Galantamine bioactivity improvement in rat brain using two novel nanodrug delivery systems as therapy for Alzheimer's disease.

***Sanaa M.R.Wahba; **Atef S. Darwish and *Sara M. Kamal.**

***** Zoology department, Women's College for Arts, Science and Education – Ain Shams University.

****** Chemistry department, Faculty of Science, Ain Shams University.

Abstract

Reminyl is one of the most common drugs presently used for treatment of mild to moderate Alzheimer. The present study is an attempt to investigate the influence of presenting two innovative nano-engineered drug carriers (Ce/Ca-HAp& CMCS/Ce/Ca-HAp) as carriers for Rem in Alz therapy. A total of 86 adult female albino Wistar rats were randomly assigned to the following groups: Control group (C) of 8 rats given saline; Reminyl group (Rem) of 8 rats treated with Rem; Alzheimer's group (Alz) of 16 ovariectomized animals inoculated orally with $AlCl_3$ (17mg/Kg b.wt/day) for 2 months after 6 weeks of surgical operation; Alzheimer's disease-induced rats treated (i.p) with Rem (2.5mg/Kg b.wt/day); Alzheimer's disease-induced rats treated (i.p) with Rem coated by Ce/Ca-HAp (2.5mg/Kg b.wt/day) and Alzheimer's disease-induced rats treated (i.p) with Rem coated by CMCS/Ce/Ca-HAp (2.5mg/Kg b.wt/day). After 2 and 4 weeks animals were sacrificed by ether inhalation anesthesia where brains were removed and processed for histological analysis by Hx&E and biochemical analysis for measuring GSH, SOD, CAT and CytoP450reductase levels. Histological and biochemical alterations designated in Rem treated rat brains were ameliorated following the use of Rem coated by Ce/Ca-HAp and not with Rem coated by CMCS/Ce/Ca-HAp. Initiation of new nano drug delivery systems may attribute in delivery of therapeutic drug to the site of action and overcome drawbacks of the drug alone.

Key words: Alzheimer (Alz), Ceria-doped calcium hydroxyapatite (Ce/Ca-HAp), Carboxymethyl Chitosan/Ceria/hydroxyapatite composite (CMCS/Ce/Ca-HAp), Reminyl (Rem).

1. Introduction

Alzheimer's disease (AD), a progressive neurodegenerative disorder that causes an irreversible degeneration in the brain ***(Brookmeyer et al., 2007),***is characterized by progressive neuronal cell dysfunction. It is accompanied by the formation of amyloid plaques in brain tissue. The major constituent of AD plaques is the amyloid β-peptide

Corresponding author: Sara Mostafa Kamal; Zoology department; Women's College for Arts, Science and Education – Ain Shams University,

email: sara.m.kamal88@gmail.com.

(A β), which is a product cleaved from the membrane-bound amyloid precursor protein (APP) by β- and γ-secretases**(Lee et al., 2011)** and intracellular neurofibrillary tangles **(Maija and Reisa, 2008).**

Around 250,000 people develop Alz annually. In Egypt more than 300,000 have the disease **(Middle East Journal, 2013).**

Marked atrophy of the cerebral cortex and loss of cortical neurons are the hallmarks of AD. The neuropathological investigations of AD, in human brain, illustrate amyloid rich senile plaques **(Selkoe, 2000)**, neurofibrillary tangles and neuronal degeneration with impairment of short-term memory.

Epidemiological studies have indicated a link between Aluminum (Al) in drinking water and AD and a variety of human and animal studies have implicated learning and memory deficits after Al exposure **(Exley, 2005).**

A comprehensive animal model imitating all the cognitive, behavioral, biochemical, and histopathological abnormalities observed in AD patients does not exist **(Rui et al., 2008).** Excessive Al intake might lead to memory impairments in rats **(Miu,2003),** deposition of amyloid protein in central nerve cells and overexpression of APP **(Campbell et al., 2000).**

Al has also been reported to alter the blood–brain barrier (BBB); as a result of which it gains an easy access to the central nervous system (CNS) under normal physiological conditions and accumulates in the different regions of brain **(Miu and Benga, 2006).** Further, Al being a potent cholinotoxin causes apoptotic neuronal loss which is related to high levels of acetyl cholinesterase (AChE) in the brain**.** The latter is associated with the loss in cognition observed during AD. Recent research shows that Al-induced neurotoxicity is also associated with apoptosis and oxidative stress **(Shati et al., 2011).**

