"Neuroprotective Role of Alpha-tocopherol in H_2O_2 induced Oxidative stress in Neonatal Sheep's brain Tissue Culture"

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Declaration of Interest

No conflict of interest to proclaim.

Abstract

Aims: Apoptosis and oxidative stress are considered as the key factors in the progression and pathogenesis of a number of neurodegenerative diseases. This research aims to assess the ameliorative influence of alpha tocopherol on oxidative stress in sheep's brain tissue. **Method:** Brain slice cultures from the frontal lobe of sheep's brain were pretreated with 10μM alpha tocopherol for 1h, followed by treatment with 2.5mM and 5mM hydrogen peroxide for an additional hour. The total reduced glutathione and lipid peroxidation levels were measured in the control and pretreated samples. The percentage of DNA damage was also measured by diphenyl and Agarose gel fragmentation assay. **Results:** Alpha tocopherol was shown to significantly increase levels of reduced glutathione and decrease the levels of "lipid peroxidation" in the brain tissue. DNA fragmentation assay showed significantly reduced DNA fragmentation in samples pretreated with alpha tocopherol, suggesting that Vitamin E has an anti-apoptotic role against the neuronal death induced by hydrogen peroxide. **Discussion:** These results suggested that alpha tocopherol has a neuroprotective role against the effects of "oxidative stress induced by hydrogen peroxide". This study further highlights the potential role of alpha tocopherol as a therapeutic agent against a wide variety of neurodegenerative diseases.

Key words: Alpha tocopherol (AT), oxidative stress, brain slice culture, neuroprotection, Lipid peroxidation.

Introduction

Oxidative stress (OS) is referred to as a principle pathogenic process behind various neurodegenerative diseases including Parkinson and Alzheimer's [1, 2]. Generally, oxygen is required by the biological tissues to fulfill the energy requirements of the body. Nevertheless, this oxygen consumption also results in free radical generation which possesses the capability to damage the cell. Due to the highly reactive nature of reactive oxygen species (ROS), it has a strong tendency to interact with biological molecules causing apoptosis through DNA fragmentation [3] as well as compromising the antioxidant defense system [4].

For the prevention of lipid peroxidationprocess and protection of oxy-radicals from the adverse influence of these free radicals, there is an "enzymaticantioxidantdefense system" in the body. Different compounds have been included in the category of non-enzymatic antioxidants, for example uricacid, carotenoids, ascorbate, and α-tocopherol. However, theenzymatic defense system includes enzymes suchascatalase, "superoxidedismutase" (SOD)andthe "glutathione-dependent enzymatic system", and "glutathioneperoxidase" (GPx).Recently much attention has been focused on Vitamin E and one of its active components alpha tocopherol, which is considered as a medicinal remedy for a number of neuro-degenerative diseases [5-7]. Vitamin E is clinically considered as a potential "free radical scavenger", which hinders the process of oxidative stress. Moreover, it also serves as the "chain-breaking antioxidant"whichprecludestheproliferationo f "freeradical reactions" [8, 9].

This research aims to assess the neuroprotective influence of alpha tocopherol on a short-term brain slice culture derived from frontal lobe of neonatal sheep's brain. It was found that the pretreatment of brain slice cultures with Vitamin E for one hour followed by treatment with hydrogen peroxide (2.5 mM and 5 mM), suggest significantly reduced "oxidative stress" (OS) induced on the

brain's tissue. It was reflected in the increased concentration of reduced glutathione and also reduced levels of lipid peroxidation. Pretreatment of the brain slice cultures with Vitamin E promoted neuronal survival and DNA integrity, as demonstrated by the "DNA fragmentation assay".

Materials and Methods

Chemicals: d-alpha-tocopherol (α-TP) was bought from Al Aesar (Cat. No. A17039). It was dissolved in Dimethylsufoxide (DMSO) and used as 100 X dilutions. All other chemicals used have been bought from the "Sigma-Aldrich Co., St Louis, MO, USA".

