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In vitro analysis of oxidative stress biomarkers and mitochondrial function in neuronal cell lines under hypoxic conditions

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Abstract

Objective: The present research examined the changes in oxidative stress biomarkers and mitochondrial functions of differentiated SH-SY5Y neuronal cells under hypoxia. **Methods:** SH-SY5Y cells that were differentiated were placed in 1% oxygen over 6, 12 or 24 h (n = 6). Cell viability, lactate dehydrogenase release, catalase activity, caspase-3 activity, intracellular Reactive Oxygen Species (ROS), malondialdehyde (MDA), protein carbonyls, glutathione status, superoxide dismutase and catalase activity, mitochondrial membrane potential (ΔPSm), ATP production, mitochondrial superoxide, oxygen consumption rate, and respiratory Complex I/IV activity were measured. **Findings:** Hypoxia contributed to the progressive stabilization of HIF-1, high levels of ROS, oxidation of lipids and proteins, loss of glutathione stores, and loss of antioxidant enzyme activity. Mitochondrial abnormalities included the loss of ΔPSm , reduction in the production of ATP, a decline in oxygen consumption, deterioration of Complex I/IV activity, and an increase in mitochondrial superoxide. Close relationships between oxidative stress and bioenergetic failure were detected as there were strong negative correlations between ROS and ΔPSm ($r = -0.94$) and between MDA and cell viability ($r = -0.93$). **Conclusion:** Persistent hypoxia induces an orchestrated oxidative stress and mitochondrial dysfunction which ultimately leads to neuronal apoptosis, making integrated redox and mitochondrial biomarkers useful tools in mechanistic studies and neuroprotective drug discovery.

Keywords: Hypoxia, Oxidative stress, Reactive oxygen species, Mitochondrial dysfunction, Glutathione, Neuronal apoptosis

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1. Introduction

The Central nervous system is very susceptible to oxidative damage. Neurons have a high level of oxidative metabolic activity, high levels of polyunsaturated lipids in membranes, relatively small antioxidant reserves – in the body, and a limited ability to regenerate, which makes them highly susceptible to damage should there be a shift in the ratio of producer to antioxidant in the body (Teleanu *et al.*, 2022). This imbalance towards the formation of pro-oxidant species is a common element of any acute or chronic neurological conditions and is

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responsible for the resulting lipid peroxidation, protein oxidation and nucleic-acid damage leading to neuronal dysfunction and death (Houldsworth, 2024; Jomova *et al.*, 2023).

Mitochondria play a central role in the overall process. They are the main intracellular source of ROS (electrons escaping the respiratory chain, especially at Complex I and III, which reduce molecular oxygen to superoxide which can then be converted to other reactive species (Onukwufor *et al.*, 2019)), and they are the main site of ATP synthesis that occurs aerobically. Mitochondria are also an abundant source of ROS, and are a principal target of ROS injury, thus creating a vicious cycle of injury – failure of bioenergetic metabolism.

Oxygen availability is the key aspect that determines this system. The transcription factor Hnf 1 HIF-1; HIF-1) stabilizes quickly in response to the hypoxic environmental factor, and is closely linked to the adaptive homeostatic metabolism to hypoxia; HIF-1 is directly coupled with the architecture, respiration and ROS (reactive oxygen species) homeostasis of mitochondria (Huang *et al.*, 2023). Paradoxically however, under acute hypoxia, mitochondrial ROS level can be dramatically increased to induce either cytoprotection via HIF-1 α signalling (or not, depending on the length and extent of acute hypoxia) or to promote pro-apoptotic signalling (under severe and/or prolonged acute hypoxia) (Yoo *et al.*, 2019).

Hypoxic and ischaemic insults will consistently produce an increase in intracellular and mitochondrial ROS, a decrease in antioxidant buffering, a loss of mitochondrial transmembrane potential and activation of mitochondrial programmed cell death pathways in models of neurons. Chemical hypoxia and OGD selectively induce cell death through programmes of cytosol and mitochondrial ROS production, caspase-dependent pathways of apoptosis and pyroptosis (Park *et al.*, 2024) whereas OGD/reperfusion leads to accumulation of ROS and morphological and functional changes of mitochondria which are responsible for cell death (Zhuge *et al.*, 2023).

