Caffeic acid phenethyl ester decreases oxidative stress index in blunt spinal cord injury in rats

咖啡酸苯乙酯減少老鼠脊髓鈍性創傷的氧化壓力指數

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Objective: The aim of this study was to investigate the total oxidant status and total antioxidant status of caffeic acid phenethyl ester and methylprednisolone in blunt spinal cord injury in rats. ***Methods:*** Twenty-four adult Wistar albino rats were randomised into three groups. Spinal cord injury was performed by the weight-drop model. Group 1 underwent laminectomy followed by spinal cord injury and received no medication (control group). Group 2 underwent laminectomy followed by spinal cord injury and received caffeic acid phenethyl ester (10 μmol/kg) by intraperitoneal injection. Group 3 underwent laminectomy followed by spinal cord injury and received methylprednisolone (30 mg/kg) by intraperitoneal injection. Twenty-four hours later, all rats were sacrified and after that total oxidant status, total antioxidant status and oxidative stress index levels were determined in spinal cord tissues and the obtained results were compared. ***Results:*** The highest total antioxidant status level was observed in the caffeic acid phenethyl ester group and the highest total oxidant status level was observed in the control group. Oxidative stress index levels in the control group were statistically higher than the caffeic acid phenethyl ester and methylprednisolone groups ($p<0.01$). ***Conclusion:*** Based on our results, it is concluded that caffeic acid phenethyl ester might be a promising neuroprotective agent after spinal cord injury via its antioxidant effects. (*Hong Kong j.emerg.med.* 2010;17:250-255)

目的：本研究旨在調查老鼠脊髓鈍性創傷後，咖啡酸苯乙酯及甲基潑尼松龍的總氧化狀態及總抗氧化狀態。**方法：**將24隻成年的威斯特白化病老鼠隨機分為3組。以墜下重物模式製造脊髓創傷。第一組經椎板切除術後弄傷脊髓及不接受藥物（對照組）。第二組經椎板切除術後弄傷脊髓及在腹腔內注射咖啡酸苯乙酯（每公斤10微分子）。第三組經椎板切除術後弄傷脊髓及在腹腔內注射甲基潑尼松龍（每公斤30毫克）。24小時後，犧牲所有老鼠，然後在脊髓組織測定總氧化狀態，總抗氧化狀態及氧化壓力指數水平及比較所得的結果。**結果：**最高的總抗氧化狀態水平見於咖啡酸苯乙酯組及最高的總氧化狀態水平見於對照組。對照組的氧化壓力指數水平在統計學上高於咖啡酸苯乙酯組及甲基潑尼松龍組（p<0.01）。**結論：**基於本研究結果，推論咖啡酸苯乙酯藉其抗氧化作用，可能有希望成為脊髓受傷後的神經保護劑。

Keywords: Antioxidants, caffeic acids, oxidants, oxidative stress, spinal cord injuries

關鍵詞：抗氧化劑、咖啡酸、氧化劑、氧化壓力、脊髓創傷

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Introduction

Spinal cord injury (SCI) is still a major public health problem.[1] The pathophysiology of acute SCI involves a complex cascade of secondary neurodegenerative events including oxidative stress, initiated by the primary injury.[2,3]

Caffeic acid phenethyl ester (CAPE), one of the major components of honeybee propolis, was recently found to be a potent free radical scavenger and antioxidant, and is used in folk medicine.[4-6] CAPE inhibits 5-lipooxygenase-catalysed oxygenation of linoleic acid and arachidonic acid in the micromolar concentration range. At a concentration of 10 µM, it completely blocks production of reactive oxygen species (ROS) in human neutrophils and the xanthine/xanthine oxidase system. Previous studies have demonstrated that CAPE also exhibits antioxidant property as well as anti-inflammatory, cytostatic, antiviral, antibacterial and antifungal properties.[5-7]

The objective of our study was to investigate the effects of CAPE on total antioxidant status (TAS), total oxidant status (TOS), oxidative stress index (OSI) levels in blunt spinal cord injury in rats. We also aimed to compare the effects of CAPE with methylprednisolone (MP).

