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Neuroprotective Role of SRT1720 Against Hydrogen Peroxide Induced Oxidative Stress in NT2 Cells

Lama N. Abdullah[a*], Ruy Tchao[b], Shanaz M. Tejani-Butt[c]

[a,b,c]*Department of Pharmaceutical Sciences, Division of Pharmacology and Toxicology, Philadelphia College of Pharmacy, University of the Sciences in Philadelphia, Philadelphia, PA, USA*

[a]*Email: ln.abdullah@hotmail.com*

[b]*Email: r.tchao@usciences.edu*

[c]*Email: s.tejani@usciences.edu*

Abstract

Neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) are characterized by a significant increase in neuronal loss. Oxidative stress (OS) plays a significant role on neuronal damage. Reactive oxygen species (ROS) generated from agents such as hydrogen peroxide (H_2O_2) leads to cell damage and reduction of cell viability. Sirtuin 1 (SIRT1) is a therapeutic target for neurodegenerative disorders because it regulates several cellular functions and biological processes that promote cellular longevity. This study was undertaken to examine the role of SRT1720 in protecting cells from H_2O_2 induced stress in Ntera-2 cl.D1(NT2), which has been proven to be a useful *in vitro* system for the investigation of functions related to human neuronal and glial systems. The results provide evidence that H_2O_2 significantly induced oxidative stress in a concentration dependent manner. Moreover, pre-treatment with low concentrations of SRT1720 for 48 hours protected against the effects of H_2O_2. Also, a combination of H_2O_2 and SRT1720 improved cell viability. Interestingly, apoptotic or necrotic cell death was not detected after H_2O_2 treatment in the cell culture model system employed.

Keywords: Oxidative stress; H_2O_2; SRT1720; NT2 cells.

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* Corresponding author.

1. Introduction

Oxidative stress plays a vital role in the pathophysiology of neurodegenerative diseases and other related conditions such as ischemia. At physiological levels, ROS is considered a signaling molecule. However, at higher levels, ROS is capable of causing protein, nucleic acid, and lipid dysfunction. The most affected part in the body is the brain due to its high oxygen demand and its large quantity of peroxidation-susceptible lipid cells [1]. H_2O_2 is a physiological oxidant that is produced in living cells by various cellular pathways has been widely studied [2]. H_2O_2 can be harmful to cells by impairing essential cellular molecules such as DNA and lipids. OS stress that induced by H_2O_2 reduces cell viability via several pathways including metabolic, apoptotic and necrotic changes. Metabolic changes initiated when oxidative stress causes mitochondrial damage including damage to the mitochondrial respiratory chain and mitochondrial membrane permeability, reduction in ATP levels, mutations in mitochondrial DNA, and disruption to Ca^{2+} homeostasis [3]. Apoptosis is initiated by altering the inner mitochondrial membrane permeability, which leads to release of pro-apoptotic factors such as cytochrome C from the mitochondria into cytosol. Therefore, cytochrome C initiates the caspase cascade and the formation of apoptosomes [4]. Furthermore, OS can activate mitochondrial-dependent process by the translocation of p53 into the mitochondria and forming complex with mitochondrial protein (cyclophilin-D) leading to mitochondrial depolarization and cell necrosis [5]. Sirtuins (silent information regulator genes, SIRTs) are extremely conserved nicotinamide adenine dinucleotide (NAD^+) dependent enzymes. The sirtuin family consists of seven proteins known as SIRT1 to SIRT7 [6]. Sirtuins are class III histone/lysine deacetylases (HDAC) which differ from other classes in that they require NAD^+ for their activity [7]. Subcellular localization, enzymatic activity, and binding targets vary depending on the binding to the catalytic domain in N or C- termini [6]. While, SIRT1, SIRT6 and SIRT7 mainly reside in the nucleus, SIRT3, SIRT4 and SIRT5 are found in the mitochondria and SIRT2 is chiefly cytosolic [6]. In the CNS, SIRT1 has a crucial role in maintaining synaptic plasticity, cognitive functions, and learning and memory function. It also modulates cellular homeostasis through improving neuron survival, insulin sensitivity, glucose metabolism, and mitochondrial biogenesis [8]. SIRT1 increases the function of peroxisome proliferator-activated receptor γ co-activator-1α (PGC-1α), which is an essential regulator of mitochondrial biogenesis and energy metabolism. SIRT1 is reported to exert anti-apoptotic effects via deacetylation of p53 (proapoptotic protein), nuclear factor kappa B (NF-κB), and Forkhead box O (FOXO) transcription factors. Therefore, activation of SIRT1 is a promising target for treatment of neurodegenerative disorders [8]. There are several plant derived metabolites and synthetic chemicals that act as sirtuin-activating compounds (STACs). Resveratrol is a natural polyphenolic compound which is reported to be an effective activator of SIRT1. On the other hand, STACs synthetic agents, including SRT1720 and SRT2183 have been found to activate SIRT1 with a potency of 1000-fold greater than that of resveratrol [9]. These activators could also activate SIRT1 indirectly by targeting several receptors, transporters, and ion channels [10]. For example, targeting serotonin (5-HT) receptors which could be involved with PGC-1α in mitochondrial biogenesis [11]. They antagonize enzymes such as cyclooxygenase (COX-1 and COX-2) reducing inflammation [10]. The present study investigated the extent of oxidative stress induced by H_2O_2 on NT2 cells, and the ability of SRT1720 to reduce this damage.