Brain Slice Culture: Neonatal sheep's skull was obtained fresh from the local slaughter house. The skull was cut open and meninges were removed in sterile conditions using sterile forceps and scissors. The Frontal lobe of the brain was sliced in small pieces of 1-2mm^3 and weighed under sterile conditions. The brain slices were kept in the bicarbonate buffered salt solution mimicking the composition of the cerebrospinal fluid which is commonly called Artificial Cerebrospinal Fluid (ACSF) and contains "12.4 mM sodium chloride, 2.5 mM potassium chloride, 1.3 mM Magnesium chloride, 2 mM Calcium chloride, 1.25 mM Dipotassium hydrogen phosphate, and 26 mM Sodium hydrogen carbonate. The pH was adjusted to 7.4". Then solution was sterilized by autoclaving and 10mM of sterile filtered Glucose (Sigma) was added before use. The buffer was gassed with carbon dioxide and oxygen (95% O_2, 5% CO_2) for 20-30 minutes to ensure saturation with oxygen (O_2) and carbon dioxide (CO_2) before using.

Treatment of Brain Culture: Approximately 1gm of brain slices were incubated in 10ml of the ACSF solution as described above at 37°C in a slow shaking water bath for each incubation type. Following are the different conditions of incubation for the brain slice cultures. The brain slice cultures (BSC) were treated as follows.

Group 1: BSC which were incubated without any treatments.

Group 2: BSC which were incubated with DMSO alone.

Group 3: BSC which were incubated with Vitamin E alone.

Group 4: BSC which were incubated for one hour without any treatment with vitamin E, followed by treatment with 2.5mM H_2O_2.

Group 5: BSC which were pre-incubated with 10µM of alpha tocopherol for one hour followed by the treatment with 2.5mM H_2O_2 for an additional one hour.

Group 6: BSC which were incubated for one hour without the treatment followed by treatment with 5mM H_2O_2 for one hour.

Group 7: BSC which were incubated in the presence of 10µM Vitamin E for one hour followed by treatment with 5mM H_2O_2.

Preparation of Brain Tissue for Biochemical Assay

After incubation, the conditioned medium was removed from the brain tissue slices and kept for reduced Glutathione assay. The brain tissue was cleaned with sterile cold PBS that was subsequently homogenized in 5ml of cold sterile PBS. About 1ml of brain homogenate was used for each biochemical assay.

"DNA Fragmentation and Quantitation Assay"

The degree of "DNA fragmentation" was assessed through the method described by Taylor [10]. Brain tissue homogenate was lysed with equal volume of lysing buffer, "5 mM Tris/HCl pH 8, 20 mM EDTA, and 0.5% triton X-100", and this lysate was centrifuged for the isolation of fragmented chromatin and intact chromatin. A concentration of "12.5% trichroacetic acid" (TCA) was used to precipitate supernatant and pellet, then the precipitated DNA was treated for 10 min at a

temperature of 90ºC in 1ml of 5% TCA. Furthermore, a reaction involving diphenylamine was conducted for quantitative analysis overnight at room temperature. The optical density was measured at 600nm against blank. The fragmentation percentage was analyzed as "the ratio of DNA in the supernatant to the total DNA (supernatant plus pellet)".

"**DNA Extraction and Electrophoresis"**

The influence of H_2O_2 on fragmentation of DNA has been analyzed through gel electrophoresis method, as demonstrated by Lee [11]. The tissue homogenate was re-suspended in a lysis buffer (10mM Tris-KCl pH 8.0, 10mM NaCl, 10mM EDTA, 100µg/ml proteinase K, and 1% SDS). Phenol/chloroform was used to extract the lysate with the ratio of 1:1, v/v, while ethanol was used to precipitate the DNA fragments. In the buffer, DNA pellets were determined as "10 mM Tris-HCl, 1mM EDTA, and pH 8.0". Then, the buffer loaded DNA samples were introduced into 1.8% agarose gel. Afterwards, the DNA was photographed followed by the visualization through ethidium bromide

"Reduced Glutathione Assay"

This assay was carried out through procedure of Beutler et al. [12]. In this assay, about 1 ml of brain homogenate and 1ml of conditioned medium were used. Then, 1.5 ml of "double distilled water" (DDW) was introduced to the tissue homogenate and conditioned medium, which was then preserved in 0.6 ml of precipitating chemical containing "1.67 g of glacial metaphosphoric acid, 0.2 g of EDTA and 30.0 g of NaCl" made up to 100 ml with DDW. The mixture was placed in the centrifugation machine for 10 minutes at a speed of 1200xg. To "0.3 ml of supernatant, 2 ml of Na2HPO4 (0.3 M) and 0.25 ml of 5, 5' dithio-bis-2-nitrobenzoic acid (DNTB), and 0.4 % in 1% sodium citrate" were added, and volume was made up to 3 ml with DDW. The OD was measured against blank at 412 nm, while the value was expressed as µg of reduced glutathione per gm of tissue.

