavenger of ROS in vitro. Several studies have investigated the total antioxidant capacity of cell culture media and identified components such as albumin, tyrosine, tryptophan, and phenol red as making significant contributions to the total antioxidant capacity of the media.[34] Other factors such as metal ions or oxygen can increase the generation of ROS.[35] Finally, antioxidant compounds such as ascorbate, flavonoids, and polyphenolic compounds can react with cell culture media to generate ROS during in vitro experimentation.[15]

The Fenton reaction is a known pathway that utilizes free iron to produce ROS. As such, it is a valid theoretical concern

that free iron in the media could be another extrinsic source of ROS. To address this potential problem, we measured free iron with a ferrozine colorimetric assay using conditioned media from cells plated at varying densities (0, 1,000, 4,000 cells/well) after an overnight incubation (data not shown). Conditioned media from all densities invariably had concentrations of free iron below the detectable limit of the assay (<5 μg/dL or <1 μM). Several studies in the literature also suggest that free iron plays an insignificant role during in vitro oxidative stress assays. In a study conducted by Fabiani et al., chelating free iron out of media via o-phenanthroline showed no significant effect on apoptosis or in lowering extracellular H_2O_2 concentrations as determined by the FOX assay.[36] Finally, the ideal reaction conditions for the formation of hydroxyl radicals via the Fenton reaction include 8.5 mM H_2O_2, 1.25 mM Fe^{2+}, and a pH of 3. These are all orders of magnitude outside of physiological conditions and of the parameters of our study.[37]

Unknown interactions between intrinsic and extrinsic factors can undermine the validity of experimental in vitro assays by creating artifactual results. For example, in 2000, Long et al. demonstrated that certain polyphenolic compounds react with cell culture media to generate substantial amounts of hydrogen peroxide.[15] Up until that point, in vitro studies reported that polyphenolic compounds induced apoptosis in several different cancer cell lines, and, for this reason, there was considerable interest in their potential use as anticancer agents. Halliwell's data, however, suggested that the cytotoxic effect resulted from the ROS generated by the interaction between the cell culture media and the polyphenolic compounds. It was an artifact of the cell culture system, not the polyphenolic compound itself, inducing the apoptosis.

Our concern is that a general lack of knowledge of the effect of cell density on oxidative stress assays may be resulting in similar artifactual results. In 2008, Watson et al. reported that human colon cancer cells were more sensitive to curcumin-induced cytotoxicity than primary human dermal fibroblasts.[38] They used this evidence to suggest that curcumin had a degree of selectivity for neoplastic cells that supported its potential use in cancer treatment. However, a close examination of their methods reveals that different cell densities were used during their cell viability assay. The human dermal fibroblasts were seeded at twice the density of the colon cancer cells with all other aspects of the assay being identical. It has been established that curcumin can exert its cytotoxic effect via generation of ROS.[39] It is therefore possible that increased sensitivity to curcumin-induced cytotoxicity was due to a lower seeding density not selectivity for neoplastic cells.

Our results suggest that largely overlooked factors have profound effects on in vitro oxidative stress assays. Pyruvate, an ingredient commonly found in cell culture media, is an extrinsic factor that masks oxidative stress. Cell density is an intrinsic factor shown here to directly correlate with resistance to oxidative stress in tissue culture. These data suggest that pyruvate status and cell density should be intentionally considered and clearly denoted in future in vitro oxidative stress experiments. In the same spirit of the Principles for Good Laboratory Practice, Good Cell Culture Practices (GCCP) have been established by the European Centre for the Validation of Alternative Methods to improve the reliability and reproducibility of results from in vitro studies.[40] Part of the GCCP's recommendations is for detailed reporting of cell culture conditions.

