Establishing an Oxidative Stress Model in the Human Mesencephalic Cell Line (LUHMES): an in vitro study

Handan Sevim Akan 1, Özgü Zuğa Örsoğlu 1 and Özer Aylin Gürpınar 1
Department of Biology, Faculty of Science, Hacettepe University, Ankara, Turkey.

ABSTRACT

Oxidative stress caused neurodegenerative diseases, such as Alzheimer’s, Parkinson’s disease, and amyotrophic lateral sclerosis, are widely recognized as the most prevalent brain and central nervous system disorders. This is attributed to the vulnerability of neurons to oxidative stress within the body. Although substantial research has been performed on these diseases, it is extremely difficult to establish an oxidative stress model for brain tissues. In primary cultures, it is difficult to obtain neurons and the continuity of the culture is limited for in vitro cell line models. By providing valuable insights into the mechanisms of oxidative stress-induced neurodegenerative diseases, these in vitro models can aid in the development of effective treatment strategies. Here, we developed an in vitro oxidative stress model utilizing hydrogen peroxide on the LUHMES cell line. Our study evaluated the impact of this model on LUHMES cell viability and the equilibrium between oxidants and antioxidants by assaying total oxidant capacity (TOC) and total antioxidant capacity (TAC). Our results provided evidence of oxidative effect of hydrogen peroxide in critical concentration and proved the efficacy of this model for further investigations.

Key Words
Oxidative stress, LUHMES cell line, total oxidant capacity, total antioxidant capacity.

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Correspondence to: H. Sevim Akan, Department of Biology, Faculty of Science, Hacettepe University, Ankara, Turkey
E-Mail: gurpinar@hacettepe.edu.tr
INTRODUCTION

The vast majority of the ATP required for physiological events in the cell is produced by a series of reactions in the mitochondria. During these reactions, volatile molecules with unpaired electrons in their final orbitals, produce free oxygen radicals, and reactive oxygen species (ROS) [1]. In order to maintain a healthy state, the antioxidant defense system plays a crucial role in maintaining a certain balance between free radicals and ROS production in the body. In this state of balance, antioxidants protect the cell against harmful reagents [2]. Disruption of this antioxidant/ROS balance in the direction of ROS is defined as oxidative stress (OS) in the cell [3]. OS is formed in cells due to the increase in endogenous and exogenous compounds that react with ROS, depletion of reserves with decreased antioxidant production, and inactivation of antioxidant enzymes. [4].

Free radicals and ROS can be extremely dangerous for biomolecules when they accumulate in high amounts. They can easily react with any molecule in the body, disrupting structural integrity. Especially lipids, proteins, carbohydrates, and DNA are the main targets for these molecules [5]. The amount of polyunsaturated fatty acids, which are sensitive to ROS and prone to oxidation, is quite high in membrane lipids in the brain [6]. In addition, neurons produce much more ATP by oxidative phosphorylation for energy production and this induces oxidative stress as a result of more ROS formation [7]. Although the brain accounts for only 5% of body weight, it is exposed to high oxygen concentrations, utilizing approximately 20% of inhaled oxygen. Under physiological conditions, 1-2% of the O₂ consumed is converted into ROS, leading to oxidative stress [8]. Therefore, the nervous system and especially brain tissue are more susceptible to oxidative stress than other tissues. This leads to the widespread incidence of various neurodegenerative diseases affecting the central nervous system such as Alzheimer’s Disease, Parkinson’s Disease, and Amyotrophic lateral sclerosis [9]. Increased DNA oxidation products like 8-hydroxy-deoxy-guanosine in mitochondria and nuclei indicate that oxidative stress is effective in Alzheimer’s Disease [10]. Also, in Parkinson’s Disease mitochondrial complex I activity gradually decreases in Substantia Nigra dopaminergic neurons with age, which may contribute to the high oxidative stress levels in the aging Substantia Nigra [11]. Further in Multiple Sclerosis, it has been reported that there is an increase in the production of ROS as a result of impaired iron metabolism and that iron plaque deposited on the myelin sheath initiates an inflammatory response that causes demyelination [12].

