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Review Article
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Specific Hypoxic Preconditioning Could Improve the Activity and Adaptability of Cells in Nutritionally Deficient Environment

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Abstract

During the treatment of endodontic diseases, multipotential dental stem cells will experience a certain degree of nutritional deficiency, such as hypoxia or ischemia, in the process of reconstructing the root canal microenvironment. It is becoming a hot area of research that how to deal with the cells to survive the harsh living environment, retaining or even improving their biological activity. This review summarized the literatures concerning the cellular adaptative responses and mechanisms to hypoxia after specific beforehand treatment, proposing that rapid extreme hypoxia precondition could activate a series of self-protective responses of cells, such as Hypoxia-Inducible Factor (HIF) pathway activation, mitochondrial Reactive Oxygen Species (ROS) production and Unfolded Protein Response (UPR). This review raised a promising pretreatment that could enhance the protective efficacy of existing vital pulp therapies.

Introduction

Cellular survival depends on adequacy of nutrition in the organism, and oxygen level to which cells are exposed is an important environmental parameter. As a result of the high metabolic rates of proliferating and differentiating cells in the developing tissues, their oxygen and nutrients demands rapidly exceeded supply, leading to local pericellular hypoxia. With the enlargement of cell clusters, some cells are gradually in a relatively low-oxygen and low-nutrient environment. Affected by signals from the pericellular environment, the cells will undergo a series of changes at the level of organelles, proteins, and molecules. The most in-depth study of this phenomenon is the response of tumor cells to hypoxia signals. Except for pathological conditions like pulpitis, cells experience similar processes in various physiological situations. For example, in the process of undifferentiated stem cells implanting into the root canal to regenerate and renew the pulpal microenvironment, cells will proliferate and differentiate in the ischemia and hypoxia environment when they are just implanted, for the vessel narrowing and occlusion in the apical part of root canal. However, it is necessary for these multipotential cells maintaining the bioactive state, to differentiate into functional cells such as vascular epithelial cells to form new blood vessels, providing nutrients transport pathway for the subsequent physiological processes. How to maximize the uptake efficiency of the limited.

Resources while avoiding the cells from initiating the apoptosis under tough conditions is the key to successful treatment. Thus, the hypothesis was put forward that whether certain pretreatments make cells present higher biological activity and differentiation ability under relatively poorer conditions? This review proposes an overview of different responses including translation, organelle alteration and metabolism to hypoxia in different intensity and duration, especially the pretreatment that may help improve the biological activity of cells, exploring the potential exploitation of this idea to figure out unsettled biological questions and meet unfulfilled biomedical needs.

HIF Showed Temporal Correlation Expression in Multiple Reactions

Typically, the cellular oxygen concentration was defined as followed: 21% O_2 as normoxia, 4% to 7.5% O_2 as tissue normoxia or physoxia, 1% to 2% O_2 as hypoxia and radiobiological hypoxia when less than 0.1% O_2 [1-3]. Hypoxia is a vital stressor of multiple species encountered under physiological or pathological conditions, triggering complicated reaction to adapt new environment. These adaptations include enhanced ventilation, blood vessel development and metabolic change, as well as adjustment of endoplasmic reticulum and mitochondria [4].

