Self-Double Emulsified Drug Delivery System of Pyridostigmine Bromide Augmented Permeation Across Caco-2 Cells.

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Abstract

Aim: The study aims to enhance the permeability of pyridostigmine bromide by developing a self-double emulsifying drug delivery system (SDEDDS) and enteric-coated spheroids.

Background: Pyridostigmine bromide is a reversible anticholinesterase used to treat Myasthenia Gravis, reverse neuromuscular blockade, and prevent nerve gas (i.e., soman) poisoning. It is readily soluble in water, but its poor and irregular intestinal absorption is responsible for its poor oral bioavailability (7.6±2.4%). Many approaches have been made to increase the bioavailability of this drug, but no significant improvement has been achieved to date. Presently pyridostigmine tablets are given orally, and a treatment schedule of multiple doses every day (3–6 times per day) is recommended for adult patients, while sustained-release pyridostigmine (Timespan®) tablets can be taken once or twice daily. An increase in permeability of pyridostigmine bromide may also result in reduced dosage frequency.

Objective: In the present work, it is proposed to develop a self-double emulsifying drug delivery system (SDEDDS) of pyridostigmine bromide which will increase its intestinal permeability and hence its oral bioavailability.

Method: For the preparation of PB-SDEDDS, the primary water in oil emulsion was mixed with the optimized concentration of Tween 80 using a magnetic stirrer. PB-SDEDDS were converted into spheroids and were then characterized.

Result: The pseudo ternary phase diagram was constructed, showing a double emulsion region. The viscosity of SDEDDS at the different shear rates was found to be 125 mPas. The optimized SDEDDS formulation formed a bright white emulsion within 2 minutes, having droplet size around 20-25 μm. In vitro uptake studies of PB-SDEDDS on Caco2 cells demonstrated the increase in Papp value from (4.38±0.27) ×10-4 cm/s to (9.488±0.182) ×10-4 cm/s (2.166 folds) that was attributed to the SDEDDS formulation. In vitro cytotoxicity studies on Caco2 cells revealed that the blank SDEDDS showed almost no toxicity after incubation for 2 hours at various dilutions tested.

Among all formulations, F3 was optimized for the concentration of adsorbent and binder at a concentration of 10% each. SEM showed that the spheroids were spherical, and 73.92% of spheroids were in between 0.595-0.841 mm of size. The optimized formulation had 70.29% spheroids retained on sieve no. 30. The angle of repose showed good flow properties with 25.20 and stability with friability of 0.52 %. The disintegration time of the developed formulation

was 3.30 minutes, and drug content was found to be 97.83%. The release studies showed that PB-SDEDDS improved the release significantly as compared to the market formulation.
Conclusion: The PB-SDEDDS solid spheroids resulted in favorable physical properties and did not affect its drug content and in vitro drug release profile. The self-double emulsifying drug delivery system of pyridostigmine bromide can be explored as a suitable alternative to its solid oral dosage form.

Keywords: Myasthenia gravis, Permeability, Poor oral bioavailability, Pyridostigmine Bromide, SDEDDS, SDEDDS-spheroids,

1. Introduction

Myasthenia gravis (MG) is a neuromuscular disorder manifested by weakness and fatigability of voluntary muscle. The muscle innervated by cranial nerves are particularly affected and usually those of the neck, trunk, and extremities. In several cases, it remains localized to the extraocular muscles. The symptoms are commonly ameliorated, although to a variable degree, by anticholinesterase compounds. The usual cause is an acquired immunological abnormality, but some cases result from genetic abnormalities at the neuromuscular junction [1]. The prevalence of myasthenia gravis in the United States is estimated at 14 to 20 per 100,000 populations, approximately 36,000 to 60,000 cases in the United States [2]. USFDA recommended various treatment modalities such as Mestinon, azathiopyrine, mycophenolate mofetil, neostigmine, regonol, soliris, ephedrine, eculizumab for the treatment of MG [3,4]. Conversely, each drug has its plus and blemish.

Pyridostigmine bromide (3-[(dimethyl carbamoyl) oxy]-1-methyl pyridinium bromide), a reversible anticholinesterase drug, is the bromide salt form of pyridostigmine and a cholinesterase inhibitor [5, 6]. It is widely indicated to treat muscle weakness and is also used in gastrointestinal motility disorders, postoperative urinary retention, and prophylaxis of muscular paralysis as a result of nerve agents [7].

Pyridostigmine bromide binds reversibly to acetylcholinesterase active sites in the peripheral nervous system, thereby preventing the breakdown of acetylcholine [8]. PB, a reversible cholinesterase (ChE) inhibitor, is a carbamate compound, specifically, the dimethyl carbamate ester of 3-hydroxy-1-methyl pyridinium bromide [9]. Compounds in this category are poorly

absorbed from the gastrointestinal tract also, excluded by the blood-brain barrier [10] which accumulates acetylcholine at cholinergic synapses and facilitates transmission of impulses across the neuromuscular junction [11,12]. Pyridostigmine bromide (PB) comes under (Biopharmaceutical Classification System) BCS class III, which is a hydrophilic drug with high aqueous solubility and poor permeability [1]. The oral bioavailability of pyridostigmine bromide is very low (approx. 7.6±2.4%) and as it is a quaternary amine, hence its intestinal absorption is poor [13].

Recently, various available dosage forms for anticholinesterase compounds such as liposomes, nanoparticles, IV dosage forms have gained more attention in the market [14].

In the United States, pyridostigmine is available in various dosage forms with the trade name Mestinon® (180 milligrams sustained release tablet), Regonol Solution for Injection® (injection 5 milligrams/ milliliter) [15]. However, this drug delivery system suffers serious stability problems. Isolation ways are too tough and show high specificity to superficially located organs and tissues but cannot be targeted to deep-seated organs. Monoclonal antibodies may sometimes cause an unwanted antigen-antibody reaction which leads to serious consequences [16].

Many approaches have been used to increase its oral bioavailability but have no significant improvement. Many types of research used various approaches to increase the oral bioavailability of PB; however, none of the studies have exercised a beneficial impact. Recently, water in oil in water double emulsion (w/o/w) has gained more potential for the enhancement of oral bioavailability of hydrophilic drugs [17]. Water in oil in water (w/o/w) emulsion is a complex polydispersed system in which oil droplets may be surrounded by an aqueous phase, which in turn encloses one or several water droplets [18]. The hydrophilic drug is present in the internal water phase of double emulsion, which acts as a storage chamber for hydrophilic drugs [19].

