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Investigation of liposome formulation effects on rivastigmine transport through human colonic adenocarcinoma cell line (CACO-2)

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Transportations of rivastigmine containing liposomes across Caco-2 cells were studied and *in vitro* test results were compared with *in vivo* results. MTT test was used for cell viability studies. Series of formulations were prepared containing rivastigmine which is used for the treatment of Alzheimer's disease. Characterization and stability studies for liposome formulations were performed. Encapsulation efficiencies of liposomes were 35.4%, 25.2% and 29.9% for rivastigmine, rivastigmine-sodium taurocholate, rivastigmine-dimethyl-beta-CD liposomes, respectively. In stability studies, particle size and size distribution, zeta potential, rivastigmine amounts were determined and shelf lives of liposomes were calculated. Penetration properties of rivastigmine through Caco-2 cells, dialysis membrane and kinetics of release from liposomes were determined. Permeability coefficients were calculated after diffusion studies. The highest value of % cumulative amount of rivastigmine passed through Caco-2 cell cultures was found to be 87.2% for rivastigmine-sodium taurocholate solution and 12.8% for rivastigmine-sodium taurocholate liposome. The highest permeability coefficient value was obtained with sodium taurocholate liposomes for -0.75. Rivastigmine liposomes and solutions were also applied to animals. Acetyl choline esterase (AChE) activity was determined by the Ellman method on mice. %AChE inhibition values were calculated using blood and brain tissue samples. The physical appearances of the brains were investigated by TEM microscope. The highest value of AChE inhibition was observed for rivastigmine and sodium taurocholate liposomes. The histological investigations and observations also supported these results.

1. Introduction

Alzheimer disease (AD) is one of the most common causes of dementia which is indicated as a neurodegenerative disorder. It is characterized by progressive loss of memory and cognitive function (Mayeux and Sano 1999; Scarpini et al. 2003; Doraiswamy et al. 2002). Dysfunction of the basal forebrain cholinergic system is thought to be the main cause of the neurodegeneration in the brains of AD patients (Mustazza et al. 2007). One of the most observed neurochemical alterations in the brains of AD patients is the decrease in cortical cholinergic markers; acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) (Eskander et al. 2005; Leonard et al. 2004). Therefore, depletion of altered cortical cholinergic transmission takes a great part in the research and treatment of AD.

Although there is no pharmacologic treatment for alteration of the disease pathology, acetylcholinesterase inhibition (AChEI) therapy manages to delay the progression in cognitive, behavioral and functional deficits and provides symptomatic improvements (Farlow 2001). Rivastigmine is a carbamate derivative and a pseudo-irreversible cholinesterase inhibitor which can inhibit both AChE and BuChE (Jann et al. 2002; Agid et al. 1998). It is indicated for the symptomatic treatment of mild to moderate dementia (Williams et al. 2003).

Liposomes are spherical, colloidal lipid vesicles that form naturally or are prepared artificially when phospholipids are exposed to an aqueous medium (Uhumwangho and Okor 2005; Lasic 1998). Their unique structural properties allow encapsulation of hydrophilic drugs in the aqueous interior, incorporation of hydrophobic core of the phospholipids barrier and it is also possible to formulate the drugs of intermediate solubility as liposomes (Allen 1997). Liposomes are a composition of biocompatible and biodegradable lipids highly similar to biological membranes (Garcia et al. 2005; Sharma and Sharma 1997).

Caco-2 cells are colorectal derivative epithelial cells. They have been used in oral absorption studies and also for the evaluation of different transport mechanisms such as: carrier mediated transport, passive diffusion, paracellular transport systems (Boulenc et al. 1993; Artursson and Karlsson 1991). Caco-2 cells are also used to estimate blood-brain barrier (BBB) permeability (Lundquist et al. 2002; Garberg et al. 2005).

In this study, liposome formulations of rivastigmine were developed. The effects of absorption enhancers (dimethyl- β -cyclodextrine and sodium taurocholate) on the permeability of rivastigmine through Caco-2 cells were investigated. Permeability coefficients were calculated after absorption studies (Mutlu et al. 2008). *In vivo* tests were also performed in mice and animals were treated with rivastigmine liposome formulations. The *in vitro* data were compared with *in vivo* results.

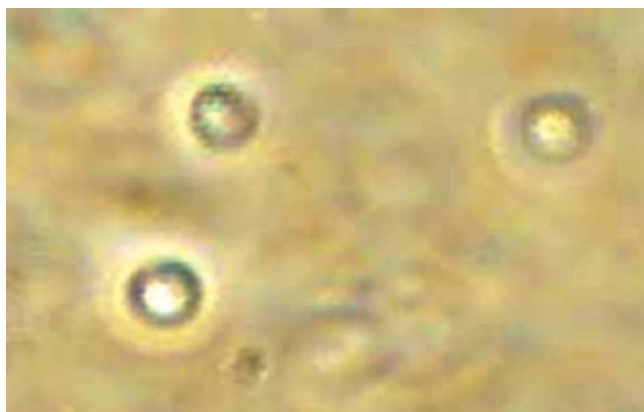


Fig. 1: Inverted microscope image of liposomes ($\times 400$)

2. Investigations and results

2.1. *In vitro* studies

The type of liposomes were determined as multilamellar vesicles (MLV) using an inverted microscope (Olympus CK2, Japan) (Fig. 1). The particle sizes of three types of liposomes were investigated by a laser diffraction particle sizer. The mean particle sizes of rivastigmine liposomes, rivastigmine and NaTC containing liposomes and, dimethyl- β -CD containing liposomes were $3.40 \pm 0.01 \mu\text{m}$, $3.37 \pm 0.00 \mu\text{m}$ and $4.51 \pm 0.04 \mu\text{m}$ ($\pm\text{SD}$, $n=6$) respectively.

Zeta potential of the liposomes were analysed by a Zetasizer-Nano ZS-Malvern (Germany). The mean zeta potentials of rivastigmine liposomes, rivastigmine and NaTC containing liposomes and, dimethyl- β -CD containing liposomes were $-3.28 \pm 0.00 \text{ mV}$, $-4.30 \pm 0.66 \text{ mV}$ and $-5.43 \pm 0.00 \text{ mV}$ ($\pm\text{SD}$, $n=6$) respectively.