Reseaching neurodegenerative diseases is a complex task, particularly when it comes to developing an oxidative-stress model involving the brain. Obtaining primary neurons in primary cultures is challenging, and the culture system’s continuity is limited. Additionally, using a large number of experimental animals is impractical. For this reason, in vitro models are necessary to be established in neuronal cell lines. The Lund human mesencephalic (LUHMES) cell line consists of neuronal precursor cells derived from ventral mesencephalic tissue of a human female fetus. These cells are capable of differentiating into dopaminergic-like neurons and expressing dopaminergic neuron-specific biochemical markers. Moreover, they able to form an extensive network of neurites. These unique properties make LUHMES cells an excellent candidate for an in vitro human cell model [13]. In this study, we developed an in vitro oxidative-stress model using the LUHMES cell line, which is a human mesencephalic neuron-like cell line. We examined the effects of oxidative stress on LUHMES viability and the oxidant/antioxidant balance.

MATERIALS and METHODS

Cell culture

LUHMES cells were incubated under standard culture conditions (CRL-2927, ATCC, USA). DMEM/F12 (Biowest B. L0092-500) medium containing 10% FBS (Biowest B. S181H-500), 2.5% glutamine, and 1% N₂ supplement in 75 cm² culture dishes with surfaces coated with Poly-L-Ornithine (PLO) [(50 PLO (v): 50 PBS (v)) / Fibronectin, DMEM/F12 (Biowest B. L0092-500) medium containing 2.5% glutamine and 1% N₂ supplement at 37°C in 5% CO₂:95% air culture conditions. 8 ng/fibroblast growth factor (FGF) was added freshly to the medium. When the cells reached approximately 80% confluency, cells were trypsinized and stocks were stored in liquid nitrogen. Cell morphology was visualized using an inverted microscope (Olympus, Japan).

Oxidative stress modeling with hydrogen peroxide and determination of cell viability

Hydrogen peroxide used for oxidative stress modeling and the cells were treated with four different doses of hydrogen peroxide (0.05 mM; 0.1 mM; 0.15 mM; 0.2 mM) to determine the reliable concentration range.
for the *in vitro* model; hydrogen peroxide-free medium was used as a control group. Each experiment was performed three times. Cell viability was determined by the MTT method 4 and 24 hours after incubation with hydrogen peroxide. Cell viability was determined by absorbance measurement with an ELISA plate reader (EZ Reader 400, Biochrome) at 570 nanometers. In the literature, it was observed that hydrogen peroxide application was performed for various periods of time (90 minutes-24 hours) to create oxidative stress [14-16]. The incubation period of 4 and 24 hours were chosen in order to measure short-term and long-term effects hydrogen peroxide.

**Total Oxidant Capacity (TOC) Assessment**

**Total Antioxidant Capacity (TAC) Assessment**

Total oxidant capacity was assessed using the TOC ELISA kit (TOC, Rel Assay Diagnostics). This method is based on the oxidation of ferrous iron ions (Fe²⁺) to ferric iron ions (Fe³⁺) by oxidants. Fe³⁺ ion, which increases with oxidation, combines with the chromogen in the kit to form a colored complex. This color change is directly proportional to the total amount of oxidant (measured 570 nm on an ELISA reader (EZ Reader 400, Biochrome), and the total oxidant capacity was measured as μmol H₂O₂ Eq/L by making the necessary calculations according to the protocol specified by the manufacturer.

