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Pregnenolone protects the PC-12 cell line against amyloid beta peptide toxicity but its sulfate ester does not

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ABSTRACT

Pregnenolone (P), the main precursor of the steroids, and its sulfate ester, pregnenolone sulfate (PS), are the major neurosteroids produced in the neural tissue. Many neuroendocrinological studies stressed the neuroprotective role of neurosteroids although it has been suggested that the inhibition of P and PS synthesis can delay neuronal cell death. The potential roles of P and PS in vital neuronal functions and in amyloid beta peptide (Aβ) toxicity are not clearly identified. This work aims to investigate the effects of P and PS on cell viability and Aβ peptide toxicity in a concentration and exposure time-dependent manner in rat PC-12 cells. The cells were treated with 20 μM Aβ peptide 25–35 and variable concentrations of P and PS ranging from 0.5 μM to 100 μM. To examine the effects of steroid treatment on Aβ peptide toxicity, 0.5 μM (low) and 50 μM (high) neurosteroids were used. The cell viability and lactate dehydrogenase release of cells were evaluated after 24, 48 and 72 h. Morphological changes of cells were also examined. The treatment with higher than 1 μM concentrations of P and PS significantly decreased the cell viability comparing to untreated cells. At lower concentrations, P and PS had no toxic actions until 72 h. The Aβ treatment resulted in a significant decrease in cell viability comparing to untreated cells. P showed a dose-dependent protective effect against Aβ peptide in PC-12 cells. But its sulfate ester did not have the same effect on Aβ peptide toxicity, even it significantly decreased cell viability in Aβ-treated cells. Consequently, the discrepant effects of P and PS on Aβ peptide toxicity may provide insight on the pathogenesis of Alzheimer's disease.

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1. Introduction

Pregnenolone (P) and pregnenolone sulfate (PS) are the most abundant neurosteroids produced in the neural tissue of rats and humans [1,2]. After synthesis of pregnenolone in mitochondria, it can be converted to its sulfate ester by hydroxysteroid sulfotransferase [1]. The concentrations of neurosteroids in neuronal tissue could be changed by ischemia, oxidative stress or neurotoxicity namely induced by amyloid beta peptide (Aβ), glutamate [2–5]. After identifying that the steroids are synthesized *de novo* in nervous system, some investigators have been focused on the interrelationship between the synthesis of neurosteroids and their potential roles on vital neuronal functions. The synthesis of P and PS was suggested as an early event of neuronal apoptosis and the inhibition of their synthesis has been shown to attenuate excitotoxicity in rat retina [6].

Alzheimer's disease is the most common cause of dementia in the elderly. The toxicity of Aβ peptide and excitotoxicity using N-methyl-D-aspartic acid (NMDA) receptors are thought to be involved in neuronal damage of Alzheimer's disease [7]. Aβ peptide is negatively correlated with the levels of PS in the brain cortex of patients with Alzheimer's disease [8]. In common opinion the neurosteroids such as P, dehydroepiandrostenodione sulfate, estradiol are accepted as protective agents against toxicity induced by Aβ peptide in vitro [9–12]. On the other hand, Cascio et al. suggested that the sulfate ester of P, PS, was a naturally excitotoxin in rat retinal cells and the antagonist of NMDA receptors could reduce the cell death induced by PS [13,14]. To the best of our knowledge, the actions of P and PS on neuronal cell viability and Aβ peptide toxicity are not clear. This work aims to investigate the effects of P and PS on cell viability and Aβ peptide toxicity in a

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concentration and exposure time-dependent manner in rat PC-12 cells.

2. Materials and methods

2.1. Chemicals

Aβ peptide (25–35) fragment, 5-pregnen-3β-ol-20-one (pregnenolone), pregnenolone sulfate, 2,4-dichloro-6-nitrophenol (DCNP) were from Sigma Chemical Co., St. Louis, USA. Dimethylsulfoxide (DMSO) was purchased from Merck Chemicals (Germany). P and PS were dissolved in DMSO and added to the medium in a few hours to obtain a final concentration 1% of DMSO. All tissue 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).

2.2. Cell culture and treatments

PC-12, a rat pheochromocytoma-derived cell line was selected as the neuronal model which was originally described by Greene and Tischer [15]. The cell line was obtained from DSMZ Cell Lines Bank (Germany).