The rivastigmine contents of liposomes were determined using HPLC, after ultracentrifugation process. The encapsulation efficiencies of rivastigmine were found to be 35.4% for rivastigmine containing liposomes, 29.9% for rivastigmine and dimethyl- β -CD containing liposomes, 25.2% for rivastigmine and NaTC containing liposomes.

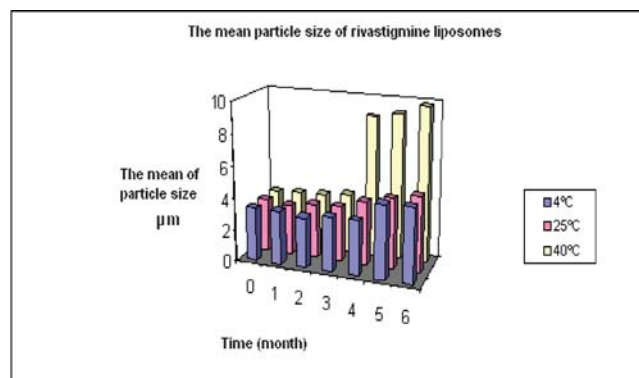
NaTC amount captured in liposomes was determined by HPLC. It was found to be 41%.

Dimethyl- β -CD amount captured in liposomes was determined by spectrophotometer. It was found as 65%.

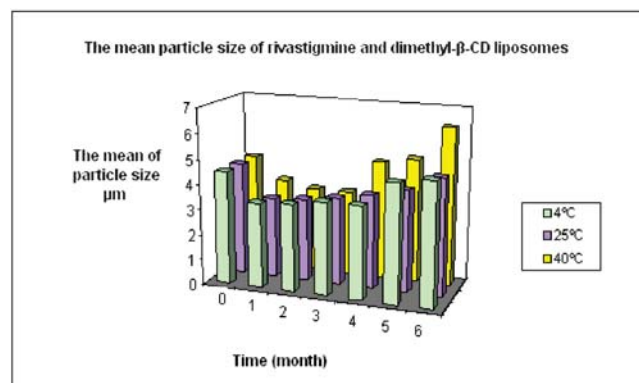
The quantitative analyses of rivastigmine, particle size and zeta potential measurements were performed for evaluating stability of rivastigmine liposomes in aqueous solutions. The stability studies were performed at 4°C , 25°C and 40°C for 180 days for three types of liposomes. The mean particle size of rivastigmine liposomes, rivastigmine and dimethyl- β -CD liposomes, rivastigmine and NaTC liposomes for 6 months are given in Fig. 2.

The mean particle sizes of rivastigmine liposomes were significantly increased after three months at 4°C , 25°C and 40°C . The most significant increase at particle size was observed at 40°C . Significant increase of particle size was observed for rivastigmine and dimethyl- β -CD liposomes after three months at 4°C , 25°C and 40°C similar to rivastigmine liposomes, Rivastigmine and NaTC liposomes were the most stable liposomes at both 4°C , 25°C and 40°C . No significant change was observed for rivastigmine and NaTC liposomes for six months.

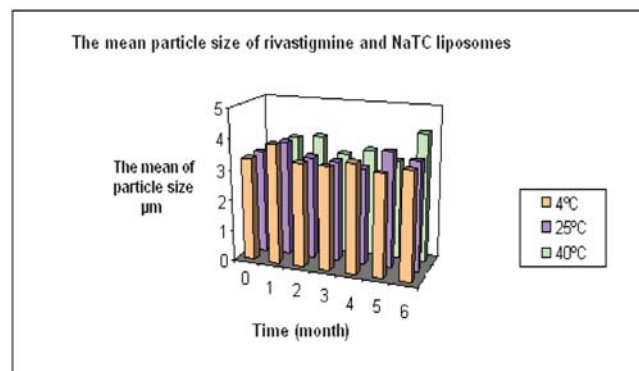
The zeta potential measurements were evaluated for 6 months. Initial zeta potentials were -3.28 ± 0.00 , -5.43 ± 0.00 , -4.30 ± 0.66 for rivastigmine, rivastigmine-dimethyl- β -CD and rivastigmine-NaTC liposomes, respectively, at 4°C . They were re-determined at same temperature after 6 months as follows: -4.03 ± 0.60 , -4.91 ± 0.35 , -4.36 ± 0.31 .



a)



b)



c)

Fig. 2: a) The mean particle size of rivastigmine liposomes stored at 4°C , 25°C and 40°C ; b) The mean particle size of rivastigmine and dimethyl- β -CD liposomes stored at 4°C , 25°C and 40°C ; c) The mean particle size of rivastigmine and NaTC liposomes stored at 4°C , 25°C and 40°C

The test samples were examined during six months and the self 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. The degradation of rivastigmine in liposomes followed second order kinetics and shelf life at 4°C was calculated as 11 days ($r^2=0.920$) and 7 days at 25°C ($r^2=0.882$). The degradation of rivastigmine in the rivastigmine and dimethyl- β -CD containing liposomes, followed first order kinetics and shelf life at 4°C was 14 days ($r^2=0.963$) and 12 days at 25°C ($r^2=0.972$). The degradation of rivastigmine in the rivastigmine and NaTC liposomes, followed first order kinetics and shelf life at 4°C was 21 days ($r^2=0.910$) and 14 days at 25°C ($r^2=0.975$).

