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# 흰쥐의 뇌 Astrocyte에서 amyloid-β 25-35로 유발된 세포 독성과 지질과산화에 대한 胡桃의 보호효과

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## Protective effects of Juglandis semen on amyloid-β-induced neuronal toxicity and lipid peroxidation in rat astrocytes

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胡桃 (Juglandis semen)가 치매에 미치는 영향을 알아보기 위하여 치매(Alzheimer's disease) 유발물질로 알려진 amyloid-β(Aβ) 25-35를 흰쥐의 뇌 신경세포의 일종인 astrocyte에 처리한 후 뇌의 신경세포에 대한 독성 및 세포막에서의 지질 과산화에 미치는 영향을 검토하였다. 胡桃는 Aβ 25-35로 인한 신경세포의 파괴를 억제하는 것으로 나타나 신경세포의 손상을 예방하고 보호하는 효과가 있었다. 그리고, 지질의 과산화 지표인 malondialdehyde 생성은 Aβ 25-35 처리로 크게 증가하였으나, 胡桃의 전처리와 후처리로 크게 감소되어 胡桃가 세포막 파괴로 인한 뇌세포의 손상을 방지하는 것으로 나타났다. 이러한 결과들을 볼 때, 胡桃는 신경세포의 하나인 astrocyte에 대한 보호효과와 세포막에서 지질의 과산화를 저해 및 Aβ 25-35 처리와 같은 치매 유발 독성에 대한 적응능력 향상을 통하여 뇌 신경세포를 보호하는 효과가 있음을 보여주는 것으로 노인성 치매 등의 임상적 응용에 그 효과가 기대된다.

**Key Word :** Juglandis semen, dementia, amyloid-β, astrocytes, lipid peroxidation

## I. Introduction

Alzheimer's disease (AD) is a neurodegerative disorder characterized by a progressive cognitive decline result ing from selective neuronal dysfunction, synaptic loss, and neuronal cell death. The well-studied neuropatho logical features of AD showed the following characters such as loss of neurons, formation of intra-neuronal neurofib rillary tangles composed of paired helical filaments of the cytoskele tal protein tau, and extracellular plaques composed primarily of diffused or compacted deposits of amyloid-β (Aβ) aggregates, with or without a compo nent of dystrophic neuritis[1,2].

It has suggested that glial cells in AD may play in the neurodegenerative cascade and leads to Alzheimer dimen tia. Neurodegerative disorders usually involve the activation of astrocytes and gliosis (microglia). Glial activation involves morphological changes (more spherical cell soma, hypertrophy of nuclei, appearance of extensive cellular processes) and changes in expression of a large number of proteins[3]. In AD, activated astrocytes surround the neuritic shell of the amyloid plaque, and activated microglia are near the center of the neuritic shell adjacent to the amyloid core[4]. There are a number of stimuli that cause glial activation. One of the best inducers of glial activation is neuronal dysfunction or injury. Although the role of glial activation in AD is uncertain, it was known that reactive glia are associated with amyloid plaques[5]. In addition, some cytokines and inflamma tory mediators produced by activated glia have the potential to initiate or excerbate the progression of neuropatho logy[5-8].

The factors responsible for inducing and maintaining the glial activation state

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in AD are unknown. However, it was suggested that Aβ is involved in the neurodegenerative process. Aβ is 39-43 amino acids long and proteolytically derived from an integral membrane protein termed amyloid precursor protein (APP)[9,10], although mechanism for APP processing is still unknown. There are many in vitro studies demonstrating that Aβ is directly neurotoxic and increase neuronal susceptibility to other toxic agents[5,10,11]. The toxic effect of Aβ is correlated with its ability to form aggregates[12]. Both oxygen species[13] and excessive $Ca^{2+}$ influx[14] are also implicat ed in the mechanism of Aβ neurotox icity. In contrast, it was also reported that Aβ promotes neurite outgrowth under certain culture conditions instead of toxic action[15].

On the other hand, the action of Aβ that affect glia, and the glial responses to Aβ exposure are not well understood. When the effects of synthetic Aβ peptides (Aβ1-42, Aβ17-42, and scram bled Aβ1-42) on cultured astrocytes were examined, Aβ 1-42 induced a robust astrocyte activation, as evidenced by morphological changes, upregulation of the interleukin-1 mRNA, and stimulation of inducible nitric oxide synthase (iNOS) mRNA and nitric oxide (NO) release[16].

