Physiology and Pharmacology

Physiol Pharmacol 20 (2016) 12-23 www.phypha.ir/ppj

Original Article

Carbon nanotube-anandamide complex exhibits sustained protective effects in an *in vitro* model of stroke

Parichehr Hassanzadeh[1,2*], Elham Arbabi[3], Fatemeh Atyabi[1,4], Rassoul Dinarvand[1,4]

1. Nanotechnology Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
2. Neurological Research Center, Tehran University of Medical Sciences, Tehran, Iran
3. Research Center for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran
4. Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences, Teheran, Iran

Abstract

Introduction: The therapeutic potential of anandamide (AEA) for the neurological disorders may be negatively affected by its short half-life or poor solubility. The superior properties of carbon nanotubes (CNTs) for controlled drug delivery, prompted us to design AEA-CNTs complex and assess its effect in in vitro model of ischemic stroke.

Methods: In this experimental study, a multi-walled CNTs (MWCNTs)-AEA complex was prepared using amino-functionalized COOH-MWCNTs and characterized by Fourier transform infrared spectroscopy and transmission electron microscopy. PC12 cells in the presence of AEA (0.5, 1, 2 µg/ml), acid- or amine-modified MWCNTs, or MWCNTs-AEA complex (2, 5, 8 µg/ml) were exposed to 1 and 3 h oxygen-glucose deprivation (OGD) followed by 24 h re-oxygenation. In vitro cytotoxicity and oxidative stress were evaluated using three-way ANOVA.

Results: AEA immobilization on the aminated MWCNTs was confirmed. OGD significantly reduced cell viability (P<0.001). After 3 h of OGD induction, COOH-MWCNTs showed higher cytotoxicity than other MWCNTs (P<0.05, P<0.01, P<0.001) and MWCNTs-AEA was more protective than AEA alone (P<0.05, P<0.01). OGD increased malondialdehyde (MDA) and decreased glutathione (GSH) and superoxide dismutase (SOD) (P<0.001). Following 1-h OGD, AEA dose-dependently reduced MDA (P<0.001), and elevated GSH (P<0.05, P<0.01) and SOD (P<0.05, P<0.01), but AEA was ineffective following 3-h OGD (P>0.05). MWCNTs-AEA complex was effective at both time points (MDA and GSH: P<0.01, P<0.001, SOD: P<0.05, P<0.01, P<0.001). This nanostructure was more effective than AEA following longer exposure periods to OGD insult (P<0.05, P<0.01, P<0.001).

Conclusion: Aminated MWCNTs are suitable carriers for AEA and provide longer-lasting effects against OGD insult.

Keywords:
Anandamide;
Carbon nanotubes;
Ischemic stroke;
Oxidative stress

Received:
3 Feb 2016
Accepted:
6 Apr 2016

***Correspondence to:**
P. Hassanzade

Tel: +98-21 66959095
Fax: +98-21 66581558

Email:
p-hassanzadeh@razi.tums.ac.ir

Introduction

Ischemic stroke is a neurological deficit which occurs following the reduced cerebral blood flow in a major cerebral artery due to the transient or permanent occlusion by the embolus or thrombosis. It is a leading cause of disability and death which affects up to 0.2% of the people in the developed countries per

year (Klijn and Hankey, 2003). Activation of a cascade of complex molecular events including oxidative stress with well-established markers such as malondialdehyde (MDA), inflammation, mitochondrial dysfunction, intracellular calcium accumulation, and excessive release of excitatory amino acids usually result in the neuronal cell death (Draper and Hadley, 1990; Moskowitz et al., 2010; Serteser et al., 2002; Xu et al., 2005). Following the ischemic damage, brain is the most susceptible organ to the oxidative stress and subsequent injuries (Chen et al., 2011). Recombinant tissue plasminogen activator (r-tPA) is the only medication for improvement of functional outcome in patients, however, it should be administered within a short period of time after the onset of symptoms. The limited efficacy of current treatment strategies against the ischemic stroke necessitates the development of novel treatment options. During the last decades, the endocannabinoid system, a group of neuromodulatory lipids and their receptors which regulate the neuronal proliferation and maturation (Ferna´ndez-Ruiz et al., 2000), synaptic plasticity, neurotensin neurotransmission (Hassanzadeh and Rostami, 2014; Hassanzadeh and Arbabi, 2012), and neurotrophin signalling (Williams et al., 2003), has emerged as a topic of great interest in neuroscience. The endocannabinoids, 2-arachidonoylglycerol and arachidonoylethanolamide (anandamide, AEA), are produced on-demand by the lipid precursors in the neuronal cell membrane and activate two types of G protein-coupled receptors, cannabinoid CB_1 and CB_2, leading to a variety of physiological and pathological processes. This ubiquitous signalling system exerts the neuromodulatory actions in various types of diseases and is implicated in the mechanisms of action of a wide variety of psychotropic agents (Hassanzadeh and Rahimpour, 2011; Hassanzadeh et al., 2016). In this respect, development of cannabinoid receptor agonists, selective inhibitors of endocannabinoid degradation, and AEA uptake blockers has attracted growing research interests (Boger et al., 2000; Casanova et al., 2003). In transsynaptic neuronal changes due to the neurodegenerative processes or nitric oxide-induced cytotoxicity, the endocannabinoid system has shown beneficial effects (van der Stelt and Di Marzo, 2005). Based on the protective effects of endocannabinoid system against neuronal insult and excitotoxic damage (van der Stelt and Di Marzo, 2005), this ubiquitous signalling system may be considered as an emerging target for therapeutic interventions in neurological disorders. As previously reported, the non-psychoactive component of cannabis, cannabidiol, inhibits the voltage-sensitive Ca^{2+} channels leading to the reduction of excitotoxicity. Cannabidiol has shown neuroprotective effects even 6 h after the cerebral ischemia (Iuvone et al., 2004). Furthermore, cannabidiol preserves the regional cerebral blood flow, reduces the number of glial fibrillary acidic protein-positive cells, and improves the motor coordination after the cerebral ischemia (Hayakawa et al., 2008) indicating the protective effects of this cannabinoid in the post-ischemic cerebrovascular events. Another cannabinoid, dexanabinol, has also shown therapeutic potential in the experimental model of focal cerebral ischemia (Lavie et al., 2001). In recent years, the broad-spectrum therapeutic effects of AEA, the first recognized endogenous ligand of cannabinoid receptors (Devane et al., 1992), have been the focus of intense research. This endoacannabinoid by activating cannabinoid receptors exerts a wide variety of effects. There are numerous reports showing the therapeutic potentials of AEA against the inflammatory conditions, pain, epilepsy, mental disorders, ischemic injury, and neurotoxicity (Eljaschewitsch et al., 2006; Walker et al., 2002; Wallace et al., 2002; Gobbi et al., 2005; Giuffrida et al., 2004; Sinor et al., 2000; Milton, 2002; van der Stelt et al., 2001). Moreover, AEA has shown anticancer effects by inhibition of the nuclear factor-κB which is implicated in the cell proliferation and cancer development (Sancho et al., 2003; Adinolfi et al., 2013). The protective effects of AEA against the ischemic neuronal damage have also been well documented (van der Stelt et al., 2001; Nagayama et al., 1999), however, poor aqueous solubility and a very short half-life of AEA due to the action of fatty acid amide hydrolase (Jarho et al., 1996) necessitates repeated administration of AEA which may negatively affect the effectiveness of AEA and elevate the side effects. Over the last decade, outstanding breakthroughs in modelling approaches and nanotechnology have led to the development of novel treatment strategies (Hassanzadeh, 2013; Hassanzadeh, 2014). Because of the multiple disadvantages associated with nanoparticulate