Methods

Twenty-four male Wistar albino rats (270-300 g) were used in the study. All the protocols were approved by the institutional animal ethics committee. The rats in each group were kept in separate cages in rooms with controlled light and temperature and were fed standard chow and water ad libitum. The animals were anesthetized by an intraperitoneal injection of 10 mg/kg xylazine (Rompun, Bayer Turk Kimya Sanayii Limited Sirketi, Istanbul, Turkey) and 50 mg/kg ketamine hydrochloride (Ketalar, Pfizer Ilacları Limited Sirketi, Istanbul, Turkey). Rats were positioned on a thermistor-controlled heating pad in the prone position and a rectal probe was inserted. Surgical procedures were performed under sterile conditions with the assistance of a surgical microscope. Following a T5-12 midline skin incision and paravertebral muscle dissection, the spinous processes and laminar arcs of T7-10 were removed. The dura was left intact. Weight-drop model was performed for SCI.[2,8] The animals were subjected to an impact of 50 g/cm to the dorsal surface of the spinal cord. The force was applied via a stainless steel rod (3-mm diameter tip, weighing 5 g) that was round at the surface. The rod was dropped vertically through a 10-cm guide tube that was positioned perpendicular to the centre of the spinal cord. Afterwards, the muscles and the incision were sutured with 6-0 polyglactin-910 sutures (Vicryl, Ethicon, Johnson & Johnson International, Lanneke Marelaan, Belgium).

The rats were randomised into three groups each having 8 rats. Caffeic acid phenethyl ester was synthesized in the physico-chemistry laboratory using the technique described by Grunberger to prepare 25 µmol/ml of CAPE solution.[9] Group 1 underwent laminectomy followed by SCI and received no medication (control group). Group 2 underwent laminectomy followed by SCI and received CAPE 10 µmol/kg by intraperitoneal injection.[4] Group 3 underwent laminectomy followed by SCI and received MP (Prednol-L, Mustafa Nevzat Ilac Sanayi Anonim Sirketi, Istanbul, Turkey) by intraperitoneal injection at a single dose of 30 mg/kg. Following the surgical procedure, the rats were placed in a warming chamber and their body temperatures were maintained at approximately 37°C until they were completely awake. In the early postoperative period, the rats received 3 ml of saline by intraperitoneal injection to compensate for the blood loss during the surgical procedure, while water intake was limited.

Biochemical analysis

All the rats were sacrificed for biochemical analysis at 24 hours after SCI. The exposed spinal cord segments and meninges were removed. The samples were immediately frozen and stored in a -20°C freezer for assays of TAS and TOS levels.

After homogenisation in phosphate buffer (in a ratio of 1/10, pH 7.4), tissue samples were centrifuged at

5000×g for 15 min at 4°C. The TAS of the samples was determined using a novel automated measurement method developed by Erel.[10] In this method, hydroxyl radical, which is the most potent biological radical, is produced. In the assay, ferrous ion solution which is present in Reagent 1, is mixed with hydrogen peroxide which is present in Reagent 2. The sequentially produced radicals such as brown-coloured dianisidinyl radical cation, produced by the hydroxyl radical, are also potent radicals. Using this method, the antioxidative effect of the sample against the potent free radical reaction, which is initiated by the produced hydroxyl radical, is measured. The protein content of the supernatants was determined using Lowry's method.[11] The results were expressed as nmol Trolox Eqv/mg protein. The TOS levels of the tissue samples were determined using a novel automated measurement method developed by Erel.[12] Tissue samples were homogenised in a ratio of 1/10 in phosphate buffer (pH 7.4) and centrifuged at 5000×g for 15 min at 4°C. The supernatant was carefully separated and used for the assay. Oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a coloured complex with xylenol orange in an acidic medium. The colour intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of nmol H_2O_2 Eqv/mg protein. The percent ratio of TOS to TAS was accepted as the oxidative stress index (OSI). The OSI value was calculated according to the following formula:[13]

OSI (arbitrary unit) = [TOS (μmol H_2O_2 Eqv/l) / TAS (μmol Trolox Eqv/l)] x 100.

Statistical analysis

For statistical evaluation, we used the software package SPSS 15.0 and probability value of less than 0.05 was accepted as statistically significant. As the data were normally distributed and independent, statistical analysis was performed using analysis of variance (ANOVA) followed by Tukey test when comparing groups. The results are given as the mean±standard deviation of the mean (SD).