The iSY5Y human neuroblastoma cell line, especially when differentiated into a neuronal phenotype with Retinoic Acid (RA), is a popular in vitro model for understanding neuronal redox biology and mitochondrial integrity and has been used to show a correlation between mitochondrial membrane-potential loss, ROS generation, and apoptosis, mitophagy and glutathione depletion under a variety of stressors (Rehfeldt *et al.*, 2021; Huang *et al.*, 2021; Pienkowska *et al.*, 2023). It is, however, less systematically described how the biomarkers of oxidative stress are affected by a quantitative measure of mitochondrial function in the same experimental setting in respect of the temporal components of a graded hypoxic exposure.

Hence, the present study presents an integrated in vitro, time resolved analysis of the neuronal response to hypoxia. We performed a panel of oxidative stress-based biomarkers together with a number of mitochondrial functional metrics and cell viability assessments on differentiated SH-SY5Y cells exposed to 1% O₂ for 6, 12 and 24 h and looked at the correlations between all these metrics to identify a coherent biomarker signature of hypoxic neuronal damage and the probable mechanistic cascade from which it originates (Kwak *et al.*, 2025).

2. Materials and methods

2.1. Cell culture and differentiation of neurons

All experiments were done in SH-SY5Y human neuroblastoma (NB) cell line that was cultured in Dulbecco Modified Eagles Medium (DMEM)/Hamns F-12 (1:1) at 37 °C with a wet and humid environment of 5% CO₂ and 10% Foetal Bovine Serum (FBS). Differentiated into a neuronal phenotype by culturing (5 days) with all-trans retinoic acid (10 μ M), in reduced-serum medium, then subjected to experiment. They were seeded in standard protocol and in all the types of differentiated cells, they were given a chance to settle down before subjected to hypoxia.

2.2. Hypoxic exposure

The hypoxia was induced by flushing a controlled atmosphere incubator/chamber with 1% O₂, 5% CO₂ and the balance as N₂. Cells were either made hypoxic for 6, 12 or 24 h or were kept in normoxic (21% O₂) conditions for the same time. Three levels of Angiogenin were used for the induction of hypoxia, and hypoxia HIF-1 α levels were verified by the stabilisation procedure. Biological experiments were repeated six times.

2.3. Cell viability, cytotoxicity and apoptosis

Cell viability was measured using a MTT reduction, and presented as a percentage relative to the normoxic control. A measurement of damage to the membrane was affected by releasing the enzyme lactate dehydrogenase (LDH) and was reported as percentage cytotoxicity. The caspase-3 enzymatic activity was measured as apoptotic activation and it was represented as fold change with respect to control. Western blots were performed to quantify levels of HIF-1 α protein and normalise to a housekeeping protein.

2.4. Oxidative-stress biomarkers

Intracellular ROS was measured with the help of 2,7- dichlorodihydrofluorescein diacetate (DCFH-DA) and expressed as a percentage of the control fluorescence. The level of lipid peroxidation (in the form of malondialdehyde-MDA) was measured with the help of thiobarbituric-acid-reactive-substances method, the content of protein carbonyls, which is a measure of protein oxidation, was measured with the help of 2,4-dinitrophenylhydrazine assay. The amounts of reduced glutathione (GSH) and oxidised glutathione (GSSG) were determined by performing enzymatic recycling assay and the proportion of GSH-GSSG ratio was calculated. The actions of catalase and superoxide dismutase (SOD) were spectrophotometrically determined. Bicinchoninic acid assay was used to determine total protein and the biochemical values were normalised to total protein.

2.5. Mitochondrial function

Mitochondrial membrane potential ($\Delta\Psi_m$) was evaluated by detecting the ratiometric probe, JC-1 (red/ green fluorescence ratio) as a percentage of control. ATP was quantitated in cells by measuring the luminescence through a luciferase reaction. MitoSOX was used to detect mitochondrial superoxide. Extracellular-flux analysis was used to measure basal Oxygen-Consumption Rate (OCR) and spectrophotometric methods employed to measure the enzymatic activity of Complex I and IV of respiration, both relative to control.

