

Encapsulated Choroid Plexus Epithelial Cells Actively Protect Against Intrahippocampal A β -induced Long-Term Memory Dysfunction; Upregulation of Effective Neurogenesis with the Abrogated Apoptosis and Neuroinflammation

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Abstract Choroid plexus epithelial cells (CPECs) as a secretory epithelium are responsible for the secretion of cerebrospinal fluid (CSF). Beyond this classical tenet, CPECs also synthesize and release many neurotrophic factors such as antioxidants into the CSF, participating in brain homeostasis. In this study, CPECs were isolated from rat's brain and encapsulated in alginate microcapsules. Firstly, functional properties of alginate microcapsules and encapsulated CPECs were examined *in vitro*. Following, micro-encapsulated CPECs were grafted into rats' brains that were pretreated with A β . The *in vivo* studies include western blotting against Caspase-3 and Terminal-Transferase dUTP Nick End Labeling test that were performed to detect apoptosis in brain tissues. The *in vivo* part also included immunohistochemistry against Iba-1, glial fibrillary acidic protein, and Brdu to detect microglial migration, gliosis, and neurogenesis, respectively. Moreover, the activity of superoxide dismutase enzyme in hippocampi also was measured, and the memory was assessed by shuttle box apparatus. Our data suggest that transplantation of encapsulated CPECs resulted in a significant decrease in apoptosis, reduced migration microglia, diminished gliosis, increased neurogenesis, and improved long-term memory as well as upregulated antioxidant activity. Since

microencapsulated CPECs do not need immunosuppression following implantation, and also we showed their neuroprotective effects against A β toxicity and oxidative stress, this may be a suitable candidate for cell therapy in neurological disorders.

Keywords Choroid plexus epithelial cells · Oxidative stress · A β · Alginate microcapsules

Introduction

Alzheimer's disease (AD) is the progressive neurodegenerative disease of aging. As the most common form of dementia, AD is characterized by neuronal loss, extensive deposition of amyloid- β peptide (A β) in the brain parenchyma (amyloid plaques) and in vessel walls, along with the presence of neuronal inclusions of abnormally phosphorylated tau (neurofibrillary tangles). These pathological findings are supposed to be related to an array of clinical manifestations including gradually and progressive cognitive deficiency, memory decline, behavioral disturbance, and neuropsychiatric symptoms (Breteler et al. 1992; Adlard and Cummings 2004). Unspecificity of neurofibrillary tangles to AD has shifted AD research towards amyloidogenic hypothesis and recently it has been revealed that A β may be the upstream of tau in AD pathogenesis (Bloom 2014).

The common explained feature of AD pathology is the neuronal loss. This pathophysiological manifestation is mostly reported in the hippocampus and cerebral cortex (Newman et al. 2007). Despite the numerous described mechanisms regarding amyloidogenic approach, the distinctive underlying molecular mechanism and signaling pathway, linking A β to AD pathophysiology, have not yet completely understood. In

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this regard, the aging axis of excessive generation of reactive oxygen species (ROS) that leads to the progression of oxidative stress with the depletion of neuronal antioxidants, such as superoxide dismutase (SOD) and catalase, appears to be one of the central mechanisms (Tsay et al. 2000). Regarding initiation factors, inflammatory reaction hypothesis of AD also has multiple features, capable of describing the vast pathophysiological evidences. In this manner, extensive gliosis with the incompetency of microglial phagocytosis has been discussed as a toxic consequence of A β deposition. Also, other remarkable A β peptide-induced dysfunctions had been illustrated ranging from protein aggregation to biometal dyshomeostasis and mitochondrial failure (Rosales-Corral et al. 2012; Kihara et al. 2010; Li et al. 2011).

The choroid plexus (CP) is a highly vascularized secretory epithelium that also serves as a selective molecular gateway (Bailey 1916). The classic role attributed to the CP is the production of the cerebrospinal fluid (CSF); however, there is a growing body of evidence over the last decade that CP epithelial cells (CPECs) are associated with various physiological aspects of brain homeostasis, beyond the classical tenet (Dohrmann 1970; Speake et al. 2001). In the recent years, the application of CPECs in neurodegenerative diseases has become an excellent candidate for cell therapy aiming to restoring brain tissue (Borlongan et al. 2004). The suggestive characteristics include secretion of growth factors, neurotrophins, numerous biologically activated enzymes, and containing a subpopulation of neural progenitor cells (Li et al. 2002; Chodobski and Szmydynger-Chodobska 2001). As such, application of conditioned medium (CM) and intracerebral implants of CPECs in AD models are the major routes that deliver potentially mentioned pro-survival and pro-growth agents. Accordingly, hippocampal implants of CPECs were recovered in vitro and in vivo AD-associated pathology via modulating A β degradation (Bolos et al. 2014). Also, the CM of the primary culture of rat CPECs displayed a significant improvement in survival and outgrowth of hippocampal neurons in vitro (Watanabe et al. 2005). In spite of some beneficial effects, direct cellular delivery and CPECs grafts permit anatomical integration between the host and implanted cells that result in immune rejection and uncontrolled migration (Dionne et al. 1996; Winn et al. 1994). Furthermore, the CM administration has the blood–brain-barrier exchange limit in systemic infusion and direct brain infusion encounters immunological responses with inadequate distribution. These and other limitations have led CPECs transplantation studies towards encapsulated cell therapy as a promising approach for drug delivery to the brain. Encapsulated cell therapy (microcapsules and macrocapsules) provides a purposive and continuous source of molecules with the safety of an implantable medical device that does not need immunosuppression. Microcapsules protect cells from the host immune system, permitting neurochemical diffusion (Emerich et al. 2014).

Alginate microcapsules, containing an alginate core surrounded by a polycation layer membrane, have been the most widely employed microcapsules (Zimmermann et al. 2007). In AD context, it has been reported that encapsulated nerve growth factor (NGF)-secreting cells prevented extensive neuronal loss in the aged rodents and restored cognitive function (Lindner et al. 1996). Moreover, cortical implantation of vascular endothelial growth factor (VEGF)-secreting cells in a bed of alginate microcapsules resulted in VEGF-dependent A β clearance and amelioration of cognitive impairment (Spuch et al. 2010).

As it was strongly evidenced, CPECs secrete numerous biologically active neurotrophic factors, including NGF, vascular endothelial growth factor (VEGF), and brain-derived neurotrophic factor (BDNF) that have been shown to counteract with AD pathology (Chodobski and Szmydynger-Chodobska 2001; Spuch et al. 2010; Diniz and Teixeira 2011; Winn et al. 1994). Recently, we showed that CPECs-CM attenuated apoptosis in H₂O₂ insulted PC12 cells and activated nuclear factor (erythroid-derived 2)-like 2/antioxidant response element (Nrf2/ARE) pathway to overwhelm H₂O₂-induced oxidative stress (Aliaghaei et al. 2014). Here, we aimed to combine the advantages of encapsulated cell therapy with the capacity of CPECs to excrete multiple neurotrophic factors, in order to hamper the A β -induced neuronal toxicity. To this end, alginate microcapsules containing CPECs implanted overlying the cerebral cortex, 1 week following intrahippocampal A β injection, and the apoptosis, neurogenesis and antioxidant enzyme activity were assessed. We also examined memory improvement following the injection of encapsulated CPECs in rats receiving exogenous A β .

