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ORIGINAL ARTICLE

Neuroprotective effects of carnosine-loaded elastic liposomes in cerebral ischemia rat model

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Abstract

Purpose The present study aims to investigate the neuroprotective effects of carnosine-entrapped elastic liposomes (CAR-ELs) against cerebral ischemia.

Methods CAR-ELs were prepared by extrusion method using egg phosphatidylcholine (eggPC) as a phospholipid and Tween 80 (TW80) as an edge activator (eggPC:TW80 = 8:2, w/w). The prepared CAR-ELs were purified by centrifugal ultrafiltration followed by characterization for particle size, polydispersity index, zeta potential and entrapment efficiency. The elasticity of CAR-ELs, the most distinct feature of elastic liposomes, was determined using a stainless steel pressure filter and compared with that of conventional liposomes. In vivo neuroprotective effects of CAR-ELs were evaluated in cerebral ischemia induced by permanent middle cerebral artery occlusion (pMCAO) in rats. CAR-ELs (250 mg/kg of CAR) were intravenously administered 20 min before pMCAO and 6 h after pMCAO, respectively. The infarct volume in brain was measured by staining with 2,3,5-triphenyltetrazolium chloride after 24 h of cerebral ischemia.

Results CAR-ELs showed nanometric particle size near 100 nm and homogeneous distribution with polydispersity index below 0.1. The elasticity of CAR-ELs was 2-fold higher than that of conventional liposomes. The brain ischemia was successfully developed with pMCAO as indicated by highly infarcted hemisphere (~50%) in saline-treated rats. The pre-treatment with CAR-ELs significantly reduced infarct volume (7.9%) compared with CAR solution (19.1%)- and saline (50.8%)-pretreated rats. CAR solution, however, showed better neuroprotective effects than CAR-ELs when administered 6 h after ischemia induction.

Conclusion The pre-treatment with CAR-ELs could be promising nanocarrier-based neuroprotective therapeutics against ischemic stroke.

Keywords Carnosine · Elastic liposomes · Neuroprotective effect · Ischemic stroke

Introduction

Stroke ranks second among the leading causes of deaths globally and is one of the primary reason of neurological disability in adults (Shah et al. 2019; Yan et al. 2013). There are two major types of stroke as ischemic stroke and hemorrhagic stroke. Ischemic stroke is characterized by the brain's cell death resulting from the blockage of blood supply to brain and accounts for more than 80% of all cases (Doyle et al. 2008; Sims and Muyderman 2010). Tissue damage in response to ischemic stroke involves multiple pathways including oxidative and nitrative stress, acidosis, excitotoxicity, inflammation, apoptosis, ionic imbalance, peri-infarct depolarization, matrix metalloproteinase activation and breakdown of blood–brain barrier (Doyle et al. 2008;

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Rajanikant et al. 2007). Restoring the blood flow by using thrombolytic such as recombinant tissue plasminogen activator (rt-PA) is the only approved treatment for ischemic stroke in most parts of the world (Green and Shuaib 2006; Min et al. 2008). However, the use of rt-PA therapy is limited due to potential side effects including short therapeutic window and increased risk of hemorrhage. In addition, rt-PA is only effective within 3 h of ischemia thus restricting its clinical applications to only less than 5% of patients (Bae et al. 2013). Neuroprotection is an alternate approach to treat ischemic stroke, which involves reduction in neurodegeneration and tissue damage by interfering with multiple pathways in the ischemic cascade (Green and Shuaib 2006).

Carnosine (CAR) is a naturally occurring endogenous dipeptide consisting of alanine and histidine (β-alanyl-L-histidine) (Shen et al. 2010). CAR is found in many tissues of human body such as skeletal and cardiac muscle, liver and central nervous system. In brain, CAR is mainly present in glial and ependymal cells (De Marchis et al. 2000). CAR is safe, well-tolerated and utilized as dietary supplement without serious adverse effects. CAR has been reported to exhibit neuroprotective activity owing to its excellent antioxidant effect, free radicals scavenging, membrane stabilizing and heavy metals chelating properties, regulation of macrophage activity, cytosolic buffering and protection against glutamate-induced excitotoxicity (Guiotto et al. 2005; Rajanikant et al. 2007; Stvolinsky and Dobrota 2000). Exogenously administered CAR might be of great potential to improve the neurological outcomes in ischemic stroke. However, delivery of therapeutic agents to brain is hampered by the relative impermeability of blood–brain barrier, which protects the brain against noxious materials by restricting entry of compound and allows essential nutrients to reach the brain cells and thus maintaining brain homeostasis (Gabathuler 2010). The blood–brain barrier is composed of endothelial cells of brain capillaries with tight junctions of cell adhesion molecules between the capillary endothelial cells (Begley 2004; Gabathuler 2010). Encapsulation of CAR in elastic liposomes (ELs) might be a promising strategy to enhance the transport of CAR into brain and protect it against possible enzymatic hydrolysis. Liposomal formulations have been reported to enhance the stability and bioavailability of peptide and proteins (Byeon et al. 2019; Colletier et al. 2002).

Liposomes are biocompatible and biodegradable nanocarriers capable of encapsulating hydrophilic and lipophilic drugs. Conventional liposomes commonly consist of phospholipids and cholesterol and are formed by one or more concentric lipid bilayers enclosing an aqueous phase (Din et al. 2017). Since conventional liposomes have got limited capabilities to cross the biomembranes, elastic liposomes (ELs) were developed to impart elasticity and to enable them to cross membrane pores smaller than their own size by incorporating edge activator into lipid bilayers (Cevc et al. 2002). ELs have also been termed or known as flexible, deformable, ultradeformable liposomes or Transfersomes™. ELs were originally developed to increase permeation of therapeutic drugs across the skin, however, their small size and elastic nature might allow ELs to cross other biological barriers like blood–brain barrier to achieve meaningful therapeutic concentration in brain. Oral administration of ELs have been reported to enhance blood levels, brain distribution and stability of (+)-catechin compared with aqueous solution (Huang et al. 2011). In another study, a direct correlation was observed between elasticity of liposomes and amount of olanzapine delivered to the brain via intranasal route (Salama et al. 2012). The edge activators in ELs cause shape transformation when exposed to space detention in biomembranes. The edge activator-induced shape transformation in ELs is attributed to the local readjustment of their bilayer components thereby permitting ELs to deliver their payload across the skin (Cevc 2012; Perez et al. 2016).

