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Research Article

The Effect of Dichloronitrophenol on Amyloid Beta Toxicity and Steroid Levels

Murat ORMEN[1], Ozlem GURSOY CALAN[1], Ugur Bekir ERGUR[2], Pinar AKAN[1]

[1]*Deparment of Medical Biochemistry, Dokuz Eylül University School of Medicine, İzmir, Turkey*

[2]*Deparment of Histology and Embryology, Dokuz Eylül University School of Medicine, İzmir, Turkey*

Summary

In Alzheimer's disease (AD), which is the most common cause of dementia, the accumulation of amyloid beta (AB) peptides has a causal role in the neurodegeneration process. AB peptides can induce synthesis of dehydroepiandrosterone (DHEA), which is a precursor of various steroids. Neurosteroids are modulators of neuronal survival and may have different effects depending on whether they are free or sulfa-conjugated.
Nitrophenols can inhibit the aggregation of AB peptides at relatively low concentrations. 2,4-dichloro-6-nitrophenol (DCNP) is a small water-soluble derivative of nitrophenols, and it is also known as the inhibitor of steroid sulfotransferase enzyme. However, the effects of DCNP on levels of distinct steroids and cell viability is not yet clearly known. This work aimed to investigate the effects of DCNP on various steroid levels and AB-induced cell death.
SH-SY5Y cells were treated with DCNP (1 µM) and aggregates of AB 1-42 peptides (10 µM) for 72 hours. Cell viabilities were evaluated using MTT reduction and immune-fluorescence microscopy. The cellular changes of DHEA, DHEA sulfate, and sex steroids were determined using liquid chromatography-mass spectrometry (LC-MS/MS). The effect of DCNP on ultrastructure of AB aggregates was also examined with transmission electron microscopy.
Seventy-two-hour treatment with DCNP significantly decreased DHEA sulfate and increased DHEA levels. Treatment with AB 1-42 aggregates decreased cell viability to approximately 50% and significantly changed DHEA and estradiol levels in SH-SY5Y cells. The ratio of cell death induced by AB 1-42 was reduced with DCNP treatment. When the AB aggregates were incubated in the aqueous solution with DCNP, the size of the aggregates became significantly smaller compared with the initial size. According to our literature knowledge, this is the first study to show the beneficial effects of DCNP on AB toxicity.

Key words: Dichloronitrophenol, Amyloid Beta Toxicity, SH-SY5Y cells, Neurostreroids

Dikloronitrofenolün Amiloid Beta Toksisitesi ve Steroid Düzeylerine Etkisi

Özet

Demansın en sık sebebi olan Alzheimer Hastalığı'nda Amiloid Beta (AB) birikimi nörodejeneratif süreçte nedensel rol oynar. AB peptid pek çok steroidin prekürsörü olan dehidroepiandrosteron (DHEA) sentezini indükler. Nörosteroidler nöronal canlılığı düzenler ve serbest ya da sülfa konjügat formda olmalarına göre farklı etkiler gösterirler. Nitrofenoller AB peptit agregasyonunu rölatif olarak düşük konsantrasyonlarda inhibe edebilirler. 2,4-dikloro-6-nitrofenol (DCNP) nitrofenollerin küçük, suda çözünebilen türevi olup steroid sülfotransferaz enziminin inhibitörü olarak da bilinir. Ancak DCNP'nin farklı steroid düzeylerine ve hücre canlılığına etkileri henüz tam olarak bilinmemektedir. Bu çalışma DCNP'nin farklı steroid düzeylerine ve AB'nın indüklediği hücre ölümüne etkilerini araştırmayı hedeflemektedir. SH-SY5Y hücrelerine 72 saat süreyle DCNP (1µM) ve agrege edilmiş AB 1-42 peptit (10 µM) uygulandı. Hücre canlılığı MTT redüksiyon metodu ve

immunfloresan mikroskop ile değerlendirildi. Hücresel DHEA, DHEA sülfat ve seks steroidlerinin değişimi LC-MS/MS ile belirlendi. DCNP'nin AB agregatlarının ultrastrüktürel yapısına olan etkisi transmisyon elektron mikroskop ile değerlendirildi. 72 saat boyunca DCNP uygulaması DHEA sülfat düzeyini anlamlı azaltırken, DHEA düzeyinde artışına neden oldu. SH-SY5Y hücrelerine AB 1-42 agregatlarının uygulanması, hücre canlılığını yaklaşık %50 azaltırken, DHEA ve östradiol düzeylerini de anlamlı değiştirmiştir. AB 1-42'nin indüklediği hücre ölüm oranı DCNP uygulaması ile azalmıştır. AB agregatları DCNP ile sulu ortamda inkübe edildiğinde agregat boyutlarında başlangıç ile kıyaslandığında anlamlı küçülme meydana gelmiştir. Bu çalışma, literatür bilgilerimize göre DCNP'nin AB toksisitesi üzerine faydalı etkilerinin gösterildiği ilk çalışma niteliğindedir.

Anahtar Kelimeler: Dikloronitrofenol, Amiloid Beta Toksisitesi, SH-SY5Y hücreleri, Nörosteroidler

INTRODUCTION

Together with improvements in socio-economic development, life span is getting longer but the frequency of dementia is also increasing in the aging society (1). Alzheimer's disease (AD) is a progressive neurodegenerative disorder and is the most common cause of dementia. It has been suggested that amyloid-beta (AB) peptide toxicity had a central role in the neurodegeneration process of AD (2-6). Previous studies on AB toxicity and AD have strongly motivated the search for therapies concerning AB peptides (7,8).

AB peptide is produced by cleavage of the amyloid precursor protein (APP) in a stepwise process mediated by β - and γ – secretases (9). According to the place where γ -secretase cleaves, short (39-40 amino acids) or long (42-43 amino acids) AB peptide isoforms can be released. Long fragments of AB peptides are more prone to form non soluble fibers and possess more neurotoxic properties than the shorter ones. AB1-42 fragment is the predominant long fragment of AB-peptide found in the brains of Alzheimer patients. It is usually used as a tool for investigating AD-related mechanisms in animal models or cell lines (10,11).

