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Neuroprotective Role of Glutathione against Hydrogen Peroxide Induced Toxicity to the Neuronal Cells in Culture

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Abstract

Neuropathophysiology research is receiving considerable attention. Studies have demonstrated that under oxidative stress, reactive oxygen species (ROS) generated at high levels inducing cellular and DNA damage, thereby resulting in apoptosis of neuronal cells. This is implicated in the etiology of several neurodegenerative and neurodevelopmental disorders. This study was undertaken to examine the role of glutathione as a Neuroprotective bioactive compound on hydrogen peroxide-induced apoptosis. Assessment of DNA damage with the help of Comet assay (single cell gel electrophoresis) and DNA fragmentation Assay were carried out on cultured SH-SY5Y neuroblastoma cells. The treatment with glutathione markedly attenuated hydrogen peroxide-induced cell viability loss and apoptotic neuronal cell death. These results provide evidence that glutathione may act as a significantly bioactive compound and support the possibility that it may be important in health and disease, and for protection against DNA damage by oxidative stress.

Keywords: Oxidative stress; Hydrogen peroxide; Glutathione(GSH); SH-SY5Y; Comet assay; DNA fragmentation assay.

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1. Introduction

Oxidative stress, which is often described as a disruption in the Pro-oxidants - oxidants balance, has been implicated in a variety of physiological and pathological processes of the nervous system including Alzheimer's disease [1], Parkinson's disease [2], amyotrophic lateral sclerosis [3] and neurodevelopmental disorders such as autism.[4,5]Oxidative damage is mediated by the generation of reactive oxygen species(ROS), such as hydroxyl (OH⁻), alkoxy (RO⁻) or peroxy (ROO⁻)radicals which are short lived, as well as medium lived radicals such as superoxide(O₂⁻) and nitroxyl radical(NO⁻). It also includes non-radical hydrogen peroxide (H₂O₂), organic hydroperoxides(ROOH) and hypochlorous acid(HOCl). Reactive oxygen species (ROS) are generated following cell lyses, oxidative burst in immune response [6] or the presence of an excess of free transition metals that can act catalytically to generate free radicals by the Fenton reaction [7]. The main source of ROS formation is primarily constituted by mitochondria during electron transport in the oxidative phosphorylation (respiratory) chain. H₂O₂ can readily diffuse out of the mitochondrial membrane and have a cytoplasmic effect. Low levels of H₂O₂ are important to the cell because they regulate physiological processes such as receptor mediated cell signaling pathways, normal cell proliferation and transcriptional activation. However overproduction of oxidants and/or dysfunction of endogenous antioxidant defenses may occur and that result in oxidative stress induced cell damage and even cell death [8].

The central nervous system is particularly vulnerable to oxidative stress due to its high energy requirement, its disproportionately low levels of oxidative defense mechanisms and its high content of easily oxidised substrate such as membrane polyunsaturated lipids and iron. The brain makes up about 2% of the body mass but consumes 20% of metabolic oxygen. The vast majority of energy is consumed by the neurons [9].Due to the lack of glutathione-producing capacity of neurons, the brain has a limited ability to detoxify ROS. Therefore neurons are the first cells to be affected by the increase in ROS and shortage of antioxidants and as a result, they are more susceptible to oxidative stress. Moreover, elevated levels of ROS may affect cerebral vasculature, which is crucial for maintaining the blood brain barrier [10].

The intensity and duration of exposure of the cells to a toxic stimulus in the form of oxidative stress determines whether a cell undergoes apoptosis or necrosis (energy dependent and energy independent phenomena respectively). While a high degree of oxidative stress can cause necrosis, low levels will trigger apoptosis [11]. The biochemical cascade that leads to apoptic cell death seems to involve the activation of one or more members of a family of cysteine proteases called caspases and the release of factors from mitochondria (such as cytochrome C). This ultimately induces nuclear DNA fragmentation and condensation [12-13]. Morphologically, apoptosis is associated with cell shrinkage, nuclear and cytoplasmic condensation, externalization of phosphatidylserine(PS) at the plasma membrane level and the formation of apoptic bodies [14].

Studies in our lab revealed significantly elevated levels of H₂O₂ and lipid peroxide in autistic patients' plasma, a marker of oxidative stress [5]. In the present study, we attempted to investigate the neurotoxic effects of H₂O₂ on neuronal cells in culture in order to develop an *in-vitro* neurodegenerative model, as well as investigating the Neuroprotective effect of glutathione, as the main antioxidants against H₂O₂ toxicity.

2. Materials and Methods

2.1: Cell Culture:

a. Primary Cortical neuronal Cell Culture: Primary cortical neuronal cultures were prepared by slight modification of the method described by Lu et al [15]. 1-2 day old western albino rats were anesthetized and decapitated. The cerebral cortex was removed and chopped into small pieces in a sterile tissue culture hood. The minced tissue was placed into sterile saline solution and repeatedly aspirated with the help of a sterile Pasteur pipette in order to break the bigger cell debris. The solution was centrifuged for 10 min at 1000 r.p.m. The resulting cell suspension was incubated at 37⁰ C in the presence of 2% trypsin and Dulbecco's modified Eagles medium (DMEM, Gibco-BRL Life Technologies) for 30 minutes with slight agitation. The cell suspension was centrifuged at 1000 r.p.m for 5 minutes and re-suspended in fresh DMEM solution with 10% fetal bovine serum (FBS, Gibco-Brl Life Technologies). The cells were centrifuged again for 5 minutes at 1000 r.p.m. The supernatant was discarded and the remaining cell pellet was washed at least three times. After final cell washing the cells were suspended in 10 ml of DMEM supplemented with 10% FBS, 2mM L-Glutamine, 100µl/ml of penicillin/streptomycin and seeded on 12 well culture plates with a plating density of 5X10⁶ cells per well. The cultures were maintained at 37⁰C in a humidified 5% CO₂-95% air atmosphere.

b. Human neuroblastoma Cell line SH-SY5Y: Cells were purchased from American Type Tissue Culture (ATTC; Manassas, VA) and cultured in DMEM, supplemented with 15% FBS, 2mM L-Glutamine, 1000 mg/ml (high glucose) D-Glucose, 1% Non-Essential Amino Acids, 100 U/ml Penicillin, and 100 µg/ml Streptomycin, in a 95% air and 5% CO₂-humidified incubator at 37⁰C. They were grown in T-25 flasks until 70-80% confluence, and then split for sub culturing [16-19].