Materials and Methods

Materials

The Dulbecco's Modified Eagle's Media (DMEM/F12) were obtained from Invitrogen (Carlsbad, CA). The Terminal-Transferase dUTP Nick End Labeling (TUNEL) assay kit and Polyvinylidene difluoride membrane (PVDF) were purchased from Millipore (Millipore, USA). Antibody against caspase-3 was purchased from cell signaling technology (Beverly, MA, USA). Antibodies against ionized calcium binding adaptor molecule 1 (Iba-1), 5-bromo-2'-deoxyuridine (BrdU), glial fibrillary acidic protein (GFAP), and goat anti mouse IgG conjugated TRITC were purchased from ABCAM (Cambridge, Massachusetts, USA). The electrochemiluminescence (ECL) kit was purchased from Amersham (Amersham, Bioscience, USA), and other materials were purchased from Sigma Aldrich (St. Louis, MO, USA).

Preparing Alginate Microcapsules Containing CPECs

CPECs were isolated and cultured according to method described previously (Aliaghaei et al. 2014). CPECs were detached from the flask's bottom using trypsin and were suspended in 1.5 % (*w/v*) alginate solution. The suspension was extruded to 60 mM CaCl₂ solution that alginate beads could be formed. Then, alginate beads incubated with poly-L-lysine solution (0.1 %) for 5 min and with alginate solution (0.1 %) for further 5 min. Finally, the microcapsules were incubated with 55 mM sodium citrate solution for 5 min and then were washed. Microcapsules were cultured in the presence of DMEM/F12 medium and serum.

Transfection of CPECs with Green Fluorescent Protein (GFP) Expression Vector

Before transfection, CPECs were maintained in culture medium supplemented with 10 % fetal bovine serum and allowed to reach 60–70 % confluency. Transfection was carried out using the calcium phosphate method (Calphos transfection kit, Clontech). The medium was replaced 4 h before the transfection. Then, 3 µg of the GFP expression vector of pWPXLd was used for transfection. Following an overnight incubation at 37 °C, the medium was removed and replaced by fresh medium and incubated at 37 °C for a further 48 h. GFP expressing cells were monitored 48–72 h post transfection.

Atomic Force Microscopy (AFM)

Microcapsules were washed using phosphate buffer saline (PBS) solution and were placed on a surface made of mica. After being dried, a drop of PBS was immediately poured on them. Microcapsules were placed on the AFM holder plate and AFM scanner tip was placed carefully onto the sample. AFM images of the surface of microcapsules were captured by means of an Explorer TMX 2000 (Topo Matrix) microscope at room temperature and using the non-contact mode model. Since alginate is a soft and elastic material, the slower scanning speed was used (linear frequency <1 Hz). The scan was performed by a liquid tripod scanner with a maximum scan speed of 100 µm in *XY*-axis and 10 µm in *Z*-axis. For proper scanning and placing the tip of the scanner on microcapsules, alginate capsules were observed using an optical microscope attached to the AFM.

Determination of the Permeability of Alginate Microcapsules

First, cell-free alginate microcapsules were prepared. Thioflavin T (ThT, 0.4 mM) solution was dissolved in ethanol (1:3 ratio). Then, ThT solution was filtered and alginate microcapsules were incubated for 8 h in Th T. Next, the

microcapsules were washed and examined under a fluorescent microscope (Olympus, IX 71, Japan).

In Vitro Biological Activity

CM was collected from encapsulated CPECs cells. Embryonic cortical neurons were isolated from Wistar rats on embryonic day 15. Briefly, the pregnant rats were killed, the embryos were removed, and their brains were extracted and washed with PBS. The cells were isolated mechanically and cultured in the presence of culture medium and serum. After reaching an appropriate confluency, the cells were detached and transferred into 6-well plates. Forty-eight hours later, medium was removed and neurons were cultured in the presence of medium containing serum (serum control) or serum-free medium and/or different concentrations of encapsulated CPECs-CM (3 or 10 %). To evaluate cell survival, trypan blue staining was done.

Injection of β -amyloid into the Rats' Brain

Under approval by the animal care committee of Shahid Beheshti University of Medical Sciences, adult male albino Wistar rats (200–220 g) were anesthetized using ketamine and xylazine (100 and 2.5 mg/kg, respectively), and their skull was fixed in a stereotaxic device. A longitudinal incision was made along the sagittal line on the scalp. A β _{1–42} was prepared freshly and administered with a Hamilton microsyringe. Two micrograms of A β _{1–42} solution in 4 µl PBS was administered over 2 min into the dorsal hippocampus bilaterally at coordinates 3.6 mm posterior and \pm 2 mm lateral to bregma, and 3.2 mm ventral to the skull surface (Paxinos and Watson 2005). The needle was remained in position for an additional 2 min after injection. The needle was slowly removed from the brain and the scalp was sutured. The animals were returned to their cages to recover.

Transplantation of Alginate Microcapsules

One week after A β _{1–42} injection, rats were anesthetized and craniotomy was performed at coordinates 3.6 mm posterior and 2 mm lateral to bregma. Dura mater was cut and seven microcapsules were implanted on each side of the cortex. Craniotomy site was covered with pieces of surgical cellulose. The following designated groups were included: (1) control group or intact, (2) the group which received A β _{1–42}, (3) the group which received A β _{1–42} and 1 week later empty capsules, and (4) the group which received A β _{1–42} and 1 week later encapsulated CPECs. The animals were killed 14 and 30 days after injection of A β _{1–42}.

BrdU Labeling

BrdU, a thymidine analogue, is incorporated into the DNA of dividing cells during S phase. BrdU labeling was used for mitotic labeling. Rats received daily intraperitoneal injections of 100 mg/kg BrdU for 4 consecutive days, starting 4 days before killing the rats.

Western Blot Test

The animals were killed, their brains were removed, and the hippocampus was extracted. Tissues were lysed by tissue lysis buffer. To determine the protein concentration of the samples, Bradford test was performed (Bradford 1976). Sixty micrograms of proteins was loaded on 12 % SDS-PAGE gel and electrophoresis followed by transfer onto PVDF. Then, blots were incubated with blocking solution followed by incubation with primary antibody against caspase-3 overnight. After being washed, blots were incubated with horse radish peroxidase conjugated antibody and finally, immunoreactivity of polypeptides was detected using ECL solution. Quantification of results was performed by densitometry. Data analysis was done by Image J software.

TUNEL Assay

TUNEL assay was performed according to the manufacturer's protocol after preparing sections of tissue using microtome, paraffin removal, and hydration. Briefly, the tissues were incubated with proteinase K which was followed by incubation with terminal deoxynucleotidyl transferase (TdT) buffer and also TdT end labeling cocktail. After washing with TB buffer, incubation with blocking solution was done. To observe the reaction, the samples were incubated with solution of avidin-FITC and after being washed, and were observed under fluorescent microscopy.

Staining with Th T

Tissues were incubated for 5 min with ThT solution (0.5 % in 0.1 N HCl) and after being washed, and were observed under fluorescent microscopy (Olympus, IX 71, Japan).

Immunohistochemistry

The brains were fixed by 4 % paraformaldehyde (PFA). Brains were cut with a microtome, and after deparaffinating, antigen retrieval process was performed. Then, the samples were washed with PBS and were incubated using normal goat serum solution containing Triton X-100 (0.3 %). Then, the samples were incubated with primary antibodies, including mouse anti Iba-1, mouse anti Brdu, and mouse rabbit anti GFAP for 3 h. After being washed, the samples were stained

with secondary antibody of goat anti mouse IgG conjugated TRITC or the DAB method. After washing the samples, they were examined under fluorescent microscopy or an optical microscope.

SOD Activity Assay

The activity of SOD was measured based on the modified method described by Kakkar et al. (1984). Accordingly, the extent of inhibition of amino blue tetrazolium (NBT) formazan formation was measured in the mixture containing tissue lysate, sodium pyrophosphate (0.052 M), phenazine methosulfate (186 μ M), and nitroblue tetrazolium (300 μ M). Upon the addition of NADH (750 μ M), the reaction was started and then, the plate was incubated at 30 °C for 90 s. Absorbance of resulted coloration was measured at 560 nm.