Estrogen has been suggested to offer protection against the onset and progression of AD. Preclinically, it has been shown to be neuroprotective against oxidative stressors such as Aβ and H2O2 as well as stimulating the α-secretase pathway resulting in increased APP secretion, thereby possibly affording neuroprotection indirectly **(Goodenough et al., 2003).**

Four medications are approved by the **U.S. Food and Drug Administration** to treat Alzheimer. Donepezil (Aricept®), rivastigmine (Exelon®), or galantamine (Razadyne®, Reminyl *Rem*) are used to treat mild to moderate Alzheimer.Memantine (Namenda®), is used to treat moderate to severe Alzheimer **(Alzheimer's association, 2015).**

Galantamine is a competitive and reversible acetylcholinesterase (AChE) inhibitor, an activator of nicotinic Acetylcholine receptors (nAChRs) as well as allosterically modulator for nicotinic receptors (to increase acetylcholine release) and is used as a reverser of neuromuscular blockade. It is believed that it works by enhancing cholinergic function by increasing the concentration of acetylcholine in

the brain and enhancing cholinergic neuro-transmission in the brain. Previous studies have reported that the stimulation of nAChRs by galantamine and/or nicotine inhibits neuronal death induced by β-amyloid-enhanced glutamate neurotoxicity **(Kihara et al., 2004).**

One approach to reduce dosing amounts, frequency of administration, and adverse side effects while maintaining the drug efficiency, is the development of new drug delivery systems with inflammatory site targeting and long circulating time **(Hwang et al., 2008)**

Hydroxyapatite (HAp) has been extensively used in medicine for implant fabrication owing to its similarity with mineral constituents found in hard tissues (i.e. teeth and bones) **(Riman et al., 2001),** which leads to formation of bonds between the bone and the implanted materials being acted as biocompatible phase reinforcement in composites as well coatings to metal implants and granular fillers for direct incorporation into human tissues.

Implementation of new species in the HAp lattice offer fundamentally new possibilities and areas of their practical applications in biology and medicine.

Among most of the metallic species, ceria (CeO_2) nanoparticles exhibit high catalytic activity and a regenerative capacity to neutralize ROS. Ceria was found to protect cells against oxidative stress, inflammation, or damage caused by radiation. The particles are small and can cross the blood brain barrier. A neuroprotective effect of nanoceriahas been shown in many types of oxidative injury models**(Estevez and Erlichman, 2011).**

Carboxymethyl chitosan (CMCS) is one of the most investigated water-soluble derivatives of chitosan (CS) that own specific biological activities such as antitumor activity, immune-stimulating effects, enhancing protective effects against infection with some pathogens in mice, antimicrobial activity and radical scavenging activity. Also, the drug delivery systems prepared from CMCS-based formulations have received increasing attentions in recent years. Because of the carboxymethylation, CMCS possesses negative charges when dissolved in water, the CMCS hydrogels seem to be successfully prepared by physical crosslink with calcium-based biopolymers**(Luo et al., 2012).** The present study is an attempt to investigate the influence of presenting two innovative nano-engineered drug carriers (Ce/Ca-HAp& CMCS/Ce/Ca-HAp) as carriers for Rem in Alz therapy.

2.Material and Methods

Eighty six female adult albino Wistar rats from the Animal breeding colony of the Medical Research Centre Ain Shams University were employed in the present study. Their weights ranged between 180-200 gm representing an age group of 6 months. Animals were allowed a one week pre-experimentation period to adapt to laboratory conditions. They received food and water ad libitum with fresh supplies presented daily. The experimental rat groups were ovariectomized prior to induction with $AlCl_3$ for 2 months after one month post-operative procedure.

Drugs:

I-Aluminium chloride ($AlCl_3$) was purchased from Fluka Chemicals (Ronkonkama, NY) and was administered orally at doses of 17mg/kg b.wt. **(Krasovskii et al., 1979)**daily for two months.

