Appendixes

List of Publications and Presentations

Publications from thesis work

1. Krishna, K.V., Saha, R.N., Singhvi, G. and Dubey, S.K., 2018. Pre-clinical pharmacokinetic-pharmacodynamic modelling and biodistribution studies of donepezil hydrochloride by a validated HPLC method. RSC advances, 8(44), pp.24740-24749.

2. Krishna, K.V., Wadhwa, G., Alexander, A., Kanojia, N., Saha, R.N., Kukreti, R., Singhvi, G. and Dubey, S.K., 2019. Design and biological evaluation of lipoprotein-based donepezil nanocarrier for enhanced brain uptake through oral delivery. ACS chemical neuroscience, 10(9), pp.4124-4135.

3. Dubey, S.K., Singhvi, G., Krishna, K.V., Agnihotri, T., Saha, R.N. and Gupta, G., 2018. Herbal medicines in neurodegenerative disorders: an evolutionary approach through novel drug delivery system. Journal of Environmental Pathology, Toxicology and Oncology, 37(3).

4. Dubey, S.K., Ram, M.S., Krishna, K.V., Saha, R.N., Singhvi, G., Agrawal, M., Saraf, S., Saraf, S. and Alexander, A., 2019. Recent Expansions on Cellular Models to Uncover the Scientific Barriers Towards Drug Development for Alzheimer's Disease. Cellular and molecular neurobiology, 39(2), pp.181-209.

5. Dubey, S.K., Lakshmi, K.K., Krishna, K.V., Agrawal, M., Singhvi, G., Saha, R.N., Saraf, S., Saraf, S., Shukla, R. and Alexander, A., 2020. Insulin mediated novel therapies for the treatment of Alzheimer's disease. Life Sciences, p.117540.

6. Krishna, K.V., Saha, R.N., Dubey, S.K., 2020. Biophysical, Biochemical and Behavioral Implications of ApoE3 Conjugated Donepezil Nanomedicine in a $A\beta_{1-42}$ Induced Alzheimer's Disease Rat Model. ACS chemical neuroscience (Under minor revision).

7. Krishna, K.V., Saha, R.N., Dubey, S.K., 2020. Development and validation of reversed phase high-performance liquid chromatographic method for the quantification of donepezil polymeric nanoparticles: Stability studies, *in-vitro* and cellular evaluation (To be communicated).

Other Publications

1. Geetika W*, K V Krishna*, Taliyan, R., Tandon N, Yadav Satyapal, Katiyar C K, Dubey S K, (2020). Pre-clinical Pharmacokinetics of Trigonelline using UPLC-MS/MS and

Pharmacological Studies Targeting Type 2 Diabetes. Journal of Separation Science. *Equal First Author Contribution. (Under review)

2. Donthi M R*, Munnangi S*, Krishna K V*, Marathae S, Saha R N, Singhvi G, Dubey S K., (2020). Experimental Design, Characterization, Molecular Modelling Studies of Sorafenib tosylate Inclusion Complexes with Beta-cyclodextrin and Auxiliary Substances. European Journal of Pharmaceutical Sciences *Equal First Author Contribution. (Under revision)

3. Mittal A, Mahala N, Krishna K V, Dubey U S, Dubey S K., (2020). Calcium Chloride Linked Camel Milk Derived Casein Nanoparticle for the Delivery of Sorafenib Drug in Hepatocarcinoma Cells. International Journal of Food Science and Technology (Communicated).

4. Wadhwa, G*., Krishna, K.V*., Taliyan, R., Tandon, N., Yadav, S.S., Katiyar, C. and Dubey, S.K., 2020. Pre-clinical pharmacokinetic and pharmacodynamic modelling study of 4-hydroxyisoleucine using validated ultra-performance liquid chromatography-tandem mass spectrometry. RSC Advances, 10(10), pp.5525-5532. (*Equal First Author Contribution)

5. Pradhan, R*., Krishna, K.V*., Wadhwa, G., Taliyan, R., Khadgawat, R., Kachhawa, G., Singhvi, G. and Dubey, S.K., 2020. QbD-driven development and validation of HPLC method for determination of Bisphenol A and Bis-sulphone in environmental samples. International Journal of Environmental Analytical Chemistry, 100(1), pp.42-54. (*Equal First Author Contribution)

6. Krishna, K.V., Saha, R.N., Puri, A., Viard, M., Shapiro, B.A. and Dubey, S.K., 2019. Pre-clinical compartmental pharmacokinetic modeling of 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH) as a photosensitizer in rat plasma by validated HPLC method. Photochemical & Photobiological Sciences, 18(5), pp.1056-1063.

7. Khosa, A., Krishna, K.V., Saha, R.N., Dubey, S.K. and Reddi, S., 2018. A simplified and sensitive validated RP-HPLC method for determination of temozolomide in rat plasma and its application to a pharmacokinetic study. Journal of Liquid Chromatography & Related Technologies, 41(10), pp.692-697.

8. Reddi, S.S., Singhvi, G., Khosa, A., Krishna, K.V. and Saha, R.N., 2019. Determination of Venlafaxine in Bulk and Pharmaceutical Formulations Using Stability Indicating RP-HPLC Method with UV Detector. Current Pharmaceutical Analysis, 15(3), pp.258-265.

9. Dubey, S.K., Jindal, M., Nagpal, S., Saha, R.N., Singhvi, G., Anand, A., and Krishna, K.V., 2019. A Systematic Review on Analytical Methods to Determine Chiral and Achiral Forms of VEN and its Metabolite o-desmethylvenlafaxine. Current Pharmaceutical Analysis (Accepted).

Patents

1. K V Krishna, S K Dubey, R N Saha, G Singhvi "Orally administrable thermosensitive liposomes for encapsulating a molecule" (IN Patent Application: 202011016481; dated 16 April 2020; Complete specifications).

Book Chapters

1. Khosa, A., Krishna, K.V., Dubey, S.K. and Saha, R.N., 2020. Lipid Nanocarriers for Enhanced Delivery of Temozolomide to the Brain. In Drug Delivery Systems (pp. 285-298). Humana, New York, NY.

2. Singhvi, G., Rapalli, V. K., Krishna, K. V., Dubey, S. K., 2019. Alginate: Versatile Polymer in Biomedical Applications and Therapeutics. Alginate: Drug Delivery and Application. Apple Academic Press, Ontario, Canada (In press).

Presentations

1. K V Krishna, R N Saha, S K Dubey., "Design, Synthesis and Neuroprotective Potency of Self-Assembling Memantine-Docosahexaenoic Acid Conjugate Nanomedicine for the Effective Treatment of Alzheimer's Disease", *presented at Alzheimer's Association of International Conference (AAIC)-2020, Amsterdam, Netherlands, held as an entirely virtual meeting on 27th – 31st July 2020.*

2. Participated in a virtual LCMS conference "virtual event designed for the masses: your fast track to confident results" organized by *Thermo-fisher Scientific on 23rd June 2020.*

3. Participated in the online international conference entitled *"Disso India 2020 Online International Conference" on 13th – 16th May 2020.*

4. K V Krishna, R N Saha, S K Dubey, "Inhibition of Amyloid beta Protein Aggregation by Self-Assembling Nanoparticles of Memantine-Alpha Lipoic Acid Conjugate", *2nd AAT-*

ADPD^{TM} 2020 Focus Meeting, Vienna, Austria held as an entirely virtual meeting on 02nd – 05th April 2020.

5. Participated in the workshop entitled *" Pharmacokinetic & Pharmacodynamic Modeling and Simulation"* at Department of Pharmacy, BITS Pilani, Pilani Campus in collaboration with School of Pharmacy, University of Maryland, USA on 24th – 25th February 2020.

6. Participated in the workshop entitled *"Advancement in Genomics, Proteomics to Drug Discovery & Development associated with Neurological Disorders"* at National Institute of Mental Health and Neurosciences, Bangalore on 05th – 08th February 2020.

7. K V Krishna, R N Saha, R Taliyan, S K Dubey, "Pre-Clinical Neuroprotective Potency of Surface Modified Donepezil loaded mPEG-PCL Nanoparticles in Amyloid-beta$_{1-42}$ Induced Cognitive Impairment", *International Brain Research Organization - Asia-Pacific Regional Committee (IBRO-APRC) Associate School on Blood-Brain-Barrier: From Basic Physiology to Neurological Disorders at Punjab University on 04th – 09th November 2019.*

8. K V Krishna, R N Saha, S K Dubey, "Behavior Analysis of ApoE3 Conjugated Donepezil Nanoparticles on Memory Impaired Rats", *International Conference on Neurological Disorders and Therapeutics (ICNDT - 2019) at NIPER-Ahmedabad on 24th – 26th October 2019.*

9. K V Krishna, Anu Puri, Bruce A. Shapiro, R N Saha, S K Dubey, "Whole Blood Analysis by UPLC-MS/MS for the Simultaneous Estimation of a Photosensitizer and Topotecan: Application to Pharmacokinetics", *6th Asia-Pacific Congress of Interventional Oncology (APCIO)-2019 at Kuala Lumpur, Malaysia on 02nd – 05th October 2019. (Received Young Investigator Award & CSIR International Travel Grant)*

10. Krishna K V, Rajesh Pradhan, R N Saha, Sunil K Dubey, "Pre-Clinical Pharmacokinetics of Memantine - Alpha-Lipoic Acid conjugate in Wistar Rat using UPLC-MS/MS", *4th Annual Conference of the Society for the Study of Xenobiotics, Bangalore on 18th – 21st September 2019.*

11. Krishna K V, Geetika Wadhwa, Sunil K Dubey, R N Saha, "Enantioselective Analysis and Molecular Modeling Studies of Tolvaptan", *Life Science Research its Interface with Engineering and Allied Sciences (LSRIEAS 2018), 01st– 03rd November 2018, BITS Pilani, Pilani Campus.*

12. K V Krishna, R N Saha, S K Dubey. Synthesis and Pre-Clinical Neuroprotective Potency of Memantine-Alpha lipoic acid Conjugate in Cognitive Disorders, *presented at Alzheimer's Association of International Conference (AAIC)-2018, Chicago, USA, 21st – 26th July 2018. (Received AAIC-2018 Travel Grant)*

13. S K Dubey, Krishna K V, Saha R N. Exploring the compartmental pharmacokinetics and bio-distribution of donepezil hydrochloride, *Presented at 9th World Congress on Bioavailability and Bioequivalence, Dubai, UAE, 16th -18th April 2018.*

14. K V Krishna, S K Dubey, R N Saha, Gautam Singhvi, Maneesh Goyal, "Analytical Method for the Isomeric Separation of Sennosides", *National Conclave "Vanoushadhi - 2018" Chhattisgarh hosted by Chhattisgarh State Medicinal Plants board in association with Ethix Pharma and Columbia College of Pharmacy at Pt. Deendayal Upadhyaya Auditorium, 3th-4th February, 2018, Vanoushadhi, Raipur, C.G.*

15. Krishna K V, Dubey S K, Saha R N, "Development and pharmacokinetic studies of donepezil loaded polymeric nano-micelles', *Nano and Functional Materials- Interface between Science and Engineering, BITS Pilani, Pilani Campus, 16th-18th November, 2017.*

