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FREE RADICALS MEDICINE (X SHI, SECTION EDITOR)

Oxidative Stress Under Ambient and Physiological Oxygen Tension in Tissue Culture

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Abstract Oxygen (O_2) levels range from 2–9 % in vivo. However, cell culture experiments are performed at atmospheric O_2 levels (21 %). Oxidative stress due to generation of reactive oxygen species (ROS) in cells cultured at higher than physiological levels is implicated in multitude of deleterious effects including DNA damage, genomic instability, and senescence. In addition, oxidative stress activates redox-sensitive transcription factors related to inflammatory and apoptotic signaling. Furthermore, several chromatin-modifying enzymes are affected by ROS, potentially impacting epigenetic regulation of gene expression. While primary cells are cultured at lower O_2 levels due to their inability to grow at higher O_2, the immortalized cells, which display no such apparent growth difficulties, are typically cultured at 21 % O_2. This review will provide an overview of issues associated with increased oxygen levels in in vitro cell culture and point out the benefits of using lower levels of oxygen tension even for immortalized cells.

Keywords Oxygen tension · Oxidative stress · Primary cells · Immortalized cells · Reactive oxygen species · Hypoxia

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Introduction

Aerobic life forms on earth adapted to progressively increasing oxidative environment and evolved with complex respiratory system to utilize and distribute oxygen to various organs and tissues. The partial pressure (pO_2) of ambient atmospheric oxygen is 150 mm/Hg, which is equivalent to 21 % oxygen (O_2; normoxia). However, the inhaled O_2 levels progressively decrease as it reaches various internal organs and tissues (Fig. 1). The level of O_2 and its distribution among the various tissues depends on the rate of capillary blood flow and its metabolic activity (i.e., oxygen utilization) [1]. For instance, in humans, under physiological conditions, the pO_2 in the arterial blood is 100 mm/Hg or 14 % O_2. When it reaches the well-irrigated parenchymal organs such as lungs [2–4], liver [5–7], and kidneys [8], the O_2 levels ranges from 4 to 14 %. In the relatively less irrigated organs and tissues, including brain [9, 10], eye [11], and bone marrow [12], the O_2 concentration ranges between 0.5 and 7 %. Moreover, gradient distributions of O_2 within organs and developmental stage-specific O_2 levels have also been observed [4, 7, 13–15]. Several pathophysiological conditions resulting from fluctuations in oxygen tension including (e.g., hyperoxia-associated lung injury, hypoxia in cancer, and wound healing) has been well documented [16].

In vitro cultured cells (both non-immortalized primary cells and immortalized cells lines) are the preferred model system for studying cellular response to specific stimuli such as drug treatment and toxicity testing and to understand basic cellular mechanisms including differentiation and disease development. Cell lines provide cellular homogeneity, which is valuable for understanding molecular mechanisms including functional genomics. Moreover, studies on certain areas such as genetic disorders, neurological disorders, and genetic polymorphisms that may be uniquely human and thus not amenable to studying using animal models. In vitro cell culture is traditionally performed at 21 % O_2, the O_2 level in ambient