REFERENCES

1. Sies H. Oxidative stress: oxidants and antioxidants. *Exp Physiol* 1997; 82: 291–5.
2. Phillips T, Stanton B, Provan A, Lew R. A study of the impact of leg ulcers on quality of life: financial, social, and psychologic implications. *J Am Acad Dermatol* 1994; 31: 49–53.
3. Chen WYJ, Rogers AA. Recent insights into the causes of chronic leg ulceration in venous diseases and implications on other types of chronic wounds. *Wound Rep Regen* 2007; 15: 434–49.
4. Wall IB, Moseley R, Baird DM, Kipling D, Giles P, Laffafian I, Price PE, Thomas DW, Stephens P. Fibroblast dysfunction is a key factor in the non-healing of chronic venous leg ulcers. *J Invest Dermatol* 2008; 128: 2526–40.
5. Singh V, Devgan L, Bhat S, Milner SM. The pathogenesis of burn wound conversion. *Ann Plast Surg* 2007; 59: 109–15.
6. Horton JW. Free radicals and lipid peroxidation mediated injury in burn trauma: the role of antioxidant therapy. *Toxicology* 2003; 189: 75–88.
7. Clark RAF. Oxidative stress and "senescent" fibroblasts in non-healing wounds as potential therapeutic targets. *J Invest Dermatol* 2008; 128: 2361–4.
8. Espin J, García-Conesa M, Tomas-Barberan F. Nutraceuticals: facts and fiction. *Phytochemistry* 2007; 68: 2986–3008.
9. O'Donnell-Tormey J, Nathan CF, Lanks K, DeBoer CJ, de la Harpe J. Secretion of pyruvate. an antioxidant defense of mammalian cells. *J Exp Med* 1987; 165: 500–14.
10. Varma SD, Devamanoharan PS. Oxidative denaturation of lens protein: prevention by pyruvate. *Ophthalmic Res* 1995; 27: 18–22.
11. Desagher S, Glowinski J, Prémont J. Pyruvate protects neurons against hydrogen peroxide-induced toxicity. *J Neurosci* 1997; 17: 9060–7.
12. Long LH, Halliwell B. Artefacts in cell culture: pyruvate as a scavenger of hydrogen peroxide generated by ascorbate or epigallocatechin gallate in cell culture media. *Biochem Biophys Res Commun* 2009; 388: 700–4.
13. Shostak A, Gotloib L, Kushnier R, Wajsbrot V. Protective effect of pyruvate upon cultured mesothelial cells exposed to 2 mM hydrogen peroxide. *Nephron* 2000; 84: 362–6.
14. Clément MV, Ramalingam J, Long LH, Halliwell B. The in vitro cytotoxicity of ascorbate depends on the culture medium used to perform the assay and involves hydrogen peroxide. *Antioxid Redox Signal* 2001; 3: 157–63.
15. Long LH, Clement MV, Halliwell B. Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (−)-epigallocatechin, (−)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. *Biochem Biophys Res Commun* 2000; 273: 50–3.
16. Fiore M, Degrassi F. Dimethyl sulfoxide restores contact inhibition-induced growth arrest and inhibits cell density-dependent apoptosis in hamster cells. *Exp Cell Res* 1999; 251: 102–10.
17. Saeki K, Yuo A, Kato M, Miyazono K, Yazaki Y, Takaku F. Cell density-dependent apoptosis in HL-60 cells, which is mediated by an unknown soluble factor, is inhibited by transforming growth factor beta1 and overexpression of Bcl-2. *J Biol Chem* 1997; 272: 20003–10.
18. Ma Q, Wang Y, Lo AS-Y, Gomes EM, Junghans RP. Cell density plays a critical role in ex vivo expansion of T cells for adoptive immunotherapy. *J Biomed Biotechnol* 2010; 2010: 386545.
19. Yuan H, Kaneko T, Matsuo M. Increased susceptibility of late passage human diploid fibroblasts to oxidative stress. *Exp Gerontol* 1996; 31: 465–74.
20. Babich H, Liebling EJ, Burger RF, Zuckerbraun HL, Schuck AG. Choice of DMEM, formulated with or without pyruvate, plays an important role in assessing the in vitro cytotoxicity of oxidants and prooxidant nutraceuticals. *In Vitro Cell Dev Biol Anim* 2009; 45: 226–33.