The *in vitro* release experiment of rivastigmine from liposome formulations were performed with dialysis membrane-Franz type diffusion cells using pH 7.4 phosphate buffer at $37 \pm 0.5^\circ\text{C}$. pH 7.4 was chosen for the experiments to mimic *in vivo* conditions. The release of rivastigmine from rivastigmine liposomes,

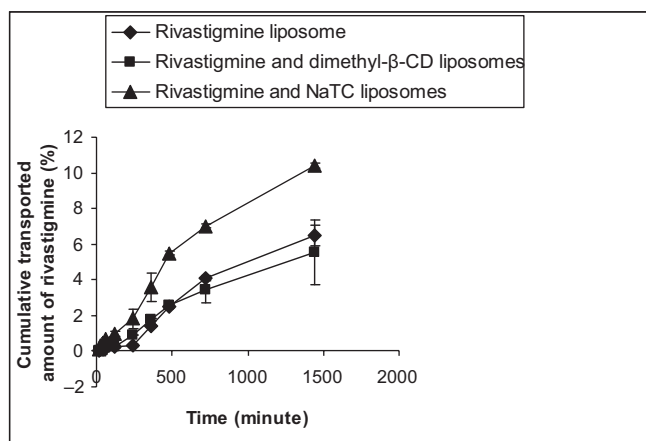


Fig. 3: *In vitro* release profiles of rivastigmine from the liposome formulations at pH 7.4 phosphate buffer at $37 \pm 0.5^\circ\text{C}$ (Error bars represent standard deviations, $n = 3$)

rivastigmine-dimethyl- β -CD liposomes and rivastigmine-NaTC liposomes followed first order kinetics, correlation coefficients were 0.967, 0.973 and 0.966, respectively. The release profiles of rivastigmine from liposomes are given in Fig. 3.

In vitro permeation studies were also performed for rivastigmine in solutions. The permeation of rivastigmine from solutions containing rivastigmine, rivastigmine-dimethyl- β -CD liposomes and rivastigmine-NaTC through dialysis membranes were also performed to understand the barrier effect of dialysis membrane and faster penetrations were observed.

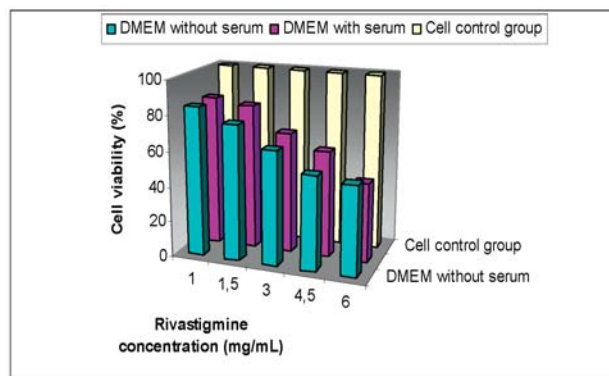
Table 1: Log k values calculated from Caco-2 transport study results

SAMPLES	LOG K
Rivastigmine solution	-1.96 ± 0.04
Rivastigmine and dimethyl- β -CD solution	-1.84 ± 0.01
Rivastigmine and NaTC solution	-1.78 ± 0.03
Rivastigmine liposomes	-1.03 ± 0.29
Rivastigmine and NaTC liposomes	-0.75 ± 0.13
Rivastigmin and dimethyl- β -CD liposomes	-1.18 ± 0.03

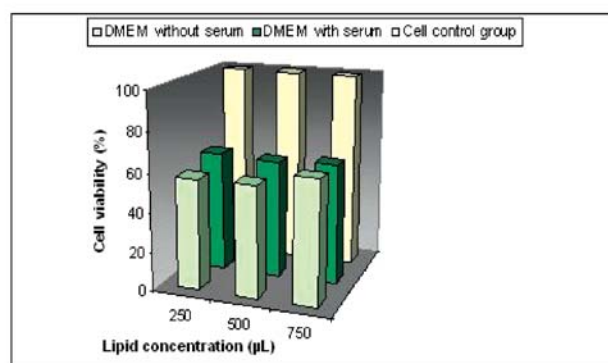
2.2. Cell culture studies

The effects of rivastigmine, NaTC, dimethyl- β -CD and lipids used in liposome formulations on Caco-2 cell viability were investigated for 24 h. Effect of serum content of Dulbecco's Modified Eagle Medium (DMEM) was also examined by using both serum containing and serum free DMEM. The cell viability percentages are given in Fig. 4.

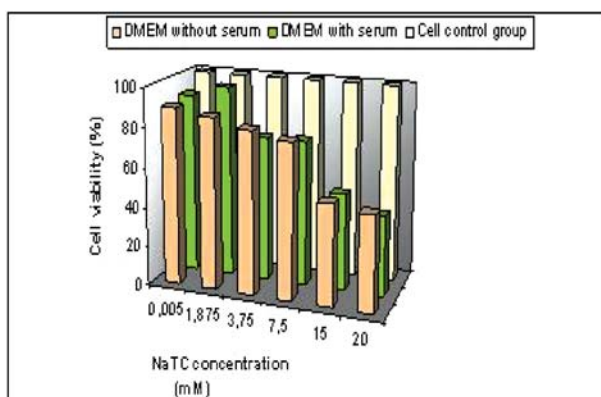
According to the MTT results, it was decided to use serum containing DMEM for further Caco-2 transport studies. The transport studies were also performed through membranes as a control, at the beginning of the study. Transport experiments of rivastigmine from solution and liposome formulations through Caco-2 cells from apical to basolateral side of the cells were evaluated. The results were shown Figures 5, 6. Cumulative amounts of rivastigmine at the end of the 24 h time period were calculated. Papp (log k) values were calculated for all groups. The log k values were given in Table 1.



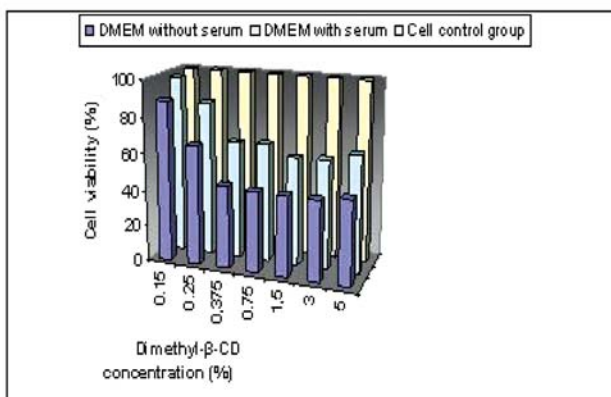
a)



b)



c)



d)

Fig. 4: a) Rivastigmine concentration and serum content effects on Caco-2 cell viability; b) Lipid concentration and serum content effects on Caco-2 cell viability; c) NaTC concentration and serum content effects on Caco-2 cell viability; d) dimethyl- β -CD concentration and serum content effects on Caco-2 cell viability