2. Materials and Methods

2.1. Cell Culture

Human teratocarcinoma Ntera-2 (NT2) cells were purchased from American Type Culture Collection (ATCC). Cells were grown in Dulbecco's modified of eagle's medium (DMEM) (ATCC) supplemented with fetal bovine serum (FBS) (Atlanta) and 1% penicillin-streptomycin (Sigma). NT2 cells were cultivated on a 75 cm^2 flask and incubated at 37^oC with 5% CO_2. The growth medium was changed every two days. The cell culture was split using Trypsin-EDTA solution (Sigma) after cells became ~ 80% confluent. The cells were seeded at a density of 5000 cells/ml and allowed to grow 48 hours prior experiments.

2.2. Hydrogen Peroxide (H_2O_2) Treatment

The stock solution of H_2O_2 (3%) were prepared by diluting with deionized water then Hanks' balanced salt solution buffer (HBSS) were used to make the final concentrations (0.1 µM, 1 µM, 10 µM, 100 µM and 1000 µM) just prior each experiment. NT2 cells were treated with either H_2O_2 or HBSS for control in 96-well microplates and incubated for 30 minutes at 37^oC.

2.3. SIRT1 Agonist (SRT1720) Treatment

SRT1720 (Selleck) were dissolved in Dimethyl sulfoxide (DMSO) then diluted in growth medium to make the final concentrations of (10 nM and 100 nM). The cells were incubated with SRT1720 alone for different time intervals (24 hours and 48 hours). The cells were also treated with SRT1720 as a post- treatment, pre-treatment, or in combination with H_2O_2.

2.4. CyQUANT Assay

The rate of cell proliferation is specified by comparing DNA content for cells treated with H_2O_2 and/or SIRT1720 with untreated controls in 96-well microplates. 200 µl of CyQUANT solution (1 ml of cell-lysis buffer, 19 ml of distilled water, and 50 µl of the CyQUANT dye) (Invitrogen) were added to cells then Incubated for 2–5 minutes at room temperature, protected from light. The cell viability was detected by using a fluorescence microplate reader with excitation at ~485 nm and emission detection ~530 nm [13].

2.5. Annexin V Assay

After treatment with H_2O_2 (100 µM), Muse Annexin V and Dead Cell Assay (MilliporeSigma) were used to detect phosphatidylserine (PS) on the external membrane of apoptotic cells. Cells were treated with 100 µM of H_2O_2 and control cells treated with HBSS for 10 or 30 minutes at 37^oC. Cells were dethatched then 100 µl of Annexin V were added to 100 µl of cell suspension for 20 minutes at room temperature, protected from light. This assay is a fluorescent-based analysis that provides quantitative analysis of live, early and late apoptosis, and cell death. Cell were analyzed using Guava Muse Cell Analyzer [14].

2.6. LIVE/DEAD Viability/Cytotoxicity Assay

This assay is a two-color fluorescence cell viability assay that measures intracellular esterase activity and plasma membrane integrity. Calcein AM and ethidium homodimer (EthD-1) are the dyes used for the assay. Calcein AM is retained within live cells forming a green florescence while EthD-1 produces a bright red color in dead cells by entering through damaged membranes. The NT2 cells were seeded at cell concentration of 5000 cells/ml in 35 mm x 10 m cell culture dishes for 48 hours. Cells were treated with 100 µM of H_2O_2 and control cells treated with HBSS for 30 minutes. LIVE/DEAD staining solution (5 ml of HBSS,10 µl of ethidium homodimer-1, and 5 µl of calceine AM) (Invitrogen) was added to cells and incubated for 20 minutes at room temperature, protected from light. Cells were rinsed with HBSS and observed using fluorescence microscope [15].

2.7. PrestoBlue Assay

PrestoBlue reagent (Invitrogen) consists of resazurin as an active ingredient which is a nontoxic, cell-permeable molecule. Resazurin is blue in color and non-fluorescent until it enters live cells that reduced it to a red and highly fluorescent compound called resorufin; in this way cell viability can be detected. The cells were seeded in 24-well microplates. After two days of cell growth, cells were treated with 100 µM of H_2O_2 or HBSS for control cells for 30 minutes at 37^{o}C. Cells washed with HBSS and then PrestoBlue solution (1 ml of PrestoBlue dye + 9 ml of HBSS) was added for 30 minutes at room temperature. Cell viability was measured using a fluorescence microplate reader with excitation wavelength at ~540 nm and emission wavelength at ~590nm [16].

2.8. Statistical Analysis

The statistical significance was obtained using unpaired student t-test and one-way ANOVA, from at least three independent experiments. Data were presented as mean ± standard error of mean (SEM). P-value <0.05 was considered significant.

