

Preliminary safety assessment of Eudragit[®] polymers nanoparticles administration in the rat brain

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ABSTRACT

Although nanotechnology can provide useful carriers to protect successfully active agents, it is necessary to determine the possible toxicity of these technological products before any extensive use. The aim of the current study was to evaluate the effects on the nervous system of Eudragit[®] polymers nanoparticles (EUD-Nps), considered as model of drug delivery carriers. Nile red-labeled EUD-NPs were prepared by a double emulsion/solvent evaporation technique. Male Sprague-Dawley rats were treated orally (PO) or intraperitoneally (IP) with a single dose of EUD-NPs (50 mg/kg bw). Animals were sacrificed after 4h, 48h, 1 week and 3 weeks and the brain of each rat was removed and dissected into the forebrain, midbrain and hindbrain regions. Acetylcholinesterase (AChE) activity, lipid peroxidation, reduced glutathione and nitric oxide were determined in different brain region homogenate. Other samples were used for the histological and fluorescent examinations. The results indicated that within the two routes of administration, all the tested parameters showed insignificant changes during the tested time intervals and all of them were in the normal range of the control by the 3rd week. Moreover, the histological examination revealed a normal histological picture of the brain tissues after 3 weeks of administration. The fluorescent examination showed that EUD-NPs were found in the brain tissues at all the studied times. These findings showed that EUD-NPs have the ability to cross the blood brain barrier but induced little toxic effects on the brain. Further studies are needed to verify the fate of these NPs in the long term before using them as a vector.

INTRODUCTION

Eudragit[®] polymers are polymers of acrylate and methacrylate available in different ionic form that have been developed by Röhm & Haas GmbH in Darmstadt. They are already authorized in the market as safe excipients to protect and/or control the time and release location of the active substances (Nagai *et al.*, 1985; Bodde *et al.*, 1990; Le Bourlias *et al.*, 1995). Eudragit[®] RL and Eudragit[®] RS are insoluble in aqueous media but they are permeable and both have a pH-independent release profiles due to the presence of quaternary

ammonium groups in their structures (Shid *et al.*, 2014). The development of polymeric nanocarriers is well-known for their beneficial characteristics enabling protection of active substances from degradation, reduction of their toxicity or side effects and sustained and controlled release of drugs locally (Padma *et al.*, 2007; Lai *et al.*, 2009; Kumari *et al.*, 2010). This was used during the formation of polymeric nanoparticles of Valsartan-loaded Eudragit[®], Carvedilol-loaded Eudragit[®] and S-nitrosoglutathione-loaded Eudragit[®] for the treatment of hypertension by reducing the dose and frequency of drug administration and increasing its bioavailability (Jensen *et al.*, 2010, Selvakumar and Yadav, 2009, Safar *et al.*, 2015). Even though these copolymers are not biodegradable, several research groups used Eudragit[®] nanoparticles (EUD-NPs) for the parenteral administration of

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drugs and they reported a good biocompatibility of this biomaterial (Schaffazick *et al.*, 2008; Basarkar and Singh, 2009; Zago *et al.*, 2013), owing to the rapid clearance from the systemic circulation by the mono-nuclear phagocytic system and their deposition in the liver (Rolland *et al.*, 1989). However, to date, nanotechnologies raise scientific questions, including their impact on health. The work accomplished over the past decade in the field of nanotoxicology is rarely oriented to the study of their cerebral impact, but they highlight the shortcomings of the protective barriers of the brain considering the nanoparticles (Bencsik, 2014). Indeed, the crossing of various biological barriers have been identified for several entrance ways and for different types of nanoparticles confirming that the nano-sized promotes inter- and trans-cellular passage within an entire organism. Thus neuronal translocation can occur directly at the nasal cavity where the nerve endings are present in the neurons of the olfactory bulb (Oberdörster, 2004), but also indirectly after passing a first barrier, lung, skin and gut. The nanoparticles reach the systemic route and they can then secondarily hit the brain through the Blood-Brain Barrier (BBB) following the opening of the tight junctions by hyper osmotic barrier (Kreuter *et al.*, 2003) and subsequently cause damage by some induction of oxidative stress or inflammatory responses (Hu and Gao, 2010). In previous work, we evaluated the effect of EUD-NPs on the hematological parameters and erythrocyte damage in rats to assess their short-term toxicity (Abdel-Wahhab *et al.*, 2014). We concluded that EUD-NPs induced moderate hematological disturbances, represented in platelet, total and differential counts of WBCs, as well as RBC counts which resulted in some degree of physiological and functional anemia, likely through oxidative stress. Moreover, the route of administration showed a major effect on the induction of these toxic effects. Despite the fast development of the nanotechnologies, little is known about a possible impact of nanoparticles on health and environment as well as their impact on human body and especially on the brain is not known. Therefore, the present study was conducted to evaluate the safety of EUD-NPs on the cerebral tissues after orally (PO) or intraperitoneally (IP) administration in order to complete our knowledge on the potential impact of NPs on the brain function. This was achieved by measuring the oxidative stress parameters in the forebrain, midbrain and hindbrain regions which is recognized as tool to evaluate toxicity mediated by small particles exposure. In addition, the effect of EUD-NPs on the cholinergic activity was estimated by assessing the acetylcholinesterase activity as a biomarker of exposure to neurotoxic compounds (Dal Forno, 2013) as well as the histological changes in the three selected brain regions.