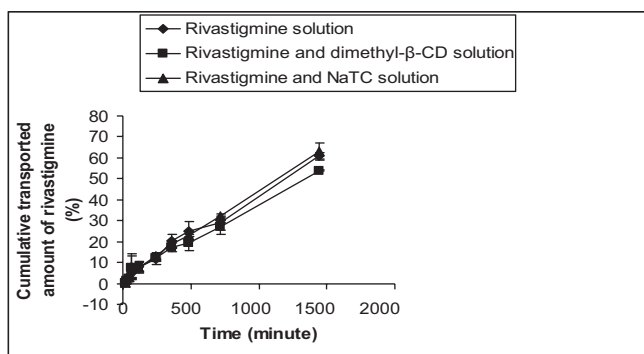


Fig. 5: Cumulative amount of rivastigmine from solutions transported through Caco-2 cells (Error bars represent standard deviations, $n = 3$)

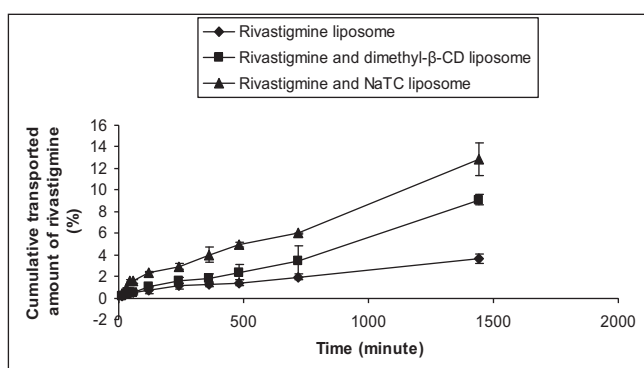


Fig. 6: Cumulative amount of rivastigmine from liposomes transported through Caco-2 cells (Error bars represent standard deviations, $n = 3$)

The electrical resistance (TEER) values were measured after the experiments for the cells of rivastigmine. TEER values for rivastigmine liposomes, rivastigmine and dimethyl- β -CD liposomes, rivastigmine and NaTC liposomes were found to be 1275, 1230 and 1070 Ω respectively.

2.3. In vivo studies

Ellman method was used for evaluating AChE inhibition in blood and brain samples. AChE inhibition percentages were given in Figures 7–10.

In vitro-in vivo correlation studies were performed with cumulative amount of rivastigmine (%) passed through apical to the basolateral side of the Caco-2 cell monolayer and AChE inhibitor (%) values calculated by animal studies for both blood and brain for all formulations. The correlations were shown in Fig. 11 below

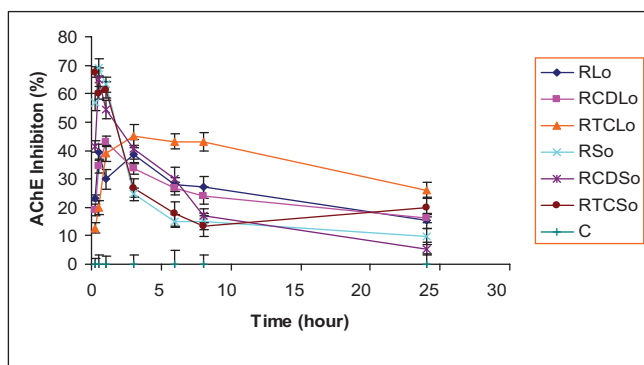


Fig. 7: AChE inhibition values in blood for orally given formulations (Error bars represent standard deviations, $n = 15$)

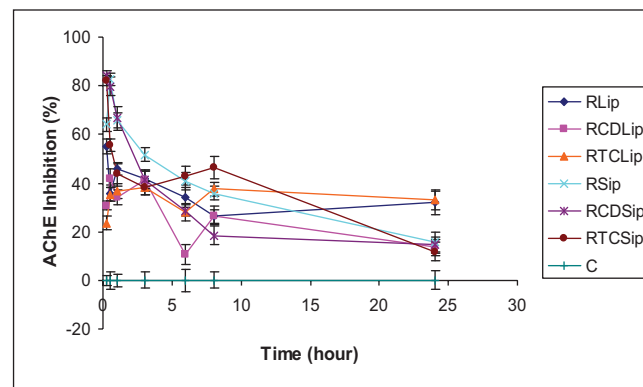


Fig. 8: AChE inhibition values in blood for intraperitoneally given formulations (Error bars represent standard deviations, $n = 15$)

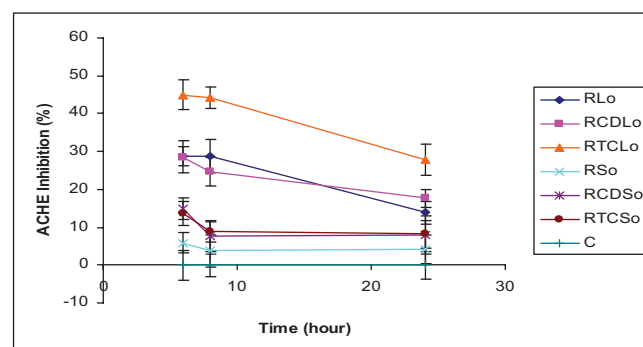


Fig. 9: AChE inhibition values in brain for orally given formulations (Error bars represent standard deviations, $n = 15$)

2.4. Histological studies

The brain tissues of different rivastigmine solutions and liposomes treated mice were investigated by ultrathin sectional preparation under TEM microscope. The TEM images of control group are shown in Fig. 12. The brain image of mice which received rivastigmine and NaTC liposomes orally is shown in Fig. 13.

No vacuolisation was observed at the brain of the control group mice.