II- Reminyl an Alzheimer's treatment derived from the bulbs of the daffodil, Narcissus pseudonarcissus and developed by the Janssen Research Foundation under a co-development and licensing agreement with the UK-based Shire Pharmaceuticals was i.p. injected at a dose of 2.5 mg/kg b.wt. for 2 and 4 weeks**(Iliev et al., 2000).**

Animals were divided according to the following design:-

a) Gonad intact animals (8) serving as normal control group (C).
b) Gonad intact animals (8) treated with Reminyl (Rem).
c) Ovariectomized animals (22) inoculated orally with $AlCl_3$ (17 mg/kg b.wt) daily for 2 months after a month of surgical operation (Alz).
d) Alzheimer's disease-induced animals (16) treated (i.p) with Rem (2.5 mg/kg b.wt.)for 2 and 4 weeks (Alz + Rem).
e) Alzheimer's disease-induced animals (16) treated (i.p) with Rem coated with Ce/Ca-HAp (2.5 mg/kg b.wt.) for 2 and 4 weeks (Alz + Rem +Ce/Ca-HAp).
f) Alzheimer's disease-induced animals (16) treated (i.p) with Rem coated with CMCS/Ce/Ca-HAp (2.5 mg/kg b.wt.) for 2 and 4 weeks (Alz +Rem + CMCS/Ce/Ca-HAP).

After 2 and 4 weeks rats were anaesthetized under ether inhalation and the brain was collected from all groups. Half of each brain was washed in saline (0.9% NaCl) and placed accordingly in 10% neutral buffered formalin for fixation and processed for histological analysis by Haematoxylin and Eosin for general histological examination **(Harris, 1900)**.

The other half was frozen at -80°C for biochemical analysis for the determination of Glutathione (GSH) by the method of **Tietze (1969);** Superoxide dismutase SOD by the method of (**Kuthan et al.,(1986);** brain catalase enzyme by the method of **Góth (1991)** and cytochrome $P_{450}$by the method **of Schenkman (1993).**

Statistical analysis

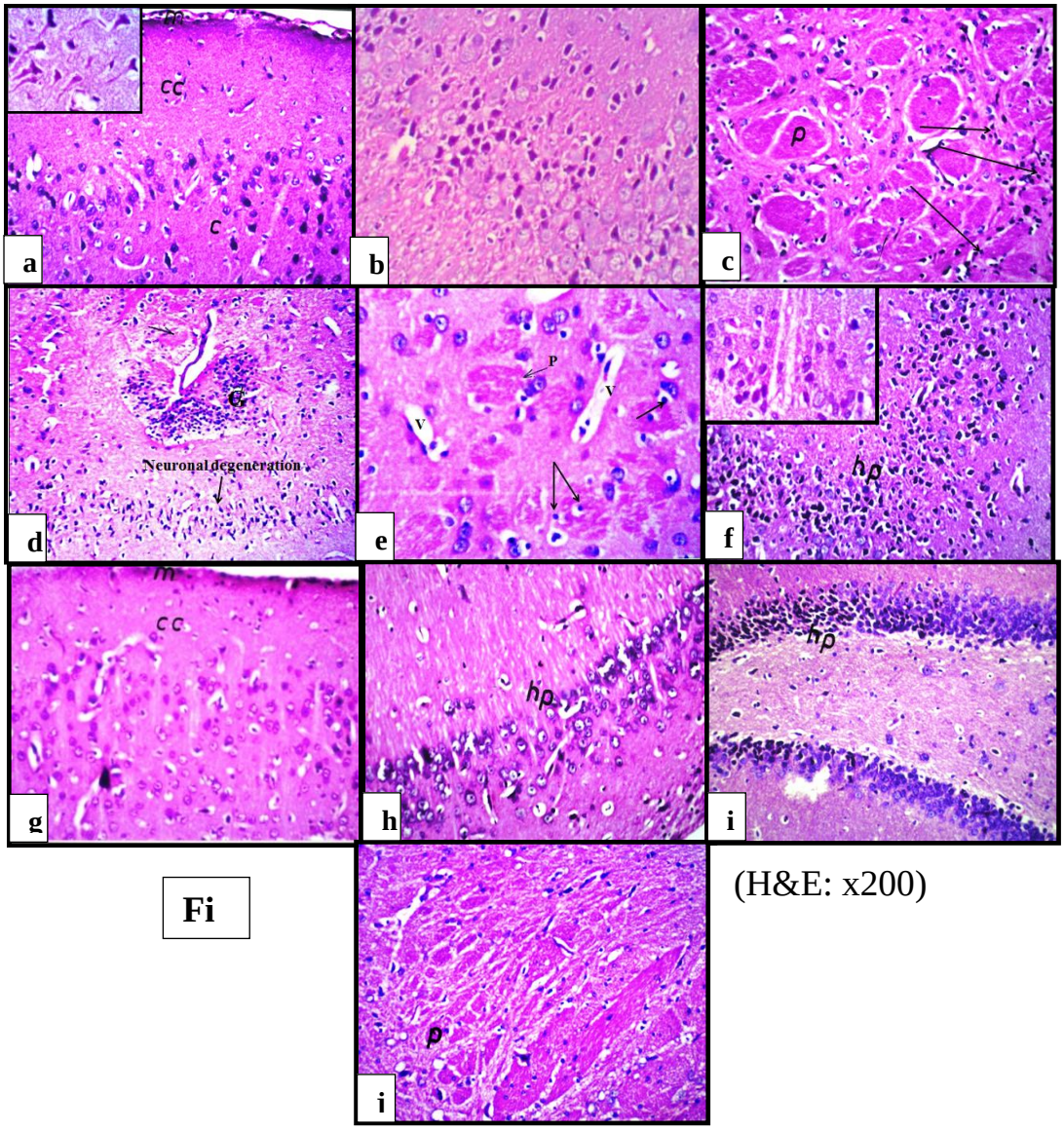
In the present study, all results were expressed as mean±S.E. of the mean. Statistical Package for the Social Sciences (SPSS) program, version 10.0 was used to compare significance between each two groups. Difference was considered significant when $P \leq 0.05$.