The reactivity of different free radicals varies and some cause severe damage to biological molecules, especially to DNA, lipids and proteins. In the presence of oxygen, free radicals can react with polyunsaturated fatty acids, resulting in highly reactive peroxyl free radicals. Peroxyl free radicals can further propagate the peroxidation of lipids or compromise the integrity of cell membranes, therefore this is thought to be involved toxic actions of some chemicals[17-19].

Recently, several reports presented that natural dietary plants may play an antioxidative role in the prevention of aging and carcinogenesis and may offer effective protection from lipid peroxid ative damage in vitro and in vivo[20,21]. Therefore, much attention has been focused on natural antioxidants, in paticular it was reported the extract of *Juglandis semen* (JS) may exert an anti-aging action and neuro-protective activity[22]. Little is yet known about the pharmacological effects or active ingredients.

As an approach to know the interac tions between Aβ and glia, we have further examined the effects of synthetic Aβ peptides on cultured astrocytes. We tested Aβ25-35 peptides aggregated under various conditions. We report here that Aβ 25-35 is responsible for the activation of the astrocytes. This study also reports the effect of JS on cytotoxicity of cultured astrocytes and lipid peroxidation in Aβ -treated conditions. Aβ which can produce intracellular free radical was used for inducer of the peroxidation of cellular lipids.

## II. Materials and Methods

### 1. Materials

The Aβ 25-35 peptide was synthe sized by Applied Biosystem's Protein Synthesizer Model 470A (Peptron Co., LTD, Taejon, Korea). Fetal bovine serum (FBS), penicillin-streptomycin were obtained from GIBCO-BRL (Grand Island, New York, USA). Dulbecco's Modified Eagle's Medium (DMEM), glutamine, 3-(4,5-dimethylth iazol-2-yl)-2,5-diphenyl tetrasolium bromide (MTT), dimethyl sulphoxide (DMSO), 2-thiobabituric acid (TBA), 1,1,3,3-tetraethoxy propane (TEP), ethylened iamine tetraacetic acid (EDTA), nitro blue tetrazolium (NBT), catalase (from bovine liver), diethylene triamine pentaacetic acid (DETAPAC), β-nicotinamide adenine dinucleotide phosphate (β-NADPH) were purc hased from Sigma Chem. Co. (St. Louis, USA). 1-chloro-2,4-dinitro benzene (CDNB), NaN3 were obtain from Aldrich Chem. Co. (Milwaukee, WI).

### 1. Extract from Juglandis semen

JS (300 g) purchased from Dongguk University College of Oriental Medicine and extracted with boiling water for 3 hrs. Then, the extract was evaporated to under reduced pressure by 75%, 85%, 95% ethanol solution. The last extracts diluted by 0.9% NaCl and filterated. The extract solution was stored at 4℃.

### 3. Cell culture and preparation of Juglandis semen

Cortical astrocyte cultures were prepared from neonatal rat (1-2 day old)

pups by the method of Levision and McCarthy[23]. Cerebral cortex was dissected from neonatal day 1-2 Sprague-Dawley rat and dissociated by gentle trituration. Cells were plated in 6-well culture plates coated with polyethy lenimine (0.2 mg/ml in sodium borate buffer, pH 8.3) at a density of 40,000 cells per well. After overnight incubation in Dulbecco' s modified Eagle' s medium (DMEM ; Gibco) supplement ed with 20% fetal bovine serum, the medium was changed to serum-free defined medium for neurons [DMEM supplemented with 2 mM glutamine, 1 mM pyruvate, penicillin-streptomycin-amphotericin B mixture (Gibco), 5 mM HEPES, 0.5% glucose, 10 μg/ml insulin, 30 nM sodium selenite, 20 nM progesterone, 100 μM putrescine, and 20 μg/ml transferrin]. The cultures were incubated at 37℃ in an atmosphere of 5% CO2/95% room air, and the medium was replaced every other day. Experi ments were performed in 6-7-day-old culture.

Depending upon the experimental group, JS was added (at 2% volume in culture medium) to or omitted from flasks. After 16～18 h, cells were washed twice with warm phosphate-buffered saline (PBS) and serum-free medium added to the flask. Then the cells were treated with 10 μM Aβ peptide for 2 h and the content of thiobar bituric acid-reactive substance (TBARS) and enzyme activities measured. 10 μM Aβ peptide was diluted in serum-free medium and added to the cultures.

