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Galantamine therapy for Alzheimer's disease by introducing nanodrug delivery systems

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Introduction
The major cause of dementia, a major public health problem, is Alzheimer's disease (AD). A reliable method for the diagnosis and follow up of AD is needed together with a specific biological marker. Galantamine (Gal), an acetylcholinesterase inhibitor for AD therapy, has several reported side effects.
Aim of the Work
One approach to reduce dosing amounts, frequency of administration, and adverse side effects while maintaining the drug efficiency is the development of drug delivery systems using nanoparticles.
Material and Methods
Presently, Galantamine with either cerium/calcium hydroxyapatite (Ce/Ca-HAp) or carboxymethyl chitosan/ceria/calcium hydroxyapatite (CMCS/Ce/Ca-HAp) was intraperitoneally injected at a dose of 2.5 mg/kg body weight for 2 and 4 weeks. A total of 86 female adult albino Wistar rats (189–200 g weight) were used. AD was induced in ovariectomized rats with aluminum chloride oral treatment at doses of 17 mg/kg body weight daily for 2 months. The rats were divided into six groups: group 1, which included normal control rats; group 2, which included rats treated with Gal injected intraperitoneally at a dose of 2.5 mg/kg body weight; group 3, which included rats with AD; group 4, which included AD-induced rats treated intraperitoneally with Gal; group 5, which included AD-induced rats treated with Gal coated with Ce/Ca-HAp; group 6, which included AD-induced rats treated with Gal coated with CMCS/Ce/Ca-HAp for 2 and 4 weeks.
Results
In the current study, AD-induced histological alterations 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. Several tests may be cumulatively used for early detection as decreased acetylcholine, B-cell lymphoma 2, tissue thromboplastin, GSH, superoxide dismutase, CAT, and cytochrome P450 and increased amyloid β, Chol, brain-type fatty acid binding protein, nitric oxide, MDA, and GSSH. Treatment with Gal coated with Ce/Ca-HAp imposed a highly significant improvement to near-to-normal levels in both histological and biochemical parameters. Gal coated with CMCS/Ce/Ca-HAp failed to encounter obvious ameliorations.
Conclusion
In conclusion, brain markers (acetylcholine, B-cell lymphoma 2, amyloid β, tissue thromboplastin, Chol, brain-type fatty acid binding protein, nitric oxide, and MDA) together with brain antioxidants (GSH, superoxide dismutase, CAT, and cytochrome P450) may be used in progressive laboratory testing method besides well-known imaging techniques. Gal therapy may impose limited improvements; thus, drug delivery systems using Gal coated with Ce/Ca-HAp may aid in minimizing dosing amounts, frequency of administration, and adverse side effects of drug while increasing its therapeutic efficacy.

Keywords:
Alzheimer's, disease, carboxymethyl chitosan/ceria/hydroxyapatite composite, ceria-doped calcium hydroxyapatite, galantamine, histology and biochemistry, rat

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Introduction

Dementia is one of the major public health problems, in which the increasing numbers of patients impose a major financial burden on healthcare systems. It is majorly related to aging, and with the increasing numbers of elderly people in the population, the

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number of patients with dementia is growing rapidly. The major cause of dementia is Alzheimer's disease (AD). Thus, more than half of the patients with dementia have AD. AD is a progressive neurodegenerative disorder of the brain that is characterized by loss of neurons because of extracellular accumulation of amyloid β (Ab) and intracellular hyperphosphorylation of the τ protein (Skoumalova and Hort, 2012). It is an etiologically heterogeneous, multifactorial disease. However, a proper pathophysiological mechanism of the disease's evolution is very complex and involves many biochemical mechanisms (Skoumalova and Hort, 2012).

Risk factors for AD are age and a positive family history of dementia, as more than one-third of AD patients have one or more affected first-degree relatives (van Duijn *et al.*, 1991a). Other risk factors that may be associated with the development of AD include severe head trauma, low levels of education, female sex, previous depression, and vascular factors (van Duijn *et al.*, 1991b; Kivipelto *et al.*, 2001).

Neuropsychological deficits in AD include changes in a variety of cognitive functions, such as episodic memory, language, semantic memory, executive abilities, attention, and visuospatial and visuoperceptual processes. Neuropsychological tests are often influenced by education (Doraiswamy *et al.*, 1995), practice (Galasko *et al.*, 1993), and sociocultural or ethnic factors (Manly *et al.*, 1998). As a screening test for dementia, the sensitivity of mini-mental state examination test has been 56–90% and specificity has varied from 85 to 95% (Koivisto, 1995).

Macroscopic changes found in AD brain include shrinkage of the gyri and widening of the sulci, especially in the frontotemporal areas, thickening of leptomeninges, and enlargement of the ventricles. The two major microscopical lesions are amyloid plaques and neurofibrillary tangles (NFTs), which are found significantly more often in AD compared with normal aging.

