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Title: Caffeic Acid Phenethyl Ester with Mesenchymal Stem Cells Improve Behavioral and Histopathological Changes in the Rat Model of Parkinson' Disease

Running title: CAPE Improves Parkinson’s Disease

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ABSTRACT

Introduction: Parkinson's disease (PD) is the result of the destruction of Dopaminergic neurons in the brain. The aim of this study was to investigate the protective effects of natural antioxidants such as caffeic acid phenethyl ester for the maintenance of these neurons.

Methods: Caffeic acid phenethyl ester (CAPE) is one of the main ingredients of Propolis. Intranasal administration of (1-methyl-4-phenyl-2;3;4;6-tetrahydropyridine) MPTP was used to generate PD model in rats. 2×10^6 bone marrow stem cells (BMSCs) were injected from tail vein. Behavioral test, Immunohistochemistry, DiI, cresyl fast violet, TUNEL staining were evaluated, 2 weeks after treatment.

Results: DiI staining method revealed in all treatment groups using stem cells, the cells migrated to the substantia nigra pars compacta after injection. Treatment with CAPE significantly protects dopaminergic neurons from MPTP. The highest number of tyrosine hydroxylase (TH) positive neurons was seen in group Pre CAPE+PD+Stem cell. The number of TH^+ cells in all groups that received CAPE was significant in compared to groups that received the stem cells only (P <0.001). Intranasal administration of MPTP significantly increase the number of apoptotic cells. The lowest number of apoptotic cells was in group Pre CAPE+PD+ Stem cell.

Conclusion: The results showed that the use of CAPE and stem cells in Parkinson's rats caused a significant reduction in the apoptotic cells.

Keywords: Parkinson disease; MPTP; Antioxidant; Caffeic acid phenethyl ester; Tunnel
**Highlights**

CAPE is a natural antioxidant and is a powerful neuronal protector due to its anti-inflammatory properties.

CAPE plus Bone marrow stem cells can reduce apoptotic cells in striatum and substantia nigra.

Pretreatment with CAPE protect dopaminergic neurons from degeneration.

**Plain language summary**

The Parkinson's model from MPTP is used as a standard model. MPTP causes a significant reduction of dopaminergic neurons in the substantia nigra. CAPE has an anti-inflammatory effect, effectively contributing to the collection of free radicals and removing inflammatory agents and subsequently improve the disease. Stem cells can regulate immune responses and differentiate into special cells to replace injured cells. By inhibiting apoptotic pathways, these cells create tropical factors to protect and repair cells.
1. Introduction

Parkinson's disease (PD) is caused by central nervous system destruction, (Badban et al., 2015; Dauer & Przedborski, 2003). PD is the result of the destruction or malfunction of dopaminergic secretory neurons in the substantia nigra pars compacta (SNpc) in the midbrain. The loss of dopamine-secreting cells in the corpus luteum depends on several factors. (Safari et al., 2016). Symptoms of Parkinson's disease appear when at least 80% of dopaminergic neurons are destroyed (Braak, Ghebremedhin, Rüb, Bratzke, & Del Tredici, 2004). The goal of treatment should be to protect the remaining neurons or to replace the damaged neurons with stem cells (Hald, van Beek, & Lotharius, 2007). There are several treatments for Parkinson's disease that are used to relieve symptoms. The most commonly used drug is levodopa. This drug in the early stages of the disease improves symptoms, but it gradually causes memory, learning and sleep disorders (Cools, Barker, Sahakian, & Robbins, 2003). Studies have shown that the use of antioxidants such as propolis to protect neurons and the use of stem cells to replace damaged cells is helpful (Dantuma, Merchant, & Sugaya, 2010; Pellegrini et al., 2003). MPTP (1-methyl-4-phenyl-2;3;4;6-tetrahydropyridine) is widely used to create an animal model of Parkinson's disease. Intranasal injection of this toxin effectively and selectively destroy the dopaminergic neurons (Prediger et al., 2006). Propolis is a waxy substance and a bee product, it has strong antibacterial, antifungal, anti-inflammatory, anti-parasitic, and antioxidant properties and the most important of its active antioxidant is caffeic acid phenethyl ester. CAPE inhibits lipid peroxidation and lipoxygenase activities (Sud'Ina et al., 1993). Stem cell use is a promising treatment for neurodegenerative diseases. The use of mesenchymal stem cells has advantages over other stem cells, including high dividing power (Jadidi et al., 2016), easy preparation, no moral problems and no transplant rejection (Jäger et al., 2010). Stem cells can self-renewal and differentiation to all types of cells, including blood, nervous and cartilage cells (Glavaski-Joksimovic & Bohn, 2013). BMSCs transplanted into the adult brain exhibit characteristics of microglia, astrocytes and neuronal-like cells (Li et al., 2001). The purpose of this study was to examine the neuroprotective effect of CAPE as pre-treatment and co-treatment with BMSCs on a dopaminergic neuron in the midbrain in a rat model of Parkinson's disease.