"Lipid Peroxidation Assay"

"Lipid peroxidation" (LP) was calculated using the procedure of Garcia et al. [13], using the TBARS (thiobarbiturate reactive substances) assay. About 1 ml of homogenate of brain was placed in incubator for one hour at 37°C. Approximately 1.5 ml of 20% TCA was added and centrifuged at 600 xg for 10 minutes, and 1 ml of newly arranged "Thiobarbituric acid" (TBA) 0.67% was added to 1ml of supernatant, and the reaction mixture was heated in a boiling water bath for 10 minutes. Absorbance was recorded at 535nm by the help of comparison with a blank reagent. The values were expressed as Mmoles of malonaldehyde formed hour/gm of the tissue.

"Statistical Analysis"

Values were expressed as mean ± S.D. The data were represented statistically in the form of numbers, standard deviation (SD), and mean. The contrast among various groups was done using an independent sample "T-Test using Microsoft Excel program 2010 for comparing two groups. A "probability value" (p value) of ≤ 0.05 was referred to be significant. For the statistical analyses, SPSS program (v22.0.0.0) was used.

Results

"Effect of alpha-tocopherol on H2O2 induced cytotoxicity": DNA fragmentation assay using diphenyl amine showed that the percentage DNA fragmentation was significantly reduced when the brain slices were pretreated and co-incubated with vitamin E at both 0.25mM and 0.5mM H_2O_2 concentrations (Fig 1). The % DNA fragmentation was reduced to 5% with the pretreatment of brain cultures with vitamin E as compared to the

control cultures. The % DNA fragmentation at 2.5mM was reduced to about 4% when the cultures were treated with 2.5mM of hydrogen peroxide in the presence and absence of alpha tocopherol. The % DNA fragmentation at 5mM was reduced to 12% when the pretreated and control cultures were compared. This study was further supported by agarose gel electrophoresis in which the genomic DNA was extracted from the samples and subjected to 1.8% agarose gel. This showed that there is extensive fragmentation of DNA with the treatment of H2O2 (Fig 1; Lane 3); however, the presence of Vitamin E protected DNA from undergoing fragmentation as shown in Fig 2 lane 4 and 5.

In all of these experiments the effect of DMSO alone was studied as well as it was used as a vehicle for the treatments. According to the experiments performed in this study, DMSO alone did not alleviate the level of oxidative stress or the amount of DNA fragmentation induced by H_2O_2. The neuroprotective effect of vitamin E on Hydrogen peroxide induced oxidative stress on the lipid peroxidation level was also investigated (Table 1). The lipid peroxidation level was calculated by the Mmoles of malandehyde formed per hour.