16. Krishna K V, Dubey S K, Saha R N. "Synthesis and Characterization of Amphiphilic Di-Block Polymer and Its Application as a Nanocarrier For Donepezil, *International Conference on Challenges in Drug Discovery and Delivery, BITS Pilani, Pilani Campus, 02rd-04th March 2017.*

17. Krishna K V, Agnihotri T, Dubey S K, Saha R N. Surface Modified Rivastigmine Loaded PCL Nanoparticles for Alzheimer's Disease, *Presented at ICMR sponsored conference at Siksha 'O' Anusandhan University at Bhubaneswar on 17th - 18th Feb 2017.*

18. S. K Dubey, K. V. Krishna, R. N. Saha, Rajeev Taliyan, Rajesh Khadgawat, Simultaneous Method for the Estimation of Endocrine Disrupting Chemicals; Bisphenol A and Bis-sulphone, *Presented at Annual Conference of Endocrine Society of India (ESICON 2016), AIIMS, New Delhi, Hotel Pullman, Aerocity, New Delhi, 21st-23rd October 2016.*

19. K.V.Krishna, M.Sneha, S.K.Dubey, R.N.Saha, Rapid and Sensitive Reverse Phase HPLC Method for Estimation of Rifaximine in Pharmaceuticals using Weighed Regression, *Presented at 4th International Conference on "Pharmacogenomics, Proteomics in Drug Discovery, Development and Clinical Therapeutics, Chitkara University, 13th-14th April 2016 (Awarded 2nd Prize for poster).*

Biography of Dr. Sunil Kumar Dubey

Dr. Sunil Kumar Dubey is an Assistant Professor in the Department of Pharmacy, Birla Institute of Technology and Science (BITS), Pilani. He earned his Master of Pharmacy from Birla Institute of Technology (BIT) Mesra, Ranchi and Ph.D. from BITS, Pilani. He has an extensive research experience in the area of pharmacokinetics, clinical pharmacology and nanomedicines. He was also invited as a Visiting Assistant Research Professor in Department of Chemical and Biomolecular Engineering at University of Maryland, USA. Dr. S. K. Dubey has about 14 years of teaching, research and administrative experience. He has supervised several postgraduate and undergraduate students. He has published several book chapters, research articles in renowned journals and presented papers in conferences in India and abroad. During his industrial tenure, he has successfully completed many projects, including development, validation, preclinical and clinical studies. He has successfully completed various projects related to formulation development, analytical method development, validation and pharmacokinetic investigation. He is presently handling several projects funded government and industrial agencies like ICMR, Slayback, EticaClin Pharm, Enaltech Pharma, Oniosomes, Axis Clinicals, Crenza Pharma, Morning Side Health Care etc.

Biography of Prof. Ranendra Narayan Saha

Prof. Ranendra N. Saha is a Senior Professor of Pharmacy and Director of BITS Pilani, Dubai Campus. He served to BITS Pilani, Pilani campus as HOD, Dean Administration and Deputy Director. He has completed his Bachelor and Master of Pharmacy from Jadavpur University, Kolkata and Ph.D. from BITS, Pilani. He has been awarded as Shri B. K. Birla and Shrimati Sarala Birla Chair Professorship for his excellence contribution in teaching, research and administration. He has vast research experience in the area of pharmaceutics, novel drug delivery systems, nanomedicine and pharmacokinetics. Prof. R. N. Saha has more than 35 years of teaching, research and administrative experience. He has supervised several doctoral, postgraduate and undergraduate students. He has published several book chapters, research articles in reputed journals and presented papers in conferences in India and abroad. He has successfully completed several government and industry sponsored projects.

Prof. R. N. Saha has transferred technologies to industries, filed patents and commercial products to his credit. He is also a member of selection committee and advisory board for several renowned Universities in India and abroad.

Biography of K V Krishna

K V Krishna is a research scholar at Department of Pharmacy, BITS Pilani, with UGC-SRF fellowship for pursuing doctoral research. He has completed his Bachelor and Master of Pharmacy from Andhra University, Vishakhapatnam. He has published articles in peer reviewed journals and delivered presentations in international and national conferences. He has qualified national level competitive exam (GPAT) and received awards including Young Investigator Award from 6th Asia-Pacific Congress of Interventional Oncology (APCIO)-2019, Malaysia, CSIR International travel award for young scientists and Alzheimer's Association Travel Grant-2018. He also has research experience at B. V. Patel Pharmaceutical Education and Research Development (PERD) Centre, Ahmedabad in formulation development, novel drug designing and pharmacokinetics before joining BITS for doctoral research. He is a professional member of Alzheimer's Association: International Society to Advance Alzheimer's Research and Treatment (ISTAART).

Publications from thesis work

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Pre-clinical pharmacokinetic-pharmacodynamic modelling and biodistribution studies of donepezil hydrochloride by a validated HPLC method†

Kowthavarapu Venkata Krishna,[a] Ranendra Narayana Saha,[b] Gautam Singhvi[a] and Sunil Kumar Dubey [ID] *[a]

A simple, sensitive and robust HPLC–PDA assay was developed and validated for rapid determination of donepezil hydrochloride (DNP), a potent acetylcholinesterase inhibitor in rat plasma and tissues. All biological samples were prepared by the solid-phase extraction method using loratadine as an internal standard. Separation of the analytes was achieved on a Waters Nova-Pak C18 column (3.9 × 150 mm, 4 μm) using an isocratic mobile phase of acetonitrile and ammonium formate (pH 6.4; 0.01 M) (62 : 38% v/v) at a flow rate of 1 mL min^{-1}. All validation parameter results were within the acceptable range described in the guidelines for bioanalytical method validation. The method showed linearity in the concentration range of 50–5000 ng mL^{-1} with LOD of 20 ng mL^{-1} and LLOQ of 50 ng mL^{-1}. Moreover, the advantage of this method over previously published methods is the short analysis run time of 6 min in HPLC itself, alongside its application not only for plasma samples but also in tissues, with low LLOQ. The method was successfully applied for studying the compartmental pharmacokinetics, tissue distribution and pharmacodynamics. A two-compartmental micro model was statistically fitted for the assessment of pharmacokinetic parameters. The tissue distribution studies suggest that the kidneys, lungs and liver are the primarily responsible organs for metabolism and elimination of DNP. Pharmacodynamic studies were performed by measuring acetylcholinesterase inhibitory activity of DNP, which indicated that the pharmacokinetic and pharmacodynamic data are in correlation with each other.

1. Introduction

Globally, ageing is accompanied by an increasing substantial social and financial impact owing to ageing-related diseases. Dementia is one among them, a syndrome which leads to deterioration in cognition, thinking, behavior and ability to carry out daily routine work.[1] As per an estimate, there were 46.8 million people worldwide living with dementia in 2015 and this number may reach 131.5 million by 2050.[2] Alzheimer's Disease (AD) is the most typical cause of dementia and it contributes to 60–70% of the reported cases. As per a recent investigation by the Alzheimer's association, every 65 s one person in the United States (US) develops AD. It is a chronic and progressive neurodegenerative disease. There are drugs approved by the US Food and Drug Administration (US FDA) to condense its symptoms, which fall under acetylcholinesterase inhibitors (AChEI) and N-methyl-D aspartate (NMDA) receptor antagonists. Tacrine was the first AChEI approved by the FDA and later it was withdrawn due to its side effects.[3] Second generation AChEIs are DNP, galantamine and rivastigmine. Memantine which belongs to NMDA receptor antagonist was approved by FDA for the treatment of moderate and severe AD.[4,5] All these drugs just delay the progression of the disease, dementia cannot be cured.[6,7] At present the aim of the treatment is to maintain the quality of life as long as possible. DNP is effective in the treatment of cognitive impairment and memory loss in patients with AD. Moreover, due to the immense therapeutic potential and pharmacological activities of DNP, it is essential to develop a quick and accurate quantification method of DNP for conducting biostudies.

To the best of our knowledge, none of the methods have been applied either for estimation of DNP after intravenous (i.v.) administration or for compartmental pharmacokinetics, tissue distribution and pharmacodynamic studies. However, some methods have been reported in literature (Table 1), which are having more run time and require large sample volume with either a complex sample preparation procedure or use of sophisticated instruments like LC-MS/MS that hamper routine analysis for various applications. Abonassif, *et al.* developed a HPLC-RF method for the estimation of DNP in human plasma using a sample volume of 1000 μL with a total run time of

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Table 1 Examples of extraction procedures and analytical techniques for estimation of DNP in biological samples[a]

S. No	Biological Sample	Extraction method	Linearity (ng mL^{-1})	System	Run time (min)	% Recovery	Reference
1	1000 μL human serum	PP by acetonitrile	5–2000	HPLC-RF	15	NR*	8
2	200 μL of rat plasma	PP by acetonitrile	50–2000	HPLC-UV	20	NR*	14
3	250 μL of human plasma	PP by acetonitrile	1–300	UPLC-MS/MS	5	79	10
4	1000 μL human plasma	LLE by *n*-hexane and IPA (97 : 3)	3–90	HPLC-UV	15	NR*	9
5	100 μL of rat plasma	LLE by *n*-hexane and IPA (95 : 5)	5–500	HPLC-RF	20	NR*	15
6	500 μL of human plasma	SPE by acetic acid and methanol (2 : 98)	0.339–51.87	LC-ESI-MS/MS	10	68.9	12
7	1000 mL of human serum	SPE by 0.025 M ammonium formate (pH:2.5) and methanol (50 : 50)	5–160	HPLC with UV detector	15	55.7	16

[a] *NR, not reported.

15 min, using protein precipitation technique (PP) by acetonitrile (ACN) as extracting solvent.[8] Furukori, *et al.* have reported a HPLC-UV method using liquid–liquid extraction (LLE) process by *n*-hexane and IPA (97 : 3), with a run time of 15 min and sample volume 1000 μL of human plasma.[9] A simultaneous method has also been developed for the estimation of DNP and memantine in plasma.[10] A UPLC-MS/MS method has been reported for the estimation of DNP in human serum, using PP by ACN.[11] Another study was performed using LC-ESI-MS/MS with a sample volume of 500 μL of human plasma, using solid-phase extraction (SPE) at a total run time of 10 min.[12] LC-MS/MS method is highly recommended procedure, significantly enhances the sensitivity of the method; but maintenance cost of the instrument is more, requires trained personnel and also not available in every research laboratory. However, DNP has been reported to be administered at a dose ranging from 5–10 mg kg^{-1} in humans.[13] Hence, the quantification upto 50 ng mL^{-1} is highly sufficient, HPLC based methods are equally helpful in estimation of DNP in pharmacokinetic samples. Moreover, the advantage of this method over previously published methods is the short analysis run time of 6 min in HPLC itself, its application not only for plasma samples but also in tissues and pharmacodynamic study.