There are studies suggesting that AB exerts its neurotoxic effect by triggering oxidative damage or increasing intracellular calcium, and activating apoptotic and inflammatory processes (5,12). It has been also shown that AB 1-42 fragments had an influence on steroid synthesis such as estradiol and dehydroepiandrosterone (DHEA) (13,14). We previously demonstrated that AB peptides affected pregnenolone and pregnenolone sulfate levels depending on cholesterol concentration in the cells and the types of AB fragments (15). According to studies in recent years, a close association between neuronal injury and steroid synthesis is expected (16-18). Exogenous treatment with some steroids such as pregnenolone, DHEA, and estradiol have favorable effects on neuronal survival (19-24). The effect of the changes in the cellular steroid levels on AB toxicity has not yet been clearly identified.

Sulfonate conjugation is an important pathway in the biotransformation of steroid hormones. Sulfotransferase enzymes (SULT) are capable of sulfonating a wide range of substrates including phenolic (17 beta-estradiol) or 3 beta-hydroxysteroids such as pregnenolone and DHEA (25). Steroid hormones, biosynthesized in nervous system, are called "neurosteroids" considering their extraordinary origins and different functions from classical steroid hormones. Besides, neurosteroids have different effects on neuronal survival and excitability depending on whether they are free or sulfa-conjugated. Some sulfo conjugated steroids, such as pregnenolone sulfate are accepted as natural excitotoxins,

whereas pregnenolone has anabolic effects (20,26).

In recent years, the control of sulfating reactions in steroid metabolism, and the advantages of pharmacologic studies targeting these reactions have been under debate (27-29). DCNP is an inhibitor of SULT and frequently used to study the role of this enzyme (30,31). It is a kind of nitrophenol. Nitrophenols have an aromatic structure and relatively low molecular weights, as such they may have hydrophobic interactions with neighboring molecules. Previously it has been suggested that some nitrophenol derivatives (2,4-dinitrophenol and 3-nitrophenol) prevented the aggregation of AB peptides (32). On the other hand, some kinds of nitrophenols are known as mitochondrial uncouplers and they can reduce ATP production (33). It has been shown that nitrophenols had protective effects against cell toxicity induced by ischemia or N-methyl-D-aspartate (NMDA) receptor activation. Nevertheless, there is no knowledge about the effects of DCNP on neurotoxicity induced by AB peptide toxicity. Also, the effects of DCNP on distinct steroids in cells is not yet clearly known.

This work aimed to investigate the effect of DCNP on AB 1-42 induced neuronal cell death, and the possible mechanisms of its action. For this purpose, the changes in the steroid levels of cells and the morphologic interactions between AB peptides and DCNP were evaluated.

MATERIAL AND METHODS

1. Chemicals

AB 1-42 peptide, DCNP, dimethyl sulfoxide (DMSO) were supplied by Sigma Chemical Co., St. Louis, USA. AB 1-42 peptides were dissolved in DMSO and

2-thiazolyl)-2,5-diphenyl-2H-tetrazolium added to the medium to obtain a final concentration of 1% DMSO. All cell culture reagents were from Seromed Biochrom FKG Germany. 3-(4,5-Dimetyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Applichem Chemica Synthesis Services (Germany). AB1-42 peptides were human origin and the purity was ≥ 95%. The amino acid sequence of the fragment was as follows: NH2-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala-COOH. This peptide prefers the beta-sheet conformation in aqueous solutions (34). Also, DCNP, which is an inhibitor of sulfotransferases, was used to evaluate its effects on AB toxicity (30).

2. Cell culture

The SH-SY5Y cell line (human neuroblastoma cell line), which is widely used in in-vitro AD models and shows neuro-endocrine characters, was developed from neural crest-derived primitive, pluripotent sympathetic cells found in the sympathetic nervous system and adrenal medulla (35-37). The cell line, which was purchased from DSMZ Cell Lines Bank (Germany), was selected as the neuronal model in our study.

SH-SY5Y cells were placed on 96- or 6-well plates at a density of $2x10^4$ or $2x10^6$ cells/well and were allowed to attach for 24 h in DMEM medium supplemented with 17% fetal bovine serum, and a mixture of 1% of penicillin/streptomycin. Cells were incubated at 37°C in a humid, 5% CO_2-95% air environment.

2.1. AB 1-42 and DCNP treatment

Before cell treatment, AB 1-42 peptides were incubated at 37°C for 72 hours to obtain aggregation and mature forms. After an initial 24 h plating, SH- SY5Y cells were treated with AB 1-42 peptide aggregates (10 μM) and DCNP (1 μM) for 24, 48, and 72 hours. Control cells were treated with AB 1-42 peptide aggregates

(10 μM) and DCNP (1 μM) for 24, 48, and 72 hours(6,38). Control cells were placed in DMEM supplemented with only 1.7% serum and 1% penicillin/streptomycin. Cell survival was evaluated using an MTT reduction assay, reverse phase light microscopy, and immunofluorescence microscopy. All experiments were run at least in triplicate in three independent experiments.

2.3. MTT reduction assay

An MTT reduction method was used to determine the cell viability and to provide information on mitochondrial function (39,40). In brief, 22 μL of a 5 mg/mL stock MTT solution in sterile phosphate-buffered saline (PBS) was added to wells containing 200 μL of medium to make the final concentration of MTT solution (0.5 mg/mL) and incubated for 2-4 h at 37°C in a humid, 5% CO_2-95% air environment. After removing the cell medium, 200 μL of DMSO solution was added and incubated for 30 min at 37°C. Then the absorbance values were determined at 562 nm using an automatic micro-titer plate reader. The results of MTT reduction for treated cells are expressed as the percentage of control (untreated) cells (40).

2.4. Immune-fluorescence microscopy

Fluorescence microscopy was used to assess the morphologic changes induced by the peptide in SH-SY5Y cells by double staining with acridine orange and ethidium bromide. These fluorescent dyes emit different fluorescence and possess a different ability to penetrate cells. Acridine orange penetrates into living cells, emitting green fluorescence after intercalation into DNA. The second dye, ethidium bromide, emits red fluorescence in cells with an altered cell membrane and intercalation into RNA and DNA. After the required incubation period (72 h), fluorescent dyes, ethidium bromide (100 μg/mL) and acridine orange (100 μg/mL) were added to cell culture dishes and they were incubated for 10 min in the dark. Cell suspension was placed on a microscope slide and at least, 200 cells/sample were evaluated and counted under a fluorescence microscope (Nikon Optiphot-2 using NB filter, 496 nm excitation and 520 nm emission wavelength) (41).