Self-Double Emulsifying Drug Delivery systems (SDEDDS) are ideally isotropic mixtures of drugs, oils, and surfactants, sometimes containing co-surfactant or co-solvents [20, 21] and are well-known for their potential to improve the permeability and oral absorption of hydrophilic drugs [22].

There are various methods to prepare SDEDDS emulsions. Among them, the best method is the emulsification of primary emulsion.

Therefore, in the present investigation, we have determined that SDEDDS for increased permeability and bioavailability of pyridostigmine bromide. PB-SDEDDS were engineered by optimizing and characterizing. A standard one-step emulsification method was adopted for the

preparation of PB-SDEDDS [24]. In addition, optimized PB-SDEDDS were characterized in vitro for particle size, zeta-potential, surface topography, crystallinity, stability, cellular uptake, cytotoxicity, and apoptosis [25].

2. Material and Methods

Materials

Pyridostigmine bromide (98% purity) was a generous gift from Samarth Pharmaceuticals (Baddi, India). Fractionated coconut oil and soyabean oil were provided by Kamani Oil Industries Pvt. Ltd., Khopoli, India. Castor oil and MTT dye were obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. Olive oil, microcrystalline cellulose, oleic acid and bean phospholipid were purchased from Central Drug House, New Delhi, India. Cross carmellose sodium was obtained as a gift from Alembic Pharmaceutical Ltd., Baddi, India. Tween 80, span 80 and silicon dioxide were purchased from Loba Chemie, Mumbai, India. All the excipients and reagents used were of analytical grade and double distilled water was freshly prepared whenever required throughout the study.

Preparation and optimization of Self Double Emulsified Drug Delivery Systems

Solubility studies:

Determination of drug solubility in various oils, surfactants, and co-surfactants (Table 2) was carried out by adding an excess amount of PB to 2 ml of the vehicle contained in a screw-capped vial. The drug was added to each then the mixture was vortexed (Spinix, Tarsons products Pvt. Ltd, India) for 10 min and was stirred for 24 h in an orbital shaker at room temperature followed by keeping them for equilibrium for 24 h. The equilibrated mixture was centrifuged at 5000 rpm for 5 min, and excess insoluble PB was removed by filtration. The filtrate was analyzed for the amount of PB by a UV-visible spectrophotometer (Perkin Elmer, Lambda 25, Champaign, IL, USA) at 269.9 nm.

Construction of pseudo-ternary phase diagram

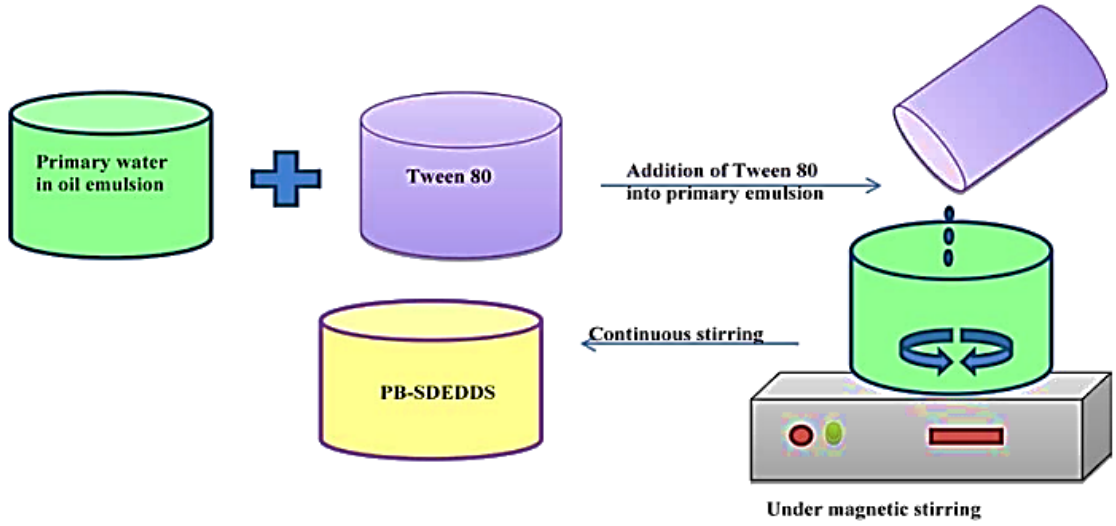
The pseudo-ternary phase diagrams were constructed by titration of a homogeneous mixture of oil, surfactants, and cosurfactant with water at ambient temperature [26, 27].

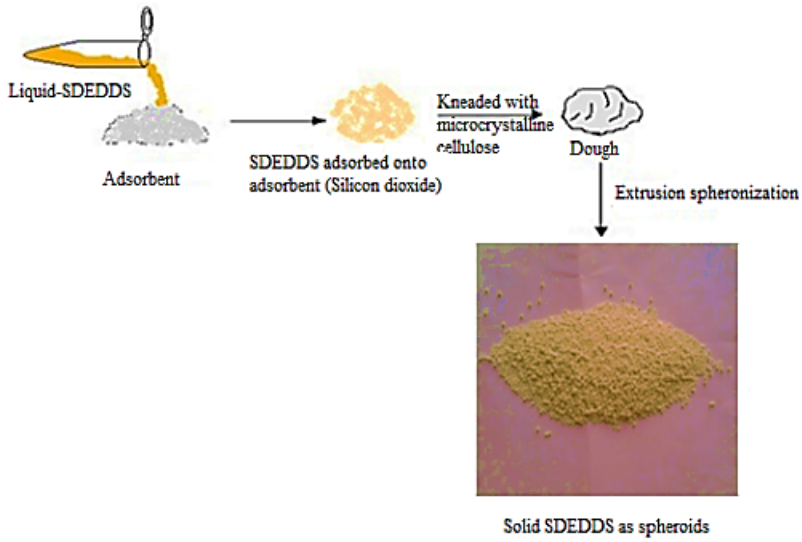
The oil (prepared water in oil emulsion) , binary mixture of surfactants with co-surfactant at different ratios (1:1, 2:1, 3:1) were dispersed at weight ratios of 10:0,9:1,8:2,7:3,6:4,5:5,4:6,3:7,2:8,1:9 and 0:10 into different vials. Each vial containing the liquid mixtures was titrated with water. Following each water addition, the mixture was homogenized by vortexing, and the sample was monitored visually for any change of optical transparency. At a critical change in transparency, the sample mixture was kept at ambient

temperature for 48 h with gentle shaking to ensure equilibrium. The percent weight composition in each vial at this stage of critical change in transparency has constituted the points between a clear single-phase and a translucent two-phase region [28]. Considering these points taking different co-surfactants, the above titrations generate the data to construct the pseudo-ternary phase diagram. The formulations (Fig.1) were prepared with desired component ratios after identification of the monophasic region.