Before treatment, PC-12 cells were placed in poly-D-lysine coated plates (96 or 6 wells) at a density of 2×10^4 or 1×10^6 cells/well and were allowed to attach for 24 h in RPMI 1640 medium supplemented with 10% donor horse serum, 5% fetal bovine serum, and a mixture of 1% of penicillin/streptomycin/L-glutamine. Cells were incubated at 37 °C in humid, 5% CO_2–95% air environment.

2.2.1. Toxin treatment

The toxin assays were performed in 96-well micro-titer plates. After an initial-24 h plating, the medium was replaced with (100 μL) RPMI 1640 containing 1% penicillin/streptomycin/L-glutamine and 1.5% horse/fetal bovine serum. 20,000 cells/well were treated for 24, 48 and 72 h of incubation in 20 μM Aβ 25–35 peptide (previously shown to induce almost 50% of neuronal cell death) [16]. The control cell groups were placed in RPMI 1640 medium supplemented with 1.5% horse/fetal bovine serum and 1% of penicillin/streptomycin/L-glutamine.

2.2.2. The dose–response curve of steroids in PC-12 cells

After initial-24 h plating, PC-12 cells in 96-well micro-titer plate were treated with variable concentrations of P and PS, separately, ranging from 0.25 μM to 100 μM (0.25, 0.5,1, 20, 40, 60, 80, 100 μM). Also, in another study group 1 (μM) DCNP, a non-specific hydroxysteroid sulfotransferase inhibitor, was added to P containing wells to inhibit the conversion of P to PS [17]. DCNP was added in culture plate together with P. Following 72-h incubation, the cell survival was evaluated.

2.2.3. The effects of steroid treatment on Aβ toxicity

To examine the possible effects of the steroids on Aβ protein toxicity, 0.5 μM (low dose) and 50 μM (high dose, moderate toxic dose) concentrations of P and PS were used. After an initial-24 h plating, the steroids and 20 μM Aβ 25–35 peptide were simultaneously added to wells. After 24, 48 and 72 h of treatments, cell viability was evaluated.

Neuronal cell survival was estimated by using three different assays: (i) cell death using the LDH release assay, (ii) cell viability using the MTT reduction assay and (iii) morphological changes of cells using fluorescence microscopic examination.

2.3. LDH release assay

Release of LDH was quantified spectrophotometrically as a measure of cell death with a commercially available kit (Cytotoxicity Detection Kit; Roche Diagnostics, USA) according to the manufacturer's instructions. Culture supernatant was collected to measure LDH release activity. To measure maximum LDH release, PC-12 cells were lysed by adding Triton-X 100. The results were expressed as the percentage of the maximum LDH activity. So that increased LDH release was accepted as an indication of neuronal injury.

2.4. MTT reduction assay

A MTT reduction method was used to determine the cell viability and to provide information on mitochondrial function [18]. Briefly, 10 μL of a 5 mg/ml MTT solution in sterile PBS was added to 100 μL of medium and incubated for 2–4 h in the incubator chamber at 37 °C. After removing the cell medium, 200 μL of DMSO solution was added and incubated for 30 min at 37 °C. Immediately before measuring, 50 μL glycine buffer was added. Then the absorbance values were determined using an automatic micro-titer plate reader at 450–650 nm. Results of MTT reduction in treated cells were expressed as the percentage of control (untreated) cells. Decreased MTT reduction was accepted as an indication of neuronal injury.

2.5. Fluorescence microscopy

Fluorescence microscopy was used to assess the morphological changes induced by P, PS and Aβ peptide in PC-12 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 (40 μL) 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) [19].

2.6. Statistical analysis

Groups were compared by Kruskal–Wallis test, followed by Mann–Whitney *U*-test. *p*-Values less than 0.05 were considered significant. All the experiments were performed at least in triplicate, and graphic results were displayed as the mean ± S.E.M. of three to nine separate experiments.

3. Results and discussion

Fig. 1A and B represents the direct effects of 20 μM Aβ 25–35 peptide on the cell viability and cell death in an exposure time-dependent manner. The 24-h treatment of PC-12 cells with Aβ, as reflected by the MTT reduction and LDH assays, had no statistically significant effect on cells. After 48 h exposure, MTT reduction results of Aβ-treated group were significantly lower than the control group (Fig. 1A, $p < 0.05$), but LDH results were not statistically different comparing to the control group (Fig. 1B). Following 72 h exposure of the cells with Aβ, a significant decrease in the cell viability and also a significant increase in the LDH release of cells