Passive Avoidance Apparatus

The apparatus used for passive avoidance (PA) training consisted of a two-compartment box. The illuminated chamber (35×20×15 cm³) made from transparent plastic was connected by an 8×8 cm² guillotine door to the dark compartment with black opaque walls and ceiling. The floors of both chambers were made of stainless steel rods (3 mm diameter) spaced 1 cm apart. The floor of the dark chamber could be electrified. Shock was delivered to the animal's feet via a shock generator.

Training Procedure

All experimental groups were first habituated to the apparatus ($n=8$). The rat was placed in the illuminated compartment, and 5 s later, the guillotine door was raised. Upon entering the dark compartment, the rat was taken from the dark compartment into the home cage. The habituation trial was repeated after 30 min and followed after the same interval by the acquisition trial during which the guillotine door was closed and a 50 Hz, 1.2 mA constant current shock was applied for 1.5 s immediately after the animal entered to the dark compartment. After 20 s, the rat was removed from the dark compartment and placed into the home cage. The rats received a foot-shock each time if reentered in the dark compartment. Training was terminated when the rat remained in the light compartment.

Retention Test

Twenty-four hours after training, a retention test was performed to determine long-term memory. Each animal was placed in the light chamber for 10 s, the door was opened, and the latency of entering to the dark compartment (step-through latency) and the time spent in the dark compartment were recorded as a measurement of retention performance. The ceiling score was 300 s. During these sessions, no electric shock was applied.

Data Analysis

All data are represented as the mean \pm SEM. Comparison between groups was made by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test to analyze the difference. The statistical significances were achieved when $P < 0.05$.

Results

Alginate Microcapsules Contained Pores on Their Surface

Our results showed that alginate microcapsules have three layers including alginate-poly-L-lysine-alginate (Fig. 1a). In our study, we showed that CPECs were able to express GFP gene and easily seen in microcapsules (Fig. 1b). Hoechst staining confirmed that nuclei of CPECs were in microcapsules (Fig. 1c). AFM has provided clear-cut evidence of the capsular surface topography clearly reveals some imperfections such as wrinkles on the capsule surface (Fig. 2a). One can see that most of the wrinkles are oriented in one direction. It is possible that the wrinkle orientation actually reflected the direction of the drop entering the cation solution and its deformation and recovery. AFM scans showed that the alginate microcapsules have numerous pores (Fig. 2b, c).

Alginate Microcapsules Containing CPECs Displayed In Vitro Functional Properties

Incubation of the alginate microcapsules in ThT solution showed the ability of ThT to enter microcapsules through the pores on their surface (Fig. 3a). When embryonic cortical neurons were cultured in the presence of serum, they had a higher survival rate than serum-deprived group. On the other hand, embryonic cortical neurons cultured in the presence of varying amounts of medium, collected from encapsulates CPECs in the absence of serum, indicated that the neurons

could survive in the presence of the CPECs conditioned medium (CPECs-CM) even in the absence of serum. This finding showed an improvement in embryonic cortical neurons survival that was dependent on the concentration of CPECs-CM (Fig. 3b, c). Cell survival in the group received encapsulated CPECs-CM 10 % was 1.6-fold higher than the group received encapsulated CPECs-CM 3 %. On the other hand, cell viability in the groups received encapsulated CPECs-CM (10 and 3 %) showed 4.6- and 2.8-fold increase, respectively, compared to serum free group.

Injection of Encapsulated CPECs to $A\beta_{1-42}$ Receiving Rats Decreased the Apoptosis Rate in Rat Hippocampus

Following the injection of $A\beta_{1-42}$ into the brains, staining with ThT revealed $A\beta$ masses in the hippocampus after 1 week (Fig. 4). Meanwhile, 14 days after injection of $A\beta$, Western blotting analysis showed that cleaved caspase-3 in the $A\beta$ -injected rats was 2.6-fold higher than the level in the control group. However, cleavage of caspase-3 in the group which received encapsulated CPECs was approximately 1.9 times lower compared to $A\beta$ -received ones (Fig. 5a, b). Thirty days after $A\beta$ injection, cleavage of caspase-3 in the group which received $A\beta$ significantly increased compared to the control (2.8-fold). In the group that received encapsulated CPECs, the amount of reduction in the level of cleaved caspase-3 was about 1.4 times compared to $A\beta$ group (Fig. 5c, d). TUNEL staining showed that the average number of TUNEL-positive cells in the $A\beta$ -injected rats was about 3.5-fold higher than the control group at day 14. Moreover, the mean number of TUNEL-positive cells in the group that received encapsulated CPECs significantly decreased compared to the $A\beta$ -injected group (2.7 times) (Fig. 6a, b). Thirty days after injection of $A\beta$, average of TUNEL-positive cells in $A\beta$ -receiving rats was about 4.5-fold higher in comparison with the control. In the encapsulated CPECs group, this value was about 2.1 times lower than the $A\beta$ -injected group (Fig. 6a, c).

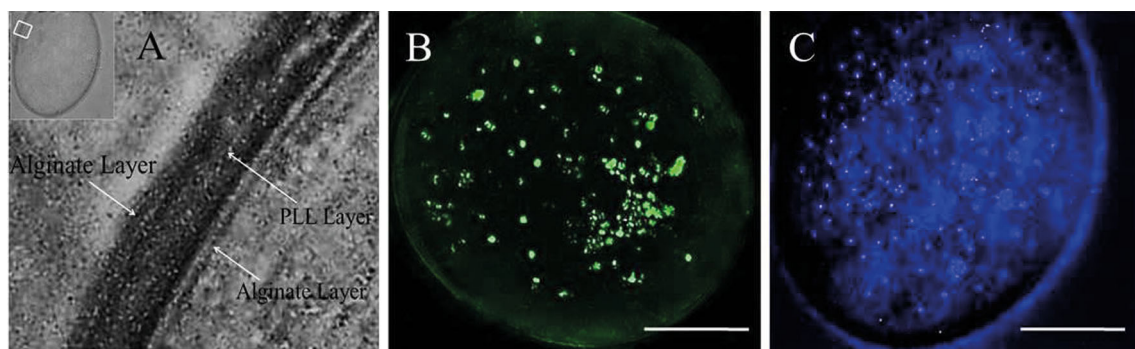


Fig. 1 **a** High magnification ($\times 60$) phase contrast image of microcapsule's surface. This shows that alginate microcapsule has three layers including Alginate-poly-L-lysine-Alginate. **b** Encapsulation of CPECs carrying GFP gene. **c** Encapsulated CPECs labeled with Hoechst 33258 (scale bar 100 μm)

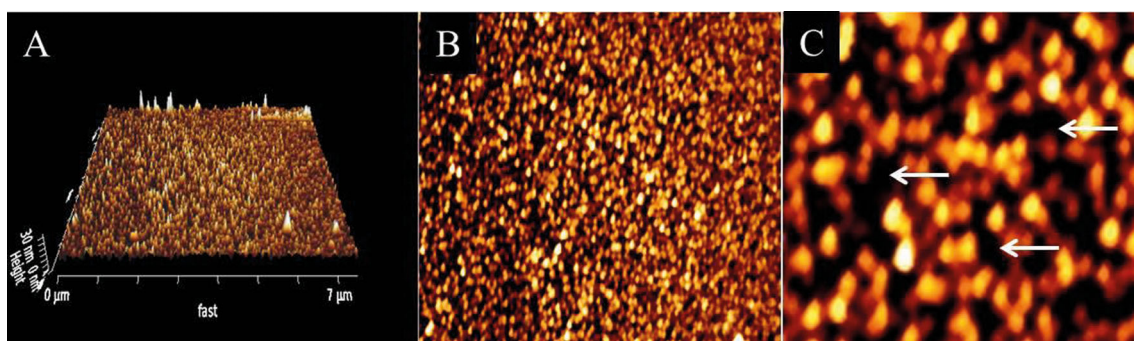


Fig. 2 Atomic force microscopy scan of alginate microcapsules surface. **a** 3D scan. **b** The surface of microcapsules. **c** The pores of microcapsules surface (white arrows)