The diagnostic usefulness of different imaging methods in AD has been widely studied. Computed tomography does not differentiate early AD from normal aging with high diagnostic accuracy (DeCarli *et al.*, 1992), but it is useful in detecting some causes of dementia. Similarly, single-photon emission computed tomography may be useful in the differential diagnosis of dementia (Talbot *et al.*, 1998). MRI offers a superior anatomic discrimination power and permits accurate imaging of the affected regions.

Thus, a reliable method for the diagnosis and follow up of AD is needed.

One possibility to improve the antemortem diagnosis, follow-up, and screening of the efficacy of therapies on AD would be to develop a specific biological marker. An ideal biomarker should detect a fundamental feature of the neuropathology of AD, be able to detect AD early in the course of the disease with high sensitivity, distinguish it from other dementias, and be validated in neuropathologically confirmed AD cases.

An ideal biological marker would identify AD cases at a very early stage of the disease, before the cognitive symptoms are found in neuropsychological tests, and before there is degeneration in brain imaging studies. A biomarker reflecting neuropathological changes at the molecular level in AD brain would be very useful in the differential diagnosis of dementia, and in distinguishing AD patients from those individuals with mild cognitive impairment who do not develop AD, and from patients with depression (Borroni *et al.*, 2004).

Studies on biochemical markers for the diagnosis of AD in cerebrospinal fluid and blood are based on the detection of inflammatory proteins, markers of cholesterol homeostasis, oxidative stress, or related to characteristic pathological alterations in AD (Teunissen *et al.*, 2002).

Cognitive deficits in AD have been widely associated with dysfunction of the cholinergic system (Francis *et al.*, 1999) as a result of degeneration of cholinergic cell bodies in the basal forebrain (Ramirez, 2004). On examining postmortem frontal cortex from AD patients, reduced levels of acetylcholine (ACh), cholinacetyltransferase, and acetylcholinesterase (AChE) activity were seen compared with controls (Gil-Bea *et al.*, 2005). Consequent to the cholinergic hypothesis of dementia, animal studies have been used to evaluate the role of cholinergic neurotransmission in learning and memory and selective lesion of cholinergic neurons remain nowadays as a widely used method to mimic some aspects of neurodegeneration in AD (Tariot, 2001).

B-cell lymphoma 2 (Bcl-2) is the founding member of the Bcl-2 family of regulator proteins that regulate cell death (apoptosis), by either inducing (proapoptotic) or inhibiting (antiapoptotic) apoptosis. Bcl-2 is

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specifically considered as an important antiapoptotic protein and is thus classified as an oncogene. The fact that Bcl-2 is expressed in neurons in the aged human brain complements observations suggesting neuronal death by apoptosis in PD and AD patients (Anglade *et al.*, 1997).

A critical role of the Ab in the pathogenesis of AD has been supported by human, animal, and in-vitro studies (Hardy and Selkoe, 2002). Intracellular and membrane-associated Ab, especially Ab-42 in the temporal neocortex, may be more closely related to AD symptoms compared with other measured Ab species (Steinerman *et al.*, 2008).

Tissue factor [tissue thromboplastin (TPL)] is the primary initiator of the extrinsic coagulation pathway. In contrast to other coagulation factors, it is an integral membrane glycoprotein that does not circulate as a plasma protein (Carson, 1984). Tissue factor was expressed diffusely in the neocortex, but in AD there was enhanced immunoreactivity in senile plaques. Although tissue factor might potentially contribute to the formation of senile plaques, it could also accumulate in the plaques as a secondary response to other biochemical perturbations (McComb *et al.*, 1991).

A previous work in cell cultures and with animals suggested that cholesterol plays an important role in promoting the deposition of amyloid in the brain (Reed *et al.*, 2014).

They reported that participants who had higher levels of low-density lipoprotein cholesterol and lower levels high-density lipoprotein cholesterol had higher levels of amyloid in the brain. Brain-derived metabolites of cholesterol also appear to be elevated in the early stages of disease before the onset of cognitive impairment, rendering them an important marker of underlying cerebrovascular disease preceding cognitive impairment and risk for developing cognitive impairment (Hughes, 2011).

Brain-type fatty acid binding proteins (B-FABPs) (Myers-Pane *et al.*, 1996) may be suitable markers for the detection of brain injury. B-FABP is a member of a family of nine distinct FABP types, each named after the tissue in which it was first detected (Glatz and van der Vusse, 1996). FABPs are 15-kDa cytoplasmic, nonenzymatic proteins involved in the intracellular buffering and transport of long-chain fatty acids. They are rapidly released from damaged cells into the circulation and are cleared from the circulation by the kidney with a plasma half-life of 20 min (Glatz *et al.*, 2002). B-FABP was first identified in the brains of rodents and showed diverse tissue production during development (Myers-Pane *et al.*, 1996). In adult-stage mice, B-FABP is produced in very low concentrations and is detected only in glial cells (presumptive astrocytes) of the white matter (Pu *et al.*, 1999).

Nitric oxide (NO) is an atypical neurotransmitter and is formed from l-arginine by the enzyme NO synthase (Shukla, 2007). NO plays a modulatory role in the brain, controlling the release of neurotransmitters, and is involved in synaptogenesis, synaptic plasticity, memory function, and neuroendocrine secretion.