2.5. Steroid analysis

SH-SY5Y cells ($2x10^6$) were plated in 6-well plates to measure steroid levels. After 72-hour treatments, cells were harvested in their culture medium and lysed using an ultrasonic homogenizer (Sonics & Materials, USA).

A known amount of internal standard solution was added to every cell lysate, calibrators and quality control materials. Samples were deproteinized according to the manufacturer's recommendations (Eureka Steroid Analysis kit, Code LC72310.) After centrifugation at 14,000 rpm for 10 minutes, the supernatants were used for steroid quantification. 17-OH-Progesterone, Dehydroepiandrosterone (DHEA), Dehydroepiandrosterone sulfate (DHEAS), androstenedione, cortisol, testosterone, dihydrotestosterone, androsterone, estrone, estradiol, and progesterone were analyzed (LC-MS /MS System, Shimadzu model 8050, Japan).

The limits of quantification of our method for each steroid were as follows: 17-OH-progesterone 15 pg/mL, androstenedione 3 pg/mL, DHEAS 0.6 ng/mL, DHEA 0.1 ng/mL, testosterone 3 pg/mL, dihydrotestosterone 20 pg/mL, androsterone 1 pg/mL, estrone 2 pg/mL, estradiol 2 pg/mL, and progesterone 300 pg/mL. The mean of the inter-runs coefficients of variation (CV %) was nearly 5.62%. The experiments were performed at least in triplicate (n=9).

2.6. Ultrastructural examinations

To obtain electron microscopic images, first AB 1-42 (10 μM) was freshly dissolved in cell culture medium with DMSO (1% final concentration). It was incubated at 37°C for 72 hours to obtain AB aggregates. Then, DCNP (1 μM) was

added into the medium containing AB aggregates and incubated for another 72 hours. Morphologic changes of peptides were examined every 24 hours for 6 days. All experiments were repeated at least three times.

Immediately before microscopic imaging, for electron microscopic investigation, the samples were dropped on formvar coated copper grids and allowed to dry. Then digital photographs were taken using a transmission electron microscope (Carl Zeiss Libra 120 EFTEM Carl Zeiss, Oberkochen, Germany). Photographs were analyzed and the particle size of AB peptides were calculated using a digital analysis program.

2.7. Statistical analysis

Groups were compared using the Mann-Whitney U test with SPSS 22.0 and P-values less than 0.05 were considered significant. All experiments were replicated at least three times, and graphic results are presented as the mean ± S.E.M.

RESULTS

Figure 1 represents the effects of 10-µM AB 1-42 peptide on cell viability in an exposure time-dependent manner. The 24-h treatment of SH-SY5Y cells with AB had no statistically significant effect on the cells. After 48-h and 72-h exposure, MTT reduction results of AB-treated cells were significantly lower than the control cells $p<0.05$. AB 1-42 decreased cell viability to approximately 50% of control cells after 72 hours. Cell viability was increased to approximately 85% with "DCNP + AB 1-42" treatment. After DCNP treatment, the cell viability was not statistically significant when compared with the control group (Figure 2). The effect of DCNP on AB toxicity was also evaluated using light and fluorescence microscopy.

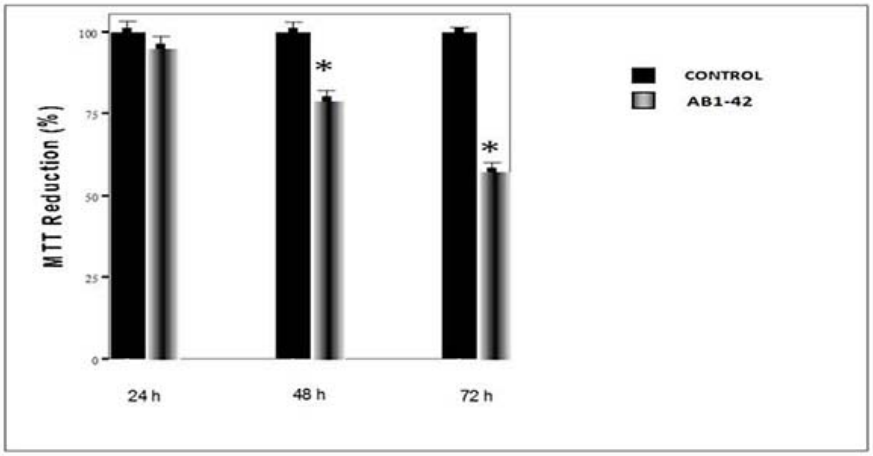


Figure 1: *The effect of amyloid beta peptide on cell viability in a time-dependent manner. 48-hour and 72-hour treatment with AB 1-42, which were incubated at 37°C previously for aggregate formation, caused a significant decrease in the viability of SH-SY5Y cells. (MTT reduction results are expressed as percentage of the control; data are given as mean ± SEM, AB: Amyloid beta peptide)*
*(*p<0.05 compared with control)*