Injection of Encapsulated CPECs into the $A\beta_{1-42}$ Receiving Rats Led to an Increase in SOD Activity

Notably, 14 days after injection of $A\beta$, the SOD activity in the $A\beta$ -receiving group achieved 71 unit/mg protein. This value in the rats received encapsulated CPECs showed an increase, up to 77 unit/mg protein. At day 30th, the activity of SOD in the rats receiving $A\beta$ was 69 unit/mg protein, whereas in the group receiving the encapsulated CPECs showed an increase, up to 76 unit/mg protein (Fig. 7).

Injection of Encapsulated CPECs into the $A\beta_{1-42}$ Receiving Rats Resulted in Decrease of Microglial Migration

Iba1 is a microglia/macrophage-specific calcium-binding protein. The results of immunohistochemistry showed that at day 14 average number of Iba-1-positive cells increased in $A\beta$ -receiving rats about 6.2-fold compared to the control. In the group receiving encapsulated CPECs, this value indicated a reduction of about 1.5 times compared to $A\beta$ -injected group (Fig. 8a, b). At day 30, average number of Iba-1-positive cells in the $A\beta$ group showed an approximate increase of 4.7-fold compared to the control. Furthermore, this value for the rats which received encapsulated CPECs showed a reduction of 2.7 times compared to $A\beta$ -injected ones (Fig. 8a, c).

Encapsulated CPECs Caused a Decrease in Gliosis in $A\beta_{1-42}$ Receiving Rats

For detection of astrocytic migration (gliosis), immunohistochemistry against GFAP was done. At day 14, migration of astrocytes in $A\beta$ -receiving group was more than the control. In the group receiving encapsulated CPECs, number of astrocytes decreased compared to $A\beta$ -injected rats. At day 30, there was a decrease in number of astrocytes in the group receiving encapsulated CPECs compared to the $A\beta$ -injected group (Fig. 9).

Injection of Encapsulated CPECs Increased the Neurogenesis in $A\beta_{1-42}$ Receiving Rats

Immunohistochemistry analysis at day 14 showed that the average of Brdu-positive cells in $A\beta$ group in comparison with control group increased by 2.5-fold. This value for the group receiving encapsulated CPECs increased approximately 2.2-fold compared to $A\beta$ -injected rats (Fig. 10a, b). At day 30, however, the mean number of Brdu-positive cells in the group receiving $A\beta$ was about 1.9-fold higher than the control rats; meanwhile for the group receiving encapsulated CPECs, this increase approximately 2.1-fold compared to $A\beta$ ones (Fig. 10a, c).

Injection of Encapsulated CPECs Improved Memory Function in Passive Avoidance Task

Thirty days after injection of $A\beta$ into the brains of rats, PA retention results indicated that the latency of the control group was more than the $A\beta$ group and the difference was statistically significant (Fig. 11a). Tukey's multiple comparison test showed that there was a significant difference in step through-latency and time spent in dark compartment between $A\beta$ group and control group ($P < 0.05$, $n = 8$ for each experimental group). Step through-latency and time spent in dark compartment between $A\beta$ group and encapsulated CPECs receiving group showed significant differences. These results suggest that the implant of encapsulated CPECs can improve behavioral functions (Fig. 11b).

Discussion

As about 30 million patients worldwide suffering, AD is currently incurable. Whether $A\beta$ or Tau is the primary cause of AD is still debated, however, the most applied immunotherapies until now, which attempted to reduce pathological manifestations, are attributed to $A\beta$ peptides (Fu et al. 2010). Our implantation of alginate microcapsules overlying the cerebral

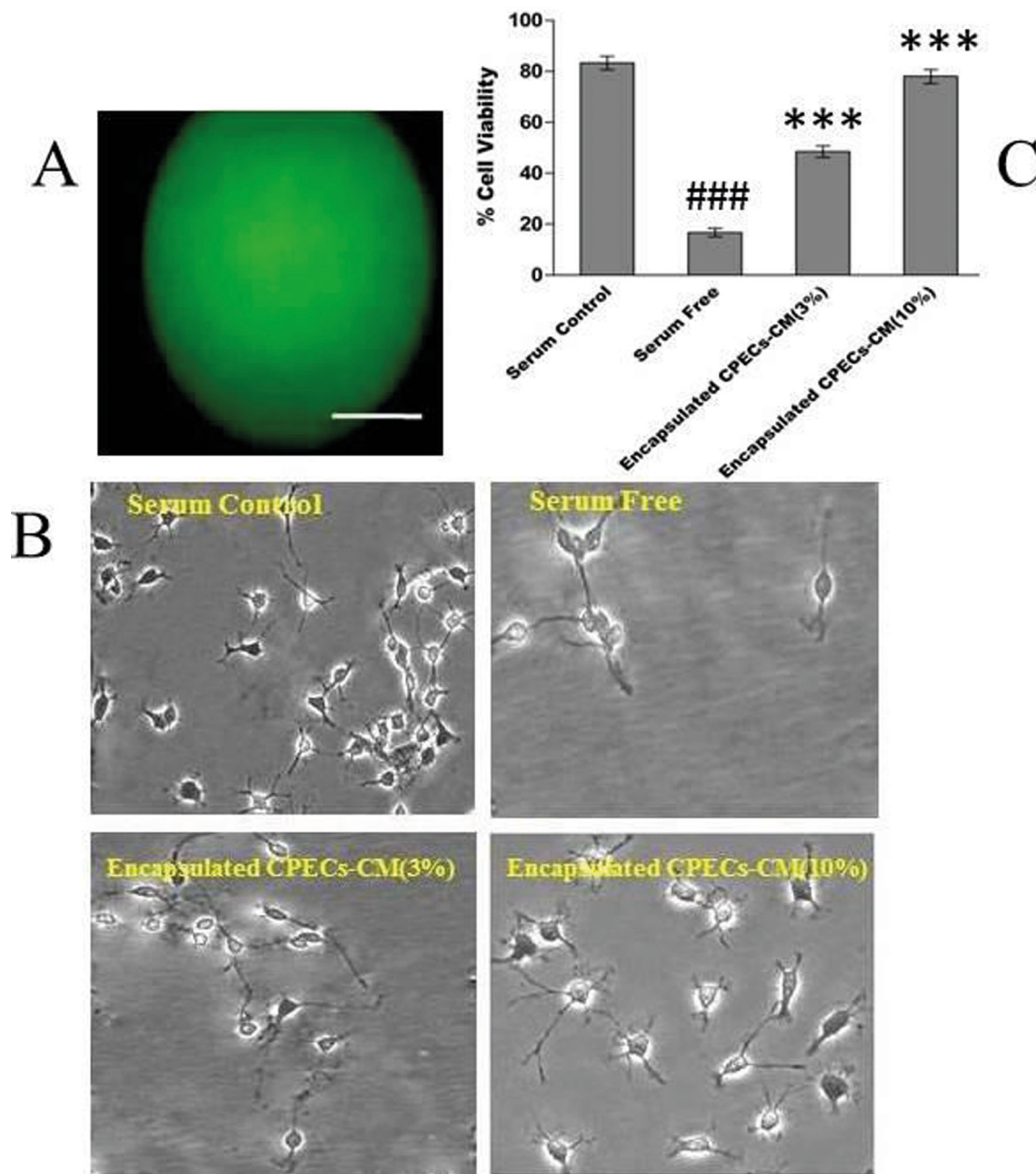


Fig. 3 Functional properties of encapsulated CPECs were determined. Firstly, **a** empty alginate microcapsules incubated with thioflavin T (ThT) and the leakage of ThT into microcapsule were examined by fluorescent microscopy (Scale bar 100 μ m). Following, **b** conditioned media of encapsulated CPECs harvested and embryonic cortical neurons cultured