NO is a free radical species, a well-known physiological signaling agent, and a pleiotropic regulator in various pathologies, including tumor growth and AD (Aliev *et al.*, 2009). NO-dependent oxidative stress results in mitochondrial ultrastructural alterations and DNA damage in cases of AD (Aliev *et al.*, 2011). In fact, it plays a crucial role in mitochondrial respiration (Moncada and Erusalimsky, 2002), as even low (nmol/l) concentrations of NO were found to reversibly inhibit the mitochondrial respiratory chain enzyme cytochrome oxidase (complex IV) and compete with molecular oxygen. Inhibition of cytochrome oxidase by NO results in the reduction of the electron-transport chain and favors the formation of the superoxide radical anions (O_2^-). NO upon reaction with superoxide radical anion forms peroxynitrite ($ONOO^-$), which is more cytotoxic than NO itself (Aliev *et al.*, 2009).

MDA arises largely from the peroxidation of polyunsaturated fatty acid. It exists either in a free form or bound to proteins. Free MDA *in vivo* is rapidly metabolized in tissues. A number of studies have documented elevated levels of MDA in AD in the plasma/serum (Greilberger *et al.*, 2008; Sinem *et al.*, 2010).

The most prevalent antioxidant in most brain cells is GSH. It can react with reactive oxygen species (ROS) and oxidized products forming glutathione disulfide (GSSG), either catalyzed by glutathione peroxidase or independently. The GSSG can then be converted back to reduced GSH by glutathione reductase. Studies of human brains have indicated that the ratio between reduced and oxidized glutathione (GSH/GSSG) is decreased in affected brain regions of AD patients compared with controls (Benzi and Moretti, 1995). Superoxide dismutase (SOD) is a part of the initial defense against ROS and catalyses the conversion of

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O_2 to H_2O_2 and O_2. The activity of SOD in serum was reduced in AD patients compared with controls and was negatively correlated to the lipid peroxidation marker MDA (Padurariu *et al.*, 2010).

Galantamine (Gal), the AChE inhibitor that is widely used for AD therapy, is a plant extract of the commercial snowdrop and snowflake that are the early spring flowers that break through the snow. It may serve to prevent hypertension from precipitating vascular dementia, which is the second most common cause for dementia after AD. The cerebrovascular injury caused by hypertension has been associated with compromised cognitive function, mainly memory dysfunction and decreased speed of information processing. It was reported that Gal works by enhancing cholinergic function by increasing the concentration of ACh in the brain and enhancing cholinergic neurotransmission in the brain (Kihara *et al.*, 2004). Nevertheless, side effects from the drug have been thoroughly reported when higher doses are gradually required.

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 the formation of bonds between the bone and the implanted materials, and acts as biocompatible phase reinforcement in composites as well as coatings to metal implants and granular fillers for direct incorporation into human tissues. Implementation of new species in the HAp lattice offers fundamentally new possibilities and areas of their practical applications in biology and medicine.

Again, 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 (Estevez and Erlichman, 2011).

Carboxymethyl chitosan (CMCS) is one of the most investigated water-soluble derivatives of chitosan that own possesses 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. Because of the carboxymethylation, CMCS possesses negative charges when dissolved in water; the CMCS hydrogels seem to be successfully prepared by means of physical crosslink with calcium-based biopolymers (Luo *et al.*, 2012).

Successful therapeutic regimens for incurring favorable pathological influence for AD necessitate reliable earlier diagnosis for the onset of clinical settings rather than the stage of dementia. They should be highly specific to AD and sensitive to changes, especially in the early stages of the disease (Skoumalova and Hort, 2012). One possibility would be to develop specific biological marker.

Thus, the aim of the present study was to identify several sensitive biological markers to detect fundamental features of the neuropathology of AD in its earlier stages. As Gal seems to impose serious side effects besides its poor therapeutic capabilities, new delivery systems using nanoparticles are given.

Materials and methods

A total of 86 female adult albino Wistar rats (189–200 g weight) from the animal breeding colony of the Medical Research Centre Ain Shams University were used. After 1 week of pre-experimentation adaptation (food and water *ad libitum*) period, AD-induced rats were ovariectomized before aluminum chloride oral treatment at doses of 17 mg/kg body weight (Krasovskii *et al.*, 1979) daily for 2 months at 1 month postoperatively.

Gal was injected intraperitoneally at a dose of 2.5 mg/kg body weight for 2 and 4 weeks (Iliev *et al.*, 2000).

Gal was coated with either cerium/calcium hydroxyapatite (Ce/Ca-HAp) or carboxymethyl chitosan/ceria/calcium hydroxyapatite (CMCS/Ce/Ca-HAp) and injected intraperitoneally at a dose of 2.5 mg/kg body weight for 2 and 4 weeks.