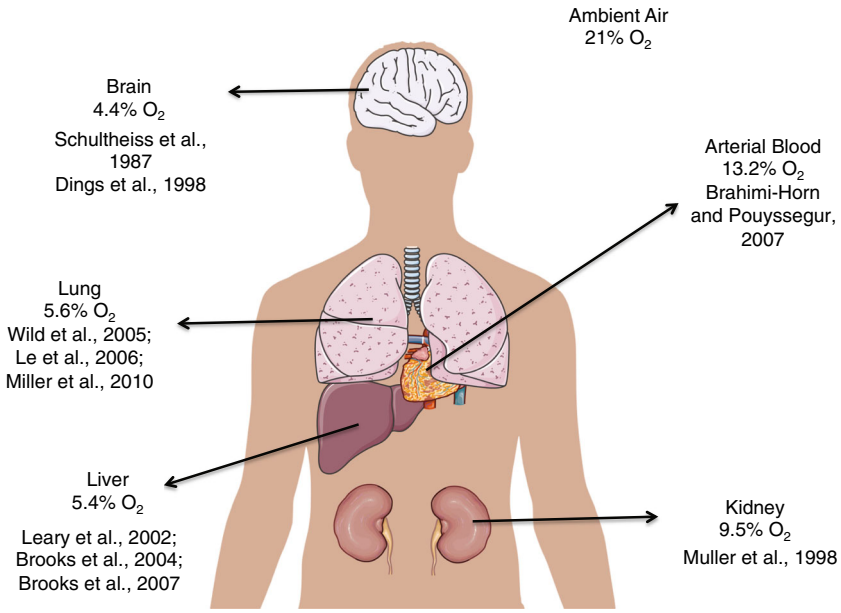

Fig. 1 Oxygen distribution in various organs: The partial pressure (pO_2) of ambient atmospheric oxygen is 150 mm/Hg (21 % O_2). The inhaled O_2 levels progressively decrease as it reaches various internal organs. The partial pressure of oxygen (pO_2) in the arterial blood is 100 mm/Hg or 14 % O_2. The O_2 tension ranges from 4 to 14 % in lungs, liver, and kidneys. In the relatively less irrigated organs and tissues, including brain, eye, and bone marrow, the O_2 concentration ranges between 0.5 and 7 %

atmospheric air. Thus, the O_2 levels in in vitro cultures are approximately 2–5-fold higher than the physiological O_2 levels encountered by tissues in vivo [17, 18]. Accumulating evidence suggests that culturing non-immortalized primary cells or stem cells at lower physiologically relevant oxygen tension (physioxia or physiological normoxia) increases the replicative life span of the cells [19–21]. Therefore, primary cells are typically cultured at lower O_2 levels [18, 22–24]. However, unlike the primary cells, immortalized cells do not exhibit apparent growth difficulties at 21 % O_2, likely due to its ability to evade replicative senescence [25, 26]. Therefore, the detrimental effects of increased oxygen levels in immortalized cell cultures have been over-looked and growing these cells at 21 % O_2 is currently a standard practice. Nevertheless, accumulating evidence suggests that abundant reactive oxygen species (ROS) and associated oxidative stress in cells cultured at high O_2 levels could affect the physiology of cells in culture [27, 28], which could potentially influence the experimental outcome. The current review will provide an overview of issues associated with increased oxygen tension in in vitro cell culture and point out the benefits of using lower levels of oxygen tension even for immortalized cells.

ROS Production in Tissue Culture

Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) and their detoxification, resulting in cellular damage. Most ROS are generated in cells by the mitochondrial respiratory chain. Mitochondrial ROS production is modulated largely by the rate of electron flow through respiratory chain complexes. Under physiological conditions approximately 1–4 % of the oxygen consumed by mitochondria is diverted for the formation of ROS [29]. Considering that the average rate of utilization of oxygen in each cell of human body is ~2.5×10–18 mol/s (i.e., 2.2×10^{10} molecules/day) [29], almost ~1 billion molecules of ROS are being produced by each cell every day in vivo [29]. This can be multiplied several fold under in vitro culture conditions which is cultured at 21 % O_2 as compared to physiological conditions (10–13 % O_2).

Although excess ROS can lead to oxidative stress, moderate to low levels of ROS function in cellular signaling pathways [30]. The mammalian system is naturally equipped to deal with oxidative stress. Antioxidants, including glutathione (GSH), vitamins C and E, and antioxidant enzymes such as catalase, superoxide dismutase (SOD), and various peroxidases help to maintain the cellular redox environment. Recent evidence suggests that lower availability of antioxidants including vitamins E and C and selenium in cell culture medium could affect the cell's ability to efficiently scavenge ROS [31]. Moreover, the photochemical oxidation of flavonoids, thiols, and polyphenolic compounds in the cell culture media can produce H_2O_2 in cell culture [32]. Therefore, overproduction of ROS at 21 % O_2 combined with impaired antioxidant system in cell culture conditions could contribute to cellular damage, inflammation, and senescence.