3. Discussion

In this study it was aimed to develop a liposome formulation for rivastigmine. Lipidisation is one of the most common ways for increasing brain penetration (Bozdağ et al. 2002). It was decided to formulate rivastigmine with lipids in liposome formulations to increase the brain penetration of rivastigmine. There

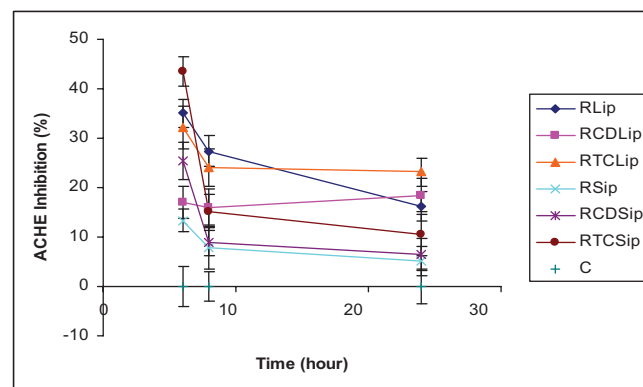


Fig. 10: AChE inhibition values in brain for intraperitoneally given formulations (Error bars represent standard deviations, $n = 15$)

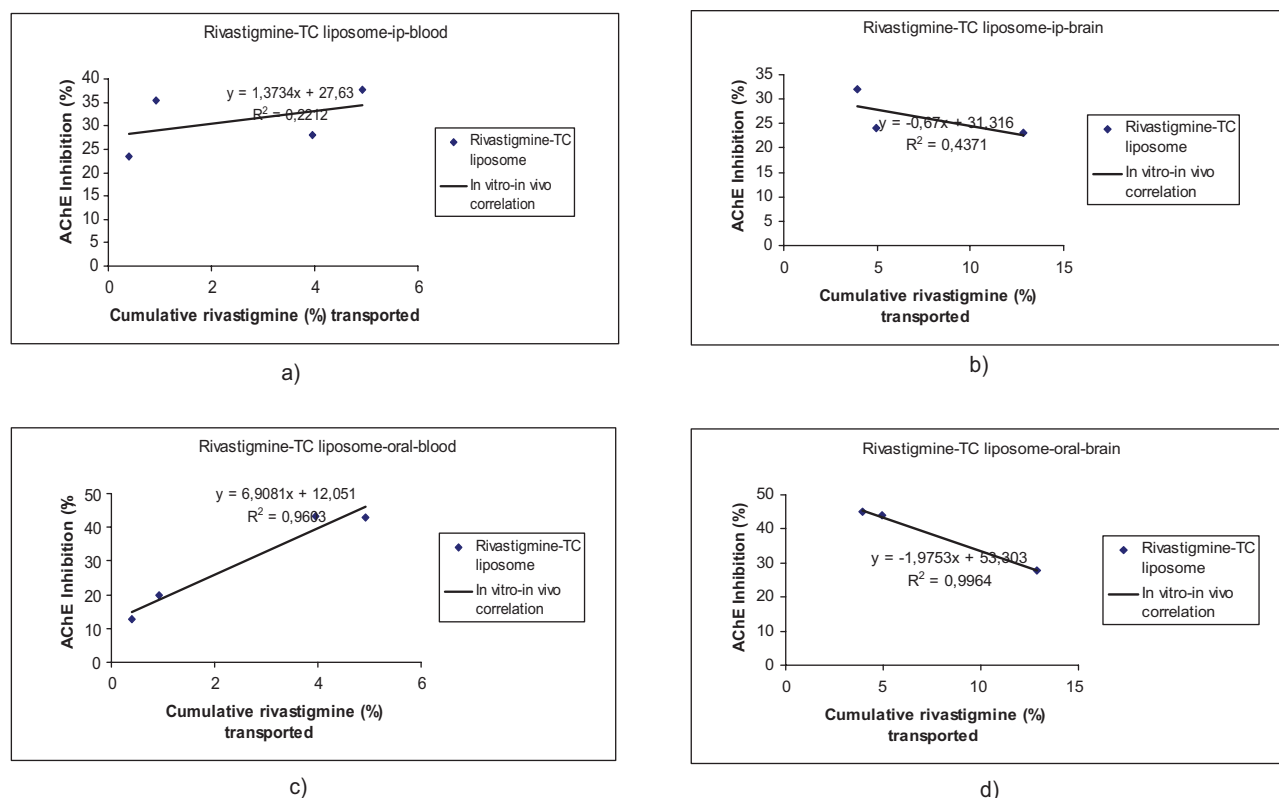


Fig. 11: *In vitro-in vivo* correlation results for rivastigmine and NaTC liposome between cumulative amount of rivastigmine (%) passed through apical to the basolateral side of the Caco-2 cell monolayer and AChE inhibition (%) values for intraperitoneally a,b) and orally c,d) treated mice for blood a,c) and for brain b,d)

is no other liposome formulation of rivastigmine or any related study found in the literature. Three types of liposome formulations were prepared containing rivastigmine and all results were compared with respective solutions.

MLV type liposomes were prepared by the film formation method using Dipalmitoylphosphatidyl choline (DPPC) cholesterol, chloroform and methanol. The shape and type of the liposomes were seen in inverted microscope (Fig. 1). In the present study, the amount of rivastigmine captured in rivastigmine liposomes, rivastigmine and NaTC containing liposomes and, dimethyl- β -CD containing liposomes were 35.4%, 25.2% and 29.9% respectively. According to Lasic (1998), it is possible to achieve an encapsulation efficiency above 70% theoretically, but this cannot be reached practically for liposomes. The encapsulation efficiency of 50% was reported as a quite high result



Fig. 12: The TEM image of the brain of a control group mice. (*) refers to neutrophile, and arrow refers to endothelial cell ($\times 8200$)

for liposomes. Rivastigmine is a hydrophilic compound, so the encapsulation efficiency obtained appeared to be high enough. In another study, Mura et. al. (2007) investigated benzocain liposomes. They prepared MLV type of liposomes by sonication method, and reported encapsulation efficiency as 26.5-29.7%. In our study, the effect of NaTC and dimethyl- β -CD on encapsulation efficiencies was also investigated. It was found to be as 41% and 65% respectively.