MATERIAL AND METHODS

Chemicals and Kits

Eudragit® RL PO (MW = 150,000 Da, CAS number: 33434-24-1), an acrylic polycationic copolymer of acrylic and methacrylic acid esters with a proportion of quaternary ammonium groups (0.5%–0.8%), was a generous gift from Evonik polymers

(Darmstadt, Germany). Pluronic F68 (CAS number: 11104-97-5) was used as a surfactant and was obtained from Sigma Aldrich (Saint-Quentin Fallavier, France). Acetylcholinesterase kit was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Kits for nitric oxide (NO), Malondialdehyde (MDA) and reduced glutathione (GSH) evaluations were purchased from Biodiagnostic (Giza, Egypt). All other chemicals used throughout the experiments were of the highest analytical grade available from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation, characterization and morphological determination of EUD-NPs

EUD-NPs were prepared using the double emulsion/solvent evaporation technique as described in our previous work (Abdel-Wahhab *et al.*, 2014). The particle sizes were determined using dynamic light scattering (DLS) with a Zetasizer™ 3000E (Malvern Instruments Worcestershire, UK). Each sample was diluted with double distilled water until the appropriate concentration of particles and dilution were kept constant to avoid multi scattering events. The particle size and size distribution of equivalent hydrodynamic spheres were calculated using the Malvern associated software by exponential sampling method. Zeta potential was calculated by *Smoluchowski's* equation (Sze *et al.*, 2003) based on nanoparticle electrophoretic mobility. All measurements were performed in triplicate at 25°C. The morphological determination of EUD-NPs was analyzed by transmission electron microscopy (2100-HR, JEOL, CA, USA) by placing one drop of the fresh EUD-NPs sample onto a carbon-coated copper grid to form a thin liquid film and was negatively stained by one drop of uranyl acetate. The excess of staining solution was removed using filter paper and the film was air-dried before the observation. The image acquisition was done with an Orius 1000 CCD camera (GATAN, Warrendale, PA, USA).

Experimental Animals

One hundred male Sprague-Dawley rats (3 months old; 140-160 g) were purchased from the Animal House Colony of National Research Centre, Giza, Egypt) and were maintained on standard lab diet (metabolizable energy: 12.08 MJ) purchased from Meladco Feed Co. (Aubor City, Cairo, Egypt). Animals were housed in filter-top polycarbonate cages in a room free from any source of chemical contamination, artificially illuminated (12 h dark/light cycle) and thermally controlled (25 °C) at the Animal House Lab., National Research Center, Dokki, Cairo, Egypt. All animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of the National Research Center, Dokki, Cairo, Egypt and the National Academy of Sciences (NIH publication 86-23 revised 1985).

Experimental design

After an acclimatization period of one week, two separate experiments (50 rats/experiment) were carried out based on the route of administration of EUD-NPs. Each experiment consisted of 5 groups of 10 rats per group. In the first experiment,

animals were treated orally (PO) while in the second experiment, animals were treated intraperitoneally (IP) with Nile Red-labeled EDU-NPs (50 mg/kg b.w) suspended in saline solution. In both experiments, the control and EUD-NPs-treated animals were sacrificed after 4h, 48 h, 1 week and 3 weeks and the brain of each rat was removed and dissected into the forebrain, midbrain and hindbrain regions. Each region was weighed and kept frozen at -20°C until analyses. The brain samples were homogenized in 5% w/v 20 mM phosphate buffer, pH 7.6. The homogenates were centrifuged at 1753x g for 15 min at 4°C using a high speed cooling centrifuge. The clear supernatants for each region of each rat were separated and used for the analysis of acetylcholinesterase (AChE) activity and levels of lipid peroxidation, reduced glutathione and nitric oxide. A sample of hindbrain (cellberum cortex) from each rat in different treatment groups was excised and fixed in natural formalin and the samples were hydrated in ascending grades of ethanol, cleared in xylene and embedded in paraffin. Sections of 5 µm thick were cut and examined by fluorescent microscopy to study the distribution of Nile Red-labeled EDU-NPs in the brain tissues after different time periods in the PO and IP treated rats.

Determination of AChE activity

AChE activity was determined spectrophotometrically according to the modified method of Ellman *et al.* (1961) as described by Gorun *et al.* (1978) using Jenway Model 67 series spectrophotometer (Camlab Limited Co., Cambridge, UK). The principle of the method is the measurement of the thiocholine produced as acetylthiocholine is hydrolyzed. The color was read immediately at 412 nm.

Determination of lipid peroxidation

Lipid peroxidation was assayed by measuring the thiobarbituric-acid-reactive substances (TBARS) in brain homogenates, using the method of Ruiz-Larrea *et al.* (1994) in which the thiobarbituric acid reactive substances react with thiobarbituric acid to produce a red colored complex having peak absorbance at 532 nm.

Determination of reduced glutathione (GSH)

GSH was determined in brain tissues by the method described by Ellman (1959). The procedure is based on the reduction of Ellman's reagent by -SH groups of GSH to form 2-nitro-s-mercaptobenzoic acid which has an intense yellow color that can be determined spectrophotometrically at 412 nm. GSH concentration was calculated by comparison with a standard curve.

Determination of nitric oxide (NO) level

NO levels, measured as nitrite, were determined using Griess reagent according to the method of Moshage *et al.* (1995), where nitrite, a stable end product of the nitric oxide radical, is primarily used as an indicator for the production of nitric oxide. Nitrite is converted to a deep purple azo compound after the

addition of Griess reagents. The purple/magenta color developed is read at 540nm. The quantity was measured by standard curve.

Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) followed by Tukey multiple range test when the F-test was significant ($p < 0.05$). All analyses were performed using the Statistical Package for Social Sciences (SPSS) software in a PC-compatible computer. All data were expressed as means \pm S.E.M and the percentage difference (% D) was calculated as follows:
 $\% D = [\text{Treated Value} - \text{Control Value} / \text{Control Value}] \times 100$

RESULTS

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) images (Fig. 1 A and B) showed nearly spherical shape for the prepared EUD-NPs with total volume ranged from 102 x 139 and 139 x 169 nm. The size distribution of EUD-NPs by DLS measurements expressed in numbers showed an average size of the particles is 185 ± 70 nm (Fig. 2). The EUD-NPs also showed a positive zeta potential value of 57.5 ± 5.47 mV. The *in vivo* results indicated that the oral administration of EUD-NPs (50 mg/kg b.w) resulted in a significant increase in AChE activity in the forebrain after 4h and 3 weeks, while 1 and 3 weeks in the midbrain and hindbrain (Table 1) although non significant increased was found after other tested periods. However, IP injection of EUD-NPs resulted in a significant increase in the forebrain only after 3 weeks, in the midbrain after 4h, 48h and 1 week and in the hindbrain of after 48h, 1 and 3 weeks. It is worthy to mention that the animals which received the IP injection of EUD-NPs showed a significant decrease in AChE in the midbrain after 3 weeks (Table 1). The results presented in Fig (3A) showed the percentage of changes of the enzyme activity compared to the control group. It is clear that both oral and IP administration of EUD-NPs disturbed AChE activity in different brain regions within different tested periods. The most obvious difference was noticed after 4h in the forebrain of rats treated orally with EUD-NPs followed by the hindbrain after 1 week in the animals treated IP and the midbrain after 3 weeks in the animals treated orally. It is also clear that the enzyme activity reduced by more than 20% compared to the control in the midbrain of animal treated IP after 3 weeks.