delivery systems (Zhang and Uludağ, 2009), the research efforts have been moving towards the development of advanced nanovectors for the delivery of compounds with short half-life or poor solubility. In this respect, carbon nanotubes (CNTs), the nanostructures with immense potential in various scientific fields, have been the focus of intense research for the protection or targeted delivery of a wide variety of compounds. The superior mechanical properties, improved biocompatibility and solubility, and high thermoelectrical conductivities have made CNTs as attractive theranostic candidates in nanomedicine (Cellot et al., 2009; Kam and Dai, 2005). CNTs may be used for biosensing, non-invasive and high-resolution imaging, regenerative medicine, and tissue engineering (Mohammadi et al., 2009; Fabbro et al., 2012). They may also be used as the nanoreserviors for controlled release of growth factors or drugs (Son et al., 2006; Bhirde et al., 2009) that may be of therapeutic value. These nanostructures modulate the synaptic plasticity and enhance neurite outgrowth (Cellot et al., 2011). Meanwhile, in order to improve the biocompatibility, solubility, and bioactivity of CNTs, they should be functionalized (Sun et al., 2002). Functionalized CNTs have been shown to promote axonal regeneration, improve the hind-limb locomotor recovery, and reduce the lesion volume in the spinal cord injury (Roman et al., 2011). The effectiveness of functionalized CNTs in the glioblastoma and stroke has also been reported (Zhao et al., 2011; Lee et al., 2011). Based on this background, we aimed to investigate; i) the suitability of functionalized CNTs-AEA complex for providing a longer-lasting effect of this cannabinoid, and ii) the therapeutic potential of CNTs-AEA complex in an *in vitro* model of cerebral stroke.

Materials and methods

Preparation of amino-functionalized MWCNTs-anandamide complex

As previously reported, amino functionalization of CNTs improves their dispersibility and reduces the toxicity (Lee et al., 2011). Meanwhile, we initially used COOH-MWCNTs instead of the direct aminization of MWCNTs, because the carboxylation of CNTs prior to the aminization increases the reactivity of CNTs and facilitates further aminization (Hamdi et al., 2015). MWCNTs were amine-functionalized as previously described (Chen et al., 2014; Hamdi et al., 2015) with some modifications. Briefly, 500 mg of COOH-functionalized MWCNTs (Plasmachem GmbH, Berlin, Germany) and 50 ml of 98% thionyl chloride ($SOCl_2$, Sigma Aldrich, Germany) were sonicated using ultrasonic system (*Tecna* 6, *Tecno-Gaz,* Italy) at 70% amplitude for 40 min and stirred using a magnetic stirrer (IKA, Germany) at 25 °C for 48 h. Then, the suspension was filtered with 0.45 µm pore-sized microporous membrane (Sartorius, *Germany*), washed with tetrahydrofuran for 5 times to remove the excess $SOCl_2$, and vacuumed for 25 min at 25 °C. The residue (MWCNTs-COCl) was reacted with 50 ml of ethylenediamine (EDA) (Sigma Aldrich, Germany) and stirred for 10 h. The suspension was filtered, washed with tetrahydrofuran for 5 times, vacuumed for 25 min, dialyzed in the deionized distilled water using a dialysis bag (MW cut-off 14 KD) for 72 h, and vacuumed for obtaining amine-modified MWCNTs. In order to prepare aminated MWCNTs-AEA complex, AEA (N-arachidonoyl-ethanolamine, Tocris Bioscience, UK) was dissolved in Tween 80 (Sigma-Aldrich, Germany), 98% ethanol, and phosphate-buffered saline (PBS) (1:2:18 v/v). Then, AEA (50 µM) was added to the mixture of aminated MWCNTs and PBS (0.25% w/v), stirred for 24 h at 25 °C, and centrifuged by *sigma-3k30 centrifuge* (*Sigma*, *Germany*) at 10,000 rpm for 20 min. Following the removal of supernatant, the precipitate was washed with PBS, re-centrifuged at 10,000 rpm for 20 min, and dispersed in 10 ml of PBS.

Characterization of MWCNTs

Fourier transform infrared (FTIR) spectroscopy which is a powerful tool for the comprehensive characterization of the chemical structures of MWCNTs, was performed using the FTIR spectrophotometer (Shimadzu, Japan). The morphologies of MWCNTs were evaluated by transmission electron microscopy (Philips CM12 TEM).

Cell culture

Rat *pheochromocytoma*-derived *cell* line *PC12* (Pasteur Institute, Tehran, Iran) were grown in

Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum, penicillin G sodium (100 U/ml), streptomycin sulfate (100 µg/ml), and 0.25 µg/ml of amphotericin B (all from Gibco) in a humidified atmosphere of 5% CO_2 at 37 °C (Wang et al., 2011). The culture media were changed every day and the experiments were carried out 72 h after seeding cells with appropriate densities.

In vitro model of ischemic stroke

Oxygen-glucose deprivation (OGD) is a widely-used *in vitro* model for stroke to investigate the molecular and cellular mechanisms underlying the brain ischemia or develop novel neuroprotective agents (Mehta et al., 2007). In this experimental study, PC12 cells were subjected to OGD injury as previously described in detail (Larsen et al., 2007). Briefly, the original culture medium was replaced with pre-warmed glucose-free Hepes buffer {in mM: Hepes (10), NaCl (150), KCl (5), $MgCl_2$ (1), $CaCl_2$ (2), pH 7.4} and antibiotic-antimycotic solution. Afterwards, the cells were transferred into the anaerobic chamber and flushed with 5% CO_2 and 95% N_2 for 1 and 3 h at 37 °C. OGD was terminated by switching back to the normal culture conditions and re-oxygenation for 24 h. Either AEA (0.5, 1, and 2 µg/ml) (Sancho et al., 2003; van der Stelt et al., 2001; Eljaschewitsch et al., 2006) or MWCNTs (2, 5, and 8 µg/ml) (Cellot et al., 2009; Hassanzadeh et al., 2015; Lee et al., 2011; Matsumoto et al., 2010) were added to cell cultures 1 h before and upon exposure to OGD. Groups receiving the equal volume of vehicle and without the OGD exposure served as controls.