The purpose of this study is to evaluate the neuroprotective effect of carnosine-entrapped elastic liposomes (CAR-ELs) in ischemic stroke model in rat. CAR-ELs were prepared by using extrusion technique followed by their characterization for physicochemical properties. The elasticity of CAR-ELs was determined and compared to that of the conventional liposomes. In vivo neuroprotective effects in both pre-treatment and post-treatment of CAR-ELs were assessed in permanent middle cerebral artery occlusion (pMCAO) rat model of ischemic stroke.

Materials and methods

Materials

L-carnosine, cholesterol, Tween 80 (TW80), sodium tetraborate, sodium sulfite, triton X-100, and *o*-phthalaldehyde (OPA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Egg yolk L-α-phosphatidylcholine (eggPC) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Isoflurane was purchased from Hana Pharm Co., Ltd. (Seoul, Republic of Korea). All other chemicals were of analytical grade and were used without further purification.

Preparation of CAR-ELs

CAR-ELs were prepared by extrusion method with minor modifications (Zeb et al. 2017). Briefly, phospholipid and edge activator were dissolved in chloroform followed by evaporation of organic solvent in rotary evaporator (Rotavapor® R-3, Büchi Labortechnik AG, Switzerland). The evaporator was operated at 40 °C under a reduced pressure of 200 mBar to leave a thin lipid film on the flask. The traces of chloroform were removed by purging the lipid film

with N_2 gas for 30 min. A completely dry lipid film was hydrated with 250 mg/mL CAR solution in normal saline by vortexing to form multilamellar vesicles. Prior to the hydration, CAR solution was maintained at 35 °C which is a temperature above the phase transition (T_m) of eggPC and close to body temperature. The resultant dispersion was finally sonicated for 30 min followed by 10 cycles of extrusion through 100 nm polycarbonate membrane using a Mini-Extruder (Avanti Polar Lipids, Inc., Alabaster, AL, USA) at 35 °C. Carnosine-entrapped conventional liposomes (CAR-CLs) were prepared by the same method as described above except that TW80 was changed to cholesterol. The composition of CAR-ELs and CAR-CLs are shown in Table 1. The prepared CAR-ELs and CAR-CLs were stored at 4 °C for further studies.

Physicochemical characterization of CAR-ELs

Particle size, polydispersity index, zeta potential and entrapment efficiency of CAR-ELs

CAR-ELs were evaluated for average particle size, polydispersity index (PDI) and zeta potential based on photon correlation spectroscopy and electrophoretic light scattering techniques using a Zetasizer Nano ZS (Malvern, Worcestershire, UK). For this purpose, 20 µL of CAR-ELs were diluted with 1 mL of filtered deionized water and the measurements were performed at 25 °C with a fixed angle of 90°.

Entrapment efficiency and loading capacity (%) of CAR-ELs was calculated from the amount of unentrapped free CAR. Centrifugation ultrafiltration method was used to separate free CAR from CAR-ELs (Li et al. 2012). The CAR-ELs dispersion was centrifuged at 14,000×*g* for 15 min at 4 °C using Ultra-0.5 centrifugal filter units. The filtrate containing free CAR was allowed to react with *o*-phthalaldehyde/sodium sulfite solution to derivatize amine group of CAR to N-alkyl-1-isoindole sulfonate for UV-spectrophotometric detection (Maldonado and Maeyama 2013). The absorbance of filtrate was measured at 340 nm using UV–visible spectrophotometer (Ultrospec 7000, Biochrom Ltd., Cambridge, UK). The amount of CAR entrapped in ELs was determined by subtracting the amount of free CAR from the total amount of CAR added.

Table 1 Composition of CAR-ELs and CAR-CLs (mg/mL)

Formulation	eggPC	TW80	CH	CAR
Blank ELs	8	2	–	–
CAR-ELs	8	2	–	250
CAR-CLs	8	–	2	250

eggPC Egg yolk L-α-phosphatidylcholine, *TW80* Tween 80, *CH* cholesterol, *CAR* carnosine, *ELs* elastic liposomes, *CLs* conventional liposomes

The entrapment efficiency and loading capacity of CAR-ELs was then calculated by using the following equation.

$$\text{Entrapment efficiency (\%)} = \frac{\text{Weight of CAR entrapped in ELs}}{\text{Total weight of CAR added}} \times 100$$

$$\text{Loading capacity (\%)} = \frac{\text{Weight of CAR entrapped in ELs}}{\text{Total weight of CAR} - \text{ELs}} \times 100$$

Transmission electron microscopy (TEM)

The morphology of CAR-ELs was examined by field emission transmission electron microcopy (FE-TEM, JEM-2100F, JEOL, Tokyo, Japan) after negative staining (Zeb et al. 2016). For this purpose, properly diluted CAR-ELs were placed and allowed to adsorb onto the carbon-coated 400 mesh copper grid and were negatively stained by adding a drop of 1% phosphotungstic acid solution. The grids were allowed to dry at room temperature and CAR-ELs were finally imaged with TEM operated at an accelerating voltage of 200 kV.