The effect of EUD-NPs treatment on GSH content in the three studied brain regions is shown in Table (2) and Fig (3B). The results indicated that oral administration of EUD-NPs induced a significant decrease in GSH in the forebrain below control value after 4h followed by non significant changes after 48h, one week and 3 weeks. The GSH content in the midbrain showed a significant decrease after 4h, 1 and 3 weeks. However, it showed a significant increase in the hindbrain only after 4h. On the other hand, IP injection with EUD-NPs resulted in non-significant changes in GSH level in the forebrain. The IP injection resulted in a significant decrease in midbrain after 4h, 1 and 3 weeks. However, it induced a significant increase in GSH in the hindbrain after 4h and 48h (Fig. 3B).

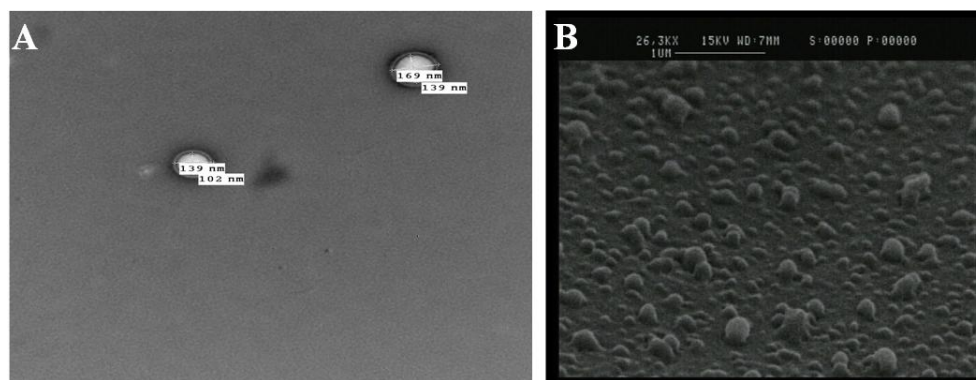


Fig.1: TEM (A) and SEM (B) images of the prepared (Eudragit® RL) nanoparticles (EDU-NPs).

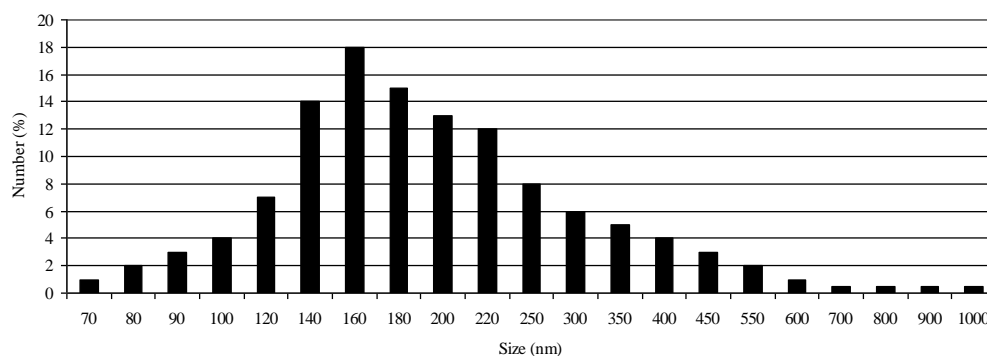


Fig. 2: The size distribution of EDU-NPs by DLS measurements.

Table 1: Effect of EUD-NPs treatment on AchE activity ($\mu\text{mol}/\text{min}/\text{g}$) in brain regions of rats.

Brain regions	Control		4 h		48 h		1 week		3 weeks	
	PO	IP	PO	IP	PO	IP	PO	IP	PO	IP
Forebrain	5.40 ± 0.64^a	5.70 ± 0.68^A	10.01 ± 1.47^b	6.49 ± 1.48^B	6.67 ± 0.84^a	6.01 ± 1.67^B	6.37 ± 0.60^a	6.21 ± 1.85^B	7.31 ± 0.66^c	7.03 ± 0.58^C
Midbrain	6.16 ± 0.18^a	7.40 ± 0.45^A	7.34 ± 0.55^b	8.94 ± 1.12^B	7.99 ± 0.61^b	8.34 ± 0.95^B	8.99 ± 1.24^c	11.88 ± 1.01^C	10.81 ± 0.77^d	5.87 ± 0.97^D
Hindbrain	3.34 ± 0.14^a	2.93 ± 0.58^A	4.17 ± 0.24^a	4.70 ± 0.13^B	3.81 ± 0.25^a	3.54 ± 0.43^C	4.93 ± 0.33^b	5.34 ± 0.24^D	4.48 ± 0.02^b	4.06 ± 0.21^B

Within each raw, means superscript with different letters are significantly difference ($P \leq 0.05$), small letters for oral treatment and Capital letters for I.P injection

Table 2: Effect of EUD-NPs treatment on GSH levels (mmol/g) in brain regions of rats.

Brain regions	Control		4 h		48 h		1 week		3 weeks	
	PO	IP	PO	IP	PO	IP	PO	IP	PO	IP
Forebrain	3.57 ± 0.07^a	3.49 ± 0.17^A	2.08 ± 0.05^b	3.69 ± 0.34^A	3.35 ± 0.05^a	3.29 ± 0.06^a	3.47 ± 0.29^A	3.58 ± 0.22^a	3.36 ± 0.18^a	3.23 ± 0.09^A
Midbrain	7.85 ± 0.59^a	6.53 ± 0.57^A	5.98 ± 0.46^b	3.69 ± 0.34^B	8.04 ± 0.48^a	6.46 ± 0.06^A	5.33 ± 0.92^b	5.87 ± 0.45^C	6.97 ± 0.41^c	5.40 ± 0.36^C
Hindbrain	5.42 ± 0.35^a	5.27 ± 0.43^A	6.01 ± 0.55^b	6.54 ± 0.25^B	5.89 ± 0.04^a	6.70 ± 0.28^B	5.83 ± 0.55^a	5.57 ± 0.48^A	5.17 ± 0.14^a	5.46 ± 0.14^A

Within each raw, means superscript with different letters are significantly difference ($P \leq 0.05$), small letters for oral treatment and Capital letters for I.P injection.