The present work describes method development and validation details for quantification of DNP in rat plasma and tissues within the range of 50–5000 ng mL^{-1} and elucidating the compartmental pharmacokinetics, tissue distribution and pharmacodynamic profiles. The proposed method has been developed using solid-phase extraction (SPE) method using methanol (2 mL) as an eluting solvent, and with a total run time of 6 min. Loratadine (LRD) was selected as internal standard (I.S) (Fig. 1). Complete validation was carried out including selectivity, lower limit of quantification (LLOQ), limit of detection (LOD), precision, accuracy, carry over effect, dilution integrity and stability as per guidelines on bioanalytical method validation. Stability studies were performed to determine the stability of stock solutions and in plasma samples that were exposed to different storage conditions which includes freeze-thaw, autosampler, bench top, short-term and long-term

storage. Moreover, using this method we have generated a complete pharmacokinetic profile for DNP in rats. Hereby we report a new HPLC–PDA method for the estimation of DNP in rat plasma and in other biological tissues also. In addition to this, we also explored pharmacodynamic studies of DNP by estimation of acetylcholinesterase (AChE) levels.

2. Materials and methods

2.1 Chemicals, materials and reagents

DNP and LRD standards were kind gift samples from Vasudha Pharma Chem Limited (Hyderabad, India). ACN, methanol (HPLC grade) and sodium phosphate were procured from Merck Limited (Mumbai, India). Ammonium formate, acetylthiocholine iodide and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were procured from Sigma Aldrich (St. Louis, MO, USA). Solid-phase cartridges, Clearnert PEP-3 SPE was procured from Agela Technologies (Wilmington, USA). Bovine serum albumin (BSA) was purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Water was produced by millipore Milli-Q Plus water treatment system (Millipore Bedford Corp., Bedford, MA, USA).

2.2 Experimental animals

All experimental animals (Wistar rats; male; 8–10 weeks; weighing around 200–220 g), were housed in polyacrylic cages, and all procedures involving sample collection and their care were conducted in conformity with the institutional guidelines that are in compliance with Institutional Animal Ethics Committee (IAEC) of BITS-Pilani university with approved protocol (IAEC/RES/22/05). All animals were housed in a temperature (20–22 °C) and humidity (65%) controlled animal facility and maintained on a 12/12 h light/dark cycle. The temperature and relative humidity were recorded daily. Both feed and water were provided ad libitum throughout the acclimatization and study period.
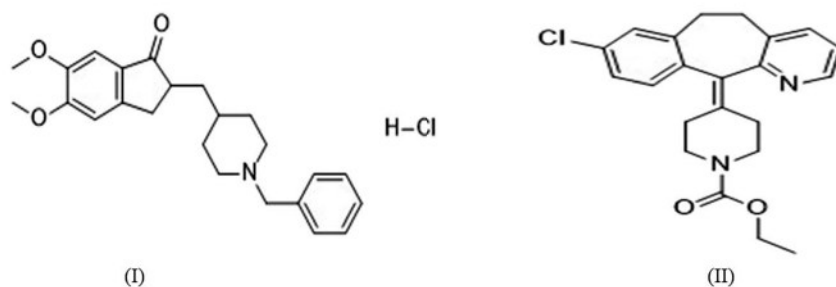
Fig. 1 Chemical structures of (I) DNP and (II) LRD.

2.3 Liquid chromatographic conditions

HPLC analysis were performed on Shimadzu liquid chromatograph system (Kyoto, Japan) equipped with a binary pump (LC-10AT), Photo Diode Array (PDA) detector (SPD-M20A), column oven (CTO-10AS) and auto sampler (SIL-HT, Shimadzu, Japan) was used to inject the samples. The HPLC system was equilibrated for approximately 30 min before starting of analysis and chromatography was carried out at 40 ± 0.5 °C. Eluents were monitored at a wavelength of 268 nm using a PDA detector. DNP and LRD were separated on Waters Nova-Pak C18 column (3.9×150 mm, 4 μm) with a mobile phase consisting of acetonitrile and ammonium formate (pH 6.4; 0.01 M) (62 : 38% v/v) in an isocratic mode at a flow rate of 1 mL min^{-1}. The injection volume was 50 μL and retention time for DNP and LRD were 3.31 and 3.99 min respectively. Control of hardware, data acquisition and elaboration were performed using LC solution software version 1.24 SP1.

2.4 Preparation of stock solutions, calibration curve standards (CS) and quality control (QC) samples

Standard stock solution of DNP was prepared by dissolving accurately weighed amount in methanol to obtain a final concentration of 5 mg mL^{-1}. Further, same volumes of DNP stock solution (5 mg mL^{-1}) was diluted with Milli-Q water to make 400 μg mL^{-1} working stock solution. From 400 μg mL^{-1} working stock solution, working standard solutions of 1, 2, 6, 20, 40, 70 and 100 μg mL^{-1} were prepared by serial dilutions in Milli-Q water. The concentrations of working QC standard solutions were prepared as 1.6, 3 and 80 μg mL^{-1}. CS and QC samples were prepared by spiking with 10 μL of corresponding working standard solutions in 190 μL of blank rat plasma. The final CS were prepared as 50, 100, 300, 1000, 2000, 3500 and 5000 ng mL^{-1} in plasma matrix. The QC were prepared by using QC standard solutions at four concentration levels, such as limit of quantification (LOQQC, 50 ng mL^{-1}); low QC (LQC, 80 ng mL^{-1}), medium QC (MQC, 1500 ng mL^{-1}) and high QC (HQC, 4000 ng mL^{-1}). All the stock and working solutions were stored in the refrigerator at −20 °C till analysis.

2.5 Sample preparation

Several extraction techniques were tested, such as protein precipitation with organic solvents (methanol, acetonitrile) and by liquid–liquid extraction using dichloromethane, n-hexane : IPA (97 : 3) and n-hexane : IPA (95 : 5). These procedures

allow good recovery for IS, but plasma interference was observed with low recovery of analyte.

SPE method was also tested in order to reduce solvent consumption, maximize the analytes recovery, and to optimize the sample clean-up process. A 200 μL aliquot of plasma sample containing DNP was taken in 2 mL capacity of polypropylene disposable tube, followed by the addition of 20 μL of 100.0 μg mL^{-1} of LRD (I.S) solution (I.S solution was prepared in ACN) and was vortexed for 1 min. Then 200 μL of ammonium formate buffer was added and vortexed for 2 min on a vortex mixer. The samples were then loaded into the SPE cartridges using a semi-automated SPE system (ESI; Table S1†). The SPE eluents were collected in glass tubes and evaporated to dryness at 40 ± 0.5 °C under a gentle stream of nitrogen gas. The residue was reconstituted with 200 μL of mobile phase and vortexed for 30 s. Finally, 50 μL of sample was injected into HPLC through auto-injector for quantification.

2.6 Method validation procedures

The proposed method was validated as per guidelines US FDA and European Medical Agency guidelines of bioanalytical method validation.[17,18]

2.6.1 System suitability. System suitability is typically a system performance test, which was carried out by injecting the same concentration sample by six times for checking the column efficiency, reproductivity and resolution. It was performed to verify the holistic function of the chromatography system on a day-to-day basis.

2.6.2 Selectivity. The selectivity of the method was carried out to evaluate potential chromatographic interference from rat plasma matrix at retention time (RT) of the drug and IS. The rat blank plasma samples were collected randomly from selected six different Wistar rats and analyzed as per described chromatographic conditions.

2.6.3 Linearity and calibration curve. The calibration curve of an analyte (DNP) was prepared using six calibration standards in a range of 50 to 5000 ng mL^{-1}. Six calibration curves were prepared for plasma and tissue samples by plotting peak area ratios (drug/I.S) on Y axis versus nominal plasma concentrations on X-axis. Highest calibration standard sample (ULOQ, 5000 ng mL^{-1}) was 100 times less than the lowest limit of calibration point (LLOQ, 50 ng mL^{-1}), so it was necessary to perform weighted linear regression on calibration data. All the different weighting factors were applied to the data obtained from least-squares regression analysis and the best weighting

factors were chosen according the percentage relative error (% RE).

2.6.4 Limit of detection (LOD) and lower limit of quantitation (LLOQ). The limit of detection (LOD) is defined as the lowest concentration of analytes that can be detected and differentiated from the noise level.

The lower limit of quantitation (LLOQ) is defined as the lowest concentration of analytes that can be quantified with accepted accuracy and precision recommendations (not exceeding ±20%).

LOD and LLOQ were determined by using signal to noise ratio method. First the signal to noise ratio was checked for standard samples at system suitability step. LOD and LLOQ were calculated based on eqn (1). Desired S/N values for LOD was 3 and for LLOQ was 10.

Concentration of LOQ and LOD

$$= \frac{\text{Concentration of Standard}}{\frac{S}{N} \text{value of Standard}} \times \text{Desired} \frac{S}{N} \text{value} \quad (1)$$

Generally, LLOQ is the first point in calibration curve and analyzed plasma samples by spiking with LLOQ concentration in six replicates ($n = 6$).

2.6.5 Precision and accuracy. The intra-day and inter-day assay precision and accuracy were determined by analyzing six replicates at three different QC levels (LQC, MQC, HQC) as described in Section 2.4 and LLOQ. The intra-day assay precision and accuracy were determined in six replicates by analyzing samples on same day, while inter-day assay precision and accuracy were determined in six replicates by analyzing each QC and LLOQ samples on five different days. The acceptance criteria for intra and inter-day precision (RSD, %) was limited to ≤15% and accuracy was required to be limited within ±15% except for LLOQ, where it should not exceed ±20% for accuracy and precision.

2.6.6 Recovery. The percentage recovery of DNP through SPE were determined by comparing the peak area of analyte spiked plasma QC samples with those of unextracted neat standards that is standard solution with equivalent concentrations.

2.6.7 Carry over effect and dilution integrity. Carry over effect was determined by injecting highest calibration standard sample (ULOQ, 5000 ng mL^{-1}) followed by blank sample. As per guidelines, the sample dilution must be performed to ensure the integrity of plasma samples. In dilution integrity, blank plasma was spiked with 2 times higher than the ULOQ concentration (10 000 ng mL^{-1}) and was diluted 5 times and 10 times with blank plasma ($n = 6$) to obtain a concentration of 2000 ng mL^{-1} and 1000 ng mL^{-1}. Dilutes samples were processed and analyzed with freshly spiked calibration curve in