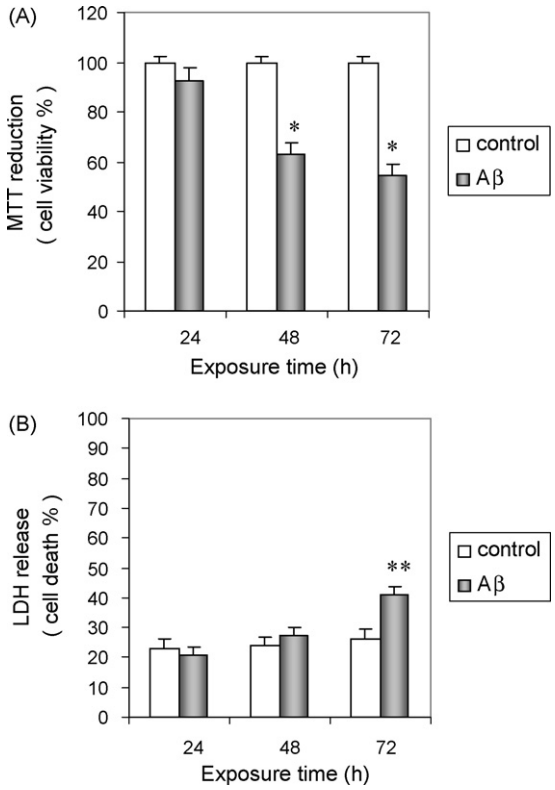


Fig. 1. The direct effects of Aβ peptide 25–35 fragment on PC-12 cells in an exposure time-dependent manner. MTT reduction results were expressed as percentage of control (untreated) cells (A), LDH results were expressed as percentage of maximum LDH release (treated with Triton X-100) (B). Values represent mean ± S.E.M. *The results were significantly lower than the control group ($p < 0.05$), **The results were significantly higher than the control group ($p < 0.05$), Aβ: amyloid beta 25–35.

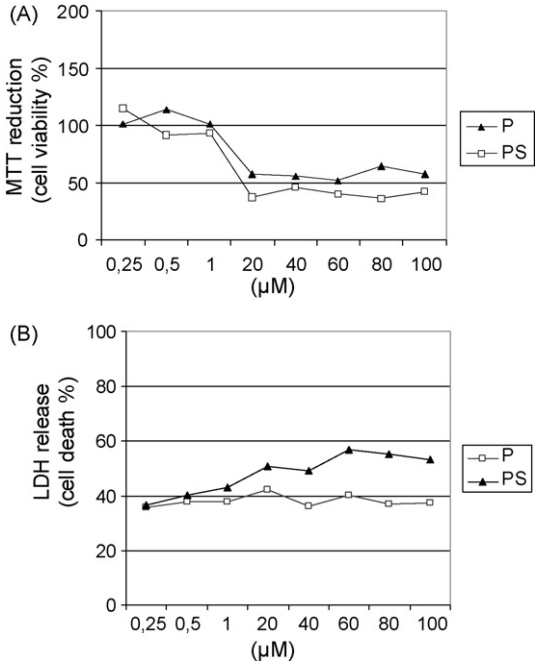


Fig. 2. The dose–response curve of pregnenolone (P) and pregnenolone sulfate (PS) for 72 h (h) exposure time. (A) The results of MTT reduction were expressed as percentage of control (untreated) cells. (B) The results of LDH release were expressed as percentage of maximum LDH release (treated with Triton X-100).

were determined. After 72 h, LDH results nearly supported the MTT reduction results.

Fig. 2 displays the effects of 72-h treatment with P and PS, separately, in a dose-dependent manner. The cell viability was significantly decreased by higher than 1 μM concentrations of P and PS comparing to untreated cells ($p < 0.05$, Fig. 2A). But, at lower concentrations, P and PS had an increasing trend in cell viability. PS induced cell death proportionally with its treatment dose (Fig. 2B) According to MTT and LDH assays, 50 μM concentrations of P and PS were accepted as a moderate toxic dose since nearly 50% of cell death was obtained at this concentration.

On the other hand, the effect of 50 μM P on the cell viability could be changed by the DCNP treatment (Fig. 3). Inhibiting sulfotransferase reaction by DCNP changed the effect of P on cell viability.

The treatment with P and PS affected Aβ protein toxicity in a dose-dependent manner. According to cell viability results, Aβ peptide toxicity could be significantly decreased by the treatment with 0.5 μM P. Interestingly PS did not have similar effect at the same concentration (Fig. 4A). Similarly, as shown by LDH results, the ratio of the cell death was significantly lower than the Aβ group in the group treated with 0.5 μM P + Aβ peptide (Fig. 4B). At high concentrations (50 μM), P and PS had no protective effect, even, PS exacerbated Aβ peptide toxicity as depicted from LDH and MTT results.