2. Methods

2.1. Animals

Adult male albino-wistar rat weighing 200-250 g were prepared from the stem cells research center of Semnan University of Medical Sciences, Semnan, Iran. All the rats were kept in separate cages. They also had free access to water and food. The room temperature and humidity were constant and on 12 h light and dark. All the principles of working with animals were per under with the National Institutes of Health Guide for Care and Use of laboratory animals authorized by the Ethics Committee (ethical code number: IR.SEMUMS.REC.1395.153) of Semnan University of Medical Sciences, Semnan, Iran. Rats were randomly assigned to 7 groups (n=7). The first group received only normal saline as a normal group or sham-control group. The second group received intranasal MPTP (2mg, 12 µl) as a Parkinson's group without receiving treatment. The third group received MPTP and one week after PD model received intravenous stem cells (in dorsal caudal vein, $2 \times 10^6$). The fourth group received intraperitoneal CAPE (10 µM) as a pre-treatment 2 weeks before PD. The
fifth group received CAPE as a pre-treatment two weeks before PD and one week after PD received intravenous stem cells. The sixth group received stem cells one week after PD and 2 weeks CAPE as a co-treatment. The seventh group received CAPE one week before PD and one week after PD as a co-treatment.

2.2. Methods

2.3. Intranasal administration of MPTP

Intranasal (i.n.) injection procedure, was done according to the method described by Prediger (Prediger et al., 2006). In summary, rats were lightly anesthetized and from PE-50 tube, 10-mm piece prepared and inserted into the nostril. The pipe was connected to a manual injection pump, and 12 µl of neurotoxin injected into the nostrils. The MPTP HCL (Sigma Chemical Co., USA) was dissolved in 0.9% NaCl (saline). After which it was infused for 8 minutes, animals were given 1-minute time to get normal breathing function and then the operation was performed by injection using the opposite nostril. To receive a 2 mg dose, 12 µl of the toxin was injected into each nostril.

2.4. Behavioral test

The pole test was originally presented by Ogawa (Ogata, Tashiro, Nukuzuma, Nagashima, & Hall, 1997; Sedelis, Schwarting, & Huston, 2001) to appraise MPTP-induced bradykinesia in an animal model of PD. The test includes an approximately 50 cm high, (1 cm diameter) with a small sphere at the above. The animals are placed on the sphere while their heads are up. In this test, the time until the animals have descended to the floor checked. This test consists of 6 degrees. Grade 1: The animal is not able to maintain balance and falls. Grade 2: The animal retains its balance on the pole, but cannot walk more than 10 centimeters. Grade 3: The animal moves through the pole using a paw and jumping on its hind legs. Grade 4: The animal passes naturally from the pole but has an a 3 or 4-foot slip. Grade 5: The animal naturally extends from the pole and has less than 3 or 4-foot slip. Grade 5 to 6 animals are healthy. Tests were taken in two steps. The first stage is one week after the creation of the Parkinson's Model as pretreatment, and the second stage is 2 weeks after the creation of the model and the end of the treatment as a post-treatment test.

2.5. TH- Immunohistochemistry

We used the immunohistochemical staining method to study the number of dopaminergic neurons of SNpc. Animals were killed at 28 days after creating the Parkinson's model, the rat brain was removed and after the tissue processing, the middle cerebral area was evaluated by immunohistochemistry. Tissue sections after washing with PBS buffer and to retrieve the antigen, 2N Hydrochloric acid was poured on the samples over minutes. Then, to penetrate the cell membrane Triton 0.3% was used. Samples were washed with PBS and to block the secondary antibody response the 10% goat serum was added for 30 minutes. The diluted primary antibody (Anti-Tyrosine Hydroxylase (1:100, Abcam) monoclonal to Tyrosine Hydroxylase) was added to the sample and placed in a temperature of 2 to 8 °C for one night. The next day, the secondary antibody (FITC-anti-mouse IgG2a-gamma chain (1:200, Abcam) was added to samples and then incubated in an incubator at 37 °C for 1 hour and 30 minutes in the darkness. Then, DAPI was used. Finally, the samples were examined by the Olympus Fluorescent Microscope (×400).