Preparation of Pyridostigmine Bromide loaded SDEDDS

PB-SDEDDS formulation was prepared by a standard one-step emulsification procedure [29]. The drug was dissolved in distilled water at ambient pH (adjusted to 6.0 ± 0.5) by a pH meter (Pico, Lab India Instruments Pvt. Ltd., India). 0.5 % gelatin solution was added to the above solution. The aqueous solution of the drug was then added drop by drop to blend of surfactant and cosurfactant in the pre-determined ratio while stirring at high speed using a magnetic stirrer (Perfit, Ambala, India) at ambient temperature. Based on the formation of maximal microemulsion region in the pseudo-ternary phase diagram, olive oil, bean phospholipid, oleic acid, and span 80 (16, 12, 46, and 26 w/w %) were selected as lipid phase. The emulsion was then homogenized (T25DS22, Cole-Parmer India Pvt. Ltd, Navi. India) at 9000 rpm for 3 min. The prepared water in oil emulsion was then mixed with tween 80 using a magnetic stirrer until PB-SDEDDS was formed (Fig. 2).





3. Characterization of SDEDDS formulation

Determination of Droplet size analysis and size distribution

The emulsion droplet size was determined by a motic microscope (Motic Microscope, Model-DMWB1-223ASC). Particle size distribution and zeta-potential of nanoparticles were measured by particle size analyzer ((DLS 4 C Beckman Coulter, USA). In brief, 1ml of each SDEDDS was dispersed in 5mL of purified water, and both size distribution, as well as zeta-potential, was measured. All measurements were made at 25℃ in triplicate (mean ± SD, n¼3) [30].

Confocal Laser Scanning Microscopy (CLSM)

The morphology of the freshly prepared PB-SDEDDS was observed using a confocal laser scanning microscope (Olympus FV-1000 (Tokyo, Japan). For the evaluation of the size of water droplets, SDEDDS containing 5 µg/mL sodium fluorescein was diluted with an aqueous phase. The sample was dropped on a plain glass slide and then covered with a coverslip and was detected using an Ar/Kr laser with an excitation line of 491 nm.

Viscosity analysis of SDEDDS formulations

The viscosity (η) of PB-SDEDDS was determined by using Brookfield Viscometer (Brookfield Rheometer, Model-RS3CPS230LS, Middleboro, USA) through spindle number CC3- 14 at 25±0.3°C by keeping the share rate of 100 rpm [31].

Self-double emulsification test:

The SDEDDS were formed of oil, Six, and water, so there might be a chance of phase separation when they go through the infinite aqueous dilution in gastrointestinal fluids, which may cause precipitation of PB due to its low aqueous solubility.

The emulsion was obtained after dilution with distilled water under gentle stirring for 2 minutes by the visual grading system. PB- SDEDDS was assessed visually after 120 min by the visual grading system [32, 33]. Furthermore, the Motic Microscope (Motic, DMWB1-223ASC) was used to observe the self-double emulsification performance/behavior/process of the optimized pyridostigmine bromide-SDEDDS formulation.

4. In vitro Caco2 studies

***In vitro* studies Cytotoxicity studies of SDEDDS (MTT assay)**

Cytotoxicity studies were carried out using MTT assay on Caco-2 cell line to assess the safety of excipients used in the preparation of SDEDDS. After 2 hours of incubation, 20 all of MTT reagent was added to each well, and then it was incubated for 4 hours. After the incubation of 4 hours in the well, 180 µl of dimethyl sulfoxide was added. The absorbance values were measured at a wavelength of 595 nm using an Elisa plate reader (Biorad, Model 680, Japan). Cell viability was calculated using the following formula:

Cell viability (%) = × 100 Eq. 1

Uptake studies of pyridostigmine bromide

Caco-2 cells were seeded onto six-well plates at a density of 5× 104 cells/cm2 for the uptake experiments. Before initiation of the study, the cells were cultured for 14 days. At the beginning of the experiment, the plates were incubated with different concentrations of PB solutions and PB-loaded SDEDDS for 2 hours. The cells were washed thrice with ice-cold PBS, frozen and thawed thrice, resuspended in water (1 mL), then it was probe sonicated 10 times to obtain cell lysates. The lysates were centrifuged at 15,000 rpm for 10 minutes, and the supernatant was analyzed through high-performance liquid chromatography (HPLC). The experiment was done in triplicate for each concentration and results were presented as an average ± standard deviation.

Transport studies of pyridostigmine bromide

The Caco-2 cells were seeded onto a 12-well polycarbonate membrane transwell (pore size of 0.4 µm) having a growth surface area of 1.12 cm2 at a density of 5 × 104cells/cm2. After 19 to 22 days of seeding, the monolayers of cells were used [4]. Transport studies were carried out by adding 120 µg/ml PB-SDEDDS and the same concentration of PB solution to the apical cell side and blank Hank's buffered salt solution to the basolateral cell side. At predetermined intervals of 15, 30, 45, 60, 90, and 120 min, the samples were collected from the basolateral

side. PB concentrations were detected using the HPLC method described below. Apparent permeability (Papp) was calculated as follows:

$$Papp = dq/dt \times 1/(AC0) \quad \text{Eq. 2}$$

Where dq/dt is the permeability rate, A is the surface area of the monolayer, and C0 is the initial concentration of the drug.

HPLC analysis of samples

All the samples were analyzed using an HPLC system (Waters® 515, USA) with a RP C18 column (4.6mm × 250mm, 5µm). The mobile phase for pyridostigmine bromide analysis was acetonitrile: 1 % triethylamine buffer (37:63). The flow rate was maintained at 1ml/ min with the column temperature 40°C while the detection was performed at a wavelength of 260 nm.