2.2: Treatments:

H₂O₂ and glutathione were purchased from Sigma (St. Louis, MO, USA). The cells were divided into two groups for the treatment.

In the first group, cells were plated and treated with different concentrations of H₂O₂(which was added to the culture media to reach the final concentrations 0.5mM and 1mM). These groups of cells were used for studying the effects of H₂O₂ toxicity.

The second groups of cells were used to study the neuroprotective effects of GSH against H₂O₂ toxicity. These cells were divided and treated with GSH (which was dissolved in the media to reach final concentrations of 2 mM). GSH treated cells were incubated in the presence and absence of 0.5mM H₂O₂ and 1mM H₂O₂ for either 24h, or 48h. Cells without any treatment of H₂O₂ and GSH were used as the control group.

All the cultures were maintained for either 24 hours or 48 hours at 37⁰C in an atmosphere of 95% air and 5% carbon dioxide.

2.3: Cell Viability assay: The cell viability was determined by visual cell count in conjunction with Trypan Blue exclusion. MTT assay was also employed to examine cell viability. Methyl thiazolyltetrazolium (MTT) purchased from Sigma (St. Louis, MO, USA) was added to the culture medium at the end of treatment at a final concentration

of 0.5mg/ml. After the cells were incubated at 37°C for 4h, the culture medium containing MTT was removed, dimethyl sulfoxide (DMSO) was then added into each well and the absorbance at 570nm was measured with a reference wavelength of 630nm.

2.4: Comet Assay (Single Cell Gel Electrophoresis SCGE):

The effect of H₂O₂ treatment induced DNA damage to the cultured neuroblastoma with and without glutathione was studied by the conventional single cell alkaline Gel electrophoresis as described originally by Singhet *al*[20]. Neuroblastoma cell lines SH-SY5Y were treated with and without H₂O₂ and GSH as described before on the slides for a standard comet assay. About 4x10⁶ cells were mixed with 80 µl of 0.7% low-melting agarose in Phosphate buffer saline (PBS) at 37°C in a microtube, and then spread over a window microscopic slide. The slides were pre-coated with 150 µl of 0.5% normal-melting agarose in PBS that were specially designed for this assay. The slides were placed immediately in cold lysis buffer (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris (pH 10), and 1% Triton X-100) at 4°C for a minimum of 1 hour. After lysis, the slides were drained and placed in a horizontal gel electrophoresis tank surrounded by ice, and filled with fresh cold electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA, pH 13). To allow DNA unwinding, the slides were kept in the high pH buffer for 20 minutes. After that, electrophoresis was carried out for 20 minutes at 25V and 300 mA. The slides were then drained and flooded slowly with 3 changes of neutralization buffer (0.4 M Tris, pH 7.5) for 5 minutes each, and then stained with 30 ml of ethidium bromide (20 mg/l) and covered with cover slips. All those steps were performed under dimmed light in order to prevent additional DNA damage caused by visible light. A total of 50 randomly selected cells per slide were analyzed. Imaging was done using a fluorescence microscope (Zeiss Axiovert L410 Inc., Jena Germany), attached to a digital camera (Olympus Inc., Tokyo, Japan), and equipped with 549 nm excitation filter, 590 nm barrier filter, and a 100-W mercury lamp. The percentage of DNA in the comet tail "DNA damage" was automatically calculated [21-23].

2.5: DNA Fragmentation Assay: The method described by Chaudhary *etal* [24] was used for DNA extraction and Agarose gel electrophoresis. After 24 and 48 hours of treatment, both attached and floating cells were collected, washed with Phosphate buffer saline and centrifuged at 1500 rpm for 5 minutes to precipitate the cells. The cell pellet was resuspended in 0.5 ml of lysis buffer, transferred to a microfuge tube and incubated for 1 hour at 37°C. Then, 4 µl of proteinase K was added to the tubes and they were incubated at 50°C for 3 hours. To separate the DNA, phenol–chloroform extraction was carried out by adding 0.5 ml of phenol: chloroform: isoamyl alcohol (25:24:1) to each tube with mixing and centrifugation at 13,000 rpm for 1 minute. The extraction was repeated twice, followed by chloroform extraction alone. The resulting DNA containing upper aqueous phase was separated, and to that, 2 volumes of ice-cold absolute ethanol and 1/10th volume of 3M sodium acetate were added, followed by incubation on ice for 30 minutes to precipitate the DNA. Centrifugation at 13,000 rpm for 10 minutes at 4°C precipitated DNA pellets, which, after discarding the supernatant, were washed with 1ml of 70% ethanol. The centrifugation step was repeated to remove the last traces of the supernatant fraction. The pellets were allowed to dry at room temperature for approximately 30 minutes, and resuspended in 50 µl of Tris-EDTA buffer. DNA quantification was conducted using UV spectroscopy. 10µg of DNA was run on 1.2% agarose gel electrophoresis, (containing ethidium bromide) in a mini gel tank containing Tris–boric acid–EDTA buffer for 2 hours at 90V. A ready-to-use 100 bp DNA Ladder (GenScript, USA) was run alongside the samples. Finally, the gel was visualized under UV transilluminator [25]. For

the quantitative analysis the fluorescent DNA bands on the photograph were scanned using digitizing densitometer as described by Freeman and Thompson, 1988. [26]

Statistical analysis: Results were obtained in general from at least three independent experiments. Results were presented as mean values and error bars represent SEM. All data were presented as mean \pm SEM. Mean \pm SEM (n=5).