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Regarding the hypoxia response, the first and most well-known adaption is the expression of Hypoxia-Inducible Factor (HIF) family genes, including HIF-1, HIF-2 and HIF-3 families. HIF is a heterodimeric basic-helix -loop-helix-PAS transcription factor consisting of HIF-α and HIF-β subunits, the regulatory α-sub-units (HIF-1α, HIF-2α, HIF-3α) are negatively controlled by cellular oxygen supply, stabilized by hypoxia and bind to the HRE sites, and the β-subunit (HIF-β) was constitutively expressed [5]. HIF-1α is expressed ubiquitously in nucleated cells; meanwhile, HIF-2α and HIF-3α have a distinct, tissue-specific expression patterns and binding partners limited to the endothelium, kidney, lung, heart, and small intestine [6]. The transcriptional response to hypoxia mainly depends on the HIF-PHD-p VHL axis. HIFs are stable when oxygen concentration is 2% to 6% [7]. When the oxygen level is sufficient, functional Prolyl Hydroxylases (PHD) promotes binding of HIF-α to the VHL (Von Hippel Lindau) tumor suppressor protein, targeting component of an E3 ligase complex, resulting in proteasomal degradation of α-subunit, preventing the dimerization between HIF-α and HIF-β, and inhibiting the transcriptional activity [6,8]. In the hypoxia situation, reduced PHD activity results in the stabilization and accumulation of cytoplasmic HIF-1α. Subsequently, HIF-1α translocates to the nucleus forming the HIF transcription factors, targets the expression of relevant genes [9,10]. Also, there is another oxygen-regulated post-translational histone acetyl transferases modification by the Factor Inhibiting HIF (FIH) towards HIF transcriptional activity [11,12]. HIF was originally identified in studies of the hematopoietic growth factor Erythropoietin (EPO). Further, more genes have been identified to be governed by mammalian HIF during O_2 deprivation, including glycolytic enzymes, glucose transporter, and vascular endothelial growth factor [13], recognized to play an important role in a variety of systemic and local adaptive responses, including angiogenesis, metabolism and so on [6]. The involvements of HIF-1α in vascular diseases such as atherosclerosis and carotid stenosis have recently been raised [14-18]. The spectrum of genes targeted by the HIF system makes it an appealing pharmacological target for treatment of diseases including anemia, ischemic stroke, and wound healing [10], proposing the therapeutic prospect of HIF in these ischemic diseases. Except for the regulating factors, the temporal expression pattern of HIF is also a potential research point, for the cardiac beneficial effect of short-term HIF overexpression and long-term HIF stabilization suggesting the association between the biological effects and response duration of HIF [19]. The expression of HIF-1increased exponentially as oxygen concentration declined from 20% to 0.5% [1, 20-23], its expression might be associate with oxygen concentration. For example, the induction of HIF-1areached peak about 30 min after 1% O_2 was applied [1], but its maximum expression appeared at 5 h under 10% O_2 [24]. Studies have shown that HIF-1α is a short-term stable marker and may be degraded in the long-term, HIF-1α expression was detected within 30 min and peaked at 4 h of continuous hypoxia (1% O_2) [24]. The HIF-1 DNA binding activity decayed rapidly when hypoxic cells were exposed to increased oxygen tension (20% O_2) within 5 min, and completely eliminated at 15 min [25]. HIF-2α showed a little bit slower response, and continuously expressing up to 6 h of exposure to hypoxia (8% O_2) [6]. Previous study demonstrated homologous cells employed different aspects of hypoxic response pathways in adjacent region, for example, HIF-2α was found induced by hypoxia in peritubular endothelial cells and fibroblasts, whereas HIF-1α was expressed in tubular cells. HIF-1α was reported expressed under normoxic baseline conditions, and its expression was transient as long as 3 h. However, HIF-2α was induced at 4 h under 1% O_2 and its protein induction reached peak at 6 h, diminished after 12 h [6]. Considering the beneficial effect of HIF pathway, the hypothesis was raised that specific pretreatment such as hypoxia attack could activate the protective scheme of cells, so that cells could survive from the rugged environment. Previous study showed 24-h-pretreatment with the PHD inhibitors followed by 24-h-reoxygenation significantly reduced the cell apoptosis in hypoxia (0.3% O_2) [10], confirming the feasibility of the novel assumption.