3. Results

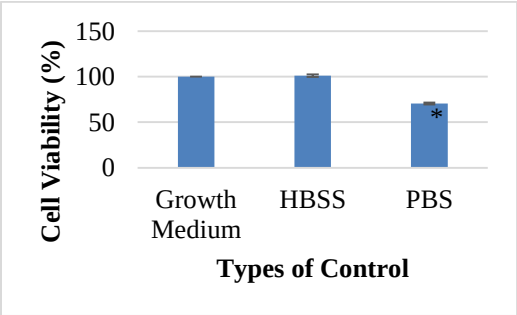


Figure 1: NT2 cell viability following treatment with growth medium, HBSS or PBS for 30 minutes. The percentage of cell viability using CyQUANT assay. (*) represents significant values comparing PBS to growth medium (P<0.05).

In preliminary studies, we observed that NT2 cells treated with PBS buffer for 30 minutes resulted in cell loss when compared to cells treated with growth medium. Based on results, we determined a better buffer for NT2 cells. HBSS contains calcium (Ca^{2+}) and magnesium (Mg^{2+}), which for cell attachment are essential elements (not found in PBS). The NT2 cells that were grown in growth medium or HBSS buffer did not alter NT2 cell viability while PBS caused a significant decline in cell viability by 30% (P=0.000026) (figure 1).

3.1. Dose Effect of H_2O_2 in NT2 cells

The effects of H_2O_2 were evaluated using HBSS buffer instead of PBS. NT2 cells were treated with H_2O_2 for 30 minutes. When the cell viability was measured immediately after treatment (figure 2A) it demonstrated a significant reduction in cell viability (P=1.09E-08). After washing the cells and incubating in growth medium for an additional 24 hours (figure 2B), a significant decrease in cell viability was detected. The results indicated that H_2O_2 significantly decrease cell viability in a concentration dependent manner and NT2 cells did not recover when observed 24 hours after H_2O_2 treatment. As the concentration of 100 µM of H_2O_2 decreased NT2 cell viability by 33% after 30 minutes, this concentration was used for subsequent experiments to detect changes (increase or decrease) in cell viability. In addition, preliminary experiments determined that incubation time of 30 minutes would allow us to uncover early effects of H_2O_2 on NT2.

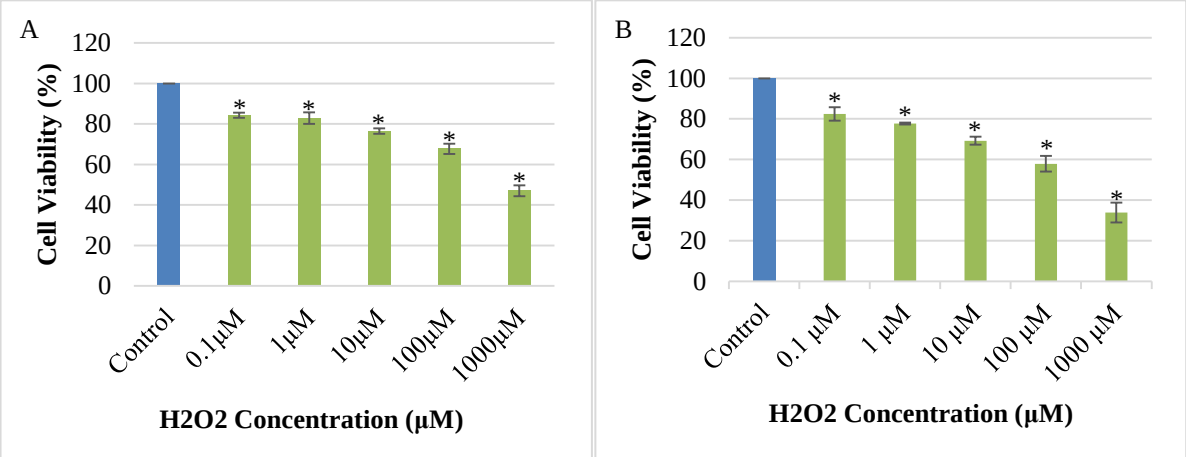


Figure 2: Effect of different concentrations of H_2O_2 on NT2 cell viability. (A) Cell viability following 30 minutes of H_2O_2. (B) Cell viability evaluated 24 hours after treatment with H_2O_2 for 30 minutes using CyQUANT assay. (*) represents significant values comparing control to treatment (P<0.05).

3.2. Effects of SRT1720 in NT2 Cells

NT2 cells were treated with two concentrations of SRT1720 (10 and 100 nM) at two time points (24 and 48 hours). Results indicated that both concentrations of SRT1720 did not significantly alter cell viability following 24 and 48 hours of treatment (figure 3).