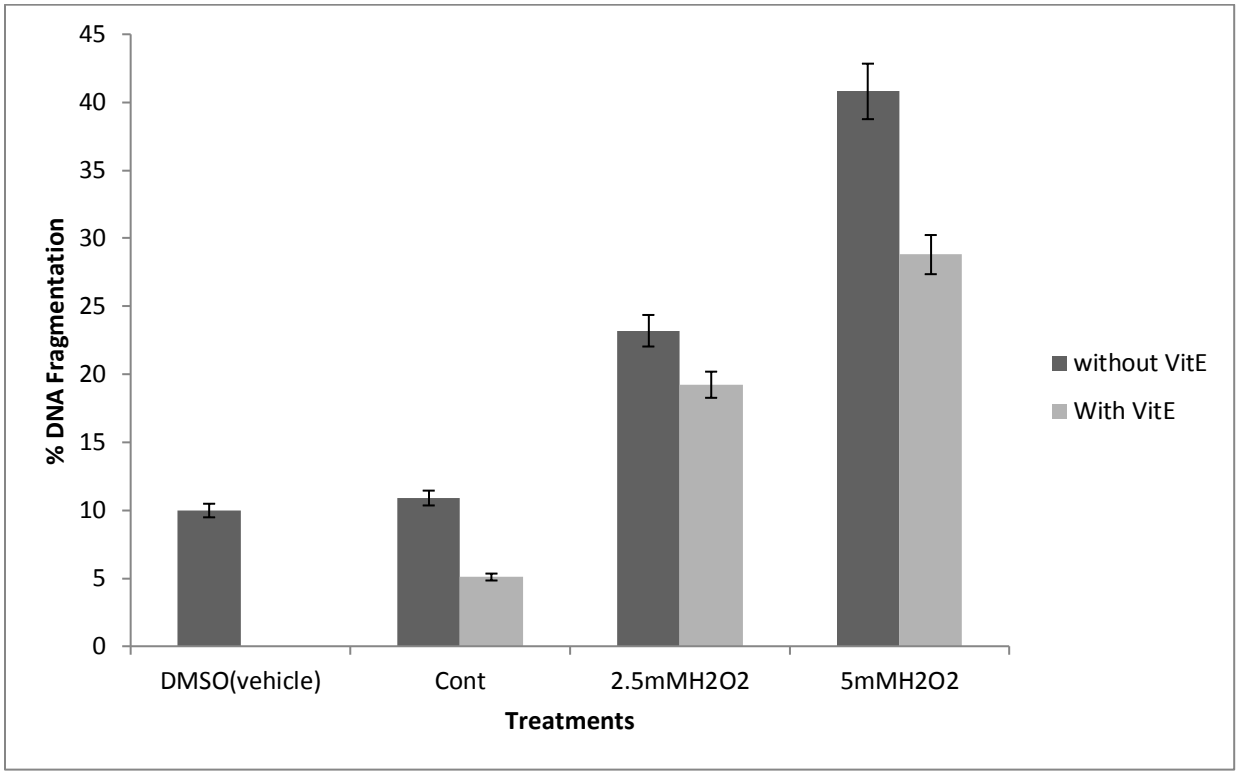

Figure1: **Histogram of %DNA fragmentation at various culture conditions.**Percentage DNA fragmentation was calculated by diphenyl amine assay method as described in materials and method section, in the control culture, with DMSO, 10µM alpha tocopherol alone and pre-treatment with 10µM alpha tocopherol followed by induced oxidative stress by 2.5mMH_2O_2 and 5mM H_2O_2.

	TBARS (Mmoles of Malondehyde formed/hour/gm (Tissues)	Reduced Glutathione (µg GSH/gm)	
		Conditioned Medium	Tissue
Group1(Control)	7.526±0.641	2.32± 0.09	1.73±.06
Group 2(With DMSO)	6.255± 0.076	2.24± 0.07	1.64±0.03
Group3(alpha-tocopherol)	3.782±0.083*	2.66± 0.1	1.865±.025
Group 4(2.5mM H_2O_2)	9.519±0.0321	0.1595±.0075	.449±.006
Group 5(2.5mM H_2O_2+ Alpha-tocopherol)	5.301± 0.378*	1.392±.006*	1.116±.106*
Group6(5mM H_2O_2)	10.183± 0.0545	.2285±.0055	.409±.034
Group 7(5mM H_2O_2 + Alpha-tocopherol)	4.375± 0.0994*	.6165±.05*	1.902±.055*

Table 1: The effect on oxidative stress (reduced GSH) and lipid peroxidation levels on pre-treatment of neonatal brain slice cultures with alpha tocopherol, in the presence and absence of induced oxidative stress by 2.5mMH_2O_2 and 5mM H_2O_2. The values were expressed as the mean of three independent experiments and ± SD were calculated. (*) represent the values are significantly ($p \leq 0.05$) different in the cultures with induced oxidative stress by hydrogen peroxide in the presence and pre-treatment with Alpha tocopherol, when compared to the hydrogen peroxide treated cultures alone.