Elasticity of CAR-ELs

The elasticity is a distinctive feature of elastic liposomes which enables them to pass through the pore smaller than their own size. The elasticity index of CAR-ELs was investigated by extrusion method using a stainless steel pressure filter device (Lipix extruder, Northern lipids Inc., Burnaby, BC, Canada) (Jain et al. 2003; Van den Bergh et al. 2001). CAR-ELs dispersion was driven through a 50 nm polycarbonate membrane with an external pressure of 0.2 MPa. The volume of CAR-ELs extruded through the membrane filter in 5 min was measured. The mean particle size of CAR-ELs after extrusion was measured by using a Zetasizer Nano ZS (Malvern, Worcestershire, UK). The following equation was used to calculate the elasticity of CAR-ELs.

$$E = J \times \left(\frac{r_v}{r_p}\right)^2$$

where E is the elasticity index of vesicles, J is the volume of vesicles extruded through membrane in a unit time, r_v is the average vesicles diameter following extrusion and r_p is the pore diameter of permeability membrane (50 nm).

In vivo neuroprotective effects of CAR-ELs

Animals

Permanent focal cerebral ischemia model was used to evaluate the in vivo neuroprotective effects of CAR-ELs in rats. The neuroprotective effects of CAR-ELs were compared with those of CAR solution. Healthy male Sprague–Dawley

rats weighing 250–300 g were purchased from Koatech laboratory animal company (Pyeongtaek, Gyeonggi, Republic of Korea) and were acclimatized to laboratory conditions for a week before the experiments. All animal experiments were conducted in accordance with the NIH policy and animal welfare act under the approval of Institutional Animal Care and Use committee (IACUC) at Hanyang University.

Induction of cerebral ischemia

Cerebral ischemia was induced by permanent middle cerebral artery occlusion (pMCAO) in rats with a slight modification (Shah et al. 2016, 2018b). The rats were kept under isoflurane anesthesia throughout the experimental procedure. The body temperatures of rats were maintained at 37 ± 1 °C by using blanket and heating lamp. The skin around the neck area was incised to expose left middle cerebral artery (MCA). The left common carotid artery (CCA) and the external carotid artery were ligated with suture and occipital artery arising from ECA was coagulated. Similarly, the pterygopalatine artery was ligated after exposing the internal carotid artery (ICA). Finally, MCA was occluded by inserting a 4-0 silicon-coated nylon monofilament into the CCA and the ICA to the origin of the MCA. The occlusion of MCA resulted in the induction of cerebral ischemia. The cerebral blood flow (CBF) of rats was measured with a laser doppler perfusion monitor (PeriFlux system 5000, Perimed AB, Järfälla, Sweden) before and after surgery to ensure the completeness of occlusion. Rats with fewer than 75% reduction in the baseline CBF were excluded from the study.

Treatment with CAR-ELs

Rats were randomly divided into three groups including saline, CAR solution and CAR-ELs group. The investigators were kept blind to the allocation of groups during the whole procedure. The formulations were injected intravenously via lateral tail vein with CAR at a dose of 250 mg/kg. Saline group was injected with sterile normal saline to serve as a control, CAR solution group was administered CAR solution (250 mg/mL) in a 0.9% sterile normal saline and CAR-ELs group was administered with CAR-ELs (equivalent to 250 mg/kg of CAR). The formulations were administered 20 min before the induction of cerebral ischemia and 6 h after cerebral ischemia induction for the evaluation of pre-treatment and post-treatment effects, respectively.

Calculation of the infarct volume

The rats were sacrificed by overdosing of isoflurane after 24 h of ischemia induction and brain was isolated immediately. The brain was washed with cold normal saline and sliced into 2 mm thick coronal sections with a tissue cutter. The brain sections were stained by incubating in 2,3,5-triphenyltetrazolium chloride (TTC) solution (2%) in PBS for 30 min and at room temperature. The stained sectioned were then fixed in 4% paraformaldehyde solution for 30 min (Shah et al. 2018a). The digital images of sections were produced by scanning using HP Scanjet 4470c scanner and the infarct area of each section was determined by using the NIH ImageJ software version 1.44 (National Institutes of Health, Bethesda, MD, USA). A total infarct volume (mm^3) of brain was calculated by summing up the infarct areas of all sections and multiplying it by section thickness (2 mm). The total infarct volume was also corrected for the brain edema (Swanson et al. 1990).

Statistical analysis

All experiments were performed at least 3 times and the data are presented as mean ± standard deviation (SD) or standard errors of mean (SEM). The statistical significant differences among the groups were determined by student's *t* test. A value of *p* less than 0.05 was considered as statistical significant difference.

Results and discussion

Preparation and physicochemical properties of CAR-ELs

CAR-ELs were successfully and reproducibly prepared by using eggPC as a lipid bilayer matrix and TW80 an edge activator. TW80 is a biocompatible and pharmaceutically acceptable surfactant and is widely used edge activator to impart elasticity to vesicular formulations for transdermal delivery (Chen et al. 2013; Zeb et al. 2018). The physicochemical properties of CAR-ELs were investigated in terms of particles size, PDI, zeta potential and entrapment efficiency and the results are presented in Table 2. The blank ELs showed a particle size of 96.9 nm, while particle size slightly increased to 104.4 nm in CAR-ELs. CAR-ELs displayed unimodal distribution curve (Fig. 1a) with PDI value below 0.1 indicating homogenous and narrow particle size distribution. PDI value less than 0.300 is generally accepted as homogeneous distribution of nanoparticulate carriers (Estella-Hermoso de Mendoza et al. 2009; Rizvi et al. 2019). Electrostatic repulsion originating from surface charge confers physical stability to colloidal dispersions by preventing particles aggregation during storage (Estella-Hermoso de Mendoza et al. 2009). Both CAR-ELs and blank ELs showed negative surface charges with zeta potential values of − 14.4 mV and − 13.8 mV, respectively. The negative zeta potential of CAR-ELs might be attributed to the partial hydrolysis of polyethylene oxide head groups

Table 2 Physicochemical properties of CAR-ELs and CAR-CLs

Formulation	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Entrapment efficiency (%)	Loading capacity (%)
Blank ELs	96.9 ± 1.3	0.076 ± 0.017	− 13.8 ± 1.2	–	–
CAR-ELs	104.4 ± 1.2	0.089 ± 0.018	− 14.4 ± 2.9	32.3 ± 0.4	89.0 ± 1.1
CAR-CLs	128.5 ± 6.5	0.101 ± 0.020	− 26.9 ± 5.0	37.4 ± 1.1	90.3 ± 2.7