Animals treated orally with EUD-NPs showed a significant increase of the NO level in the forebrain after 4h, 1 and 3 weeks and after 1 and 3 weeks in the midbrain in animal treated orally with EUD-NPs (Table 3). However, this treatment resulted in a significant increase of the NO level in the hindbrain after 4h, 1 and 3 weeks. IP injection resulted in a significant decrease of the NO level in the forebrain after 4h and 48h but it induced a significant increase of NO after 3 weeks in the forebrain. However, it induced a significant decrease of NO in the midbrain after 4h, 48 h and 3 weeks. The NO level was found to be increased in the hindbrain of animal treated IP with EUD-NPs after 4h but increased significantly after 1 and 3 weeks (Fig. 3C).

The effect of EUD-NPs treatment on the lipid peroxidation (LPO) is presented in Table (4) and Fig. (3D). The results revealed that in animals treated orally with EUD-NPs, LPO was significantly decreased in the forebrain after 48h, 1 and 3 weeks. A significant increase was noticed in LPO in the midbrain after 4h and a significant decrease was noticed after 1 week. However, LPO was decreased significantly in the hindbrain after all tested period. On the other hand, IP injection resulted in a significant increase in LPO in the forebrain after 4, 48h and 3 weeks. A significant increase was found in LPO in the midbrain after 4 and 48 h but it was decreased significantly after 1 and 3 weeks. Moreover, LPO was decreased significantly in the hindbrain after 48h and 3 weeks.

Table 3: Effect EUD-NPs treatment on NO levels ($\mu\text{mol/g}$) in brain regions of rats.

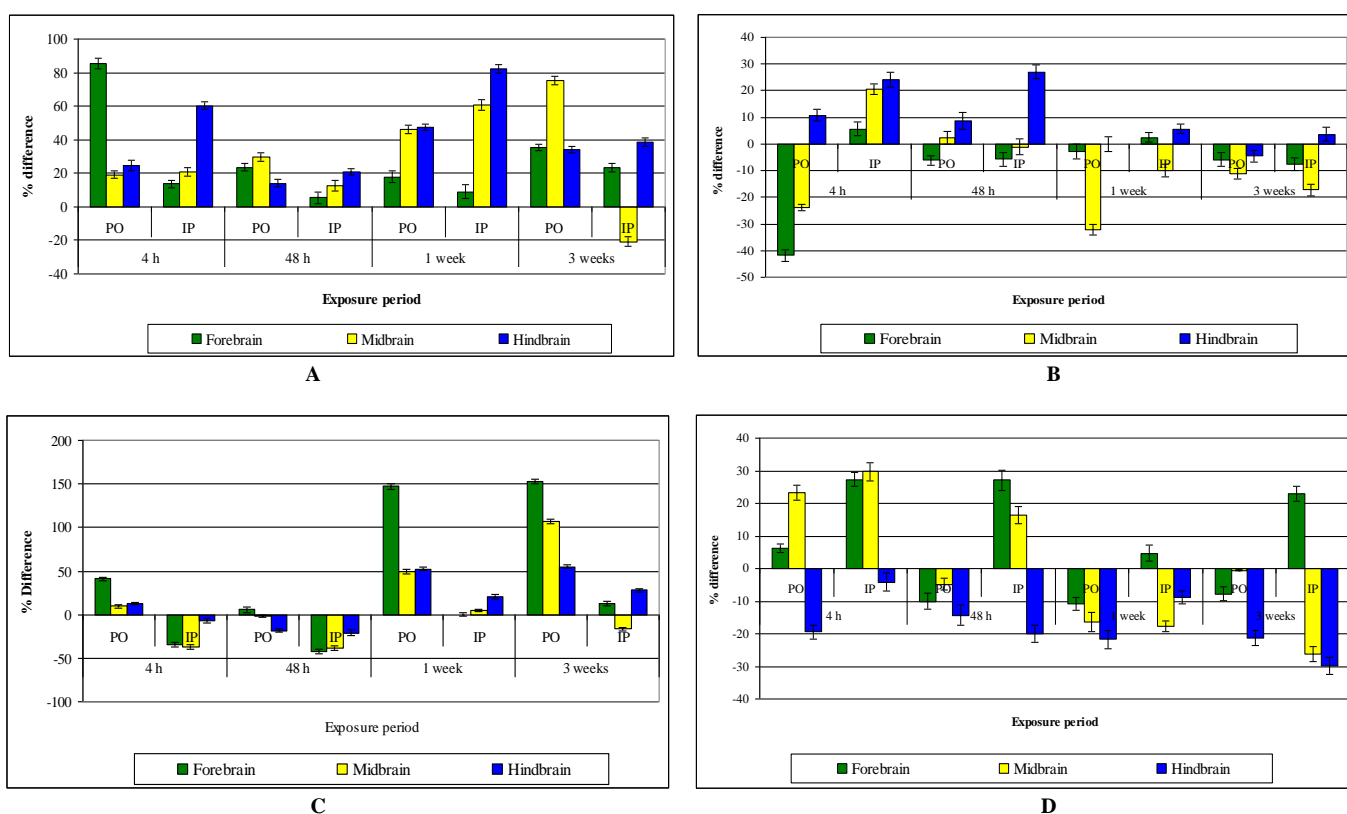
Brain regions	Control		4 h		48 h		1 week		3 weeks	
	PO	IP	PO	IP	PO	IP	PO	IP	PO	IP
Forebrain	0.17 \pm 0.04 ^a	0.38 \pm 0.11 ^A	0.24 \pm 0.02 ^b	0.25 \pm 0.04 ^B	0.18 \pm 0.03 ^a	0.22 \pm 0.04 ^B	0.42 \pm 0.08 ^c	0.38 \pm 0.09 ^A	0.43 \pm 0.02 ^c	0.43 \pm 0.04 ^C
Midbrain	0.53 \pm 0.003 ^a	1.08 \pm 0.04 ^A	0.58 \pm 0.12 ^a	0.68 \pm 0.13 ^B	0.52 \pm 0.07 ^a	0.66 \pm 0.04 ^B	0.79 \pm 0.11 ^b	1.13 \pm 0.30 ^A	1.10 \pm 0.15 ^c	0.90 \pm 0.07 ^C
Hindbrain	0.38 \pm 0.05 ^a	0.43 \pm 0.09 ^A	0.43 \pm 0.05 ^b	0.40 \pm 0.06 ^A	0.31 \pm 0.12 ^a	0.34 \pm 0.01 ^B	0.58 \pm 0.08 ^c	0.52 \pm 0.08 ^C	0.59 \pm 0.09 ^c	0.55 \pm 0.04 ^C