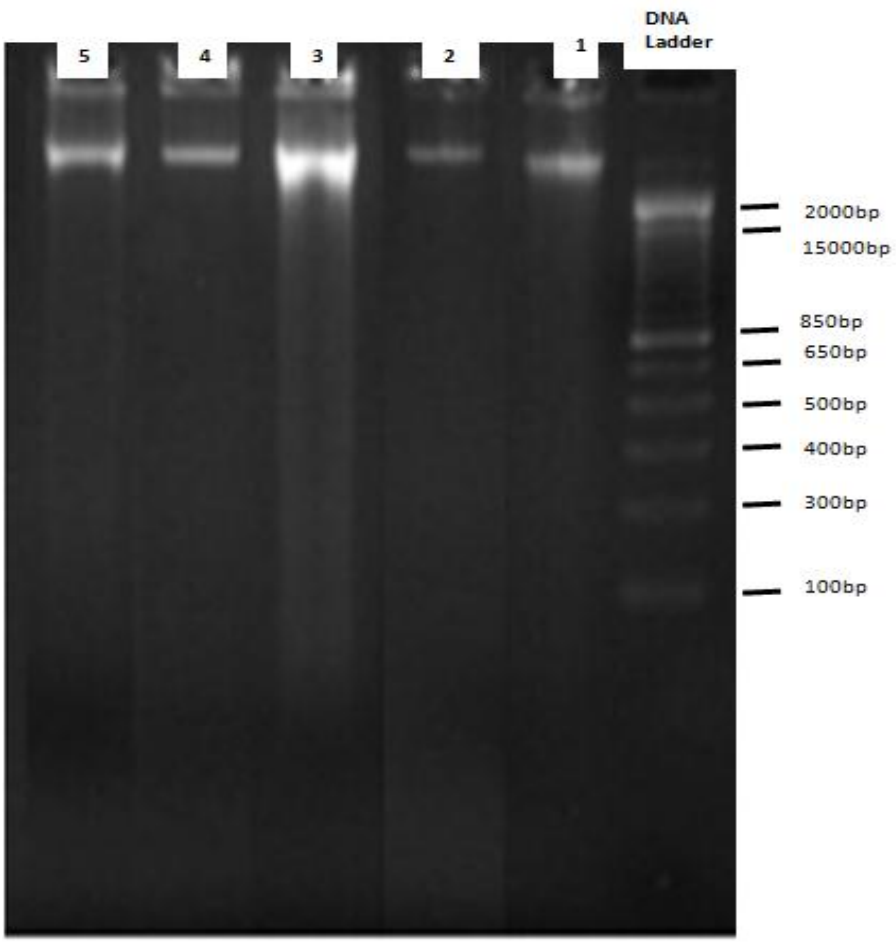

Figure 2: **1.8% agarose gel electrophoresis.** Genomic DNA was isolated from different treatments and run on 1.8% agarose gel. DNA bands were visualized by ethidium bromide staining and photographed. **Band 1**is the genomic DNA isolated from Group 1, **Band 2** is the genomic DNA isolated from Group 2 (With alpha tocopherol only), **Band 3** is genomic DNA isolated from Group 5(with 5mM H_2O_2 treatment alone), **Band 4and 5** is genomic DNA isolated from Group 6 and 7(pre-treatment with alpha tocopherol followed by 2.5mM, 5mM H_2O_2) respectively.

Treatment with vitamin E alone showed reduced amount of lipid peroxidation when compared with the control cultures. However, the lipid peroxidation measured in the control cultures as the amount of malandehyde formed per hour was not significantly ($p \leq 0.05$) different from 2.5mM and 5mM hydrogen peroxide treated cultures for 2 hours. The lipid peroxidation level was significantly reduced ($p \leq 0.05$) with the pretreatments of the brain slice cultures with Vitamin E and then exposed to H_2O_2 treatments when compared to the hydrogen peroxide treated cultures alone both at 2.5mM and 5mM concentrations.

Another marker of oxidative stress, i.e. reduced glutathione levels (GSH) were also investigated in this study. Reduced glutathione levels were measured in the conditioned medium as well as present intracellularly. Reduced glutathione levels were significantly increased in the brain cultures that were pretreated and co-incubated with Vitamin E and H_2O_2 compared to the H_2O_2 treated cultures alone (Table 1). The levels of reduced GSH intracellularly were increased eight-fold in the cultures pretreated with vitamin E and then co-incubated with 2.5mM H_2O_2, when compared to 2.5mM hydrogen peroxide treated cultures alone. The levels of reduced glutathione were also shown to have increased to three-fold at 5mM hydrogen peroxide concentration. Reduced glutathione levels were also observed in the conditioned medium, as the levels of reduced GSH were significantly increased ($p \leq 0.05$) to two-fold in 2.5mM and 5mM hydrogen peroxide treated cultures in the presence of vitamin E respectively when compared with hydrogen peroxide treated cultures alone.

