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RESEARCH ARTICLE

New perspective for the treatment of Alzheimer diseases: Liposomal rivastigmine formulations

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Abstract

The aim of this study was to determine the transportations of rivastigmine containing from various liposome formulations through Madin Darby Canine Kidney (MDCK) cells monolayer and to compare the *in vitro* test results with *in vivo*. There is no other liposome formulation of rivastigmine and the transportations of rivastigmine through MDCK cell monolayers or related study available in the literature. Cytotoxicity (MTT) test was used to determine cell viabilities. The effect of sodium-taurocholate or dimethyl-beta-cyclodextrine as penetration enhancer was also investigated. Characterization and stability studies for liposome formulations were performed. Permeation experiments of rivastigmine were performed through MDCK cells and dialysis membrane. The kinetic of release from liposomes was also investigated. The highest apparent permeability coefficient (log. values) was obtained with sodium-taurocholate liposomes for −1.15 ± 0.16 for MDCK cell. Rivastigmine liposomes and solutions were also administered to mice orally and intraperitonally. Acetylcholinesterase (AChE) activity was determined by Ellman method. AChE% inhibition values were calculated for both blood and brain after administration of rivastigmine solution and liposomes. The highest AChE inhibition was observed for rivastigmine-sodium-taurocholate liposomes. Histological observations of the mice' brains were performed under transmission electron microscope (TEM). The histological results were also indicated and supported all these findings.

Keywords: Rivastigmine, liposome, MDCK, alzheimer disease

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and known to be the most common cause of dementia with aging[1,2]. Loss of short-term memory, language impairment, and disorientation of time are the cognitive symptoms of the disease. In the early stages of the disease, the symptoms look like depression symptoms, and may be observed. At the later stages, behavioral and psychiatric symptoms develop subsequent to the decline in the motor functions[3]. Two main microscopic changes observed to be occurred in the brain in AD: plaque development between neurons and neurofibrillary tangle development within the neurons[4]. Today, the pathology of the disease is clarified but the main reason of the disease is still not known clearly and AD is thought to be more than a single etiological entity[5]. There is no drug, which has been shown and proved to treat AD completely, that inhibit the degradation of acetylcholine within synapse are the main group of drugs used in the treatment[4]. Cholinesterase inhibitors are the only agents, approved by the FDA for AD treatment[6]. Rivastigmine tartrate is a second-generation reversible carbamate derivative cholinesterase inhibitor indicated for treatment of mild-to-moderate dementia[7,8]. Rivastigmine is also reported to be a pseudoirreversible inhibitor with brain region selectivity and exhibits long duration action[2,9–11]. Rivastigmine has a short plasma elimination half-life (1.5 h). It is well absorbed (96%) with bioavailability of 36–40% for 3 mg dose and its log P is predicted as 2.45 (DrugBank). The reason of exhibiting low bioavailability is claimed to be extensive first-pass effect of rivastigmine. The bioavailability of the drug is largely affected by the

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administration route because of the first-pass metabolism in liver[12].

Brain is exactly the most difficult organ for the delivery of active agents[13]. Despite its high blood flow, blood–brain barrier (BBB) limits the brain penetration of many substances and molecules including drugs to the central nervous system (CNS)[13,14]. BBB is the interface, which regulates and controls the specific transport of endogenous/exogenous substances selectively to the brain[15]. BBB is characterized by tight junctions (TJs), which are the most specific structures in the brain endothelial cells and different from the endothelial cells of other tissues/organs of body. TJs inhibit paracellular movement by their high transepithelial electrical resistance (TEER) and divide the endothelial cell membranes into two distinct sides; luminal side (blood) and abluminal side (brain)[16–18].

İn vitro cell culture systems are adequate model systems, which are used in drug design and development to understand absorption, transportation, and distribution of drugs throughout the body. There are several systems that have been used as a tool for the prediction of *in vivo* transportation of compounds[19,20]. Various methods have been searched for CNS drug penetration screening, including *in vitro* cell culture models, *in vivo* methods, and *in silico* prediction[21]. Brain originated cells possess the main group of cell culture models but nonbrain originated epithelial cells such as Caco-2 (human colon carcinoma cells). Madin Darby Canine Kidney (MDCK) cells have also been used as *in vitro* BBB model to estimate the BBB permeability of drugs[18,22,23]. MDCK cells are derived from dog kidney[24] and represents similar protein structure (occludin, ZO-1) of TJs in BBB cells. They have high TEER values[25–27].

It is generally known that the transport of lipophilic agents to brain is easier than hydrophilics. Rivastigmine is a highly hydrophilic compound and its brain penetration is restricted by TJs[28]. Liposomes are spherical, colloidal lipid vesicles that form naturally or prepared artificially when phospholipids are exposed to an aqueous medium[29–33]. Their unique structural properties allow encapsulation of hydrophilic drugs in the aqueous interior, incorporation of hydrophobic core of the phospholipid barrier, and also it is possible to formulate drugs having intermediate or low solubility as liposomes[34].

The objective of this study was to develop liposome formulations of rivastigmine and to determine their absorption properties through MDCK cells. The effects of absorption enhancers [dimethyl-β-cyclodextrine (dimethyl-β-CD) and sodium taurocholate (NaTC)] on permeability of rivastigmine through MDCK cells and the *in vitro* data were compared with *in vivo* results. Rivastigmine liposomes were administered to mice orally and intraperitonally and also acetylcholinesterase (AChE) inhibition effects of liposomes were determined and highest effect was obtained with rivastigmine-NaTC liposomes. Transmission electron microscope (TEM) studies of brain tissues after exposure to formulations were also performed.

Materials and methods

Materials

Rivastigmine tartrate was purchased from Dr. Reddy's, India. Cholesterol, acetylcholine iodide, dimethyl-β-CD, NaTC, and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were all purchased from Sigma, USA. Dipalmitoylphosfotidyl choline (DPPC) was provided from Across Organics, Belgium. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Bichrom, Germany, and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was provided from Serva, USA. Methyl cellulose (MC) was purchased from Colorcon, England. All other chemicals were also of high pressure liquid chromatography (HPLC) grade.

Methods

In vitro studies

Preparation of rivastigmine liposomes. Multilamellar rivastigmine liposomes were prepared using film formation method. Lipids (cholesterol and DPPC in 1:1 ratio), rivastigmine and MC (20 mg), absorption enhancers [dimethyl-β-CD (0.15%) or NaTC (1.875 mM)] for only enhancer added to formulations dissolved with chloroform and evaporated under vacuo at 43–44°C and dry film were obtained. The film was hydrated by NaCl solution (0.9%) and liposome suspensions were formed after vortexing for 5 min and ultrasonication for 25 min. Liposome suspensions were centrifuged at 20,000*g* at 25°C for 10 min. Supernatants and liposomes were separated.

Measurement of the particle size of liposomes. The particle size of liposomes was determined using Smpatec GmbH-Partikel Technick (Germany) laser diffraction particle sizer.

Measurement of the zeta potential of the liposomes. Zeta potential measurements of liposomes were determined by using Zetasizer-Nano ZS-Malvern (Germany).

Determination of type liposome. The type of the liposomes was investigated by using inverted microscope (Olympus CK2, Japan).

Determination of encapsulation efficiency. The rivastigmine contents of liposomes were determined using HPLC. The method was adapted from literature[35]. Mobile phase was sodium heptane sulfonate:acetonitrile (72:28) pH 3.0. Ace 5 C18, 4.6×250 mm column was used. The encapsulation efficiency was calculated as it was reported in the literature[36–38].

$$\%EE = (TD - UED) \times 100/TD$$

where %EE is the percent encapsulation efficiency, TD is the total drug concentration, and UED is the concentration of unencapsulated drug.

Determination of NaTC amount in liposomes. Determination of NaTC amount captured in liposomes was determined by HPLC. The method was adapted from literature[39]. Mobile phase was 0.3% ammonium carbonate:acetonitrile (68:32) and detections were carried out at 210 nm.

Determination of dimethyl-β-CD amount in liposomes. Determination of dimethyl-β-CD amount in liposomes was determined by spectrophotometric method. The samples at pH 11.0 was colored with phenolphthalein and immediately detected at 554 nm[40].

In vitro release studies of rivastigmine from liposome formulations. Drug release studies were performed using Franz diffusion cell with a dialysis membrane (pore size was 12,000 Da). About 1.5 ml rivastigmine liposome suspension was placed in donor compartment of cells. Receiver compartment was involving 2 ml of phosphate buffer (pH 7.4). *In vitro* release studies were performed for three types of liposomes: Rivastigmine liposomes, rivastigmine and NaTC, and rivastigmine and dimethyl-β-CD-containing liposomes. The studies were performed for 24 h at 37 ± 0.5°C and 1.5 ml sample was removed and predetermined time periods and replenished with fresh buffer. The samples were then analyzed.

Physical stabilities of liposomes. The stability of the three types of rivastigmine liposome formulations was investigated for 6 months (180 days) under three different conditions at 4°C (refrigerator humidity conditions), 25°C (60% relative humidity), and 40°C (75% relative humidity). Rivastigmine contents, zeta potentials, and particle size of liposomes were determined periodically.

Cell culture studies

Cell culture. MDCK cells were provided from Food and Mouth Diseases Institute. MDCK cells were seeded on semipermeable polycarbonate filter inserts for 7 days (1.2 cm diameter, 0.4 μm pore size) with 60,000 cells/ml density.

Cytotoxicity assay. MTT assay is a colorimetric method for the determination of cell viability[41]. The effects of rivastigmine, NaTC, dimethyl-β-CD, and liposomes without active substance on MDCK cell viability were investigated by using serum-containing and serum-free DMEM for 24 h time period. Various rivastigmine (1, 1.5, 3, 4.5, and 6 μg/ml), NaTC (20, 15, 7.5, 3.75, 1.875, and 0.005 mM), dimethyl-β-CD (5%, 3%, 1.5%, 0.75%, 0.375%, 0.25%, 0.15%) concentrations were studied in DMEM. At the end of the 24-h time period, plates were emptied and fresh DMEM and MTT solutions were added to the wells and incubated for further 3 h. The plates were emptied and 100 μl isopropanol was added to dissolve the Formosan precipitate. The color developed was measured in 570 nm with a multiwell Eliza reader. The results were calculated as a percentage of the control group values.