Results

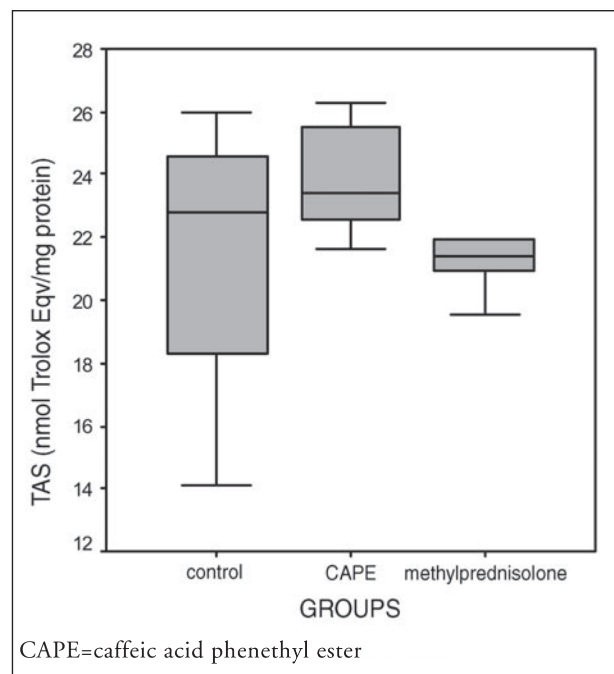
The highest TAS level was observed in the CAPE group (Figure 1) and the highest TOS level was observed in control group (Figure 2). OSI levels in the control group were statistically higher than the CAPE and MP groups ($p<0.01$) (Figure 3).

The TAS, TOS and OSI levels for all groups are shown as mean±SD in Table 1.

Discussion

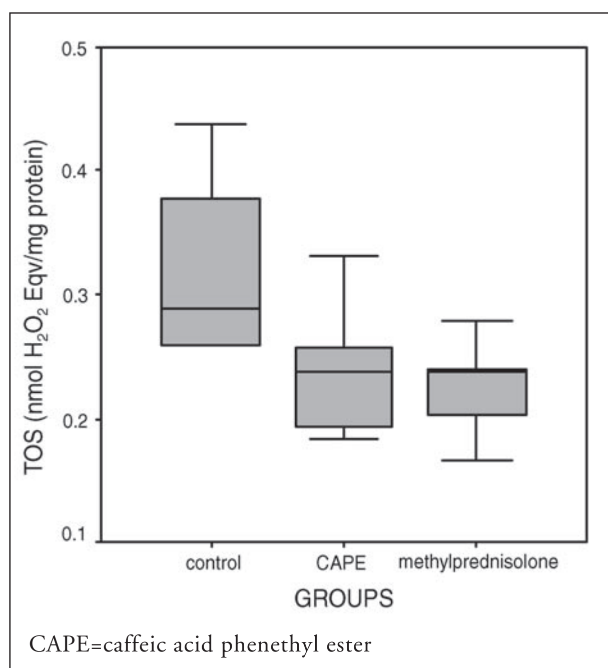
In our study, treatment of rats with CAPE (10 μmol/kg) and MP significantly decreased OSI in SCI.

Measurement of TAS provides information about the antioxidant capacity of the organism.[14] TOS reflects present oxidative status. The percent ratio of TOS to