in the presence of different conditions and **c** cell viability was determined by trypan blue staining [### $P < 0.001$ significant different between serum free and control group; *** $P < 0.001$ significant different between encapsulated CPECs-CM and serum free group]

cortex, containing viable CPECs, following intrahippocampal $A\beta$ injection resulted in recovered memory of adult male albino Wistar rats. Intracerebral administration of $A\beta$, as an approach to model AD, has been undergone great advancement since the first introduction. One of the compelling improvements was the application of fibrillar $A\beta$ instead of oligomers, although the latter ones have shown significantly more toxicity in vitro. $A\beta$ fibrils act as nucleation centers, speeding

up the assembly of monomers into toxic oligomers, so that fibril forms have more sustained effects with greater intensity. It should be noted that transgenic rat models of AD have been developed to partially resolve exogenous $A\beta$ limitations; however, they failed to display some of typical pathophysiology of disease such as lack of overt neurodegeneration and weak inflammatory state. Here, the molecular study showed a significant response 14 days following $A\beta$ injection (1 week

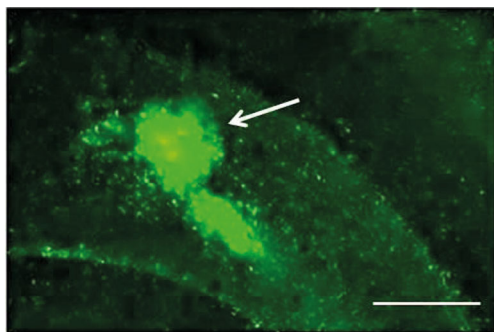


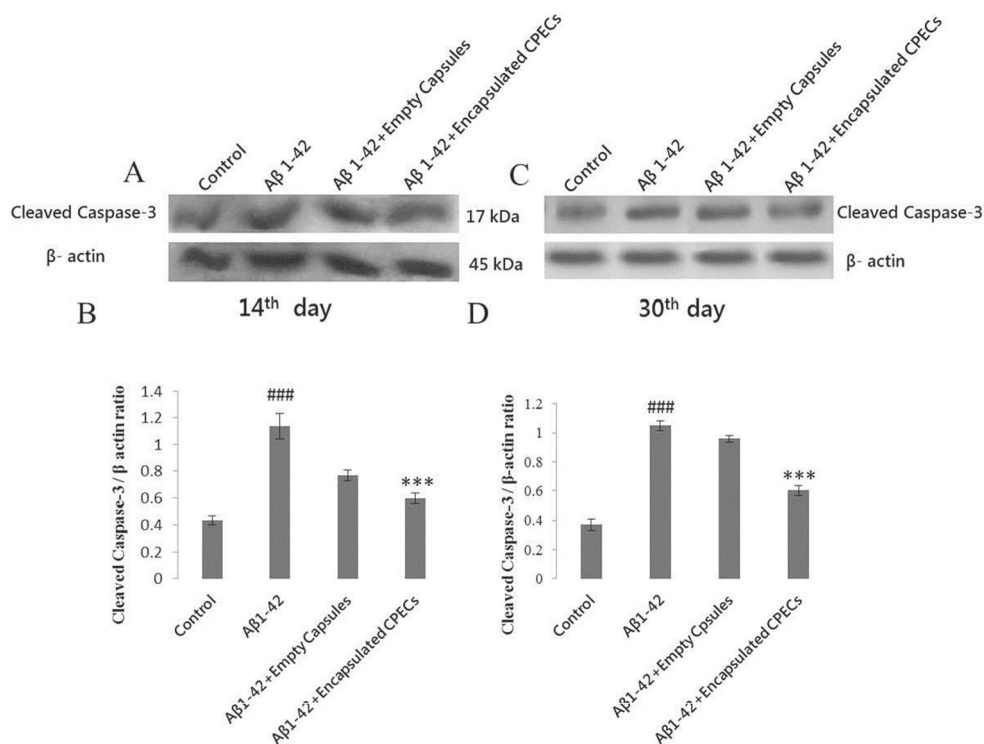
Fig. 4 Fluorescent image of Thioflavin T (ThT) staining for detection of injected $A\beta_{1-42}$ in rat hippocampus. Tissues were incubated with 0.5 % of ThT solution. Aggregation of $A\beta_{1-42}$ showed by white arrow. Scal bar 100 μm

following encapsulated CPECs implantation) that lasts up to 23 days thereafter (30 days of $A\beta$ exposure). However, the behavioral alteration markedly developed after 30 days of $A\beta$ insult, so that we also chose this point to discuss molecular data. Behind the context of behavioral manifestation, the molecular data suggest reduced hippocampal apoptosis and gliosis that accompanied with elevated antioxidant enzyme, SOD, and enhanced neurogenesis.

Excessive apoptosis causes hippocampal atrophy that can be found in AD. In an $A\beta$ viewpoint, there are several hypotheses in $A\beta$ toxicity linking to neuronal apoptosis such as mitochondrial dysfunction, protein conformational changes, and endoplasmic reticulum stress (Ferreiro et al. 2006; Lin and Beal

2006). Oxidative stress has been proposed to be an important factor in the different stages of AD pathogenesis that can be linked to and lied behind of many other independent $A\beta$ -induced apoptotic signaling (Perry et al. 2002). On the other hand, there is a growing body of evidence that hippocampal apoptosis might occur in and contribute to onset and progression of AD cognitive/memory impairment (Aliev 2011; Butterfield and Boyd-Kimball 2004; Christen 2000). Our data suggest that implantation of encapsulated CPECs significantly decreased intrahippocampal $A\beta$ -induced apoptotic markers and morphology with an improvement of memory function as was assessed by PA task. The SOD activity, a marker of anti-oxidative activity, also increased rationally in the rats with CPECs microcapsule grafts. As we demonstrated in our previous work, CPECs-CM possesses growth factors that increase the stabilization of Nrf2 as a major cellular antioxidant trans-activating transcription factor (Aliaghaei et al. 2014). As an example, NGF, secreted by CPECs, has been indicated to be confidently interconnected with Nrf2/ARE system and its downstream factors like thioredoxin and Heme oxygenase-1 (Mimura et al. 2011). The interplay between Nrf2 and VEGF has been also considered extensively (Kweider et al. 2011). Apart from Nrf2, diverse neurotrophin and growth factor profile in CPECs-CM have been described in oxidative stress modulation, independent of Nrf2, as shown by BDNF, VEGF, and NGF (Salinas et al. 2003; Hao and Rockwell 2013; Numakawa et al. 2009).