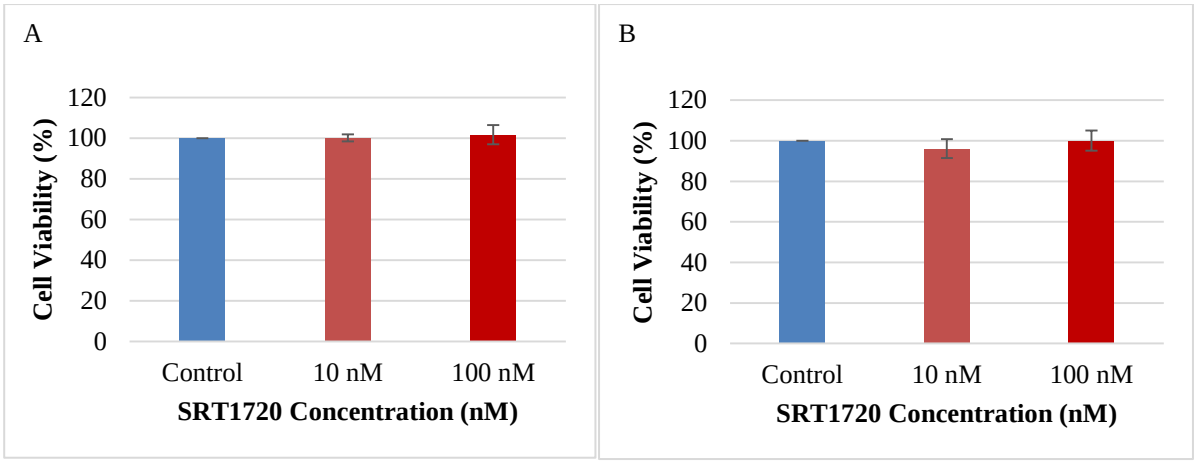


Figure 3: Effect of SRT1720 on NT2 cells. (A) Cell viability following incubation with SRT1720 for 24 hours, (B) Cell viability following incubation for 24 hours 48 hours using CyQUANT assay.

3.3. Effects of SRT1720 and H_2O_2 in NT2 Cells

These experiments were conducted to investigate whether SRT1720 would exert a protective effect against H_2O_2 induced damage.

3.3.1. Pre-treatment with SRT1720

NT2 cells pre-treated with SRT1720 (10 and 100 nM) for either 24 hours or 48 hours before treating with H_2O_2 (100 µM) for 30 minutes. Pre-treatment with SRT1720 (100 nM) for 24 hours significantly improved cell viability (P=0.04). Moreover, both concentrations of SRT1720 significantly improved NT2 cell viability (P=0.03) compared to treatment with H_2O_2 (figure 4).

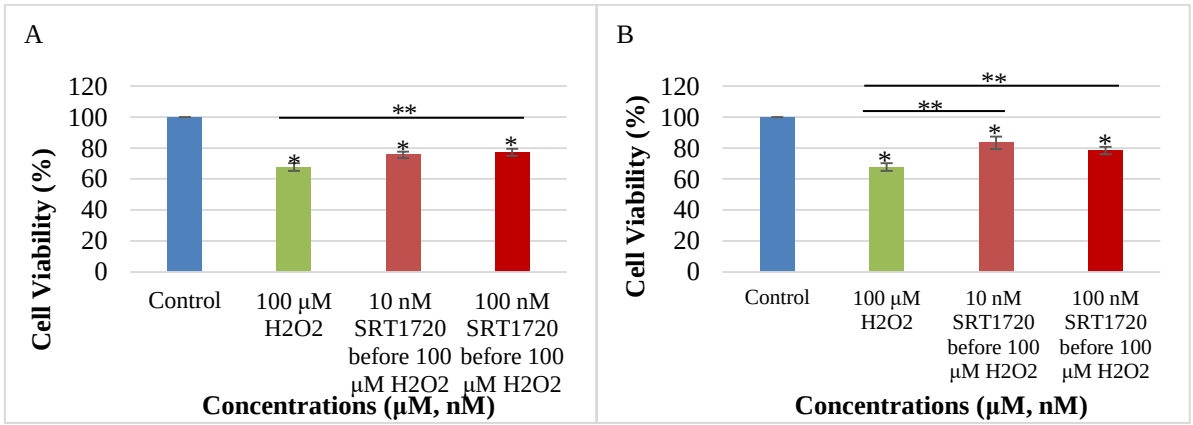


Figure 4: Effect of pre-treatment with SRT1720 hours on NT2 cells. Cells were first incubated with SRT1720 for (A) 24 hours or (B) 48 hours then H_2O_2 was added and incubated for 30 minutes. The percentage of cell viability was measured using the CyQUANT assay. (*) represents significant values comparing control to treatment (P<0.05). (**) represents the significant difference between H_2O_2 alone and cells pre-treated with SRT1720.

3.3.2. Post-treatment with SRT1720

H_2O_2 (100 uM) was administered for 30 minutes and then removed before adding SRT1720 for either 24 hour or 48 hours. The results indicate that post-treatment with SRT1720 did not protect NT2 from H_2O_2 induced damage (figure 5).