5. Preparation of solid SDEDDS

Solid SDEDDS was prepared by adsorbing a fixed percentage of PB-SDEDDS (30%) onto silicon dioxide (10%) until the liquid SDEDDS were adsorbed completely. Thereafter, the adsorbed mixture was blended with MCC (Microcrystalline cellulose) (50%), cross carmellose sodium (2%), and/ or polyvinylpyrrolidone (PVP-K30) (10%) followed by the addition of water until a suitable mass for extrusion was obtained (water: total mixture = 0.5: 1, w/w). Eventually, the wet mass was extruded at 100 RPM in an axial screen extruder (WL 350, Wenzhou, China) equipped with a die of 0.5 mm diameter circular holes. The extrude was spheronized for 15 min at 800 RPM on a 120 mm radial plate spheronizer (Cronimach, Gujarat, India) using a cross-hatch frictional plate of 3 mm× 3mm pitch and 1.2mm depth. The produced spheroids were then dried. Eight different formulations (F1-F8) were prepared using different concentrations of silicon dioxide, PVP K-30, and MCC, as shown in Table 1.

Table 1: Composition of different formulations (F1-F8) of solid SDEDDS

Ingredients (%)	Formulation number							
	F1	F2	F3	F4	F5	F6	F7	F8
SDEDDS	30	30	30	30	30	30	30	30
Silicon dioxide		10	10	10	5	7.5	10	12.5
CCS			2	2	2	2	-	2
MCC	70	52.5	50.5	48	50.5	50.5	50	45.5
PVP	5	7.5	10	12.5	10	10	10	10

Physical Evaluation of Solid SDEDDS

PB-SNEDDS tablet formulation was subjected to the following physical characterization tests [34, 35].

Spheroids size distribution

The size distribution of the spheroid formulations (F1-F8) was determined by the sieving method. A sample of 20 g was sieved through sieves No. 16, 22, 30, and 60, having the mesh opening size of 1.19, 0.841, 0.595, and 0.250 mm, respectively. The percentage weight of the pellets retained on each sieve was calculated. Shape evaluation of the spheroid samples was performed on the size fraction 0.841-0.595 using a microscope (DMBA 450 Motic Digital Microscope, China) with an optical zoom of 40× /0.17 and an eyepiece of 10×/22 [36].

Disintegration test

The mean disintegration time of the spheroid formulations (F1-F8) was determined using a modified disintegration test apparatus (MSI-42, Meta Lab Scientific Industries, Mumbai, India) containing six baskets previously maintained at 37± 2°C. The time taken complete disintegration of the spheroids followed by passing through the sieves of all the baskets [36] was documented.

Friability test

Friability of the spheroid formulation (F1-F8) was conducted on 2g of the spheroids combined with 2 g of glass beads (2 mm diameter) using a Roche friabilator (Swastika Electric and Scientific Works, Ambala Cantt., India) [36]. The experiment was carried out in triplicates for each formulation. Percentage friability was calculated using the following formula:

Eq. 3

Where, Wa= initial weight of spheroids, and Wb= final weight of spheroids after 4 minutes

Angle of repose

The angle of repose of the spheroid formulations (F1-F8) was determined by the Funnel method. Accurately weighed spheroids sample was allowed to flow through the funnel freely onto the surface. The diameter of the spheroids cone was measured, and the angle of repose was calculated using the following equation [36]. The experiment was carried out in triplicates for each of the formulations.

$$\tan \text{ø} = h/r \quad \text{Eq. 4}$$

Scanning Electron Microscopy

Solid PB-SDEDDS spheroids (F3) were examined by scanning electron microscope (SEM) (Jeol Fine coat, Ion sputter, JFC 1100, Japan) to visualize the surface topography. The sample was fixed on a brass specimen club using Double-sided adhesive tape and made electrically conductive by coating in a vacuum (10 Torr) with gold using an ion sputter. The photographs were taken at an excitation voltage of 10 kV.

Reconstitution study

In a 250 ml beaker, 640 mg of the PB SDEDDS spheroids (F3) (equivalent to 3mg pyridostigmine bromide) was dispersed in 200 ml distilled water. The mean droplet size and polydispersity index (PDI) of the resultant double emulsion obtained from spheroid subjected to a stirring for 15-30 min was determined using Delsa nanoparticle analyzer (DLS 4 C, Beckman Coulter, USA) by withdrawing 1ml sample after 30 min of dissolution testing and filtering through a Whatman filter paper. The experiment was carried out in triplicates [7].

Drug content uniformity

A sample of 640 mg spheroids (F3) was taken in a 10 ml volumetric flask. A volume of 10 ml water was added into the volumetric flask; the spheroids were crushed and kept for 30 min. The resultant suspension was centrifuged at 3500 rpm for 15 min, and the supernatant was analyzed for drug concentration by UV spectrophotometer at λmax 270 nm. The same experiment was repeated in triplicate.

In vitro drug release study

PB-SDEDDS and spheroids (F3) were filled in hard gelatin capsules (size “00”), and in vitro drug release studies were performed using a USP Dissolution Test apparatus II at 37 ± 0.50 ◦C with a rotation speed of 50 RPM. A volume of 900 ml, 0.1 N HCl buffer (pH 1.2) was used as the dissolution medium. Aliquots of 5ml were withdrawn at various time intervals of 5, 15, 30, 60, 120, and 240 min and replaced by an equal volume of fresh dissolution medium. The amount of pyridostigmine bromide released in the dissolution medium was determined spectrophotometrically at 270 nm.

6. Result and Discussion

Initial studies for screening of excipients

Phase solubility analysis Among various oils investigated for equilibrium solubility studies, viz fractionated coconut oil, soybean oil, castor oil, olive oil, and oleic acid the maximum solubility of oleic acid (0.4053±0.01mg/mL) was observed. The minimum solubility (0.0261±0.001mg/mL), however, was found in coconut oil. Table 2 depicts the capacity of different oils to solubilize

Pyridostigmine Bromide. As the solubility of PB was found to be maximum in higher solubility in oleic acid, it was preferred for further investigation. The higher drug solubility in the oil phase is essential for keeping the drug in the solubilized form in an o/w Nanoemulsion as the lesser contribution of surfactant or co-surfactant to drug solubilization upon dilution in GIT. As it was reported that Oleic acid was also used for its enhanced intestinal absorption of drugs [9, 10].

Tween 20 and Tween 80 were found to be the desirable surfactants because of their high drug solubility among the other surfactants. Although Tween 20 has a higher solubility than Tween 80, as a hydrophilic surfactant, was chosen due to its better emulsion forming ability than Tween 20 [11].