Within each row, means superscript with different letters are significantly difference ($P \leq 0.05$), small letters for oral treatment and Capital letters for I.P injection.

Table 4: Effect of EUD-NPs treatment on lipid peroxidation levels (nmol/g) in brain regions of rats.

Brain regions	Control		4 h		48 h		1 week		3 weeks	
	PO	IP	PO	IP	PO	IP	PO	IP	PO	IP
Forebrain	24.24 \pm 2.43 ^a	20.26 \pm 5.34 ^A	25.74 \pm 2.17 ^a	25.80 \pm 0.99 ^B	21.81 \pm 2.84 ^b	25.76 \pm 0.44 ^B	21.60 \pm 3.36 ^b	21.22 \pm 4.05 ^A	22.34 \pm 3.91 ^b	24.90 \pm 0.79 ^B
Midbrain	19.83 \pm 2.52 ^a	20.87 \pm 1.2 ^A	24.47 \pm 1.65 ^b	27.08 \pm 1.84 ^B	18.84 \pm 4.37 ^a	24.29 \pm 2.83 ^C	16.55 \pm 1.75 ^c	17.18 \pm 1.36 ^D	19.71 \pm 3.29 ^a	15.40 \pm 1.56 ^E
Hindbrain	14.02 \pm 1.66 ^a	14.97 \pm 0.19 ^A	11.28 \pm 2.70 ^b	14.31 \pm 0.06 ^A	11.99 \pm 0.94 ^b	11.96 \pm 0.25 ^B	10.96 \pm 1.15 ^c	13.65 \pm 0.79 ^A	11.03 \pm 2.53 ^b	10.48 \pm 0.03 ^C

Within each row, means superscript with different letters are significantly difference ($P \leq 0.05$), small letters for oral treatment and Capital letters for I.P injection.

**Fig. 3:** Percentage of differences in AChE activity (A), GSH (B), NO (C) and LPO (D) between oral or I.P injected groups and control group.

The histological examination for the brain sections revealed that either PO or IP administration of EUD-NPs did not show severe histological changes in the cerebrum tissues. The microscopic examination of the cerebellar cortex brain sections of the control rats showed normal molecular layer, Purkinje cells with regular homogenous cytoplasmic cytoskeletal elements and granular layer containing small granule nerve cells with dark nuclei and Golgi type II cells with vesicular nuclei (Fig. 4a). After 4h of oral administration of EUD-NPs, the examination of brain sections showed distortion of Purkinje cells with irregular shape and eosinophilic homogenization of its cytoplasm with some neuroglial cells accumulated around it (Fig. 4b). After 48h of the

treatment with EUD-NPs, the brain sections showed distortion of Purkinje cell layer with displacement of Purkinje cells as well as cellular shrinkage. The surrounding neuropil tissue is vacuolated and the neuroglial cells accumulated around it (Fig. 4c). However after 1 week of the oral administration of EUD-NPs, the brain sections showed shrinkage of Purkinje cells and deeply stained. The surrounding neuropil tissue is vacuolated and some neuroglial cells accumulated around it (Fig. 4d). After 3 weeks of oral administration of EUD-NPs, the rat's cerebellar cortex showed marked distortion of Purkinje cells layer with cellular shrinkage. The surrounding neurophil tissue is vacuolated and the neuroglial cells accumulated around it (Fig. 4e).