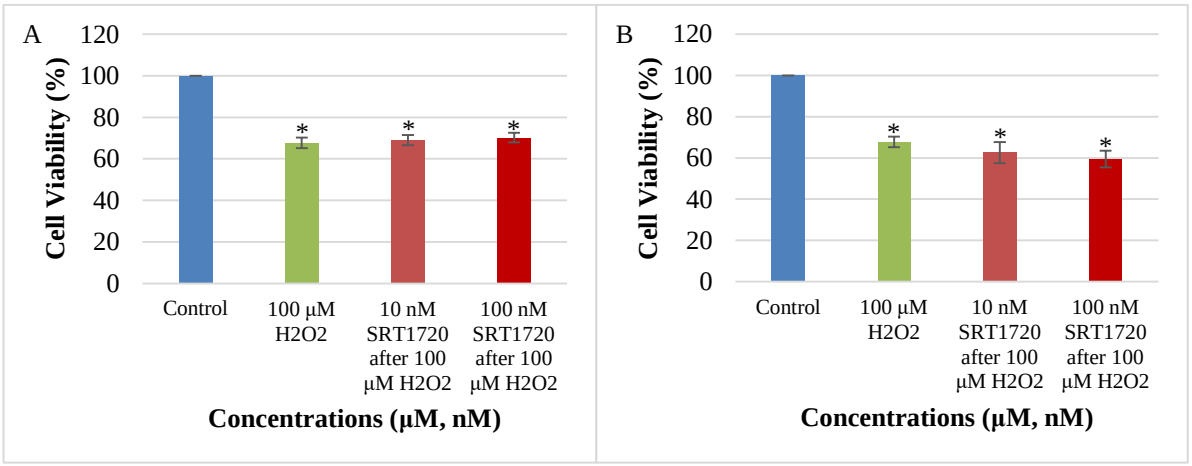


Figure 5: Effect of post-treatment with SRT1720 for 24 hours on NT2 cells. Cells were first incubated with H_2O_2 for 30 minutes then SRT1720 were added and incubated for (A) 24 hours or (B) 48 hours. The percentage of cell viability was measured using the CyQUANT assay. (*) represents significant values comparing control to treatment ($P<0.05$).

3.3.3. SRT170 and H_2O_2 in Combination

Both concentrations of SRT1720 significantly altered cell viability when incubated in combination with H_2O_2 for 30 minutes (figure 6). A statistical increase of 24% ($P=0.001$) in cell viability was observed.

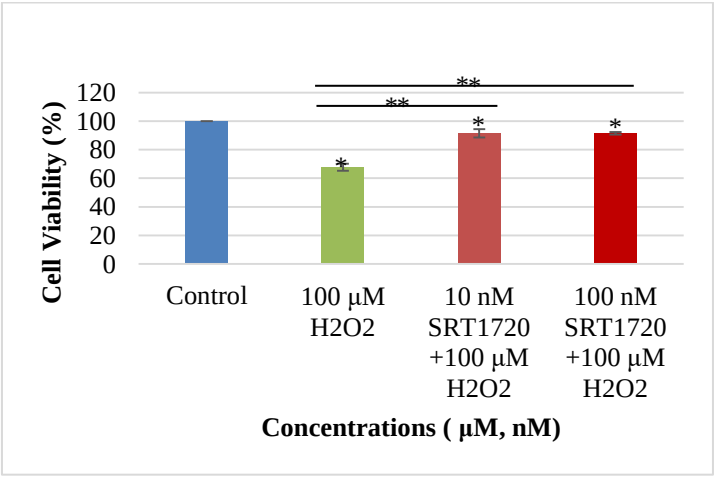
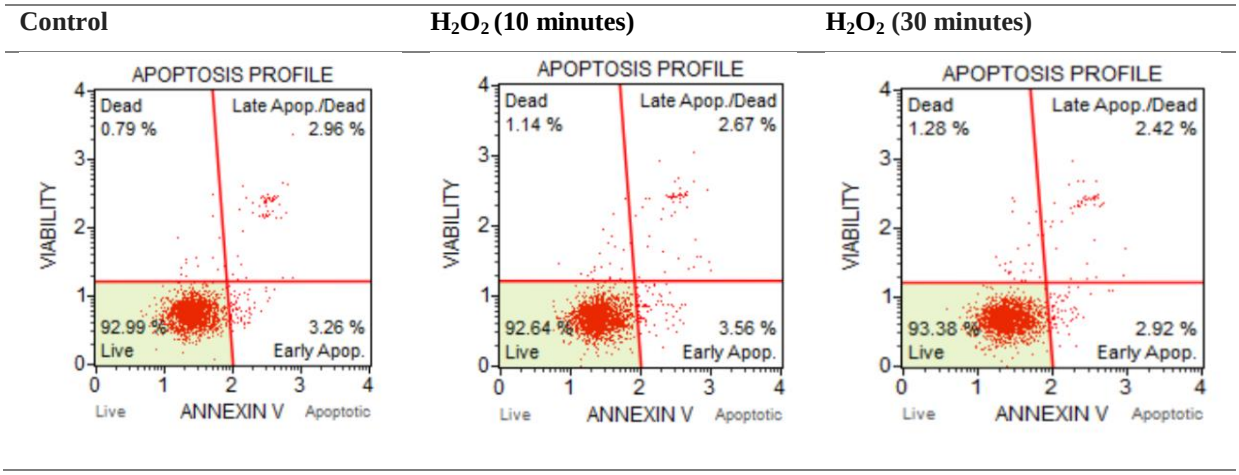


Figure 6: Effect of combination treatment of SRT1720 and H_2O_2 for 30 minutes on NT2 cells using CyQUANT assay. (*) represents significant values comparing control to treatment ($P<0.05$). (**) represents the significant difference between H_2O_2 alone and combination therapy.

