Effects of Levodopa loaded chitosan nanoparticles on cell viability and caspase-3 expression in PC12 neural like cells

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Levodopa (L-DOPA) is one of the most valuable agents for the symptomatic treatment of Parkinson's disease (PD).[1] However, this drug has very low water solubility, causing problems in drug formulation and administration. Also, some studies have demonstrated that L-DOPA accelerates the death of dopaminergic neurons and hastens progression of the disease and apoptosis.[2] The evidence supports that "apoptosis" is an evolutionarily conserved form of cell death, and one of the predominant signaling pathways involved in pathophysiology of neurodegenerative diseases. Caspases play a major role in cells undergoing apoptosis and can be an appropriate candidate for apoptosis evaluation studies.[3] On the other hand, in recent years, chitosan (CS) nanoparticles (NP) have been intensively investigated for drug administration because of its favorable features in terms of biocompatibility, non-toxicity, and bioadhesion.[4] Chitosan and its derivatives are promising neuroprotective agents, and they have shown some neuroprotective properties such as: suppression of beta-amyloid formation, acetylcholinesterase inhibition, anti-neuroinflammatory activity, and apoptosis inhibition. Studies have shown that CSNPs effectively reduce cell membrane damage, secondary oxidative stress, and lipid peroxidation in neural like cells.[5] Therefore, in this study we evaluated the effects of L-DOPA loaded CSNP on cell viability, and caspase-3 expression in PC12 neural like cells.

The CSNPs were prepared according to a modified ionic gelation method.[4] Briefly, 1.5 ml of a low molecular weight CS (Sigma-Aldrich, Munich, Germany) solution (0.2%, w/v, in acetic acid 0.1%, v/v) was maintained under magnetic stirring (IKA, Staufen, Germany) in 150 rpm conditions. Then 1.6 ml of pentasodium tripolyphosphate (TPP) (Sigma-Aldrich, Munich, Germany) aqueous solution (0.07%, w/v) was sprayed into the CS solution leading to NP formation. For L-DOPA adsorbing CSNPs evaluation, 1 ml of L-DOPA (Ramopharmine, Tehran, Iran) aqueous solution (1 mg/ml) was prepared. Then, 0.5 mL of CSNPs were added and incubated at 25°C in a water bath for 3 hours under mild stirring and the resulting NPs were isolated by centrifugation (Sigma 2-16pk, Osterode, Germany) at 14000 rpm for 40 minutes. Finally, the supernatant was removed and residual parts were re-suspended in ultrapure water (w/v) by manual shaking. The loading capacity of the NPs was determined by the spectrophotometrical method. The amount of free L-DOPA was determined in the supernatant (previous step) by UV spectrophotometry (LAMBDA 35, PerkinElmer, Waltham, MA, USA) at 279 nm. The loading capacity was calculated using the equation below:

$$\text{Loading capacity} = \frac{\text{Total amount of drug - Free amount of drug}}{\text{CSNP weight}} \times 100$$

The range of 3 independent experiments under the same conditions was similar.

The CSNPs and CSNP-L-DOPA were characterized using the following measurements. Particle size was determined using Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) and the analysis was performed at a scattering angle of 90° and room temperature. For each sample, one mg of NPs was diluted in de-ionized water, then vortexed and sonicated for a few minutes. Each sample was measured in triplicate. The experimental subjects were PC-12 cells. The cells were cultured at 37°C, in 5% CO_2 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, and streptomycin in 75 cm^2 culture flasks, and the culture medium was replaced every 48 hours. In the evaluation step, the cells were seeded in culture flasks and treated with (200 µM) L-DOPA,[1] CSNP-L-DOPA (corresponding to 200 µM L-DOPA concentration) and CSNP (in the same volume) separately. The test materials were diluted with culture medium to yield the desired final concentrations and obtain the best results. After 24 hours of incubation, cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay.[3]

Expression of caspase-3 was investigated by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Briefly, total RNA was isolated according to the AccuZol kit protocol provided by the manufacturer (BioNeer, Daejeon, Korea). For RT-PCR, 1 µg of RNA was used for cDNA synthesis. The PCR was performed with 5 pmole of primers,[6] according

Disclosure. The authors declare no conflicting interests, and this study was not supported or funded by any drug company.

to the AccuaPower kit protocol (BioNeer, Daejeon, Korea). The PCR product was run on a 1% agarose gel, and quantitative analysis was carried out by band intensity read out (Kodak GL 200 imaging Cabinet, Acquire software). All genes were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The mean of 3 independent experiments was recorded.