Drug release experiments for liposomes are generally performed using a dialysis membrane and buffer solutions (Shabbits et al. 2002). In this study the reason of choosing pH 7.4 as a medium was to mimic *in vivo* conditions. The pH of DMEM, the cell culture medium, was measured as 7.36. The kinetics of rivastigmine release were found to be first order for rivastigmine liposomes ($r^2 = 0.967$, SST = 1.33), dimethyl- β -CD containing liposomes ($r^2 = 0.973$, SST = 0.846) and NaTC containing liposomes ($r^2 = 0.970$, SST = 3.35). Absorption enhancers especially NaTC, increased rivastigmine transport through membrane for liposomes. Absorption enhancers possibly increased the membrane partition coefficients for rivastigmine. According to the literature, when partition coefficients increases, the transport of compound increases (Potts and Guy 1992). This can be explained by Fick's second law (Higuchi 1960). When absorption enhancers were used together with active compounds in formulations, they increase the membrane partition coefficients and thermodynamic activity coefficients (α) so they enhance the transport of active compounds through membranes.

MTT tests were also carried out to determine the effects of rivastigmine, NaTC, dimethyl- β -CD and liposome content (cholesterol, DPPC, MC). We also investigated the serum content effect of cell culture medium. It is known that serum has a positive effect on cell viability and integrity (Freshney 1994; Keenan et al. 1998). Rivastigmine was used with the dose of 1.5 mg/mL, cell viability was measured as 82%. NaTC concentration was 1.875 mM, with cell viability of 97%. Dimethyl- β -CD concentration was 0.15% cell viability was

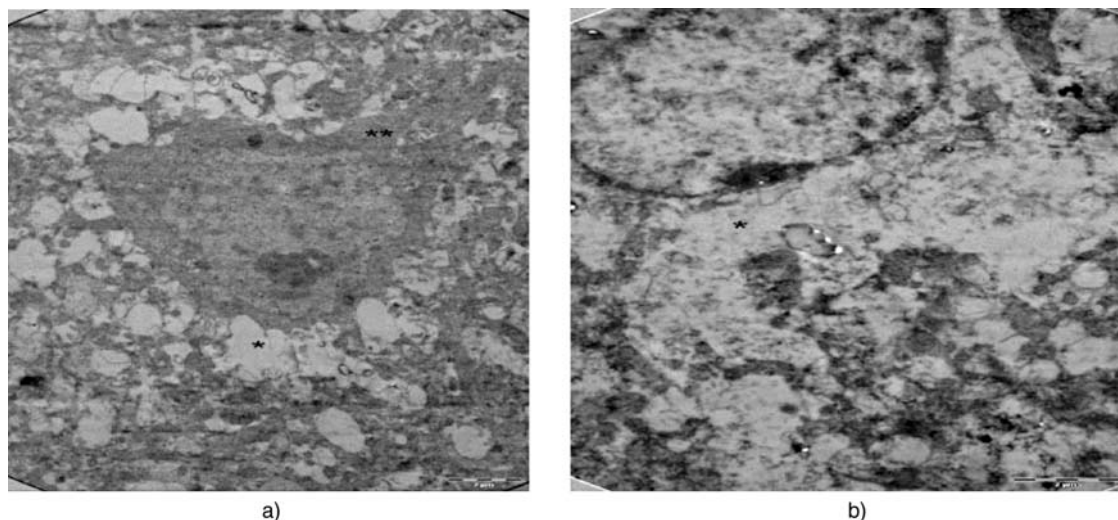


Fig. 13: a) The TEM image of the mouse brain which was treated orally with rivastigmine and NaTC containing liposome. (*) refers the intracellular space, and (**) refers neuronal shrinkage. (x6000); b) The TEM image of the mouse brain which was treated orally with rivastigmine and NaTC containing liposome. (*) refers to vacuolization (x8200)

98%. Liposome contents had no clear effect on cell viability in our study, so liposomes were not found to be toxic to cells at any concentrations. These materials were used at given concentrations with serum including DMEM in transport studies.

The transport studies were performed for solutions and liposome formulations. The data of all transport studies were given in Table 1. It's clearly observed for liposomes that NaTC and dimethyl- β -CD increases the rivastigmine transport for both solution and liposome formulations. NaTC and dimethyl- β -CD increased the cumulative transported amount of rivastigmine significantly ($p < 0.01$) for all experiment groups. NaTC was found to be most effective on enhancing rivastigmine transport from Caco-2 cells comparing to dimethyl- β -CD. The effect of filter inserts used in transport studies. Transport studies were also performed with no Caco-2 monolayer including filter inserts. There was a significant difference and the data suggested that Caco-2 cells formed a monolayer on filter inserts and this monolayer limited the rivastigmine transport with its tight junctions forming a second barrier.

Permeability coefficients were also calculated from solutions data, the highest value was found to be -1.78 cm/h, for rivastigmine and NaTC containing solution. The medium value was -1.84 cm/h, for rivastigmine and dimethyl- β -CD containing solution. The lowest value, -1.96 cm/h was found for rivastigmine containing solution. According to the liposome transport data the highest log k value was -0.75 cm/h, for rivastigmine and NaTC containing liposomes. The log k value calculated for dimethyl- β -CD containing liposome transport data (-1.18 cm/h) was lower than the log k value calculated for only rivastigmine containing liposome transport data (-1.03 cm/h). The cumulative amount of transported rivastigmine was higher for dimethyl- β -CD containing liposome than only rivastigmine containing liposome. In the first one hour the amount of rivastigmine transported was higher for only rivastigmine containing liposomes. The rivastigmine release from dimethyl- β -CD containing liposomes was delayed and found to be slower in comparison with only rivastigmine containing liposome.

TEER measurements were performed before and after experiments. The data showed that absorption enhancers, especially NaTC, decreased TEER values. This result indicated that absorption enhancers had opened or loosened the tight junction regions and though increased rivastigmine transport.