Cytotoxicity assay

The viability of PC12 cells exposed to the various concentrations of MWCNTs and AEA was evaluated using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma Aldrich, Germany) colorimetric assay (Mosmann, 1983). In this respect, 20 µl of MTT stock solution (5 mg/ml) was added to each well (10^4 cells/well) 4h prior to the completion of re-oxygenation period. Afterwards, the culture medium was carefully aspirated and 100 µl of dimethyl sulfoxide was added to each well in order to dissolve the formazan crystals. The plate was subjected to low-speed oscillation for 10 min to completely dissolve the crystals and absorbance at 570 nm was measured using a microplate reader (Anthos 2020, Anthos Labtec Instruments, Austria). The cell viability was expressed as the percentage relative to the untreated control cells assuming the survival rate of 100% and presented as the mean ± SEM of six independent experiments (n=6).

Assessment of malondialdehyde (MDA) and reduced glutathione (GSH) contents and superoxide dismutase (SOD) activity

PC12 cells (5×10^5) were homogenized and centrifuged at 2000×g for 15 min at 4 °C and the supernatant was stored at -20 °C for further analysis by corresponding kits (Sigma Aldrich, Germany). MDA, the end product of lipid peroxidation, reacts with thiobarbituric acid (TBA) leading to the formation of a pink-coloured chromophore. MDA content was determined at 530 nm and expressed as nM/mg protein (Ohkawa et al., 1979). Protein levels were determined by the Bradford method (Bradford, 1976). Determination of GSH content is based on the reaction between GSH and 5,5'-dithiobis(2-nitrobenzoic acid) leading to the formation of a yellow-coloured product with absorbance at 412 nm (Jollow et al., 1974) and expressed as nM/mg protein. SOD activity was assessed based on the extent of the inhibition of amino blue tetrazolium formazan formation in the mixture of nicotinamide adenine dinucleotide, phenazine methosulphate, and nitroblue tetrazolium. The colour intensity was measured at 560 nm and the quantityof enzyme which caused 50% inhibition of nitroblue tetrazolium reduction/mg protein was considered as one unit of enzyme activity (Kakkar et al., 1984).

Statistical analysis

Three-way ANOVA followed by Tukey's post hoc test was used for data analysis. Data are presented as mean ± SEM. The level of significance was set at $P<0.05$.

Results

Characterization of MWCNTs

In acid-functionalized MWCNTs, FTIR spectroscopy

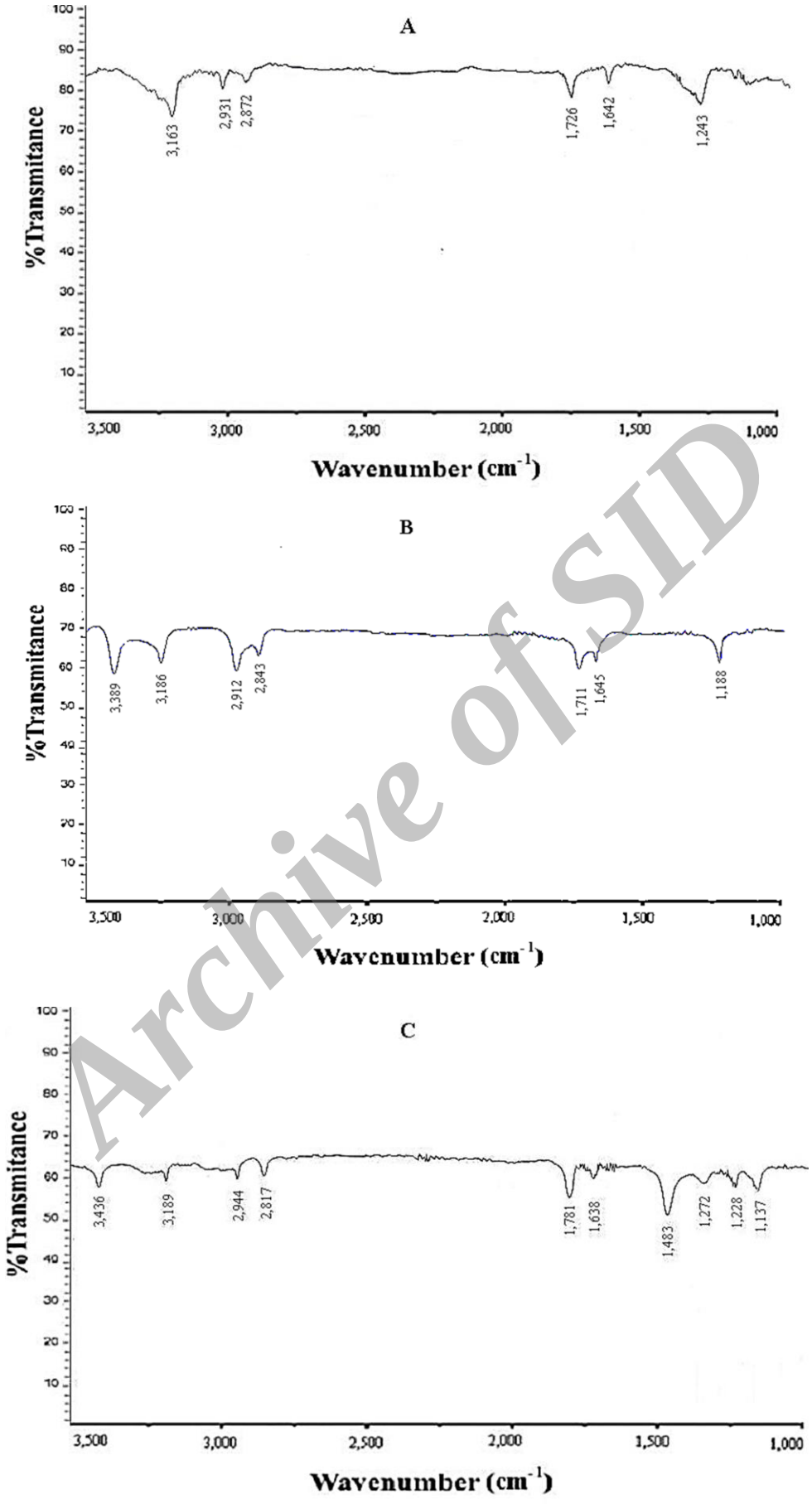

Fig.1. FTIR spectra of A: COOH-MWCNTs, B: EDA-MWCNTs, and C: EDA-MWCNTs-AEA complex. (MWCNTs: multi-walled carbon nanotubes, EDA: ethylenediamine, AEA: anandamide)