3.4. The Role of H_2O_2 in Altering NT2 Cell Viability

3.4.1. Apoptosis study

A



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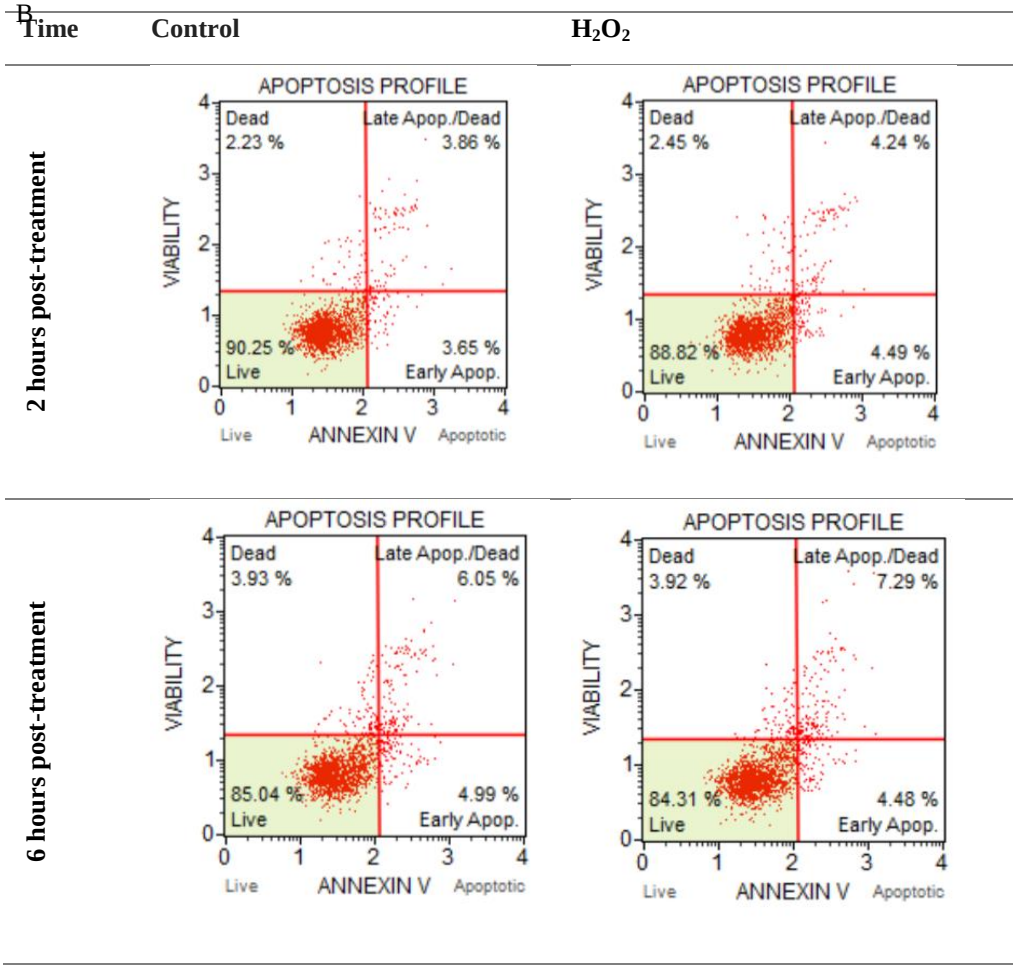

Figure 7: Apoptosis effect of 100 µM of H_2O_2 on NT2 cells. (A) NT2 cells were incubated with H_2O_2 for 10 minutes or 30 minutes. (B) H_2O_2 were added for 30 minutes the cells washed and incubated with growth medium for either 2 hours or 6 hours. Apoptosis was assessed using Annexin V assay.

To determine the early apoptotic effect of H_2O_2 (100 uM), we incubated NT2 cells with H_2O_2 for short times (10 minutes or 30 minutes) and assessed changes using the Annexin V assay. The data show an absence of significant apoptotic changes following treatment with 100 µM of H_2O_2 at both periods of time (figure 7A). To determine if changes occurred at later time points, the Annexin V assay was performed 2 hours and 6 hours after NT2 cells were incubated with H_2O_2 for 30 minutes. No significant apoptotic changes were detected (figure 7B).

3.4.2. Necrosis Study

As H_2O_2 treatment did not uncover significant apoptotic changes in NT2 cells as shown in figure 7, we investigated whether treatment with H_2O_2 (100 µM) for 30 minutes led to necrotic changes using the LIVE/DEAD assay. The results show that there was no difference between control and H_2O_2 treated cells (figure 8).