Fig. 5 The evaluation of apoptotic factor caspase-3 in rats receiving $A\beta_{1-42}$ after transplantation of encapsulated CPECs. Blots were probed with anti caspase-3 antibody and reprobred with β -actin antibody. The densities of cleaved caspase-3 and β -actin were measured, and their ratio was calculated. Bands of caspase-3, 14 days (a) and 30 days (c) after transplantation. Densities of cleaved caspase-3, 14 days (b) and 30 days (d) after transplant of microcapsules. (One representative Western blot is shown; $n=3$). Each point shows the mean \pm S.E.M. [#### $P<0.001$ different from the control group. *** $P<0.001$ different from the $A\beta$ -injected group]



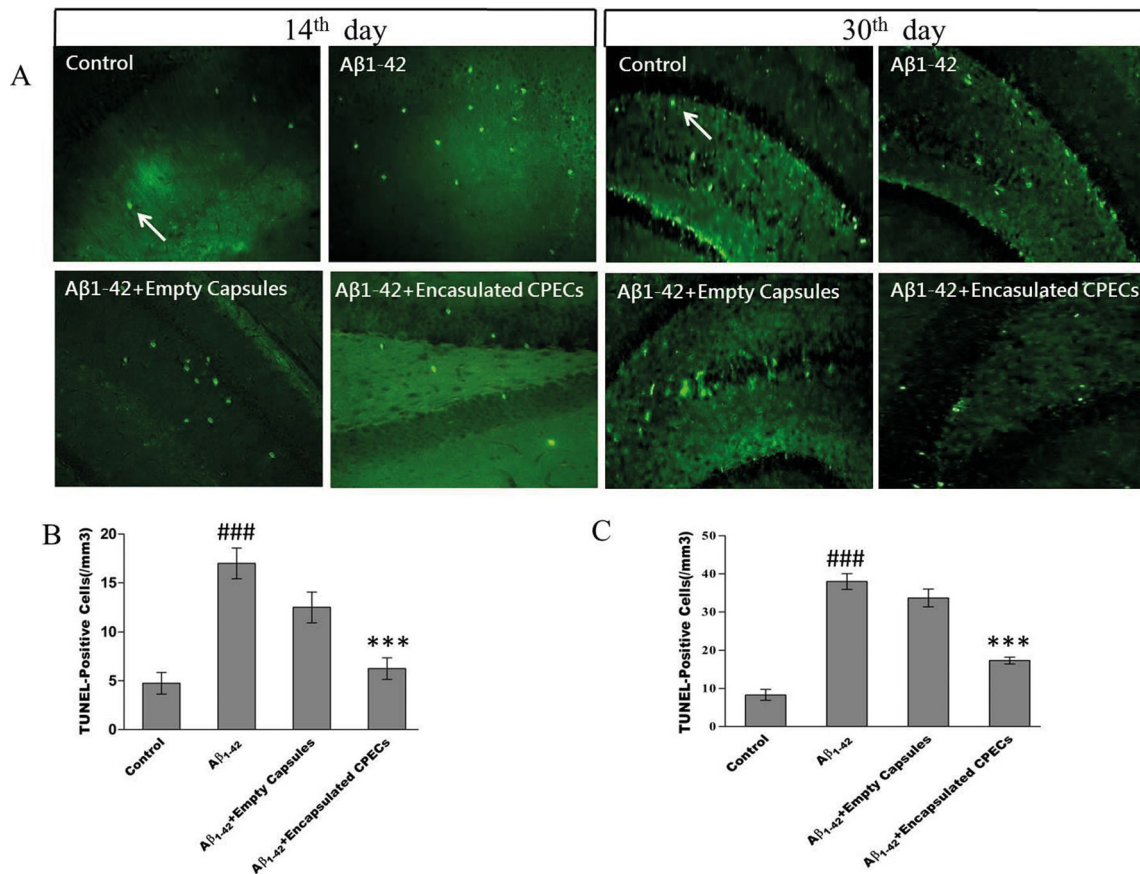


Fig. 6 The evaluation of number of apoptotic cells by TUNEL staining 14 and 30 days after transplantation of encapsulated CPECs. **a** TUNEL-positive cells shown by the white arrows. Number of apoptotic cells were counted in each field after 14 days (**b**) and 30 days (**c**) after

transplantation. Each point shows the mean ± S.E.M. [### $P < 0.001$ different from the control group. *** $P < 0.001$ different from the Aβ-injected group]

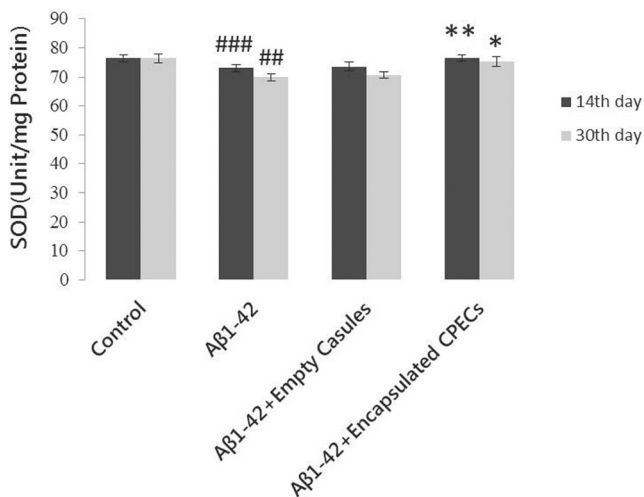


Fig. 7 Effect of encapsulated CPECs on SOD activity in rats receiving Aβ₁₋₄₂. **a** Fourteen days after transplant of microcapsules. **b** Thirty days after transplant. (One representative SOD activity is shown; $n=3$). Each point shows the mean ± S.E.M. [### $P < 0.001$; ## $P < 0.01$ different from the control group. ** $P < 0.01$; * $P < 0.05$ different from the Aβ-injected group]

Excluding oxidative stress modulation, CPECs-CM has several other anti-apoptotic features. Interestingly, Williams and coworkers have concluded that attenuated expression of some immunomodulatory molecules, including β-defensins, in the setting of neurodegenerative diseases may be linked to diminished anti-apoptotic activity (Williams et al. 2012). Accordingly, our observation of cognitive improvement, following encapsulated CPECs implantation, is consistent with the recently reported administration of non-capsulated CPECs grafts in the hippocampus of transgenic mouse model of AD (Bolos et al. 2014). Very recently, in a report by Baruch and colleagues, they showed a great shift toward type I interferon (IFN-I) hyperactivity in the aging murine choroid plexus, which was concomitant with the increased expression of IFN-I-dependent genes. Subsequently, neutralizing IFN-I signaling within the brain restored cognitive function in aged mice (Baruch et al. 2013).

The close relation between neurogenesis and the performance of learning and memory has been extensively discussed. Aβ can promote neurogenesis, both in vitro and in vivo, by inducing neural progenitor cells to differentiate