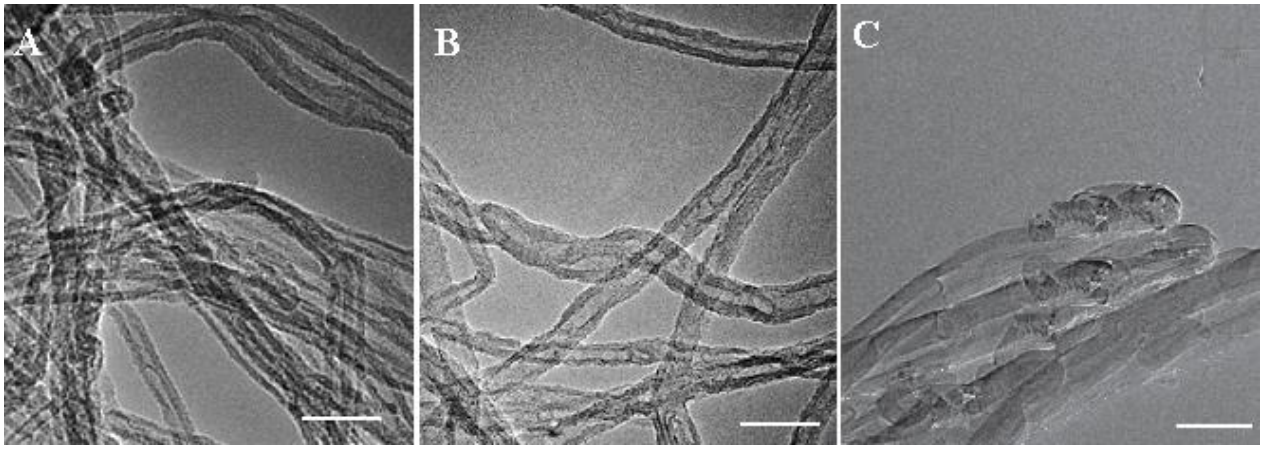

Fig.2. TEM images of MWCNTs. A: COOH-MWCNTs, B: EDA-MWCNTs, and C: EDA-MWCNTs-AEA complex (scale bars: 50 nm).
(MWCNTs: multi-walled carbon nanotubes, EDA: ethylenediamine, AEA: anandamide)

detected the carboxyl groups at 3163 and 1726 cm $^{-1}$ and C-H stretching at 2931 and 2872 cm $^{-1}$ (Fig. 1, curve A). Peaks at 1642 and 1243 cm $^{-1}$ corresponded to C=O and C-O stretching, respectively (Fig. 1, curve A). After EDA treatment, two peaks at 3389 and 3186 cm $^{-1}$ were observed which corresponded to N-H stretching (Fig. 1, curve B). Peaks at 2912 and 2843 cm $^{-1}$ corresponded to C-H stretching and peaks at 1711 and 1645 cm $^{-1}$ attributed to C=O stretching due to the formation of amide linkage (Fig. 1, curve B). The peak at 1188 cm $^{-1}$ corresponded to C-N stretching of amide group (Fig. 1, curve B). AEA immobilization effect on amine-modified MWCNTs has been shown in the curve C (Fig. 1) with characteristic peaks at 1483, 1272, and 1228 cm $^{-1}$. Other peaks were also observed in the curve C including those at 3436 and 3189 cm $^{-1}$ (N-H stretching), 2944 and 2817 cm $^{-1}$ (C-H stretching), 1781 and 1638 cm $^{-1}$ (C=O stretching), and 1137 cm $^{-1}$ (C-N stretching).

The morphology of MWCNTs

Based on transmission electron microscopy (TEM) evaluations, MWCNTs-AEA complex (Fig. 2C) was thicker and rougher than acid- or amino-functionalized MWCNTs (Figs 2A and 2B, respectively) and no visible damages was observed in the *structure* of MWCNTs.

MTT assay

A significant decrease in cell viability was observed in OGD and OGD+vehicle groups (P<0.001 *vs*. control). After 3 h of OGD induction, EDA-MWCNTs and EDA-MWCNTs-AEA complex showed less cytotoxicity than COOH-MWCNTs (P<0.05, P<0.01, and P<0.001, Table 1). EDA-MWCNTs-AEA complex increased cell viability for a longer time period compared with AEA solution (P<0.05 and P<0.01, Table 1). OGD-induced reduction of cell viability was not affected by the lowest dose of AEA solution or EDA-MWCNTs-AEA complex (P>0.05).

The effects of anandamide solution and various types of MWCNTs on MDA and GSH contents and SOD activity in PC12 cells exposed to OGD for 1 and 3 h

As shown in Table 2, OGD resulted in a significant enhancement of MDA level and reduced GSH content and SOD activity (P<0.001 *vs*. control). Following 1 h of OGD induction, AEA solution reduced MDA (P<0.001 *vs*. OGD and OGD+vehicle groups), and elevated GSH content (P<0.05, P<0.01) and SOD activity (P<0.05, P<0.01) in a dose-dependent manner, however, AEA was ineffective following 3 h of OGD induction (P>0.05). EDA-MWCNTs-AEA complex showed dose-dependent effects against OGD insult at both time points (MDA: P<0.01, P<0.001; GSH: P<0.01, P<0.001, SOD: P<0.05, P<0.01, P<0.001 *vs*. OGD and OGD+vehicle groups). This nanostructure was significantly more effective than AEA alone following longer exposure to OGD (P<0.05, P<0.01, P<0.001). No significant effect

Table 1: Cytotoxicity assays in PC12 cell cultures.

Groups	Cell viability after 1h of OGD (% of control)	Cell viability after 3h of OGD (% of control)
Control	100	100
OGD+ vehicle	77.93±4.29***	65.81±4.23***
OGD	78.82±3.07***	63.54±3.51***
AEA solution:		
0.5 µg/ml +OGD	77.83±4.01***	71.83±4.64*
1 µg/ml + OGD	93.51±3.31ϒ	68.50±3.91**
2 µg/ml + OGD	95.13±2.29 ʎ	67.36±2.39**
COOH-MWCNTs:		
2 µg/ml + OGD	71.53±6.88 *	58.83±3.07***
5 µg/ml + OGD	74.81±6.02 **	55.67±3.38***
8 µg/ml + OGD	70.33±4.43 **	52.33±4.58***
EDA-MWCNTs:		
2 µg/ml + OGD	79.53±5.34*	64.33±3.61***
5 µg/ml + OGD	76.33±6.69*	72.84±3.85¥
8 µg/ml + OGD	80.17±5.28*	76.67±3.87Σ
EDA-MWCNTs-AEA complex:		
2 µg/ml + OGD	78.51±5.42Ï	78.47±5.43Θ
5 µg/ml + OGD	93.83±3.29∞	81.98±4.15$
8 µg/ml + OGD	94.54±3.0 β	84.08±4.2 ^