Data are expressed as mean ± SD (n = 3)

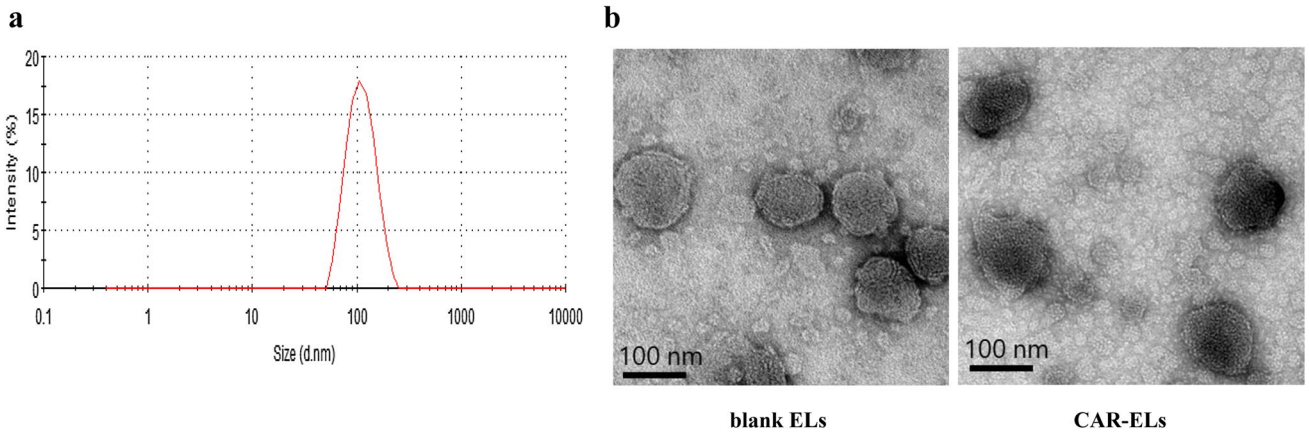

Fig. 1 Particle size distribution (**a**) and TEM image of CAR-ELs (**b**)

$(CH_2–CH_2–O)_n$ of TW80 at the surface of vesicles (Lee et al. 2005; Tasi et al. 2003; Yang et al. 2007). TEM was used to investigate the morphology of blank ELs and CAR-ELs and images are presented in Fig. 1b. TEM images revealed their vesicular structure, spherical and uniform shape. TEM results also confirmed the nanometric vesicle size (~ 100 nm) of CAR-ELs measured by photon correlation spectroscopy.

The entrapment efficiency (%) of CAR-ELs was measured after derivatization of amine group in CAR for UV-spectrophotometric detection. The spectrophotometric detection of most amino acids is difficult as they are week chromophores and show very little light absorbance. Pre-column derivatization was usually performed using UV chromophore or fluorophore reagents to analyze such amino acids (Soga and Heiger 2000). *o*-Phthalaldehyde (OPA) is the most commonly employed reagent for pre-column derivatization of amino acids owing to its sensitivity, simplicity and reliability (Tcherkas et al. 2001). In the current study, OPA/sodium sulfite reaction was employed to derivatize CAR and the entrapment efficiency of CAR into CAR-ELs was calculated as 32.3%. A relatively low entrapment efficiency of CAR-ELs is attributed to small vesicles size with low aqueous compartment volume to accommodate CAR (El Maghraby et al. 2001). In contrary to water soluble drugs, a high entrapment efficiency in elastic liposomes is usually observed for lipid soluble drugs which find place in lipid bilayers of the vesicles (Nii and Ishii 2005). On the other hand, CAR-ELs showed a high loading capacity of 89% as highly concentrated CAR solution (250 mg/mL) was initially added to hydrate thin lipid film during the preparation of CAR-ELs.

Elasticity of CAR-ELs

Elasticity imparted by edge activators is a unique property which enables ELs to bring about shapes transformation and to pass through small pores. The shape transformation in ELs occurs at low energy state since edge activators possess a high radius of curvature and mobility. Upon exposure to space confinement or mechanical stress, edge activators relocate themselves in the zones of higher curvature/stress, while eggPC are positioned in bilayer region with smaller curvature. The repositioning of edge activator and eggPC allows ELs to drive through the pores smaller than their own size without being disassembled (Cevc 2012; Perez et al. 2016). Tween 80 was selected as an edge activator in the current study because of the superior elasticity index to cholic acid and its derivatives. Tween 80 has long and flexible hydrocarbon chains in its chemical structure which results in more flexible lipid bilayers compared with cholic acid and its derivatives having bulky and less flexible steroidal rings (El Zaafarany et al. 2010; Jain et al. 2003; Zeb et al. 2016).

The elasticity of CAR-ELs was measured in terms of elasticity index and was compared with that of CAR-CLs. The elasticity index of blank ELs and CAR-ELs was 97.87 and 95.49, respectively, and the elasticity of blank ELs and CAR-ELs was two times higher than that of CAR-CLs (Fig. 2). CAR-CLs are composed of eggPC and cholesterol instead of edge activator, which makes their lipid bilayer membranes more rigid than ELs with high elastic modules (Tseng et al. 2007). The presence of surfactant (TW80) and elasticity of CAR-ELs would contribute to efficient transport of CAR across the blood–brain barrier and improve its neuroprotective effects in ischemic stroke.

Physiological parameters of animals during ischemia model

The physiological parameters of animals play a vital role in successful cerebral ischemia induction, producing highly reproducible outcomes and minimizing inter-laboratory variations (Liu et al. 2009). The physiological variables of body weight, body temperature and cerebral blood flow were monitored during cerebral ischemia model and the results are presented in Table 3. The body weight of rats in saline, CAR solution and CAR-ELs was found to be 265–270 g, indicating uniformity of body weight among the experimental groups. The body weight of rats affect their arterial dimensions and thereby determine the optimum suture diameter to occlude artery and cause cerebral ischemia (Fluri et al. 2015). Therefore, matching the occluder diameter and coating length with animal weight improve animal model consistency. The physical features of the occluding filament have been reported to influence ischemia induction rate by causing insufficient occlusion, premature reperfusion and filament dislodgement (Liu et al. 2009; Spratt et al. 2006). In the current study, infarct variations were reduced by utilizing uniform body weight rats and accordingly optimized silicon-coated nylon monofilament for cerebral ischemia induction.