Transport experiments. The transport studies were performed from apical to basolateral side of the diffusion cells at 37°C. The concentrations of rivastigmine and absorption enhancers were considered according to the MTT test results. The samples were withdrawn at predetermined time periods replaced with fresh DMEM. Rivastigmine that passed through the basolateral side was analyzed with HPLC and Papp values were calculated according to the following Equation 1[42–44]:

$$P_{app} = \frac{dQ}{dt}\frac{1}{AC_0} \quad (1)$$

Measurement of TEER values. The TEER values recorded at $t = 0$ h were taken as initial values. TEER values were measured after the permeation studies completed at 24 h point.

In vivo studies

Design of animal experiments. Balb-C type, 14–16 weeks of aged male mice were used for our *in vivo* experiments. All animal studies were conducted under the protocol approved by the Animal Care and Use Ethical Committee of Gazi University (G.Ü.ET-07.011).

The animals were divided into 14 groups, 15 animals were used in each group. Rivastigmine solution, rivastigmine, and absorption enhancers including solutions, rivastigmine liposomes, rivastigmine and absorption enhancers including liposomes were administrated to the animals both orally (o) and intraperitonally (ip) . Rivastigmine dose was 2300 μg/kg for all formulations[45]. The design of the animal groups was shown in Table 1.

Preparation of animals. All animal groups except control group were treated intraperitonally or orally with an appropriate concentration of rivastigmine (121 μg) in different formulations. About 1 ml of blood samples were collected intracardially at predetermined time points (0.25, 0.5, 1, 3, 6, 8, and 24 h). Brain samples were taken from animals at 6, 8, and 24 h after blood sampling. All blood and brain samples were stored at deep freezer (–80°C) until analyzed[46,47].

Cholinesterase inhibitory activity measurement. AChE inhibitory measurement studies were conducted spectrophotometrically by the method of Ellman[48] for both blood and brain samples. Spectrophotometric determinations were performed at 412 nm.

Histological studies. Histological examinations were conducted using TEM. Brain tissues were fixed in 2.5% glutaraldehyde-containing phosphate buffer solution for 2 h. Postfixation was done in 1% osmium tetraoxide

(OsO_4) and dehydration was achieved in a series of different graded alcohols. Tissues were kept in propylene oxide for 10 min and then embedded to Araldyt CY212, 2-dodecen-1-yl succinic anhydride (DDSA), and benzyldimethyl amine (BDMA)-containing embedding flasks. Thin and ultra-thin sections were then taken out and examined with light microscope after painting with toluidine blue. Ultra-thin sections were investigated by TEM after staining with uranyl acetate and lead citrate.

Statistical analysis

All our data in this study were considered as means ± SD, and one-way ANOVA was used for statistical analysis.

Results

In vitro studies

Determination of the size and type of liposomes

Inverted microscope (Olympus CK2, Japan) was used to investigate the physical appearance of liposomes. The type of liposomes was observed to be multilamellar vesicles (MLVs) (Figure 1). The particle sizes of three types of liposomes were analyzed by laser diffraction particle sizer. The mean particle sizes of rivastigmine liposomes, rivastigmine and NaTC-containing liposomes, and dimethyl-β-CD-containing liposomes were found to be 3.40 ± 0.01 μm, 3.37 ± 0.00 μm, and 4.51 ± 0.04 μm (±SD, $n = 6$) respectively.

Determination of zeta potential of liposomes

The mean zeta potentials of rivastigmine liposomes, rivastigmine and NaTC-containing liposomes, and dimethyl-β-CD-containing liposomes were found to be –3.28 ± 0.00 mV, –4.30 ± 0.66 mV and –5.43 ± 0.00 mV (±SD, $n = 6$), respectively.

Determination of encapsulation efficiency of liposomes

The encapsulation efficiencies of rivastigmine in liposomes were found to be 35.4% for only rivastigmine-containing liposomes, 29.9% for rivastigmine and dimethyl-β-CD-containing liposomes, and 25.2% for rivastigmine and NaTC-containing liposomes.

Determination of NaTC amount in liposomes

It was found to be 41%.

Determination of dimethyl-β-CD amount captured in liposomes

It was found to be 65%.

Physical stability of liposomes

The quantitative analyses of rivastigmine, particle size, and zeta potential measurements were performed to evaluate stability of rivastigmine liposomes in aqueous solutions at 4°C, 25°C, and 40°C for 180 days.

Table 1. Design of experimental animal groups.

Group numbers and codes	Treatment
1 (C)	Control group (no formulation)
2 (RSip)	Rivastigmine solution (ip)
3 (RFLip)	Rivastigmine-free liposomes (ip)
4 (RLo)	Rivastigmine liposomes (o)
5 (RTCLo)	Rivastigmine + NaTC-containing liposomes (o)
6 (RCDLo)	Rivastigmine + dimethyl-β-CD-containing liposomes (o)
7 (RFLo)	Rivastigmine-free liposomes (o)
8 (RTCLip)	Rivastigmine + NaTC-containing liposomes (ip)
9 (RCDLip)	Rivastigmine + dimethyl-β-CD-containing liposomes (ip)
10 (RLip)	Rivastigmine liposomes (ip)
11 (RCDSip)	Rivastigmine + dimethyl-β-CD-containing solution (ip)
12 (RTCSip)	Rivastigmine + NaTC-containing solution (ip)
13 (RCDSo)	Rivastigmine + dimethyl-β-CD-containing solution (o)
14 (RTCSo)	Rivastigmine + NaTC-containing solution (o)

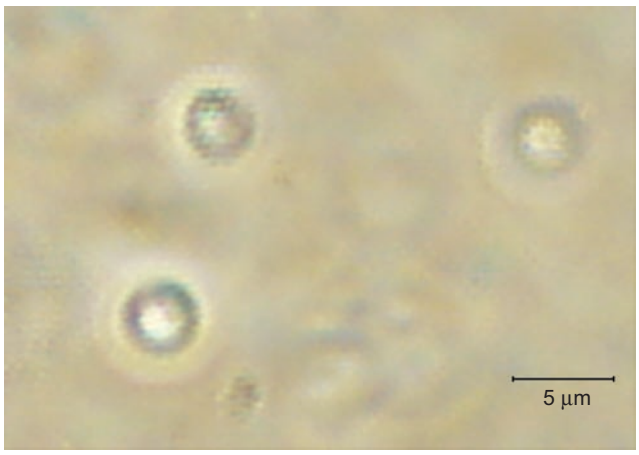

Figure 1. The inverted microscope image of liposomes (×400).

Table 2. The mean particle sizes of liposomes for 6 months at 4°C, 25°C, and 40°C.

	Time (Months)	Mean particle size (μm) ± SD (4°C)	Mean particle size (μm) ± SD (25°C)	Mean particle size (μm) ± SD (40°C)
Rivastigmine liposomes	0	3.40 ± 0.01	3.40 ± 0.01	3.40 ± 0.01
	6	4.66 ± 0.05	4.78 ± 0.63	9.74 ± 0.02
Rivastigmine-dimethyl-β-CD liposomes	0	4.51 ± 0.04	4.51 ± 0.04	4.51 ± 0.04
	6	4.89 ± 0.05	4.83 ± 0.11	6.78 ± 1.07
Rivastigmine-NaTC liposomes	0	3.37 ± 0.00	3.37 ± 0.00	3.37 ± 0.00
	6	3.49 ± 0.01	3.54 ± 0.02	4.19 ± 0.18

Table 3. The zeta potential values of liposomes for 6 months at 4°C, 25°C, and 40°C.

	Time (month)	Zeta potentials (mV) ± SD (4°C)	Zeta potentials (mV) ± SD (25°C)	Zeta potentials (mV) ± SD (40°C)
Rivastigmine liposomes	0	−3.28 ± 0.00	−3.28 ± 0.00	−3.28 ± 0.00
	6	−4.03 ± 0.60	−6.17 ± 0.20	−9.02 ± 0.49
Rivastigmine-dimethyl-β-CD liposomes	0	−5.43 ± 0.00	−5.43 ± 0.00	−5.43 ± 0.00
	6	−4.91 ± 0.35	−3.80 ± 0.65	−2.97 ± 0.46
Rivastigmine-NaTC liposomes	0	−4.30 ± 0.66	−4.30 ± 0.66	−4.30 ± 0.66
	6	−4.36 ± 0.31	−7.40 ± 0.58	−14.2 ± 1.19

The mean particle sizes of rivastigmine liposomes, rivastigmine and NaTC-containing liposomes, and dimethyl-β-CD-containing liposomes were measured as shown in Table 2 (mean particle size ± SD, $n = 6$), respectively.

The mean particle sizes of rivastigmine liposomes were significantly increased after 3rd months at 4°C, 25°C, and 40°C. The most significant increase was observed at 40°C. Significant increase at particle size was also observed for rivastigmine and dimethyl-β-CD liposomes after the third month. Similar to rivastigimine liposomes, rivastigmine and NaTC liposomes were found to be the most stabile liposomes. No significant change on particle size was observed for rivastigmine and NaTC liposomes for 6 months.

The zeta potential measurements were also evaluated for 6 months (Table 3). Prepared liposome samples were examined during 6 months and the shelf lives of liposomes were calculated for 4°C, 25°C, and 40°C using the Arrhenius equation. The degradation profiles of rivastigmine in liposome formulations were examined and degradation profiles were evaluated. The degradation of rivastigmine in liposomes found to be with second-order kinetic. Shelf life at 4°C was 11 days (correlation coefficient, $r^2 = 0.920$) and 7 days at 25°C ($r^2 = 0.882$). The degradation of rivastigmine in rivastigmine and dimethyl-β-CD liposomes was found to be with first order. Shelf life at 4°C was 14 days (correlation coefficient, $r^2 = 0.963$) and 12 days at 25°C ($r^2 = 0.972$). The degradation of rivastigmine in rivastigmine and NaTC liposomes was found to be with first order. Shelf life at 4°C was 21 days (correlation coefficient, $r^2 = 0.910$) and 14 days at 25°C ($r^2 = 0.975$).