EDA-MWCNTs-AEA complex elevates cell viability for longer time period. Data are presented as mean ± SEM (n=6). *P<0.05, **P<0.01, ***P<0.001 vs. control; ϒP<0.05, ʎP<0.01 vs. OGD and OGD+ vehicle; Ï P<0.01 vs. AEA (2 µg/ml +OGD); ∞P<0.05 vs. OGD, OGD+ vehicle, and AEA (0.5 µg/ml +OGD), βP<0.05 vs. OGD and OGD+ vehicle; ¥P<0.001 vs. control, P<0.05 vs. COOH-MWCNTs (5 µg/ml + OGD),and P<0.01 vs. COOH-MWCNTs (8 µg/ml + OGD); ΣP<0.001 vs. control, P<0.05 vs. COOH-MWCNTs (2 µg/ml + OGD), P<0.01 vs. COOH-MWCNTs (5 µg/ml + OGD), and P<0.001 vs. COOH-MWCNTs (8 µg/ml + OGD); ΘP<0.01 vs. control, P<0.05 vs. COOH-MWCNTs (2 µg/ml + OGD), and P<0.01 vs. COOH-MWCNTs (5 and 8 µg/ml + OGD),$$P$<0.05 vs. control, OGD and OGD+ vehicle, P<0.01 vs. COOH-MWCNTs (2 and 5 µg/ml + OGD), P<0.001 vs. COOH-MWCNTs (8 µg/ml + OGD), and P<0.05 vs. AEA (1 and 2 µg/ml +OGD); ^P<0.01 vs. OGD, OGD+ vehicle, COOH-MWCNTs (2 µg/ml + OGD), P<0.001 vs. COOH-MWCNTs (5 and 8 µg/ml + OGD), and P<0.05 vs. AEA (1 and 2 µg/ml +OGD).
(MWCNTs: multi-walled carbon nanotubes, EDA: ethylenediamine, AEA: anandamide)

was observed in cultures receiving COOH-MWCNTs or EDA-MWCNTs (P>0.05 vs. OGD and OGD+vehicle groups).

Discussion

Ischemic stroke following the loss of blood supply to the brain is one the major causes of mortality and morbidity worldwide (Klijn and Hankey, 2003) and the consequent activation of a cascade of events, including the inflammation, oxidative stress, and mitochondrial dysfunction usually results in neuronal death (Moskowitz et al., 2010; Serteser et al., 2002; Xu et al., 2005). The limited efficacy of current treatment strategies (Green and Shuaib, 2006) has provoked considerable research efforts to design more efficient therapeutic agents. Over the last decade, the pivotal role of endocannabinoid signaling in the formation of neuronal networks and survival signalling pathways has been well-documented (Pacher et al., 2006). This ubiquitous signalling system exerts neuromodulatory actions in various types of diseases (Di Marzo et al., 1998; McCormick and Contreras, 2001) and plays a protective role

Table 2: The effects of anandamide and various types of MWCNTs on MDA and GSH contents and SOD activity in PC12 cells exposed to OGD for 1 and 3 h

Groups	MDA (nM/mg protein)		GSH (nM/mg protein)		SOD (U/mg protein)	
	1h	3h	1h	3h	1h	3h
Control	0.63±0.04	0.61±0.07	9.33±0.49	9.48±0.62	43.12±3.46	41.73±2.82
OGD	1.72±0.17***	2.13±0.23***	5.17±0.31***	4.25±0.41***	28.33±1.99***	21.38±2.40***
OGD+ vehicle	1.68±0.09***	2.18±0.19***	5.33±0.61***	4.07±0.46***	28.47±2.09***	20.75±1.89***
AEA solution:	1.53±0.15***	2.03±0.18***	5.61±0.59$^{\Sigma}$	4.42±0.36***	25.83±1.87***	19.82±1.91***
0.5 µg/ml + OGD	0.98±0.08$^{£}$	1.85±0.22***	7.96±0.54^{3}	4.21±0.42***	37.91±1.79$^{\lambda}$	21.53±1.71***
1 µg/ml + OGD	0.78±0.06$^{¥}$	1.96±0.17***	8.46±0.77$^{\Theta}$	4.61±0.37***	39.86±2.30$^{\Upsilon}$	21.65±2.05***
2 µg/ml + OGD	1.85±0.08***	2.07±0.15***	5.02±0.51***	4.45±0.57***	26.92±2.33***	18.58±1.73***
COOH-MWCNTs:	1.66±0.15***	2.18±0.22***	5.35±0.56***	4.17±0.21***	22.15±1.99***	17.50±1.45***
2 µg/ml + OGD	1.73±0.18***	2.35±0.28***	4.97±0.41***	4.58±0.35***	20.67±2.40***	16.33±2.03***
5 µg/ml + OGD	1.73±0.09***	2.21±0.18***	5.88±0.66***	4.75±0.48***	28.17±2.14***	17.28±1.57***
8 µg/ml + OGD	1.68±0.17***	2.07±0.19***	5.67±0.54***	4.88±0.37***	25.67±1.43***	18.65±2.36***
EDA-MWCNTs:	1.77±0.23***	2.11±0.28***	5.83±0.33***	5.18±0.64***	26.32±1.94***	19.32±1.83***
2 µg/ml + OGD	1.48±0.14$^{\$}$	1.73±0.16***	6.03±0.43$^{\infty}$	4.13±0.31***	26.32±1.65$^{\neq}$	21.05±1.72***
5 µg/ml + OGD	1.02±0.08$^{\wedge}$	1.07±0.16^{X}	8.08±0.59$^{\beta}$	8.28±0.64$^{\varepsilon}$	39.17±2.46§	30.17±1.85‡
8 µg/ml + OGD	0.74±0.05$^{@}$	0.93±0.08$^{\Delta}$	8.92±0.78$^{\phi}$	8.96±0.53$^{\Upsilon}$	40.38±2.05†	33.50±2.14$^{\Omega}$
EDA-MWCNTs-AEA complex:						
2 µg/ml + OGD						
5 µg/ml + OGD						
8 µg/ml + OGD						