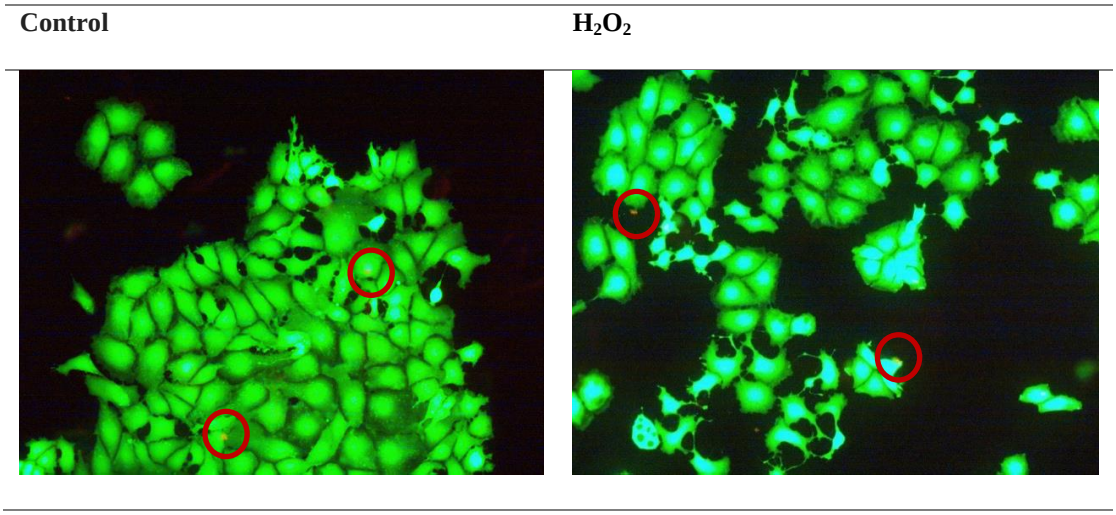

Figure 8: Necrosis effect of 100 µM of H_2O_2 on NT2 cells using LIVE/DEAD assay. Pictures were obtained using a fluorescence microscope. The green fluorescence represents live cells and the red fluorescence represents dead cells.

3.4.3. Metabolic Activity Study

As NT2 cells did not appear to show apoptotic or necrotic changes after incubation with 100 µM H_2O_2, the metabolic activity of cells was measured 30 minutes, 2 hours, and 6 hours after H_2O_2 using PrestoBlue assay. The results demonstrated that 30 minutes of exposure to H_2O_2 reduced the metabolic activity by 6% (P=0.04). When the metabolic activity was measured 2 hours later, metabolic activity was reduced by approximately 10% (P=0.02) in comparison to 30 minutes. Surprisingly, 6 hours post-treatment showed higher metabolic activity in comparison to 2 hours by 22% (P=0.007) (figure 9).

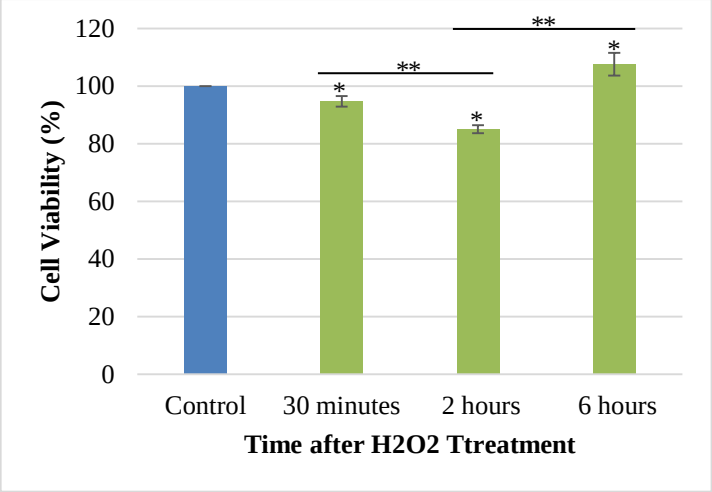

Figure 9: Effect of H_2O_2 on NT2 cells metabolism. H_2O_2 were added to cells and incubated for 30 minutes then growth medium was added for 2 hours or 6 hours. The percentage of cell viability was measured using the PrestoBlue assay. (*) represents significant values comparing control to treatment ($P<0.05$). (**) represents the significant difference between treatments.

4. Discussion

The present study was undertaken to examine the role of SRT1720 in protecting cells against H_2O_2 induced stress in NT2cells. Studies revealed that OS affects undifferentiated NT2 and differentiated NT2 neurons (NT2N) differently. NT2N cells are highly sensitive to ROS and express low levels of antioxidants such as glutathione (GSH) and enzymes such as glutathione peroxidase (GPx) and catalase [12]. Thus, in the current study we attempted to use undifferentiated NT2 cells to investigate OS effect of H_2O_2. Given that cell adhesion is dependent on many factors (pH, temperature, and divalent cations including Ca^{2+} and Mg^{2+}) [17], it was realized early on that Ca^{2+} and Mg^{2+} found in HBSS buffer and growth medium were important elements required for NT2 cell attachment. Treatment of NT2 cells with different concentrations of H_2O_2 (0.1 µM-1000 µM) led to a significant decline in cell viability, with the highest concentrations of 1000 µM showing the greatest reduction in cell viability. Our results are in agreement with Whittemore and his colleagues (1995) who reported that the concentration of 100 µM of H_2O_2 caused a 50% reduction in cell viability, 3 hours post exposure with further decline at 6 and 12 hours of treatment with H_2O_2. This study suggested that oxidative stress leads to decline in mitochondrial electrochemical potential which induces mitochondrial calcium release and loss of adenosine triphosphate (ATP) production [18].

4.1. The Effects of SRT1720 in NT2 Cells

Several studies have suggested that SIRT1 plays a crucial role in gene expression and cellular homeostasis in response to stress [19], including DNA repair, apoptosis, neurogenesis, and aging [9]. Yeung and his colleagues (2004) have suggested that SIRT1 activity can also trigger cell death through acceleration of NAD^+ depletion [20] and reducing NF-κB activation [21]. In the present study, SRT1720 did not alter NT2 cell viability at the concentrations and time points used.