The animals were allotted as follows:

(1) Group 1 (control group) included gonad intact animals (Koivisto, 1995).
(2) Group 2 (Gal group) included gonad intact animals (Koivisto, 1995) treated with Gal.
(3) Group 3 (AD group) included ovariectomized animals (Reed et al., 2014) treated orally with

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aluminum chloride (17 mg/kg body weight) daily for 2 months after 1 month from surgery.

(4) Group 4 (AD+Gal group) included AD-induced rats (Tariot, 2001) treated intraperitoneally with Gal (2.5 mg/kg body weight).
(5) Group 5 (AD+Gal+Ce/Ca-HAp group) included AD-induced animals (Tariot, 2001) treated (intraperitoneal) with Gal coated with Ce/Ca-HAp (2.5 mg/kg body weight).
(6) Group 6 (AD+Gal+CMCS/Ce/Ca-HAp group) included AD-induced animals (Tariot, 2001) treated intraperitoneally with Gal coated with CMCS/Ce/Ca-HAp (2.5 mg/kg body weight).

After 2 and 4 weeks, the rats were anesthetized by means of ether inhalation and brains were collected from all groups. One half of each brain was homogenized immediately to obtain 10% (w/v) homogenate in ice-cold medium containing 50 mmol/l Tris–HCl (pH 7.4) and 300 mmol/l sucrose. The homogenate was centrifuged at 3000 rpm for 10 min at 4°C. The supernatant (10%) was separated for biochemical estimations.

The quantitative measurement of brain markers included ACh levels (Ellman and Courtney, 1961), Bcl-2 (Barbareschi *et al.*, 1996), Ab (David *et al.*, 2000), TPL (Bolhuis *et al.*, 1982), Chol (Sidel *et al.*, 1983), B-FABP (Liu *et al.*, 2010), NO (Nathan and Matthew, 2007), and MDA (Moore and Roberts, 1998). Brain antioxidants included GSH (Tietze, 1969), GSSH, SOD (Kuthan *et al.*, 1986), CAT (Góth, 1991), and cytochrome P450 reductase (Schenkman, 1993).

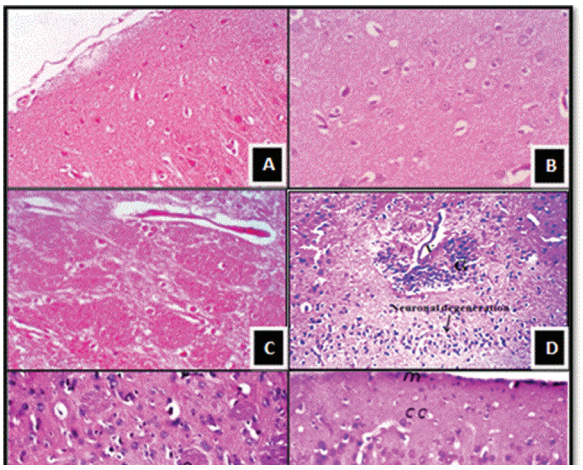
The other half was washed in saline (0.9% NaCl) and placed accordingly in 10% neutral-buffered formalin for fixation and processed for histological analysis with hematoxylin and eosin for general histological examination (Harris, 1959).

Results

Histological investigations

Hematoxylin and eosin staining data are shown in Fig. 1 for the control (Fig. 1a) and experimental groups (Fig. 1b–h). Sections of rat brain from Gal animals manifested no alterations from control (Fig. 1b). In contrast, following AD 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 and d). Following Gal therapy, sections of rat brain showed mild attenuation in amyloid plaques, which persisted in a less aggressive manner with the progress of time, and perivascular edema accompanied by diffused gliosis and degeneration in some of the hippocampal neurons (Fig. 1e). On using Gal coated with Ce/Ca-HAp, near-to-normal patterns were observed following 4 weeks of treatment (Fig. 1f). However, Gal coated with CMCS/Ce/Ca-HAp failed to induce ameliorative levels of recovery (Fig. 1g and h).

Figure 1



Photomicrograph of brain sections of rats from the control and experimental groups. (a) Normal control. (b) The galantamine (Gal)-treated group showing no alterations from controls. (c, d) Alzheimer's disease-induced rats showing amyloid plaques of different sizes, perivascular edema, and diffused gliosis. (e) Alzheimer's disease-induced rats after Gal therapy showing persistence of amyloid plagues and diffused gliosis. (f, g) Alzheimer's disease-induced rats treated with Gal coated with cerium/calcium hydroxyapatite showing near-to-normal patterns in the cerebral cortex and hippocampus. (h) Alzheimer's disease-induced rats treated with Gal coated with carboxymethyl chitosan/ceria/calcium hydroxyapatite showing persistence of lesions. Hematoxylin and eosin, ´200. cc, the cerebral cortex; hp, hippocampus; p, amyloid plaques.

Biochemical investigations

Brain marker profile

There were significant decreases in ACh, Bcl-2, and TPL in brain tissue from AD-induced rats as compared with normal control animals. In contrast, significant increases in Ab, Chol, B-FABP, NO, and MDA were recorded in the same group of rats. All parameters were significantly ameliorated to near-to-normal figures following treatment with Gal coated with Ce/Ca-HAp and not Gal coated with CMCS/Ce/Ca-HAp, which failed to impose ameliorative figures (Table 1).