Each value represents the mean ± SEM of six independent experiments. $^{***}P$<0.001 vs. control; $^{£, ¥}P$<0.001 vs. OGD and OGD+vehicle; $^{\$}P$<0.05 vs. AEA (1 µg/ml + OGD), P<0.001 vs. AEA (2 µg/ml + OGD), and P<0.001 vs. control; $^{\wedge}P$<0.01 vs. OGD and OGD +vehicle, and P<0.05 vs. AEA (0.5 µg/ml + OGD); $^{@}P$<0.001 vs. OGD, OGD +vehicle, and AEA (0.5 µg/ml + OGD); ^{X}P<0.01 vs. OGD, OGD+ vehicle, AEA (0.5 µg/ml + OGD), and AEA (2 µg/ml + OGD), and P<0.05 vs. AEA (1 µg/ml + OGD); $^{\Delta}P$<0.001 vs. OGD, OGD+ vehicle, and AEA (0.5 µg/ml + OGD), P<0.01 vs. AEA (1 µg/ml + OGD) and AEA (2 µg/ml + OGD); $^{\Sigma}P$<0.01 vs. control; ^{3}P<0.05, $^{\Theta}P$<0.01 vs. OGD and OGD +vehicle; $^{\infty}P$<0.01 vs. control; $^{\beta}P$<0.01 vs. OGD and OGD+ vehicle; $^{\phi}P$<0.001 vs. OGD, P<0.01 vs. OGD+ vehicle, P<0.01 vs. AEA (0.5 µg/ml + OGD); $^{\varepsilon}P$<0.001 vs. OGD, OGD+ vehicle, and AEA (0.5, 1, and 2 µg/ml + OGD); $^{\Upsilon}P$<0.001 vs. OGD, OGD+ vehicle, and AEA (0.5, 1, and 2 µg/ml + OGD); $^{\lambda}P$<0.05, $^{\Upsilon}P$<0.01 vs. OGD and OGD+ vehicle; $^{\neq}P$<0.01 vs. control and AEA (2 µg/ml + OGD), and P<0.05 vs. AEA (1µg/ml + OGD); $^{\S}P$<0.05 vs. OGD, OGD+ vehicle, and P<0.001 vs. AEA (0.5 µg/ml + OGD); $^{\dagger}P$<0.01 vs. OGD, OGD+ vehicle, and P<0.001 vs. AEA (0.5 µg/ml + OGD); $^{\ddagger}P$<0.01 vs. control, P<0.05 vs. OGD and OGD+ vehicle, P<0.01 vs. AEA (0.5 µg/ml + OGD), P<0.05 vs. AEA (1 and 2 µg/ml + OGD); $^{\Omega}P$<0.001 vs. OGD, OGD+ vehicle, and AEA (0.5 µg/ml + OGD), and P<0.01 vs. AEA (1 and 2 µg/ml + OGD).

(MWCNTs: multi-walled carbon nanotubes, EDA: ethylenediamine, AEA: anandamide; OGD: oxygen-glucose deprivation, MDA: malondialdehyde, GSH: reduced glutathione, SOD: superoxide dismutase).

against excitotoxic damages and neuronal insults (van der Stelt and Di Marzo, 2005). Therefore, endocannabinoid signalling has emerged as a promising target for therapeutic interventions in neurological disorders. In this respect, AEA with a wide range of actions has been the focus of intense research (Eljaschewitsch et al., 2006; Walker et al., 2002; Wallace et al., 2002; Gobbi et al., 2005; Giuffrida et al., 2004; Sinor et al., 2000; Milton, 2002; van der Stelt et al., 2001), however, the short half-life and poor aqueous solubility (Jarho et al., 1996) limits the effectiveness of this endocannabinoid. In the present study, we have used functionalized MWCNTs as the nanoreserviors in order to provide longer

lasting effects for AEA and evaluate the therapeutic potential of this nanostructure in OGD, a widely used *in vitro* model of stroke which shares some similarities with *in vivo* models of brain ischemia (Mehta et al., 2007).

After FTIR confirmation of amino functionalization of COOH-MWCNTs (the presence of N-H and C-N bands; Fig. 1, curve B) and successful AEA immobilization on the aminated MWCNTs (the presence of characteristic peaks; Fig. 1, curve C), we found that there is no remarkable difference between the morphologies of acid- and amine-modified MWCNTs, while, AEA-MWCNTs complex may be easily recognized by their increased diameter and rougher appearance. Furthermore, proper functionalization does not damage the structures of MWCNTs (Fig. 2). As shown in Table 1, EDA-MWCNTs and EDA-MWCNTs-AEA complex induce less cytotoxicity than COOH-MWCNTs, suggesting that amine modification improves the biocompatibility of MWCNTs leading to the increased cell viability. This finding is in accordance with previous report indicating the neuroprotective effects of aminated CNTs (Matsumoto et al., 2010). EDA-MWCNTs-AEA complex significantly prevented loss of cell viability after 3h of OGD induction, while, AEA was ineffective in this regard (Table 1) which might be due to its short half-life and/or rapid degradation by the intracellular located enzyme, fatty acid amide hydrolase (McKinney and Cravatt, 2005). As shown in Table 2, AEA and EDA-MWCNTs-AEA complex dose-dependently suppressed OGD-induced oxidative stress as revealed by their ability to reduce the production of MDA, an indicator of free radical generation, oxidative stress, and tissue injury (Draper and Hadley, 1990), and increase the content of GSH, a free radical scavenger and an essential component of cellular defence mechanism against the oxidative stress (Spitz et al., 1991), and activity of SOD, an essential enzyme which plays a critical role in protecting cells against the oxidative injury (Warner et al., 2004). These findings indicate the antioxidant effects of AEA and EDA-MWCNTs-AEA complex. In this context, the enhancement of PC12 viability by AEA solution and EDA-MWCNTs-AEA complex (Table 1) may be due to their ability to suppress oxidative stress. Since oxidative stress contributes to the pathophysiology of neurodegenerative disorders (Marsicano et al., 2002), therefore, prevention of OGD-induced oxidative stress by AEA and EDA-MWCNTs-AEA complex might be of therapeutic significance in neurological disorders such as cerebral stroke. EDA-MWCNTs-AEA complex, but not AEA alone, exhibited beneficial effects after longer exposure to OGD (Table 2) suggesting the ability of this nanostructure to provide a sustained concentration of AEA. Based on the protective effects of AEA against the neuronal injury (Milton, 2002; van der Stelt et al., 2001), the extended activity of AEA might be of therapeutic importance against the cellular dysfunction in both acute and chronic forms of neural injury. Furthermore, it may result in the reduced dosage frequency and psychoactive side effects which may be observed following the repeated administration of cannabinoids (Whan et al., 2006). Based on the regulatory effects of AEA on the synaptic plasticity (Gerdeman and Lovinger, 2003) and the ability of CNT scaffolds to re-establish the synaptic contacts and modulate the synaptic plasticity (Cellot et al., 2011), using MWCNTs-AEA complex might be of therapeutic value in diseases associated with abnormal synaptic plasticity that merits further investigation.

Conclusion

In conclusion, aminated MWCNTs appear as the promising carriers for AEA which are able to provide a sustained concentration of this endocannabinoid leading to the longer-lasting effects against the OGD insult in PC12 cells, a model of neuron-like cells. EDA-MWCNTs-AEA complex by increasing the cell viability and suppressing oxidative stress may be a valuable therapeutic agent for the ischemic stroke or other neurodegenerative pathologies.