PHDs Improve Cellular Adaptation to Hypoxia in Certain Condition

Seven In Absentia Homologue (SIAH) proteins are E3 ubiquitin ligases [26], which could be induced by hypoxia within 2 h, regulating the expression of HIF-1α *via* controlling the half-life of PHD3. Under 10% O_2 for 5 h, Siah2 transcript up-regulated significantly while HIF1α did not change that much. Thus, it was reported as a positive feed-forward mechanism to increase HIF-1α stability through remaining sufficient oxygen for PHD3 activity in hypoxia [24]. The expression ofsiah1 was found linear dependent with the expression of HIF-1a and tumor size in Oral Squamous Cell Carcinoma (OSCC), and the hypothesis was raised that silencing Siah function in carcinomas could be an effective approach to impairing vascular formation [27]. Preconditioning was proven effective in preventing cardiac ischemia injury in mice via increasing HIF-1α transcriptional activity under normal conditions [28]. PHD2 was associated with protection of the heart from an acute ischemic insult, as it could regulate the formation of capillary area [29]. Specific PHD inhibitors (PHDi) had been investigated to applied for the treatment of ischemia-reperfusion injury [29-33], for their abilities of down regulating hepcidin, improving iron absorption and increasing the endogenous production of erythropoietin [5], for example, DMOG was applied to mice to activate HIF-1α 2 h in advance, the attenuated myocardial infarction size was observed [28]. These studies suggested the potential of preconditioning through HIF/PHD pathway to protect cells from hypoxia injury [33].

ROS Activate the Cellular Self-Protective Pathway Under Hypoxia

Oxygen is known responsible for most of Adenosine Triphosphate (ATP) production as the major electron acceptor in oxidative phosphorylation [34]. In fact, 20% to 70% of the oxygen in mammalian cells was consumed by the plasma membrane-localized Sodium Potassium ATPase (Na/K-ATPase) [35]. As the largest oxygen consumption pathway in prokaryotic and eukaryotic organisms, the electron transport chain is most sensitive to changes in oxygen conditions and has a profound impact. When the oxygen level is greater than 3%, as long as the mitochondrial Electron Transport Chain (ETC) reaction is not affected, the cell will adjust the metabolic level for hypoxia, usually within a few minutes to a few hours [4]. Hypoxia can quickly and reversibly inhibit the endocytosis of plasma membrane α-subunits. The activity of Na/K-ATPase [36-38]. Acute hypoxia does not change the AMP/ATP ratio, increases the intracellular calcium level, activates the CAMMK/AMPK pathway within 1 h under 1.5% O_2 [39], and generally changes the metabolic level. The hypoxic stabilization of HIF-1α and HIF-2α required the functionality of complex III of ETC, and mitochondria functioned as O_2 sensors released Reactive Oxygen Species (ROS) to the cytosol [40,41]. ROS are oxygen-containing reactive molecules that can be naturally generated from biological procedures, including