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2.6. DiI staining

This staining has been developed to help the morphological recognition of neurons. In this method, for $10^6$ cells suspended in the culture medium (DMEM), add 5 μl / ml DiI (1, 1′-dioctadecyl-3, 3′, 3′-tetramethylene-docarbocyanine perchlorate) and incubate for 20 minutes. Then cells centrifuged for 5 min and 1200 rpm. Remove the supernatant and rinse the pellet slowly with PBS and dissolve with 15 μl of culture medium. The solution should be injected as soon as possible in this study, $2\times10^6$ cells injected in the dorsal caudal vein.

2.7. TUNEL staining

Detection Kit of POD from (Roche Company) was used. At first, the samples were incubated in proteinase K for 15-30 minutes at 37˚C, for permeabilization of the samples used (0.1% Triton x-100, 0.1% sodium citrate). After that sections were incubated in main TUNEL solution containing (450 μl of label solution with 50 μl of enzyme solution) for 1 hour at 37˚C. Finally, after washing the samples were observed under a fluorescent microscope (×400). In negative control groups, sections incubated only with label solution instead of the TUNEL reaction mixture.

2.8. Cell culture

To produce mesenchymal stem cells, the tibias and femurs rat bone marrow were first removed under anesthesia. The cells incubated in culture media Dulbecco's Modified Eagle Medium (DMEM, 5ml, Invitrogen), 5% CO2 at 37˚C in an incubator that supplemented with fetal bovine serum (FBS, 10%, 1ml), Pen-strep (50 λ). After 72h, supernatant was removed and adherent cells cultured again to produce BMSCs (Badban et al., 2015). After the third passage, fat and fibroblast were removed and only the BMSCs were able to survive (Badban et al., 2015). Finally, $2\times10^6$ cells were used for injection after counting.

2.9. Statistical analysis

All data were reported as Mean±SEM. Statistical analysis was done by computer using SPSS, post hoc multiple comparison tests. The p-values of less than 0.05 were significant. For each tissue was analyzed by one-way ANOVA and followed by Tukey post hoc test.

3. Results

3.1. Behavioral examination

Results of behavioral test shown the grade of test after treatment were increased in all treatment groups ($F_{2,12} = 15.36$, $P \leq 0.05$; Figure 1). Post-treatment grade on the MPTP group was (2.4±0.2) that was the smallest amount among the entire groups. The highest degree was in the group of Pre CAPE+PD+Stem Cells therapy (4.6±1.2). In all groups, animals could hardly maintain their balance at the top of the rod. In groups using only stem cells or CAPE, the results were not significant compared to the Parkinson's group (2.9±0.6, vs 2.4±0.2) ($P \leq 0.01$). In groups using CAPE as a pre-treatment, the results were significant compared to the Parkinson's group (3.5±0.4 vs 2.4±0.2) ($P \leq 0.001$). All treatment groups were compared with the PD group. In all treatment groups except BMSCs, after treatment have a significant difference with the PD group ($P \leq 0.05$).
3.2. DiI staining

DiI labeling was carried out for evaluation of the site of injected cells. The results showed that the injected cells were replaced in different areas, but most of them were located in the affected areas (Fig. 2). The use of stem cells along with CAPE has led to an increase in the number of injected cells in the affected area. The average injected cells in the SNpc in the treatment groups that used CAPE in comparison to stem cells only were higher and significant (P ≤ 0.05).