**Total Antioxidant Capacity (TAC) Assessment**

Total antioxidant capacity was measured using the TAS ELISA kit (TAS, Rel Assay Diagnostics). This kit is based on the principle of reduction of 2,2’-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS-+), a radical cation, to ABTS by antioxidants. The color change resulting from this reduction was measured at 620 nm on an ELISA reader (EZ Reader 400, Biochrome), and the total antioxidant capacity was calculated as mmol Trolox Eq/L by making the necessary calculations according to the protocol specified by the manufacturer.

**RESULTS and DISCUSSION**

**Cell culture and cell viability in oxidative stress model**

The LUHMES cells displayed a round shaped and dispersed at the beginning of the culture. However, after 24 hours of incubation, they began to adhere to the surface of the culture dish and formed clusters. When the cells reached approximately 80% confluence, they were passaged and multiplied (Figure 1).

Cell viability was evaluated at 4 and 24 hours of incubation using MTT method. The cell viability results indicated that concentrations of 0.05 and 0.1 mM exhibited similar viability compared to the control and the difference was not significant (p > 0.05). However, 0.15 and 0.2 mM concentrations showed reduced cell viability compared to control and lower concentrations. Additionally, the difference between the control group and these experimental groups was statistically significant (p < 0.05). (Figure 2a). The viability results obtained after 24 hours of incubation were consistent with the results for 4 hours. (Figure 2b). As a result, after treating the LUHMES cell line with four different doses of hydrogen peroxide, it was found that the cell viability was higher at 0.05 and 0.1 mM doses compared to the 0.15 and 0.2 mM doses at both time points. Results indicated that the safe high dose of hydrogen peroxide is 0.1 mM.
Total Oxidant Capacity (TOC) and Total Antioxidant Capacity (TAC) Determination

**Total Oxidant Capacity (TOC) Determination**

The TOC value increased following hydrogen peroxide treatment during both the 4 and 24-hour incubation periods. (Figures 3a and 3b). In both time periods, TOC values were low concentrations were similar to each other and the control (p>0.05). In concentrations 0.15 and 0.2 mM, the TOC value was higher than in 0.05 and 0.1 mM; the difference between these two groups was significant (p<0.05). Also, the TOC value of 0.15 and 0.2 mM was higher compared to the control and the difference between them was statistically significant (p<0.05).

**Total Antioxidant Capacity (TAC) Determination**

The TAC value is higher in 0.05 and 0.1 mM, which are lower concentrations (Figures 4a and 4b). TAC values in these two concentrations were similar to each other and to the control and the difference was not statistically significant (p>0.05). TAC values in 0.15 and 0.2 mM show a decrease compared to lower concentrations and the difference between them is statistically significant (p<0.05). Further, while the difference between 0.15 and 0.2 mM was not significant; TAC values in these two concentrations were lower than in the control and the difference was significant (p<0.05).

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**Figure 2.** LUHMES cell viability after hydrogen peroxide treatment. a. 4 hours incubation b. 24 hours incubation. Low concentrations were similar to each other with no significant difference (p > 0.05). 0.15 and 0.2mM concentrations showed reduced cell viability compared to control and lower concentrations. Error bars indicate standard deviations over six replicates. All experiments repeated three times. SPSS Version 23.0 for Windows used for analysis.

**Figure 3.** Total Oxidant Capacity (TOC) of LUHMES cells after hydrogen peroxide treatment. a. 4 hours incubation b. 24 hours incubation. Low concentrations were similar to each other and the control group (p>0.05). 0.15 and 0.2 mM, the TOC value was higher than in 0.05 and 0.1 mM; the difference between these two groups was significant (p<0.05). Error bars indicate SD over six replicates. All experiments repeated three times. SPSS Version 23.0 for Windows used for analysis.
Discussion

The susceptibility of brain tissue to oxidative stress leads to irreversible neurodegenerative diseases [17]. Hydrogen peroxide is a major contributor to oxidative stress and is widely used in in vitro studies to simulate oxidative stress [18,19]. The literature contains several studies on the dosage and effect of hydrogen peroxide on various types of cells. Tsai et al. showed that treatment with 0.25 mM hydrogen peroxide for three hours induced oxidative stress in L929 cells [7]. In their 2015 study, Wang et al. showed that oxidative stress occurred in SH-SY5Y cells when treated with 0.2 mM hydrogen peroxide for 24 hours [14]. Parallel to our study, Kang et al. also showed that oxidative stress occurred in SH-SY5Y cells after 0.3 mM hydrogen peroxide treatment [15].