the mitochondrial metabolic and ionic changes caused by the lack of oxygen in the ischemia procedure [42]. To maintain metabolic levels, HIF up regulate glucose transporter genes and glycolytic enzymes, and inhibit oxidative phosphorylation by preventing the conversion of pyruvate to acetyl-Co-A, reducing glucose oxidation and inhibiting β-oxidation of fatty acids [43-47]. Hypoxia reduces the formation of aspartic acid from oxaloacetate in the TCA cycle (necessary for nucleotide synthesis and cell proliferation), as well as affects cell proliferation [4]. Low level ROS acts as important signaling molecules involved in multiple activities such as immune response, muscle contraction, including the protective HIF pathway of Ischemia Preconditioning (IPC) [42], while elevated ROS levels might cause a variety of damages [48]. Despite these negative effects, a small amount of ROS generation is critical for normal cell function, appropriate amount of ROS was demonstrated as a signaling molecules in the existed therapies such as pharmacological interventions and post-conditioning to improve cell survival [49], hold great potential in attenuating Ischemia Reperfusion (IR) injuries [42], including the activation of Ca^{2+} intracellular cascades [42], Extracellular Signal-Regulated Kinases (ERK)1/2 and Jun Amino-Terminal Kinases (JNK) [50,51]. Hypoxia increases mitochondrial ROS to activate HIF-dependent induction of Human Telomerase (hTERT) gene expression. Some scholars have proposed that mitochondrial ETC produces superoxide activation signaling pathways, and the ROS produced by mitochondria explode and are distributed in mitochondria and cytoplasm. Trigger cells adaptive response to hypoxia, but long-term exposure to hypoxia can cause cell damage, decrease ETC activity, and decrease ROS production. HIF-1 also regulates the composition of Cytochromec Oxidase (COX) subunits to optimize respiration efficiency during hypoxia, and reduces ROS by promoting ROS clearance, and inhibits mitochondrial ROS production through HIGD1A/PDK1/COX4I2/NDUFA4L2. This may also indicate that in the short-term hypoxia response, the HIF-1 response accounts for the main part [38,42,52]. Nuclear factor erythroid-2-Related Factor 2 (NRF2) is the master transcriptional regulator of the antioxidant response [53]. NRF2 was activated following multiple oxygen concentrations, including anoxia (0 h to 12 h, 3 to 6 h maximum) [54], acute hypoxia (24 h at 1% O_2) or chronic hypoxia (4 h at 1% O_2 for 7 days) [55], but only chronic hypoxia pre-treatment protected cells from doxorubicin-induced DNA damage by inducing PARP1 gene expression [56]. In another study, the level of NRF2, GR, and catalase were significantly increased in Human Lens Epithelial Cells (HLECs) treated for 3 h, protecting cells from stress through inhibiting ROS. In contrast, HLECs treated for 5 h induced the apoptotic Unfolded Protein Response (UPR) and significant levels of the production of ROS and resulted in apoptosis [57]. The increasing mitochondrial ROS under hypoxia leads to dissociation of NRF2 from its negative regulator KEAP1, then NRF2 translocates to the nucleus to regulate the transcription of antioxidant response genes [53]. Except that, experiments with iNOS knockout mice suggest that NO plays a key role in mediating the cardio protection observed following HIF-1α activation [58]. Cardiolipin was reported as a key phospholipid component of inner mitochondrial membranes, which reduced during ischemia, and ROS-mediated cardiolipin peroxidation significantly impaired complex III activity within the respiratory chain during IR [59,60]. Having said all of above, rational utilization of ROS production may be a step to improve the adaptive capacity of cells in hypoxia process.

UPR and UPRmt Involve in the Cellular Self-Regulation in Hypoxia Condition

Endoplasmic Reticulum (ER) is a central organelle for protein synthesis; modifications and transport, the protein bonds formation during the post-translational folding in the ER are oxygen dependent [61]. Since mitochondria are separated from the cytosol and ER by their bilayer membranes, the mitochondrial stress response mechanisms for translation, folding and refolding proteins were self-controlled. The accumulation of unfolded/misfolded proteins in the mitochondria, the impairment of energy dependent mitochondrial protein import, and the disturbances in mitochondrial protein synthesis and folding all lead to the activation of a mitochondrial Unfolded Protein Response (UPRmt) [62-65]. Despite the HIF-related responses reduce the detrimental effects of anaerobic glycolysis and energy availability, this metabolic change eventually disturbs cellular homeostasis. The maintenance of ion homeostasis and the related redox potential was limited by this energy deficiency, affecting the protein and lipid synthesis, protein folding capabilities as well as ROS activity [43,66], resulting UPR activation to preserve ER homeostasis [67]. The stressors activated the UPR include hypoxia, viral infection, starvation, calcium depletion, hyper- or hypothermia and acidosis [68]. Low glucose condition (<1.5 mM) for 1 h in hypoxia (1% O_2) environment was sufficient to induce the protective UPR, returned to the 5 mM glucose-DMEM in 4% O_2 for 20 h did not produce ROS and apoptosis [57]. Short-term hypoxia often results in the oxidative stress, usually alleviating protein load by reducing protein synthesis within a few hours [4]. Controlling ER homeostasis depends on the interplay between UPR signaling pathways initiated by three distinctive transmembrane sensors [69]. The accumulation of misfolded proteins in the ER induces the expression of multiple chaperone proteins including BIP (binding immunoglobin protein). BIP dissociates from luminal domains of three proteins, Protein Kinase RNA-like Endoplasmic Reticulum Kinase (PERK), the Inositol-Requiring Enzyme 1α (IRE1α), and Activating Transcription Factor 6 (ATF6) [62,69,70]. The activation of PERK pathway was reported in normal cells [71,72].