3.3. TH-immunohistochemistry

TH-immunohistochemistry staining was done for the evaluation of the dopamine-secreting neurons. In the normal group, the number of TH positive neurons in the SNpc was significantly higher than in the striatum area (F5,24 = 55.08, 28± 2/3 vs F5,24 = 41.03, 23±1/9; P ≤ 0.001). Intranasal administration of MPTP significantly reduced the number of TH-positive cells in SNpc and striatum of all groups (P ≤ 0.001) (Figure 3 & 4). The highest number of neuronal reductions was seen in the Parkinson's group (11±1.94; P ≤ 0.001). This neuronal decline was seen in all groups, but the number of neurons in all therapeutic groups was higher than the PD group. The highest number of TH+ neurons was seen in the group of Pre CAPE+PD+Stem cells (SNpc: 23/40±1/14, striatum: 21±1/19; P ≤ 0.001) and pre-CAPE (SNpc: 23/2±1 striatum 19±1/1; P ≤ 0.001). In the groups that used stem cells with CAPE, the number of TH+ neurons were more than those using just stem cells (15/80±1/64; P ≤ 0.001). There was a significant difference in the number of TH+ cells in therapeutic groups that received pre-CAPE compared to stem cells only (P <0.001). In the treatment group that received only CAPE, the number of TH+ neurons were also higher than the group of stem cells only (15/1±1/13 vs 14±1/64; P ≤ 0.001). This can indicate that CAPE has a great protective role for tyrosine hydroxylase neurons against MPTP.

3.4. TUNEL staining

This protocol is used for the detection and quantification of apoptotic cells in SNpc and striatum. The intranasal administration of MPTP significantly increases the number of apoptotic cells in the SNpc and striatum (F5,24 = 62.75, SNpc: 24/8± 2/9 F5,24 = 44.43 striatum: 22/8± 3/1; P ≤ 0.001). Apoptotic cells were observed in all groups (Figure 5). The highest number of apoptotic cells was in the Parkinson's group (24/8±2/9; P ≤ 0.001) and the lowest number was in group Pre-CAPE+PD+Stem cell (SNpc: 13/40±1/14, striatum: 16/8±2/6; P ≤ 0.001). The results show that the use of CAPE as a pre-treatment will significantly reduce the number of apoptotic cells in the treatment groups (P ≤ 0.001). In other treatment groups, a decrease in the number of apoptotic cells was observed, but the results were not significant.

4. Discussion

Numerous studies have shown that the main cause of Parkinson's disease is the destruction of dopamine-secreting neurons in the substantia nigra. Dopamine plays a role as a neurotransmitter in motion, so motor disorders occur in Parkinson's disease (Braak et al., 2004). One of our goals in this study is to show, whether the CAPE as a power antioxidant can protect dopaminergic neurons against the MPTP neurotoxin. MPTP neurotoxin decreases dopaminergic neurons and creates behavioral and non-behavioral defects. The present study shows that the intranasal administration of MPTP causes a significant reduction of dopaminergic neurons in the substantia nigra. In the brain, MPTP is rapidly converted to MPP+ (1-methyl-4-phenylpyridinium) by the enzyme MAO-B, the majority in glial cells. This ion
does not cross the cytoplasmic membrane freely. This toxin has a strong tendency to bind to the dopamine transporter (DAT). Therefore, they are captured in dopaminergic terminals. In dopamine-secreting neurons, MPP+ cumulate in mitochondria and then inhibit complex I of the electron transport chain, and lead to reduce the level of ATP and increase the level of reactive oxygen species (ROS) particularly superoxide. ROS production appears to be one of the first incidents of MPP+ neural toxicity. Although it will not cause cell death independently, it can cause cell death by stimulating some of the mechanisms (Prediger et al., 2010). In this study, results showed that CAPE can protect neurons against MPTP. This neuroprotection is due to the strong antioxidant properties. Oxidative stress plays a role in the pathogenesis of Parkinson's disease. Therefore, CAPE probably protects neurons by removing free radicals (Song et al., 2012).