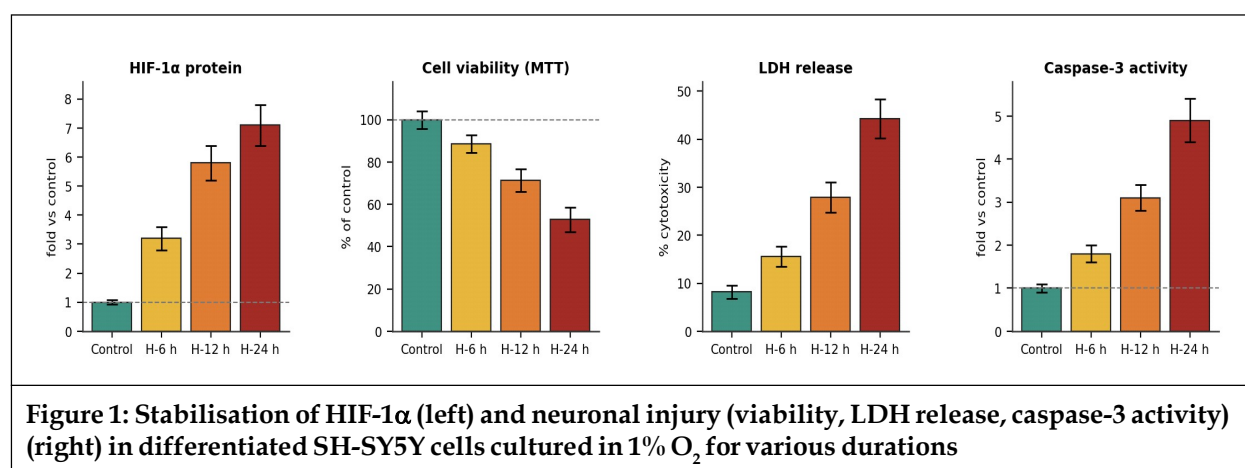
2.6. Statistical analysis

All data are mean $\pm$ standard deviation (SD) of six separate experiments. The differences between groups were tested using one way analysis of variance (ANOVA) followed by Tukey's post hoc test. Pearson's correlation coefficient was used to determine association between oxidative-stress and mitochondrial parameters and each parameter and the duration of hypoxia. A p-value of < 0.05 was deemed statistically significant for both-tailed tests. GraphPad Prism analysed the data.

3. Results

3.1. Hypoxia validation and neuronal viability

1% O₂ exposure induced a strong (3.2, 5.8 and 7.1-fold) and time-dependent stabilisation of HIF-1 α at 6, 12 and 24 h, respectively, demonstrating an efficient induction of hypoxia. The viability of the neurons decreased to 88.6% at 10 H, 71.4% at 20 H, and 52.8% at 40 H, compared with the control, and the release of LDH increased (cytotoxicity: 8.2% to 44.3%) at the same time, suggesting that the cell deaths were mainly apoptotic deaths (Figure 1); the activity of caspase-3 increased fourfold after 24 h.



3.2. Oxidative-stress biomarkers

A progressive pro-oxidant transition was brought about by hypoxia and not hyperoxia. At 6, 12 and 24 h, levels of intracellular ROS were 168%, 242% and 318% higher compared to the control, respectively; the incorporation MDA and protein carbonyls, on the other hand, is increasingly between 1.24 nmols/mg protein and 4.82 nmols/mg protein, and 0.86 nmols/mg protein and 3.15 nmols/mg protein, respectively, indicating progressive oxidation of lipids and proteins. The reduced glutathione levels were also reduced by 63% (38.6 to 14.3 nmol/mg protein) and GSH/GSSG ratio had decreased by 75 (18.4 to 4.6). The biphasic pattern of increase at 6 h (15.9+/-1.8 U/mg protein) and subsequent decrease than control at 24 h was observed in SOD activity whereas catalase activity only decreased at 24 h (Table 1).

Table 1: Oxidative-stress biomarkers under hypoxia				
Parameter	Control	Hypoxia 6 h	Hypoxia 12 h	Hypoxia 24 h
Intracellular ROS (% control)	100 ± 6.5	168 ± 12*	242 ± 18*	318 ± 24*
Malondialdehyde (nmol/mg protein)	1.24 ± 0.15	2.18 ± 0.22*	3.46 ± 0.31*	4.82 ± 0.44*
Protein carbonyls (nmol/mg protein)	0.86 ± 0.11	1.42 ± 0.16*	2.28 ± 0.24*	3.15 ± 0.30*
Reduced glutathione (nmol/mg protein)	38.6 ± 3.4	30.2 ± 2.9*	21.7 ± 2.4*	14.3 ± 2.0*
GSH/GSSG ratio	18.4 ± 1.8	12.6 ± 1.4*	7.9 ± 1.1*	4.6 ± 0.8*
SOD activity (U/mg protein)	12.8 ± 1.2	15.9 ± 1.5*	10.4 ± 1.3*	6.8 ± 0.9*
Catalase activity (U/mg protein)	24.6 ± 2.3	22.1 ± 2.4	16.8 ± 2.0*	11.2 ± 1.6*

3.3. Mitochondrial function

All mitochondrial function deteriorated proportionate to the occurrence of the oxidative changes. By 24 hours, the mitochondrial membrane potential had decreased to 43.7% of control, and ATP levels had dropped to 45.2%, while mitochondrial superoxide (MitoSOX) had increased to 336% of control. Basal oxygen consumption also decreased to 39.4% of control, while enzymatic activities of respiratory Complex I and Complex IV decreased to 44.8% and 41.3% respectively, suggesting a combined depolarisation, bioenergetic failure and dysfunction of electron-transport-chain (Table 2).