Phosphorylated eukaryotic Initiation Factor 2α (eIF2α) binds the guanine nucleotide exchange factor eIF2B with high affinity interfering with the assembly of a 43S translation initiation complex [73,74], activated PERK phosphorylates the eIF2α, leading to general translation attenuation [62]. In response to a specific level of UPR, PERK protected cells and restored to normal proliferative activity within 2 h [74], within a few minutes after exposure to acute hypoxia (less than 0.1% O_2), PERK-mediated phosphorylation of eIF2 was observed in the cells, and this reaction rate continued to decrease as the oxygen concentration increased [75]. Cells with impaired UPR, such as those whose PERK and eIF2α signals are abolished, are more sensitive to ER-induced cell death than their wild-type counterparts, which may be due to protein toxicity. In short, the activation of UPR plays an important role in the response and adaptation of cells to hypoxia [76]. PERK and eIF2α could be potential targets to enhancing adapting ability of cells in hypoxia environment. Considering HIF-1 signaling is partially sustained during the transition from HIF-1 to HIF-2 expression, the activation of the PERK axis was suggested regulated by HIF-1, which is the replacement mechanism of HIF-2 in long-term hypoxia [21,77,78]. The PERK/ATF4 axis has been reported as a limiting factor for Erythropoietin (EPO) production, which might limit cell adaptation to hypoxia [79]. Upon the dissociation of

BIP, IRE1α undergoes oligomerization and autophosphorylation and thus gains endoribonuclease activity [80]. To decrease the ER load, activated IRE1α degrades RNAs and miRNAs through Regulated IRE1α-Dependent Decay (RIDD). Previous study suggested that under low ER stress conditions, IRE1α preferentially splices XBP1, while at higher stress it also causes ER-localized mRNA decay [80]. IRE1α also performs splicing of XBP1 mRNA to release transcriptionally active XBP1s, which involved in ER membrane biosynthesis, disulfide bond formation and ER-Associated Degradation (EDEM), to restore ER homeostasis [81]. The ER stress inductive agent was used to treat cells, within 2 h, IRE1α was induced significantly, and the splice of XBP1s was at peak 4 h to 8 h after IRE1α activation [82]. Moreover, IRE1α could activate a proapoptotic kinase JNK1 [52,62]. ATF6 is a member of the leucine zipper protein family, constitutively inducing the promoter of glucose regulated protein genes through activation of the Endoplasmic Reticulum Stress Element (ERSE) [83]. ATF6 translocation to Golgi is induced by BIP dissociation, where cleavage of this protein results in release of transcriptionally active ATF6f, inducing BIP expression, lipid synthesis, N-glycosylation, CHOP expression and ERAD [52,84-86]. Also, ATF6 was reported as a target of p38 Mitogen-Activated Protein Kinase (MAPK) phosphorylation [87]. ATF6 is constitutively expressed as a 90-kDa protein in non-stressed cells, within 2 h of UPR-inducer treatment, endogenous ATF6 protein level dropped initially [88], at 4 h following stress, a soluble nuclear protein form of ATF6 was detected. With prolonged inducing treatment, the total amount of ATF6 also increased [83]. Another research towards glucose intake and insulin resistance set up a mice model for intermittent hypoxia (IH, normoxia 15s+hypoxia 15s), after 1 day, IH group showed significant insulin resistance and impaired glucose tolerance [55]. Several *in-vivo* studies suggest that whereas acute exposure to Continuous Hypoxia (CH) can induce insulin resistance, chronic exposure to CH may be associated with normalization, or even an enhancement of metabolic function [89,90]. Some studies have studied the relationship between UPR and DNA damage through meta-analysis, and screened out some representative regulatory factors [91]. For example, SRC is a protein-tyrosine kinase that is over expressed in a variety of cancers and induced in hypoxia [92], and the serine-threonine kinase, GSK-3β, contributes to the hypoxia-induced UPR [93], suggesting UPR could protect cells from apoptosis through this way. Specificity protein 1 (Sp1) is a transcription factor involved in cell cycle and proliferation [94]. Sp1 and HIF collaborate upon exposure to 1% O_2 for 16 h to activate genes needed to adapt to the hypoxic microenvironment, most of these genes regulated lipid metabolism [95]. Clusterin (CLU) is a pro-survival chaperone-like protein stabilizing unfolded proteins, demonstrated essential in DNA repair and cell cycle regulation [96,97]. It has previously been shown that HIF-1α directly bound to Hypoxic Response Elements (HRE) in the CLU promoter under 1% O_2 for 6 h [98]. And CLU was found required for the activation of pro-survival autophagy pathway when the human proximal tubular epithelial cells were exposed to 1% O_2 [99]. Nuclear CLU was reported contained a conserved BH3 domain and this domain binded to anti-apoptotic Bcl-2 family proteins Bcl-XL and Bcl-2 in a similar manner to those of other BH3-onlyproteins [100]. Tunicamycin was used as a UPR mimic, as it could block glycosylation and disturb the folding of newly synthesized protein, resulting in the accumulation of misfolded proteins. The proliferative activity of cells could recovery under tunicamycin treatment within 1.5 h, suggesting the self-recovery ability of cells function normally in short-term UPR interference [74]. The modifications of misfold/unfold proteins include the expansion of the endoplasmic reticulum membrane, controlling the folding and quality control of key protein components, and reducing protein influx. However, prolonged hypoxia would aggravate the intensity of UPR, which could damage the normal functional protein synthesis, especially in protein secreting cells. For example, the chondrocyte survived from ER stress lost its terminal differentiation ability, producing a chondrodysplasia phenotype in mice [101]. Moreover, if the ER stress is not relieved, UPR will trigger apoptosis [102]. Thus, it is crucial for cell fate and function to control the stimulation intensity.