Table 2: Solubility of pyridostigmine bromide in various vehicles (n=3).

Vehicles (oils/surfactants/aqueous buffer)	Solubility (mg/ml)
Fractionated coconut oil (MCT)	0.0261±0.001
Fractionated soybean oil (MCT)	0.2732±0.002
Fractionated olive oil	0.1169±0.010
Fractionated castor oil	0.1072±0.01
Oleic acid	0.4053±0.01
Span 20	0.318±0.01
Span 80	0.5708±0.02
Tween 20	1.018±0.01
Tween 80	0.8630±0.02
Kolliphor	0.2513±0.02
Aqueous buffer (pH 1.2)	105.41±3.82
Aqueous buffer (pH= 7.0)	120.86±1.28

Construction of pseudo-ternary phase diagram

Figures 1 show phase diagrams constructed to identify the area of stable micro/Nanoemulsion in the presence of PB. Based on the various combinations, (w/o primary emulsion and tween 80) was selected for the formulation of optimized liquid SDEDDS.

To ensure the spontaneity of SDEDDS to form the Nanoemulsion within the GI conditions, the construction of pseudo-ternary plots is considered to be a vital exercise [12].

As indicated in Figures 1, the stability of Nanoemulsion was reduced after increasing the oil in water emulsion to Surfactant, which restricted the lowest amount of the surfactant to be added to the formulation.

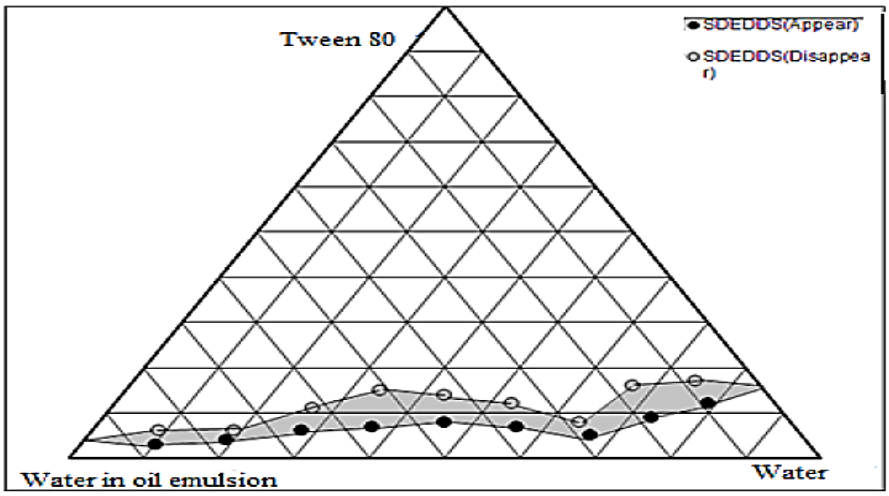


Fig. 1. Pseudo-ternary phase diagram showing double emulsion region (oil: w/o emulsions; SA: Tween 80 and distilled water) at room temperature.

Droplet size analysis and polydispersity index (PDI) determination

The mean particle size of the formulation is as depicted in Fig. 2. Clearly portrays the spherical edifice of the emulsion droplets together with the presence of internal water phase and drug. Additional dilution of the emulsion resulted a bright and white appearance (visual grading system). The PDI were found to be 0.267±0.01.

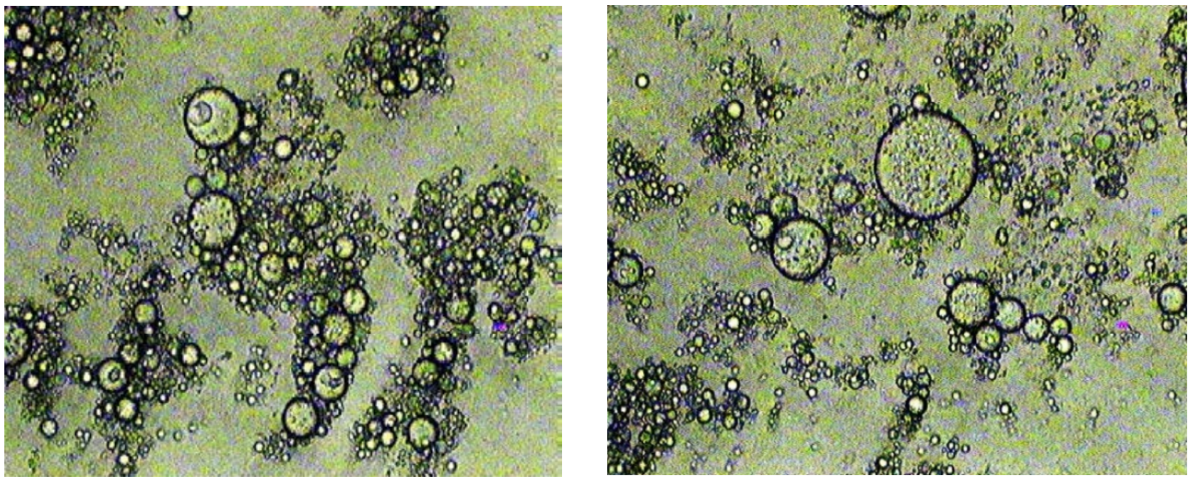


Fig. 2. Microscopy images of freshly prepared PB-SDEDDS transformed into w/o/w double emulsions after 2-minute dilution with dispersion medium.

The optimized SDEDDS formulation formed bright white emulsion within 2 minutes having droplet size ranging between 20-24µm, which is considered ideal for a low emulsification time and enhanced micellar absorption [4].

Viscosity analysis of SDEDDS formulations

The viscosity of prepared SDEDDS formulation was in the range of 115.19 ± 0.825 cps. Results shows that is direct function of oil and surfactant used. As the viscosity of PB-SDEDDS is less than 10,000 cps it implies that the developed SDEDDS can be filled in hard gelatin capsules by commercial liquid filling equipment.