The apoptotic and necrotic cell death induced by Aβ, PS and neuroprotection of low-dose P were also observed by immunofluorescence microscopy. According to double-dying results, the necrotic cell death in group treated with Aβ plus 50 μM PS was more than the others (~necrotic cells 77.8% of total cell count, apoptotic cells 10% of total cell counts). While the cells treated only with PS were approximately 67.8% necrotic, 10.0% apoptotic, the cells treated only with P were 7.8% necrotic and 17.4% apoptotic, after 72 h exposure to 50 μM concentration. The apoptotic cells and necrotic cells induced by Aβ was 18.2 and 30% of total cell count, respectively. The apoptotic cell death in control group was almost 3% of the total cell count. Fig. 5 shows alive, apoptotic and necrotic PC-12 cells.

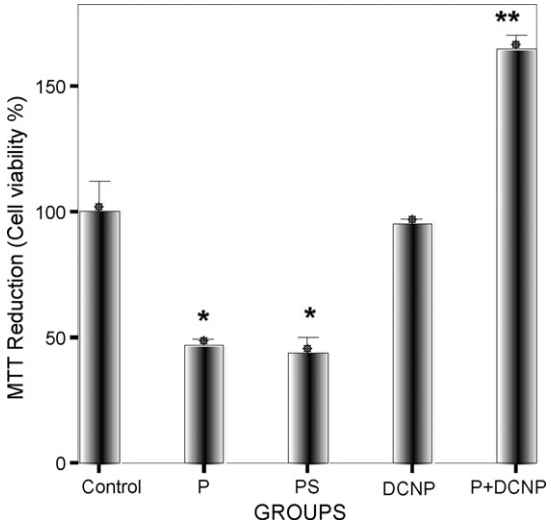


Fig. 3. DCNP changed the effects of P (50 μM) on the cell viability. At 50 μM concentration, P and PS decreased the viability of PC-12 cells after 72 h exposure. DCNP (1 μM) increased the cell viability of the cells treated with P. *MTT reduction levels of P and PS group were significantly lower than the control group. ** Significantly higher than P group ($p < 0.05$). Values represent mean ± S.E.M. Control: untreated cell group, P: pregnenolone, PS: pregnenolone sulfate, DCNP: 2,4-dichloro-6-nitrophenol, the inhibitor of sulfotransferase enzyme.

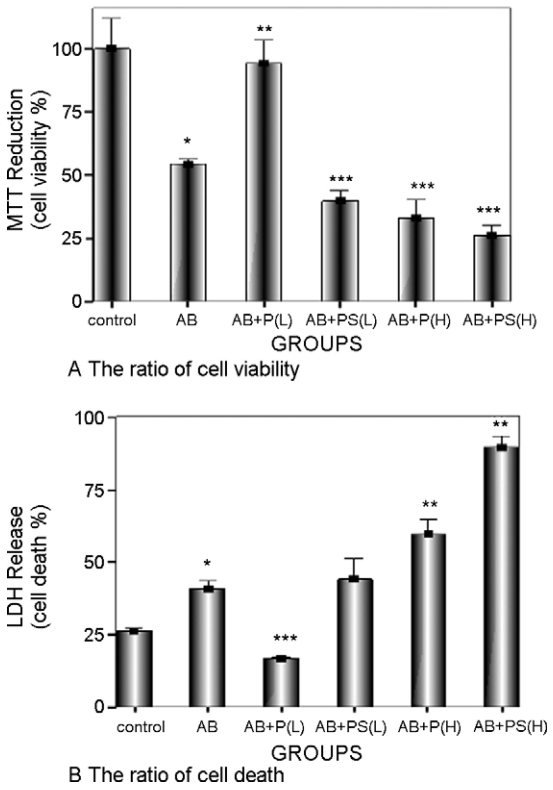


Fig. 4. The effects of the treatment with P and PS on Aβ protein toxicity regarding their treatment dose; (A) the ratio of cell viability and (B) the ratio of cell death. Aβ + P/PS (L): the cells were treated simultaneously with 20 μM Aβ 25–35 and 0.5 μM steroids during 72 h. Aβ + P/PS (H): the cells were treated simultaneously with 20 μM Aβ 25–35 and 50 μM steroids during 72 h. Values represent mean ± S.E.M. *The results were significantly different comparing with the control group ($p < 0.005$). **The results were significantly higher than Aβ-treated cell groups ($p < 0.05$). ***The results were significantly lower than Aβ-treated cell groups ($p < 0.05$). Aβ: amyloid beta 25–35. Control: untreated cell group; P: pregnenolone; PS: pregnenolone sulfate.