3.Results and Discussion:

3.1 Results

Histological investigations:

Hx&E staining data are shown in Fig (1) in control (Fig 1a) and experimental groups (Fig 1b-1j). Sections of rat brain from Rem group manifested no alterations from control. On the other hand following Alz induction, amyloid plaques of different sizes, congestion with perivascular edema, degenerated neurons with diffused gliosis, loss of pyramidal cells, separation of cortical tissue and formation of fibrous glial scar were manifested (Fig 1c & 1d). Following Rem therapy rat brain sections presented mild attenuation in amyloid plaques which persisted in a less aggressive manner with the progress of time; peri-vascular edema accompanied by diffused gliosis and degeneration in some of the hippocampal neurons (Fig. 1e & 1f). Using Rem coated by Ce/Ca-HAp designated near to normal patterns following 4 weeks of treatment (Fig 1g & 1h). While Rem coated by CMCS/Ce/Ca-HAp failed to induce ameliorative levels of recovery (Fig 1i & 1j).



Fi (H&E: x200)

Biochemical investigations:

In the present investigation, a significant depletion in the contents of GSH, SOD, CAT and $CytoP_{450}$reductase was shown in Alz-induced rats group as compared with the control. By treatment with Rem, limited improvement in each of GSH, SOD, CAT and $CytoP_{450}$reductase was recorded. On the other hand, treatment with Rem coated by Ce/Ca-HAp managed to improve the previous parameters to near to normal levels, while, Rem coated by CMCS/Ce/Ca-HAp failed to encounter any obvious ameliorations. These results validate fore mentioned histopathological alterations **(Table 1).**

Table (1): Therapeutic role of Reminyl or Reminyl coated by nanoparticles in brain tissue of control and experimental groups

		C	Rem	Alz	Alz+Rem	Alz+Rem+Ce/C	Alz+Rem+CMC
GSH (U/mg protein)	2 weeks	41.17^{A}_{a}± 1.423	39.20^{B}_{a}± 1.121	26.89^{C}_{b}± 0.832	30.26^{D}_{a}± 0.957	35.07^{E}_{a}± 0.988	32.79^{F}_{a}± 0.972
	4 weeks	40.93^{A}_{a}± 1.418	39.45^{A}_{a}± 0.931	25.47^{C}_{b}± 0.795	31.21^{D}_{b}± 1.086	38.54^{E}_{b} ±1.30	34.11^{F}_{b}± 1.117
SOD (U/mg protein)	2 weeks	3.08^{A}_{a} ±0.071	3.28^{A}_{a} ±0.071	1.59^{B}_{a} ±0.034	1.72^{C}_{a} ±0.045	2.12^{D}_{a} ±0.052	1.94^{E}_{a} ±0.045
	4 weeks	2.93^{A}_{b} ±0.068	2.83^{A}_{b} ±0.068	1.48^{B}_{b} ±0.029	1.97^{C}_{b} ±0.057	2.55^{D}_{b} ±0.071	2.01^{E}_{b} ±0.069
CAT (U/mg protein)	2 weeks	6.52^{A}_{a} ±0.173.	6.01^{B}_{a} ±0.073	4.39^{B}_{b} ±0.101	4.81^{C}_{a}±0.136	5.58^{D}_{a} ±0.148	5.09^{E}_{a} ±0.133
	4 weeks	6.49^{A}_{a} ±0.168	5.90^{B}_{a} ±0.168	4.80^{C}_{b} ±0.092	5.03^{D}_{b}± 0.149	6.43^{A}_{b} ±0.171	5.87^{B}_{b} ±0.152
$CytoP_{450}$ (ng/mg protein)	2 weeks	2.19^{A}_{a} ±0.023	1.90^{B}_{a} ±0.020	0.63^{C}_{a} ±0.017	1.02^{D}_{a}± 0.021	1.71^{E}_{a} ±0.029	1.25^{F}_{a} ±0.025
	4 weeks	2.11^{A}_{a} ±0.024	2.01^{A}_{b} ±0.025	0.958^{B}_{b}± 0.012	1.32^{C}_{b}± 0.024	2.04^{A}_{b} ±0.034	1.64^{D}_{b} ±0.028