Cellular Damage by ROS

The deleterious effects of 21 % O_2 on primary cells have been well documented [33]. Oxidative stress induces production of

highly reactive free radicals, which cause irreversible damage to all biomolecules including DNA, proteins, lipids, and sugars [34]. The formation of peroxyl radicals and oxidation of cysteine residues during oxidative stress causes protein conformational changes, which ultimately affect their functions [35]. For instance, damage to protein kinases or phosphatases leads to dysregulation of signaling cascades, affecting a multitude of cellular functions. Protein folding in endoplasmic reticulum (ER) is catalyzed by disulfide isomerase that requires GSH for its functioning [36]. Over utilization of GSH during oxidative stress results in ER stress, causing in accumulation of unfolded proteins in the cytoplasm [36] and autophagy [37]. The most destructive effect of ROS is their ability to cause DNA damage [22, 38–41]. ROS can oxidize nucleotides and can cause single- and double-strand DNA breaks (SSBs and DSBs), and oxidatively generated non-DSB clustered DNA lesions (OCDLs) [38]. The addition of hydroxyl radical to the C8 position of guanine ring, produces the 8-hydroxy-7,8-dihydroguanyl radical that is further oxidized to 8-oxo-7,8-dihydroguanine (8-oxodG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapydG) [38]. The OCDLs are resistant to DNA repair process and can result in irreversible DNA damage [38, 41]. Interestingly, mouse stem cells cultured at 3 % O_2 showed less oxidative stress and lower aneuploidy, compared to the cells cultured at 21 % O_2 [42]. Increased neuronal cell survival and differentiation of human neural progenitor cells is observed in human umbilical cord Wharton jelly derived mesenchymal stem cells (hWJ-MSCs) cultured at lower oxygen tension as compared to normoxia [43, 44]. On the contrary, culturing cells at 21 % O_2 is known to induce chromosomal abnormality/aberration [41, 45, 46], aneuploidy [42], telomere shortening [47], and DNA damage [22] at genome levels in primary cells.

Activation of Redox-Sensitive Inflammatory Signaling

Prolonged generation of reactive oxygen species (ROS) contributes to chronic inflammation [34]. Alternatively, chronic inflammation can also induce oxidative stress and reduce the cellular antioxidant capacity [34]. Freshly isolated human PBMCs at atmospheric oxygen levels (21 % O_2) sent out inflammatory signals as though they were fighting off an infection. Interestingly, the same cells grown at lower oxygen levels (5 and 10 % O_2) displayed considerably reduced inflammatory signaling [17, 18]. Human PBMCs response to stimulation with mitogens including conA and CD3/CD28 crosslinking is observed to be significantly higher at 20 % oxygen tension, compared to 5 and 10 % oxygen tensions [17, 18]. The mitogen-induced PBMCs show increased intracellular nitric oxide levels and reduced intracellular glutathione levels at 20 % oxygen [17, 18]. Haddad et al., showed the increase in expression of genes involved in cell death, cellular repair, and stress response in primary T cells maintained at atmospheric O_2 levels indicating increased oxidative stress response [48]. Cultivation of rat liver sinusoidal endothelial cells (LSECs) at 5 % O_2, as opposed to 21 % O_2 improved the survival of LSECs and scavenger receptor-mediated endocytic activity, reduced the production of the pro-inflammatory mediator, interleukin-6, and increased the production of the anti-inflammatory cytokine, interleukin-10 [49]. Expression of the cell adhesion molecule, ICAM-1 at the cell surface was slightly more elevated in cells maintained at 21 % O_2 [50], indicating activation of an inflammatory response.