21. Moran EC, Kamiguti AS, Cawley JC, Pettitt AR. Cytoprotective antioxidant activity of serum albumin and autocrine catalase in chronic lymphocytic leukaemia. *Br J Haematol* 2002; 116: 316–28.
22. Rahman I, Kode A, Biswas SK. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat Protoc* 2006; 1: 3159–65.
23. Burdon RH, Gill V, Alliangana D. Hydrogen peroxide in relation to proliferation and apoptosis in BHK-21 hamster fibroblasts. *Free Radic Res* 1996; 24: 81–93.
24. Gurjala AN, Liu WR, Mogford JE, Procaccini PSA, Mustoe TA. Age-dependent response of primary human dermal fibroblasts to oxidative stress: cell survival, pro-survival kinases, and entrance into cellular senescence. *Wound Repair Regen* 2005; 13: 565–75.
25. Abercrombie M. Contact inhibition and malignancy. *Nature* 1979; 281: 259–62.
26. Maisch B. Extracellular matrix and cardiac interstitium: restriction is not a restricted phenomenon. *Herz* 1995; 20: 75–80.
27. Thannickal VJ, Horowitz JC. Evolving concepts of apoptosis in idiopathic pulmonary fibrosis. *Proc Am Thorac Soc* 2006; 3: 350–6.
28. Santiago B, Galindo M, Rivero M, Pablos JL. Decreased susceptibility to Fas-induced apoptosis of systemic sclerosis dermal fibroblasts. *Arthritis Rheum* 2001; 44: 1667–76.
29. Mathieu C, Jozan S, Mazars P, Côme MG, Moisand A, Valette A. Density-dependent induction of apoptosis by transforming growth factor-beta 1 in a human ovarian carcinoma cell line. *Exp Cell Res* 1995; 216: 13–20.
30. Preobrazhensky S, Malugin A, Wentz M. Flow cytometric assay for evaluation of the effects of cell density on cytotoxicity and induction of apoptosis. *Cytometry* 2001; 43: 199–203.
31. Ohnuma T, Arkin H, Holland JF. Effects of cell density on drug-induced cell kill kinetics in vitro (inoculum effect). *Br J Cancer* 1986; 54: 415–21.
32. Long H, Han H, Yang B, Wang Z. Opposite cell density-dependence between spontaneous and oxidative stress-induced apoptosis in mouse fibroblast L-cells. *Cell Physiol Biochem* 2003; 13: 401–14.
33. Gomer RH, Jang W, Brazill D. Cell density sensing and size determination. *Dev Growth Differ* 2011; 53: 482–94.
34. Ermilov A, Diamond MP, Sacco AG, Dozortsev DD. Culture media and their components differ in their ability to scavenge reactive oxygen species in the plasmid relaxation assay. *Fertil Steril* 1999; 72: 154–7.
35. Halliwell B. Biochemistry of oxidative stress. *Biochem Soc Trans* 2007; 35(Pt 5): 1147–50.

36. Fabiani R, Fuccelli R, Pieravanti F, De Bartolomeo A, Morozzi G. Production of hydrogen peroxide is responsible for the induction of apoptosis by hydroxytyrosol on HL60 cells. *Mol Nutr Food Res* 2009; 53: 887–96.

37. Chang CY, Hsieh YH, Cheng KY, Hsieh LL, Cheng TC, Yao KS. Effect of pH on Fenton process using estimation of hydroxyl radical with salicylic acid as trapping reagent. *Water Sci Technol* 2008; 58: 873–9.

38. Watson JL, Hill R, Lee PW, Giacomantonio CA, Hoskin DW. Curcumin induces apoptosis in HCT-116 human colon cancer cells in a p21-independent manner. *Exp Mol Pathol* 2008; 84: 230–3.

39. Kelly MR, Xu J, Alexander KE, Loo G. Disparate effects of similar phenolic phytochemicals as inhibitors of oxidative damage to cellular DNA. *Mutat Res* 2001; 485: 309–18.

40. Coecke S, Balls M, Bowe G, Davis J. Guidance on good cell culture practice. *Altern Lab Anim* 2005; 33: 261–87.