3.2 Discussion

Alzheimer's disease is a neurological disorder in which the death of brain cells causes memory loss and cognitive decline. It is a neurodegenerative type of dementia; the disease starts mild and gets progressively worse ***(MNT,2014).***

In the present investigation, following Alz induction, several histological alterations were manifested as amyloid plaque formation of different sizes; congestion with perivascular edema; degenerated neurons with diffused gliosis; loss of pyramidal cells; separation of cortical tissue and formation of fibrous glial scar.

Similar changes were perceived by ***Rebai and Djebli (2008)*** who stated that the brains of experimental animals, studied by optical microscopy, displayed massive cellular depletion in the hippocampal formation with neurofibrillary degeneration.

They observed numerous ghost-like neurons with cytoplasmic and nuclear vacuolations.

It was claimed that Al can interact with the peripheral sites of AChE and modify its secondary structure and eventually its activity **(Kakkar andKaur, 2011).** Aluminum salt has been found to induce the overexpression of APP.

Accordingly, present manifestations may be due to inflammatory responses that are known to play an important role in neurodegenerative disease **(Rebai and Djebli, 2008)**.

Vallés et al. (2008) have demonstrated that Aβ peptide causes oxidative stress in the neurons and inflammation in the astrocytes in the primary culture indicating that this toxic peptide can affect not only neuronal cells but astrocytes too.

Wu et al. (2012) who suggested that, the amyloid plaques caused by Al administration plays a role in the pathology of AD by directly inducing neuronal cytotoxicity and stimulating microglia to secrete cytokines and reactive oxygen species (ROS) which also damage neurons. Amyloid plaques could activate astrocytes and oligodendrocytes to produce chemokines, in particular Monocytes chemotactic protein(MCP-1), which serves as potent in vitro microglial and macrophage chemo attractants. Plaques have been shown to activate astrocytes to upregulate proinflammatory cytokine expression, therefore plaques mediated astrocytes activation that initiates the inflammatory cascade.

Various inflammatory mediators under neuronal injury exacerbate the neurotoxic environment. Thus, anti-inflammatory strategy therapies have continually been considered to reduce the risks of various neuropathologies**(Nam et al., 2010).**

Although, there is no cure**.** for AD, there are five prescription drugs approved by the U.S. Food and Drug Administration (FDA) to treat its symptoms. Donepezil, Galantamine (Rem), Rivastigmine and Tacrine which belong to "cholinesterase inhibitors".**(Alzheimer's association, 2015).**

The benefits of galantamine were consistent across the different studies. AD treatment guidelines in the UK and USA agree that only galantamine has demonstrated benefits on cognitive, functional, global and behavioral outcomes **(Clegg et al., 2001).**

In the concurrent study, it was noticed that Alz-induced rats treated with Rem showed mild attenuation of amyloid plaques which persisted in a less aggressive manner with the progress of time; peri-vascular edema accompanied by diffused gliosis and degeneration in some of the hippocampal neurons**Bhattacharya et al. (2014).**

Galantamine inhibits the breakdown of ACh by binding competitively and reversibly to the active site on AChE. Of particular importance are the inhibitory effects of galantamine on AChE in the frontal cortex and hippocampal regions of the brain. The two areas in which cholinergic neuro transmission is most affected in patients with AD and also galantamine makes nAChRs more sensitive to ACh, potentiating their response to available ACh. Moreover, electrophysiological

experiments have consistently shown that,of the actively marketed drugs, galantamine is the only cholinergic treatment for AD that allosterically modulates nAChRs in this way**(Lilienfeld, 2002).**

Thus, present attenuation in amyloid plaques and persistence of some of histological lesions may be attributed to the advanced levels of the disease that interfere with the release of neurotransmitters. This may also attenuate its apoptotic action.