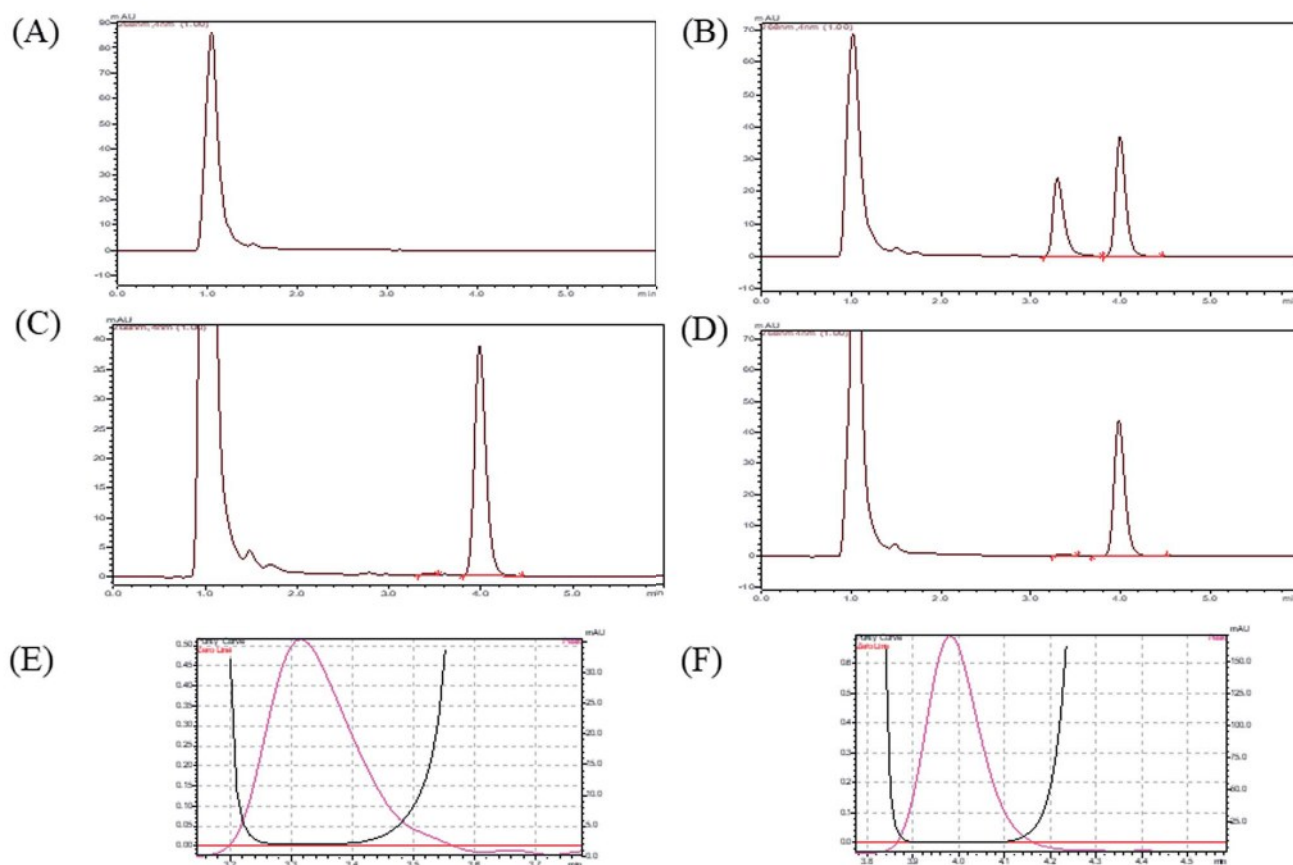**Fig. 2** HPLC chromatograms for (A) blank plasma, (B) calibration standard, (C) LOD (20 ng mL^{-1}), (D) LLOQ (50 ng mL^{-1}), (E) peak purity index of DNP, (F) peak purity index of I.S.

Table 2 Precision and accuracy data of back calculated concentrations of calibration standard samples of DNP in rat plasma ($n = 6$)[a]

Component	Linear equation and coefficient of regression	Nominal concentration (ng mL^{-1})	Measured concentration (Mean ± SD, ng mL^{-1})	Precision (% CV)	Accuracy (% bias)
Plasma	$y = 0.000384x - 0.0088$, $r^2 = 0.9999$	50	51.87 ± 3.21	6.19	3.74
		100	95.30 ± 4.57	4.80	−4.70
		300	304.26 ± 16.99	5.58	1.42
		1000	999.58 ± 2.96	0.30	−0.04
		2000	1983.08 ± 12.60	0.64	−0.85
		3500	3464.12 ± 5.27	0.15	−1.03
		5000	5042.00 ± 8.58	0.17	0.84
Brain	$y = 0.0003x - 0.0058$, $r^2 = 0.9998$	50	55.26 ± 1.15	2.08	10.52
		300	289.54 ± 15.48	5.35	−3.49
		1000	939.40 ± 87.62	9.33	−6.06
		2000	1950.68 ± 13.60	0.70	−2.47
		3500	3335.07 ± 30.75	9.18	−4.71
		5000	4872.22 ± 19.75	0.41	−2.56
Heart	$y = 0.0003x - 0.0075$, $r^2 = 0.9991$	50	56.24 ± 6.54	11.63	12.48
		300	314.36 ± 1.10	0.35	4.79
		1000	1011.78 ± 18.88	1.87	1.18
		2000	2147.01 ± 51.93	2.42	7.35
		3500	3547.65 ± 4.37	0.12	1.36
		5000	5262.01 ± 43.58	0.83	5.24
Lungs	$y = 0.0003x - 0.0099$, $r^2 = 0.9999$	50	56.78 ± 4.23	7.45	13.56
		300	316.62 ± 28.83	9.11	5.54
		1000	1041.39 ± 93.03	8.93	4.14
		2000	2168.05 ± 106.81	4.93	8.40
		3500	3749.94 ± 206.25	5.50	7.14
		5000	5372.00 ± 291.26	5.42	7.44
Spleen	$y = 0.0003x + 0.0089$, $r^2 = 0.9996$	50	55.31 ± 3.32	6.00	10.62
		300	289.15 ± 8.66	2.99	−3.62
		1000	1049.53 ± 80.29	7.65	4.95
		2000	2148.97 ± 146.01	6.79	7.44
		3500	3600.13 ± 269.69	7.49	2.86
		5000	5247.77 ± 32.88	0.63	4.96
Kidney	$y = 0.0003x - 0.0008$, $r^2 = 0.9995$	50	46.42 ± 4.12	8.88	−7.16
		300	309.21 ± 3.51	1.14	3.07
		1000	1050.37 ± 85.23	8.11	5.04
		2000	2243.01 ± 223.27	9.95	12.15
		3500	3804.79 ± 163.19	4.29	8.71
		5000	5355.65 ± 51.11	0.95	7.11
Liver	$y = 0.0003x - 0.0126$, $r^2 = 0.9999$	50	52.94 ± 1.68	3.17	5.88
		300	281.24 ± 25.88	9.20	−6.25
		1000	935.92 ± 29.60	3.16	−6.41
		2000	1880.22 ± 135.81	7.22	−5.99
		3500	3240.65 ± 18.66	0.58	−7.41
		5000	4703.54 ± 5.48	0.12	−5.93

[a] SD, standard deviation; CV, coefficient of variation.

plasma. The accuracy and precision values should be within ±15%.

2.6.8 **Stability studies.** Stability of analytes in plasma matrix were evaluated before and after subjecting to different conditions and temperatures that could encounter during regular analysis. Stability in plasma was evaluated in terms of freeze-thaw stability, bench top stability, short-term stability and autosampler stability. All stability studies were conducted in three replicates at each concentration of the three different QC levels (LQC, MQC, and HQC). Freeze thaw stability was performed after freezing (−80 ± 10 °C for 48 h) and thawing QC samples for three consecutive cycles within 2 days. Bench top stability was analyzed at 25 ± 2 °C for 24 h, short-term stability was evaluated at −80 ± 10 °C for 15 days and long-term stability

was examined at −80 ± 10 °C for 3 months were performed in rat plasma with all QC levels. Replicate injections of extracted plasma samples were analyzed after 48 h to estimate auto sampler stability.

All QC samples were extracted and quantified against fresh calibration curves and fresh QC samples. The acceptance criteria of accuracy and precision for all stability samples should be within ±15%.

2.7 Pharmacokinetic study

To investigate the developed method, pharmacokinetic studies of DNP was performed on young Wistar rats weighing around 200–220 g. An i.v. administration, at a dose of 5 mg kg^{-1} of DNP were given to animals. After dosing, blood samples were collected in

Table 3 Precision (% CV) and accuracy (% bias) of DNP in rat plasma samples at QC levels ($n = 6$)

		Inter-day			Intra-day		
Level	Nominal Conc. (ng mL^{-1})	Measured Conc. (Mean ± SD, ng mL^{-1})	Precision (% CV)	Accuracy (% bias)	Measured Conc. (Mean ± SD, ng mL^{-1})	Precision (% CV)	Accuracy (% bias)
LLOQ	50	53.54 ± 2.43	4.54	7.08	50.21 ± 3.19	6.35	0.42
LQC	80	79.74 ± 4.45	5.58	−0.33	77.51 ± 5.36	6.92	−3.11
MQC	1500	1543.40 ± 14.82	0.96	2.89	1513.29 ± 22.85	1.51	0.89
HQC	4000	4025.35 ± 25.97	0.65	0.63	3926.75 ± 65.42	1.67	−1.83

1.5 mL capacity of polypropylene disposable tubes for each time point at 0, 0.083, 0.166, 0.5, 0.75, 1, 3, 6, 9, 12, 15, 18, 24, 30 and 36 h. Blood samples were collected from the retro-orbital plexus into heparinized micro centrifuge tubes at pre-determined time interval. Plasma samples were harvested by centrifuging the blood at 6000 rpm for 15 min and stored at −80 ± 10 °C until analysis. DNP plasma concentration-time profiles were plotted.

2.8 Pharmacokinetic modeling

Pharmacokinetic parameters for DNP were performed by using compartmental analysis. Model selection was guided by visual inspection of the observed plasma profiles and Akaike's information(AIC) criterion.[19] The lowest AIC value suggests best fit model. Various pharmacokinetic parameters such as concentration maximum (C_{max}), area under curve (AUC), volume of distribution in central compartment ($V1$), volume of distribution in peripheral compartment ($V2$), plasmatic clearance (Cl), intercompartmental clearance (CLD2), alpha half-life ($t_{1/2}\ \alpha$), beta half-life ($t_{1/2}\ \beta$), and elimination rate constants of two compartmental model (K10, K12, K21) were estimated Phoenix WinNonlin Certara™ (Pharsight, U.S.A; version:8.0).

2.9 Tissue distribution study in rats

Wistar rats were randomly divided into 2 groups, one group as control and for the other group DNP was given i.v. at a dose of 5 mg kg^{-1}. Tissues (brain, heart, lungs, kidney, spleen and liver) were promptly harvested at 0.166, 0.75, 2, 6 h (three rats/time point) after the administration of dose and thoroughly rinsed in ice-cold saline to remove blood and other content and blotted dry with filter paper. An accurately weighed amount of each tissue samples were homogenized in 1 : 2 w/v of phosphate buffer saline solution by using tissue homogenizer. After homogenization, centrifugation was done at 10 000 rpm for 15 min, clear supernatant was collected and stored at −80 °C until analysis.