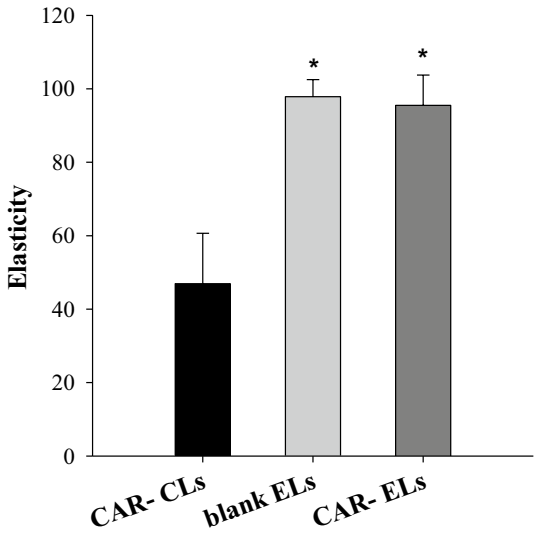

Fig. 2 Elasticity index of CAR-ELs and CAR-CLs. Data are presented as the mean ± SD (n = 3). *$p < 0.05$ vs. CAR-CLs

Monitoring and controlling of animal's body temperature is crucial to eliminate harmful effects of hyperthermia and neuroprotective effects of hypothermia in cerebral ischemia, which lead to variable results in different treatment groups. It has been reported that hyperthermia exacerbates ischemic brain injury by accentuating ischemic mechanisms within the penumbra (Zaremba 2004). On the other hand, hypothermia reduces ischemic brain injury by affecting apoptosis, excitotoxicity and neuroinflammation (Yenari and Han 2012). Fluctuating body temperatures will therefore produce variable infarct volume in different rats. The body temperatures of rats before and after cerebral ischemia induction were maintained in the normal range of 37.5 ± 0.5 °C in each group, thus excluding the chances of outcomes variation from body temperature. To verify the completeness of occlusion, CBF of rats before and after cerebral ischemia was measured.

The results indicated that CBF was reduced to 15.5 ± 3.1, 14.0 ± 3.2, 14.3 ± 3.3 from the base line (100%) after the occlusion of MCA in saline, CAR solution and CAR-ELs groups, respectively, representing about 96% reduction of CBF from their baseline levels. These results confirm successful induction of cerebral ischemia as 75% reduction from the baseline CFA is widely accepted indicator of successful cerebral artery occlusion in stroke models (Schmid-Elsaesser et al. 1998). Taken together, it is evident

Table 3 Physiological parameters of rats during cerebral ischemia model

Group	Body weight (g)	Body temperature (°C) before pMCAO	Body temperature (°C) after pMCAO	CBF before pMCAO	CBF after pMCAO	Reduction of CBF (%)
Saline	268.0 ± 8.8	37.7 ± 0.7	37.6 ± 0.7	431.5 ± 40.0	15.5 ± 3.1	96.41
CAR solution	270.2 ± 4.5	38.0 ± 0.7	38.0 ± 1.0	451.3 ± 41.7	14.0 ± 3.2	96.89
CAR-ELs	265.5 ± 7.7	37.7 ± 0.5	37.7 ± 0.6	435.8 ± 45.6	14.3 ± 3.3	96.72

CAR Carnosine, *pMCAO* permanent middle cerebral artery occlusion, *CBF* cerebral blood flow, *CAR-ELs* carnosine-entrapped elastic liposomes

that physiological parameters were uniformly maintained throughout the ischemic model and ischemia was successfully and reproducibly induced in all experimental groups.

Neuroprotection by CAR-ELs in cerebral ischemia

The neuroprotective effects of CAR-ELs were investigated in rats by measuring infarct volume in cerebral ischemia using permanent occlusion. CAR has previously been shown to protect the rodents against ischemia brain injury via different mechanisms (Min et al. 2008). In current study, we utilized ELs to further improve brain delivery and neuroprotective effects of CAR in contrary to previous reports where ELs were mainly studied to enhance drug delivery via skin owing to their stress dependent adaptability (Zeb et al. 2018). The results showed that CAR-ELs and CAR solution were able to reduce brain damage compared with normal saline when administered 20 min before the induction of cerebral ischemia (Fig. 3). Treatment with CAR-ELs reduced the infarct volume to 7.9% in comparison to CAR solution (19.1%) and normal saline (50.8%) as a control, indicating better neuroprotection of CAR when administered in the formulation of CAR-ELs. The improved neuroprotection of CAR-ELs might be attributed to their superior permeability across the blood–brain barrier due to nanometric size and elastic capabilities. Several mechanisms have been proposed to explain nanoparticles-medicated transport of drugs across the blood–brain barrier including endocytosis, transcytosis and opening of tight junctions between the endothelial cells by surfactant molecules of nanoparticles (Saraiva et al. 2016). The presence of TW80 as edge activator in ELs might also adsorb plasma proteins thereby facilitating their binding to lipoprotein receptors and transport into the brain (Lim et al. 2014; Reimold et al. 2008). The combining effects of proposed mechanisms might be ascribed to better neuroprotection of CAR-ELs in ischemic rats.