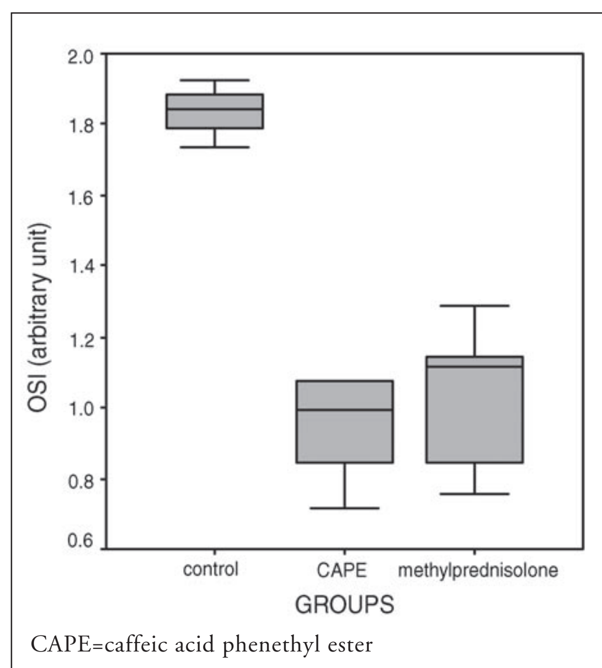
CAPE=caffeic acid phenethyl ester

Figure 1. Total antioxidant status (TAS) levels in different groups.



CAPE=caffeic acid phenethyl ester

Figure 2. Total oxidant status (TOS) levels in different groups.



CAPE=caffeic acid phenethyl ester

Figure 3. Oxidative stress index (OSI) levels in different groups.

Table 1. Total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI) levels (mean±SD)

	TAS (nmol Trolox Eqv/mg protein)	TOS (nmol H_2O_2 Eqv/mg protein)	OSI (arbitrary unit) *
Group 1 (control)	21.13±4.89	0.31±0.08	1.83±0.09
Group 2 (CAPE)	23.81±1.80	0.23±0.05	1.01±0.25
Group 3 (MP)	21.52±1.41	0.22±0.04	1.02±0.22

*P values: Group 1 and Group 2, p<0.001; Group 1 and Group 3, p<0.001
CAPE=caffeic acid phenethyl ester; MP=methyprednisolone.

TAS was accepted as the OSI as an indicator of the degree of oxidative stress.[15]

SCI is a very destructive process for both patients and society and no satisfactory treatment is available at present.[2] CAPE prevents lipid peroxidation induced by injury and has a potent antioxidant property and ROS-scavenging capabilities.[16]

In our study, alterations in the state of oxidative stress were determined by measuring the spinal cord tissue TAS, TOS and OSI. Oxidants and antioxidants have additive effects. Although the concentration of oxidant and antioxidant components can be measured individually, these measurements are time and cost consuming and require sophisticated systems. In addition, it may not accurately reflect the TAS and TOS.[17] Thus, we measured the total oxidants and antioxidants.

Spinal cord trauma results in rapid and extensive oxidative stress.[18] It has long been established that oxidative stress plays a critical role in the pathophysiology of spinal cord injury, and represents an important target of therapeutic intervention following the initial trauma.[19] Decreasing the level of oxidative stress will minimise secondary destruction after traumatic injury.[20] Although it is difficult to limit the primary injury, there is increasing evidence on the possibility of lowering the impact of secondary injury, using pharmacological strategies.[21]

Several studies have demonstrated that SCI initiates systemic inflammatory reactions, ROS and finally leads

to peroxidation. The underlying mechanism of this event is the deformation of cell membrane phospholipids by oxidizing radicals. Cell membranes are rich sources of polyunsaturated fatty acids (PUFAs). The oxidative destruction of PUFAs is known as lipid peroxidation which causes tissue injury. An intense affiliation has been demonstrated between the quantity of lipid peroxidation and the degree of SCI. ROS are produced constantly in cells with normal metabolism. On the other hand, cells are able to defend themselves from the destructive potential of oxygen radicals in normal physiologic condition by way of their own antioxidant mechanisms including enzyme systems, vitamins, elements, and some antioxidant molecules. There is an exquisite balance between production and destruction of ROS. When this equilibrium is destroyed, ROS are produced excessively and all tissues are exposed to oxidative injury.[22] Lipid peroxidation products increase immediately after SCI and the peak concentrations of reactive oxygen and nitrogen species occur within the first 24 hours.[2] Antioxidant defence mechanisms are important to prevent the potential harmful effects of oxidative stress.[2] Much experimental and clinical research have focused on oxidative stress mechanisms in an effort to improve neurological outcome following SCI.