3. Results

In this study, the DNA damage incurred by the treatment of H₂O₂ and the possible neuroprotective effect of GSH was investigated.

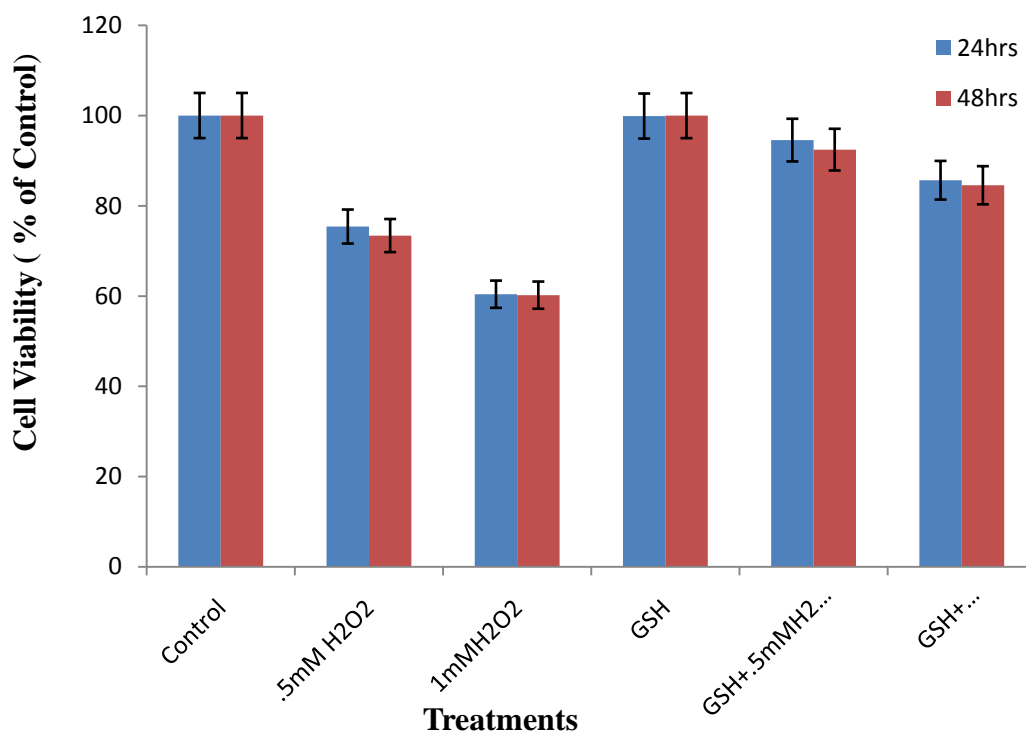


Fig.3.1.A. Effect of H₂O₂ (0-1mM) and 2mM GSH on cellular viability assessed by Cell counting. 5X10⁶ cells per well were treated with 0-1mM H₂O₂ for 24h. and 48 in the presence and absence of 2mM GSH and assessed for viability using morphological cell counting in conjunction with Trypan blue exclusion. For each condition three random fields of cells from triplicate wells were counted. Values are normalized by the value in control group. Mean \pm S.E.M. (n=5)

3.1: Cell Viability:

Cell viability was determined by morphological cell counting using Trypan Blue exclusion Method and MTT assay. Treatment of primary cortical cells with 1mM H₂O₂ in culture showed significantly decreased cell viability by about 40% at 48hrs.(**Fig 3.1A**). This damage was reversed by 2mMGSH (This concentration of GSH was used as it gives the optimum amount of neural cells growth response when added to the culture medium in our preliminary studies

on primary neural culture). The cells which were treated with 1mM H₂O₂ in the presence of 2mM GSH showed about 50 % improvement in cell viability when compared to the cells without any treatment of GSH, after 24 h, and 48 h, in culture, which clearly indicated the neuroprotective nature of GSH.