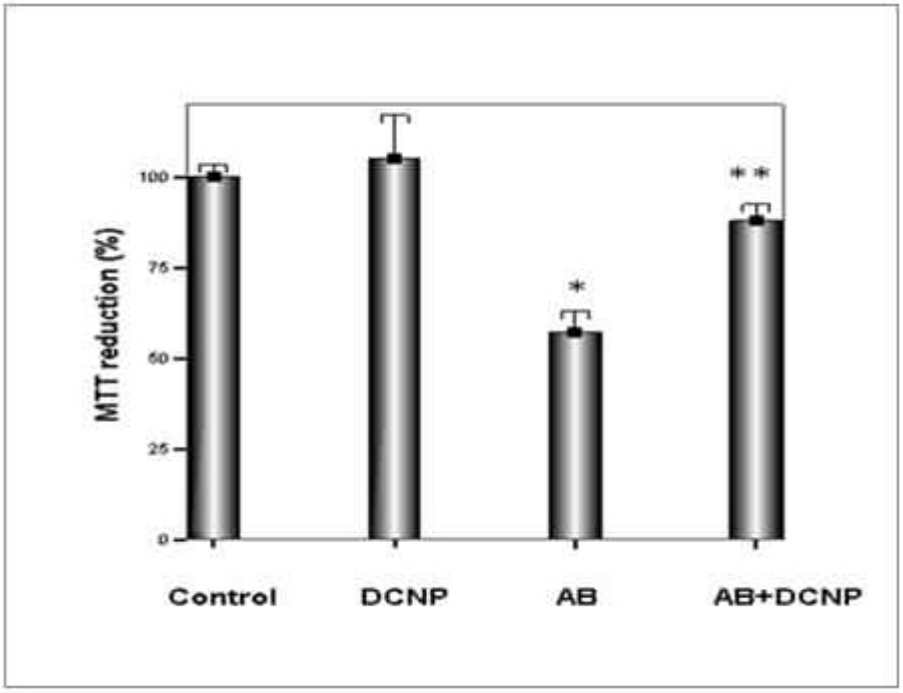


Figure 2: *The effect of DCNP on the viability of SH-SY5Y cells. AB 1-42 + DCNP treatment for 72 hours increased cell viability when compared with the AB 1-42 group. (MTT reduction results are expressed as percentage of the control; data are given as mean ± SEM, AB: Amyloid beta peptide; DCNP: dichloronitrofenol)*
*(*p< .05 compared with the control group)*
*(**p< .05 compared with the AB groups)*

According to the light microscopic examination, the most recognizable morphologic changes in AB-treated groups were cytoplasmic condensation, cell shrinkage, and aggregation of nuclei into dense masses. It was observed that disrupted cells were less in the DCNP-treated group (Figure 3). To verify MTT assay results, treated SH-SY5Y cells were stained with acridine orange/ethidium bromide to immune-fluorescence microscopic analysis (Figure 4). In the first 24 hours, the ratios of viable, apoptotic and necrotic cells were ~89%, 7%, and 4%, respectively, in SH-SY5Y cells (n=6). After 24 hours, ~15 % of cells treated with AB 1-42 developed green fluorescence with nuclear fragmentation, which indicated apoptotic cell death. Apoptotic cells can be seen in Figure 4-B. After 72 hours, the majority of cells exhibited green fluorescence in the control group (~80 % of total cell count), whereas red or orange-colored nuclei were significantly high in AB 1-42 treated cells, indicating membrane disruption and necrotic cell death (~60% of total cell count). Necrotic cells can also be seen in Figure 4-C. Necrotic cells were lower in the "DCNP + AB 1-42" treatment group (~40% of total cell count). In the group treated only with DCNP, the majority of cells exhibited green fluorescence, indicating viability (~80% of total cell count), after 72 hours (Graphic 1).

Figure 5 shows the effects of DCNP and AB 1-42 aggregates on the levels of DHEA and DHEA sulfate in SH-SY5Y cells. Treatment with AB 1-42 enhanced DHEA levels but it resulted without statistically

significant changes in levels of DHEA sulfate. After 72-h treatment with DCNP, the levels of DHEA were also significantly increased but DHEA sulfate levels were significantly decreased when compared with the control group ($p<0.05$). The levels of DHEA sulfate were also reduced when the cells were treated together with AB and DCNP.

The changes in the levels of female and male sex steroids are represented in Table 1. The levels of estradiol, which is a female sex steroid, were significantly decreased with AB treatment when compared with the control group ($p<0.05$) (Figure 6). However, there was no statistically difference between hydroxyl-progesterone levels. Progesterone levels could not be determined exactly because the levels were below the quantification limits of our assay method. The levels of male sex steroids (androsterone, androstenedione and DHT) were not significantly changed with all treatments. Only testosterone level was reduced by DCNP treatment ($p<0.05$, Table 1)

In electron microscopic examinations, it was seen that the soluble AB 1-42 monomers in cell culture medium changed to non-soluble aggregates after 72-h incubation at 37°C. The sizes of AB 1-42 monomers were in average ~127 nm in the first 24 hours of the incubation. After 72 hours AB aggregates covered almost the whole grid. When DCNP was added over the incubation medium containing AB aggregates, it was observed that DCNP significantly dissolved the aggregates at the end of 72 hours. Small particles of AB peptides with the average size of ~50-60 nm were formed. The electron microscopic images of AB peptides are shown in Figure 7.

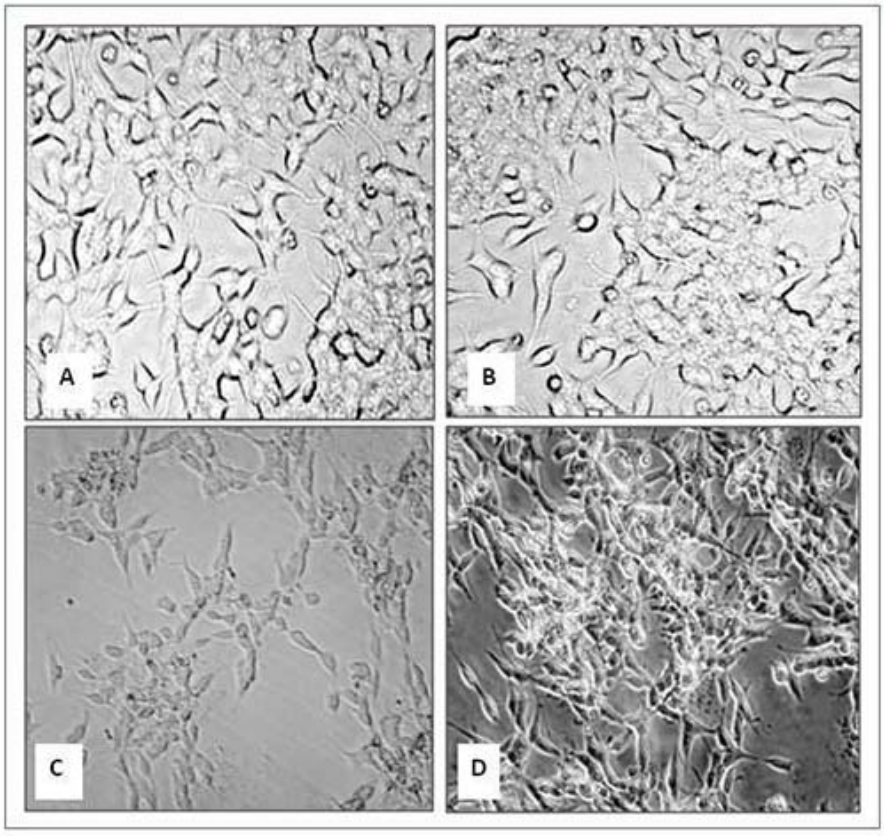


Figure 3: *Morphologic changes of SH-SY5Y cells after 72-hour treatments. According to light microscopic examination, there was no difference between the control and DCNP groups (A,B), whereas in the AB-treated group, there was cytoplasmic condensation, cell shrinkage, and aggregation of nuclei into dense masses (C). In the DCNP-treated group, disrupted cells were fewer than those in the AB-treated group (D).*
(A: Control group, B: DCNP-treated group, C: AB-treated group, D: AB+DCNP-treated group, AB: Amyloid beta peptide)