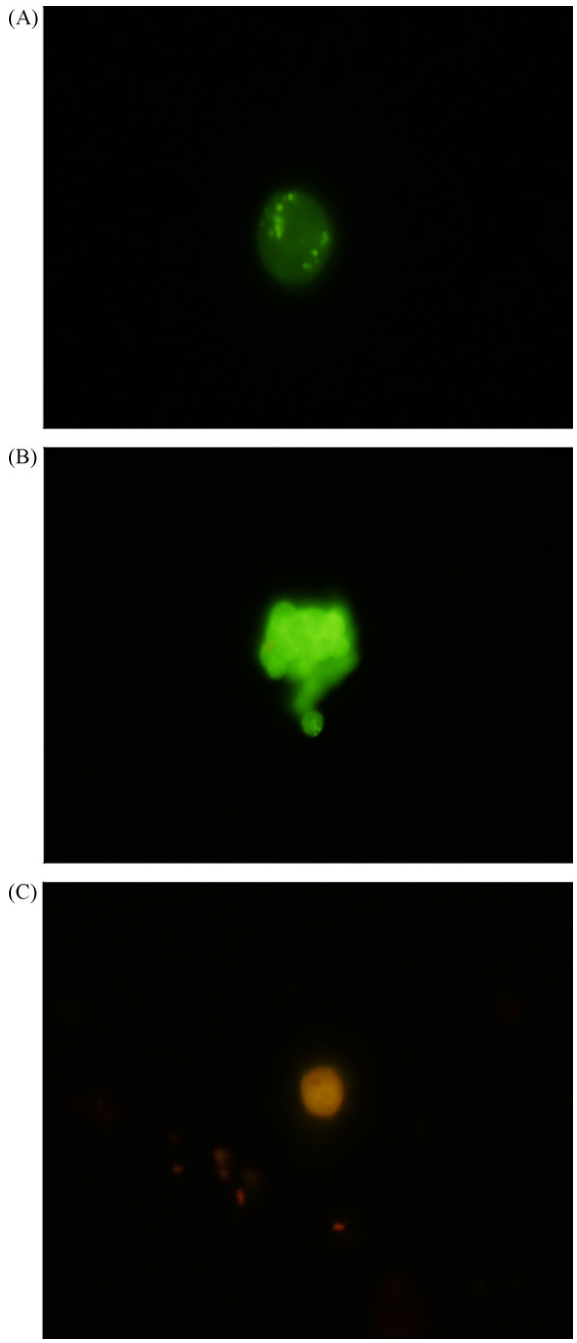


Fig. 5. The fluorescent images of PC-12 cells under fluorescence microscopy, (A) an apoptotic PC-12 cell, (B) alive PC-12 cells and one of apoptotic PC-12 cells, and (C) a necrotic PC-12 cell.

Aβ is a 4 kDa amino acid peptide which can accumulate as insoluble extracellular deposits in senile plaque and has a causal role in pathogenesis of Alzheimer's disease [20]. Aβ 25–35 is often used for modeling different aspects of AD [20–25]. The 11 amino acid fragment Aβ 25–35 is a C-terminal peptide of Aβ 1–42 located in its hydrophobic functional domain [26]. The presence of Aβ 25–35 antigen in AD brain as well as a principal ability of brain protease-mediated conversion of non-fibrillar, soluble and non-toxic Aβ 1–40 to fibrillar and toxic fragment Aβ 25–35 have been reported [27]. The data give support to use of Aβ 25–35 as a tool for investigating AD-related mechanisms in animal models or animal-derived cell lines.

The results of our study supported the toxic effect of Aβ 25–35 peptide on cell viability in PC-12 cells. Gursoy et al. showed that 0.5 μM concentration of pregnenolone protected hippocampal cell line against neuro-toxins until 24 h and they proposed that to have hydroxyl (OH) group on the C3 position of steroids such as P, estradiol and dehydroepiandrosterone was responsible for neuroprotective effects [11]. In our study we also demonstrated that P, at 0.5 μM concentration, protected PC-12 cell line against Aβ protein toxicity until 72 h, and this finding was similar to Gursoy's.