Confocal Laser Scanning Microscopy (CLSM)

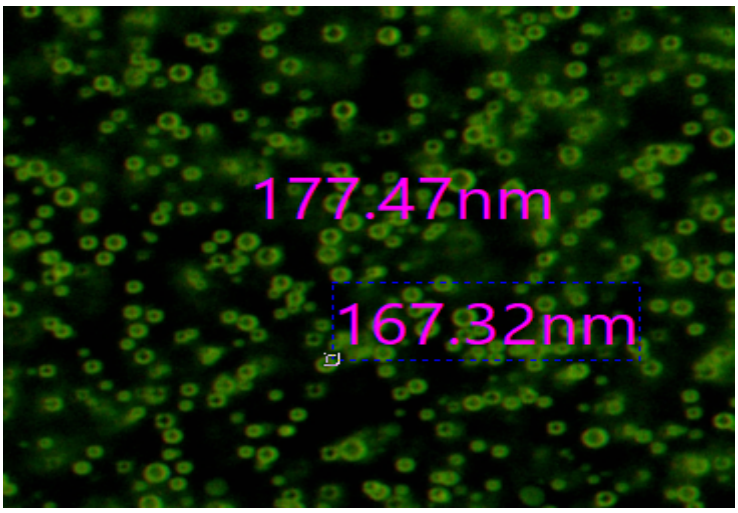


Fig. 3. Confocal images of formulated fine w/o/w double emulsions after 2-minute dilution with dispersion medium.

The imageries of CLSM (Fig. 3) revealed that the emulsion had spherical droplets with narrow particle size distribution. Overall, the prepared emulsion showed characteristics of a desired double emulsion i.e. the structure of dispersed oil droplets containing smaller dispersed aqueous droplets.

In vitro Caco2 studies

Cytotoxicity studies of SDEDDS (MTT assay)

Finally, we evaluated the cytotoxic activity of prepared SDEDDS in **Caco2** cells by dissolving the formulations in phosphate buffer saline (pH 7.4). The cytotoxic activity was evaluated using the standard MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) cell viability assay. The results of cytotoxicity studies on Caco2 cells (Fig. 4) revealed that the blank SDEDDS, when diluted 10, 30 and 50 folds, showed almost no toxicity after 2hrs incubation. . The blank SDEDDS shows almost no toxicity on Caco2 cells.

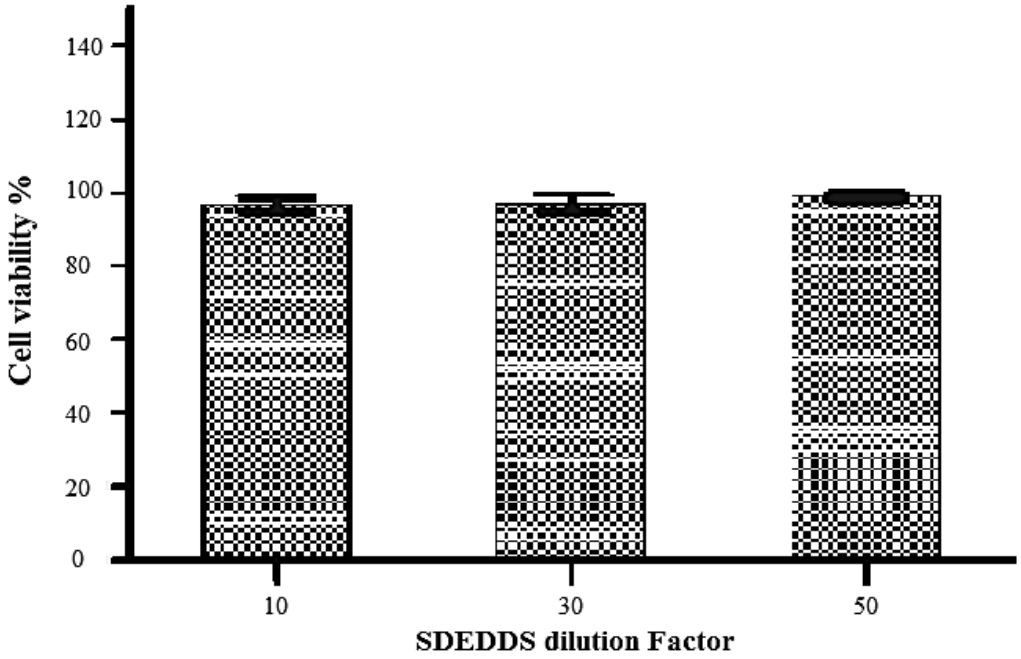


Fig. 4. Effect of blank SDEDDS on the cytotoxicity of Caco-2 cells after incubation for 2 hours.

Uptake studies of pyridostigmine bromide

The results of uptake and transport studies are illustrated in Fig. 5 and Fig. 6. The uptake studies showed that permeability of PB was low but SDEDDS significantly increased the permeability of drug across Caco2 cell monolayer by four folds in comparison to plain drug. The results confirmed that the intestinal membrane permeability of pyridostigmine bromide alone was low.

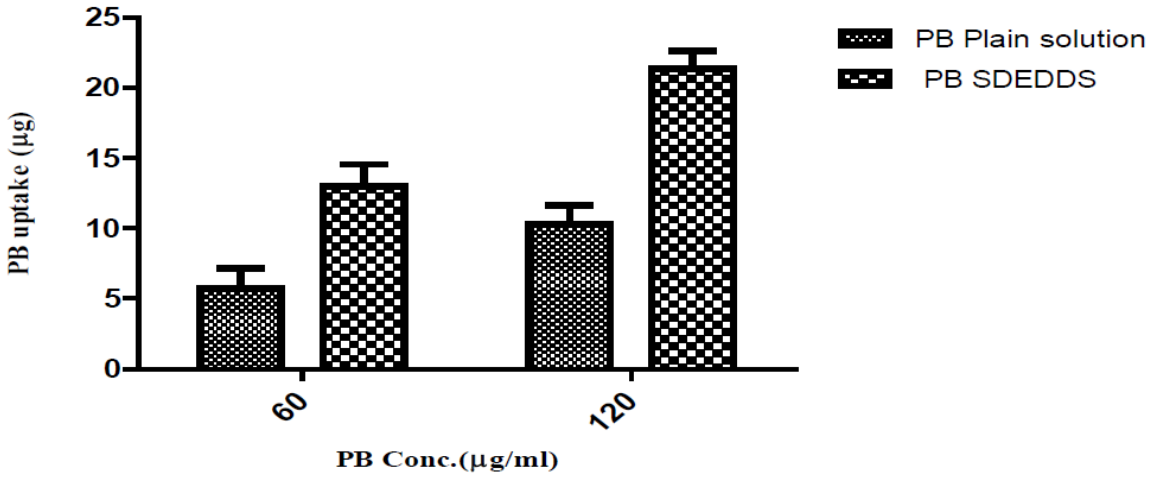


Fig. 5. Effect of PB-SDEDDS on Caco-2 cellular uptake.