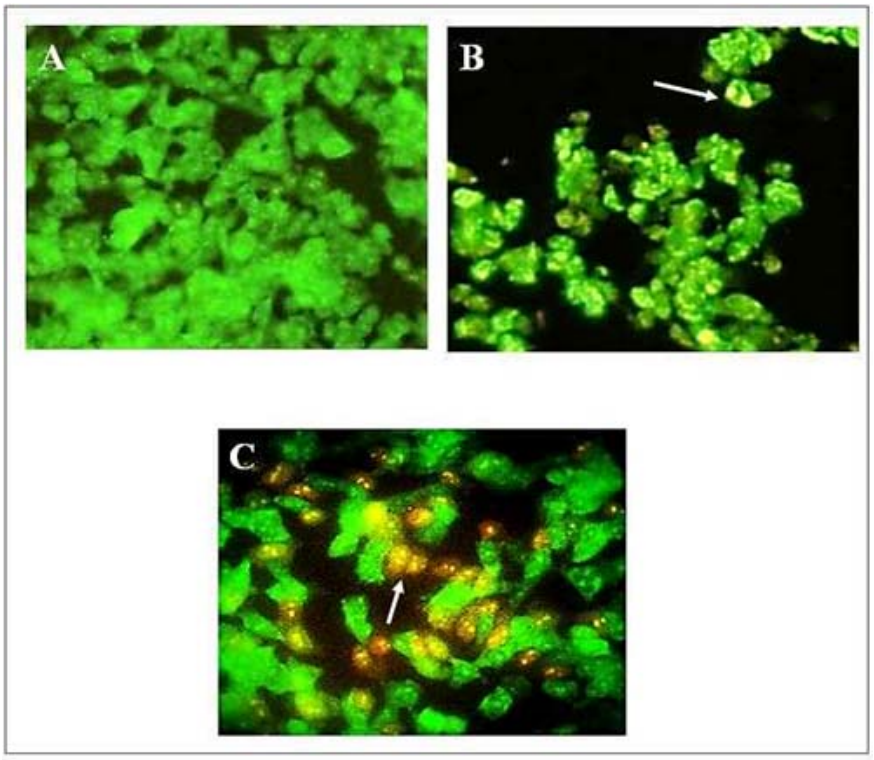


Figure 4 *Immunofluorescence Microscopic Images of SH-SY5Y cells.*
SH- SY5Y cells stained with acridine orange/ethidium bromide after incubation with AB1-42 and AB 1-42 +DCNP were observed under fluorescence microscopy (x40). 24-hour treatment with AB 1-42 increased apoptotic cell death when compared with the control (B). Bright and condensed (apoptotic) cells are indicated with arrows. After 72 hours, orange-red colored nuclei were significantly higher in AB 1-42 treated cells indicating membrane disruption and necrotic cell death (C). Necrotic cell death was significantly lower in the DCNP + AB 1-42 treatment group.
(A; Control group 24 hour, live cells, B; AB-treated group 24 hour, apoptotic cells C; AB- treated group 72 hour, necrotic cells; AB; amyloid beta, DCNP: dichloronitrophenol)

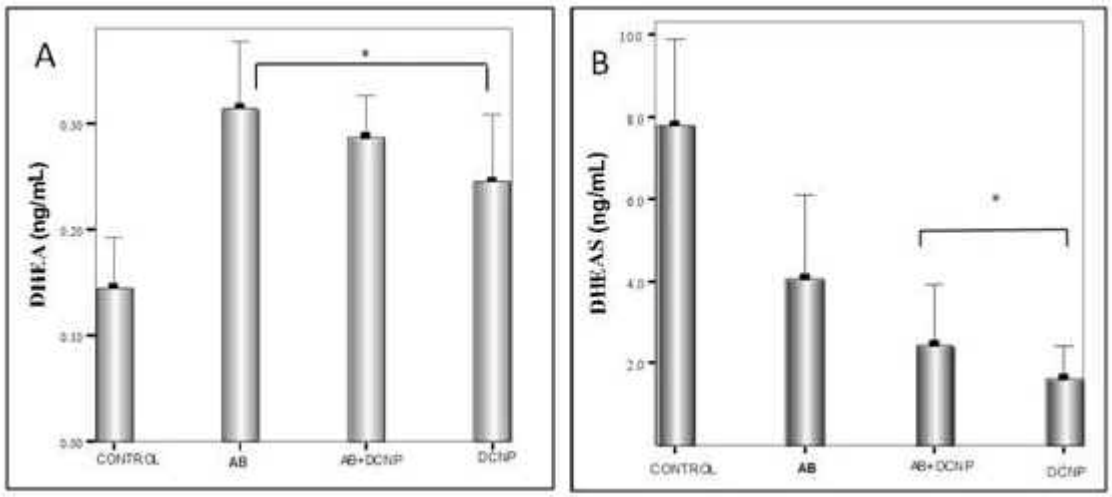
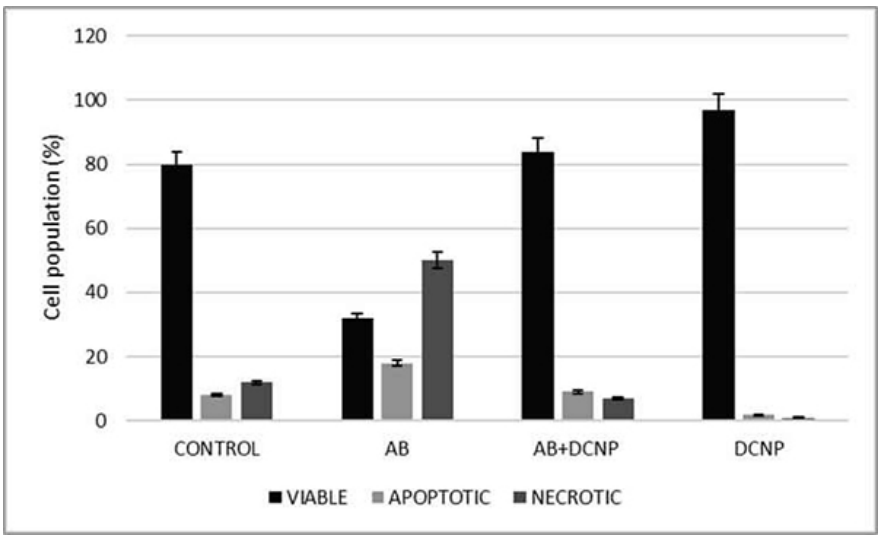