Increase in secretion of pro-inflammatory cytokines in senescent cells suggests a crucial link between oxidative stress and inflammatory signaling [34]. A likely reason is that several inflammatory signaling events and transcription factors are redox-sensitive and would get manipulated by the alteration in the cellular redox potential [34, 51]. Interestingly, the activation of different redox-sensitive transcription factors and the resultant biological response is correlated with the levels of ROS in the cells [34, 51] (Fig. 2). Low-sustained ROS level mainly induces the antioxidant and cellular detoxification program like the Kelch-like ECH-associated protein 1 (Keap1)–NF-E2-related factor-2 (NRF2) regulatory pathway, which plays a key role in the protecting cells against oxidative and xenobiotic damage [52]. An intermediary amount of ROS triggers inflammatory response through the activation of NF-κB, STATs, and AP-1, transcription factors involved in the regulation of a wide array of genes involved in stress response, inflammation, immune function, differentiation, apoptosis, cell survival, and growth [53]. Finally, high levels of oxidative stress trigger disruption of electron transfer by inducing mitochondrial pore formation, thereby resulting in apoptosis or necrosis (Fig. 2). The effects of ROS on the cell are also partly mediated through activation of mitogen-activated protein kinases (MAPKs) [54, 55]. Recent studies show non-hypoxic activation of HIF-1α via mitochondrial-derived ROS [56]. The ROS-induced activation of HIF-1α might cross talk with redox sensitive transcription factors and signaling mechanisms, which could lead to tumor initiation.

Cellular Immortality Requires Development of Oxidative Stress Resistance

Primary cells exhibit growth difficulty at 21 % O_2 due to the overproduction of ROS and the subsequent bio-molecular damage [39, 47, 57–59]. This poor growth response in high oxygen-treated cultures was abrogated by administration of catalase, which scavenges excessive H_2O_2 produced at 21 % O_2. This suggests that the toxic effects observed in high O_2 environments are largely caused by endogenous production of H_2O_2 [39]. Moreover, treatment with low doses of H_2O_2 is