In vitro release studies of rivastigmine

The *in vitro* release experiment of rivastigmine from solution and liposome formulations were performed using Franz-type diffusion cells dialysis membrane, receptor medium was pH 7.4 phosphate buffer at 37 ± 0.5°C. The release of rivastigmine from rivastigmine liposomes, rivastigmine and dimethyl-β-CD liposomes, rivastigmine and NaTC liposomes was determined to be with first-order kinetic, correlation coefficients were 0.967, 0.973, and 0.966, respectively. The release profiles of rivastigmine from liposomes were given in Figure 2.

In vitro release studies were also done using rivastigmine solutions. To put forward the effect of liposomes on the release of rivastigmine from solution, rivastigmine and dimethyl-β-CD solution, rivastigmine and NaTC-containing solutions were determined to be with RRSWB kinetics (correlation coefficients were 0.994, 0.994, and 0.996, respectively). The release profiles of rivastigmine from solutions were given in Figure 3.

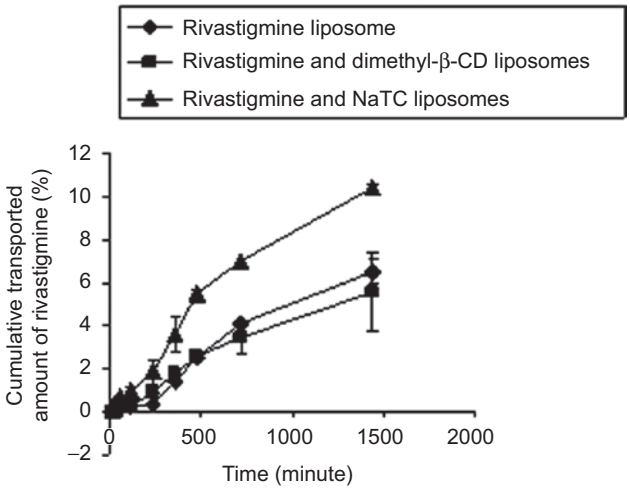

Figure 2. *In vitro* release profiles of rivastigmine from the liposome formulations at 37 ± 0.5°C (medium: pH 7.4 phosphate buffer) (error bars represent standard deviations, $n = 2$).

Cell culture studies

Cytotoxicity assay

The effects of rivastigmine, NaTC, dimethyl-β-CD, and lipids used in liposome formulations on MDCK cell viability were investigated for 24 h. Effect of serum content of DMEM was also examined by using both serum-containing and serum-free DMEM. Rivastigmine was used with the dose of 1.5 mg/ml, and cell viabilities were measured as 55% for MDCK cells. NaTC amount in the formulations was 1.875 mM and cell viabilities with this dose were found to be 69% for MDCK cells. Dimethyl-β-CD concentration was 0.15% and cell viabilities with this dose were determined to be 56% for MDCK cells. Liposome contents had no clear effect on cell viabilities in our study, so liposomes were not found to be toxic to any cells at any concentrations. These materials were used at given concentrations with serum including DMEM in all transport studies.

Transport experiments

The transport studies were also performed through only membranes, which did not contain any cells as a control, at the beginning of the study. Transport experiments

for rivastigmine for solution and liposome formulations through MDCK cells from apical to basolateral side of the cells were evaluated. Cumulative amounts of rivastigmine at the end of the 24-h time period were calculated using Equation 1[42–44]: The calculated log k values were given in Table 4. The cumulative amounts of rivastigmine at the end of the 24-h time period were calculated and results for MDCK cells transportation are given in Figure 4.

Determination of TEER values

The TEER values recorded at $t = 0$ h were found 1315 ± 10, 1030 ± 20, and 1005 ± 25 Ω.cm²as initial values and TEER values were measured after the permeation studies completed; at 24 h point, these values were found as 1308 ± 15, 1010 ± 20, and 990 ± 20 Ω.cm² for rivastigmine liposomes, rivastigmine + dimethyl-β-CD liposomes, rivastigmine-NaTC liposomes formulations, respectively. The TEER values at 24 h were expressed as percent of initial values.

In vivo studies

Ellman method was used for evaluating AChE inhibition effect of rivastigmine in blood and brain samples for liposome and solution formulations[48]. AChE inhibition percentages were given in Figures 5–8.

In vitro–in vivo correlation studies were performed between penetrated amounts of rivastigmine (%) passed through apical to the basolateral side of the MDCK cell monolayer and AChE inhibition (%) values calculated by animal studies for both blood and brain for all formulations. The graphics were shown in Figure 9A–D) below.

Histological studies

Brain tissues of mice, which received different rivastigmine solutions and liposomes, were investigated by ultrathin sectional preparation under TEM. The brains of the control group were also considered. The TEM images of different treatment groups were given in Figures 10–20.

Discussion

AD is known to be the most common cause of dementia with aging. It causes loss of memory function and affects the daily lives of patients. Cholinesterase inhibitors are the main group of therapeutics used in AD treatment. Rivastigmine is a pseudoirreversible hydrophilic cholinesterase inhibitor with low bioavailability. Liposomes resemble cell membrane compounds in their compositions. Brain uptake of lipids/lipophilic substances is possible by passive diffusion, while hydrophilic compound cannot cross BBB[49,50]. Lipidization is one of the most

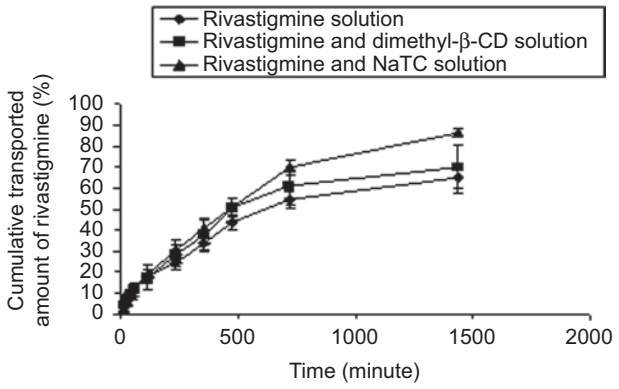

Figure 3. *In vitro* release profiles of rivastigmine from the solutions at 37 ± 0.5°C (medium: pH 7.4 phosphate buffer) (error bars represent standard deviations, $n = 3$).

Table 4. Log k values calculated from MDCK transport study results.

Samples	log k (cm/hour) (MDCK)
Rivastigmine solution	−1.80 ± 0.01
Rivastigmine and dimethyl-β-CD solution	−1.72 ± 0.03
Rivastigmine and NaTC solution	−1.65 ± 0.06
Rivastigmine liposomes	−1.26 ± 0.12
Rivastigmine and dimethyl-β-CD liposomes	−1.29 ± 0.03
Rivastigmine and NaTC liposomes	−1.15 ± 0.16

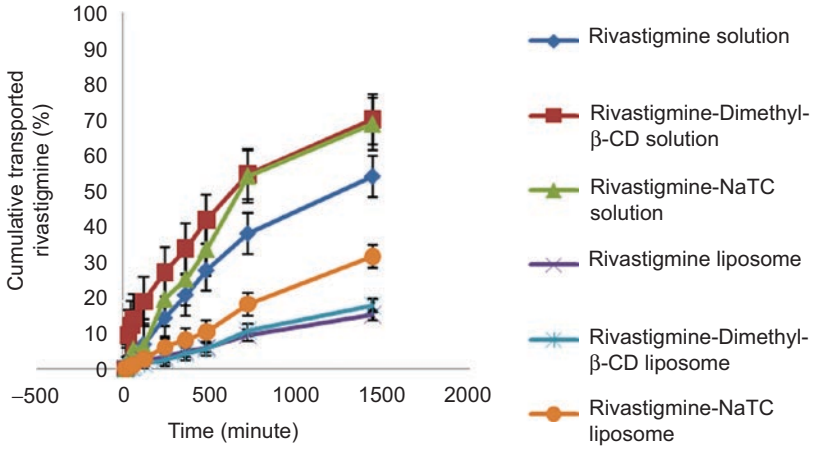

Figure 4. Cumulative amounts of rivastigmine from various formulations transported through MDCK cells (error bars represent standard deviations, $n = 3$).

common ways for increasing brain penetration[14,51,52]. The aim of our study was to increase the brain penetration of rivastigmine by formulating rivastigmine as liposomes. Three types of liposome formulations of rivastigmine were developed and the absorption properties of liposomes were investigated.

MLV-type liposomes were prepared by the film formation method, using DPPC and cholesterol (1:1 w/w). MLV type of rivastigmine liposomes were observed under inverted microscope as seen in Figure 1. Arumugam and his group prepared rivastigmine liposomes as a carrier system to deliver rivastigmine to the brain by administering it through the intranasal route[53].

They formulated liposomes with soya lecithin and cholesterol (4:1 molar ratio) by lipid layer hydration

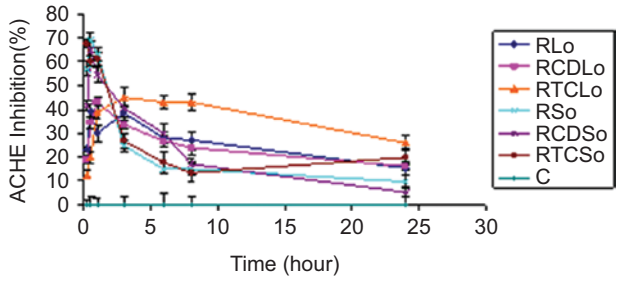

Figure 5. AChE inhibition blood values of orally given formulations (error bars represent standard deviations, $n = 15$).

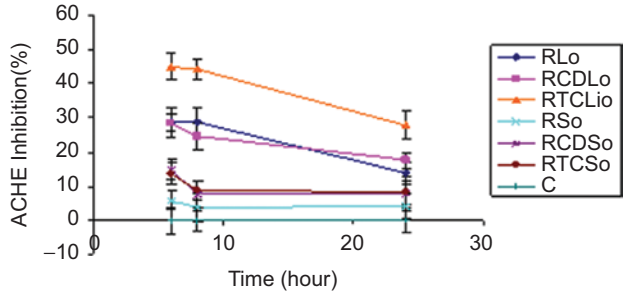

Figure 7. AChE inhibition brain values of orally given formulations (error bars represent standard deviations, $n = 15$).