Acknowledgment

This work was funded by a grant from Tehran University of Medical Sciences. Authors wish to thank Dr. Mohsen Amini, Department of Medicinal Chemistry, Tehran University of Medical Sciences, for helpful discussion.

Conflict of Interest

None of the authors has any conflict of interest to disclose.

References

Adinolfi B, Romanini A, Vanni A, Martinotti E, Chicca A, Fogli S, et al. Anticancer activity of anandamide in human cutaneous melanoma cells. Eur J Pharmacol 2013; 718: 154-59.

Bhirde AA, Patel V, Gavard J, Zhang G, Sousa AA, Masedunskas A, et al. Targeted killing of cancer cells in vivo and in vitro with EGF-directed carbon nanotube-based drug delivery. ACS Nano 2009; 3: 307-16.

Boger DL, Sato H, Lerner AE, Hedrick MP, Fecik RA, Miyauchi H, et al. Exceptionally potent inhibitors of fatty acid amide hydrolase: the enzyme responsible for degradation of endogenous oleamide and anandamide. Proc Natl Acad Sci U S A 2000; 97: 5044-49.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-54.

Casanova ML, Blazquez C, Martinez-Palacio J, Villanueva C, Fernandez-Acenero MJ, Huffman JW, et al. Inhibition of skin tumor growth and angiogenesis in vivo by activation of cannabinoid receptors. J Clin Invest 2003; 111: 43-50.

Cellot G, Cilia E, Cipollone S, Rancic V, Sucapane A, Giordani S, et al. Carbon nanotubes might improve neuronal performance by favouring electrical shortcuts. Nat Nanotechnol 2009; 4: 126-33.

Cellot G, Toma FM, Varley ZK, Laishram J, Villari A, Quintana M, et al. Carbon nanotube scaffolds tune synaptic strength in cultured neural circuits: novel frontiers in nanomaterial-tissue interactions. J Neurosci 2011; 31: 12945-53.

Chen H, Yoshioka H, Kim GS, Jung JE, Okami N, Sakata H, et al. Oxidative stress in ischemic brain damage: mechanisms of cell death and potential molecular targets for neuroprotection. Antioxid Redox Signal 2011; 14: 1505-17.

Chen W, Xiong Q, Ren Q, Guo Y, Li G. Can amino-functionalized carbon nanotubes carry functional nerve growth factor? Neural Regen Res 2014; 9: 285-92.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 1992; 258: 1946-1949.

Di Marzo V, Melck D, Bisogno T, De Petrocellis L. Endocannabinoids: endogenous cannabinoid receptor ligands with neuroremodulatory action. Trends Neurosci 1998; 21: 521-28.

Draper HH, Hadley M. Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol 1990; 186: 421-31.

Eljaschewitsch E, Witting A, Mawrin C, Lee T, Schmidt PM, Wolf S, et al. The endocannabinoid anandamine protects neurons during CNS inflammation by induction of MKP-1 in microglial cells. Neuron 2006; 49: 67-79.

Fabbro A, Bosi S, Ballerini L, Prato M. Carbon nanotubes: artificial nanomaterials to engineer single neurons and neuronal networks. ACS Chem Neurosci 2012; 3: 611-18.

Ferna´ndez-Ruiz J, Berrendero F, Herna´ndez ML, Ramos JA. The endogenous cannabinoid system and brain development. Trends Neurosci 2000; 23: 14-20.

Gerdeman GL, Lovinger DM. Emerging roles for endocannabinoids in long-term synaptic plasticity. Br J Pharmacol 2003; 140: 781-89.

Giuffrida A, Leweke FM, Gerth CW, Schreiber D, Koethe D, Faulhaber J, et al. Cerebrospinal anandamide levels are elevated in acute schizophrenia and are inversely correlated with psychotic symptoms. Neuropsychopharmacology 2004; 29: 2108-14.

Gobbi G, Bambico FR, Mangieri R, Bortolato M, Campolongo P, Solinas M, et al. Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. Proc Natl Acad Sci U S A 2005; 102: 18620-25.

Green AR, Shuaib A. Therapeutic strategies for the treatment of stroke. Drug Discov Today 2006; 11: 681-93.

Hamdi H, De La Torre-Roche R, Hawthorne J, White JC. Impact of non-functionalized and amino-functionalized multiwall carbon nanotubes on pesticide uptake by lettuce (Lactuca sativa L.). Nanotoxicology 2015; 9: 172-80.

Hassanzadeh P, Rahimpour S. The cannabinergic system is implicated in the upregulation of central NGF protein by psychotropic drugs. Psychopharmacology 2011; 215: 129-41.

Hassanzadeh P, Arbabi E. Cannabinoid CB_1 receptors mediate the gastroprotective effect of neurotensin. Iran J Basic Med Sci 2012; 15: 803-10.

Hassanzadeh P. Computational modelling: Moonlighting on the neuroscience and medicine. Biomed Rev 2013; 24: 25-31.

Hassanzadeh P, Rostami F. CB_1 Cannabinoid receptors are involved in neuroleptic-induced enhancement of brain neurotensin. Iran J Basic Med Sci 2014; 17: 181-88.

Hassanzadeh P. Nanopharmaceurticals: Innovative theranostics for the neurological disorders. Biomed Rev 2014; 25: 25-34.

Hassanzadeh P, Arbabi E, Atyabi F, Dinarvand R. The endocannabinoid system and NGF are involved in the mechanism of action of resveratrol: A multi-target nutraceutical with therapeutic potential in neuropsychiatric disorders. Psychopharmacology 2016; 233: 1087-96.

Hayakawa K, Mishima K, Irie K, Hazekawa M, Mishima S, Fujioka M, et al. Cannabidiol prevents a post-ischemic injury progressively induced by cerebral ischemia via a high-mobility group box1-inhibiting mechanism. Neuropharmacology 2008; 55: 1280-86.

Iuvone T, Esposito G, Esposito R, Santamaria R, Di Rosa M, Izzo AA. Neuroprotective effect of cannabidiol, a non-psychoactive component from Cannabis sativa, on beta-amyloid-induced toxicity in PC12 cells. J Neurochem 2004; 89: 134-41.

Jarho P, Urtti A, Pate DW, Suhonen P, Ja¨rvinen T.

Increase in aqueous solubility, stability and in vitro corneal permeability of anandamide by hydroxypropyl-ß-cyclodextrin. Int J Pharm 1996; 137: 209-16.

Jollow DJ, Mitchell JR, Zampaglione N, Gillette JR. Bromobenze induced liver necrosis: protective role of glutathione and evidence for 3,4-bromobenzenoxide as the hepatotoxic metabolite. Pharmacology 1974; 11: 151–69.

Kakkar P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. Indian J Biochem Biophys 1984; 21: 130-32.

Kam NW, Dai H. Carbon nanotubes as intracellular protein transporters: generality and biological functionality. J Am Chem Soc 2005; 127: 6021-26.