Table 2: Basal oxygen-consumption rate, ATP, mitochondrial membrane potential, Complex I/IV activities and mitochondrial superoxide are measured across hypoxic time points				
Parameter	Control	Hypoxia 6 h	Hypoxia 12 h	Hypoxia 24 h
Mitochondrial membrane potential, $\Delta\Psi_m$ (% control)	100 ± 5.8	82.4 ± 5.1*	61.3 ± 5.6*	43.7 ± 5.2*
Cellular ATP (% control)	100 ± 5.2	84.7 ± 4.8*	63.9 ± 5.5*	45.2 ± 5.1*
Mitochondrial superoxide, MitoSOX (% control)	100 ± 6.0	172 ± 14*	258 ± 20*	336 ± 26*
Basal oxygen-consumption rate (% control)	100 ± 5.5	79.8 ± 5.0*	58.6 ± 5.4*	39.4 ± 4.8*
Complex I activity (% control)	100 ± 6.1	81.2 ± 5.4*	62.4 ± 5.7*	44.8 ± 5.3*
Complex IV activity (% control)	100 ± 5.9	78.6 ± 5.2*	57.9 ± 5.6*	41.3 ± 5.0*

3.4. Correlation analysis

Across the full dataset (n = 24), hypoxic duration correlated strongly and positively with intracellular ROS (r = +0.96), MDA (r = +0.97) and MitoSOX (r = +0.96), and strongly and negatively with the GSH/GSSG ratio (r = -0.95), $\Delta\Psi_m$ (r = -0.98), ATP (r = -0.97) and cell viability (r = -0.98). There was an inverse correlation between intracellular ROS and $\Delta\Psi_m$ (r = -0.94), MDA (r = -0.93) and viability (r = +0.95) and a significant inverse correlation of intracellular ROS with all. The link between ROS accumulation and the activation of this series

of events, including mitochondrial depolarisation, energetic failure and cell death, was of a mechanistic type, in line with the aforementioned interrelationships.

3.5. Proposed mechanistic sequence

The temporal and correlative patterns support together an integrated cascade: hypoxia stabilizes HIF-1 α and initiates impairment of the ETC, resulting in a burst of mitochondrial ROS and lipid and protein oxidation and glutathione depletion, collapse of mitochondrial membrane potential, failure of ATP production, and finally, activation of caspase-3 dependent neuronal death (Figure 3).