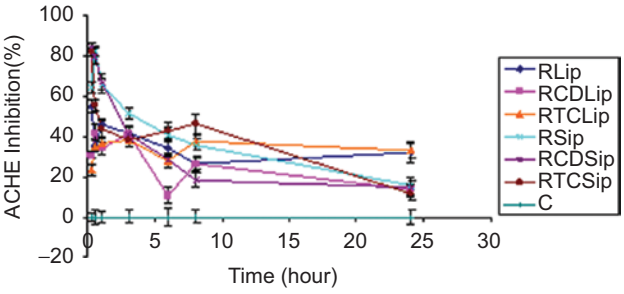

Figure 6. AChE inhibition blood values of intraperitonally given formulations (error bars represent standard deviations, $n = 15$).

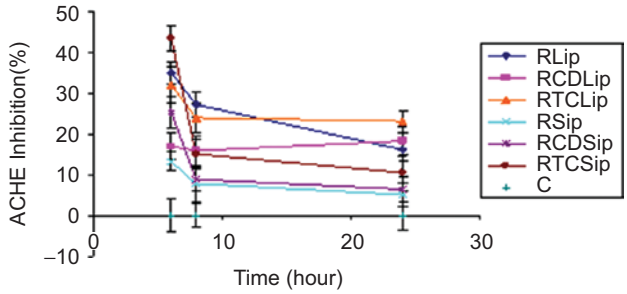

Figure 8. AChE inhibition brain values of intraperitonally given formulations (error bars represent standard deviations, $n = 15$).

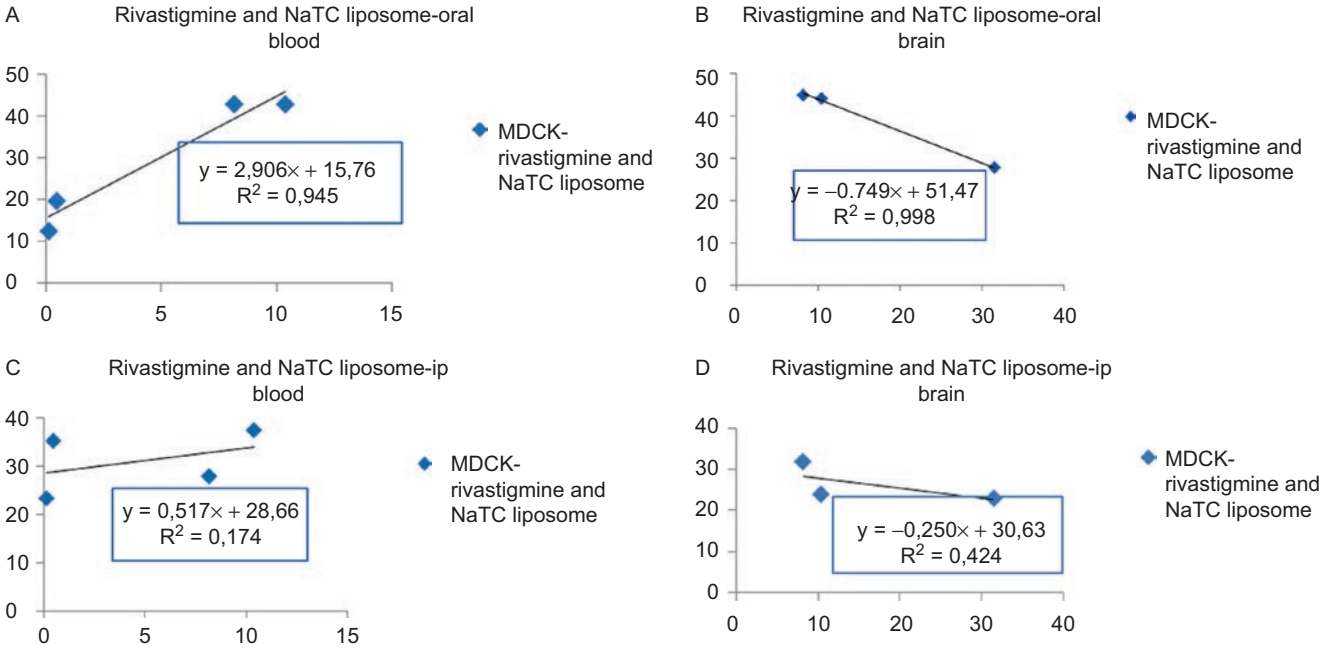

Figure 9. *In vitro–in vivo* correlation results for rivastigmine and NaTC liposomes between penetrated amount of rivastigmine (%) passed through apical to the basolateral side of the MDCK cell monolayer and AChE inhibition (%) values for orally (A, B) and intraperitonally (C, D) treated mice for blood (A, C) and for brain (B, D).

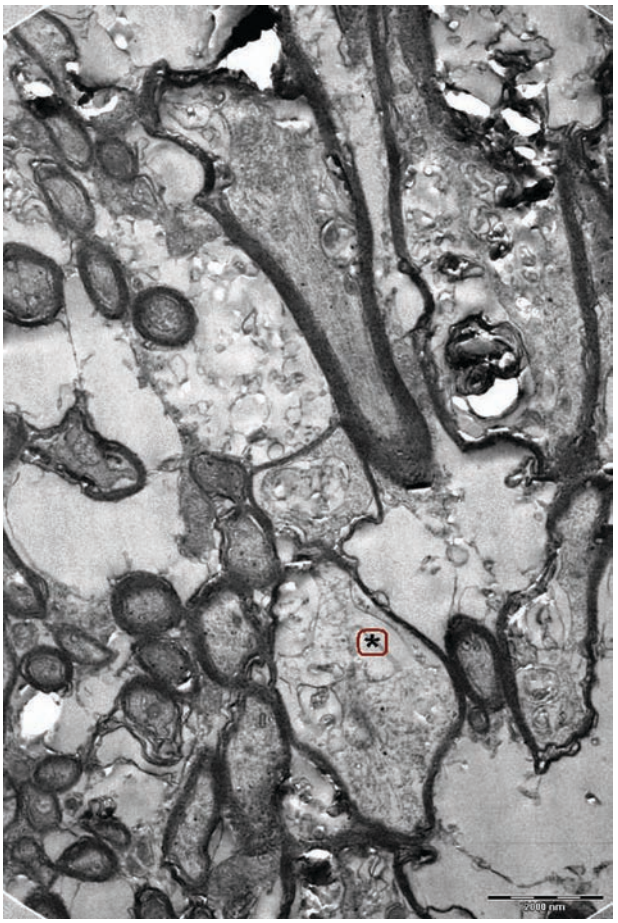

Figure 10. Rivastigmine and NaTC-containing solution intraperitonally applied mice brain.*refers to myelinated neuronal ending (×8200).

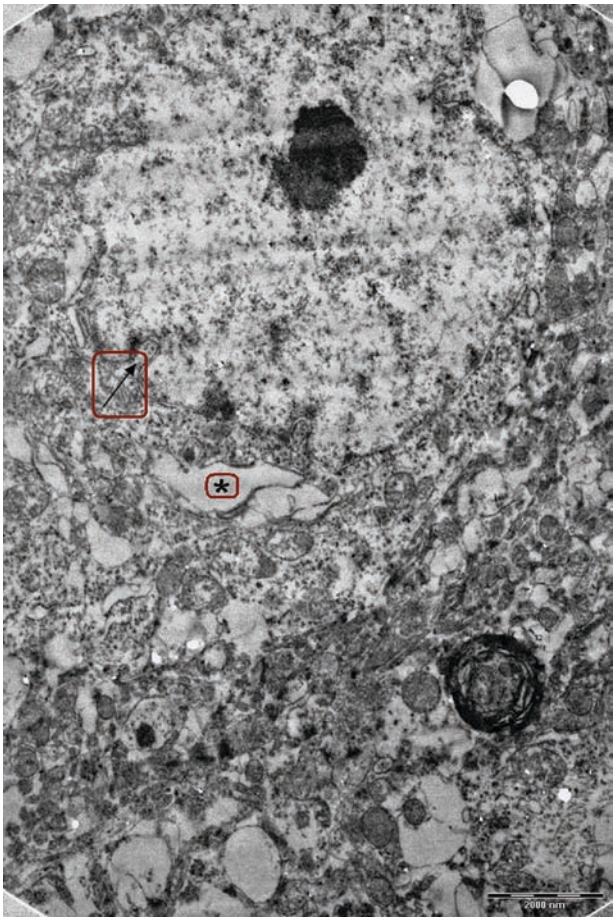

Figure 11. Rivastigmine and NaTC-containing liposome intraperitonally applied mice brain.*refers to endoplasmic reticulum cisterna, and → refers to nucleus concavity (×8200).

method with an encapsulation efficacy of 80%. The reason of getting high encapsulation efficacy might be because of the particle size (10 μm). In our study, the amount of rivastigmine in liposomes, dimethyl-β-CD-containing liposomes, and rivastigmine and NaTC-containing liposomes was found to be 35.4%, 29.9%, and 25.2%, respectively. According to Lasic[30], although it is possible to achieve encapsulation efficiency above 70% theoretically, it cannot be reached practically to this value for liposomes. The encapsulation efficiency of 50% was reported as a quite high and good enough value for liposomes. In another study, Mura and her group formulated MLV-type benzocain liposomes by sonication and encapsulation efficiency reported as 26.5–29.7%[54]. We also investigated NaTC and dimethyl-β-CD amounts in liposomes (41% and 65%).

In vitro rivastigmine release experiments from liposomes were performed with dialysis membrane using Franz-type diffusion cells at pH 7.4 phosphate buffer at 37 ± 0.5°C. pH 7.4 was chosen because the pH of DMEM, the cell culture medium, was measured as 7.36. The liposome suspensions in pH 7.4 phosphate buffer were placed to the donor compartment and pH 7.4 phosphate buffer was added to the receptor compartment. The rivastigmine amount was analyzed by HPLC. The kinetics of rivastigmine release from liposomes was also studied. The release profiles of formulations were different for solutions and liposomes. Rivastigmine in solutions were passed through the dialysis membrane according to RRSWB kinetic. r^2 and SD values for rivastigmine release from RS, RTCS, and RCDS solutions were 0.994, 0.996, and 0.994 and 47.2, 31.7, and 87.3, respectively. When liposomes were considered, the release kinetic model for RL ($r^2 = 0.967$, SD = 1.33), RCDL ($r^2 = 0.973$, SD = 0.846), and RTCL ($r^2 = 0.970$, SD = 3.35) was found to be with first-order kinetic. Absorption enhancers especially NaTC were increased rivastigmine transport through dialysis membrane for both solutions and liposomes. Absorption enhancers were possibly increased the membrane partition coefficient of rivastigmine or thermodynamic activity might be altered. It is known that when partition coefficient increases, the transport of compound also increases[55]. This can be explained by also Fick's second law[56]. When absorption enhancers were used together with active compounds in formulation, increased membrane partition coefficient and thermodynamical activity coefficient (α) can enhance the transport of active compounds through the membrane.