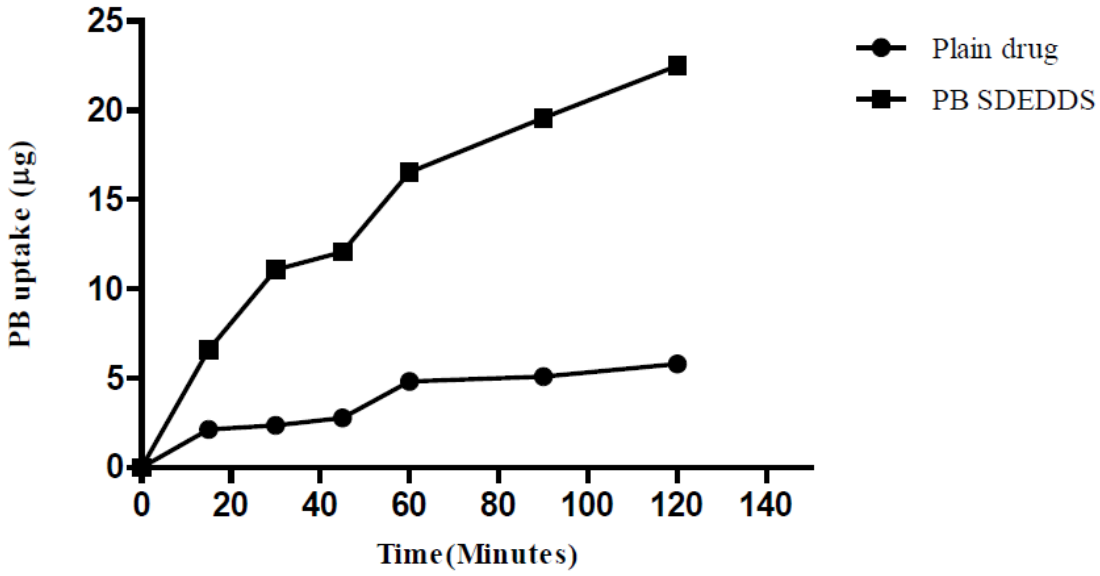


Fig. 6. Effect of PB-SDEDDS on Caco-2 cellular transport.

Transport studies of pyridostigmine bromide

However, PB-SDEDDS potentially enhanced the permeability of pyridostigmine bromide across Caco-2 cell monolayers irrespective of the drug concentration. The Papp of pyridostigmine bromide (120µg/ml) was (4.38±0.27) ×10-4cm/s. But the same concentration of PB-SDEDDS, when was applied to Caco2 cells, Papp gradually increased to (9.488±0.182) × 10-4cm/s. The increased value of Papp of pyridostigmine bromide by 2.166 fold was attributed to the SDEDDS formulation. The phospholipid used in the formulation increases the membrane fluidity and hence results in the increased permeability of the hydrophilic drug. Oleic acid was also reported for its enhanced absorption drugs [2, 7]. Certain excipients such as tween 80 used in SDEDDS were reported to inhibit p-glycoprotein and therefore increased the drug absorption [7]

Physical characterization of Solid SDEDDS

The prepared PB-SDEDDS was then converted into spheroids (F1-F8) as shown in Table 1 and further were evaluated based on size distribution, percentage of spheroids retained on Sieve no. 30 (0.595 mm), disintegration time, friability, and angle of repose. The results are accessible in Table 3 where the spheronization speed and time were optimized to 800 and 15 minutes, respectively having only 0.56% percentage of fines and 70.05% of total yield retained on sieve no. 30. Among all formulations, F3 was optimized for the concentration of adsorbent and binder at a concentration of 10% each. SEM showed that the spheroids were spherical and 73.92% of spheroids were in between 0.595-0.841 mm of size. The optimized formulation had

70.29% spheroids retained on sieve no. 30, the angle of repose showed good flow properties with 25.20 and stability with friability of 0.52 %. The disintegration time of the developed formulation was 3.30 minutes F3 formulation was found to have desirable characteristics and the same was selected as the optimized formulation for the solid SDEDDS.

Table 3: Evaluation of spheroid formulations

Formulation code	% retained on Sieve no. 30 (0.595 mm)*	Angle of Repose*	Friability (%)	Disintegration time (min)
F1	25.81±1.63	38.00±0.04	1.45±0.14	8.0±2.13
F2	48.27±3.27	29.23±0.16	0.82±0.06	12.46±1.71
F3	**70.29±1.05**	**25.20±0.02**	**0.52±0.02**	**3.30±1.28**
F4	65.25±1.94	32.57±0.61	0.67±0.01	6.28±2.36
F5	51.35±2.08	27.00±0.08	0.74±0.02	7.98±1.14
F6	60.71±1.73	31.56±0.0.14	0.39±0.01	5.38±2.92
F7	66.56±2.51	35.21±0.417	0.54±0.06	10.48±1.85
F8	68.34±1.18	26.71±0.039	0.45±0.02	4.28±0.91

*Values were expressed as Mean ± SD (n=3)

Scanning Electron Microscopy

We next employed SEM to visualize the surface texture of the formulation Fig. 7 shows the surface morphology of the optimized (F3) solid PB-SDEDDS. The SEM micrograph confirmed that the spheroids had a spherical shape of size 0.59 mm. The PB-SDEDDS appears to be dispersed on the surface of the spheroids and also entrapped in the matrices. (Fig. 7).