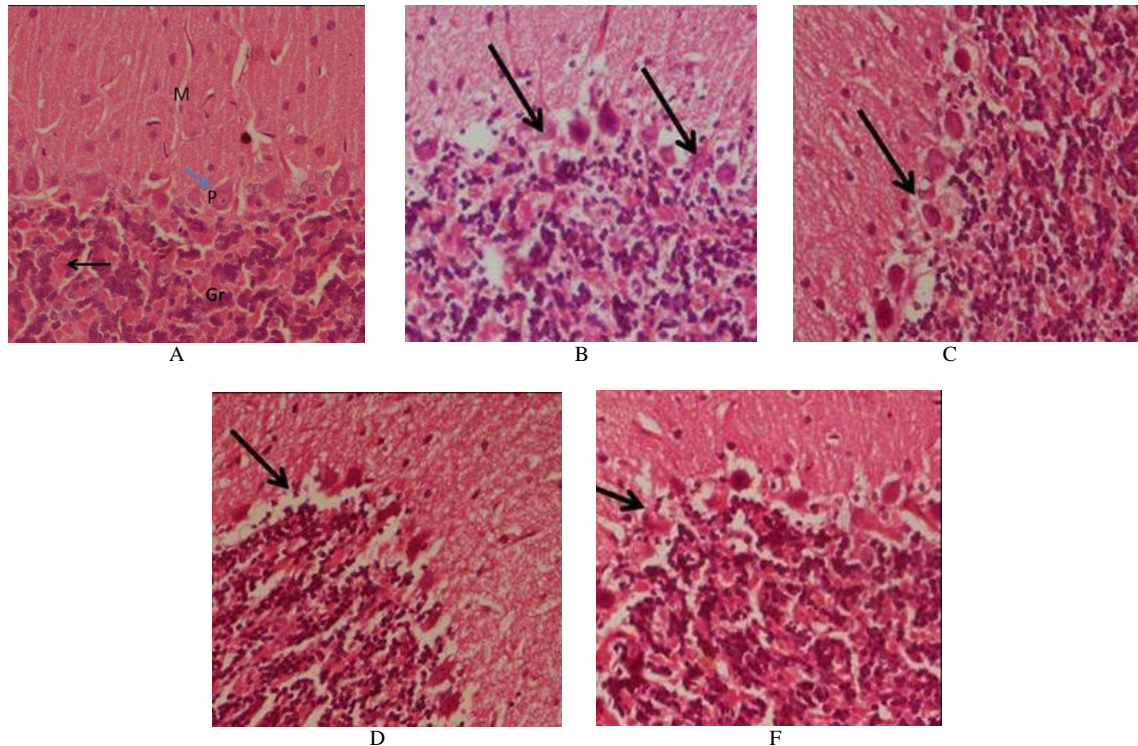


Fig. 4: Photomicrographs of (a) control rat's cerebellar cortex showing molecular layer (M), Purkinje cells with regular homogenous cytoplasmic cytoskeletal elements and granular layer (Gr) containing small granule nerve cells with dark nuclei and Golgi type II cells with vesicular nuclei. Notice the glomeruli in between cells of the granular layer, (b) rat's cerebellar cortex after 4 h of PO administration of EUD-NPs showing distortion of Purkinje cells with irregular shape and eosinophilic homogenization of its cytoplasm. Some neuroglial cells accumulated around it, (c) rat's cerebellar cortex after 48h of PO treatment showing distortion of Purkinje cell layer with displacement of Purkinje cells as well as cellular shrinkage. The surrounding neuropil tissue is vacuolated and the neuroglial cells accumulated around it, (d) rat's cerebellar cortex after 1wk of PO treatment showing shrinkage of Purkinje cells and deeply stained. The surrounding neuropil tissue is vacuolated and some neuroglial cells accumulated around it and (e) rat's cerebellar cortex after 3 wks showing marked distortion of Purkinje cell layer with cellular shrinkage. The surrounding neuropil tissue is vacuolated and the neuroglial cells accumulated around it.

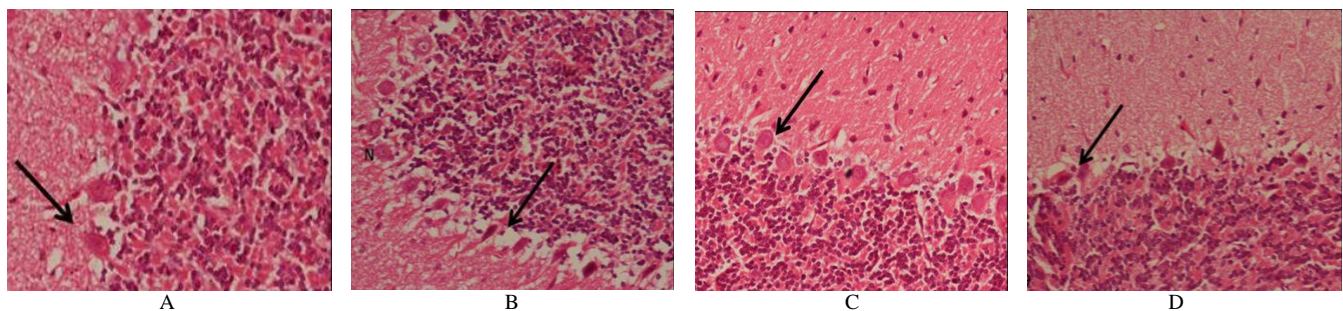


Fig. 5: Photomicrographs of (a) rat's cerebellar cortex after 4 h of IP administration of EUD-NPs showing normal flask shape Purkinje cell with apical dendrites and central vesicular nuclei, (b) cerebellar cortex after 48 h of IP administration of EUD-NPs showing normal flask shape of Purkinje cell with apical dendrites and central vesicular nuclei (N) and some cells appeared shrinkage with vacuolation in neuropil tissue (arrow), (c) rat's cerebellar cortex after 1 wk of administration showing normal Purkinje cell with apical dendrites and central vesicular nuclei (arrow) and (d) rat's cerebellar cortex after 3 wks showing normal Purkinje cells with irregular shape and pyknotic or fragmented the surrounding neuropil tissue is vacuolated (arrow).

On the other hand, IP treatment with EUD-NPs was safe compared to PO treatment since no histological changes were observed in the brain tissue. The microscopic examination of the rat's cerebellar cortex after 4h of IP treatment showed normal flask shape Purkinje cells with apical dendrites and central vesicular nuclei (Fig 5a). After 48h of IP treatment, animals showed the same features but in some cells appeared shrinkage with vacuolation in neuropil tissue (Fig. 5b). The cerebellar cortex of rats after 1 wk of IP administration of EUD-NPs showed normal

flask shape of Purkinje cells with apical dendrites, central vesicular nuclei and for some cells appeared shrinkage with vacuolation in neuropil tissue (Fig. 5c). However, after 3 wks of administration of the nanoparticles, the cerebellar cortex showed normal Purkinje cells with irregular shape and pyknotic or fragmented and the surrounding neuropil tissue was vacuolated (Fig. 5d). The fluorescent microscopy examination revealed that red Nile-labeled EUD-NPs were detected in all brain region after various tested periods in both exposed groups (Fig 6a,b).