Brain enzymes antioxidants profile

Significant depletion was obvious in GSH, SOD, CAT, and cytochrome P450 reductase in the brain tissue of AD-induced rats as compared with normal controls. Partial amendment in above

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Table 1 Concentration of different brain markers in brain tissue samples from AD-induced rats and rats treated with Gal, Gal+Ce/Ca-HAp and Gal+CMCS/Ce/Ca-HAp

	Control	AD	AD+Gal	AD+Gal+Ce/Ca-HAp	AD+Gal+CMCS/Ce/Ca-HAp
ACh (μmol/mg protein)					
2 weeks	$100.67\pm3.22^{A}_{a}$	$71.68\pm2.24^{B}_{a}$	$83.29\pm2.84^{C}_{a}$	$90.54\pm2.93^{D}_{a}$	$84.01\pm2.87^{E}_{a}$
4 weeks	$101.42\pm3.32^{A}_{a}$	$67.55\pm2.16^{B}_{a}$	$94.68\pm3.09^{C}_{b}$	$97.76\pm3.23^{D}_{a}$	$94.88\pm3.11^{E}_{b}$
Bcl-2 (pg/mg protein)					
2 weeks	$92.33\pm2.91^{A}_{a}$	$58.93\pm1.78^{B}_{a}$	$71.45\pm2.31^{C}_{a}$	$77.31\pm2.47^{D}_{a}$	$72.04\pm2.35^{E}_{a}$
4 weeks	$92.42\pm2.94^{A}_{a}$	$54.27\pm1.49^{B}_{b}$	$80.73\pm2.55^{C}_{b}$	$86.55\pm2.69^{D}_{b}$	$81.26\pm2.58^{E}_{b}$
Amyloid peptide (pg/mg protein)					
2 weeks	$13.26\pm0.53^{A}_{a}$	$25.77\pm0.93^{B}_{a}$	$19.05\pm0.76^{C}_{a}$	$17.25\pm0.69^{D}_{a}$	$18.79\pm0.71^{E}_{a}$
4 weeks	$13.21\pm0.51^{A}_{a}$	$29.02\pm1.12^{B}_{b}$	$15.82\pm0.62^{C}_{b}$	$14.44\pm0.58^{D}_{b}$	$15.63\pm0.64^{E}_{b}$
TPL (μg/mg protein)					
2 weeks	1277.4±21.59	785.41±15.78	953.68±19.25	1077.43±21.03	961.22±19.43
4 weeks	1259.8±20.87	694.33±14.25	1104.55±22.17	1269.55±22.86	1127.38±22.06
Chol (μg/mg protein)					
2 weeks	$648.66\pm13.16^{A}_{a}$	$1008.29\pm23.56^{B}_{a}$	$870.56\pm18.82^{C}_{a}$	$801.21\pm17.41^{D}_{a}$	$863.98\pm18.77^{E}_{a}$
4 weeks	$652.51\pm13.19^{A}_{a}$	$1152.65\pm25.78^{B}_{b}$	$749.91\pm15.33^{C}_{b}$	$682.32\pm14.62^{D}_{b}$	$736.72\pm15.18^{E}_{b}$
B-FABP (pg/mg protein)					
2 weeks	$39.91\pm2.14^{A}_{a}$	$75.99\pm3.86^{B}_{a}$	$58.54\pm2.78^{C}_{a}$	$53.76\pm2.52^{D}_{a}$	$57.88\pm2.69^{E}_{a}$
4 weeks	$40.52\pm2.23^{A}_{a}$	$81.31\pm4.09^{B}_{b}$	$50.27\pm2.39^{C}_{b}$	$46.56\pm2.28^{D}_{b}$	$49.55\pm2.37^{E}_{b}$
NO (pg/mg protein)					
2 weeks	$36.43\pm1.97^{A}_{a}$	$70.67\pm2.94^{B}_{a}$	$51.31\pm2.28^{C}_{a}$	$47.22\pm2.11^{D}_{a}$	$50.82\pm2.23^{E}_{a}$
4 weeks	$35.99\pm1.95^{A}_{b}$	$76.55\pm3.18^{B}_{b}$	$43.77\pm2.04^{C}_{b}$	$39.81\pm1.96^{D}_{b}$	$43.49\pm2.02^{E}_{b}$
MDA (nmol/mg protein)					
2 weeks	$4.07\pm0.101^{A}_{a}$	$8.02\pm0.206^{B}_{a}$	$6.28\pm0.154^{C}_{a}$	$5.79\pm0.146^{D}_{a}$	$6.08\pm0.151^{E}_{a}$
4 weeks	$4.13\pm0.103^{A}_{a}$	$8.61\pm0.273^{B}_{b}$	$5.51\pm0.138^{C}_{b}$	$5.05\pm0.123^{D}_{b}$	$5.39\pm0.134^{E}_{b}$

Values are expressed as mean±SE. AD, Alzheimer's disease; ACh, acetylcholine; Bcl-2, B-cell lymphoma 2; B-FABP, brain-type fatty acid binding protein; Ce/Ca-HAp, cerium/calcium hydroxyapatite; CMCS/Ce/Ca-HAp, carboxymethyl chitosan/ceria/calcium hydroxyapatite; Gal, galantamine; NO, nitric oxide; TPL, tissue thromboplastin. $^{A, B, C, D}$A common superscript within a row are statically significantly different ($P<0.05$). $^{a, b}$A common subscript within a column are statically significantly different ($P<0.05$).