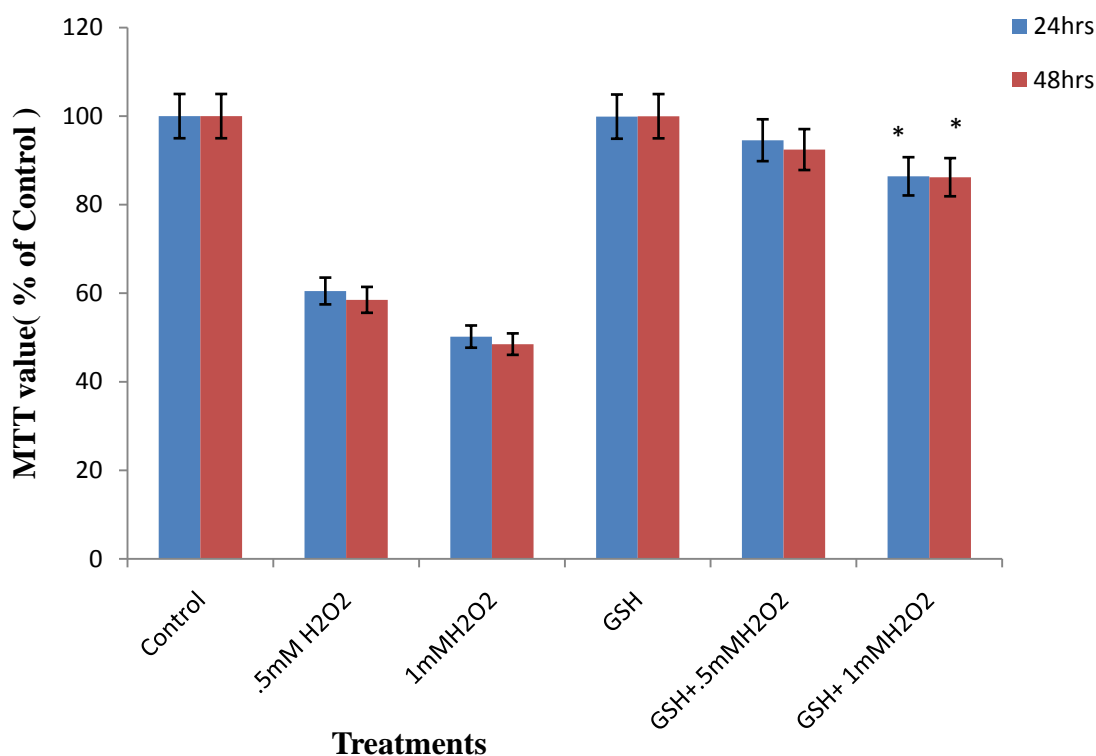


Fig. 3.1 B. Effect of H₂O₂ (0-1mM) and 2mM GSH on cellular viability was determined using MTT Assay. Triplicate wells were treated with (0-1mM) H₂O₂ in the absence and presence of 2mM GSH. As above data were analyzed statistically using ANOVA. MTT values were normalized by the value in control group. Mean ±SEM (n=5). *P < 0.05 represents significant values

MTT assay was also employed to assess the cytotoxicity of H₂O₂. Treatment of primary cultured cortical neurons with 1mM H₂O₂ significantly decreased cell viability by about 50% (**Fig.3.1B**). However the cells that were treated with 1mM H₂O₂ in the presence of 2mMGSH showed significant protection against H₂O₂-induced cytotoxicity.

3.2: H₂O₂ toxicity to the cells:

The DNA damage incurred by the treatment of H₂O₂ was further investigated by using comet assay (SCGE) and DNA fragmentation assay using neuroblastoma cell line SH-SY5Y. An image analysis system was used to measure damage parameters such as the tail length and the percentage of DNA in the tail and tail moment which was an index of DNA damage as described by Tice *et al* [27].

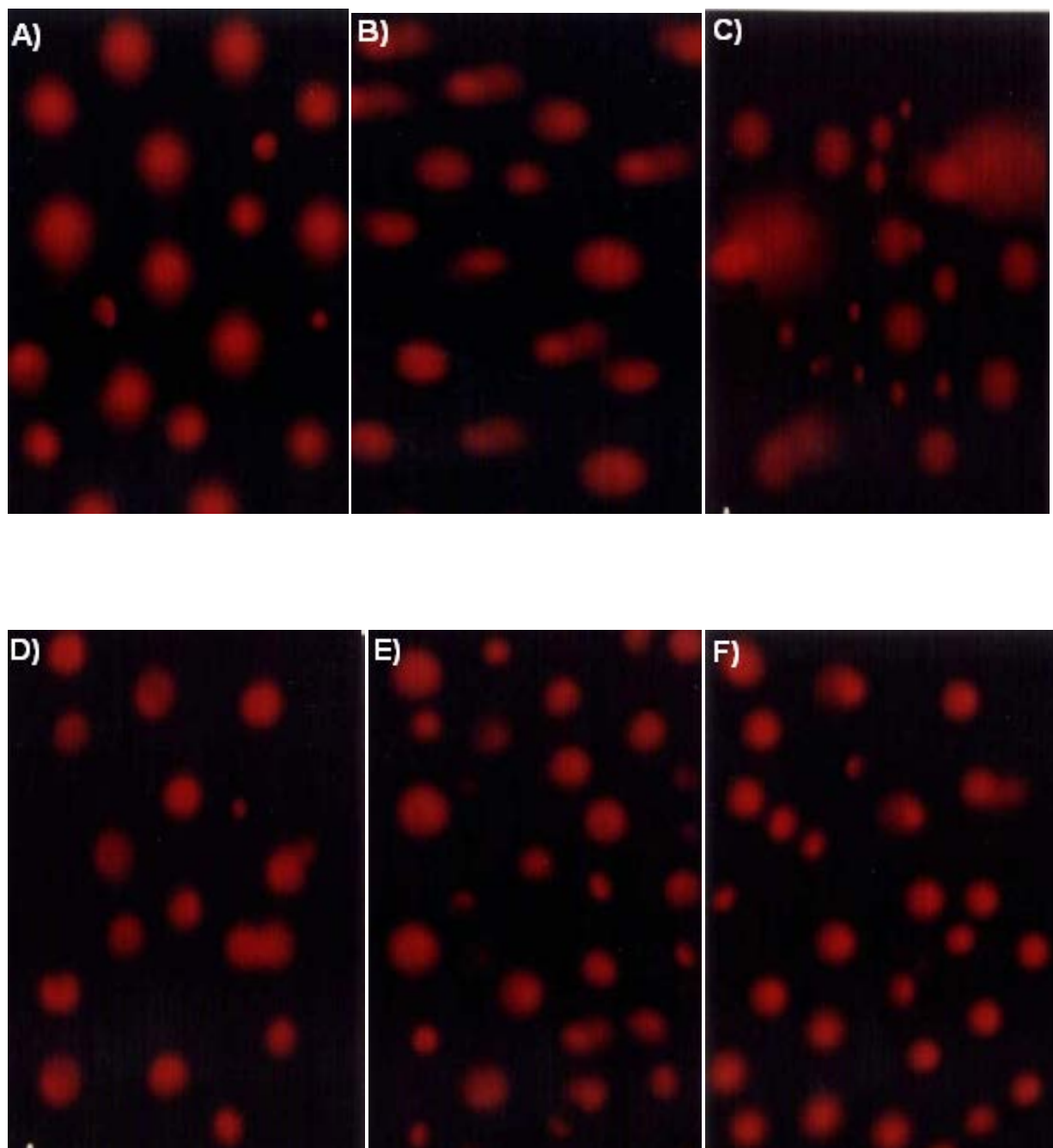


Fig 3.2.(A)