In contrary to treatment before ischemia induction, CAR solution showed significantly less infarct volume than CAR-ELs and saline solution in post-treatment ischemic model (Fig. 4). The infarct volume of rats treated with CAR solution after 6 h of ischemia induction was reduced to 17.1% while control and CAR-ELs showed 50.3% and 30.7% of infarct volume, respectively. The results showed that CAR solution displayed similar neuroprotective effects when administered before and after ischemia induction. CAR solution is immediately available for its neuroprotective effects on intravenous administration, thereby resulting in a significantly reduced infarct volume even after 6 h of ischemia. It has been previously reported that CAR exhibits 9 h therapeutic time window in pMCAO model at a dose of 1000 mg/kg (Bae et al. 2013). In contrary, time lag is required for CAR-ELs to show their neuroprotective effects. Therapeutic time window might have been elapsed for CAR-ELs, resulting in higher infarct volume when administered 6 h post ischemia. Taken together, the pretreated CAR-ELs improved the neuroprotective effects via effective brain delivery in cerebral ischemia rat model.

Conclusion

Elastic liposomes have been widely investigated to deliver their payloads across the skin. Their potential to permeate other bio-membranes including blood–brain barrier remained unveiled. In this study, CAR-ELs were successfully formulated to improve its brain delivery in cerebral ischemia. CAR-ELs displayed 2-folds higher elasticity than CAR-CLs. CAR-ELs significantly improved neuroprotective effects of CAR compared with CAR solution

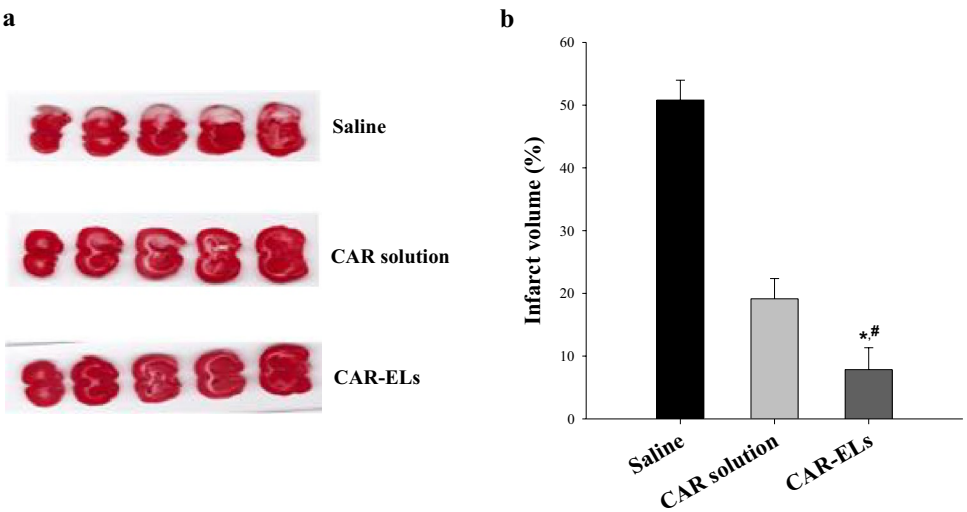

Fig. 3 Pre-treatment effects of CAR-ELs against brain damage during ischemic stroke: TTC-stained brain sections (**a**) and infarct volumes (**b**). Data is presented as the mean ± SEM (n = 5). $^{*}p < 0.001$ compared with saline group as the control and $^{\#}p < 0.05$ vs. CAR solution

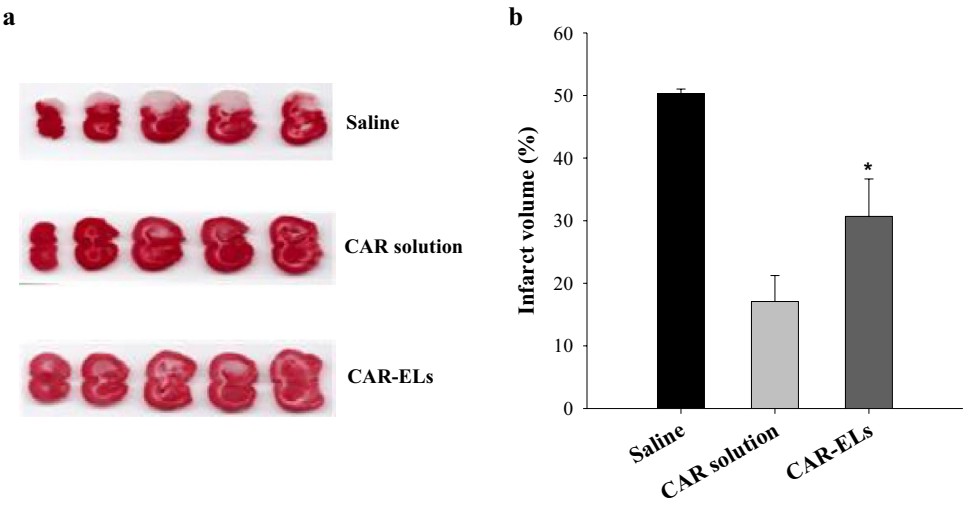

Fig. 4 Post-treatment effects of CAR-ELs against brain damage during ischemic stroke: TTC-stained brain sections (**a**) and infarct volumes (**b**). $*p < 0.05$ compared with saline group as the control. Data is presented as the mean ± SEM (n = 5)

when intravenously administered before ischemia induction. Consequently, CAR-ELs might be of great potential to enhance the therapeutic potential of CAR and to minimize the risk of brain damage in ischemic stroke. Further studies to explore transport mechanism across blood–brain barrier and a reduced neuroprotective effects of CAR-ELs in post-treatment model are highly encouraged.

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Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest in this work.

Ethical approval All animal experiments were conducted in accordance with the NIH policy and animal welfare act under the approval of Institutional Animal Care and Use committee (IACUC) of Hanyang University (IACUC approval No. 2014-0152A).