Table 2 Concentration of different brain enzyme antioxidants in brain tissue samples from AD-induced rats and rats treated with Gal, Gal+Ce/Ca-HAp, and Gal+CMCS/Ce/Ca-HAp

	Control	AD	AD+Gal	AD+Gal+Ce/Ca-HAp	AD+Gal+CMCS/Ce/Ca-HAp
GSH (U/mg protein)					
2 weeks	$41.17\pm1.423^{A}_{a}$	$26.89\pm0.832^{B}_{a}$	$33.26\pm0.957^{C}_{a}$	$35.07\pm0.988^{D}_{a}$	$33.79\pm0.972^{E}_{a}$
4 weeks	$40.93\pm1.418^{A}_{a}$	$23.47\pm0.795^{B}_{b}$	$37.91\pm1.086^{C}_{b}$	$40.54\pm1.302^{D}_{b}$	$38.11\pm1.117^{E}_{b}$
GSSG (μmol/l/mg protein)					
2 weeks	$0.63\pm0.008^{A}_{a}$	$1.16\pm0.018^{B}_{a}$	$0.82\pm0.012^{C}_{a}$	$0.74\pm0.011^{D}_{a}$	$0.81\pm0.013^{E}_{a}$
4 weeks	$0.64\pm0.008^{A}_{a}$	$1.42\pm0.024^{B}_{b}$	$0.74\pm0.011^{C}_{b}$	$0.65\pm0.009^{D}_{b}$	$0.74\pm0.012^{E}_{b}$
SOD (U/mg protein)					
2 weeks	$3.08\pm0.071^{A}_{a}$	$1.69\pm0.034^{B}_{a}$	$2.55\pm0.045^{C}_{a}$	$2.79\pm0.052^{D}_{a}$	$2.55\pm0.045^{E}_{a}$
4 weeks	$2.9\ 3\pm0.068^{A}_{a}$	$1.48\pm0.029^{B}_{b}$	$2.87\pm0.057^{C}_{b}$	$2.95\pm0.071^{D}_{b}$	$2.91\pm0.069^{E}_{b}$
CAT (U/mg protein)					
2 weeks	$6.502\pm0.173^{A}_{a}$	$5.09\pm0.101^{B}_{a}$	$5.78\pm0.136^{C}_{a}$	$6.08\pm0.148^{D}_{a}$	$5.69\pm0.133^{E}_{a}$
4 weeks	$6.49\pm0.168^{A}_{a}$	$4.78\pm0.092^{B}_{b}$	$6.03\pm0.149^{C}_{b}$	$6.43\pm0.171^{D}_{b}$	$5.97\pm0.152^{E}_{b}$
Cytochrome P450 reductase (ng/mg protein)					
2 weeks	$2.19\pm0.023^{A}_{a}$	$1.23\pm0.017^{B}_{a}$	$1.82\pm0.021^{C}_{a}$	$2.11\pm0.029^{D}_{a}$	$1.96\pm0.025^{E}_{a}$
4 weeks	$2.11\pm0.024^{A}_{a}$	$0.958\pm0.012^{B}_{b}$	$2.06\pm0.024^{C}_{b}$	$2.34\pm0.034^{D}_{b}$	$2.16\pm0.028^{E}_{b}$

Values are expressed as mean±SE. AD, Alzheimer's disease; Ce/Ca-HAp, cerium/calcium hydroxyapatite; CMCS/Ce/Ca-HAp, carboxymethyl chitosan/ceria/calcium hydroxyapatite; Gal, galantamine; GSSG, glutathione disulfide; SOD, superoxide dismutase. $^{A, B, C, D}$A common superscript within a row are statically significantly different ($P<0.05$). $^{a, b}$A common subscript within a column are statically significantly different ($P<0.05$).

parameters was reached following Gal treatment. Treatment with Gal coated with Ce/Ca-HAp imposed highly significant improvement to near-to-normal levels in the previous parameters. However, Gal coated with CMCS/Ce/Ca-HAp failed to encounter any obvious ameliorations. These results validate aforementioned histopathological alterations (Tables 1 and 2).

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Discussion

AD is a progressive neurodegenerative disorder that is the most common form of dementia, accounting for ~50–60% of all cases (Querfurth and LaFerla, 2010). It represents a major public health concern, with important social and economic outcomes (Wimo *et al.*, 2010). The disorder usually starts in a mild form and progressively gets worse because of late discovery of onset together with the lack of specific efficient therapeutic regimens and due to its progressive course.