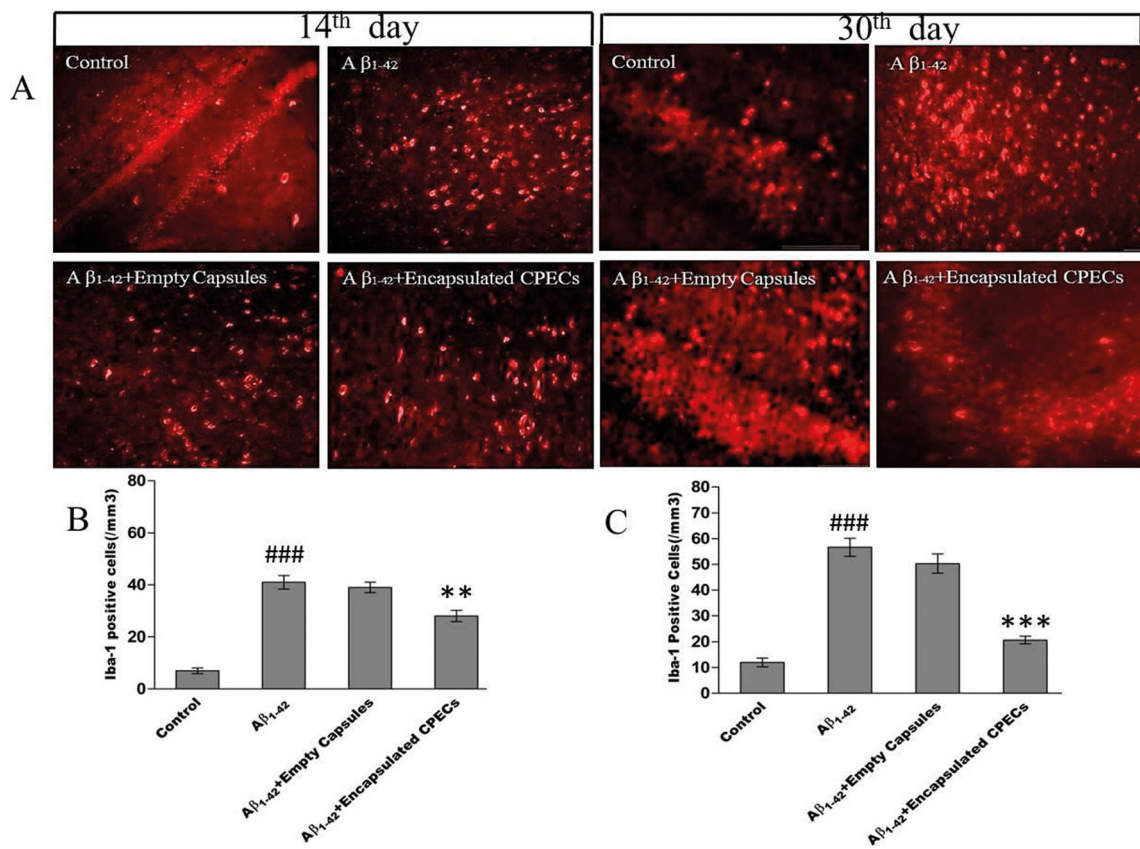


Fig. 8 Immunohistochemistry against Iba-1 as a marker for microglia migration. **a** Iba-1 positive cells in rats' hippocampus shown by the white arrow. **b** Number of Iba-1 positive cells was counted each field at 14 days

(b) and 30 days **(c)** after transplantation. Each point shows the mean ± S.E.M. [###*P*<0.001 different from the control group. ***P*<0.01; ****P*<0.001 different from the Aβ-injected group]

into neurons (López-Toledano and Shelanski 2004; Calafiore et al. 2012). Contrarily, Haughey et al. reported that Aβ has

the potential to oppose human neurogenesis of neural progenitor cells and induces their apoptosis (Haughey et al. 2002). In

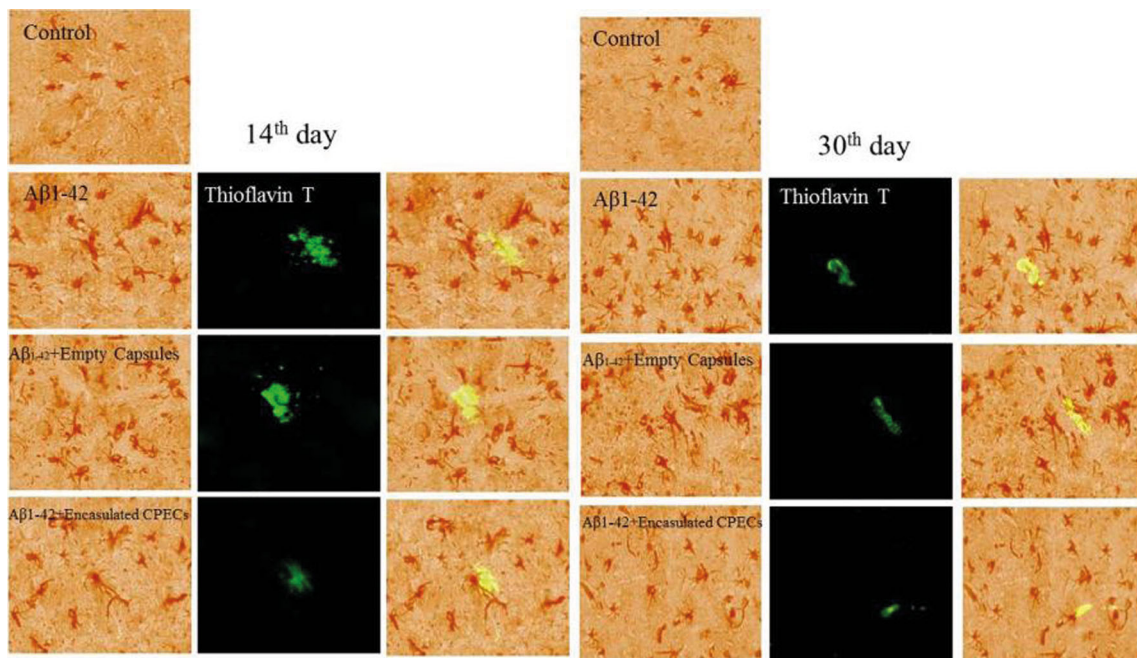


Fig. 9 Immunohistochemistry against GFAP as an astrocytic marker was done 14 and 30 days after microcapsules transplantation