The nanotreatment methods for AD are numerous. They are categorized as neuroprotective methods from toxicity of amyloid-β peptide (Aβ) oligomers, oxidative stress of free radicals and nanocarriers for targeted drug delivery **(Nazem&Mansoori, 2011).**

Present results manifested cortical tissue separation with limited changes in glial cells where some showed pyknotic changes; neuronal degeneration and congestion in blood vessels following Rem coated by Ce/Ca-HAp. After 4 weeks of treatment, near to normal cerebral patterns; normal neuronal cells; complete disappearance of amyloid plaques were manifested. On the other hand, treatment with Rem coated by CMCS/Ce/Ca-HAp failed to induce ameliorative levels of recovery. Presently, persistence of amyloid plaques; diffused gliosis; degenerative changes in neuronal & glial cells; clear signs of pyknosis were shown by some cells. Similar results were encountered after 4 weeks of treatment with congestion & perivascular edema.

Occasionally, various modification procedures for hydroxyapatite (HAp) solids become of great necessity allowing new generations of hydroxyapatites to be used for the first time as galantamine drug delivery carrier in curing of Alzheimer's disease.

To raise the effectiveness stature of biomedical applications of hydroxyapatites, it could be doped by various metallic species like Fe, Cu, Zn, Ni and gold**(Dominguez et al., 2008)** among most of the metallic species, ceria (CeO_2) nanoparticles is used. Another strategy for modification of HAp lattice has been based on developing some uncommon composites that gather hydroxyapatite lattice by specific polymeric matrices of vital biomedical features such as carboxymethyl chitosan hydrogels**(Kong et al., 2006).**

In the present investigation, a significant depletion in the contents of GSH, SOD, CAT and CytoP_{450}reductase was designated in Alz-induced rats group as compared with the control group.

Presently as Alz-induced rats were treated with Rem limited improvement in each of GSH, SOD, CAT and CytoP_{450}reductase was recorded. On the other hand, treatment with Rem coated by Ce/Ca-HAp manages to improve the previous parameters to near to normal levels, while, Rem coated by CMCS/Ce/Ca-HAp failed to encounter any obvious ameliorations. Such results confirm re-mentioned histological parameters.

SOD presents the first line of defense against the produced super oxides, as it dismutase the superoxide anion to H_2O_2 and O_2**.** Because the SOD enzyme

generates H_2O_2, it works in collaboration with H_2O_2 removing enzymes e.g. catalase which converts H_2O_2 to water and oxygen. Catalase is present in the peroxisomes of mammalian cells, and probably serves to destroy H_2O_2 generated by oxidase enzymes located within these sub cellular organelles **(Kakkar andKaur, 2011).** Finally, glutathione peroxidases catalyze the conversion of hydrogen and lipid peroxides into less harmful metabolites **(Estevez and Erlichman, 2011)**

5. Conclusion

The present study confirms that Reminyl, which is one of the most common used drugs in treatment of AD showed mild recovery. So, initiation of new drug delivery systems may attribute in delivery of the drug to the site of action and overcomesuch therapeutic drawback. Thus comes the role of nanotechnology with its different nano-carriers in such systems allowing a possible potential decrease of dose intake and inducing successful therapeutic role.

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تحسين الفاعلية الحيوية لعقار الجلانتامين فى مخ الفئران باستخدام اثنان من الانظمة النانونية المستحدثة كعلاج لمرض الالزهايمر

*سناء محمد رفعت وهبة- **عاطف سمير درويش-*سارة مصطفى كمال

*قسم علم الحيوان- كلية البنات للاداب والعلوم والتربية – جامعة عين شمس

**قسم الكيمياء- كلية العلوم – جامعة عين شمس

مرض الالزهايمر هو مرض عصبى ويعتبر عقار الريمينيل وهو المنتج التجارى للجلانتامين من أهم الادوية المستخدمة لعلاج الحالات الخفيفة والمتوسطة للمرض مما يؤدى الى تحسن الذاكرة والوعى والقدرة على أداء المهام اليومية. ومن احدى طرق تثبيط جرعات التعاطى ومدتها وتقليل الاعراض الجانبية مع المحافظة على فاعلية العقار هى تطوير نظم توصيل جديدة تستهدف بالعقار الى مكان الالتهاب مباشرة ويعمل على زيادة فترة دوران العقار. ان من المتوقع أن استخدام ناقلات حجم النانو فى توصيل العقار تعمل على زيادة الفاعلية الحيوية للعقار, وبالتالى تقليل الاثار الجانبية عن طريق خفض الجرعة المستخدمة من العقار.