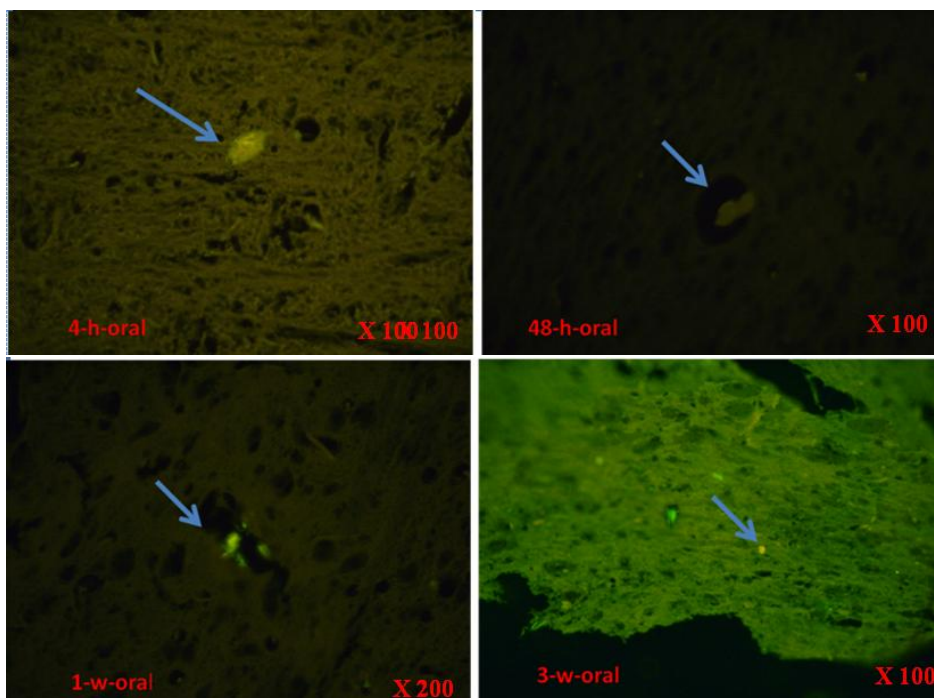


Fig. 6a: The distribution of EUD-NPs in brain tissues in oral-treated rats.

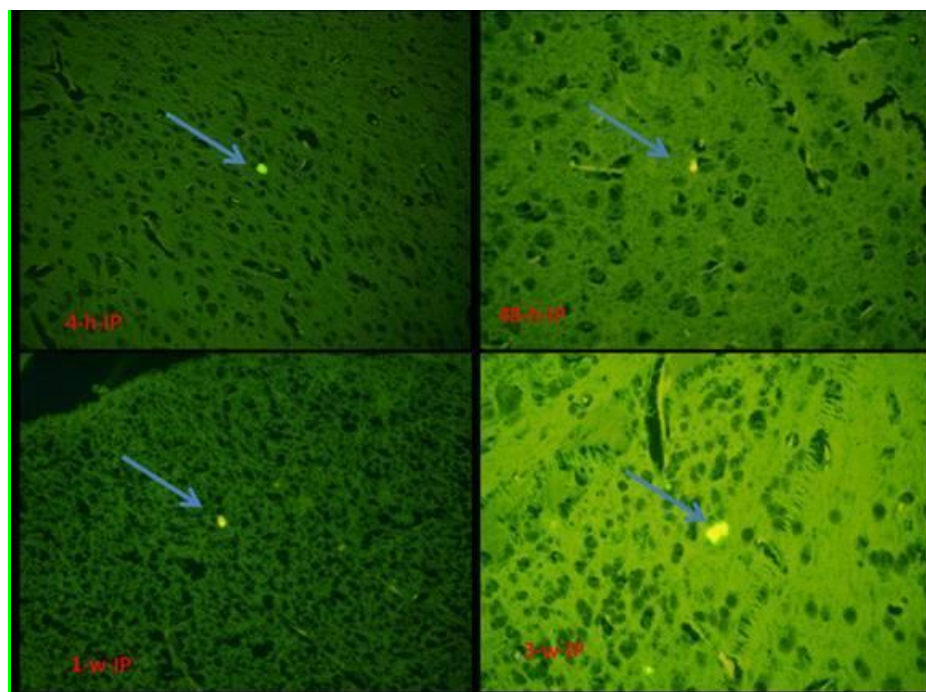


Fig. 6b: The distribution of EUD-NPs in brain tissues in IP-treated rats.

DISCUSSION

At present, only few studies have evaluated possible neurotoxic effects of nanoparticles exposure *in vivo*. The objective of this work was to evaluate the safety of EUD-NPs in some cerebral tissues. The selected dose and the tested durations were based on our previous work (Abdel-Wahhab *et al.*, 2014). The toxicity

profile was evaluated by analyzing four biomarkers of nanoparticles toxicity AChE activity, GSH, NO levels and lipid peroxidation. The importance of the cholinergic system in learning and memory processes is undeniable, and thus, alteration in AChE activity, as well as in acetylcholine neurotransmitter levels, is neurochemically associated with cognitive deficits (Das *et al.*, 2005a,b). Moreover, it has been reported that normal AChE

activity is essential for brain healthy function and the changes in AChE activity are accompanied by clear signs of neurobehavioral toxicity. Therefore, this parameter can be used as a neurotoxicity index in animals and human (Yousefi Babadi *et al.*, 2014). As for example, AChE activation has been demonstrated as a result of neurotoxic compounds exposure such as aluminum (Senger, 2011). However, an inhibition of AChE activity as a biomarker of the exposure to organophosphate and carbamate insecticides as reported by Van Dyk (2011). On the other hand, AChE is the neurotransmitter enzyme that terminates neurotransmission at cholinergic synapses by splitting acetylcholine neurotransmitter into choline and acetate (Tripathi *et al.*, 2008), thereby ending the cholinergic response (Milatovic *et al.*, 2006). Besides the catalytic properties, AChE has potent effects in cellular adhesion, neurite extension and postsynaptic differentiation and has been accepted as the most important biochemical indicator of cholinergic signaling in the central nervous system (Silman and Sussman, 2005). Previous studies have provided evidence that nanoparticles have different effects on AChE activity. For example, metal oxide ones induced an inhibition of this activity (Xia *et al.*, 2013), while fullerene C60 increased it (Dal Forno, 2013). The present findings indicated that a single dose of EUD-NPs (50 mg/kg b.w) given either orally or IP increased AChE activity after different time of exposure in either oral or I.P injected rats. Either oral or I.P injection of EUD-NPs induced a significant increase in AChE in forebrain. Accordingly, this may reflect the effect of EUD-NPs on the forebrain functions related to cholinergic activity such as learning, memory and cognition. Moreover, in the midbrain and hindbrain, EUD-NPs could affect the level of acetylcholine in these two regions. Since AChE is the enzyme responsible for the degradation of acetylcholine and the termination of its effect at its receptors, the present increase in AChE observed in the midbrain and hindbrain could be a compensatory mechanism to maintain the level of acetylcholine at its normal values. Thus we can deduce that the effect of EUD-NPs on cholinergic activity is tolerable. Consequently, it could be postulated that the increased AChE activity might be a compensatory response to these biochemical events. It is well known that acetylcholine is an ester of acetic acid and choline and its effects on its receptors are terminated by the degradable action of acetylcholinesterase. Eudragit® RL 100 and RS 100 are copolymers of acrylic and methacrylic acid esters with a low content in quaternary ammonium groups (Shid *et al.*, 2014). Accordingly, another explanation of the increased activity of acetylcholinesterase recorded in the present study due to the EUD-NPs administration could arise from the ability of AChE to act on EUD-NPs as a substrate.