References

Bae O-N, Serfozo K, Baek S-H, Lee KY, Dorrance A, Rumbeiha W, Fitzgerald SD, Farooq MU, Naravelta B, Bhatt A (2013) Safety and efficacy evaluation of carnosine, an endogenous neuroprotective agent for ischemic stroke. Stroke 44:205–212

Begley DJ (2004) Delivery of therapeutic agents to the central nervous system: the problems and the possibilities. Pharmacol Ther 104:29–45

Byeon JC, Lee S-E, Kim T-H, Ahn JB, Kim D-H, Choi J-S, Park J-S (2019) Design of novel proliposome formulation for antioxidant peptide, glutathione with enhanced oral bioavailability and stability. Drug Deliv 26:216–225

Cevc G (2012) Rational design of new product candidates: the next generation of highly deformable bilayer vesicles for noninvasive, targeted therapy. J Control Release 160:135–146

Cevc G, Schätzlein A, Richardsen H (2002) Ultradeformable lipid vesicles can penetrate the skin and other semi-permeable barriers unfragmented. Evidence from double label CLSM experiments and direct size measurements. Biochim Biophys Acta 1564:21–30

Chen J, Lu W-L, Gu W, Lu S-S, Chen Z-P, Cai B-C (2013) Skin permeation behavior of elastic liposomes: role of formulation ingredients. Expert Opin Drug Deliv 10:845–856

Colletier J-P, Chaize B, Winterhalter M, Fournier D (2002) Protein encapsulation in liposomes: efficiency depends on interactions between protein and phospholipid bilayer. BMC Biotechnol 2:9

De Marchis S, Modena C, Peretto P, Migheli A, Margolis FL, Fasolo A (2000) Carnosine-related dipeptides in neurons and glia. Biochem Mosc 65:824–833

Din FU, Aman W, Ullah I, Qureshi OS, Mustapha O, Shafique S, Zeb A (2017) Effective use of nanocarriers as drug delivery systems for the treatment of selected tumors. Int J Nanomed 12:7291–7309

Doyle KP, Simon RP, Stenzel-Poore MP (2008) Mechanisms of ischemic brain damage. Neuropharmacology 55:310–318

El Maghraby GMM, Williams AC, Barry BW (2001) Skin delivery of 5-fluorouracil from ultradeformable and standard liposomes in-vitro. J Pharm Pharmacol 53:1069–1077

El Zaafarany GM, Awad GAS, Holayel SM, Mortada ND (2010) Role of edge activators and surface charge in developing ultradeformable vesicles with enhanced skin delivery. Int J Pharm 397:164–172

Estella-Hermoso de Mendoza A, Campanero MA, Mollinedo F, Blanco-Prieto MJ (2009) Lipid nanomedicines for anticancer drug therapy. J Biomed Nanotechnol 5:323–343

Fluri F, Schuhmann MK, Kleinschnitz C (2015) Animal models of ischemic stroke and their application in clinical research. Drug Des Dev Ther 9:3445–3454

Gabathuler R (2010) Approaches to transport therapeutic drugs across the blood–brain barrier to treat brain diseases. Neurobiol Dis 37:48–57

Green AR, Shuaib A (2006) Therapeutic strategies for the treatment of stroke. Drug Discov Today 11:681–693

Guiotto A, Calderan A, Ruzza P, Borin G (2005) Carnosine and carnosine-related antioxidants: a review. Curr Med Chem 12:2293–2315

Huang YB, Tsai MJ, Wu PC, Tsai YH, Wu YH, Fang JY (2011) Elastic liposomes as carriers for oral delivery and the brain distribution of (+)-catechin. J Drug Target 19:709–718

Jain S, Jain P, Umamaheshwari R, Jain N (2003) Transfersomes-a novel vesicular carrier for enhanced transdermal delivery: development, characterization, and performance evaluation. Drug Dev Ind Pharm 29:1013–1026

Lee EH, Kim A, Oh Y-K, Kim C-K (2005) Effect of edge activators on the formation and transfection efficiency of ultradeformable liposomes. Biomaterials 26:205–210

Li G, Fan Y, Fan C, Li X, Wang X, Li M, Liu Y (2012) Tacrolimus-loaded ethosomes: physicochemical characterization and in vivo evaluation. Eur J Pharm Biopharm 82:49–57

Lim WM, Rajinikanth PS, Mallikarjun C, Kang YB (2014) Formulation and delivery of itraconazole to the brain using a nanolipid carrier system. Int J Nanomed 9:2117–2126

Liu S, Zhen G, Meloni BP, Campbell K, Winn HR (2009) Rodent stroke model guidlines for preclinical stroke trials (1st Edition). J Exp Stroke Transl Med 2:2–27

Maldonado M, Maeyama K (2013) Simultaneous electrochemical measurement method of histamine and Nτ-methylhistamine by high-performance liquid chromatography–amperometry with o-phthalaldehyde–sodium sulfite derivatization. Anal Biochem 432:1–7

Min J, Senut MC, Rajanikant K, Greenberg E, Bandagi R, Zemke D, Mousa A, Kassab M, Farooq MU, Gupta R, Majid A (2008) Differential neuroprotective effects of carnosine, anserine, and N-acetyl carnosine against permanent focal ischemia. J Neurosci Res 86:2984–2991

Nii T, Ishii F (2005) Encapsulation efficiency of water-soluble and insoluble drugs in liposomes prepared by the microencapsulation vesicle method. Int J Pharm 298:198–205

Perez AP, Altube MJ, Schilrreff P, Apezteguia G, Celes FS, Zacchino S, de Oliveira CI, Romero EL, Morilla MJ (2016) Topical amphotericin B in ultradeformable liposomes: formulation, skin penetration study, antifungal and antileishmanial activity in vitro. Colloids Surf B Biointerfaces 139:190–198

Rajanikant G, Zemke D, Senut M-C, Frenkel MB, Chen AF, Gupta R, Majid A (2007) Carnosine is neuroprotective against permanent focal cerebral ischemia in mice. Stroke 38:3023–3031

Reimold I, Domke D, Bender J, Seyfried CA, Radunz H-E, Fricker G (2008) Delivery of nanoparticles to the brain detected by fluorescence microscopy. Eur J Pharm Biopharm 70:627–632