Figure 5: *DHEA, DHEAS levels of SH SY5Y cells treated with AB and DCNP. 72-hour treatment with AB 1-42 or DCNP or combination significantly increased DHEA levels of the cells (A). 72-hour treatment with AB+DCNP and DCNP significantly decreased DHEAS levels of cells when compared with the controls. (Results are expressed as ng/mL per $1x10^6$ cells; data are given as mean ± SEM, DHEA; dehydroepiandrostenedione, DHEAS; dehydroepiandrostenedione sulfate, AB; amyloid beta, DCNP; dichloronitrophenol)*
*(*p<0.05 compared with the control group)*



Graphic 1: *The ratios of viable, apoptotic and necrotic cells. SH-SY5Y cells treated with AB 1-42 or/and DCNP for 72 hours. Viable cells were higher and necrotic cells were lower in DCNP + AB 1-42-treated group than the AB-treated group.*

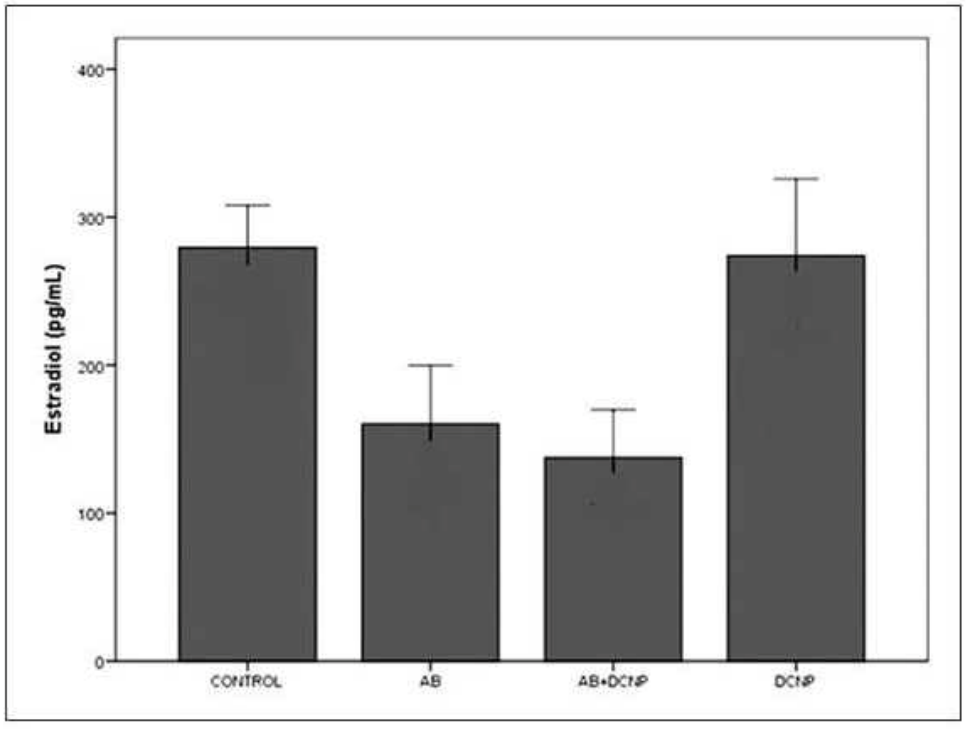


Figure 6: *Estradiol levels of SH SY5Y cells. Estradiol levels in the group treated with DCNP were significantly higher than other groups. (Results are expressed as pg/mL per $1x10^6$ cells; data are given as mean ± SEM, AB; amyloid beta, DCNP; dichloronitrophenol)*
*(*p<0.05 compared with the other groups)*

Table 1: Various Steroid Levels in Treated SH SY5Y Cells (Results are expressed as pg/mL per $1x10^6$ cells; data are given as mean ± SEM, AB; amyloid beta, DCNP; dichloronitrophenol)

(pg/mL)	CONTROL (n=9)	AB (n=9)	AB+DCNP (n=9)	DCNP (n=9)
Testosterone	14.6 ±0,8	15 ±3,5	12 ±2	**7 ±1***
Dihydrotestosterone	114 ±46	58.5 ±26	68 ±21	118.5 ±40
Androstenodione	12.6 ±2	7 ±2	7 ±1,6	6.2 ±2
Androsterone	486.5 ±12	447.7 ±49	452 ±89	538 ±39
Progesterone	ND	ND	ND	ND
Hydroxyprogesterone	83.3 ±18	117.5 ±28	160 ±34	89 ±20
Estradiol	279.6 ±14	**160.5 ±19***	**98 ±26***	274 ±25

(*p<0.05 compared with control group)