Fig 3.2 A, .Comet assay (SCGE) images for neuroblastoma SH-SY5Y cells cultured at 24h

A) Control cells. B) 0.5mM H₂O₂ C)1mMH₂O₂, D)Controlwith GSH, E)0.5mM H₂O₂ +GSH . F) 1mM H₂O₂ +GSH

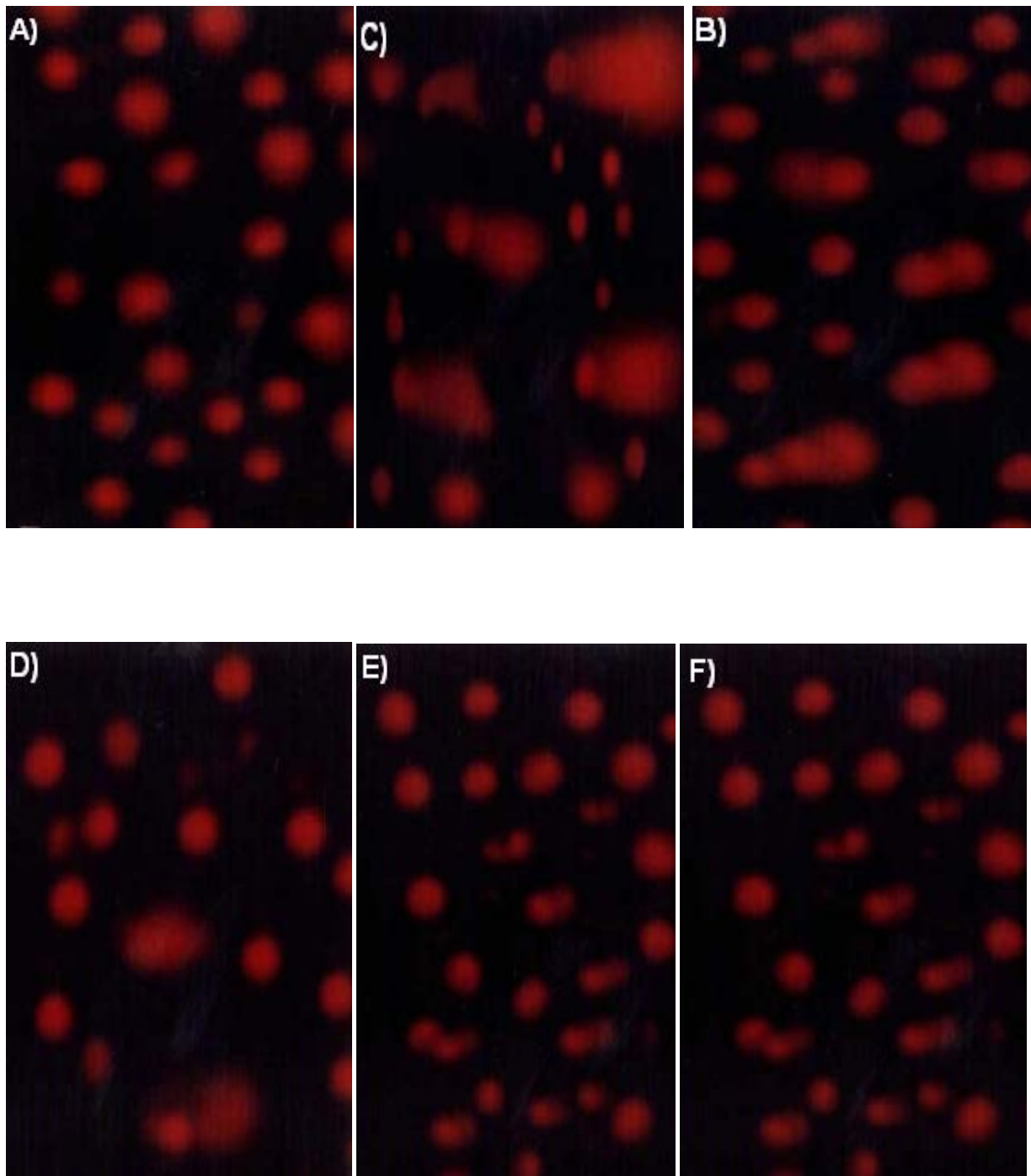


Fig 3.2 (B).

Fig 3.2 B. Comet assay (SCGE) SH-SY5Y cells cultured at 48h.The images were taken using fluorescent microscope (Zeiss Axiovert L410, Germany) after staining the cells with ethidium bromide (20mg/L)