Rizvi SZH, Shah FA, Khan N, Muhammad I, Ali KH, Ansari MM, Din Fu, Qureshi OS, Kim K-W, Choe Y-H, Kim J-K, Zeb A (2019) Simvastatin-loaded solid lipid nanoparticles for enhanced anti-hyperlipidemic activity in hyperlipidemia animal model. Int J Pharm 560:136–143

Salama HA, Mahmoud AA, Kamel AO, Abdel Hady M, Awad GA (2012) Brain delivery of olanzapine by intranasal administration of transfersomal vesicles. J Liposome Res 22:336–345

Saraiva C, Praça C, Ferreira R, Santos T, Ferreira L, Bernardino L (2016) Nanoparticle-mediated brain drug delivery: overcoming blood–brain barrier to treat neurodegenerative diseases. J Control Release 235:34–47

Schmid-Elsaesser R, Zausinger S, Hungerhuber E, Baethmann A, Reulen HJ (1998) A critical reevaluation of the intraluminal thread model of focal cerebral ischemia: evidence of inadvertent premature reperfusion and subarachnoid hemorrhage in rats by laser-Doppler flowmetry. Stroke 29:2162–2170

Shah FA, Gim SA, Sung JH, Jeon SJ, Kim MO, Koh PO (2016) Identification of proteins regulated by curcumin in cerebral ischemia. J Surg Res 201:141–148

Shah FA, Park DJ, Koh PO (2018a) Identification of proteins differentially expressed by quercetin treatment in a middle cerebral artery occlusion model: a proteomics approach. Neurochem Res 43:1608–1623

Shah FA, Zeb A, Ali T, Muhammad T, Faheem M, Alam SI, Saeed K, Koh P-O, Lee KW, Kim MO (2018b) Identification of proteins differentially expressed in the striatum by melatonin in a middle cerebral artery occlusion rat model-a proteomic and in silico approach. Front Neurosci 12:1–15

Shah FA, Liu G, Al Kury LT, Zeb A, Abbas M, Li T, Yang X, Liu F, Jiang Y, Li S (2019) Melatonin protects MCAO-induced neuronal loss via NR2A mediated prosurvival pathways. Front Pharmacol 10:1–11

Shen Y, He P, Fan Y-Y, Zhang J-X, Yan H-J, Hu W-W, Ohtsu H, Chen Z (2010) Carnosine protects against permanent cerebral ischemia in histidine decarboxylase knockout mice by reducing glutamate excitotoxicity. Free Radic Biol Med 48:727–735

Sims NR, Muyderman H (2010) Mitochondria, oxidative metabolism and cell death in stroke. Biochim Biophys Acta 1802:80–91

Soga T, Heiger DN (2000) Amino acid analysis by capillary electrophoresis electrospray ionization mass spectrometry. Anal Chem 72:1236–1241

Spratt NJ, Fernandez J, Chen M, Rewell S, Cox S, van Raay L, Hogan L, Howells DW (2006) Modification of the method of thread manufacture improves stroke induction rate and reduces mortality after thread-occlusion of the middle cerebral artery in young or aged rats. J Neurosci Methods 155:285–290

Stvolinsky S, Dobrota D (2000) Anti-ischemic activity of carnosine. Biochem Mosc 65:849–855

Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR (1990) A semiautomated method for measuring brain infarct volume. J Cereb Blood Flow Metab 10:290–293

Tasi L-M, Liu D-Z, Chen W-Y (2003) Microcalorimetric investigation of the interaction of polysorbate surfactants with unilamellar phosphatidylcholines liposomes. Colloids Surf A Physicochem Eng Asp 213:7–14

Tcherkas YV, Kartsova LA, Krasnova IN (2001) Analysis of amino acids in human serum by isocratic reversed-phase high-performance liquid chromatography with electrochemical detection. J Chromatogr A 913:303–308

Tseng L, Liang H, Chung T, Huang Y, Liu D (2007) Liposomes incorporated with cholesterol for drug release triggered by magnetic field. J Med Biol Eng 27:29

Van den Bergh BAI, Wertz PW, Junginger HE, Bouwstra JA (2001) Elasticity of vesicles assessed by electron spin resonance, electron microscopy and extrusion measurements. Int J Pharm 217:13–24

Yan BC, Park JH, Shin BN, Ahn JH, Kim IH, Lee JC, Yoo KY, Hwang IK, Choi JH, Park JH, Lee YL, Suh HW, Jun JG, Kwon YG, Kim YM, Kwon SH, Her S, Kim JS, Hyun BH, Kim CK, Cho JH, Lee CH, Won MH (2013) Neuroprotective effect of a new synthetic aspirin-decursinol adduct in experimental animal models of ischemic stroke. PLoS ONE 8:e74886

Yang T, Cui F-D, Choi M-K, Cho J-W, Chung S-J, Shim C-K, Kim D-D (2007) Enhanced solubility and stability of PEGylated liposomal paclitaxel: in vitro and in vivo evaluation. Int J Pharm 338:317–326

Yenari MA, Han HS (2012) Neuroprotective mechanisms of hypothermia in brain ischaemia. Nat Rev Neurosci 13:267–278

Zaremba J (2004) Hyperthermia in ischemic stroke. Med Sci Monit 10:148–153

Zeb A, Qureshi OS, Kim HS, Cha JH, Kim HS, Kim JK (2016) Improved skin permeation of methotrexate via nanosized ultradeformable liposomes. Int J Nanomed 11:3813–3824

Zeb A, Qureshi OS, Yu C-H, Akram M, Kim H-S, Kim M-S, Kang J-H, Majid A, Chang S-Y, Bae O-N, Kim J-K (2017) Enhanced anti-rheumatic activity of methotrexate-entrapped ultradeformable liposomal gel in adjuvant-induced arthritis rat model. Int J Pharm 525:92–100

Zeb A, Arif ST, Malik M, Shah FA, Din FU, Qureshi OS, Lee E-S, Lee G-Y, Kim J-K (2018) Potential of nanoparticulate carriers for improved drug delivery via skin. J Pharm Investig 48:1–33