Many studies of putative biomarkers have been conducted, but none of the antemortem tests has fulfilled all of the criteria for an ideal biomarker. This necessitates the use of several methodology for the early identification, together with the different diagnostic imaging methods such as single-photon emission computed tomography and MRI. Such biomarkers include ACh, Bcl-2, amyloid peptide, TPL, Chol, B-FABP, NO, and MDA in addition to the brain antioxidant enzymes GSH, GSSH, SOD, CAT, and cytochrome P450. Biomarkers seem to be more sensitive to AD changes in its earlier stages as presently designated that would impose a reliable method for early diagnosis.

In the current study, AD was reported to induce serious histological alterations 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. Similarly, neuropathological examination of the AD rat brain showed extensive neuronal loss, accumulation of fibrillary Ab plaques, and NFTs within neurons (Praticò and Trojanowski, 2000). Mahdy *et al.* (2012) and Yassin *et al.* (2013) observed necrosis of the brain, spongy appearance, plaques, loss of normal structure, various sizes of amyloid plaques in the hippocampus, NFTs, and fatty changes in AD-induced rat brain tissue.

Aluminum salt has been found to induce the overexpression of APP. Present manifestations may be primarily a sequel to inflammatory responses that are known to play an important role in neurodegenerative disease.

Vallés *et al.* (2008) demonstrated that Ab peptide causes oxidative stress in the neurons and inflammation in the astrocytes, indicating that this toxic peptide can affect not only neuronal cells but also astrocytes. Wu *et al.* (2012) also suggested that the amyloid plaques caused by aluminum administration plays a role in the pathology of AD by directly inducing neuronal cytotoxicity and stimulating microglia to secrete cytokines and ROS, which also damage neurons. Amyloid plaques could activate astrocytes and oligodendrocytes to produce chemokines, in particular monocyte chemotactic protein 1, which serves as potent in-vitro microglial and macrophage chemoattractants. Plaques have been shown to activate astrocytes to upregulate proinflammatory cytokine expression; therefore, plaques mediated astrocytes activation initiates the inflammatory cascade.

The challenges for establishing an early diagnosis of AD have created a need for biomarkers that reflect the core pathology of the disease. There has been increasing effort to find new biomarkers that could make earlier diagnosis possible in a clinical setting. They should be highly specific to AD and sensitive to changes, especially in the early stages of the disease (Skoumalova and Hort, 2012).

Presently, brain markers have been studied for the possible early detection of AD. Recorded results signify a considerable decrease in ACh content and a significant increase in Bcl-2 and TPL in AD-induced rats. In contrast, a limited increase in Ab was recorded and a significant increase in Chol, B-FABP, NO, and MDA was recorded.

Cognitive deficits in neuropsychiatric disorders have been closely related to cholinergic deficits, in which total ACh levels in both humans and rats were reduced (Gil-Bea *et al.*, 2005). The cholinergic hypothesis was initially presented over 20 years ago and suggests that a dysfunction of ACh-containing neurons in the brain contributes substantially to the cognitive decline observed in those with advanced age and AD (Terry and Buccafusco, 2003).

Decreased expression of Bcl-2 is observed in AD's dementia in neurons with NFTs (MacGibbon *et al.*, 1997). Studies conducted on Bcl-2 localization (mitochondria and endoplasmic reticulum) suggest that it inhibits generation of ROS or lipid peroxidation (Hockenberry *et al.*, 1993). It is also reported to modulate calcium fluxes from intracellular stores in hippocampal neurons *in vitro* (Prehn *et al.*, 1994). Such observations may be crucial as increased oxidative stress and intracellular calcium levels have been put forward as possible mechanisms implicated in neuronal death in AD (Smith *et al.*, 1995).

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Overproduction of amyloidogenic forms of the Ab, a peptide that is generated by a sequential cleavage of APP by the β-secretase and γ-secretase (Selkoe, 2001), is the hallmark of AD. Similarly, present studies have reported a significant increase in Ab in AD rats. Ab is a complex biological molecule that interacts with many types of receptors and/or forms insoluble assemblies, and, eventually, its nonphysiological depositions alternate with the normal neuronal conditions (Sadigh-Eteghad *et al.*, 2015). Under normal conditions, there is an equilibrium between the production and elimination of Ab that maintains Ab at constant levels. However, in aging and pathological conditions such as metabolic disorders and excitotoxicity, the formation and clearance of Ab are disturbed, leading to an accumulation of Ab and senile plaque formation. Neurodegeneration in AD is mediated in part through soluble forms of Ab. Increased soluble Ab concentration correlates with cognitive decline in AD-affected individuals. Hence, it may be considered as a reliable predictor of AD (Sadigh-Eteghad *et al.*, 2015).