2.10 Pharmacodynamic studies

The levels of AChE and butyl cholinesterase are the biological markers for AD.[20] Cholinesterase inhibitors (ChEIs) enhances the breakdown of AChE in synaptic cleft, thus enhancing ACh levels. Pharmacodynamic effects of DNP on AChE activity in rats was explored. The estimation of AChE activity in plasma samples were performed as per method described by Ellman et al.[21] In brief, the assay sample contained 0.05 mL of plasma, 3 mL of 0.01 M sodium phosphate buffer (pH 8), 0.10 mL of

acetylthiocholine iodide and 0.10 mL of DTNB (Ellman reagent). The change in absorbance was measured immediately at 412 nm spectrophotometrically. Protein content in plasma samples was measured by the method of Lowry et al.[22] using BSA (1 mg mL^{-1}) as a standard. Results were calculated using molar extinction coefficient of chromophore (1.36×104 M^{-1} cm^{-1}) and expressed as nanomoles per milligram protein.[23] Maximal AChE inhibition time of DNP was estimated.[24,25]

3. Results

3.1 Method development

3.1.1 Optimization of liquid chromatographic conditions. A methodological approach was followed for the method development so as to obtain analyte in less time using simplest possible chromatographic conditions for obtaining an appropriate recovery, symmetric peaks and high resolution of the drugs. For that, liquid chromatographic conditions such as choice of mobile phase, composition of mobile phase, pH of buffer, column selection, flow rate and injection volume were optimized (ESI; Table S2†). After successive optimization, acetonitrile–ammonium formate (pH: 6.4; 0.01 M) was selected as mobile phase which gave best peak shape and good peak intensities. Different columns were used to optimize better resolution, selectivity and retention time. Waters Nova-Pak C18 column (3.9 × 150 mm, 4 μm) with acetonitrile–ammonium formate (pH: 6.4; 0.01 M) mobile phase in ratio of 62 : 38% v/v in isocratic mode at 1 mL min^{-1} flow rate was found to be most suitable for DNP analysis with a total run time of 6 min. The injection volume was 50 μL and optimized retention time for DNP and LRD (I.S) were 3.31 and 3.99 min respectively at a wavelength 268 nm.

Table 4 Absolute recoveries (%) of DNP in rat plasma samples from QC levels[a]

			Recovery (%)	
Level	Nominal concentration (ng mL^{-1})	n	Mean ± SD ($n = 3$)	% CV
LLOQ	50	3	80.22 ± 1.44	1.80
LQC	80	3	80.28 ± 1.18	1.47
MQC	1500	3	78.68 ± 1.62	2.06
HQC	4000	3	80.28 ± 1.95	2.43
	Mean	12	79.86 ± 1.55	1.94

[a] n, number of samples; SD, standard deviation; CV, coefficient of variation.

Table 5 Stability of DNP in rat plasma at QC levels

Stability	Nominal concentration (ng mL^{-1})	Measured Concentration (ng mL^{-1}) $\pm$ SD	Precision (% CV)	Accuracy (% bias)
0 h (for all)	50	53.54 $\pm$ 2.43	4.54	7.08
	80	79.97 $\pm$ 4.45	5.58	−0.33
	1500	1543.40 $\pm$ 14.82	0.96	2.89
	4000	4025.35 $\pm$ 25.97	0.65	0.63
Autosampler (48 h)	50	51.72 $\pm$ 2.60	5.03	3.44
	80	83.48 $\pm$ 4.06	4.86	4.35
	1500	1446.35 $\pm$ 8.49	0.59	−3.58
	4000	3972.38 $\pm$ 5.35	0.13	−0.69
Bench-top (24 h, RT)	50	54.12 $\pm$ 1.42	2.62	8.24
	80	84.49 $\pm$ 4.94	5.85	5.61
	1500	1548.91 $\pm$ 7.95	0.51	3.26
	4000	3951.87 $\pm$ 24.88	0.63	−1.20
Freeze-thaw (−80 °C, 3 cycle)	50	54.84 $\pm$ 2.50	4.56	9.68
	80	82.64 $\pm$ 6.28	7.60	3.30
	1500	1463.00 $\pm$ 15.13	1.03	−2.47
	4000	3840.44 $\pm$ 18.39	0.48	−3.99
Short-term (−80 °C, 15 days)	50	49.74 $\pm$ 2.68	5.39	−0.52
	80	78.01 $\pm$ 2.62	3.36	−2.49
	1500	1414.08 $\pm$ 54.99	3.89	−5.73
	4000	3722.16 $\pm$ 12.29	0.33	−6.95
Long-term (−80 °C, 3 months)	50	47.81 $\pm$ 2.47	5.17	−4.38
	80	84.54 $\pm$ 2.78	3.29	5.62
	1500	1337.55 $\pm$ 12.59	0.94	−10.83
	4000	3655.47 $\pm$ 13.88	0.38	−8.61

3.1.2 Optimization of plasma sample preparation. Once the chromatographic conditions were optimized, extraction recovery of analyte was optimized by selecting an appropriate extraction method. The plasma sample clean-up procedure was also an important step in the method as it is directly proportional to sensitivity and selectivity of method. Higher recovery of analyte from plasma matrix can be obtained by minimum sample preparation processing, as well as proper choice of extraction method. Various problems have been encountered during the method optimization. Different methods for extracting drug from plasma matrix have been tried such as protein precipitation (with ACN and methanol), liquid–liquid extraction (with hexane, methylene chloride, IPA) and solid-phase extraction (SPE) (ESI; Table S3†). Out of all, SPE was found to produce higher recoveries, reproductivity and cleaner chromatogram.

3.2 Method validation

3.2.1 System suitability, selectivity and chromatographic separation. By performing the system suitability test, we conformed that system was working fine and it showed reproducibility with proper resolution. In described chromatographic condition, analytes were successfully separated with optimum retention time less than 6 min. (DNP and I.S retention times were 3.31 and 3.99). The sample clean-up procedure was so selective as there was no significant plasma matrix interference at retention time of analyte. A representative chromatogram of blank plasma, (plasma sample without analyte and IS), plasma sample with analyte and IS, LOD plasma sample (20 ng mL^{-1}) and LLOQ plasma sample (50 ng mL^{-1}) are shown in Fig. 2.

3.2.2 Linearity, calibration curve, LOD, LLOQ. All the calibration standard curves for plasma and tissue samples showed linearity and reproducibility over the selected concentration range of 50–5000 ng mL^{-1} with r^2 values more than 0.9999. The r^2 values, slopes and intercepts were calculated from five intra and inter day calibration curves using weighted linear regression analysis ($1/var$, $1/x$, $1/x^2$, $1/\sqrt{x}$, $1/y$, $1/y^2$, $1/\sqrt{y}$) wherein, best weighting factors were chosen as $1/x^2$ as these showed minimum percentage relative error (% RE) (ESI; Table S4†). The observed mean back calculated concentrations with accuracy (% bias) and precision (% CV) are presented in Table 2. At LLOQ ($n = 6$) accuracy (% bias) was in the range of 3.74% with a % CV of 6.19%. LOD and LLOQ were established as 20 ng mL^{-1} and 50 ng mL^{-1} respectively.

Table 6 Pharmacokinetic parameters for DNP after i.v. bolus (5 mg kg^{-1}) administration in rats ($n = 4$)

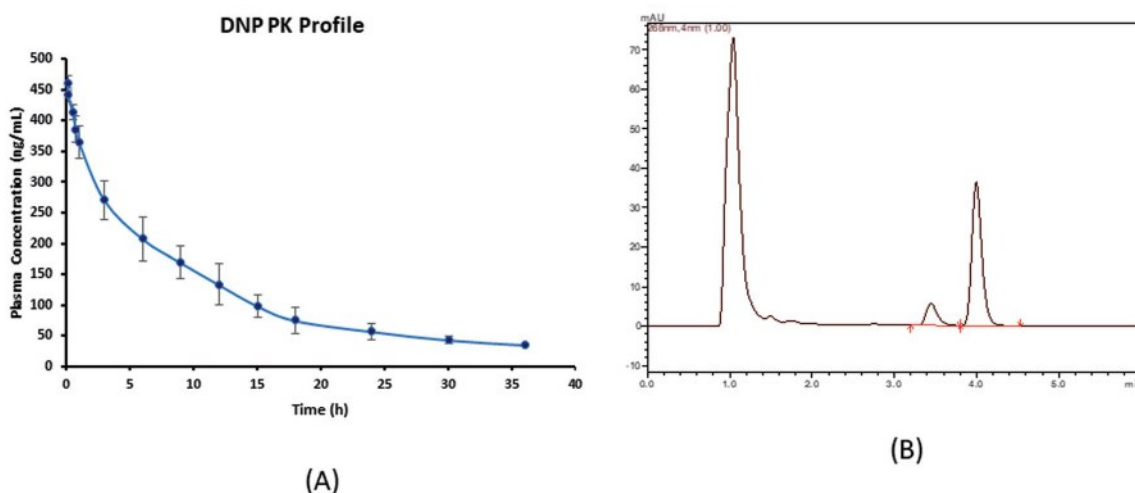| Pharmacokinetic parameters	DNP 5 mg kg^{-1}; i.v. (Mean $\pm$ SEM)
AIC of one compartment	129.86 $\pm$ 2.61
AIC of two compartment	114.06 $\pm$ 5.36
C_0 (ng mL^{-1})	468.02 $\pm$ 11.84
AUC$_{0-\infty}$ (ng h mL^{-1})	4655.05 $\pm$ 352.53
V1 (mL kg^{-1})	10 697.06 $\pm$ 274.35
V2 (mL kg^{-1})	4424.51 $\pm$ 816.59
Cl (mL h^{-1} kg)	1087.58 $\pm$ 89.00
CLD2 (mL h^{-1} kg)	2070.95 $\pm$ 599.09
$T_{1/2}$ α (h)	1.21 $\pm$ 0.46
$T_{1/2}$ β (h)	10.43 $\pm$ 0.53
MRT (h)	13.94 $\pm$ 0.95
$K10$ (h^{-1})	0.101 $\pm$ 0.0058
$K12$ (h^{-1})	0.196 $\pm$ 0.06
$K21$ (h^{-1})	0.554 $\pm$ 0.24

(A)

(B)

Fig. 3 (A) Plasma concentration–time profile of DNP (mean ± SD, $n = 4$) and (B) representative chromatogram of plasma sample obtained after 30 min of post drug administration.* *In these studies, DNP was administered by i.v. bolus at a single dose of 5 mg kg^{-1} each in rat ($n = 4$).

3.2.3 Precision and accuracy. The intra and inter day accuracy, precision data obtained from QC samples at four different concentration levels (LLOQC, LQC, MQC, HQC) are shown in Table 3. The inter and intra-day precision values did not exceed 6.92%. The inter and intra-day % bias ranged from −3.11 to 7.08%. All the values were within the recommendation of international guidelines.

3.2.4 Recovery, carry over, dilution integrity. The mean recovery values from rat plasma samples are listed in Table 4. The mean absolute recovery for DNP was found to be 79.86 ± 1.55% ($n = 12$). No carry over effect was observed, there was absence of peaks of analytes in blank sample was injected after ULOQ. Dilution integrity was carried out by diluting 5 times and 10 times with the blank plasma ($n = 6$). The accuracy value after 5 times and 10 times dilution were found to be 98.60 ± 3.47 and 101.75 ± 2.81 respectively.

3.2.5 Stability studies. The stability studies for DNP were performed as per the procedure described in Section 2.6.8. Different stability studies such as stock solution stability, freeze-thaw stability, bench top stability, short-term stability and autosampler stability results indicated that DNP was stable under different storage conditions that may be faced during routine study sample analysis and there was no significant degradation observed (Table 5).