## 4. Determination of cell viability and toxicity assay

Cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the coloured formazan product by mitochondrial enzymes in viable cells[24]. Cell were cultured in polyethylenimine-coated 24 or 96 well culture plates at a density of 10,000 cells per well for lactate dehydrogenase (LDH) assay or 40,000 cells per well for MTT reduction assay. LDH activities in the medium were measured by a Cytotox 96 nonradioactive cytotoxicity assay kit (Promega) according to the manufac turer' s instructions. The results were expressed as percentages of peak LDH release on complete cell lysis (control).

The MTT reduction was measured essentially as described previously[10] with a slight modification. In brief, after incubating cells for 48 hrs with vaious samples, t-BHP and Aβ peptides, MTT (Sigma) solution in PBS was added to a final concentration of 0.5 mg/ml, and the cells were further incubated for 4 hrs at 37℃. After incubation, the plate were centrifuged at 90 x g for 10 min to obtain the resulting insoluble formazan precipitates. To dissolve the crystal precipitates, 150 l or 600 l of a 1:1 mix of ethanol and DMSO were added to each well. Each plate was gently shaken for approximately 20 min before reading on the Enzyme-Linked Immunosorbent Assay (ELISA) reader (measurement 570 nm, reference 620 nm). Absorbance of converted dye was measured. Assay values obtained on addition of vehicle were taken as 100%, and complete inhibition of MTT reduction (0%) was defined as the value obtained following addition of 0.9% Triton X-100.

To examine whether JS could attenuate the cytotoxicity of Aβ peptides, cultures were pretreated with indicated concentrations of JS for 4 hrs. Thereafter either 10 μM Aβ 25-35 was added to cultures and incubated for 48 hrs. LDH activity in the culture medium was determined as described above. To investigate the effect of pretreatment with Aβ peptides on the cytotoxicity induced by hydrogen peroxide or glutamate, cells were pretreated with 10 μM A peptide for 48 hrs, and then 100 μ M hydrogen peroxide or 100 μM glutamate was added to cultures and incubated further for 4 hrs or 1 hr, respectively. For all findings, each condition represents five separate wells per experiment and is repeated in two or five independent experiments.

## 5. Treatment of astrocytes with Aβ peptides

Confluent astrocytes were trypsinized and plated into T-75 tissue culture flasks at a density of 5 x $10^6$ cells/flasks (for lipid peroxidation and antioxidative enzyme activity), or into 96-well plates at a density of 5 x $10^4$ cells/well (for MTT reduction assays). After 24 hrs, cells were washed with PBS to remove serum, and cultures were incubated in DMEM free FBS for an additional 12 hrs before addition of Aβ peptides or control buffer.

### 6. Lipid peroxidation assay

The release of TBARS into incubation medium was measured by the method of Glascott et al.[25]. Depending upon the experimental group, JS was added (at 2% volume in culture medium) to or omitted from flasks and then overnight incubation. Overnight (16 ~18 hrs) cells were washed twice with warm PBS buffer and 10 ml of serum-free medium were added to the flask, and the cells were treated with Aβ peptide. After Aβ peptide treatment for 2 hrs, the cells were removed by scraping, then trichloroacetic acid (TCA) was added to scraped cell and medium (4.5% final concentration). The scraped cells were sonicated for 20 sec and centrifuged to pellet the protein. In brief, 1 ml of TCA supernatant was added to 2 ml of TBA solution (composed of 0.45%, w/v, TBA and 7.5%, v/v, acetic acid, pH to 4.15 with 10 N NaOH). This reactive solution was placed in a boiling water bath for 15 min, cooled to room temperature and read on a Gilford Response spectrophotometer with excitation wavelength of 532 nm. TEP was dissolved in 0.01 N HCl to produce malondialdehyde (MDA), and this was used to generate a TBARS standard curve. The data was expressed as nmol of TBARS/mg protein.

### 7. Protein determination

Protein was determined on each sample by the method of Smith et al.[26] (using bicinchoninic acid), using bovine serum albumine as the standard.

### 8. Statistical analysis

Standard procedures were used to calculate means and standard deviation of the mean. Mean values were compared using Duncan's Multiple Range Test with on SAS program (SAS Institute, Cary, NC) ; $P<0.05$ was considered significant.