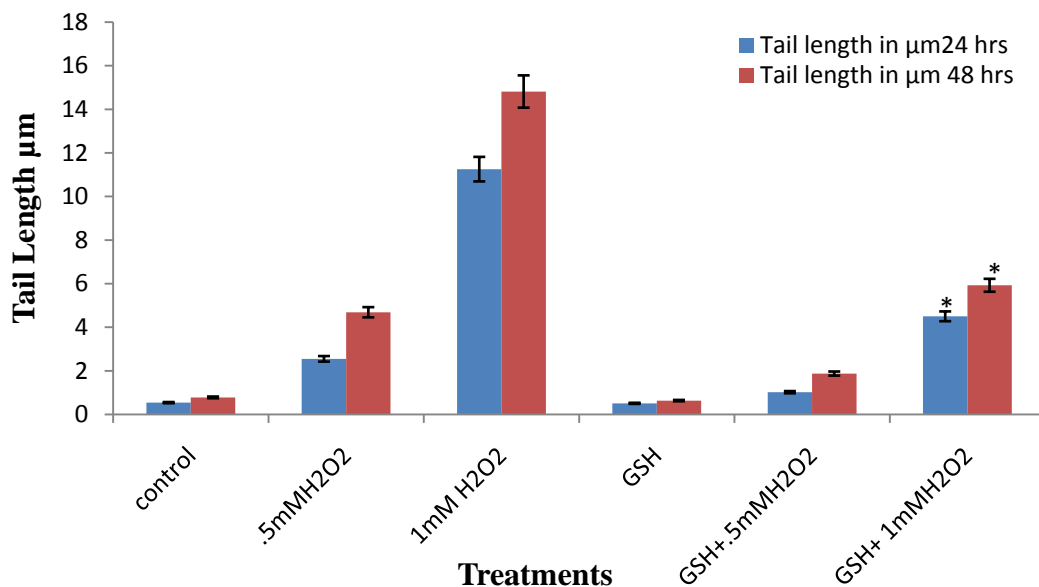


Fig. 3.2 C : Histogram showing the average tail length calculated in μm for the neuroblastoma cells SH-SY5Y treated with and without 0.5mM and 1mM H_2O_2 in the presence and absence of 2mM GSH for 24h and 48 h.*P < 0.05 represent significant values

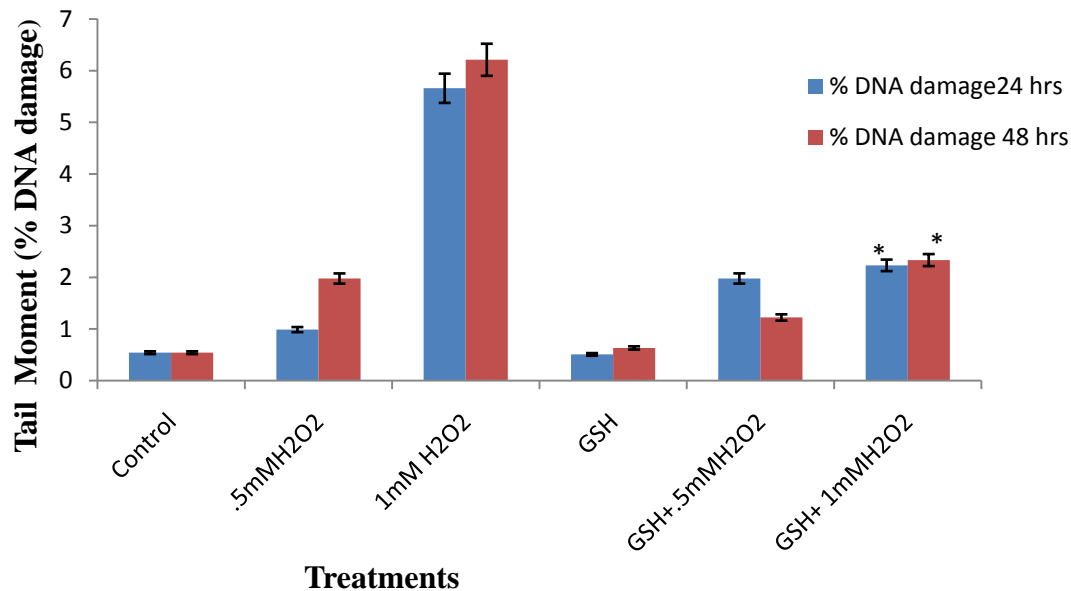


Fig 3.2 D Histogram showing the tail moment which is an index of DNA damage incurred by the treatment of neuroblastoma cells SH-SY5Y with and without H_2O_2 in the presence and absence of 2mM GSH for 24 h and 48 h. There is a significant difference between the DNA damage in the control and when H_2O_2 is added. The percentage of DNA damage was calculated by the DNA tail length and the distribution of the DNA in the tail called tail moments as described by [21].

*P < 0.05 represent significant values

The cells treated with various concentrations of H₂O₂ (0.5mM and 1 mM) had significant damage to their DNA in a dose and time dependent manner **Fig 3.2 (A, B)**. Significant DNA damage was observed, as shown in the by the formation of the comet at 24 hours after the exposure of cells to H₂O₂ and the tail length, as calculated in μm, increased significantly after 48 hours (**Fig.3.2 C, D**).