Primary cultures are the preferred choice for in vitro studies as they accurately represent the target tissue [20]. However, preparing primary cultures for all tissues at all times is a labor-intensive process, and in the case of brain tissue, it is nearly impossible to establish human primary cultures [21]. Consequently, cell lines are popularly used for disease modeling.

The majority of cell lines are derived from cancerous tissues such as neuroblastoma or glioblastoma. One of these cell lines, DBTRG-05MG, is a cell line derived from human glioblastoma and is frequently used in studies examining the effects of anti-cancer drugs. Othman et al. investigated the effects of andrographolide on cell cycle, apoptosis, and related signaling pathways in DBTRG-05MG cells used in glioblastoma multiforme (GBM), the most common and the most lethal brain tumor [22]. Another neuronal cell line, D341Med, was isolated from cerebellar medulloblastoma. In their studies with this cell line, Zhou et al. examined the effects of matrine, a Chinese herbal medicine, on proliferation and apoptosis in this cell line [23]. The D341Med cell line, isolated from the cerebellum, was utilized to investigate the apoptotic effects of Lovastatin in cholesterol treatment [24]. The study examined the effects of diallyl disulfide and garlic oil in treating gliomas, a type of malignant brain tumor, using astroblastoma-derived cell line CHLA-03-AA [25]. The D238Med cell line is derived from peritoneal metastasis of medulloblastoma and has been frequently used in cancer stem cell research and drug efficacy studies [26,27]. The PFSK-1 cell line is a cell line derived from a cerebral malignant neuroectodermal tumor and is mostly preferred in cancer research [28]. The Doay cell line isolated from cerebellar neuroblastoma is also predominantly used in cancer research and various neurobiological studies [29,30]. In general, when we examine the scope of these studies, we observe that they primarily focus on cancer research and the investigation of the effects of anti-cancer drugs.

The LUHMES cell line is a neuronal cell line isolated from healthy tissue and is a good model for the study of neurodegenerative diseases. In parallel with our study, there are studies in the literature using the LUHMES cell line, especially in neurodegenerative diseases and neurotoxicity. Zhang et al. differentiated LUHMES cells into dopaminergic neurons and studied neurotoxicity in a Parkinson’s cell model induced by 1-methyl-4-phenylpyridinium (MPP+), a dopaminergic neuron-specific toxin [31]. Smirnova et al. investigated rotenone
toxicity in the LUHMES Parkinson’s model [32]. In 2017, Höllerhage et al. investigated alpha-synuclein toxicity using LUHMES cells [33].

In the literature various in vitro cell culture models were created using hydrogen peroxide. Sameti et al. investigated the effect of hydrogen peroxide on bioenergetic mechanism in astrocytes and LUHMES cell line [34]; in another study, they investigated the effect of hydrogen peroxide directly on DNA damage [35]. Schlachetzki et al. examined the effects of hydrogen peroxide on neurodegenerative diseases in cell culture models [36].

Our study aimed to investigate the direct effect of hydrogen peroxide on oxidative stress parameters using the LUHMES cell line as an in vitro model for neurodegenerative diseases. Our findings demonstrate a significant decrease in cell viability as hydrogen peroxide concentration increases. The Total Oxidant Capacity (TOC) value increases as expected with increasing concentration of hydrogen peroxide, while the Total Antioxidant Capacity (TAC) value decreases. Our results suggest that this in vitro model would be a suitable option for studying oxidative stress-induced neurodegenerative diseases.

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