Cellular Adapt to Hypoxia Condition via Autophagy

As mentioned before, autophagy was a normal pathway for cell survival, selectively degrading specific organelles including mitochondria (mitophagy), peroxisomes (pexophagy), ER (ER-phagy or reticulophagy), nucleus (nucleophagy) and aggregate-prone proteins (aggrephagy) [103]. Above these, ER-phagy was proved associated with hypoxia, for it was activated by UPR under severe ER stress. Obviously, autophagy is mostly delayed and occurs when cellular organelles or protein molecules are damaged. An important mechanism for UPR to bypass hypoxia and oxidative stress damage is the induction of autophagy. Autophagy is a conservative intracellular pathway in which dysfunctional organelles are "Digested" by endogenous enzymes to recover and reuse their components [104-107]. Autophagy activation secondary to UPR stimulation reduces apoptosis during hypoxia/preoxygenation injury [108]. In the early stage of hypoxia, the reduction of ER and oxidative stress leads to a decrease in macrophage apoptosis [108]. FAM134B, one of the ER-phagy receptor, was demonstrated induced after 24-h-hypoxia (1% O_2), promoting this protective pathway through binding to the ER chaperone BIP [108]. CLU was proved to be a key cytoprotective factor, the CLU-mediated autophagy pathway was activated in renal cells after incubating in 1% O_2 for 24 h, and CLU knockdown led to a reduction in UPR related gene expression [99]. However, autophagy mostly occurs in the middle or late stage of cell adaptation, inevitably accompanied by some programmed apoptosis processes, which is not, reviewed much in this paper.

Conclusion and Future Perspectives

With the more theoretical study developed into hypoxia, researchers have discovered that specific hypoxic conditions could make cells transit into a state of adaptive phase. Through pre and post-transcriptional change, cells modulate the response intensity to adapt to the external stimuli and metabolic pattern. From this review, rapid extreme hypoxia pretreatment could induce plenty of cellular adaptative response such as ROS generation, UPR activation and metabolic change, to survive in a scarce environment, and associated therapeutic molecules have been put into practice. During the treatment of endodontic diseases, multipotential dental stem cells will experience hypoxia procedure like above, it would be astounding if the appropriate pretreatment of implanting dental cells to enhancing the cellular biocompatibility is figured out. Future research is warranted to explicit the treatment modalities of precondition as well as to develop reliable pharmacological agents to enhance the protective efficacy of existing vital pulp therapies.

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