MTT tests were carried out to understand the effects of rivastigmine, NaTC, dimethyl-β-CD, and liposome content (cholesterol, DPPC, MC) on living cells for 24-h

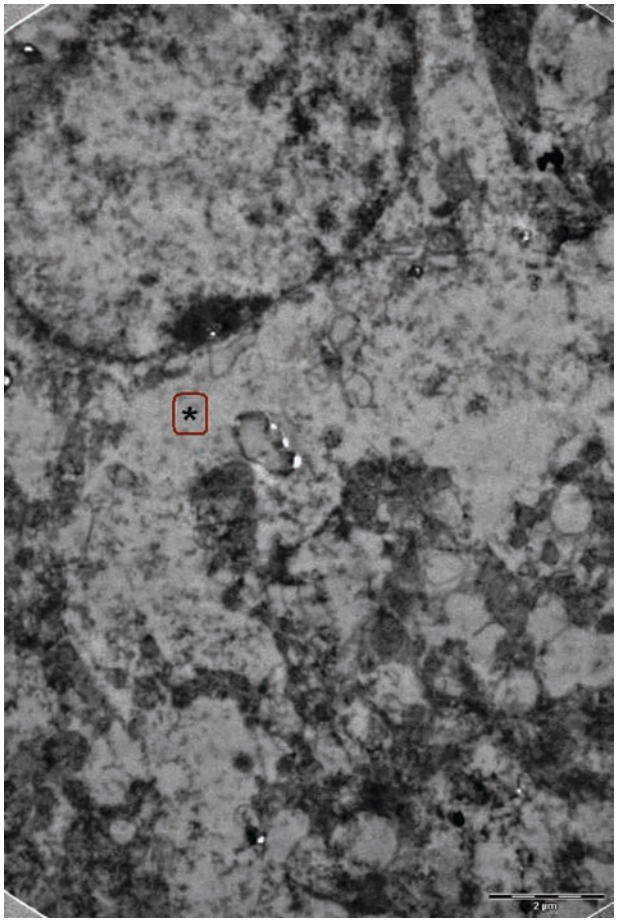

Figure 12. Rivastigmine and NaTC-containing liposome orally applied mice brain.*refers to vacuolization. (×8200).

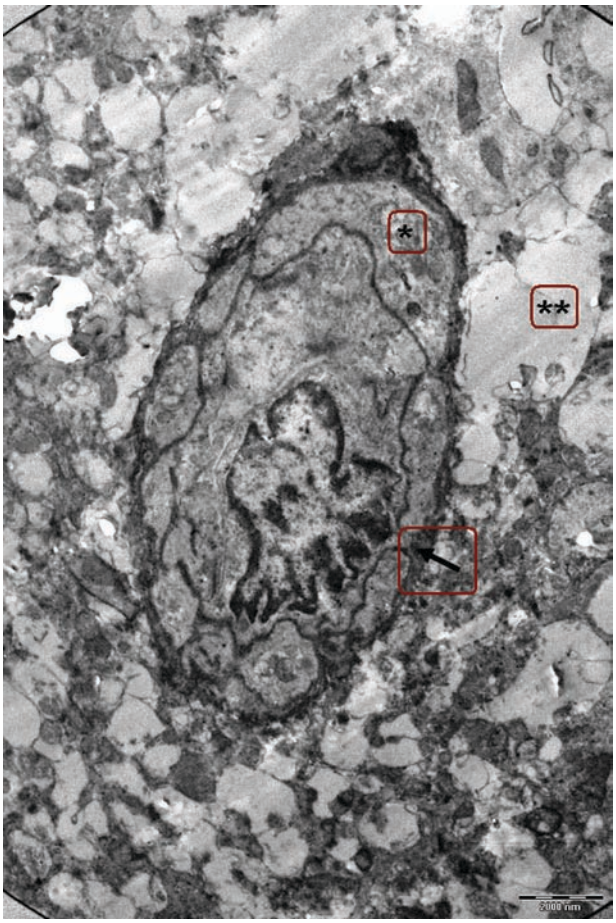

Figure 13. Rivastigmine and dimethyl-β-CD-containing solution intraperitonally applied mice brain. *refers to neuronal vacuolization, **refers to vacuolization and → refers to neuronal ending (×6000).

time period. MTT tests were evaluated on MDCK cells. The effect of serum content of DMEM on cell viability was also investigated. It is known that serum has a positive effect on cell viability and their integrity[57]. MTT test was important to be sure about the cell viability during transport studies[58]. The cell viability should be above 50% for acceptable cell culture studies. According to MTT results, we decided to use rivastigmine below 1.5 mg/ml because cell viability was 55% with this dose. NaTC concentration was selected to be 1.875 mM (cell viability was %69). Dimethyl-β-CD concentration was chosen to be 0.15% (cell viability was 56%). MTT test results showed that liposome content namely cholesterol, DPPC, and also MC did not have significant reverse effect on cell viability, so the contents of liposomes were not toxic to cells at any concentrations. Enhancers with used dose had no toxic effect on cells.

The transport studies were performed through MDCK cells for solutions and liposome formulations during 24-h time period. MDCK is one of the *in vitro* models for BBB based on the use of continuous cell line, and to investigate the specificity of this model. In spite of the documented value of cultured brain endothelial cells as BBB models, they have not been universally popular with pharmaceutical companies. This is largely due to the labor-intensive nature of their preparation, and the fact that the expertise needed to prepare reproducible cultures is greater than for commonly used epithelial models, such as MDCK. Therefore, MDCK cells are reproducible cultures in terms of preparation. The best-performing and widely used models were Caco-2 cells and bovine brain endothelial cells cocultured with rat astrocytes; however, even for these models the correlation with the *in vivo* data was poor. MDCK cells give the best separation of passively permeating and effluxed compounds. The two most commonly employed systems are LLC-PK1, a renal epithelial line derived from porcine kidney, and MDCK, for which there are two derivative kidney cell lines derived from the dog. Not only does the relatively inert background of these cell lines provide a good basis for the estimation of passive membrane permeability but this also allows for assessment of specific transporters following transfection with the appropriate carrier system[22,59]. Therefore, considering all these MDCK cell line was chosen.

The data of all transport studies were given in Figure 8 for MDCK cell transport of rivastigmine formulations. When the data was examined, it was observed that NaTC and dimethyl-β-CD increase the rivastigmine transport for both solution and liposome formulations through MDCK cells. NaTC and dimethyl-β-CD increased the cumulative

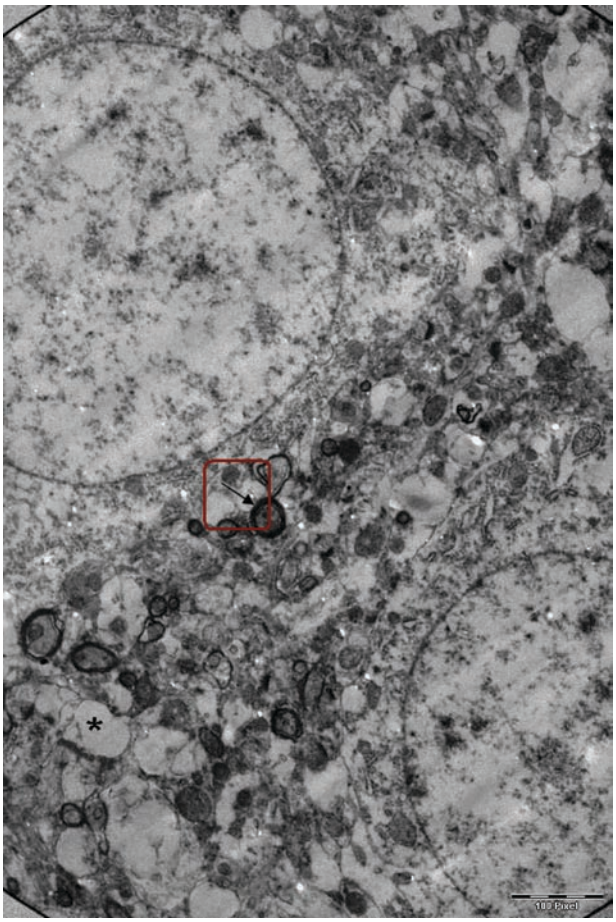

Figure 14. Rivastigmine and dimethyl-β-CD-containing liposome intraperitonally applied mice brain.*refers to intracellular space (×6000).

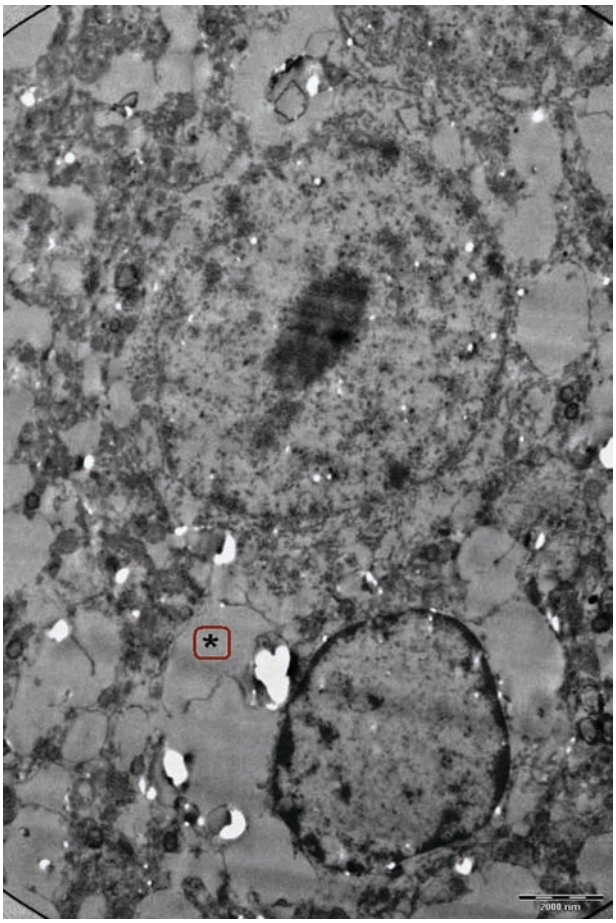

Figure 15. Rivastigmine and dimethyl-β-CD-containing solution orally applied mice brain. *refers to endothelial cell (×6000).

transported amount of rivastigmine significantly ($P < 0.01$) in all experimental groups. NaTC was most effective on enhancing rivastigmine transport from MDCK cells comparing to dimethyl-β-CD, especially more significant for liposomes. The effect of filter inserts which was used in transport studies also controlled before the transportation studies. To understand the filter effect, transport studies were performed with no cell monolayer including filter inserts. There was a significant difference between the results. This data suggested that MDCK cells formed a confluent monolayer on filter inserts and this monolayers significantly limited the rivastigmine transport with TJs between the cells forming a second barrier on the filters.