It is well known that liposomes are physically and chemically unstable and this is the most important disadvantage for long term stability of liposomes. The stability of liposomes were also

considered by examining the particle size, zeta potential and rivastigmine amount of liposomes when stored at 4°C , 25°C and 40°C for six months. Significant particle size increase was observed initially in rivastigmine liposomes and dimethyl- β -CD containing liposomes when stored at 4°C after 5 months ($p < 0.001$). There was no significant particle size increase at rivastigmine and NaTC containing liposomes at the end of the six months time period ($p > 0.05$). Liposomes were found to be quite stable in terms of particle size and distribution. Significant zeta potential change was observed in rivastigmine liposomes and dimethyl- β -CD containing liposomes when stored at 4°C after 3 months ($p < 0.001$). NaTC containing liposomes kept their zeta potentials at the end of the six months period ($p > 0.05$). Rivastigmine is not a problematic compound when stored at room temperature protected from light. Rivastigmine content of liposomes was analyzed every month by HPLC and the degradation kinetics of rivastigmine containing in liposomes were investigated at 4°C . The degradation kinetics for dimethyl- β -CD ($r^2 = 0.972$, SST = 113) and NaTC ($r^2 = 0.910$, SST = 355) containing liposomes was first order. The shelf lives for rivastigmine liposomes, dimethyl- β -CD containing liposomes, NaTC containing liposomes at 4°C were found to be 11, 14, 21 days respectively. The highest shelf life was found to be for NaTC containing liposomes. The shelf life of liposomes was decreased with the increase of temperature.

The *in vivo* experiment results were also considered in terms of AChE inhibitor (%). The blood data of intraperitoneally treated solutions caused high inhibition values within the first hour after treatment. The results were found to be similar for time period but lower for oral treatment with solutions. This data suggested that the bioavailability of treatment with rivastigmine was better than after oral administration. The AChE inhibition was found to be highest for NaTC containing solution. The AChE inhibition caused by liposomes was lower when compared with the first AChE inhibition data of solutions but the effect was found to be prolonged. When liposomes were incorporated into the blood stream, they need some time to release the active compound. The rivastigmine in solution is free, so it can immediately show the effect and cause AChE inhibition. Rivastigmine shows a first pass effect, when liposome formulations were administered intraperitoneally, they were distributed lymphatically to a higher extent. They possibly went through Peyer patches and reach to the blood stream without disintegration. MC was also added to liposomes to increase their stability after oral treatment and in GI track degradation. All results showed

that liposomes can reach to the bloodstream without disintegration and released rivastigmine. The AChE inhibition data of liposomes orally and intraperitoneally administered, showed no significant difference, but the effect of intraperitoneal treatments appeared to be rapid. The highest effect was found with NaTC containing liposomes for both intraperitoneal and oral ways. They showed slow, prolonged and enhanced effect on AChE inhibition in both blood and brain. The brain AChE inhibition values were also similar. The liposome formulations were found to be more effective than solutions in terms of AChE inhibition in brain, because it is well known that lipid content provides higher penetration through lipophilic barriers. Dimethyl- β -CD containing liposomes were compared with rivastigmine containing liposomes, and no significant difference was observed with in sixth and eight hours but the action of dimethyl- β -CD liposomes were continued throughout 24 h. Dimethyl- β -CD prevented rivastigmine from elimination in brain and prolonged the action. Absorption enhancers especially NaTC increases brain penetration of rivastigmine. It was also observed that significant decrease in AChE inhibition brain results of solutions after six hours and this data suggested that rivastigmine in solution had a faster elimination in the body compared with 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 action. Correlation studies were also performed between *in vitro* cumulative transported rivastigmine amount (%) and *in vivo* AChE inhibition (%) data. A high correlation was found especially for liposome formulations. The best correlation was found for rivastigmine and NaTC containing liposomes for both blood and brain.

According to TEM images of brains except for the control group, there was a significant increase at the intercellular spaces and vacuolisation. The vacuolisation and dilatation was significant in dimethyl- β -CD and NaTC containing liposomes. This data suggested that they enhanced the brain penetration of rivastigmine and caused dilatation. The best, most significant images were found to be NaTC containing liposomes after oral treatment. Caco-2 cells are used for blood-brain mimicking (Garberg et al. 2005; Wang et al. 2005). A high correlation was also found between *in vitro* and *in vivo* results for rivastigmine. This suggests that Caco-2 cells are a choice for a blood-brain model. In conclusion, the most stable and effective liposome formulation was found to be with rivastigmine and NaTC. This formulation was also found to be effective in both *in vitro* cell culture transportation studies and *in vivo* animal studies. *In vitro-in vivo* correlation studies showed that Caco-2 cell monolayer is a good model for drugs to predict their brain permeability. Liposome formulation was also found to be useful for rivastigmine to enhance brain permeability.

4. Experimental

4.1. Materials

Rivastigmine tartrate was purchased from Dr. Reddy's, India. Cholesterol, acetylcholine iodide, dimethyl- β -cyclodextrine (dimethyl- β -CD), sodium taurocholate (NaTC) and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT) were all purchased from Sigma, USA. Dipalmitoylphosphatidyl choline (DPPC) was provided from Across Organics, Belgium. Dubelco's Modified Eagle's Medium (DMEM) was purchased from Bichrom, Germany. 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) was provided from Serva, USA. Methyl cellulose (MC) was purchased from Colorcon, England. All other chemicals were HPLC grade.

4.2. Methods

4.2.1. *In vitro* studies

4.2.1.1. Preparation of rivastigmine liposomes. Multilamellar rivastigmine liposomes were prepared using dry film hydration method. Cholesterol

and DPPC were added to the round-bottomed flask in 1:1 molar ratios. Rivastigmine and MC were added to the flask. Absorption enhancers (dimethyl- β -CD or NaTC) were also added to the flask while preparing absorption enhancer included liposomes. After dissolving with chloroform, chloroform was evaporated under rotavapor at 43–44 °C until the observation of dry film. The film was hydrated by NaCl solution (%0.9) and liposome suspensions were formed after vortexing 5 min and ultrasonication for 25 min. Liposome suspensions were ultracentrifuged at 20000 g at 25 °C for 10 min. Supernatants and liposomes were separated. Liposome formulations were freshly prepared and used just before the experiments to avoid any degradation. Three types of liposomes: rivastigmine liposomes, rivastigmine-NaTC and rivastigmine-dimethyl- β -CD.

4.2.1.2. Measurement of the particle size of liposomes. The particle size of liposomes were determined by using Smpatec GmbH-Partikel Technik (Germany) laser diffraction particle sizer.

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

4.2.1.4. Determination of the type of liposomes. The type of the liposomes were determined by using an inverted microscope (Olympus CK2, Japan).