Data were examined using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) version 19 for Windows. Comparisons were made using descriptive statistics, and one-way ANOVA followed by Tukey post hoc test. The α level in our analysis was set to 0.01%.

The CSNP-L-DOPAs had an average particle size diameter of 250 nm with positive zeta potential, but the CSNP particles were smaller than CSNP-L-DOPAs at a 160-210 nm size range. The loading capacity of the CSNPs for L-DOPA was 92%. The MTT assay results were calculated as the percentage of living cells in treated cultures compared with control cultures. No significant differences were observed for cell viability, between CSNP and control. The L-DOPA had a significant effect on decreasing cell viability (p<0.01). However, there was a significant increase in the CSNP-L-DOPA treatment group (p<0.01) (**Table 1**). The Caspase-3 expression levels in the control and test groups are presented in **Table 1**. The expression of caspase-3 increased with treatment with L-DOPA (p<0.01), but the CSNP-L-DOPA group expression was significantly decreased compared with the L-DOPA treatment group (p<0.01).

The NPs made from CS were successfully employed for the administration of various drugs in the CNS. Ngwuluka et al's study[7] demonstrates the feasibility of fabricating of L-DOPA-loaded methacrylate copolymer/CS NPs. The obtained NPs were hollow, capsular, nanoparticulate complexes with 93% drug loading efficiency. These NPs seem to be useful for drug delivery to the brain as they can transport some neuroactive compounds across the brain blood barrier, and have some neuroprotective properties.[4] The obtained CSNP-L-DOPA size in the present study was 250 nm, and the loading capacity was 92%; a rate suitable for drug delivery systems.

In vitro experiments show that L-DOPA has toxic effects (decreased viability and deformed morphology) on dopaminergic neurons.[1] In Du et al's study,[8] a significant decrease in viability was found in the neurons treated with 10, 100, or 200 μM of L-DOPA. In the present study, cells viability was decreased in the L-DOPA treated group.

The caspase family is one of the most important apoptotic activators. In particular, caspase-3 is considered to play an important role in the final common pathway of apoptosis. In Park et al's study,[2] the cleaved caspase-3 increased in PC12 cells treated with 200 μM L-DOPA. In the present study, L-DOPA treatment increased caspase-3 expression by around 7 fold relative to the control cells.

Levodopa might accelerate the rate of nigral degeneration, because it undergoes oxidative metabolism[1] and CS is one of most important preventers of oxidative stress.[3] In a study that evaluated CSNPs effects on acrolein-induced cell injury,[5] treatment with CS/TPP showed a very significant increase in cell viability, corresponding to 120.5 ± 26.6% of control values.

According to results of this study, cell viability in the CSNP-L-DOPA treated cultures was 83.1±1.8% in comparison with 98.7±2.6% in the control culture. However, this rate in the L-DOPA treated group was 42.8±1.4 and statistically significant (p<0.01). In the caspase-3 evaluation, CSNP-L-DOPA significantly decreased gene expression in comparison with the L-DOPA group. The band of caspase-3 is weaker in CSNP-L-DOPA group compared with the L-DOPA treated cells by approximately 2.9 fold, demonstrating the ability of CSNP to reduce caspase-3 expression in PC12 cells.

Table 1 - The MTT assay and caspase-3 expression results. Gene expression was normalized to GAPDH. The mean of 3 independent experiments was recorded (mean ± standard error).

Variable	Control	CSNP	L-DOPA	CSNP - L-DOPA
Percentage of living cells	98.7±2.6	99.3±3.2	42.8±1.4*	83.1±1.8#
Mean fold change Caspase-3/GAPDH	0.19±0.01	0.17±0.02	1.49±0.02*	0.51±0.01#

*Significantly different from control and CSNP (p<0.01), # Significantly different from L-DOPA (p<0.01)
MTT - 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, GAPDH - glyceraldehyde-3-phosphate dehydrogenase, CSNP - chitosan nanoparticles, L-DOPA - levodopa

In conclusion, these results reveal the ability of CSNPs as a potential drug carrier and neuroprotective agent in neural cells, and represent as an interesting nanotechnological platform for L-DOPA delivery. It may provide a useful strategy for PD treatment via neuroprotection and reduction of L-DOPA-induced neurotoxicity.

Acknowledgement. *The authors are thankful to the Research Office of Tabriz University of Medical Sciences, Tabriz, Iran for spiritual auspices.*

Received 21st November 2012. Accepted 24th March 2013.

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