3.3 Pharmacokinetic study in rats

The developed assay was productively implemented for the pharmacokinetic study of DNP after the i.v. administration of DNP at a dose of 5 mg kg^{-1} in Wistar rats. The calibration range (50–5000 ng mL^{-1}) of this assay method fitted to estimate all pharmacokinetic samples. The various pharmacokinetic parameters of DNP including observed maximum concentration (C_0), half-life ($t_{1/2}$) and area under the curve (AUC$_{0-t}$) were calculated using compartmental pharmacokinetic analysis and represented in the Table 6. Mean ± SD plasma concentration *versus* time profile of DNP is shown in Fig. 3.
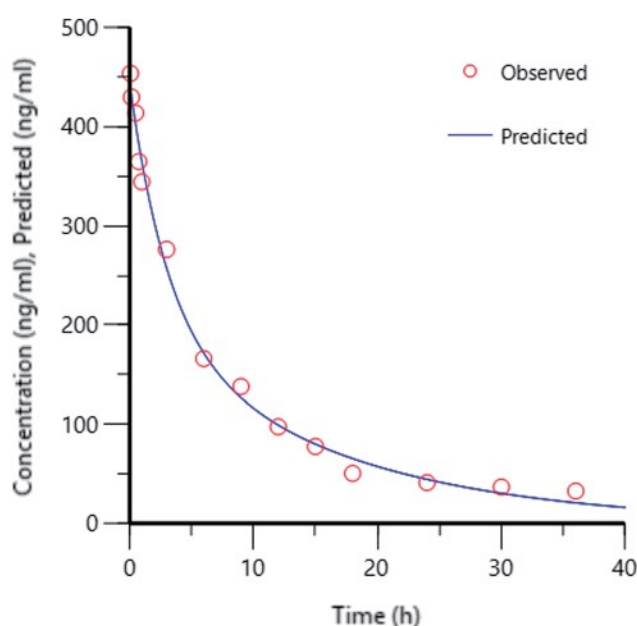

Fig. 4 Concentration plot of observed *versus* predicted.

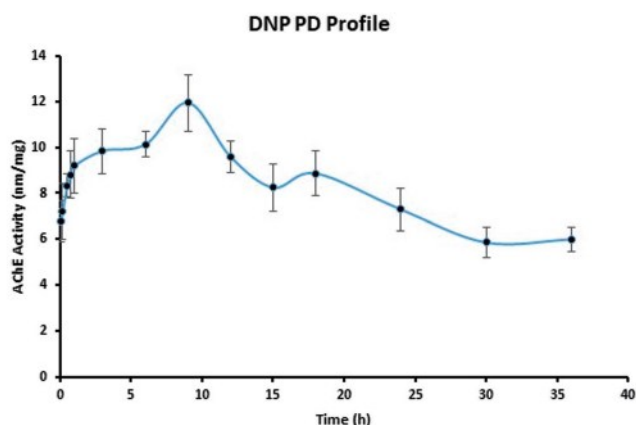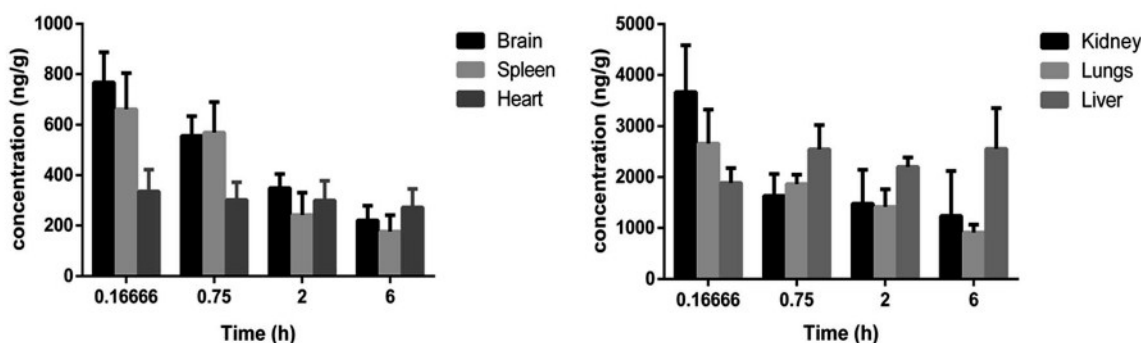**Fig. 5** Pharmacodynamic profile of DNP.

Fig. 6 Concentration (mean ± SD) levels of DNP in rat tissues at 0.1666, 0.75, 2 and 6 h.** **In these studies, DNP was administered by i.v. bolus at a single dose of 5 mg kg^{-1} each in rat (three/time point).

3.4 Pharmacokinetic modeling

Experimental plasma concentration-*versus*-time profiles were fitted to a two-compartmental micro model with i.v. bolus input and linear first order elimination from the central compartment using iterative weighted non-linear least squares regression using Phoenix WinNonlin Certara™ (Pharsight, U.S.A; version:8.0). The observed *versus* predicted concentrations did not differ from the line of identity, and no bias was observed (Fig. 4). DNP have shorter distribution half-life ($T_{1/2}\,\alpha$) of 1.21 h, which indicates rapid distribution from systemic circulation into the extravascular tissue spaces. Moreover, smaller elimination rate constant (K_{10}) of 0.1 h from central compartment, longer elimination half-life ($T_{1/2}\,\beta$) of 10.43 indicates behaved slow distribution and elimination characteristics.

3.5 Pharmacodynamic study

To examine the pharmacodynamic changes of DNP, the AChE inhibitory activity was determined (Fig. 5). Results have shown the correlation between pharmacokinetic profile, as the inhibitory activity was observed till 9 h thereafter, decrease in activity was noticed. The maximal effect of AChE inhibition was at 9.5 h, it clearly indicates the action of the DNP was matching with the pharmacokinetic data. Therefore, the established data in this study was reliable and highly suitable for pharmacokinetic-pharmacodynamic modeling.

3.6 Tissue distribution study

Tissue distribution study was carried out to identify the major distribution sites of DNP after i.v. administration at a dosage of 5 mg kg^{-1}. The distribution of DNP in the various rat tissues are represented in Fig. 6 and the representative of chromatographs are shown in Fig. S1.† The results demonstrated that DNP has exhibited wide range of distribution to tissues within the time course examined. As per the data obtained, the DNP concentration in various organs showed the following order: kidney > lungs > liver > brain > spleen > heart. By comparing organ and plasma samples, the distribution level in the kidney was 7.95 folds higher than that in plasma, 5.77 folds higher in the lungs than that in plasma and 4.09 folds higher in the liver than that in plasma. This clearly suggests that kidney, lungs and liver were the organs primarily involved in metabolism of DNP, leading to deposition at

these sites. Moreover, the lowest concentration was found in heart, which indicates DNP has less perfusion rate in heart. In summary, the distribution of DNP was highest in kidney and lowest in heart.

4. Conclusion

The aim of the present study was to develop an assay method for DNP in plasma matrix with simple extraction preparation techniques. Chromatographic separation was achieved on Waters Nova-Pak C18 column (3.9 × 150 mm, 4 μm) using isocratic mobile phase of acetonitrile and ammonium formate (pH 6.4; 0.01 M) (62 : 38% v/v) at flow rate of 1 mL min^{-1}. Solid-phase extraction was selected as an optimized method for higher recovery and proper resolution. The assay recovery was ≥79 ± 1.55% with insignificant matrix effect. The calibration curves in plasma and other tissues were linear in a concentration range of 50–5000 ng mL^{-1} with LOD and LOQ was 20 ng mL^{-1} and 50 ng mL^{-1} for plasma samples, respectively. The advantages of the method include simple and rapid sample preparation process, freedom from matrix interference and requirement of small plasma sample volume (200 μL), can be applied within a wide concentration range (5000 ng mL^{-1}) and a short total run time (06 min). Experimental plasma concentration-*versus*-time profiles were fitted to a two-compartmental micro model with i.v. bolus. The tissue distribution studies suggest, that kidney, lungs and liver are the primarily responsible organs for metabolism and elimination of DNP. Moreover, pharmacodynamic studies were conducted by estimating the inhibitory action of AChE was in correlation with pharmacokinetic profile. Our constructive studies provide valuable information to evaluate pharmacokinetic and pharmacodynamic studies for DNP.

Conflicts of interest

The authors have no conflict of interest.

Acknowledgements

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Design and Biological Evaluation of Lipoprotein-Based Donepezil Nanocarrier for Enhanced Brain Uptake through Oral Delivery

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S *Supporting Information*

ABSTRACT: Alzheimer's disease (AD) is a progressive neurodegenerative disorder associated with memory and cognitive impairment. Donepezil is an acetylcholinesterase inhibitor used for the symptomatic treatment of AD. However, high dose of donepezil is prescribed to achieve effective concentration in the brain, which leads to significant side effects, gastrointestinal alterations, and hepatotoxicity. In the present study, ApoE3 conjugated polymeric nanoparticles derived from diblock copolymer methoxy poly(ethylene glycol)−polycaprolactone (mPEG−PCL) have been used to boost the delivery of donepezil to the brain. mPEG−PCL is an amphiphilic diblock polymer with a tendency to avoid nanoparticle uptake by phagocytic cells in the liver and can significantly reduce the gastric mucosal irritations. Moreover, ApoE3-based nanocarriers showed a promising ability to enhance brain uptake, binding to amyloid beta with high affinity and accelerating its clearance. Donepezil-loaded polymeric nanoparticles were performed by using a nanoprecipitation method and further surface modified with polysorbate 80 and ApoE3 to increase the brain bioavailability and reduce the dose. Optimization of various process parameters were performed using quality by design approach. ApoE3 polymeric nanoparticles were found to be stable in simulated gastric fluids and exhibited a sustained drug release pattern. Cellular uptake studies confirmed better neuronal uptake of the developed formulation, which is further corroborated with pharmacokinetic and biodistribution studies. Orally administered ApoE3 polymeric nanoparticles resulted in significantly higher brain donepezil levels after 24 h (84.97 ± 11.54 ng/mg tissue) as compared to the pure drug (not detected), suggesting a significant role of surface coating. Together, these findings are promising and offer preclinical evidence for better brain availability of donepezil by oral administration.