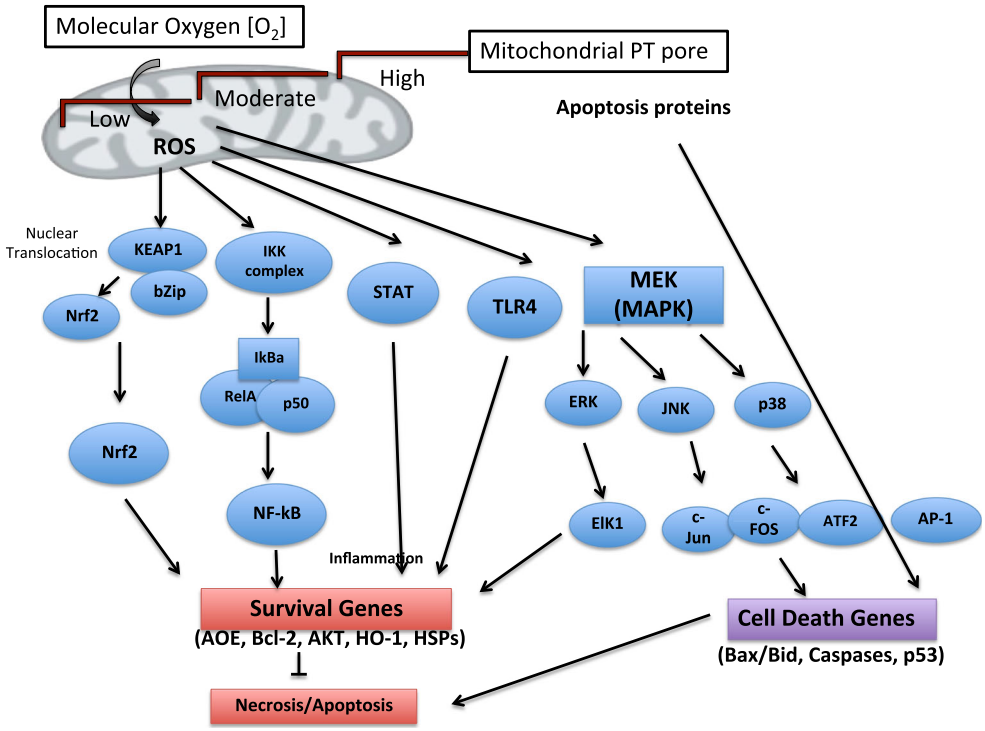

Fig. 2 Activation of redox sensitive inflammatory signaling: ROS activates inflammatory signaling via activation of redox sensitive transcription factors and signaling. The activation of different redox-sensitive transcription factors and the resultant biological response is correlated with the levels of ROS in the cells. Low-sustained ROS level mainly induces the antioxidant and cellular detoxification program like the Kelch-like ECH-associated protein 1 (Keap1)–NF-E2-related factor-2 (NRF2) regulatory pathway. An intermediary amount of ROS triggers inflammatory response through the activation of NF-κB, STATs, and AP-1 transcription factors associated gene expression. Finally, high levels of oxidative stress trigger disruption of electron transfer by inducing mitochondrial pore formation, thereby resulting in apoptosis or necrosis. The effects of ROS on the cell are also partly mediated through activation of mitogen-activated protein kinases (MAPKs). All these mechanism can induce multitude of downstream processes including inflammation, survival and apoptosis

sufficient to induce replicative senescence in primary mouse embryonic cells [39]. Unlike primary cells, the effect of high O_2-induced ROS in immortalized cells is less well studied. However, it is clear that immortalized cells suffer less oxidative damage compared to primary fibroblasts when cultured under 20 % O_2 [60, 61]. Moreover, immortalized cells are shown to exhibit higher resistance to deleterious effect of H_2O_2 than primary cells [60, 61]. Kondoh et al., showed an induction of immortality in primary cells selected for increased phosphoglycerate mutase (PGM) activity, which showed enhanced glycolytic-influx and resistance to oxidative stress [61, 62]. Similarly, tumor cells show remarkable tolerance towards oxidative stress and utilize excessive glucose via anaerobic glycolysis to prevent the oxidative damage [60]. These studies suggest association of cellular immortalization with the development of resistance to oxidative stress, allowing them to grow at high O_2 levels for extended periods. However, immortalized cells cultured at ambient atmospheric O_2 levels showed redox imbalance, cell proliferation defects, and activation of NF-κB/RelA-mediated inflammatory signaling. Furthermore, the cells cultured at physiological O_2 levels showed better ability to handle oxidative stress as compared to the cells cultured at ambient 21 % O_2 levels [63]. Therefore, although immortalized cells acquire the ability to grow under high O_2 levels, the cellular damage due to oxidative stress and inflammation is not completely eliminated [63].

ROS Mediates the Effects of Both Hypoxia and Hyperoxia

While increasing oxygen concentration can influence the production of free radicals, decreasing it can lead to hypoxia. Hypoxia can lead to the production of oxygen radicals in a variety of experimental systems via electron attack of molecular oxygen in the inactive mitochondria [64–66]. It is interesting that oxidative stress and hypoxia, which are quite different scenarios in terms of total oxygen tension, can result in similar outcome due to production of the common mediator, ROS. Recently, it has become clear that under hypoxic conditions, the mitochondrial respiratory chain also produces nitric oxide (NO), which can generate other reactive nitrogen species (RNS) [30]. Oxidative stress and hypoxia are serious consequences of many diseases such as cancer, heart disease, inflammation, COPD, and many others.

Hypoxia plays a major role in the pathology of several human diseases, including cancer, diabetes, aging, and stroke/ischaemia [67, 68]. Under hypoxia, the hypoxia inducible factors (HIF) family of transcription factors becomes activated. The members of the HIF family transcription factors, HIF-1α, HIF-2α, and HIF-3α, are oxygen labile and can heterodimerize with the oxygen insensitive HIF-1β, also known as aryl hydrocarbon nuclear translocator (ARNT) [68]. Under normal oxygen tension (normoxia), the proline residues in the oxygen-dependent domain of HIF is hydroxylated by a family of dioxygenases, called prolyl-hydroxylases (PHDs) (oxygen sensors) [69]. The hydroxylated proline residues are recognized by von Hippel-Lindau (VHL), which subsequently recruit an E3 ubiquitin ligase complex and target HIF for degradation [70]. Another dioxygenase, factor inhibiting HIF (FIH) can also influence HIF activity. The enzymatic activities of both PHDs and FIH require oxygen, α-ketoglutarate, iron (Fe^{2+}), and ascorbate as cofactors. Depletion of any of these cofactors, as well as oxidative stress mediated Fe oxidation, can inhibit enzymatic activity and stabilize the HIFs.