When the permeability coefficients calculated from solutions, it was clearly seen in Table 4 that NaTC significantly increased the transport of rivastigmine from both solutions and liposomes through MDCK cells (log k value was –1.65 ± 0.06 cm/h). The permeability coefficient value for RCDS was –1.72 ± 0.03 cm/h. The lowest value, –1.80 ± 0.01 cm/h was calculated to be for RS. The highest log k value was –1.15 ± 0.16 cm/h RTCL. The cumulative amount of transported rivastigmine was higher for RTCL than RCDL and RL for MDCK cells.

It is well known that physical and chemical stability problems of liposomes are the most important disadvantage. In our study, liposomes' stability were considered by examining the particle size, zeta potential, and rivastigmine amounts into liposomes when stored at 4°C, 25°C, and 40°C. Significant particle size increase was observed in RL and RCDL when stored at 4°C after 5 months ($P < 0.001$). There was no significant increase in particle size for RTCL at the end of 6 months ($P > 0.05$). Prepared liposomes were found to be quite stable in terms of particle size and distributions. Zeta potential results were similar to particle size and distribution data. Significant zeta potential change was observed in RL and RCDL when stored at 4°C after 3 months ($P < 0.001$). NaTC-containing liposomes kept their zeta potential during 6 months ($P > 0.05$). Rivastigmine is not a problematic compound when stored at room temperature if protected from light. We analyzed the rivastigmine amounts of liposomes every month by HPLC and the degradation kinetic of rivastigmine was investigated at 4°C. The degradation of rivastigmine was found to be with second-order kinetic in liposomes ($r^2 = 0.920$, SD = 591). The kinetic was different for dimethyl-β-CD ($r^2 = 0.972$, SD = 113) and NaTC ($r^2 = 0.910$, SD = 355)-containing liposomes, which was found to be first order. The shelf lives for RL, RCDL, and RTCL at 4°C were 11, 14, and 21 days, respectively. The highest shelf life was found to be with NaTC-containing liposomes. The shelf life of liposomes was found to be

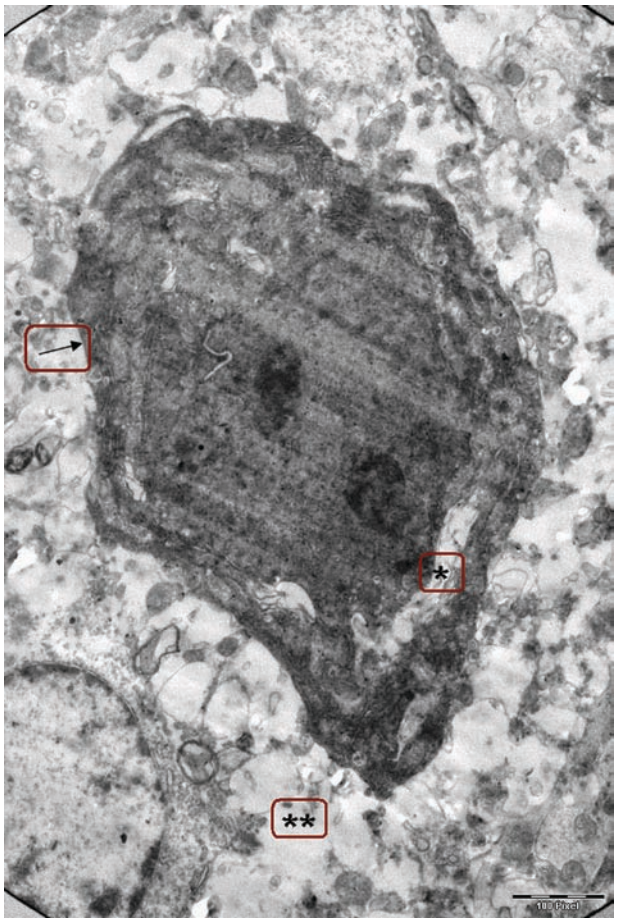

Figure 16. Rivastigmine solution intraperitonally applied mice brain.*refers to endoplasmic reticulum cistern, **refers to intracellular space, and → refers to shrunken neuron (×6000).

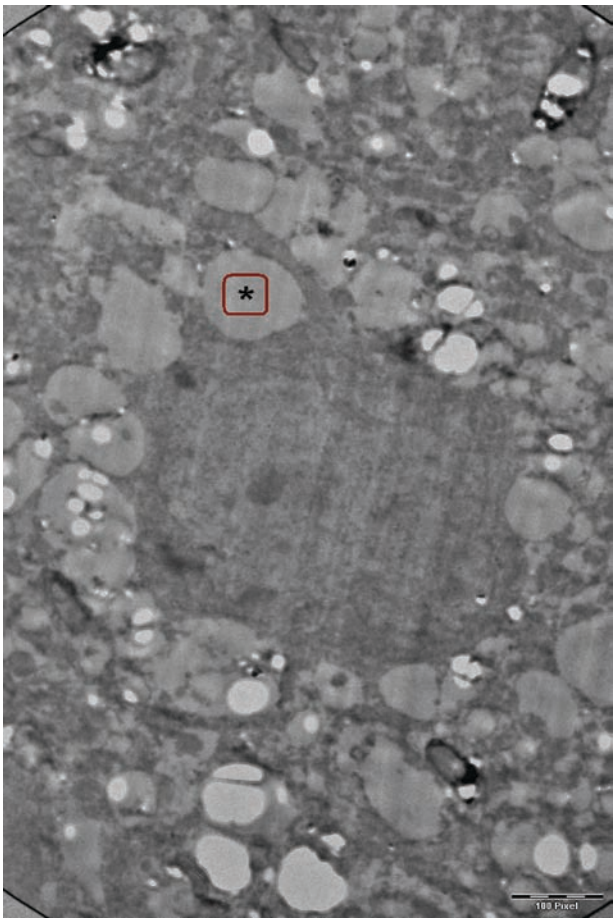

Figure 17. Rivastigmine liposome intraperitonally applied mice brain.*refers to neutrophil vacuolization. (×6000).

decreased with the temperature. NaTC was increased the liposome stability significantly ($P < 0.001$).

The *in vivo* experiment results were performed considering AChE inhibition (%). The blood data of intraperitonally administered solutions caused high inhibition values within the first 1 h after the treatment. The results were similar but lower when solution was orally administered. This data suggested that the bioavailability of intraperitoneal treatment of rivastigmine was better than the oral for solution. AChE inhibition results were the highest for RTCS. When liposomes were entered into bloodstream, they needed more time to release the active compound . The rivastigmine in solution was free, so it can immediately show the effect. Rivastigmine has a first-pass effect in liver. When liposomes were given intraperitonally to the body, they possibly distribute lymphatically in higher extend. They can go through lymphatic vessels may be even through Peyer's patches[60,61]. It is well known that if any small antigenic particle reaches to the Peyer's patches, they are taken up by phagocytic cells which are also bases for oral vaccination, and it has been used successfully for years. Liposomes made by phospholipids are antigenic, and their size is suitable for internalization for phagocytic cells. Therefore whenever they met with phagocytic cell, they undergo phagocytosis through lymphatic vessels. This has been known as a way to protect active substance from pH and enzymatic degradation. This is also known why higher plasma levels of protein can be obtained when they introduce in liposomal form as an example. In our experiment, we used a liposomal form and we also provide MC to increase mucoadhesive properties and viscosity of the microenvironment; therefore, we observed a prolonged % AchE inhibition effect. They indeed possibly went through Peyer's patches and reach the blood stream without disintegration. It has also been stated in the literature for especially oral vaccines[60,61]. MC was also added to our liposomes to increase their stability for oral treatment. Our results showed that our liposomes entered bloodstream without disintegration and release rivastigmine. The AChE inhibition data of liposomes for orally and intraperitonally applied had no significant difference, but the effect of intraperitoneal treatments was appeared to be high when compared to oral treatment. When all blood data was considered the most determined effect was belong to NaTC-containing liposomes after both intraperitonal and oral administration. They had a slow, prolonged, and determined effect on AChE inhibition in both blood and brain. The brain AChE inhibition values were also found to be similar. The liposome formulations were appeared to be more successful than solutions for brain penetration. The reason was found to be high lipid

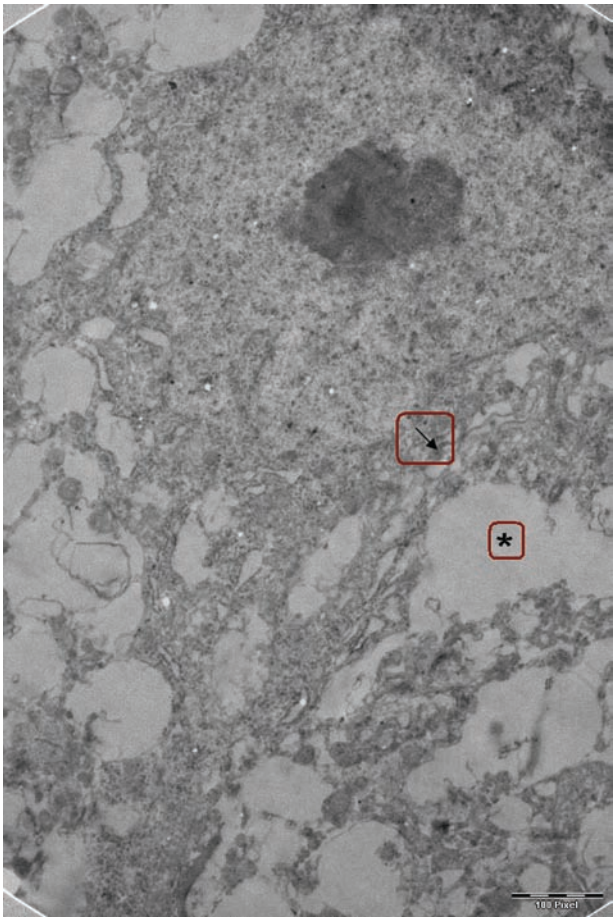

Figure 18. Rivastigmine liposome orally applied mice brain.*refers to neutrophil vacuolization and → refers to golgi body cistern (×8200).