وقد أجريت الدراسة على ٨٦ من اناث الجرذان البيضاء البالغة. وتم تقسيم الجرذان عشوائيا الى مجموعتين (المجموعة الظابطة والمجموعة التجريبية) ثم تم ازالة المبيضين من الحيوانات التجريبية. ثم تم تقسيم الجرذان الى ٦ مجموعات تجريبية: المجموعة الظابطة, مجموعة عوملت بعقار الريمينيل وهى حيوانات سليمة الغدد التناسلية, مجموعة حيوانات مستأصلة المبيضين وحقنت عن طريق الفم ب ١٧ مجم الومنيوم كلوريد لمدة شهرين بعد ٦ أسابيع من العملية الجراحية لاحداث المرض, مجموعة مريضة عولجت ب 2.5 مجم ريمينيل/ كجم/يوم لمدة اسبوعين وأربع أسابيع, مجموعة مريضة عولجت ب 2.5 مجم ريمينيل محمل على نانو هيدروكسي اباتيت الكالسيوم المطعم بالسيريوم/ كجم/يوم لمدة اسبوعين وأربع أسابيع, مجموعة مريضة عولجت ب2.5مجم ريمينيل محمل على نانو هيدروكسي اباتيت الكالسيوم المطعم بالسيريوم مع الكربوكسي ميثيل كيتوسان/ كجم/يوم لمدة اسبوعين وأربع أسابيع.

ثم بعد أسبوعين من الحقن تم اختيار نصف عدد الجرذان عشوائيا من كل مجموعة وبالمثل استخدمت بقية الجرذان بعد مرور أربع أسابيع. وأجريت عمليات التشريح وازالة الرؤوس لاخذ المخ لاجراء دراسة نسيجية باستخدام صبغة الهيماتوكسلين والايوسين ودراسة بيوكيميائية تحليلية لقياس معدلات الجلوتاثيون وثنائى كبريتيد الجلوتاثيون والكتالاز والسوبر أكسيد ديسموتاز وسيتو كروم ب٤٥٠ ريدكتاز.

أظهرت النتائج النسيجية لمخ الجرذان المصابة بالمرض الى تدهور الخلايا العصبية ووجود ترسبات من لويحات الاميلويد مع وجود انتفاخ واستسقاء حول الأوعية ودباق بؤري في القشرة المخية والهيبوكامبس مقارنة مع المجموعة الظابطة. وهذه الخصائص الهيستولوجية من أهم الصفات المميزة لمرض الالزهايمر.وأدى العلاج باستخدام عقار الريمينيل منفردا أو محملا على نانو هيدروكسي اباتيت الكالسيوم المطعم بالسيريوم مع الكربوكسي ميثيل كيتوسان الى انخفاض بطئ للظواهر السابقة. وبدأ نسيج المخ فى العودة الى الانماط الطبيعية عند المعالجة باستخدام عقار الريمينيل محملا على نانو هيدروكسي اباتيت الكالسيوم المطعم بالسيريوم.

كما أظهرت مجموعة الجرذان المصابة بالمرض عن وجود ارتفاع ملحوظ فى معدلات الجلوتاثيون والكاتلاز وسوبر اكسيد ديسموتاز وسيتوكروم ب٤٥٠ريدكتازز ظهر تحسن معتدل عند العلاج باستخدام عقار الريمينيل منفردا أو محملا على نانو هيدروكسي اباتيت الكالسيوم المطعم بالسيريوم مع الكربوكسي ميثيل كيتوسان وتحسن طبيعيا الى حد ما عند استخدام عقار الريمينيل محملا على نانو هيدروكسي اباتيت الكالسيوم المطعم بالسيريوم. وبالتالى أكدت هذه النتائج النتائج النسيجية المذكورة سابقا.