The brain is highly sensitive to oxidative stress because of its high consumption of oxygen, energy and glucose, large amount of oxidizable polyunsaturated fatty acids and low antioxidant capability (Halliwell *et al.*, 1992; Dringen *et al.*, 2005). Thus we studied the effect of EUD-NPs on the oxidative status in the forebrain, midbrain and hindbrain by measuring the levels of lipid peroxidation, reduced glutathione and nitric oxide as indicators of oxidative status. Lipid peroxidation gives evidence

for the attack of cell membranes by the evolved free radicals (Ferrari, 2000). In addition, glutathione in its reduced form is the most abundant intracellular antioxidant and is involved in scavenging free radicals or serving as a substrate for glutathione peroxidase enzyme that catalyzes the detoxification of H₂O₂ (Dua and Gill, 2001). In the present study, significant changes in GSH were observed in the three studied brain regions after the two routes of EUD-NPs administration. The significant decrease in forebrain GSH after 4 h of EUD-NPs of oral or I.P administration could be attributed to its exhaustion against the evolved free radicals. In addition, the significant decrease in GSH in the midbrain after EUD-NPs administration may be a compensatory mechanism to mitigate the accompanied lipid peroxidation. Fortunately, these effects of EUD-NPs on GSH returned to non significant changes in the forebrain and hindbrain after 3 weeks reflecting to somewhat the extent effect of EUD-NPs on GSH.

Nitric oxide in its constitutive level has a neuroprotective effect and mediates physiological signaling whereas higher concentrations are considered as toxic. It has been observed that disruption of NO pathways underlie many of the mechanisms underlying brain injury (Toda *et al.*, 2009). The NO overproduction is linked to excitotoxicity where excessive glutamate causes a state of hyperexcitability and consequently the massive influx of calcium ions and production of free radicals including NO (Wang and Qin, 2010). NO is widely regarded as a messenger molecule that participates in diverse physiological processes in the central nervous system (CNS), including brain development, pain perception, neuronal plasticity, memory and behavior (Ray *et al.*, 2007). It reacts rapidly with superoxide and produces a peroxynitrite which is highly reactive and has been shown to mediate much of the toxicity of nitric oxide (Bolanos *et al.*, 1997; Lipton *et al.*, 1998). In addition to its vasoactive and immunological properties, NO has significant neurophysiological functions; however, it can also be neurotoxic primarily due to its free radical properties (Law *et al.*, 2001). It is clear from the present data that the increase in NO levels in the forebrain after one and 3 weeks of oral EUD-NPs administration and in the midbrain after 3 weeks of oral administration was not associated with any corresponding changes in GSH and lipid peroxidation. Therefore, the recorded increase of nitric oxide due to EUD-NPs administration is tolerable and has no damaging effect. As no signs of brain damage was observed with the increased NO level due to the effect of oral administration of EUD-NPs on the forebrain and midbrain, therefore, this increase could have neuroprotective effects since NO can cause cerebral vasodilatation and improve the blood flow to the brain. Moreover, the increase in LPO level after different period of exposure to either PO or IP route suggested a damaging effect of EUD-NPs on lipids especially in the cell membrane.

The effect of EUD-NPs administration by the two routes was further confirmed by the histological examination of the brain tissues. Our results revealed that no significant pathological changes were observed. More importantly, the fluorescent microscopy examination revealed that EUD-NPs can reach the

brain after 4 h of administration, and the nanoparticles were also found 3 weeks after administration *via* the two routes. In this concern, Semete *et al.* (2010) demonstrated that polymeric PLGA poly(lactide-co-glycolide) nanoparticles of the same range size of EUD-NPs were found after one week in the brain without toxic effects in comparison to industrial metallic nanoparticles (Semete *et al.*, 2010). Previous reports suggested that the main mechanism of metal nanoparticles toxicity is due to an increase of the production of reactive oxygen species (ROS) resulting in oxidative stress, inflammatory cytokines production and consequent damages to proteins, membranes, DNA and in the end cell death (Nel *et al.*, 2006). However, in the case of polymer nanoparticles derived from copolymer with quaternary ammonium groups, the mechanism of oxidative stress may be different. The cytotoxicity of copolymer nanoparticles could be attributed to the molecular structure of the utilised monomers. The elongation of the monomer alkyl chain by four methylene groups increased the toxicity of the resulting nanoparticles. Moreover, the integrity of cell membranes is influenced by the substitution of the amino group. Therefore, it was concluded that the toxic effects of the investigated particle derivatives resulted from the incorporation of aminoalkyl chains into the lipid and/or lipoprotein (Zobel *et al.*, 1999). Similar to the current observation, Campbell *et al.* (2005) reported that some inflammatory biomarkers such as IL1 α and TNF α were increased in the lungs of mice exposed to ambient air particulate matter compared to controls.

CONCLUSION

From the results of the current study, it could be concluded that EUD-NPs of size about 200 nm nearly have the ability to cross the BBB and to reach the brain after oral or IP administration and that they stay for at least three weeks. They induced a little toxic effects. Despite these results, we cannot confirm their safe use as a drug carrier. This study is considered the first step of evaluation and further studies will be needed to verify the fate of these NP in the long term before using them as a vector.

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