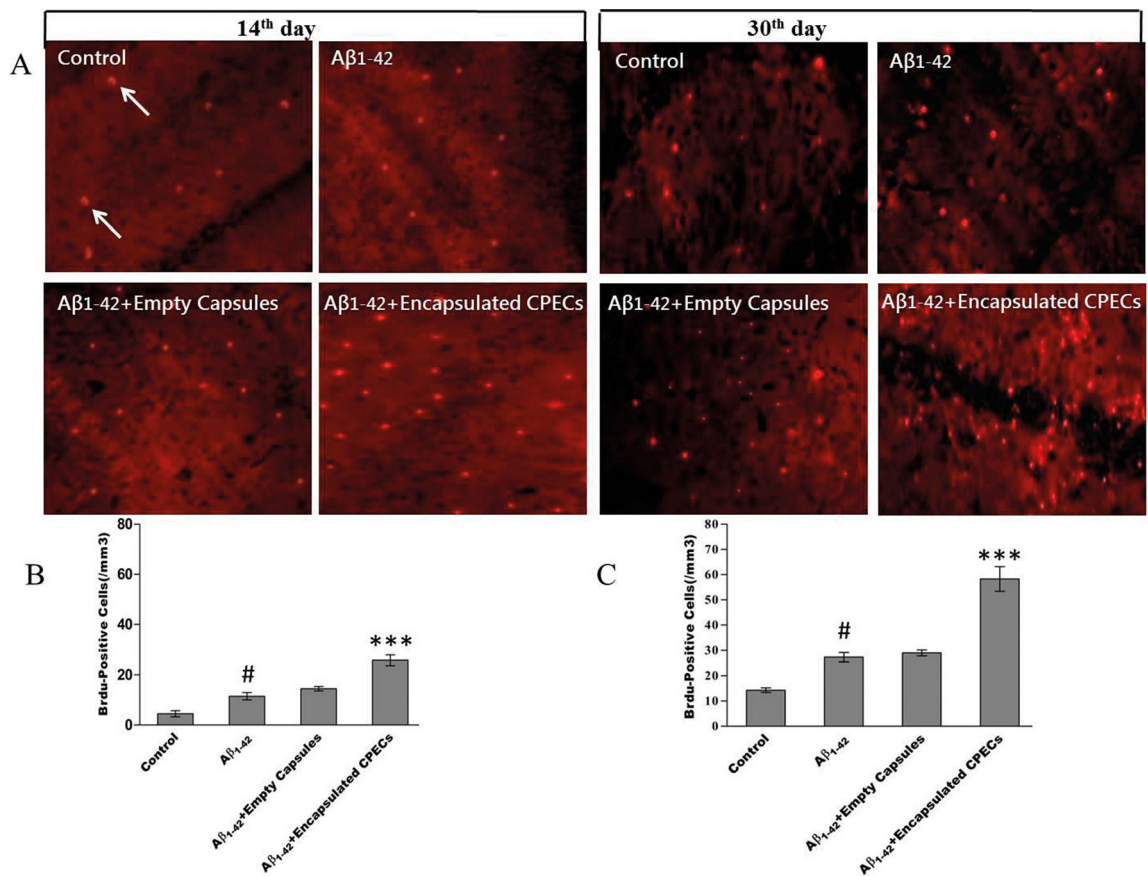


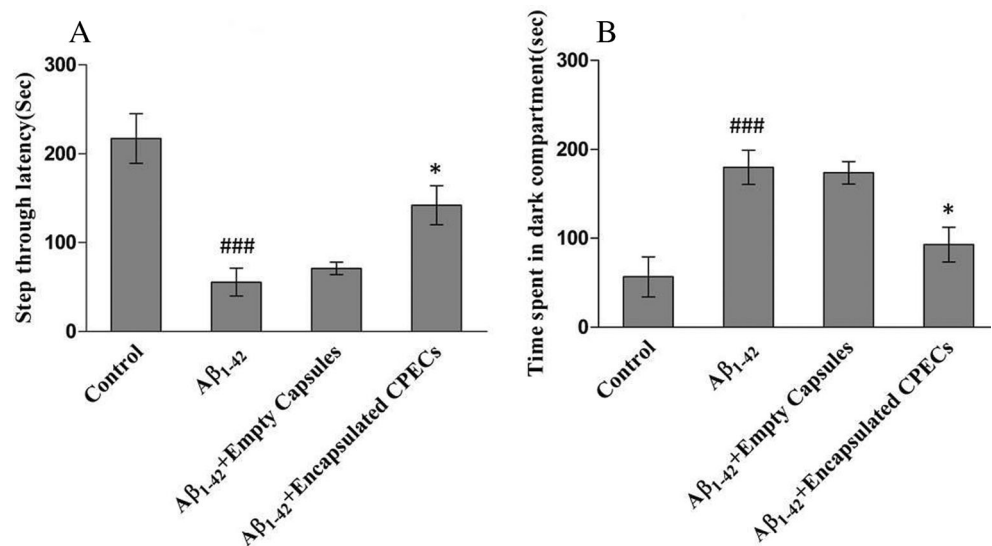
Fig. 10 Immunohistochemistry against Brdu at 14 and 30 days after transplantation, **a** Brdu-positive cells in rat hippocampus shown by white arrows. Number of Brdu-positive cells at 14 days (**b**) and 30 days

(**c**) after transplantation of alginate microcapsules containing CPECs. Each point shows the mean ± S.E.M. [[#]*P*<0.05 different from the control group. ^{***}*P*<0.001 different from the Aβ-injected group]

a study by Bolos et al., it was indicated that although Aβ increased proliferation and differentiation of neuronal progenitor cells in CPECs, newly differentiated neurons showed diminished survival (Bolos et al. 2013). As another point of view, hippocampal neurons displayed promoted neurite

outgrowth by close contact with the cell surface of cultured CPECs (Kimura et al. 2004). Consistently, our data indicated that intrahippocampal injection of Aβ significantly increased neurogenesis of hippocampal neurons, albeit, administration of encapsulated CPECs considerably intensified Brdu-

Fig. 11 The effect of transplantation of encapsulated CPECs on passive avoidance retention at 30th day. **a** Step-through latency and **b** time spent in dark compartment. Each point shows the mean ±S.E.M. [^{###}*P*<0.001 different from the control group. **P*<0.05 different from the Aβ-injected group]



positive cells, representing neurogenesis. Since we encapsulated the CPECs, this logically prevented direct contact between CPECs and hippocampal neurons. As a matter of fact, not only touching CPECs by neurons improves their neurite outgrowth, but also CPECs-CM has been reported to enhance neuronal survival and outgrowth (Watanabe et al 2005). On this subject, amphiregulin as a growth factor is secreted from CPECs and promotes the mitosis of neural stem cells and subsequently enhances neurogenesis (Falk and Frisén 2002).

Astrogliosis and microglial migration is the markers of neuroinflammation in the central nervous system (Frautschy et al. 1998; Bates et al. 2002). The A β deposition in the hippocampus and other brain regions is associated with a robust inflammatory response (Kitazawa et al. 2004). Neuroinflammation has been considered as a major cause in the progression of AD and may be responsible for degeneration in vulnerable brain regions such as the hippocampus. Intriguingly, Frackowiak and Wisniewski firstly discovered that microglia cells are at the bottom of A β phagocytosis in the brain that suggests a protective role for the microglial migration and neuroinflammation in AD (Frackowiak et al. 1992). Moreover, the fact that microglia and astrocytes secrete several neurotrophins and growth factors in an inflammatory environment recommends the enhancement of neurogenesis in an inflammatory context (Russo et al. 2011). On the other hand, the non-specific activation of microglia could alternatively cause harm as neuroinflammation (Mandrekar-Colucci and Landreth 2010). Here, we indicated that robust microglial migration towards the A β deposits with the elevated astrogliosis at this site significantly modulated by the encapsulated CPECs. In the searching for possible interrelation between proneurogenic and antineurogenic features of A β -induced neuroinflammation, previous study reported that microglial phenotype changes in the AD brain (Jimenez et al. 2008). As such, hyper-inflammatory environment has been shown to negatively affect the capacity of microglial cells to engage in phagocytosis. So that endogenous microglia are inefficient to affect plaque clearance (Mandrekar-Colucci and Landreth 2010). Supportingly, increased GFAP-positive astrocytes in the hippocampus were shown to be in accordance with the impairment of spatial memory. Besides, in an AD context, administration of anti-inflammatory cytokines has been demonstrated to reverse any phagocytic deficit provoked by hyper-inflammation (Mandrekar-Colucci and Landreth 2010). This is consistent with our result that modulation of inflammatory state in hippocampus by CPECs subsequently improved cognitive function, which was primarily abrogated by A β . In the support of this context, CPECs' excretion of anti-inflammatory and antioxidative factors, such as autotaxin, has an evidenced role in the alleviation of inflammatory state in specific stresses, including traumatic injury and neurodegenerative diseases (Awada et al. 2014). Moreover, CPECs are a source of TGF- β that can suppress

proliferation and migration of microglia in vitro with the promotion of A β clearance (Mandrekar-Colucci and Landreth 2010).

Overall, our present findings indicated that encapsulated CPECs recovered exogenous A β -induced memory impairment. Our discussion underlying signaling encompasses CPECs' ability to hamper in vivo A β -mediated oxidative stress, specifically by increasing SOD activity, and also blocking the detrimental neuroinflammation occurred in hippocampus that interdependently reduce overall hippocampal apoptosis. The futile neurogenesis by A β transformed to more significant and effective neurogenesis that was activated by encapsulated CPECs. Recently, it has been identified that CPECs induce efflux of A β from the brain, which can be seen in specific conditions, such as intravenous immunoglobulin utilization. Our study provides a novel role of choroid plexus in the processes of A β neurotoxicity; however, the precise mechanism linking choroid plexus to detailed manifestation of the most common form of dementia still remains as an open question.

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