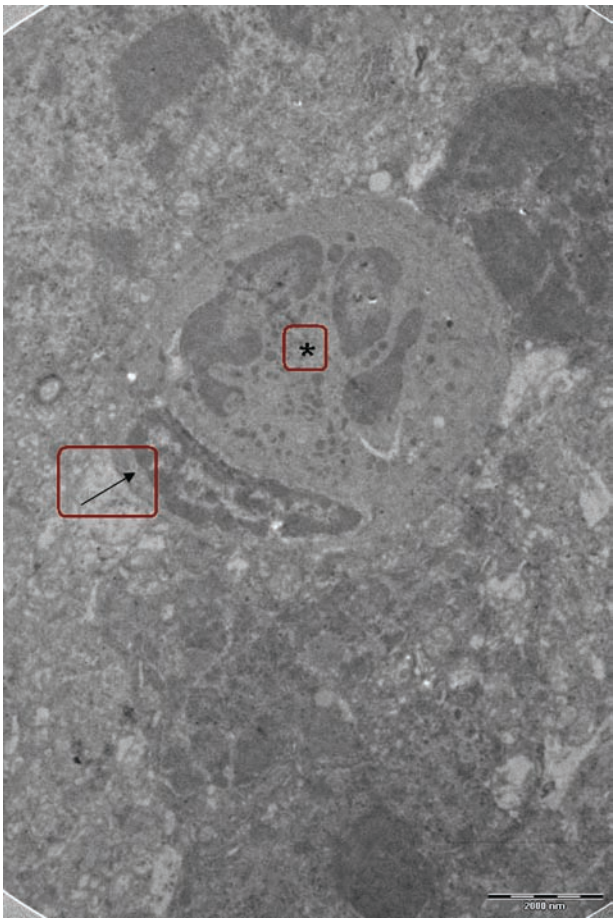

Figure 19. Brain of a control group mice.*refers to neutrophil, and arrow refers to endothelial cell (×8200).

content of liposomes. Dimethyl-β-CD-containing liposomes were compared with only rivastigmine-containing liposomes, no significant difference was observed at 6th and 8 h, but the action of dimethyl-β-CD liposomes was continued to be less during 24 h. Dimethyl-β-CD prevented rivastigmine in brain from elimination by AcHE in synaptic spaces and prolonged the action. BBB is characterized by TJs, which are the most specific structures in the brain endothelial cells and different from the endothelial cells of other tissues/organs of body[16–18]. Absorption enhancers especially NaTC had opened or loosened the TJ regions and increased brain penetration of rivastigmine. It was found that absorption enhancers especially NaTC increased brain penetration of rivastigmine. Although significant decrease was also observed in AChE inhibition in brain results of solution after 6h, rivastigmine solution had a faster elimination in body compared to liposomes. The best AChE inhibition results in brain were obtained with rivastigmine and NaTC-containing liposomes. NaTC increased the brain penetration of rivastigmine and prolonged the time of action.

It was found a good correlation between *ex vitro* penetrated amount of rivastigmine (%) and *in vivo* AChE inhibition (%) data. The correlation was high especially for liposome formulations. Best correlation was obtained with RTCL for both blood and brain after oral application.

According to TEM images (Figures 10–20) of mice' brains, there was an increase at the intercellular spaces and vacuolization was observed in all animal groups except control group. The vacuolization and dilatation was strikingly significant with dimethyl-β-CD and NaTC-containing liposomes. There was a dilatation on endoplasmic reticulum system observed in oral empty liposomes applied group. In intraperitoneal empty liposomes applied group, there was a neuronal vacuolization (enlargement of neuronal cells). There was a dilatation and vacuolization on endoplasmic reticulum system observed in intraperitoneally applied rivastigmine solution group. In intraperitoneally applied rivastigmine liposomes group, there was an increase on vacuolization in neutrophils. When rivastigmine liposomes were administered orally, an increase on vacuolization in neutrophils and golgi systems was observed.

In intraperitoneal RCDS applied group, neuronal vacuolization and some nerve ends were observed. Orally applied RCDS applied group also showed some vacuolization. In intraperitoneally RCDLip applied group, there was an increase at the intercellular space. Orally applied RCDLo applied group showed a relaxation on endothelial connections. In intraperitoneal rivastigmine

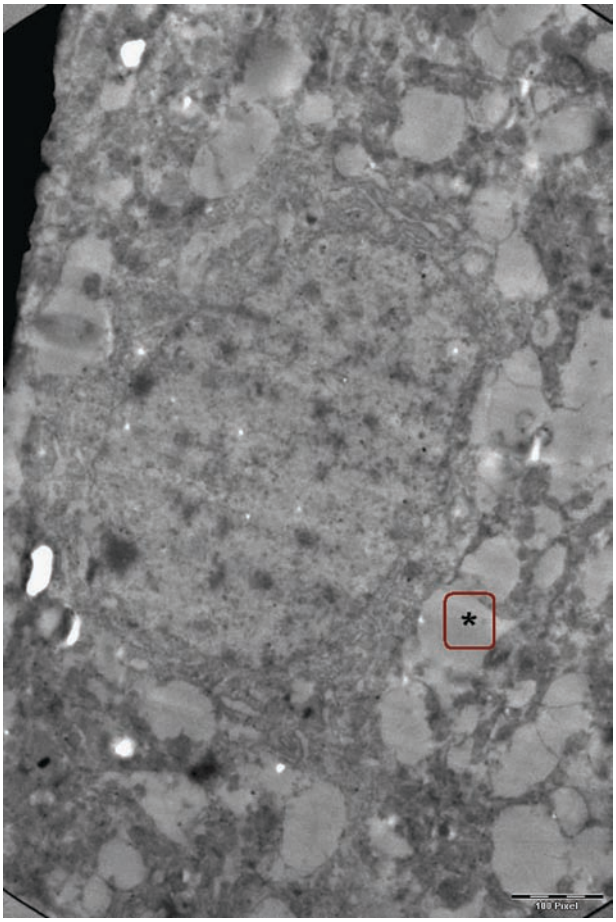

Figure 20. Only lipid-containing liposome intraperitonally applied mice brain.*refers to neuronal vacuolization (×6000).

and NaTC-containing solution applied group, degenerations were observed at myelinated nerve ends. In orally applied RCDSo applied group, an increase at intercellular space was noticed. In intraperitoneally RCDLip applied group, there were changes on the shape of the nucleus and dilatation on endoplasmic reticulum and an increase at the intercellular space. In orally applied RCDLo applied group, an increase at the intercellular space was observed and neurons were found to be shrunk.

Degeneration at myelinated nerve end was observed in intraperitoneally applied RTCSip group. When RTCSo group was administered orally, an increase at then intercellular space was noticed. When RTCLip group was administered intraperitoneally, degeneration at cell nucleus and a dilatation on endoplasmic reticulum were observed. When these liposomes (RTCLo group) were administered orally, an increase at intercellular space and a shrink on neurons were observed.

When all results are evaluated, in all groups intercellular spaces were found to be increased except control group. Dimethyl-β-CD and NaTC-containing formulation vacuolizations, an increase at the intercellular space and dilatations were found to be more noticeable. The degree of vacuolization, dilatation, and increase in intercellular space were found to be the highest for orally applied rivastigmine and NaTC liposomes applied groups (RTCLo group) among all.

This data suggested that they enhanced the brain penetration of rivastigmine and caused dilatation, TJs might be loosened. Our best, most significant images were belonged to rivastigmine-NaTC-containing liposomes after oral treatment.

Conclusion

MDCK cells have been used for BBB mimicking[22,58] because of their high TEER values and similar protein structure with brain originated cells. The high correlation found between *in vitro* and *in vivo* with this study suggests that MDCK cells are successful as blood–brain model. NaTC appeared to be a good choice for rivastigmine's absorption enhancement for liposomal drug carriers. Rivastigmine liposomes with NaTC may be hope giving in oral treatment of AD in future.

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Declaration of interest

This study was supported by a research grant from Gazi University (02/2006-17). The authors declare here that there is no conflict of interest with anybody or any organizations.

References

1. Scarpini E, Scheltens P, Feldman H. (2003). Treatment of Alzheimer's disease: current status and new perspectives. Lancet Neurol, 2:539–547.
2. Polinsky RJ. (1998). Clinical pharmacology of rivastigmine: a new-generation acetylcholinesterase inhibitor for the treatment of Alzheimer's disease. Clin Ther, 20:634–647.
3. Alloul K, Sauriol L, Kennedy W, Laurier C, Tessier G, Novosel S et al. (1998). Alzheimer's disease: a review of the disease, its epidemiology and economic impact. Arch Gerontol Geriatr, 27:189–221.
4. Delagarza VW. (2003). Pharmacologic treatment of Alzheimer's disesase: an update. Am Fam Phys, 68:1365–1372.
5. Smith MA, Perry G. (1998). What are the facts and artifacts of the pathogenesis and etiology of Alzheimer disease? J Chem Neuroanat, 16:35–41.
6. Standridge JB. (2004). Pharmacotherapeutic approaches to the treatment of Alzheimer's disease. Clin Ther, 26:615–630.
7. Williams BR, Nazarians A, Gill MA. (2003). A review of rivastigmine: a reversible cholinesterase inhibitor. Clin Ther, 25:1634–1653.
8. Farlow MR, Lilly ML; ENA713 B352 Study Group. (2005). Rivastigmine: an open-label, observational study of safety and effectiveness in treating patients with Alzheimer's disease for up to 5 years. BMC Geriatr, 5:3.
9. Agid Y, Dubois B, Anand R,Gharabawi G. (1998). Efficacy and tolerability of rivastigmine in patients with dementia of the Alzheimer type. Curr Ther Res, 59:833–845.
10. Yüksel N. (2000). Alzheimer hastalığının ilaçla tedavisi. Klin Psikiyatr, 3:137–141.