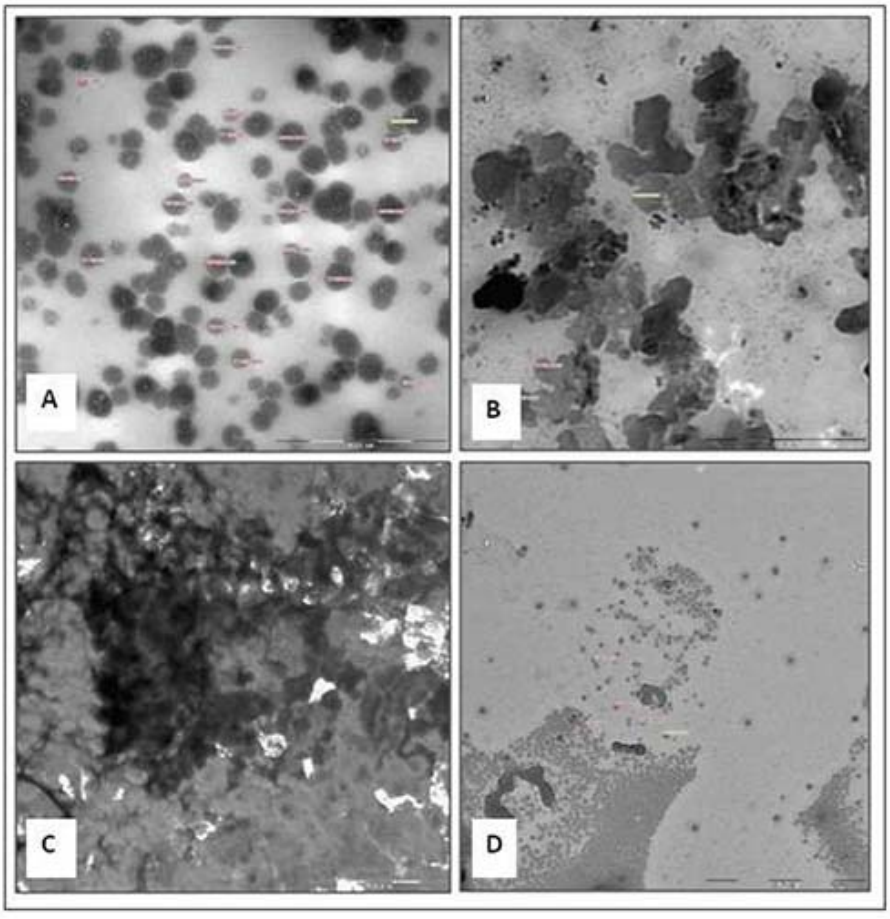


Figure 7: *Electron microscopic images of AB 1-42 peptides during incubation and after DCNP treatment. AB 1-42 peptides were dissolved in DMSO and added to DMEM medium to obtain a final concentration of 1% DMSO. Immediately after preparation, the sizes of AB 1-42 monomers were measured in average ~127 nm (A) After 24 hour-incubation at 37°C, the monomers started to come closer and at 48. hours, AB aggregate forms were observed plainly (B). AB aggregates covered almost the whole grid at 72 hours (C). At the end of 72 hours of incubation, DCNP was added to the medium containing AB aggregates, then it was incubated for 72 hours at 37°C. At the end of incubation with DCNP, the aggregates of AB dissolved into small particles (D).*
(Magnification: 10,000X; The scale bar (1000 nm) is shown in the lower right of the images)

DISCUSSION

Nitrophenols are controversial chemicals. Whether they are useful to human health is being questioned (32). At first, these chemicals were used as a photographic developer, dye, wood preserver, and herbicide. After discovering dinitrophenols could reduce ATP production via mitochondrial uncoupling and increase the body's metabolic rate, they were used as weight loss drugs in past years. However, their use was abandoned because of sudden death and toxicity cases in uncontrolled conditions (33). Genotoxic effects of nitrophenols in microorganisms were also reported. These genotoxic effects associated with DNA methylation occur at relatively high (mM) concentrations (4). On the other hand, there are some studies on the protective effects of nitrophenol derivatives. It has been shown that the application of 2,4-dinitrophenol (DNP) ensured mitochondrial homeostasis and reduced tissue damage related to focal cerebral ischemia (43). It has been suggested that nitrophenols increased the life span of yeast and drosophila by decreasing mitochondrial reactive oxygen release (44). It was also demonstrated that rats treated intraperitoneally with dinitrophenol showed significant protection against brain damage caused by the N-methyl-D-aspartate (NMDA) receptor agonist quinolinic acid (45). In 2001, Felice et al. suggested that nitrophenol derivatives had protective effects against AB neurotoxicity. They showed that DNP and 3-nitrophenol (NP) at micromolar concentrations caused disaggregation of AB fibrils and decreased neurotoxicity. In their study, treatment with DNP or 3-nitrophenol caused no toxic effect in neuronal cells (46). In our study, DCNP was used at a micromolar concentration and this application created no toxic effects on SH-SY5Y cells during 72 hours.

According to our MTT results, treatment with AB 1-42 aggregates at 10 μM concentration decreased cell viability to approximately 50% of the controls. The ratio of cell viability in the AB toxicity groups was enhanced to approximately 85% by treatment with DCNP (1 μM). Our MTT results were in consonance with data obtained in microscopic examinations. After staining the cells with acridine orange-ethidium bromide, it was observed that the ratio of necrotic cell death in the group treated together with DCNP and AB 1-42 was significantly lower than the group treated with only AB 1-42 in immune-fluorescence microscopic images. Under the same in-vitro conditions, the levels of DHEA and its possible derivatives, which may have influence on cell survival, were also evaluated.

In peripheral tissues, DHEA is produced from cholesterol through two cytochrome P450 enzymes. Cholesterol, which is main precursor of all steroids, is converted to pregnenolone by the enzyme P450 scc (side chain cleavage); then another enzyme, CYP17A1, converts pregnenolone to 17α-hydroxypregnenolone and then to DHEA(47). In the brain, an alternative pathway is used via a CYP17A1, independent of DHEA formation. In neural tissue, DHEA synthesis can be regulated by oxidative stress, iron, and AB peptides (48,49). After synthesis, DHEA can be locally converted to its more stable sulfate ester, DHEA sulfate, by catalyzing with the enzyme steroid sulfotransferase (50).

According to the LCMS/MS results, we found that treatment with DCNP and AB 1-42 significantly increased DHEA levels in SH-SY5Y cells. DCNP treatment also reduced DHEA sulfate levels. Considering that DCNP is a steroid sulfotransferase enzyme inhibitor, these effects of DCNP can be expected. To DHEA formation, some alternative sources may occur during oxidative stress induced by AB peptides. The increasing effect of AB 1-42 treatment

on DHEA levels is in concordance with previous data published by Brown et al. (14). At toxic concentrations, AB 1-42 may induce the synthesis of hydroperoxides or oxidized steroids, which can be used in DHEA creation (48). In our study, DHEA levels in cells treated together with DCNP and AB were similar to the values in the cells when the treatments were applied one by one. Thus, it can be considered that DCNP and AB 1-42 had no synergistic effect on DHEA formation.