4.2. The Effects of SRT1720 and H_2O_2 in NT2 Cells

While SRT1720 alone did not have an effect on NT2 cells, pre-treatment with SRT1720 resulted in improved cell viability. Combining SRT1720 with H_2O_2 for 30 minutes also resulted in improved cell viability when compared to cells treated with H_2O_2 alone, suggesting that SIRT1720 may be exerting a protective effect. In contrast, post-treatment with SRT1720 failed to improve NT2 cell viability from H_2O_2 induced toxicity. At the present time, the mechanism by which SRT1720 alters cell viability in NT2 cells is not known. While SRT1720 can promote mitochondrial turnover, studies show that it can also destroy aged or damaged mitochondria through mitophagy. Nicotinamide reduced mitochondrial mass and increased mitochondrial membrane potential can result in mitophagy. Since NAD^+/NADH ratio and SIRT1 activation may mediate the mitophagic effect of nicotinamide, it is possible that this effect is mimicked by SRT1720. The mitophagy occurs by hyperactivation of the DNA damage sensor PARP-1, resulting in reduced SIRT1 activity [22]. It is possible that pre-treatment with SRT1720 or a combination treatment with H_2O_2 may protect NT2 cells through deacetylation of PGC-1α, which play a crucial role in mitochondrial biogenesis. In addition, 5-HT receptors could be inhibited by SRT1720 which also increases mitochondrial function. However, post-treatment with SIRT1720 may fail to improve NT2 cell viability as a result of mitophagy.

4.3. The Role of H_2O_2 in Altering NT2 Cell Viability

Studies in PC12 cells have demonstrated that 400 μM of H_2O_2 caused apoptosis by increasing caspase-6 gene expression. In addition, H_2O_2 also increases the apoptotic gene PIN1 which manages the activities of p53 family members to control p53 buildup and apoptotic function in the cells exposed to stress conditions [23]. These data indicated that cell death by apoptosis may be contributing to the effects of H_2O_2 in a time, concentration, and cell type dependent manner. However, in the present study, treatment with H_2O_2 (100 μM) did not uncover early apoptotic changes when the exposure lasted for 10 or 30 minutes. Nor did longer times of exposure to H_2O_2 provide any evidence of apoptotic changes. A recent study suggested that oxidative stress due to high concentration of H_2O_2 (250 μM) for 48 hours could activate mitochondrion-dependent necrosis pathways (programmed necrosis). This necrosis pathway is initiated by translocation of p53 into mitochondria, which associates with cyclophilin-D (Cyp-D). Cyp-D is a protein that found in mitochondrial permeability transition pore (mPTP). The complex of p53-Cyp-D causes depolarization of mitochondria and cell necrosis. Moreover, H_2O_2 induced lactate dehydrogenase (LDH) in a dose-dependent manner, which is an indicator of cell necrosis [5]. These results indicated that the necrotic changes due to H_2O_2 are dose and time dependent. Interestingly, in the present study, treatment with H_2O_2 for 30 minutes did not appear to induce necrotic changes in NT2 cells under the experimental conditions employed. Previous study using SH-SY5Y cells showed about 10% reduction on cell viability when treated with 100 μM of H_2O_2 for 12 hours [24]. Another study performed on isolated mitochondria of NT2 and SH-SY5Y cells after treatment with 100 μM of H_2O_2 for 1 hour showed a decline in ATP levels. Furthermore, mitochondrial ROS and caspase-3 increased after H_2O_2 which induced mitochondrial dysfunction and mediate apoptosis [25]. Thus, we used PrestoBlue assay to determine the metabolic activity of NT2 cells. The results demonstrated that 100 μM of H_2O_2 for 30 minutes and 2 hours after H_2O_2 led to a modest loss in metabolic activity. However, the metabolic activity of NT2 cells modestly increased with longer incubation time. Studies of cultured primary cortical neurons showed that the mitochondria could recover after

exposure to metabolic and oxidative stress via the oxoguanine DNA glycosylase (OGG1) [26]. 8-oxoguanine (8-oxoG), a product of oxidation of guanine induced by ROS, accumulated in mitochondrial DNA (mDNA) leading to mitochondrial dysfunction observed in neurodegenerative diseases [27]. OGG1 is a key enzyme for the repair of 8-oxoG that is involved in base excision repair (BER), nuclear and mitochondrial DNA repair pathway [26, 28]. These data suggested that NT2 mDNA recovery could be the reason for the increase in metabolic activity 6 hours after exposure to H_2O_2.

5. Limitations of the Study

To the best of our knowledge, the effects of SRT1720 on NT2 cells, and the effects of SRT1720 with or without H_2O_2 in NT2 cells has not been studied previously, thus preventing any comparison with similar studies in the literature. While this study looked at NT2 cells, other cell lines, sample sizes, doses and times of treatment were not considered.

6. Conclusion

In conclusion, our findings that H_2O_2 significantly induced oxidative stress in a concentration dependent manner, suggest that H_2O_2 may be exerting its effect in NT2 cells at the mitochondrial level. In addition, SRT1720 exerted a protective effect in that pre-treatment with SRT1720 or a combination of H_2O_2 and SRT1720 increased NT2 cell viability. Future studies investigating the mechanism of SRT1720 in NT2 cells would improve our understanding of how SIRT1 is considered an important target for the treatment of neurodegenerative disorders.

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