## III. Results & Discussion

### 1. Effect of Aβ25-35 peptide on cultured astrocytes by MTT assays

The present study was done to investigate the effects of JS on cultured astrocyte cell system and lipid peroxidation in Aβ25-35 treatment conditions. As shown in Fig. 1, cell killing was significantly enhanced by addition of increasing concentrations of Aβ compared to those of untreated group. When we measured the amount of MTT reduction from cultured rat cortical astrocyte cells at 48 hrs after peptide treatment, the Aβ25-35 decreased MTT reduction by 55% of the control value at 0.1 μM concentra tion. Aβ25-35 reduced MTT reduction by 30% and 20% at 50 μM and 100 μM, respectively.

### 2. Effect of Aβ 25-35 peptide on cultured astrocytes by LDH assays

On the other hand, cellular toxicity of Aβ25-35 was observed during cell culture by means of decrease of LDH activity. Following the appropriate incubation time with various peptides, LDH activities in the medium were measured by a Cytotox96 nonradioa ctive cytotoxicity assay kit (Promega) according to the manufacturer's guidances. The results were expressed as percentage of peak LDH release obtained on complete lysis. The Aβ25-

**Table 1.** LDH activity in the culture medium of cultured rat cortical astrocytes at 48 hrs after treatment with indicated concentrations of Aβ,25-35 and protective effects of Juglandis semen (JS) treatment

| Concentration (μM) | LCD (% of maximal release) | | |
|---|---|---|---|
| | Without | Pretreatment of JS (100 μg/ml) | Posttreatment of JS (100 μg/ml) |
| 0.1 | 2.52±0.23 | 2.33±0.23 | 2.65±0.26 |
| 0.5 | 2.56±0.25 | 2.34±0.23 | 3.43±0.34 |
| 1.0 | 3.54±0.3 | 4.34±0.43 | 4.65±0.46 |
| 5.0 | 4.23±0.43 | 6.31±0.66 | 4.87±0.42 |
| 10 | 5.87±0.65 | 7.34±0.69 | 5.32±0.56 |
| 25 | 10.65±1.07 | 8.76±0.67* | 11.53±1.21 |
| 50 | 26.20 ±2.63 | 22.23±2.23** | 32.14±3.77* |
| 100 | 48.65±4.54 | 40.74±3.76** | 42.54±4.22* |

The results are expressed as percentage of maximal LDH release that was obtained on complete cell lysis. Data are mean ± SEM values obtained from five culture wells per experiment, determined in three to five independent experiments. *, $P<0.05$. **, $P<0.01$.

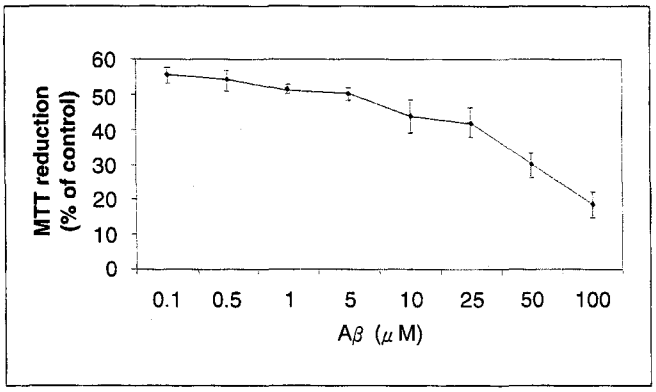


**Fig. 1.** Effects of various concentrations of Aβ25-35 on MTT reduction in cultured rat cortical astrocytes. Cells were incubated with the indicated concentrations of Aβ25-35 for 48 hrs. Assay values obtained on addition of vehicle were taken as 100% and complete inhibiion of MTT reduction (0%) was defined as the assay value obtained following addition of 0.9% Tritone X-100 to lyse the cells completely.

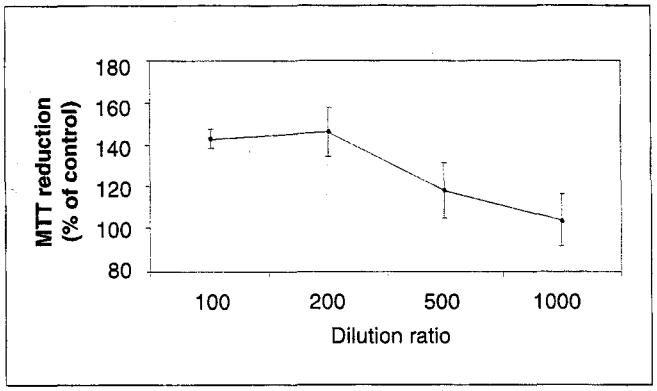


**Fig. 2.** Effects of various concentrations of *Juglandis semen* (JS) on MTT reduction in cultured rat cortical astrocytes.