Discussion

In this study, the short-term brain slice culture was used to study the therapeutic potential of Vitamin E. Brain slice culture has a unique advantage over other "*in-vitro* and *in vivo* experimental models" as this maintains the neuronal activity with complete functional "local synaptic circuitry" and permits direct treatments through pharmacological agents that are capable to modulate the response [14-16]. The alpha tocopherol has the ability of crossing the "blood brain barrier" (BBB) and protecting the neuronal cells through the "oxidative stress" (OS), which can be considered as a principle contributing factor in various neurodegenerative diseases, as elucidated in a number of studies [5].

Most studies that were previously conducted, have examined the influence of Vitamin E on the OS induced by co-incubation of Vitamin E with the agonist. In contrast, this study examined a hypothetical function of vitamin E for neuroprotection, so brain slice cultures remained pre-incubated with vitamin E before treating them with H_2O_2 inducing oxidative stress. This is of particular interest as the neuroprotective property of vitamin E is not only primarily attributed to its inherent antioxidant action but may also be mediated through other cellular functions. The concentration chosen for the treatment is 10µM which has been shown to be the optimum concentration in other *in-vitro* studies [17].

H_2O_2 is considered as a principle reactive oxygen species, also known as ROS that is produced by the process of redox reaction and acts in "intracellular signaling cascade" as a messenger [18]. This compound is also used as the initiator of OS in the in–vitro models, as it causes "mitochondrial membrane dysfunction", lipid peroxidation, and immense damage in the DNA structure [19].

Furthermore, the dietary antioxidants are also significant for the prevention of

toxiceffect induced by "endogenousreactive oxygen species", while many studies assert that vitamin E possesses a potential role against OS. In addition, the vitaminE deficiency may elevate the vulnerability to OS-induced neuronal toxicity. A number of researches have investigated the vitamin Esupplementation function to reduce DNA damage and OS [20-22]. Vitamin E is composed of alpha- tocopherol, beta-tocopherol, gamma- tocopherol, and delta-tocopherol isomers. However, in this research, alpha-tocopherol was selected for investigation, which is one of the isomers of Vitamin E. Alpha-tocopherol is generally considered as a powerful anti-oxidant because of its high bioavailability and bioactivity. Structurally, alpha-tocopherol has electron-donating methyl groups attached to its chromanol ring, which act to inhibit lipid peroxidation [23].

As far as the "free radical scavenging properties" of alpha tocopherol are concerned, the current research has been conducted to assess the vitamin E neuroprotective potential in the neonatal sheep brain slice culture. This research has explored the neuroprotection potential of Vitamin E in terms of its effect on neuronal cell viability using the DNA fragmentation assay by diphenylamine method. In measuring apoptosis, "diphenylamine assay" is considered as a significant tool to determine the fragmentation percentage of known DNA amounts in oligosomal-sized fragments. One more benefit is that this diphenylamine assay is the apoptotic analysis of DNA fragmentation in both shed and adherent cells prior to the experimentation or treatment. This assay was first described in 1930's but modified considerably by other researchers [10, 24]. These modifications have resulted in an improved susceptibility of almost five folds by the addition of acetaldehyde and sulfuric acid, as well as by permitting the "colorimetric reaction" to take place at room temperature overnight. Such alterations were shown to reduce an interface through certain substances which were considered initially as a drawback in an originally labeled method, which in turn improved sensitivity of assay. Meanwhile, a reaction of diphenylamine took place, emphasizing the benefits of deoxyribose and purines bonding that were immensely labile. As soon as the bonds were broken, there was liberation of inorganic phosphates throughout the DNA to give off the substrate product that was assessed through this reaction. This study was further supported by DNA fragmentation assay on agarose gel, which clearly showed the integrity and stability of the genomic DNA derived from Vitamin E treated cultures although they induced oxidative stress with H_2O_2 as compared to the cultures that were exposed to H_2O_2 alone.

Such deleterious effect of ROS in this "Lipid peroxidation" can be significantly implemented to treat several acute as well as chronic brain disorders [25,26]. The most common and prevalent "lipid peroxidation assay" is regarded as TBARS assay that involves the measurement of end products' reactivity as a result of lipid peroxidation, i.e. MDA that reacts with the TBA to give off red adduct which can be quantified spectrophotometrically. In this research, "lipid peroxidation" was found to have decreased in the conditions where Vitamin was present. Vitamin E has been suggested as primary antioxidant reacting with peroxyl radicals produced by lipid peroxidation in other studies [27].