In 1990 the results of the Second National Acute Spinal Cord Injury Study (NASCIS II) were published, which showed that the administration of a high-dose regimen of the glucocorticoid steroid MP, which had been previously shown to inhibit post-traumatic lipid peroxidation in animal models of SCI, could improve neurological recovery in spinal cord-injured humans. This resulted in this treatment quickly became the standard of care for acute SCI in many countries. In recent years, the use of high-dose MP in acute SCI has become controversial largely on the basis of the risk of serious adverse effects versus what is perceived to be on average a modest neurological benefit. Subsequently, it has been demonstrated that CAPE may be an available agent to protect the spinal cord from ischemia-reperfusion injury in rabbits.[5] Our results suggested that CAPE might have promising neuroprotective effects in spinal cord-injured humans that could either replace or be used in combination with high-dose MP. CAPE is an active component of honeybee propolis extracts and has been used in traditional medicine for many years. For this study we used CAPE synthesized by Dr Ilhan[5] from bees of our region. Recent studies have shown that CAPE has anti-inflammatory, antioxidant, immunomodulatory, antimitotic and anticarcinogenic properties.[23-28] Up to now, there is no documented harmful effects of CAPE on normal cells.[29] We hypothesized that CAPE would effectively protect spinal cord by its antioxidant effects on spinal cord injury. Our results demonstrate that CAPE will be able to reduce the damage to rat spinal cord injury. Although CAPE has potent antioxidant property, its effects on TOS, TAS and OSI in SCI have not been investigated to date. In our study, CAPE treatment in SCI decreased OSI compared to the control group, statistically. There were no statistical differences between the CAPE and MP groups.

All of these findings suggest that CAPE and MP provide neuroprotection against SCI through their antioxidant action.

References

1. Saunders LL, Selassie AW, Hill EG, Nicholas JS, Varma AK, Lackland DT, et al. Traumatic spinal cord injury mortality, 1981-1998. J Trauma 2009;66(1):184-90.
2. Yucel N, Cayli SR, Ates O, Karadag N, Firat S, Turkoz Y. Evaluation of the neuroprotective effects of citicoline after experimental spinal cord injury: improved behavioral and neuroanatomical recovery. Neurochem Res 2006;31(6):767-75.
3. Hall ED, Springer JE. Neuroprotection and acute spinal cord injury: a reappraisal. NeuroRx 2004;1(1):80-100.
4. Bilen BT, Kilinc H, Alaybeyoglu N, Celik M, Iraz M, Sezgin N, et al. Effect of caffeic acid phenethyl ester on survival of axial pattern flaps in rats with ischaemia-reperfusion injuries. Scand J Plast Reconstr Surg Hand Surg 2006;40(2):73-8.
5. Ilhan A, Koltuksuz U, Ozen S, Uz E, Ciralik H, Akyol O. The effects of caffeic acid phenethyl ester (CAPE) on spinal cord ischemia/reperfusion injury in rabbits. Eur J Cardiothorac Surg 1999;16(4):458-63.
6. Fidan H, Sahin O, Yavuz Y, Kilbas A, Cetinkaya Z, Ela Y, et al. Caffeic acid phenethyl ester reduces mortality and sepsis-induced lung injury in rats. Crit Care Med 2007;35(12):2822-9.
7. Armutcu F, Gurel A, Hosnuter M, Pabuccu O, Altnyazar C. Caffeic acid phenethyl ester improves oxidative erythrocyte damage in a rat model of thermal injury. J Burn Care Rehabil 2004;25(2):171-8.
8. Bilginer B, Onal MB, Narin F, Ustun H, Kilinc K,