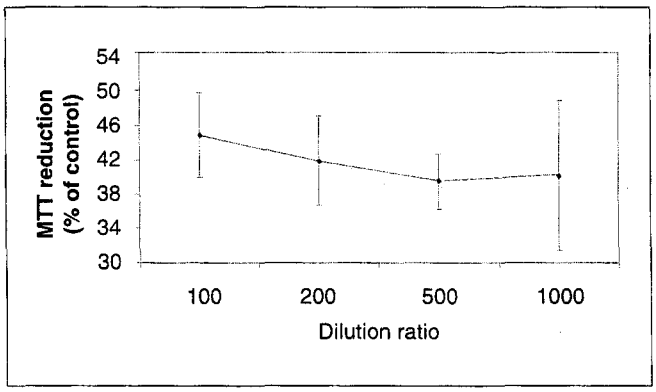


**Fig. 3.** Effects of pretreatment of *Juglandis semen* (JS) on A 25-35-induced cytotoxicity in cultured rat cortical astrocytes. Cultures were pretreated with various concentrations of JS for 24 hrs before application of 50 μM Aβ25-35. At 48 hrs after 50 μM Aβ25-35 treatments, MTT reduction was assayed.

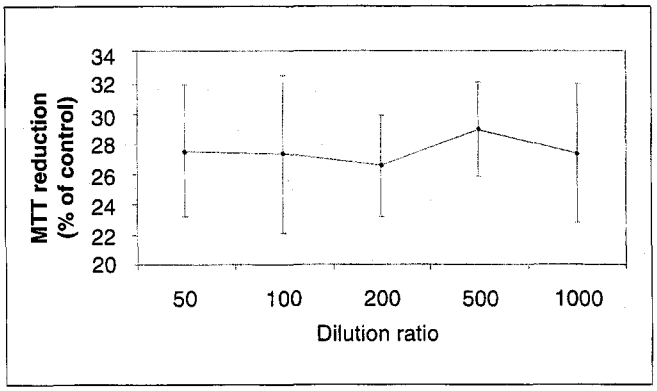


**Fig. 4.** Effects of *Juglandis semen* (JS) on Aβ25-35-induced cytotoxicity of cultured rat cortical astrocytes. Cultures were treated with 50 μM Aβ25-35 for 48 hrs before application of various concentrations of JS. At 24 hrs after JS treatment, MTT reduction was assayed.

35 peptide increased LDH release by 48.65% of the maximal value at 100 μM concentration. Aβ25-35 induced LDH release only 11.4 and 26.2%, respectively, even at 25 and 50 μM (Table 1). However, non-toxic fragment of Aβ, had little effect on LDH release up to 100μM.

On the other hand, we measured the protective and proliferative effects of JS on LDH activity in 24 hrs before treatment with indicated concentrations of Aβ25-35. The treatment of 100 μg/ml of concentrated JS solution (10-times diluted from 1000 μg/ml in Table 1) reduced the LDH activity by 75% of control group when compared at concentration of 100 μM Aβ25-35 (48.65 vs 40.74). Also, when the protective and proliferative effects of JS on LDH activity in 48 hrs after treatment with indicated concentrations of Aβ25-35, the treatment (100 μg/ml) of JS solution reduced the LDH activity by 90% of control group of 100 μM Aβ25-35 (48.65 vs 42.54). This result indicates that the pretreatment of JS is much more effective for astrocyte protection than posttreatment of JS.