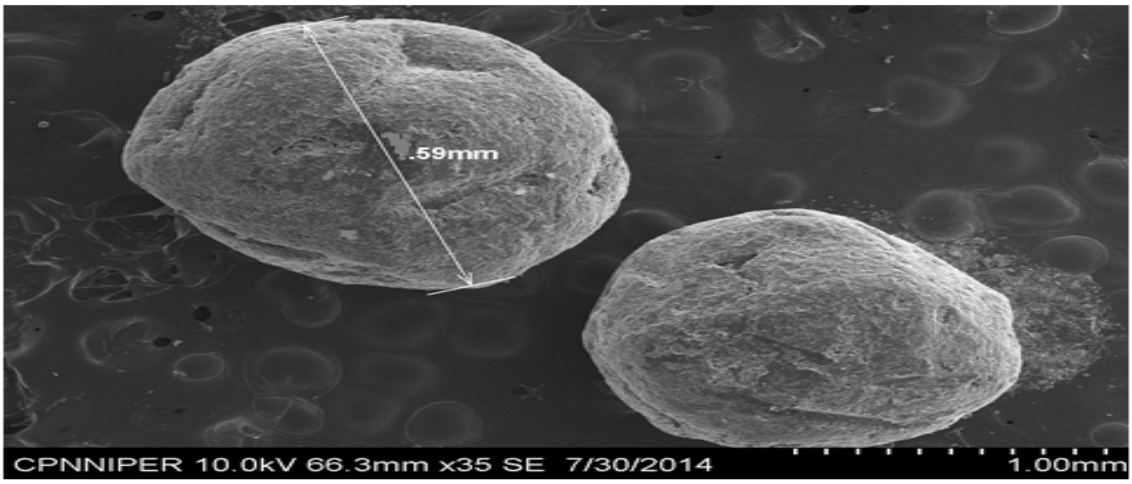


Fig. 7. Surface morphology of the optimized (F3) solid PB-SDEDDS.

The visual assessment of self-emulsification of solid SDEDDS (F3) ensured that spheroids spontaneously get emulsified to form a double emulsion. The mean droplet size of reconstituted solid SDEDDS was found to be 28.69±3.15 µm with the polydispersity index (PDI) value of 0.215± 0.037. The lower value of PDI (less than 1) confirms that the droplets were uniformly distributed throughout the formulation.

***In vitro* drug release studies**:

The drug content of the spheroids (F3) was found to be 97.83± 0.0614 % as shown in Fig 8. The percentage cumulative drug release for PB-SDEDDS, PB-SDEDDS spheroids and the marketed formulation in 0.1N HCl buffer (pH 1.2) was found to be 99.52±0.7055, 97.75±0.5322 and 94.07±0.946 respectively. This established that drug release from SDEDDS was higher as compared to SDEDDS spheroids and the marketed formulation. (Fig. 8). Around 99% drug was released in 60 min in case of PB SDEDDS, whereas the spheroids and marketed formulation recorded 93% and 85% drug release, respectively, at the same duration. The reason behind the lower drug release in the spheroids at the beginning may be due to the disintegration step involved for the spheroids.

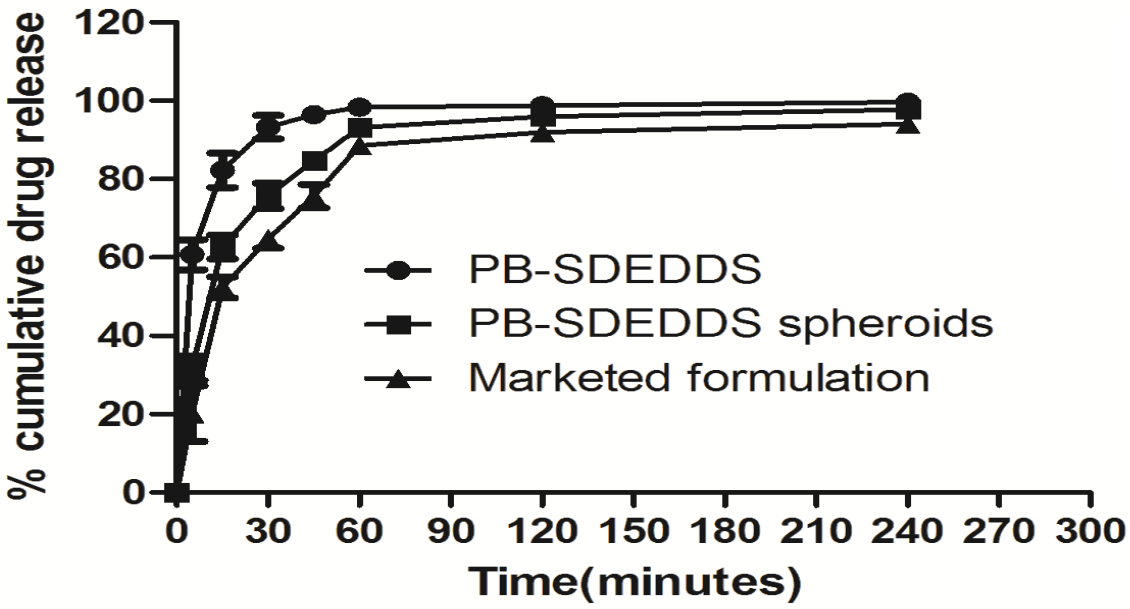


Fig. 8. *In vitro* dissolution profiles of PB-SDEDDS, PB-SDEDDS spheroids and marketed formulation in 0.1N HCl (pH 1.2), data expressed as mean ±SD, n=3.

The release studies showed that PB-SDEDDS improved the release significantly as compared to market formulation. Enteric coated spheroids prevented its release in stomach and released the drug in intestine, which avoided the degradation of drug in stomach.

7. Conclusion

The present research work aims to development of SDEDDS for improved bioavailability of pyridostigmine bromide (PB). PB belongs to BCS class III drugs therefore have poor permeability and high solubility. The oral bioavailability of PB was low (7.6 ± 2.4%) due to its poor and irregular intestinal absorption. A self-double emulsifying drug delivery system (SDEDDS) of pyridostigmine bromide was successfully developed and characterized. The utility of SDEDDS for enhancing the absorption of hydrophilic drugs was demonstrated. The increased Papp value of pyridostigmine bromide via SDEDDS results in the improvement of absorption of PB across Caco2 cells and developed PB-SDEDDS showed no cytotoxicity. Conversion of PB-SDEDDS into solid spheroids did not affect the drug content as well as a formulation in vitro release profile. The preliminary in vitro study confirms that the novel solid SDEDDS of pyridostigmine bromide has high potential as an alternative oral drug delivery system of the drug to address its poor bioavailability.

8. Ethical approval and consent to participate

The work was approved by Institutional ethical commity to carry out cell line study.

9. Human and animal study

No human and animal study were used in this study.

10. Consent for publication

Not applicable

11. Availability of Data

The data carries masters project of first author.

12. Funding

NA

13. Conflict of Interest

The authors report no conflict of interest for this article.

14. Acknowledgements

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