ROS Mediates Epigenetic Dysregulation in Both Hypoxia and Hyperoxia

ROS produced during both hypoxia and hyperoxia has profound effects on epigenetic modification. A number of 2-oxoglutarate dependent dioxygenases that demethylate histone tails or hydroxylate the 5-position cytosine of DNA use oxygen for their enzymatic activity. These enzymes sense oxygen, since the oxygen levels are critical for mediating their modification of the epigenetic program of a cell. Hypoxia inhibits these enzymes thus causing an increase in histone methylation and 5-methylcytosine (5mC) and a decrease in hydroxymethylcytosine (5hmC) [71] During periods of low oxygen tension, the messenger RNA (mRNA) and protein levels of a number of histone demethylases are increased via the hypoxia response elements (HREs) in their promoters [71]. Similar to hypoxia, inhibition of demethylases also occurs due to oxidative stress since the iron at the active site of these enzymes can be oxidized rendering the enzyme inactive [71] At the same time, oxidative stress decreases reduced ascorbate levels, which further attenuates enzymatic activity since ascorbate is a cofactor for these enzymes [72]. Finally, if the oxidative stress arises from dysfunctional mitochondria there will be less of 2-oxoglutarate, which is an essential cofactor for these enzymes [66].

Methylation of histone lysines and arginines in the N terminal tails, which protrude from the nucleosomes is involved in regulating a wide range of cellular processes [73–75]. Histone methylation can be involved in both activation and repression of gene expression depending on the residue modified and degree of methylation. For example, while histone 3 lysine 4 di- (H3K4me2) and trimethylation (H3K4me3) and histone 3 lysine 9 monomethylation (H3K9me1) are associated with transcriptionally active, open chromatin regions, histone 3 lysine 27 (H3K27) di- and trimethylation and H3K9 di- and trimethylation are associated transcriptionally silent, closed chromatin regions [71, 74, 76–78]. Histone tail methylation is a dynamic process maintained by the histone methyltransferases (HMTs) and demethylases. The majority of HMTs contain a conserved catalytic domain called SET (Suppressor of variegation, Enhancer of Zeste, Trithorax) [79]. Histone demethylases, on the other hand, can be divided into two classes: (1) KDM1 (Lysine (K) Demethylase1) family, also known as LSD1, are FAD-dependent amine oxidases, and (2) the Jumonji C (JmjC) domain containing demethylases (JHDMs), which are members of the dioxygenase superfamily of enzymes containing iron and are 2-oxoglutarate-dependent enzymes [73] [80]. The demethylation of lysines by JHDMs occurs by catalyzing the generation of oxidized Fe (reactive oxygen species) in the presence of oxygen, α-ketoglutarate and ascorbate. These resultant species attack the methyl groups on histone lysines and produce unstable oxidized intermediates that spontaneously release formaldehyde, resulting in the removal of methyl groups from histone lysines [81]. Since LSD1 and JmjC histone demethlyases and TET family of DNA hydroxylases require oxygen to function, it is likely that oxygen concentration influences histone and DNA methylation.

Although the functional significance of these enzymes in relation to hypoxic response is not fully understood, a number of studies have demonstrated their involvement. It was recently shown that decreased H3K4me3 levels in clear-cell renal carcinoma were due to VHL inactivation, which was dependent on the constitutively active HIF-2α and Jumonji/ARID Domain Containing Protein 1C (JARID1C). In VHL−/−cells, HIF induced *JARID1C* expression, resulted in altered expression of hypoxia-responsive genes (HRGs) and reduced H3K4me3 levels at the promoters of *IGFBP3*, *COL6A1*, *DNAJC12*, and *GDF15* [82]. Interestingly, many 2-oxoglutarate dioxygenases histone demethylases have hypoxia response elements (HREs) in their promoters and are induced by HIF-1 [83]. For example, JARID1B (KDM5B), JMJD1A (KDM3A), JMJD2B (KDM4B), and JMJD2C (KDM4C) are known to be direct HIF-1 target genes with robust HIF-1 binding to HREs in their promoters and upregulated expression under hypoxic conditions [84–87]. Under hypoxia, cells ectopically expressing JARID1B had decreased levels of H3K4 methylation [88]. Furthermore, JMJD1A was shown to regulate a subset of hypoxia-induced genes, including *ADM* and *GDF15*, by maintaining a lower level of H3K9me2 at their promoter regions. JMJD1A (KDM3A) was also important for tumor growth in the hypoxic microenvironment of tumor xenografts [89]. In addition to