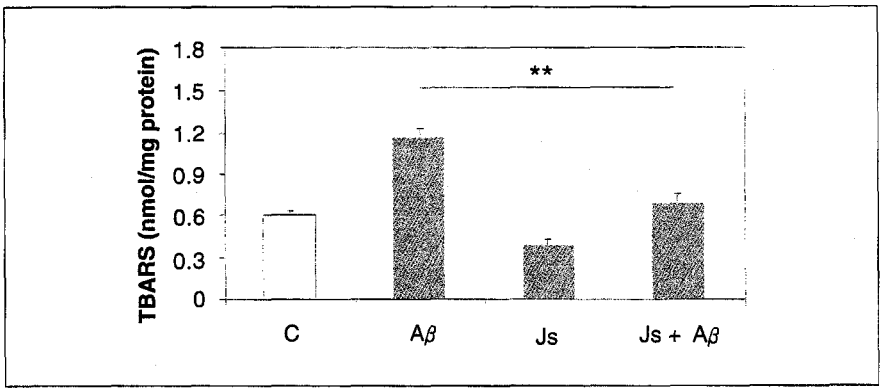


**Fig 5.** Effects of *Juglandis semen* (JS) on lipid peroxidation induced by Aβ25-35 treatment in cultured rat cortical astrocytes. Cultured cells were pretreated with JS for 24 hrs before application of 50 μM Aβ25-35. After 48 hrs, the release of TBARS into incubation medium was measured. **, P<0.01.

### 3. Effect of *Juglandis semen* on cultured astrocytes

We measured the protective and proliferative effects of JS on MTT reduction in cultured astrocytes at 48 hrs after JS treatment (Fig. 2). The treatment of 100 μg/ml of concentrated JS solution (10-times diluted from 1000 μg/ml in Fig. 2) increased the MTT reduction activity by 140% of control group. Interestingly, 20 μg/ml (50-times diluted) and 10 μg/ml (100-times diluted) concentrations of the JS solution resulted in by 165% and 370% increase of the control group, respectively, being maximal MTT reduction activity at 10 μg/ml. Upon further dilution of the JS solution up to 0.1 μg/ml concentration, the reduction activity was higher than that of vehicle. Thus, it was possibly concluded that the JS is highly effective for the protection and proliferation of the rat cortical astrocytes.

On the other hand, pretrea tment of JS attenuated in a cell killing enhanced by exposed to increas ing concentrations of Aβ25-35 (Fig. 3). This indicates that cells pre treated with JS allowed its resis tance against the toxic effects of increasing concentrations of Aβ25-35 peptides. However, gradual dilution of the JS concentration increased cell killing activity of Aβ25-35. By the pre treatment with 1000-times diluted JS, about 70% of the cells were killed within 48 hrs by 50 μM Aβ25-35. In contrast, fewer than 60% of the 100-times diluted JS pretreated cells were killed by the same concentrations of Aβ25-35. Seemingly, post-treatment with serially diluted JS showed similar cell killing activity in the with or without JS pretreatment (Fig. 4).

### 4. Effect of *Juglandis semen* on lipid peroxidation induced by Aβ25-35 in cultured astro cytes

Fig. 5 shows the effect of JS on MDA level in cultured rat astrocyte cell exposed to Aβ25-35. The accumulation of MDA, as measured by TBARS in the medium, is a sensitive index of the peroxidation of cellular lipids in cultured cell intoxicated with Aβ25-35. At present assay, TBARS levels of Aβ25-35 treatment group were significantly higher than other groups. This increased level was significantly reduced by JS pretreatment. In the case of JS+Aβ25-35 group, MDA level induced by Aβ25-35 was significantly reduced by JS pretreatment. These results of cell killing and MDA level by Aβ25-35 treatment are in agreement with those of Glascott et al[25,27].

The present study showed that Aβ25-35 strongly increased MDA level and the level was enhanced by addition of increasing concentrations of Aβ25-35 or by time-related exposure to Aβ25-35. In addition, lipid peroxidation was prevented or greatly reduced by addition of antioxidants (Vit E, Vit C, DPPD or deferoxamine)[28]. For example, addition of antioxidants in cell culture medium significantly reduced cell killing and content of intracellular antioxidants.

## IV. Summary

Neurotoxic and Alzheimer' s induc ing peptide, Aβ25-35 was shown to be harmful for the neuronal astrocyte' s growth and proliferation as assessed by mitochondrial respirative enzyme' s MTT assay and cytoplasmic enzyme, LDH. Furthermore, it was shown that A β25-35 is not only potent lipid peroxide inducer, but also cause protection of neurodegeneration induced by Aβ25-35. Interestingly, the medical extracts *Juglandis semen* (JS) protected the cell

killing and cytotoxicity induced by Aβ 25-35. The JS also reduced the MDA level. It can be concluded that the activation of antioxidative enzymes may be related to the inhibition of lipid peroxidative reactions. We cannot fully explain the effects of JS at present ; however, the ability of JS to reduce cell killing and MDA level induced by Aβ 25-35 suggest that JS may be a protective agent for free radical generating compounds such as Aβ 25-35.

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