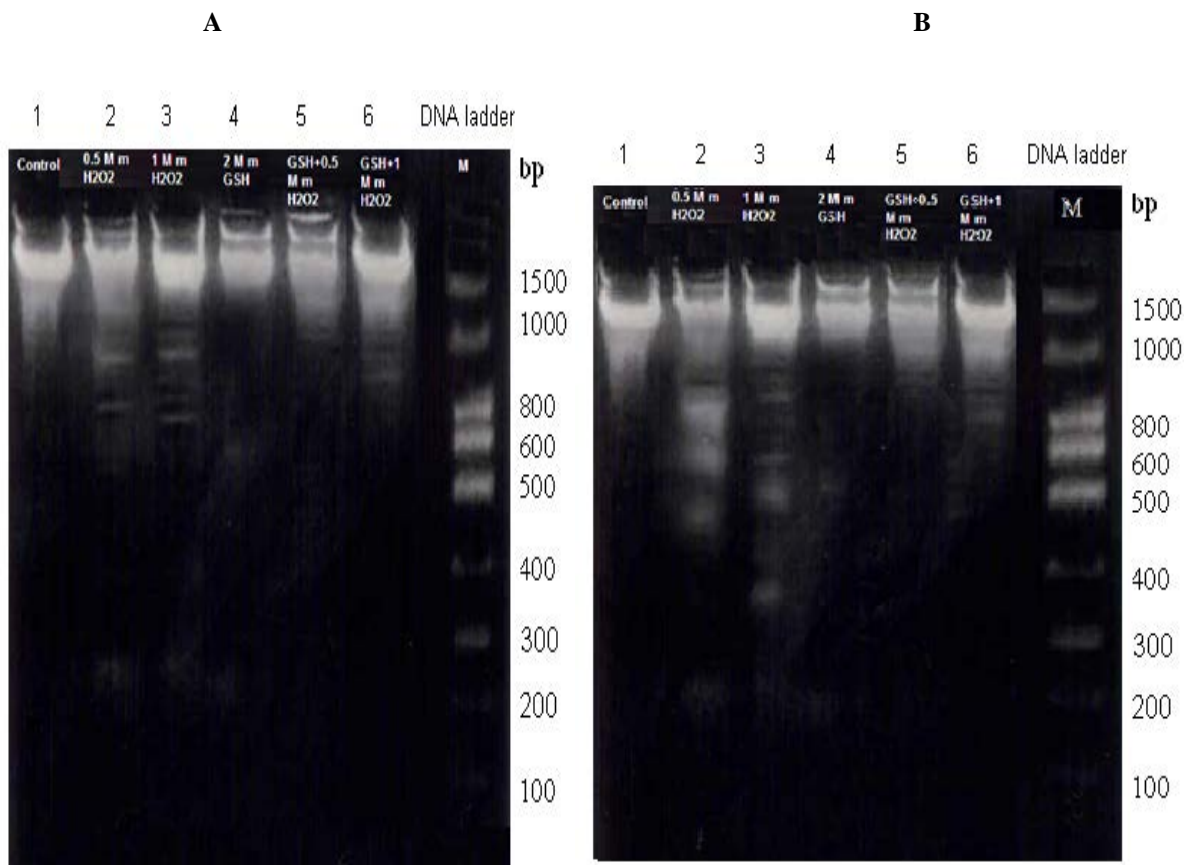


Fig 3.3 A B. 1.2% Agarose Gel of genomic DNA extracted, following exposure of neuroblastoma cell SH-SY5Y for 24h (A) and 48h (B) with and without H₂O₂ in the presence and absence of 2mM GSH. Lane 1 is DNA isolated from the control cells, Lanes 2 and 3 is the SH-SY5Y cells treated with 0.5mM and 1mM H₂O₂ respectively. Lane 4 is the SH-SY5Y cells treated with 2mM GSH alone, Lanes 5 and 6 are SH-SY5Y cells that were treated with 0.5mM and 1mM H₂O₂ respectively in the presence of 2mM GSH. Lane 7 is the 100bp DNA ladder (GenScript,USA).

The DNA fragmentation assay also supported our comet assay results, which showed degradation of DNA in the presence of 0.5mM and 1mM H₂O₂. (**Fig3.3 A, B, C, D**). After 24 hours rapid degradation of nuclear chromatin takes place even at 0.5mM H₂O₂ resulting in the formation of high molecular DNA fragments. This degradation was exasperated with time and dose and after 48 hours extensive fragmentation of nuclear DNA was observed.

3.3: Protective Role of GSH:

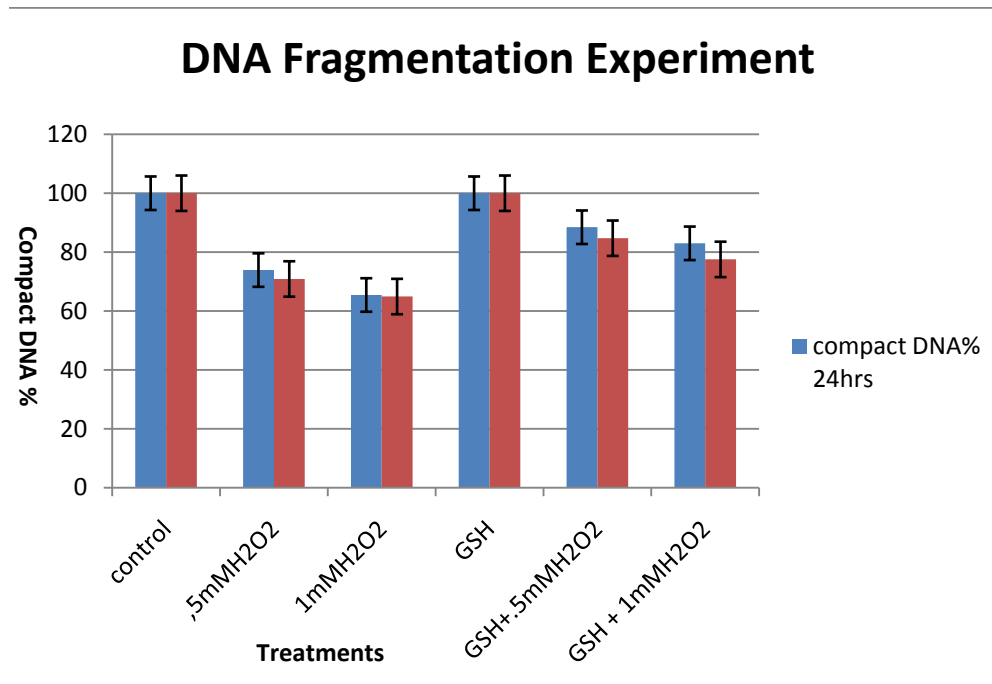


Fig 3.3 C: Histogram showing the percentage of compact DNA obtained for the control and treated cells after 24 h with 0.5mM and 1mM H₂O₂ in the presence and absence of 2mM GSH. The photograph containing fluorescent distribution of DNA on the gel is scanned by a digitizing densitometer as described by Freeman and Thompson, 1988.[26] The percentage of compact DNA was calculated and expressed in the form of a histogram.