11. Ercan S. (2002). Alzheimer hastalığı tedavisinde kullanılan kolinesteraz inhibitörleri. Demans, 2:5–9.
12. Tse FL, Laplanche R. (1998). Absorption, metabolism, and disposition of [14C]SDZ ENA 713, an acetylcholinesterase inhibitor, in minipigs following oral, intravenous, and dermal administration. Pharm Res, 15:1614–1620.
13. Scherrman JM. (2002). Drug delivery to brain via the blood-brain barrier. Vasc Pharmacol, 38:349–354.
14. Pardridge WM. (2007). Blood-brain barrier delivery. Drug Discov Today, 12:54–61.
15. Jeffrey P, Summerfield SG. (2007). Challenges for blood-brain barrier (BBB) screening. Xenobiotica, 37:1135–1151.
16. Gumbleton M, Audus KL. (2001). Progress and limitations in the use of *in vitro* cell cultures to serve as a permeability screen for the blood-brain barrier. J Pharm Sci, 90:1681–1698.
17. Hawkins RA, O'Kane RL, Simpson IA, Viña JR. (2006). Structure of the blood-brain barrier and its role in the transport of amino acids. J Nutr, 136:218S–226S.
18. Lundquist S, Renftel M, Brillault J, Fenart L, Cecchelli R, Dehouck MP. (2002). Prediction of drug transport through the blood-brain barrier in vivo: a comparison between two *in vitro* cell models. Pharm Res, 19:976–981.
19. Braun A, Hämmerle S, Suda K, Rothen-Rutishauser B, Günthert M, Krämer SD et al. (2000). Cell cultures as tools in biopharmacy. Eur J Pharm Sci, 11 Suppl 2:S51–S60.
20. Wilson G. (1990). Cell culture techniques for the study of drug transport. Eur J Drug Metab Pharmacokinet, 15:159–163.
21. Lemaire M, Desrayaund S. (2005). The priorities/needs of the pharmaceutical industry in drug delivery to the brain. Int Cong Ser, 1277:32–46.
22. Garberg P, Ball M, Borg N, Cecchelli R, Fenart L, Hurst RD et al. (2005). *In vitro* models for the blood-brain barrier. Toxicol in Vitro, 19:299–334.
23. Degim Z, Mutlu NB, Yilmaz S, Essiz D, Nacar A. (2010). Investigation of liposome formulation effects on rivastigmine transport through human colonic adenocarcinoma cell line (CACO-2). Pharmazie, 65:32–40.
24. Shah P, Jogani V, Mishra P, Mishra AK, Bagchi T, Misra A. (2008). *In vitro* assessment of acyclovir permeation across cell monolayers in the presence of absorption enhancers. Drug Dev Ind Pharm, 34:279–288.
25. Wen H, Watry DD, Marcondes MC, Fox HS. (2004). Selective decrease in paracellular conductance of tight junctions: role of the first extracellular domain of claudin-5. Mol Cell Biol, 24:8408–8417.
26. Shi LZ, Zheng W. (2005). Establishment of an *in vitro* brain barrier epithelial transport system for pharmacological and toxicological study. Brain Res, 1057:37–48.
27. Alavijeh MS, Chishty M, Qaiser MZ, Palmer AM. (2005). Drug metabolism and pharmacokinetics, the blood-brain barrier, and central nervous system drug discovery. Neurorx, 2:554–571.
28. Micklus MJ, Greig NH, Tung J, Rapoport SI. (1993). Targetting of liposomes to the blood-brain barrier in rats. Drug Deliv, 1:21–26.
29. Uhumwango MU, Okor RS. (2005). Current trends in the production and biomedical applications of liposomes: a review. J Med Biomed Res, 4:9–21.
30. Lasic DD. (1998). Novel applications of liposomes. Trends Biotechnol, 16:307–321.
31. Degim Z. (2008). Use of microparticulate systems to accelerate skin wound healing. J Drug Target, 16:437–448.
32. Alemdaroglu C, Degim Z, Celebi N, Sengezer M, Alömeroglu M, Nacar A. (2008). Investigation of epidermal growth factor containing liposome formulation effects on burn wound healing. J Biomed Mater Res A, 85:271–283.
33. Degim Z, Unal N, Essiz D, Abbasoglu U. (2004). The effect of various liposome formulations on insulin penetration across Caco-2 cell monolayer. Life Sci, 75:2819–2827.
34. Allen TM. (1997). Liposomes. Opportunities in drug delivery. Drugs, 54 Suppl 4:8–14.
35. Rao BM, Srinivasu MK, Kumar KP, Bhradwaj N, Ravi R, Mohakhud PK et al. (2005). A stability indicating LC method for rivastigmine hydrogen tartrate. J Pharm Biomed Anal, 37:57–63.
36. El-Gazayerly ON, Hikal AH. (1997). Preparation and evaluation of acetazolamide liposomes as an ocular delivery system. Int J Pharm, 158:121–127.
37. Li H, Song JH, Park JS, Han K. (2003). Polyethylene glycol-coated liposomes for oral delivery of recombinant human epidermal growth factor. Int J Pharm, 258:11–19.
38. Cong W, Liu Q, Chen X, Gao R, Lu J, Wang Y et al. (2010). Characterization and pharmacokinetics of a novel pirarubicin liposome powder. Drug Dev Ind Pharm, 36:1186–1194.
39. Yeh YH, Hwang DF. (2001). High-performance liquid chromatographic determination for bile components in fish, chicken and duck. J Chromatogr B Biomed Sci Appl, 751:1–8.
40. Basappa C, Rao P, Rao DN, Divakar S. (1998). A modified colorimetric method for the estimation of β-cyclodextrin using phenolphthalein. Int J Food Sci Tech, 33:517–520.
41. Mosmann T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, 65:55–63.
42. Raiman J, Törmälehto S, Yritys K, Junginger HE, Mönkkönen J. (2003). Effects of various absorption enhancers on transport of clodronate through Caco-2 cells. Int J Pharm, 261:129–136.
43. Zerrouk N, Corti G, Ancillotti S, Maestrelli F, Cirri M, Mura P. (2006). Influence of cyclodextrins and chitosan, separately or in combination, on glyburide solubility and permeability. Eur J Pharm Biopharm, 62:241–246.
44. Meaney CM, O'Driscoll CM. (2000). A comparison of the permeation enhancement potential of simple bile salt and mixed bile salt:fatty acid micellar systems using the CaCo-2 cell culture model. Int J Pharm, 207:21–30.
45. Duplay D. (2004). Physicians' Desk Reference, 58th edn. Montvale: Thomson PDR, pp. 2252–2260.
46. Enz A, Gentsch C. (2004). Co-administration of memantine has no effect on the *in vitro* or ex vivo determined acetylcholinesterase inhibition of rivastigmine in the rat brain. Neuropharmacology, 47:408–413.
47. Geerts H, Guillaumat PO, Grantham C, Bode W, Anciaux K, Sachak S. (2005). Brain levels and acetylcholinesterase inhibition with galantamine and donepezil in rats, mice, and rabbits. Brain Res, 1033:186–193.
48. Ellman GL, Courtney KD, Andres V Jr, Feather-Stone RM. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol, 7:88–95.
49. Begley DJ, Bradbury MW, Kreuter J. (2000). The blood-brain barrier and drug delivery to the CNS. New York: Marcel Dekker.
50. Fagerholm U. (2007). The highly permeable blood-brain barrier: an evaluation of current opinions about brain uptake capacity. Drug Discov Today, 12:1076–1082.
51. Sakamoto A, Ido T. (1993). Liposome targeting to rat brain: effect of osmotic opening of the blood-brain barrier. Brain Res, 629:171–175.
52. Visser CC, Stevanovic S, Voorwinden LH, van Bloois L, Gaillard PJ, Danhof M et al. (2005). Targeting liposomes with protein drugs to the blood-brain barrier in vitro. Eur J Pharm Sci, 25:299–305.
53. Arumugam K, Subramanian GS, Mallayasamy SR, Averineni RK, Reddy MS, Udupa N. (2008). A study of rivastigmine liposomes for delivery into the brain through intranasal route. Acta Pharm, 58:287–297.
54. Mura P, Maestrelli F, González-Rodríguez ML, Michelacci I, Ghelardini C, Rabasco AM. (2007). Development, characterization and *in vivo* evaluation of benzocaine-loaded liposomes. Eur J Pharm Biopharm, 67:86–95.
55. Potts RO, Guy RH. (1992). Predicting skin permeability. Pharm Res, 9:663–669.
56. Higuchi T. (1960). Physical chemical analysis of percutaneous absorption from creams and ointments. J Soc Cosmet Chem, 11:85–97.

57. Freshney RI. (1994). Culture of Animal Cells, 4th edn. Canada: Wiley-Liss, Inc.
58. Wang Q, Rager JD, Weinstein K, Kardos PS, Dobson GL, Li J et al. (2005). Evaluation of the MDR-MDCK cell line as a permeability screen for the blood-brain barrier. Int J Pharm, 288:349–359.
59. Kusuhara H, Sugiyama Y. (2005). Efflux transport systems at blood-brain barrier and blood CFS barrier, Int Cong Ser, 1277:111–122.
60. Borges O, Cordeiro-da-Silva A, Romeijn SG, Amidi M, de Sousa A, Borchard G et al. (2006). Uptake studies in rat Peyer's patches, cytotoxicity and release studies of alginate coated chitosan nanoparticles for mucosal vaccination. J Control Release, 114:348–358.
61. Degim IT, Gümüsel B, Degim Z, Ozçelikay T, Tay A, Güner S. (2006). Oral administration of liposomal insulin. J Nanosci Nanotechnol, 6:2945–2949.