TPL has one recognized function: it forms a tight complex with coagulation factor VII, and thereby functions as a cofactor in the enzymatic activation of coagulation factors IX and X. TPL is an integral membrane protein that is normally sequestered from circulating factor VII and other coagulation factors. Tissue injury exposes TPL to circulating factor VII, resulting in complex formation and initiation of blotting cascade through the extrinsic pathway (McComb *et al.*, 1991). In the current study, marked decrease in TPL was recorded.

It has been suggested that increased levels of free cholesterol in neuronal cell membranes may provoke Ab formation (Casserly and Topol, 2004). Highly significant augmented levels for cholesterol have been presently well documented. Several related studies have reported concomitant findings (Hughes, 2011; Reed *et al.*, 2014). Experimental in-vitro and in-vivo data indicate that brain cholesterol homeostasis is coupled with brain amyloid metabolism, although the mechanism is not known (Eckert *et al.*, 2007). However, the causative role of cholesterol in the pathogenic cascade of excessive Ab deposition in the brain of AD patients is not proven. Cell culture studies demonstrate that membrane cholesterol controls the direction of the processing of the APP *in vitro* (Eckert *et al.*, 2007). Again, under in-vitro conditions, cholesterol has been shown to influence a number of processes involved in the generation of the neuritic plaques (which predominantly consists of Ab peptides of 40 or 42 residues and NFTs) (Simons *et al.*, 2001).

B-FABP levels were elevated in the serum of 29% of the patients with AD. B-FABP expression was observed in reactive astrocytes in brain sections. Thus, it could be used in an attempt to reveal a sensitive marker for various neurodegenerative diseases and can therefore have importance for defining subgroups of these patients (Teunissen *et al.*, 2011).

Endothelial cells in the blood vessels are one of the major sites of NO production. NO is one of the most important signaling molecules in the body that affects virtually every organ in the body, allowing effective communication between cells and providing a wide array of health benefits. It is a protective molecule that maintains immune, cardiovascular, nervous, kidney, stomach and intestinal, skin, and other beneficial effects. Accordingly, lack of NO production by endothelial cells due to increase in Ab peptide aggregate formation and/or free radical generation only contributes to the pathology of AD. It was verified by Cho *et al.* (2009) that Ab produced NO, which resulted in a change to the protein Drp1, called SNO-Drp1. This change, which was the attachment of NO to the protein, assisted by cysteine at the 644th position, caused an increase in mitochondrial fragmentation and cell death in AD brains (Naditz, 2011).

Increased levels of MDA in AD brain have been confirmed by several studies (Skoumalova and Hort, 2012). One of the main reasons for high malondialdehyde (lipid peroxidation) in elder patients could be melatonin deficiency, as decrease in melatonin seems to be related to aging. The authors emphasized that low melatonin levels could be explained not only by a decrease in melatonin due to aging but also by a decrease in melatonin due to AD, which is probably much larger.

Oxidative stress is a significant element in AD pathogenesis. Possibly, lack of protection against ROS production in the aging brain could be one triggering cause of AD. Oxidative stress is one of the first consequences of Ab overproduction in the brain. The pressure from oxidative stress in the aging brain in combination with a lowered antioxidant defense creates a harmful combination that could disturb functions and damage organelles such as mitochondria. This would eventually lead to loss of

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synapses and cell death that give rise to the clinical symptoms associated with AD (Persson *et al.*, 2014).

The activity of SOD is a sensitive index in oxidative damage as it scavenges the superoxide anion to form hydrogen peroxide, leading to diminished toxic effects. Moreover, glutathione reduced, glutathione reductase, and glutathione-S-transferase are closely related to the direct elimination of ROS. Therefore, the reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide, linked with neurodegenerative diseases (Khan *et al.*, 2012).

Gal, the AChE inhibitor, has long been the drug of choice for the treatment of AD patients for its recorded beneficial role in the slowing down of the degenerative outcomes on the brain that cause impaired memory. Pathways of Gal therapy is through preventing AChE from breaking down ACh in the brain. As a result, an increased concentration of ACh leads to increased communication between nerve cells. This may temporarily alleviate or stabilize some symptoms of AD.

In the present study, therapeutic doses of Gal to AD rats signified limited ameliorations in the studied parameters of brain markers and antioxidant. Similarly, Gal coated with CMCS/Ce/Ca-HAp failed to encounter any obvious improvement parameters. Nevertheless, on coating Gal with Ce/Ca-HAp, all previous parameters retreated to near-to-normal levels. These results were simultaneously confirmed by prementioned histological parameters.

Conclusion

According to the present findings, multiple biomarkers would indeed improve diagnostic tools for the early detection of the onset of AD. Brain markers (ACh, Bcl-2, Ab, TPL, Chol, B-FABP, NO, and MDA) together with brain antioxidants (GSH, SOD, CAT, and cytochrome P450) may be used in progressive laboratory testing methods besides well-known imaging techniques. In addition, as Gal therapy may impose limited improvements, the necessity for novel drug delivery systems to minimize dosing amounts, frequency of administration, and adverse side effects of drug while increasing its therapeutic efficacy.

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Conflicts of interest
There are no conflicts of interest.

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