According to our results, the cell viability was significantly decreased and LDH release of cells was significantly increased by Aβ treatment. But, significant increase of LDH level was only obtained after 72 h exposure. MTT results (the cell viability) were significantly decreased compared to control group after 48 h. It can be proposed that LDH assay may not be suitable to detect early damage in PC-12 cells. In our experimental model, MTT assay was found more sensitive than the LDH assay to detect Aβ toxicity. PC-12 cells released high amount of LDH enzyme without any exogenously steroid treatment. To be able to measure LDH activities in the detection limits of the LDH kit (cell culture assay kit) we had to make dilutions of culture supernatant (1/10 dilution). PC-12 cells are derived from rat pheochromocytoma cells. As it is known neoplastic cells are adapted to anaerobic glycolysis [28]. For this reason an intracellular increase in the enzyme of this pathway, LDH, which converts pyruvate to lactate is expected. As a con-

sequence, release of LDH from PC-12 cells to medium was also high.

The treatment with Aβ peptide not only reduces the number of NMDA receptors but also increases NMDA response in neuronal tissue [7,29]. Cascio et al. reported that 50 μM concentrations of PS induced cell death modulating NMDA receptors [13]. In our study, 50 μM PS decreased cell viability and exacerbated Aβ peptide toxicity. NMDA receptors may be responsible from this effect. But interestingly, lower concentrations of PS (0.5 μM) also exacerbated Aβ toxicity as shown as MTT reduction assay. This assay quantifies mitochondrial activity by measuring the formation of a dark blue formazan product formed by the reduction of the tetrazolium ring of MTT and uses to accurately measure neuronal injury [30]. The reduction of MTT is thought to mainly occur in the mitochondria through the action of succinate dehydrogenase, therefore providing a measure of mitochondrial function [18,30,31]. It can be proposed that PS may have a partial role on Aβ peptide toxicity via mitochondrial dysfunction. On the other hand the sensitivity of NMDA receptors to PS might be increased by Aβ peptide or Aβ might affect the PS response of cells.

The highest concentrations of P and PS in the central nervous system are less than 1 μM level at non-pathologic conditions [1]. However their level can be increased by some insults such as NMDA, acute shock and proconvulsant agents or increased cholesterol values [6,32,33] and the inhibition of P and PS synthesis attenuated neuronal cell death [6]. In our study, supra-physiologic concentrations of P and PS reduced PC-12 cell's viability. On the other hand, physiologic concentrations (<1 μM) had no toxic effect until 72 h exposure. Consequently, it can be proposed that increased P synthesis or its sulfate ester may play a negative critical role on neuronal cell survival.

The turnover of brain cholesterol is very low and the blood–brain barrier permits only a very small lipoprotein-mediated flux of cholesterol from the plasma, most of the cholesterol being synthesized within the brain [34]. After all Sooksawate and Simmonds suggested that cholesterol modulate the effect of the neurosteroids via GABA-A receptors and the hypothalamic area may be vulnerable to changes in plasma cholesterol [35]. Taking into account that cholesterol is the precursor for pregnenolone, during fluctuations of plasma cholesterol or in people who have very high levels of cholesterol, pregnenolone content of the neuronal tissue may reach to supra-physiologic levels and cause neuronal death as demonstrated in our study.

Oligodendrocytes can synthesize P *de novo* and Aβ stimulated DHEA synthesis from P [5]. Also, Aβ 25–35 and Aβ 1–42 decrease the progesterone synthesis while Aβ 1–42 increases DHEA synthesis in human neuroblastoma cells [36]. These data suggest that Aβ affects P metabolism but it is not clear that how Aβ affects the synthesis of P and PS. Further studies are needed to elucidate the effects of Aβ peptide on PS metabolism and vice versa.

P is converted to its sulfate ester in neuronal tissue by sulfotransferase [1]. In our study, the exogenously high concentration of P (50 μM) significantly decreased the viability of PC-12 cells and DCNP which is a non-specific inhibitor of sulfotransferase altered this effect of P. From the MTT results it can be speculated that the toxic effect of P at high concentration may be via the conversion of P to PS.

In conclusion, our study demonstrated that the effects of P and PS against Aβ peptide toxicity were emerged in a concentration and exposure time-dependent manner in rat PC-12 cells. While PS had a potential role on toxicity, P, at low doses acted as a protective agent preventing cell damage induced by Aβ peptide. Further studies are needed to explain the potential role of PS in Alzheimer's disease and the interrelationship between the neurosteroid synthesis and the toxicity process induced by Aβ peptide.

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