KEYWORDS: Alzheimer's disease, Donepezil, ApoE3, mPEG−PCL, pharmacokinetic, biodistribution
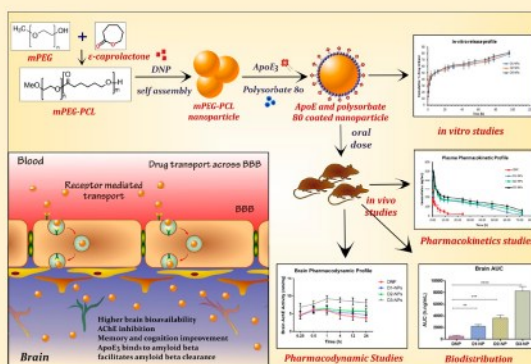
1. INTRODUCTION

Alzheimer's disease (AD) is the most predominant neurodegenerative disorder and a type of dementia that causes major issues for patients memory, thinking, and behavior.[1−3] As per a survey conducted by the Alzheimer's Association, there were 46.8 million people worldwide living with dementia in 2015 and this number may reach 131.5 million by 2050.[4] AD is a chronic and progressive neurodegenerative disease associated with the impaired level of acetylcholinesterase (AChE) in the brain region and the formation of β-amyloid plaques and neurofibrillary tangles; the management of cognitive deficit is still remaining elusive.[3,5,6] Donepezil hydrochloride (DNP) is an AChE inhibitor approved by the U.S. Food and Drug Administration (US-FDA) for treatment of AD in all stages.[7−9] Currently, DNP is marketed as an oral tablet or capsule under various brand names Aricept, Aricep, Depzil, and so forth. The daily repeated administration is essential at a dosage of 5−23 mg based upon the severity of the patient.[10] The major challenges associated with AChE inhibitors are gastrointestinal alterations and hepatotoxicity.[11,12] Further, usage of high doses

Table 1. Design and Characterization of mPEG−PCL Polymeric Nanoparticles by Nanoprecipitation Technique

		responses					
independent factors		experimental values			predicted values		
drug/polymer	solvent/nonsolvent	particle size (nm)	PDI	% DL[a]	particle size (nm)	PDI	% DL[a]
0.1	0.25	85.52	0.17	7.15	86.70	0.15	9.72
0.25	0.4	121.48	0.22	16.15	126.49	0.23	16.70
0.25	0.1	187.25	0.17	11.28	178.57	0.19	11.64
0.5	0.25	99.65	0.27	15.87	103.06	0.24	16.11
0.5	0.25	102.79	0.26	16.48	103.06	0.24	16.11
0.5	0.25	96.87	0.21	15.36	103.06	0.24	16.11
0.5	0.25	104.47	0.25	16.12	103.06	0.24	16.11
0.5	0.25	109.75	0.23	17.07	103.06	0.24	16.11
0.5	0.02	343.94	0.39	16.87	354.89	0.38	16.59
0.5	0.48	234.52	0.34	22.78	227.61	0.34	22.25
0.75	0.1	348.22	0.52	17.15	338.46	0.51	17.53
0.75	0.4	220.58	0.44	19.28	224.52	0.42	19.85
0.9	0.25	290.51	0.55	17.33	293.04	0.57	16.94

[a]% DL = drug loading.

of DNP results in painful urination, seizures, chest pain, and gastric-associated problems.[13]

Nanoparticulate drug delivery systems are highly explored by the researchers to enhance the drug permeation across the brain thereby resulting in reduced toxicity.[14−16] Several approaches have been reported to enhance the brain uptake of AChE inhibitors including nanocarrier delivery systems like liposomes, solid lipid nanoparticles, and polymeric nanoparticles which have attracted a significant interest.[17,18] However, most of the reported methods have used an alternate route for administration like intravenous, intranasal, or transdermal. DNP-loaded nanosuspension improved brain delivery by direct olfactory administration.[19] Polysorbate 80 coated with poly(n-butylcyanoacrylate) nanoparticles and liposomes decorated with cell penetrating peptide exhibited a significant improvement in the brain uptake via intravenous administration for rivastigmine.[20,21] To date, very few methods have been reported to increase the brain uptake by oral administration. Double-coated poly(butylcyanoacrylate) nanoparticles and polysorbate 80-coated polylactide-co-glycolide nanoparticulate delivery systems have shown significant brain targeting efficacy for oral delivery.[22,23] In the previous reports, paclitaxel was conjugated with a shuttle peptide and stapled RGD peptide to improve its brain delivery.[24,25] Recent studies found that the multifunctional monosialotetrahexosyl ganglioside (GM1)-modified reconstituted high density lipoprotein (GM1-rHDL) has a therapeutic potential not only in accelerating Aβ clearance but also in inhibiting the subsequent pathological cascades.[26] Moreover, lipoprotein (ApoE3)-based nanocarriers exhibited a promising ability to improve brain uptake, binding to amyloid beta (Aβ) with high affinity and accelerating its clearance.[27,28] Therefore, our aim in this study was to develop a suitable lipoprotein nanocarrier system for effective targeting to the brain, which results in dose reduction and improved therapeutic efficacy for AD. Herein, we have opted to develop a polymeric nanoparticle surface coated with ApoE3 for enhanced brain uptake and amyloid beta clearance by oral route.[27,28] Polymeric nanoparticles showed better tissue permeability and stability, however, their utility is limited by scale up issues, biodegradation, and toxicity. Thus, self-assembled polymeric nanoparticles composed of biodegradable amphiphilic block copolymers have emerged out as an

attractive approach. It has been reported that small-sized methoxy poly(ethylene glycol)-block-poly(lactic-co-glycolic acid) [mPEG−PLGA] nanoparticles can improve the transport of drugs across brain capillary cells, oral bioavailability, brain uptake, and also bioactivity.[29] Destabilizing effect on amyloid fibril formation and the ability to cross blood brain barrier has been investigated on DNP-loaded PLGA-b-PEG nanoparticles.[30] Several reports have concluded that poly(ethylene glycol) corona of long-circulating polymeric nanoparticles favors interaction with the Aβ peptide both in solution and in serum.[31] Amphiphilic block copolymers also have a hydrophilic corona, which could result in the formation of stealth properties and colloidal stability. Moreover, polymeric nanoparticles are excellent nanocarriers which can easily be altered by surface modifications for targeting and better therapeutic efficacy. Methoxy poly(ethylene glycol)−polycaprolactone (mPEG−PCL) has a tendency to avoid nanoparticle uptake by phagocytic cells in the liver and can significantly reduce the gastric mucosal irritations.[32,33]

Keeping the above stated facts in view, we have selected mPEG as the hydrophilic segment and ε-caprolactone as the hydrophobic segment to form mPEG−PCL amphiphilic polymer synthesized in-house and used for the delivery of DNP for the first time. The fabrication of DNP-loaded mPEG−PCL nanoparticles were optimized by quality by design approach (QbD) to achieve higher drug loading and lower particle size (<200 nm) and polydispersity index. Optimized formulation was further surface modified with brain targeting lipoprotein and tested for gastric stability, cellular studies, pharmacokinetics, brain uptake, and therapeutic efficacy after oral administration. Additionally, in vitro in vivo correlation (IVIVC) studies were performed for the prepared formulations using Phoenix WinNonlin 8.0 with an add-on IVIVC toolkit (CertaraTM, U.S.A.) software.

2. RESULTS AND DISCUSSION

2.1. Synthesis and Characterization of Amphiphilic Diblock Copolymers. mPEG−PCL was synthesized by simple ring opening polymerization reaction of ε-caprolactone by using tin(II) 2-ethylhexanoate as a catalyst. The ^{1}H NMR data confirmed the synthesis of mPEG−PCL diblock copolymer (Supporting Information, Figure S1). As per [1]H
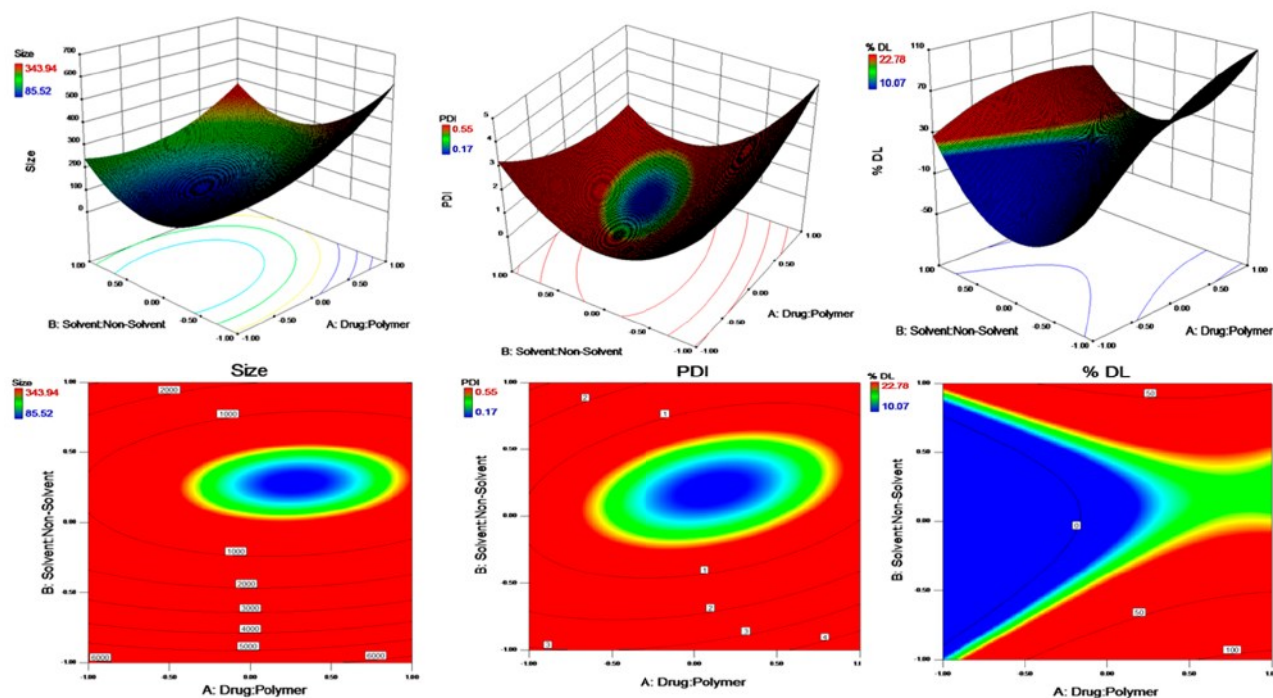
Figure 1. Surface response plots showing the effect of drug/polymer ratio (w/w) and solvent/nonsolvent ratio (v/v) on particle size (nm), PDI, and % drug loading; respective figures below each indicate their contour plots.

NMR spectrum, the presence of peaks at δ 1.39, 1.66, 2.32, and 4.07 represents the formation of polycaprolactone and characteristic peaks at δ 3.66 and 3.39 indicate the presence of methylene units of PEG. Molecular weight of diblock copolymer was determined by ^{1}H NMR and GPC. ^{1}H NMR data represents molecular weight of the polymer to be ~15 207 Da whereas GPC data showed Mn and Mw of 12 147 and 14 955 Da respectively with molecular weight distribution of 1.98 (Supporting Information; Figure S2). Yield = 89%.

2.2. Optimization of Polymeric Nanoparticles. The optimization of drug-loaded polymeric nanoparticles was fabricated by quality by design (QbD) approach using design of experiments. Moreover, it suggests the optimized batch using a minimum number of trials by statistically varying process parameters. Response surface methodologies such as CCD and Box-Behnken design are normally explored for evaluating the differences between the variables. However, we have used CCD design for optimizing polymeric nanoparticles. Polymeric nanoparticulate system prepared by a nano-precipitate technique mainly relies on the rapid diffusion of solvent across the interface between the solvent−polymer and aqueous phase. From the preliminary studies, the drug/polymer and the solvent/nonsolvent ratios showed significant effect on selected responses such as mean particle size, PDI, and percent drug loading. Five levels of independent variables were selected (Supporting Information; Tables S1 and S2) and the results obtained are represented in Table 1. This design gives information about polynomial equations involved in interaction effects of independent variables and its responses. The validation of these polynomial equations generated by software were evaluated statistically by using analysis of variance. Response surface plots generated were used to study the effects of the predesigned responses such as particle size, PDI, and percent drug loading. The attentive observation of polynomial equations and surface plots results in under-

standing the effects of each variable independently and in combination with other variables on each response.