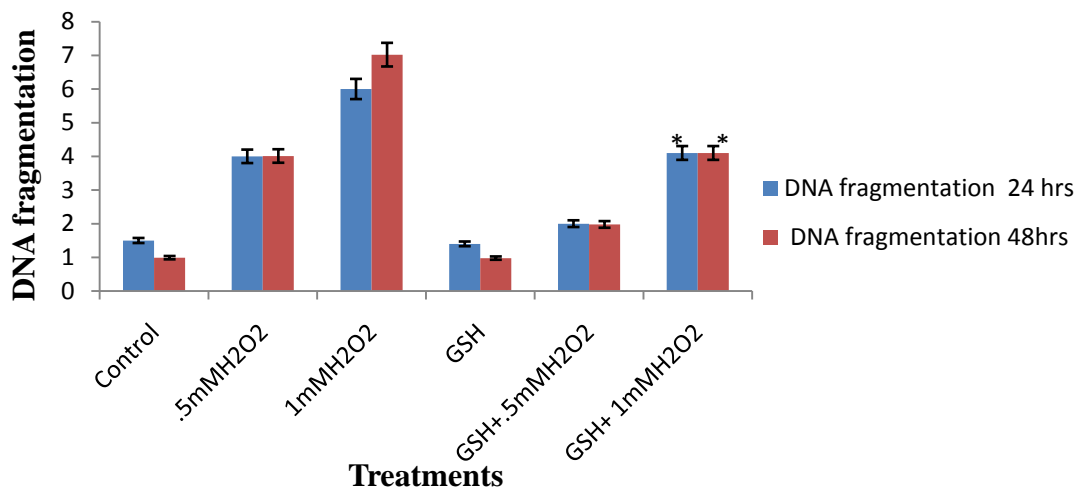


Fig 3.3 D: Histogram showing the number of fragmented DNA bands obtained for the control and treated neuroblastoma cells SH-SY5Y. Neuroblastoma cells SH-SY5Y were treated for 24h and 48h without and with 0.5mM and 1mM H₂O₂ in the presence and absence of 2mM GSH. The Genomic DNA were isolated from the above cells and run on 1.5% Agarose gel electrophoresis, followed by staining with ethidium bromide and photographed under UV light. The number of fragmented DNA bands was calculated by scanning the fluorescent DNA bands observed on the photograph of the gel using densitometer as described in section 2.5. *P < 0.05 represents significant values

The role of GSH in attenuating the toxic effect induced by H₂O₂ was investigated by the Comet assay as described before and also by DNA fragmentation assay.

Treatment of cells with 2mM GSH significantly reduced the DNA damage induced by H₂O₂ (Fig 3.2 C, D) Within 48 hours, the average tail length and tail moments (index of DNA damage) was reduced to about half of their value suggesting that the possible Neuroprotective role of GSH was also dose and time dependent.

DNA fragmentation assay further supported the Neuroprotective role of GSH as shown in (Fig 3.3). Histograms of the compact and fragmented DNA fragments (Fig 3.3 C, D) clearly showed extensive degradation of the DNA strand when the cells were cultured without GSH. However, the cells that were treated with 2mM GSH showed less than half the amount of degradation.

4. Discussion

Oxidative stress is a major contributor to many of the Neuropathophysiology of neuronal diseases. Increased free radical production with deficits in antioxidants has been reported in several brain disorders [28]. The current idea is that it could have a causative role, instead of being an epiphenomenon of the pathological processes [29.-30].

H₂O₂ has been shown to induce cell death in primary neuronal culture [31]. The exposure of cultured cells to H₂O₂ results in an imbalance in energy metabolism and the deleterious effects of hydroxyl and peroxyradicals on membrane lipids and proteins. As a result, excessive reactive oxygen species ultimately lead to apoptotic or necrotic cell death [33]. This H₂O₂-induced oxidative stress may trigger neuronal cell death with more or less necrotic and/or apoptotic depending on the concentration of H₂O₂ used [34]. Our study clearly showed that treatment of neuronal cells with H₂O₂ greatly compromised the viability of neuronal cells and resulted in their death. This apoptosis of neuronal cells was due to condensation and fragmentation of DNA.

The ability of glutathione to prevent the oxidant-induced cell injury is well documented in various cell types [35]. Glutathione with the enzymes GSH peroxidase and GSSG reductase serve to detoxify H₂O₂ to water and help maintain the cysteinyl-thiol (R-CH₂-SH) group of proteins in the reduced state, which is required for their functional integrity. Previous studies have shown that depletion of GSH could significantly affect the survival of dopamine neurons, particularly if they are under oxidative stress [36]. In future *in-vitro* studies of H₂O₂ induced neuronal cell cultures toxicity could prove a valuable tool to understand the mechanisms of oxidative injury to the CNS and will also help to elucidate various protective strategies against neurodegenerative and neurodevelopmental diseases, especially autism. Moreover, the Neuroprotective effect of GSH observed in our studies suggests a potential use of GSH and its derivatives, in preventing the progression of brain diseases.

5. Conclusion

We conclude that direct exposure to pro-oxidant H₂O₂ can induce neuronal death in culture which can be attenuated considerably in the presence of GSH. Also the *in-vitro* studies on primary neuronal culture described here offers an invaluable Cell culture model, which has a great potential to be used in future to study the effect of various neurotoxic environmental pollutants and subsequently its attenuation by beneficial compounds. That could help us to

understand the pathology and etiology of neurodegenerative disorder as well as investigating the effect of various therapeutic agents.

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