4.2.1.5. Determination of encapsulation efficiency. The rivastigmine contents of liposomes were calculated from supernatant phase after ultracentrifugation. The rivastigmine amount at the supernatant phase was subtracted from the total rivastigmine. Rivastigmine amounts were determined using high pressure liquid chromatography (HPLC) at the wavelength of 217 nm using mobile phase containing sodium heptane sulfonate:acetonitrile (72:28) at pH 3. C18 column (5 μ m, 4.6 $\times$ 250 mm) was used.

4.2.1.6. Determination of NaTC amount in liposomes. Determination of NaTC amount in liposomes was determined by HPLC method. The mobile phase consisted of % 0.3 ammonium carbonate:acetonitrile (68:32) at 210 nm.

4.2.1.7. Determination of dimethyl- β -CD amount in liposomes. Determination of dimethyl- β -CD amount in liposomes was determined by spectrophotometric method. Phenolphthalein added to the samples at pH 11 was colored with and they were immediately analyzed at 554 nm.

4.2.1.8. *In vitro* release studies of rivastigmine from liposome formulations. The release studies were performed using Franz diffusion cells with a dialysis membranes having 12000 Dalton pore size. 1.5 mL rivastigmine liposome suspension was placed in donor compartment of diffusion cells. Receiver compartment was filled with 2 mL of phosphate buffer (pH 7.4). *In vitro* release studies were performed for prepared liposomes. The release studies were continued for 24 h at 37 $\pm$ 0.5 °C. 1.5 mL samples were removed at predetermined time periods and fresh buffer was replenished at the same volume. The samples were analyzed by HPLC at 217 nm.

4.2.1.9. Physical stability of rivastigmine and/or absorption enhancer containing liposomes. The stability of three types of rivastigmine liposome formulations were investigated for six months (180 days) under three different conditions (4 °C, 25 °C and 40 °C). Rivastigmine analyses were done by HPLC. The zeta potential and particle size of three types liposomes were determined periodically.

4.3. Cell culture studies

4.3.1. Cell culture

Caco-2 cells were provided from American Type Culture Collection (US). Cells were cultured and passaged in plastic tissue culture flasks in DMEM containing 25 mM glucose, 5 mM glutamine supplemented with 10% fetal bovine serum, 1% gentamicine and 7.5% sodium bicarbonate in an incubator at 37 °C under 5% CO₂ atmosphere. The cells were grown for 21 days, the medium was changed with fresh DMEM every 48 h. The presence of a confluent monolayer was controlled with a microscope and the cells were subcultured by trypsinization. The cells were seeded to Costar filter bottom cups at the density of 80000 cells/mL for transport studies (Othman et al. 2007; Stevenson et al. 1999).

4.3.2. Cytotoxicity assay

The effects of rivastigmine, NaTC, dimethyl- β -CD and lipids used in liposome formulations on Caco-2 cell viability were also investigated. Effect of

Table 2: Design of experimental animal groups

GROUP NUMBERS-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)

serum content of DMEM was also examined by using both serum containing and serum free DMEM. The color density was measured in 570 nm with a multiwell Elisa reader. The results were calculated as a percentage using the control group values.

4.3.3. Transport experiments

The transport studies were performed for all types of prepared liposomes through 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 the predetermined time periods from the basolateral compartment. The volume removed was immediately replaced with fresh DMEM. The integrity of the monolayers was also determined and checked by measuring the electrical resistance at the end of each experiment. Rivastigmine contents of samples were analyzed by HPLC and Papp values were calculated using the following equation (Meaney and O'Driscoll 2000; Raiman et al. 2003; Zerrouk et al. 2006):

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

where P_{app} is the coefficient of apparent permeability, dQ/dt is the permeability rate, A (cm^2) is the diffusion area of the membrane and C_0 (mg/mL , mg/cm^3) denotes the initial concentration in the donor compartment.

4.3.4. In vivo studies

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

The animals were divided to 14 groups, 15 animals were used in each group. Rivastigmine solution, rivastigmine and absorption enhancer including solutions, rivastigmine liposomes, rivastigmine and rivastigmine and absorption enhancer including liposomes were administered to the animals both orally (o) and intraperitoneally (ip). Rivastigmine dose was 2300 $\mu\text{g}/\text{kg}$ for all formulations (Duplay et al. 2004). The design of the animal groups were shown in Table 2.

4.3.4.2. Preparation of animals. All animal groups except control group were treated intraperitoneally or orally with an appropriate concentration of rivastigmine (121 μg) in different formulations. Blood samples 1 mL were collected at specific time points (0.25, 0.5, 1, 3, 6, 8, 24 h). Brain samples were taken from some animals at 6, 8, and 24 h time points after blood sampling. All blood and brain samples were collected at deep freezer (-80°C) until the analysis time.

4.3.4.3. Cholinesterase inhibitory activity measurement. AChE inhibitory measurement studies were evaluated spectrophotometrically by the method of Ellman (Ellman et al. 1961; Snape et al. 1999; Alcalá et al. 2003) for both blood and brain samples. Acetylthiocholine iodide was used as substrate (0.075 M), dithiobisnitrobenzoic acid (DTNB) was used as reagent and the buffer that blood samples and other materials were suspended/soluted was phosphate buffer pH 8.0 (0.1 M). The method is similar for blood and brain with a few changes in sample preparing and concentration of substrate and DTNB reagent. The samples were analyzed spectrophotometrically at 412 nm wavelength. Changes in absorbance values were saved for 6 min and ΔA values were calculated.

4.3.4.4. Histological studies. Transmission electron microscope (TEM) was used to investigate the effects of formulations in the brains and animal groups shown in Table 2. Brain tissues were fixed in 2.5% glutaraldehyde containing phosphate buffer solution for 2 h. Postfixation was evaluated in 1% osmium tetroxide (OsO_4) and dehydration evaluated 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 taken out and examined with light microscope after painting with toluidine blue, and ultra-thin sections were investigated by electron microscope after staining with uranyl acetate and lead citrate.

4.3.4.5. Statistical analysis. All our data in this study were considered as means $\pm$ SD, and one way ANOVA was also used for statistical analysis of the data.

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