Klijn CJ, Hankey GJ. Management of acute ischaemic stroke: new guidelines from the American Stroke Association and European Stroke Initiative. Lancet Neurol 2003; 2: 698-701.

Larsen EC, Hatcher JF, Adibhatla RM. Effect of tricyclodecan-9-yl potassium xanthate (D609) on phospholipid metabolism and cell death during oxygen–glucose deprivation in PC12 cells. Neuroscience 2007; 146: 946-61.

Lavie G, Teichner A, Shohami E, Ovadia H, Leker RR. Long term cerebroprotective effects of dexanabinol in a model of focal cerebral ischemia. Brain Res 2001; 901: 195-201.

Lee HJ, Park J, Yoon OJ, Kim HW, Lee do Y, Kim do H, et al. Amine-modified single-walled carbon nanotubes protect neurons from injury in a rat stroke model. Nat Nanotechnol 2011; 6: 121-25.

Marsicano G, Moosmann B, Hermann H, Lutz B, Behl C. Neuroprotective Neuroprotective properties of cannabinoids against oxidative stress: role of the cannabinoid receptor CB1. J Neurochem 2002; 80: 448-56.

Mato S, Chevaleyre V, Robbe D, Pazos A, Castillo PE, Manzoni OJ. A single in-vivo exposure to Δ^9THC blocks endocannabinoid-mediated synaptic plasticity. Nat Neurosci 2004; 7: 585-86.

Matsumoto K, Sato C, Naka Y, Whitby R, Shimizu N. Stimulation of neuronal neurite outgrowth using functionalized carbon nanotubes. Nanotechnology 2010; 21: 115101.

McCormick DA, Contreras D. On the cellular and network bases of epileptic seizures. Annu Rev Physiol 2001; 63: 815-46.

McKinney MK, Cravatt BF. Structure and function of fatty acid amide hydrolase. Annu Rev Biochem 2005; 74: 411-32.

Mehta SL, Manhas N, Raghubir R. Molecular targets in cerebral ischemia for developing novel therapeutics. Brain Res Rev 2007; 54: 34-66.

Milton NG. Anandamide and noladin ether prevent neurotoxicity of the human amyloid-β peptide. Neurosci Lett 2002; 332: 127-30.

Mohammadi A, Moghaddam AB, Dinarvand R, Rezaei-Zarchi S. Direct electron transfer of polyphenol oxidase on carbon nanotube surfaces: Application in Biosensing. Int J Electrochem Sci 2009; 4: 895-905.

Moskowitz MA, Lo EH, Iadecola C. The science of stroke: mechanisms in search of treatments. Neuron 2010; 67: 181-98.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65: 55-63.

Nagayama T, Sinor AD, Simon RP, Chen J, Graham SH, Jin K, et al. Cannabinoids and neuroprotection in global and focal cerebral ischemia and in neuronal cultures. J Neurosci 1999; 19: 2987-95.

Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351-58.

Pacher P, Batkai S, Kunos G. The endocannabinoid system as an emerging target of pharmacotherapy. Pharmacol Rev 2006; 58: 389-62.

Piomelli D. The molecular logic of endocannabinoid signalling. Nat Rev Neurosci 2003; 4: 873-84.

Roman JA, Niedzielko TL, Haddon RC, Parpura V, Floy CL. Single-walled carbon nanotubes chemically functionalized with polyethylene glycol promote tissue repair in a rat model of spinal cord injury. J Neurotrauma 2011; 28: 2349-62.

Sancho R, Calzado MA, Di Marzo V, Appendino G, Muñoz E. Anandamide inhibits nuclear factor-kappaB activation through a cannabinoid receptor-independent pathway. Mol Pharmacol 2003; 63: 429-38.

Serteser M, Ozben T, Gumuslu S, Balkan S, Balkan E. The effects of NMDA receptor antagonist MK-801 on lipid peroxidation during focal cerebral ischemia in rats. Prog Neuropsychopharmacol Biol Psychiatry 2002; 26: 871-77.

Sinor AD, Irvin SM, Greenberg DA. Endocannabinoids protect cerebral cortical neurons from in vitro ischemia in rats. Neurosci Lett 2000; 278: 157-60.

Son SJ, Bai X, Nan A, Ghandehari H, Lee SB. Template synthesis of multifunctional nanotubes for controlled release. J Control Release 2006; 114: 143-52.

Spitz DR, Sullivan SJ, Malcolm RR, Roberts RJ. Glutathione dependent metabolism and detoxification of 4- hydroxy-2-nonenal. Free Radic Biol Med 1991; 11: 415-23.

Sun YP, Fu K, Lin Y, Huang W. Functionalized carbon nanotubes: properties and applications. Acc Chem Res 2002; 35: 1096-104.

van der Stelt M, Veldhuis WB, van Haaften GW, Fezza F, Bisogno T, Bar PR, et al., Exogenous anandamide protects rat brain against acute neuronal injury in vivo. J Neurosci 2001; 21: 8765-71.

van der Stelt M, Di Marzo V. Cannabinoid receptors and their role in neuroprotection. Neuromolecular Med 2005; 7: 37-50.

Walker JM, Krey JF, Chu CJ, Huang SM. Endocannabinoids and related fatty acid derivatives in pain modulation. Chem Phys Lipids 2002; 121: 159-72.

Wallace MJ, Martin BR, DeLorenzo RJ. Evidence for a physiological role of endocannabinoids in the modulation of seizure threshold and severity. Eur J

Pharmacol 2002; 452: 295-301.

Wang J, Sun P, Bao Y, Liu J, An L. Cytotoxicity of single-walled carbon nanotubes on PC12 cells. Toxicol In vitro 2011; 25: 242-50.

Warner DS, Sheng H, Batinic-Haberle I. Oxidants, antioxidants and the ischemic brain. J Exp Biol 2004; 207: 3221-31.

Whan LB, West MC, McClure N, Lewis SE. Effects of Δ-9-tetrahydrocannabinol, the primary psychoactive cannabinoid in marijuana, on human sperm function in vitro. Fertil Steril 2006; 85: 653-60.

Williams EJ, Walsh FS, Doherty P. The FGF receptor uses the endocannabinoid signaling system to couple to an axonal growth response. J Cell Biol 2003; 160: 481-86.

Xu L, Sun J, Lu R, Ji Q, Xu JG. Effect of glutamate on inflammatory responses of intestine and brain after focal cerebral ischemia. World J Gastroenterol 2005; 11: 733-36.

Zhang S, Uludağ H. Nanoparticulate systems for growth factor delivery. Pharm Res 2009; 26: 1561-80.

Zhao D, Alizadeh D, Zhang L, Liu W, Farrukh O, Manuel E, et al. Carbon nanotubes enhance CpG uptake and potentiate antiglioma immunity. Clin Cancer Res 2011; 17: 771-82.