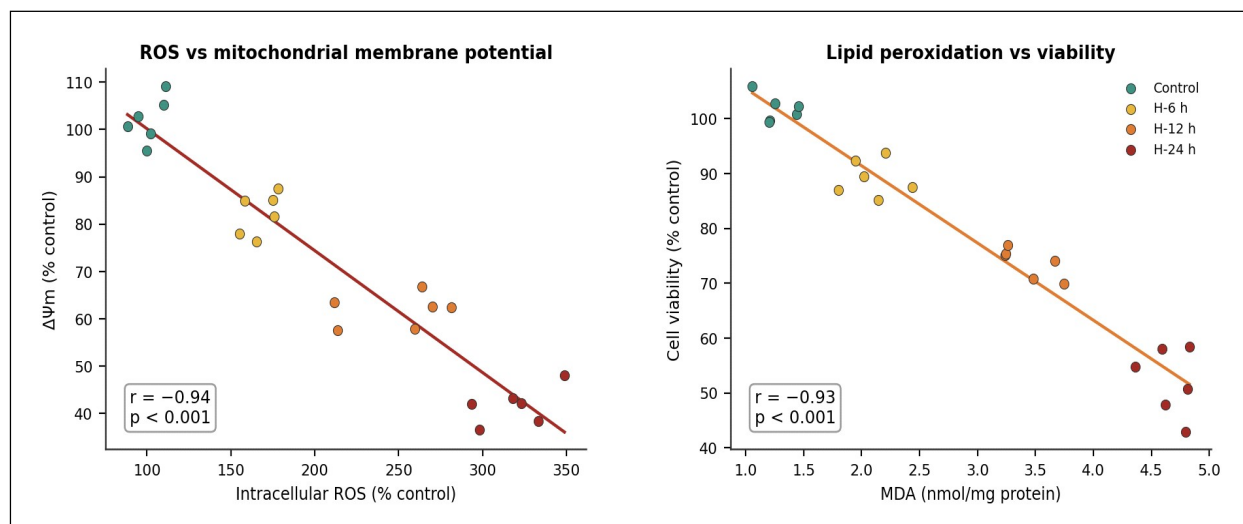


Figure 2: Correlations linking intracellular ROS to mitochondrial membrane potential (left) and lipid peroxidation to cell viability (right), with fitted regression lines

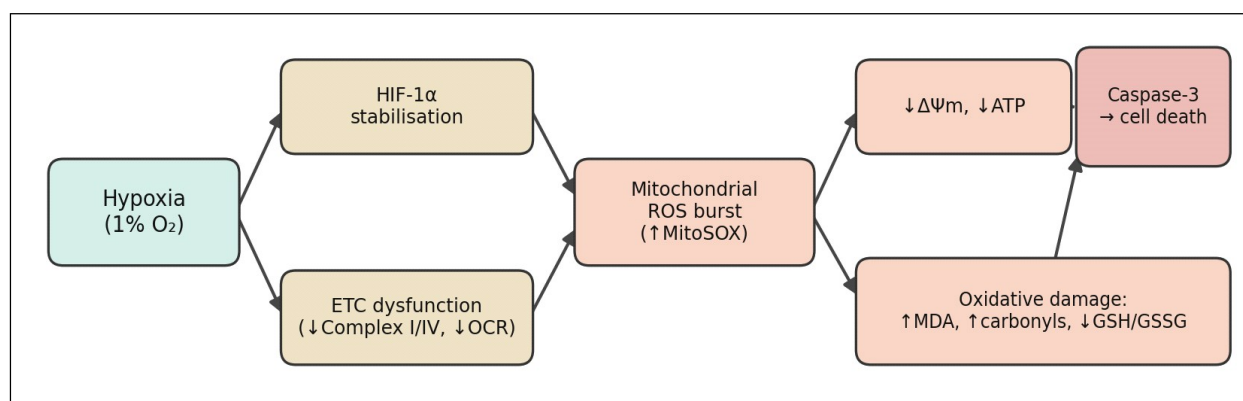


Figure 3: Proposed mechanistic cascade of hypoxia-induced oxidative injury and mitochondrial dysfunction in neuronal cells

4. Discussion

In this study, we use a model of differentiated SH-SY5Y cells to provide an integrated, time-resolved characterization of the neuronal responses to hypoxia, showing that responses consist of a tightly coordinated sequence from HIF-1 α stabilisation to ROS accumulation, to antioxidant depletion, to mitochondrial dysfunction, and to caspase-mediated cell death, that occur as a graded hypoxic exposure. The extent of the correlations between these endpoints suggest that oxidative stress and mitochondrial failure are linked processes of a common injury cascade.

The increase and subsequent maintenance of HIF-1 α at low oxygen tension is indicative of effective hypoxic induction, and supports the hypothesis that HIF-1 α , as the master oxygen sensor, links hypoxia with mitochondrial and metabolic reprogramming. Comparable HIF-1 α stabilisation, ROS accumulation and caspase activation have been reported in SH-SY5Y cells subjected to chemical hypoxia and oxygen-glucose deprivation, where hypoxia-driven ROS trigger caspase-dependent apoptotic and pyroptotic death programmes. The

progressive loss of viability, rise in LDH release and marked increase in caspase-3 activity observed here reproduce this apoptotic trajectory and align with the ROS-dependent mitochondrial injury described following oxygen–glucose deprivation/reperfusion.

The apparently paradoxical increase in ROS under low oxygen is well explained by mitochondrial biology. Even as oxygen becomes limiting, impaired electron flow through the respiratory chain–reflected here in the decline of Complex I and Complex IV activities and of basal oxygen consumption–favours single-electron reduction of the remaining oxygen to superoxide, principally at Complexes I and III. The steep rise in MitoSOX signal localises a substantial part of the oxidative burden to the mitochondria themselves, consistent with the concept of mitochondria as both the source and the target of hypoxic ROS. The resulting oxidative damage was evident across multiple biomarkers, with accumulation of MDA and protein carbonyls and depletion of the GSH pool and GSH/GSSG ratio marking a progressive collapse of cellular redox buffering.