Akalan N. Antiapoptotic and neuroprotective effects of mycophenolate mofetil after acute spinal cord injury in young rats. Childs Nerv Syst 2009;25(12):1555-61.
9. Grunberger D, Banerjee R, Eisinger K, Oltz EM, Efros L, Caldwell M, et al. Preferential cytotoxicity on tumor cells by caffeic acid phenethyl ester isolated from propolis. Experientia 1998;44(3):230-2.
10. Erel O. A novel automated method to measure total antioxidant response against potent free radical reactions. Clin Biochem 2004;37(2):112-9.
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193(1):265-75.
12. Erel O. A new automated colorimetric method for measuring total oxidant status. Clin Biochem 2005;38 (12):1103-11.
13. Harma M, Harma M, Erel O. Increased oxidative stress in patients with hydatidiform mole. Swiss Med Wkly 2003;133(41-42):563-6.
14. Rabus M, Demirbag R, Sezen Y, Konukoglu O, Yildiz A, Erel O, et al. Plasma and tissue oxidative stress index in patients with rheumatic and degenerative heart valve disease. Turk Kardiyol Dern Ars 2008;36(8):536-40.
15. Sirmatel O, Sert C, Sirmatel F, Selek S, Yokus B. Total antioxidant capacity, total oxidant status and oxidative stress index in the men exposed to 1.5 T static magnetic field. Gen Physiol Biophys 2007;26(2):86-90.
16. Ozeren M, Sucu N, Tamer L, Aytacoglu B, Bayri O, Dondas A, et al. Caffeic acid phenethyl ester (CAPE) supplemented St. Thomas' hospital cardioplegic solution improves the antioxidant defense system of rat myocardium during ischemia-reperfusion injury. Pharmacol Res 2005;52(3):258-63.
17. Horoz M, Bolukbas C, Bolukbas FF, Sabuncu T, Aslan M, Sarifakiogullari S, et al. Measurement of the total antioxidant response using a novel automated method in subjects with nonalcoholic steatohepatitis. BMC Gastroenterol 2005;5:35.
18. Xiong Y, Hall ED. Pharmacological evidence for a role of peroxynitrite in the pathophysiology of spinal cord injury. Exp Neurol 2009;216(1):105-14.
19. DeRisseau LR, Recca DM, Mogle JA, Zoccolillo M, DeRuisseau KC. Metallothionein deficiency leads to soleus muscle contractile dysfunction following acute spinal cord injury in mice. Am J Physiol Regul Integr Comp Physiol 2009;297(6):R1795-802.
20. Yazihan N, Uzuner K, Salman B, Vural M, Koken T, Arslantas A. Erythropoietin improves oxidative stress following spinal cord trauma in rats. Injury 2008;39 (12):1408-13.
21. Michael-Titus AT. Omega-3 fatty acids and neurological injury. Prostaglandins Leukot Essent Fatty Acids 2007;77(5-6):295-300.
22. Hosnuter M, Gurel A, Babuccu O, Armutcu F, Kargi E, Isikdemir A. The effect of CAPE on lipid peroxidation and nitric oxide levels in the plasma of rats following thermal injury. Burns 2004;30(2): 121-5.
23. Gurel A, Armutcu F, Hosnuter M, Unalacak M, Kargi E, Altinyazar C. Caffeic acid phenethyl ester improves oxidative organ damage in rat model of thermal trauma. Physiol Res 2004;53(6):675-82.
24. Ozer MK, Parlakpinar H, Vardi N, Cigremis Y, Ucar M, Acet A. Myocardial ischemia/reperfusion-induced oxidative renal damage in rats: protection by caffeic acid phenethyl ester (CAPE). Shock 2005;24(1):97-100.
25. Barber SC, Higginbottom A, Mead RJ, Barber S, Shaw PJ. An in vitro screening cascade to identify neuroprotective antioxidants in ALS. Free Radic Biol Med 2009;46(8):1127-38.
26. Mohamadin AM, Hammad LN, El-Bab MF, Abdel Gawad HS. Attenuation of oxidative stress in plasma and tissues of rats with experimentally induced hyperthyroidism by caffeic acid phenylethyl ester. Basic Clin Pharmacol Toxicol 2007;100(2):84-90.
27. Ozyurt H, Ozyurt B, Koca K, Ozgocmen S. Caffeic acid phenethyl ester (CAPE) protects rat skeletal muscle against ischemia-reperfusion-induced oxidative stress. Vascul Pharmacol 2007;47(2-3):108-12.
28. Koksel O, Kaplan MB, Ozdulger A, Tamer L, Degirmenci U, Cinel L, et al. Oleic acid-induced lung injury in rats and effects of caffeic acid phenethyl ester. Exp Lung Res 2005;31(5):483-96.
29. Ozyurt B, Ozyurt H, Akpolat N, Erdogan H, Sarsilmaz M. Oxidative stress in prefrontal cortex of rat exposed to MK-801 and protective effects of CAPE. Prog NeuroPsychopharmacol Biol Psychiatry 2007;31(4); 832-8.