In a study conducted in 2007 (McGeer & McGeer, 2007) in the pathogenesis of Parkinson's disease, chronic inflammation occurs in the basal ganglia. In inflammatory conditions, activated glial cells produce large amounts of free radicals and toxins for neurons. Since the dopaminergic neurons are very sensitive to free radicals. Due to the release of the free radicals by active glial, dopaminergic neurons can be seriously damaged. CAPE has an anti-inflammatory effect, effectively contributing to the collection of free radicals and removing inflammatory agents and subsequently improve the disease. CAPE has a different mechanism of action, for example, CAPE prevents the formation of oxygen free radicals (Russo, Longo, & Vanella, 2002). Blocks MPTP-increase amount of midbrain iNOS and caspase 1. CAPE can block the MPTP-induced loss of striatal DA, it block MPP+ induced neurotoxicity, block of the MPP+ induced cytochrome C release (Fontanilla et al., 2011). In this study, CAPE could improve pathologic and behavioral symptoms in PD groups. This study showed that CAPE has better efficacy in pre-treatment. The other aim of this project is to use stem cells to transfer them to the damaged black body and replace them with damaged dopaminergic neurons. However, the simultaneous use of CAPE and stem cells can be used to protect stem cells using the different mechanisms mentioned. Stem cells have significant properties. Stem cells are proliferative cells that can be divided into a variety of cells and these cells also can self-renew (Elbana, Abdel-Salam, Morad, Ibrahim, & Omran, 2015). Stem cells play a role in the treatment of various diseases, including neurodegenerative diseases such as Parkinson's (Dantuma et al., 2010; Maltman, Hardy, & Przyborski, 2011). Stem cells can regulate immune responses (Zhou et al., 2014) and differentiate into special cells to replace injured cells (Zeng et al., 2011). By inhibiting apoptotic pathways, these cells create tropical factors to protect and repair cells (Jäger et al., 2010). We observed that the labeled cells have entered the midbrain and located in different areas. Most of the cells were located in the substantia nigra. The results indicate that these cells can cross the blood-brain barrier (Jadidi et al., 2016) and be located in damaged areas. It is not well-known that these cells can differentiate into other cells or produce dopamine. Probably, the secretion of chemical factors such as cytokines from damaged cells causes to drag the stem cells toward the affected area. Migrated cells under the influence of chemical factors differentiated into dopaminergic neurons and produce. Due to the above characteristics, stem cells will be able to replace the damaged dopaminergic neurons of the substantia nigra in the brainstem. The use of CAPE with these cells can play an important role in repairing the damaged area in Parkinson's disease.
5. Conclusion

The results of this study showed that the use of CAPE can protect dopamine-secreting neurons in the SNpc of the brain. This effect can be achieved by inhibiting inflammatory factors.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles were considered in this article

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Authors’ Contribution

All authors contributed equally in preparing all parts of the research.

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Figure 1. Improving behavioral pole test before and after treatment with CAPE.

The results of one-way ANOVA and Tukey post hoc test (n = 7) showed that behavioral improvement was significantly higher in pre-treatment and CAPE groups than other treatment groups. All values are (mean ± SEM). * compared with the MPTP (PD) group (p ≤ 0.001).
**Figure 2.** Migration of injected cells to the damaged area of the SNpc.

Arrows represent BMSCs ($2 \times 10^6$) that labeled with DiI in the substantia nigra of the mid brain in rats ($\times20$: Scale bar 400 µm, $\times40$: scale bar 20 µm). The results show that in the pre CAPE+ PD+stem cells treatment group, the number of stained cells with DiI is higher than other treatment groups. The images were at magnification of $\times20$ and $\times40$. 
Figure 3. CAPE increases the number of dopamine-positive cells in the SNpc.

The results of one-way ANOVA and Tukey post hoc test (n = 7) showed that the number of tyrosine hydroxylase positive neurons in the pretreatment treatment group increased significantly and decreased significantly in the Parkinson’s group compared to the other groups. All values are (mean ± SEM). * compared with the MPTP (PD) group (p ≤ 0.001).
Figure 4. CAPE and BMSCs increases the number of dopamine-positive cells in the SNpc.

Immunohistochemical images showed that using CAPE before PD could significantly protect dopamine secreting neurons. Also, in the groups that received CAPE or stem cells separately after PD, there was no significant difference in the number of positive dopamine secreting neurons. The squares in all the pictures show the border of the SNpc. Groups in figures included: A: PD group, B: PD plus Stem cell, C: Pre CAPE plus PD, D: Pre CAPE+PD+Stem cell, E: PD+ Stem cell+ CAPE, F: PD+CAPE. The images were at magnification of x20. The results were evaluated using the one-way ANOVA and the Tukey post hoc (n=7)
Figure 5. CAPE reduce the number of apoptotic cells in SNpc.

The results of one-way ANOVA and Tukey post hoc test (n = 7) The results showed that in the pretreatment groups with CAPE alone or together with stem cells, the number of apoptotic cells will be significantly reduced. In other treatment groups, although there is a decrease in apoptotic cells, but there is no significant difference with Parkinson's group. * compared with the MPTP (PD) group (p≤ 0.001).