upregulating the expression of certain demethylases, hypoxia also directly inhibits their enzyme activity because they require oxygen to carry out their enzymatic function. The compensatory upregulation of the histone demethylase expression under hypoxia and possibly with oxidative stress affords a way to identify the affected enzyme.

Inhibition of the Jumonji histone demethylases JMJD1A-C (KDM3A-C) and JMJD2A–D (KDM4A-D) with hypoxia as well as with the treatment of dioxygenase inhibitors, such as DMOG (N-(methoxyoxoacetyl)-glycinemethyl ester, DETA-NO (2,20-(hydroxynitrosohydrazono)bis-ethanimine) and ROS, resulted in increased levels of H3K9me2/me as well as H3K36me3 [90]. Exposure to nickel, an oxidative stress-causing agent can result in the distortion of a number of different post-translationally modified histone marks. A non-toxic dose of nickel was shown to significantly increase global levels of H3K4me3 and H3K9me2 [91] as well as cause spreading of H3K9me2 causing aberrant gene silencing [78]. Thus, ROS produced during both hypoxia and hyperoxia can alter gene expression by affecting key enzymes involved histone post-translational modifications and DNA methylation, in addition to activating redox sensitive transcription factors.

Conclusion

In vitro cell culture at ambient atmospheric O_2 levels (21 %) contribute to oxidative stress in both primary cells and immortalized cells. Furthermore, the pro-oxidant nature of the cell culture medium and decreased availability of antioxidants, combined with higher than normal physiological oxygen tension would result in net increase in ROS and inhibit several key O_2 sensitive cofactors and transcription factors. However, decades of research have proven the utility of in vitro cell culture system, which is a rapid and cost-effective research tool [92–94]. The genetic and epigenetic changes observed in cancer cells in vivo has been accurately observed in in vitro studies [92–94]. Genome-wide studies have shown striking similarity in genome folding, DNA methylation, and histone modification profiles between in vitro cultures and the corresponding tumor samples [94, 95].

Although culturing the cells at the physiological O_2 levels could potentially avoid the deleterious effects of high O_2 levels, several practical difficulties exist. The distribution of O_2 in vivo is organ and tissue specific. Moreover, organs such as skin [13], liver [5–7], and lung [2–4] show gradient distribution in oxygen level depending on extent of capillary blood supply. Furthermore, developmental stage specific oxygen levels are observed in placenta [14, 15]. In addition, oxygen consumption rate by mammalian cells in in vitro culture ranges significantly between <1 and >350 amol $cell^{-1}$ s^{-1} [29]. The cellular O_2 consumption rate in vitro depends on cell type, function, and metabolic activity and shows loose linear correlation with cell volume and protein content [29]. Moreover, the actual oxygen level the cell is exposed to in a culture dish is much lower than the surrounding atmospheric levels and the O_2 levels further decrease when the cells proliferate [96]. Given the huge variation in O_2 consumption rate of mammalian cells in vitro [29] and the broad physiological O_2 levels in vivo [1], culturing cells at actual physiological O_2 levels pose huge problems and is impractical. Alternatively, addition of multiple-time matched controls, gain or loss of function experiments, and usage of different cell types for the same treatment could attenuate experimental bias to some extent and would be a practically possible strategy. Immortalized cells cultured under high oxygen tension might show higher inflammatory response and redox imbalance. Therefore, care should be taken while studying inflammatory signaling and antioxidant activity of dietary molecules in immortalized cells as they could potentially exhibit elevated activity under in vitro culture conditions.

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Compliance with Ethical Standards

Conflict of Interest Authors declare no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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