The biphasic behaviour of SOD—an early compensatory rise followed by a decline – together with the steady fall in catalase, suggests that the antioxidant defence system is initially mobilised but is ultimately outstripped and depleted by sustained oxidative load. This pattern of transient adaptation preceding failure mirrors the redox dynamics reported in neuronal oxidative-stress models and underscores the limited resilience of the neuronal antioxidant apparatus. Recent evidence further indicates that hypoxia-induced oxidative injury is closely linked to impaired mitochondrial quality-control mechanisms. Excessive ROS disrupt mitochondrial dynamics by promoting mitochondrial fragmentation while suppressing mitophagy, resulting in the accumulation of dysfunctional mitochondria and amplification of oxidative stress. Restoration of mitochondrial turnover has therefore emerged as an important strategy for preserving neuronal survival under hypoxic and ischemic conditions (Xie *et al.*, 2019).

The mitochondrial data demarcate the consequences of this oxidative injury on the bioenergetics. Combined depolarisation and impaired oxidative phosphorylation are suggested by the parallel decreases of membrane potential, ATP content, oxygen consumption and respiratory-complex activity; mitochondrial depolarisation is at the centre of the mechanisms of hypoxic neuronal death as suggested by inverse correlation between the ROS and the $\Delta\Psi_m$ ($r = -0.94$), and by positive correlation between the $\Delta\Psi_m$ and the viability ($r = +0.95$). A relationship between ROS production, loss of $\Delta\Psi_m$ and cell death was also observed here and is similar to that described for other stressors of SH-SY5Y that are MITO-induced. The accumulation of ROS, loss of $\Delta\Psi_m$ and the resulting cell death observed here are all the hallmarks of the intrinsic apoptotic pathway, and restoration of $\Delta\Psi_m$ has been demonstrated to be cytoprotective in SH-SY5Y models of oxidative stress.

Beyond ATP depletion, disruption of mitochondrial bioenergetics also interferes with calcium homeostasis and intracellular signaling, thereby accelerating activation of intrinsic apoptotic pathways. Recent studies have shown that maintaining mitochondrial integrity through regulation of mitochondrial dynamics and oxidative phosphorylation substantially improves neuronal resistance to hypoxic injury, supporting the mechanistic relationship observed between ROS accumulation, $\Delta\Psi_m$ loss, and reduced cell viability in the present study (Pickles *et al.*, 2018).

The results found are clinically relevant to situations involving central neuronal hypoxia (e.g., ischaemia, hypoxic–ischaemic injury), as well as for the wider issues of oxidative stress and mitochondrial dysfunction in neurodegeneration. They also serve to highlight the importance of mitochondria-directed and antioxidant-based neuroprotective interventions, and the potential for targeting ROS alone to be limited in efficacy if the fundamental mitochondrial dysfunction is not addressed. An integrated biomarker signature (combined redox and bioenergetic endpoints) outlined here could offer a valuable *in vitro* tool to assess candidate neuroprotective compounds.

Emerging neuroprotective strategies increasingly target mitochondrial function rather than ROS scavenging alone. Pharmacological approaches aimed at enhancing mitochondrial biogenesis, improving respiratory chain efficiency, or activating endogenous antioxidant pathways have demonstrated superior neuroprotective effects in experimental models of cerebral hypoxia and ischemia. These findings support the use of integrated oxidative stress and mitochondrial biomarkers, as employed in the present study, for evaluating novel mitochondria-targeted therapeutic interventions (Singh *et al.*, 2019).

5. Conclusion

Time-dependent co-ordinated oxidative and mitochondrial injury occurs in differentiated SH-SY5Y neuronal cells upon hypoxia. Concomitant with progressive HIF-1 α stabilisation are increased levels of intracellular and mitochondrial ROS, increased lipid- and protein-oxidation products, reduced glutathione levels, loss of mitochondrial membrane potential and ATP production, and loss of activity of mitochondrial respiratory complexes and caspase-mediated cell death. These correlations are robust, highlighting mitochondrial ROS generation and loss of membrane potential as key bridges between oxidative stress and bioenergetic failure and neuronal death, and indicate a mosaicked integrated profile of hypoxic neuronal injury for mechanistic interrogation and/or neuroprotective screening.

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