A number of Lipoxygenases, including 15-Lox, 12-Lox, and 5-Lox possess the capacity of inserting the molecular O_2 on the 5^{th}, 12^{th}, or 15^{th} atom of carbon in the arachidonic acid, as a result of which distinct hydroperoxyeicosatetraenoic

(HPETE) acid is formed [28]. The "12 - Lox produces 12 (S) - HPETE" is additionally metabolized to produce 4 different products including an "alcohol [12(S)-HETE]", a "ketone; 12 – ketoeicosatetraenoic acid", and two "epoxy alcohols; hepoxilin A3 and B3" [29]. Moreover, the formation of 12 - Lox in the neuron is further confirmed by a number of immune-histochemical studies which particularly involved nucleus, glial, olivary, striatum, hippocampus, and cerebral endothelial cells [30]. By using the HT neural cells and immature cortical neurons, a decrease was estimated in the GSH which triggered stimulation in neuronal 12-Lox that resulted in an influx of Ca2+ and production of peroxides, which eventually leads to the death of cell [31]. The principle role of 12-Lox has been formerly reportedin the "glutamate-induced death of primary neuronal cultures" by presenting that "12 - Lox deficient neurons" were reluctant to the "glutamate-α-Tocopherol" for the delivery of cell protection [32]. This protection will depend upon the concentration against "paraquat - induced cytotoxicity". However, it will not influence the toxicity induced by H_2O_2. At this stage, the molecular oxygen will dismutate into H_2O_2, and later generate the OH molecule in existence of metal ions, by breaking down H_2O_2 [33].

Therefore, it can be assumed from the present findings that there is a reaction between the oxygen molecule and α-tocopherol prior to the development of OH molecule. However, thorough destruction of intracellular oxygen is considered to represent a significant neuroprotective action mechanism including a-tocopherol in the striatal cultures among rats. Moreover, it was also noticed that the α-tocopherol has efficiently prevented the "oxidized low-density lipoprotein-mediated apoptosis" through a decrease in "nuclear factor-nB activation" and InB degradation, as well as by a decrease in "Jun kinase cascade activation" and "mitogen-activated protein kinase".

Furthermore, these results have also suggested that α-tocopherol also possess the non-antioxidant properties which are involved in an anti-apoptotic action. Such non-antioxidant actions take place at an average concentration of 10 – 100 AM. Therefore, in accordance with these researches, an "ameliorated staurosporine-induced apoptosis" is susceptible at the concentration of 10 – 100 AM which has been revealed in the current research. Hence, the "anti-apoptotic neuroprotection by α-tocopherol" can be attributable to the non-antioxidant property at least partly in the striatal cultures of rats.

The neuroprotective effect of alpha tocopherol is by both antioxidant and non-antioxidant pathways in vivo [9]. It has been observed that hydrogen peroxide treatments can cause depletion of glutathione levels and accumulation of intracellular peroxidases [34]. However, the reduction of H_2O_2 cytotoxicity through pretreatments with Vitamin E suggests that neuroprotection mediation is through the cellular effect which may include modulation of glutathione metabolism.

Over all, this study showed that pretreatment of brain slice cultures with Vitamin E may substantially increase a viability of neuronal cell by increasing the antioxidants enzymes such as amount of reduced GSH and remarkably reduce the "Lipid peroxidation levels".

Conclusion

Apoptosis and oxidative stress play an important role in the pathogenesis of a number of neurodegenerative disorders. A neuro-protectiveeffect of alpha tocopherol on H_2O_2-induced OS in short-term neonatal sheep's brain tissue culture was investigated. The results showed that the DNA fragmentation and apoptosis caused by H_2O_2

treatments to the brain cultures was attenuated significantly in the occurrence of vitamin E. Moreover, these findings also suggest that the levels of "lipid peroxidation" were significantly reduced in the presence of Vitamin E, suggesting a possible neuroprotective role of vitamin E against oxidative stress.

The Glutathione levels were also increased significantly ; thereby, mitigating hydrogen peroxide induced oxidative stress. This study suggests that α-tocopherol may be directly involved in neuronal survival by reducing the oxidative stress *in- vitro*.

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