It has been suggested that DHEA and DHEA sulfate were neuroprotective steroids according to both in vivo and in vitro studies (22,24,51,52). Hence, the increase of DHEA levels induced by AB 1-42 treatment may contribute to self-defense response of SH-SY5Y cells. However, it has been also reported that DHEA had conflicting effects on neuronal cell viability. It has been shown that DHEA could induce neurotoxicity when administered during ischemia and early reperfusion in a rat model (53). DHEA is an excitatory neurosteroid to potentiate responses to NMDA and can enhance Ca^{2+} influx through NMDA receptors (54,55). It has been also shown that DHEA exposure induced apoptosis in hypothalamic neurons (56). After considering all the reports described above, it can be suggested that the increase in levels of DHEA may be a part of a neurotoxicity process. In our previous study, we demonstrated that AB peptides could affect pregnenolone and pregnenolone sulfate levels depending on the cellular cholesterol content. Treatment with AB fragments (AB 25-35, AB 1-40 and AB 1-42) increased pregnenolone levels up to two fold. However, pregnenolone levels were increased ~ six fold by treatment together with AB peptides and cholesterol. Also, PS levels were increased only ~ 2.5 fold in PC 12 and SH-SY5Y cell lines (15). Pregnenolone and pregnenolone sulfate can antagonize the potentiation of the NMDA response induced by DHEA (54). Hence, in our present study, DCNP may have had protecting influence on AB toxicity, which lead to its enhancing effect on pregnenolone levels, although it can increase DHEA levels. Further studies are needed about DHEA and NMDA receptor-related signal mechanisms in AB toxicity.

DHEA and DHEAS, which together represent the most abundant steroid hormones in the human body, are also defined as "mother steroids" (57). They serve as precursors to ~50% of androgens in adult men, ~75% of active estrogens in premenopausal women, and nearly 100% of active estrogens in postmenopausal women. In neural tissue, androgens and estrogens can also be synthesized from DHEA (58-60). Lower circulating and higher neuronal tissue levels of DHEA in patients with Alzheimer's disease were reported by Aldred and Mecocci (61,62).

In our study, numerous steroids that may be affected by levels of DHEA or DHEA sulfate were examined. Seventy-two-hour treatment with DCNP statistically significantly decreased testosterone levels compared with the control group. Intriguingly, the increase in levels of DHEA, which is known as a precursor for androgens and estrogens, did not result in the enhancement of these steroids. On the contrary, AB treatment significantly decreased estradiol levels.

Estrogens, which are defined as female sex steroids, have protective effects against neuronal cell death (16,19,23). Their exogenous treatment can also reduce apoptotic cell death in ovarian granulosa cells (63).

Previously, Schaeffer et al. showed that extracellular application of aggregated AB 25-35 fragments revealed a decreased synthesis of progesterone at 100 nM, but the formation of 17 hydroxyprogesterone and estradiol was not altered by different AB 25-35 concentrations in SH-SY5Y cells. In 2008, the same investigators suggested that AB peptides had a selective and amino-acid sequence-dependent action

on neurosteroid synthesis. They also advised that AB 1-42 had the ability to exert either protective or deleterious effects on nerve cells. AB 1-42 peptide at non-toxic concentrations could enhance estradiol synthesis, but at higher concentrations it inhibited estradiol levels when pregnenolone was used as the precursor molecule (13,37). Similarly, in our in-vitro model, estradiol formation was reduced by AB 1-42 treatment. Pregnenolone conversion into estradiol requires complementary activities of various enzymes such as 3beta-hydroxysteroid dehydrogenase (pregnenolone to progesterone), cytochrome P450c17 (progesterone to androstenedione), 17beta-hydroxysteroid dehydrogenase (androstenedione to testosterone) and aromatase (testosterone to estradiol).Schaeffer et al suggested that the involvement of steroidogenic enzyme activities in the mediation of AB effected on neurosteroidogenesis in SH-SY5Y cells (13). The decrease in the levels of estradiol induced by AB 1-42 treatment may contribute to SH-SY5Y cell death.

Our steroid levels were generally found lower than values published in previous studies, which may be related to the absence of precursor molecules in our study model. The limits of quantification in our LC-MS/MS method allowed us to observe the steroids at pg/mL concentrations. In this study, AB 1-42 had an increasing effect on the level of DHEA although no precursor molecule was used for steroid synthesis.

In our study, we also assessed the effects of DCNP on AB aggregates. The sizes of AB 1-42 monomers were ~127 nm in the first 24 hours of preparation. AB peptides were incubated in the solution together with DCNP at 37°C and then the aggregates were evaluated under electron microscopy for six days. DCNP treatment significantly degraded the AB peptide aggregates after 72 hour-incubation. DCNP caused the aggregates to separate into pieces and form small particles (~50-60 nm). This favorable effect of DCNP on AB aggregates is in concordance with a previous study with other nitrophenol derivatives, 2,4-dinitrophenol and 3-nitrophenol (46). The disaggregation process induced by DCNP might be helpful to protect against AB toxicity.

Consequently, the beneficial effect of DCNP on AB toxicity in SH-SY5Y cells has been shown in our study. DCNP treatment significantly decreased DHEA sulfate and increased DHEA levels. The ratio of cell death induced by AB 1-42 was reduced with DCNP treatment. According to our literature knowledge, this is the first study to show the protective effect of DCNP on AB toxicity. This influence of DCNP on AB toxicity may be related to breaking effects on the integrity of AB aggregates besides its effects on protective steroid levels. AD is a progressive neurodegenerative disease, and it has no known cure. Nitrophenols are controversial chemicals but they may be used as an effective agent for serious pathologies such as AD under strict monitoring. Further well-controlled in vivo studies are needed to evaluate their effects on the neurodegeneration process.

Correspondence to:
Murat Ormen
E-mail: murat.ormen@deu.edu.tr

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