The full quadratic model equations for particle size (Supporting Information, S1), PDI (Supporting Information, S2) and % drug loading (Supporting Information, S3) were generated by response surface regression process by using design expert software as follows

$$\text{Particle size} = +355.408 - 196.75A - 1865.09B - 345.8AB + 536.732A^2 + 3537.891B$$

$$\text{PDI} = +0.218 + 0.016A - 0.779B - 0.866AB + 0.717A^2 + 2.261B^2$$

$$\% \text{ drug loading} = +5.805 + 30.972A - 9.887B - 18.266AB - 17.363A^2 + 62.651B^2$$

where, A is drug/polymer ratio (w/w) and B is solvent/nonsolvent ratio (v/v)

By clearly examining the coefficient of equations and response surface plots (Figure 1), it shows that the particle size, PDI, and percent drug loading are highly dependent on both the selected independent variables. The positive and negative signs in the polynomial equation suggest the increase and decrease in particle size, PDI, and percent drug loading with respect to the factor. As there is an increase in the drug/polymer ratio, an increase in the particle size, PDI, and percent drug loading was observed. Herein, the amount of drug was kept constant by varying the amount of polymer. From the response surface plots, the amount of polymer increase resulted in a decrease in particle size and PDI. This kind of behavior might be due to the higher concentration of hydrophilic moiety (mPEG) as the polymer concentration increases. The role of mPEG in nanoparticle is to act as a capping agent which in turn behaves as a shield on the growing particle hence the particle cannot grow after certain extent. Increasing the concentration of mPEG in turn reduces the average size of particles and PDI. Moreover, with the increase in drug/polymer ratio, enhancement of percent drug loading

was observed but above the ratio of 0.5 the particle size and PDI increased in steady increments.

An increase in solvent/nonsolvent ratio, the particle size, PDI, and percent drug loading were found to decrease until the ratio of 0.25 and after that the increase was observed. This kind of alternations may be because of a change in the viscosity of the dispersion formed during processing. Increased viscosity of the dispersion by alternation of solvent/nonsolvent ratio resulted in high viscous resistance against the shear force during the forming of nanoparticles.

The overlay plot was generated for the prediction of optimized formulation, and the independent variables were optimized by numerical and graphical methods, whereas optimized formulation was selected from design space in the overlay plot (Supporting Information; Figure S3). The selected criterion for the optimized region was to obtain minimal particle size, PDI and maximum percent drug loading. Moreover, the predicted results obtained for the responses such as particle size, PDI and percent drug loading were validated by comparing them to the actual experimental values. The differences between the predicted and experimental values were found to be from −6.34 to 5.11. The predicted versus experimental values demonstrated correlation with each other indicating good reliability of the model (Table 2).

Table 2. Experimental Design Model Validation

responses	predicted values	experimental values	% residuals
particle size (nm)	100.76	107.15 ± 3.64	−6.34
polydispersity index	0.22	0.21 ± 0.08	4.55
% drug loading	17.43	16.54 ± 1.21	5.11

The morphological characteristics and absolute size of the nanoparticles were determined by FE-SEM and HR-TEM (Figure 2). The absolute size was found to be between 100 and 120 nm for both characterization techniques which is concordant with dynamic light scattering (DLS) size. Nanoparticles were found to be uniformly spherical in shape without showing any aggregation. Redispersibility index was found to be 1.02 ± 0.02 with 5% trehalose. The residual content analysis of the organic solvent acetonitrile and triethylamine was estimated by headspace-gas chromatography. As per guidelines, acetonitrile belongs to class 2 solvents and triethylamine belongs to class 3 solvents and the content of organic solvent in the lyophilized formulation was found to be below the acceptable limits (Supporting Information, S4).

2.3. Surface Coating of Optimized Nanoparticles by Polysorbate-80 and ApoE-3 Conjugation to the Coated Nanoparticles. The particle size of D1-NPs before coating was found to be 107.15 ± 1.48 with a zeta potential of 0.79 ± 0.26, whereas after coating with 1% polysorbate-80 the particle size was 124.26 ± 2.19 with a zeta potential of −6.97 ± 0.99. However, the D3-NPs showed the particle size of 137.65 ± 2.69 with a zeta potential of −2.08 ± 0.36. The increased particle size of the polymeric nanoparticles provides a coating over the surface. The conjugation efficiency of ApoE3 was found to be 27.21 ± 2.58%.

2.4. Protein Binding Assay. Protein adsorption plays an important role in stability of nanoparticles in the blood. The nanoparticles with a lipophilic surface have a tendency to adsorb proteins on their surface, which can result in promoting their opsonization and macrophage recognition, therefore increasing the clearance rate leads to elimination from systemic
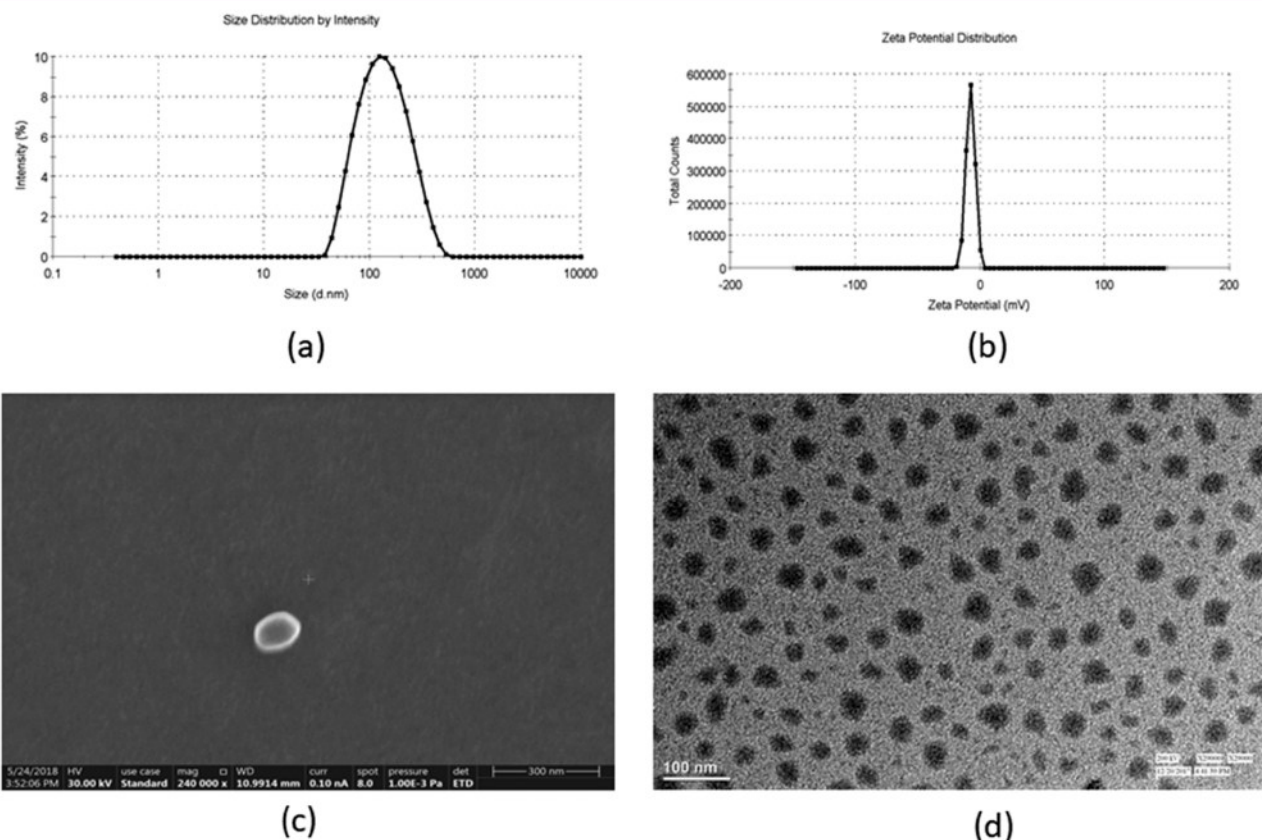

(a)

(b)

(c)

(d)

Figure 2. Characterization of polymeric nanoparticles by DLS (a) size, (b) zeta potential, and absolute size by (c) FE-SEM and (d) HR-TEM.

circulation. By utilizing hydrophilic moieties like PEG or poly(ethylene oxide) (PEO), researchers have tried to reduce the protein adsorption of the nanoparticles. PEG and PEO helps in the formation of "stealth" nature to the nanoparticles, resulting in decreased clearance rate leading to increased blood circulation by restricting interactions with the components of bloodstream.[34,35] These compounds are nontoxic and already classified as Generally Regarded as Safe (GRAS) by the United States Food and Drug Administration. In this study, we have used PEG in the polymeric system and incubated D1-NPs, D2-NPs, and D3-NPs with 4% BSA (physiological concentration). The protein adsorption for D1-NPs, D2-NPs, and D3-NPs were found to be 4.15 ± 2.12, 3.89 ± 1.79, and 3.59 ± 2.08%, respectively. The presence of PEG units over the surface of nanoparticles resulted in the low protein adsorption, thereby increasing the circulation half-life in blood.

2.5. In Vitro Drug Release Studies. The in vitro drug release pattern for the nanoformulations were studied using a dialysis method, and the drug release profile is shown in Figure 3. DNP loaded nanoparticles observed a biphasic release
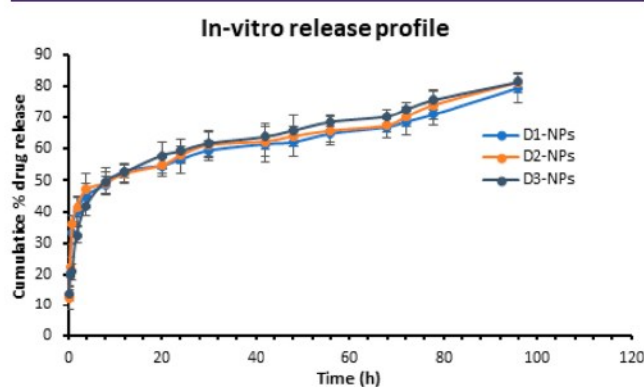

Figure 3. In vitro drug release profile of DNP at pH 7.4 (mean ± SD, $n = 3$)

pattern, and an initial burst release within 1 h of ~40% of drug was released followed by a sustained release pattern until 96 h. It can be concluded from the release studies that the mPEG–PCL nanoparticles exhibit sustained a drug release pattern. The sustained drug release from all of the nanoparticles can be ascribed to better encapsulation of the drug in the interiors of the nanoparticles, as shown from percent entrapment studies. To predict the release pattern mechanism, the data was fitted in different mathematical models like zero order, first order, Higuchi, Korsmeyer–Peppas, and Hixson–Crowell. Table 3 shows the release constants and its regression coefficient values for the respective model. On the basis of the regression coefficient values, it can be concluded that all the nanoparticles exhibit Korsmeyer–Peppas model with a Fickian diffusion release pattern ($n < 0.43$). This release mechanism might be

due to degradation of the nanoparticles and leads to creation of fine pores through which the DNP can be diffused.

2.6. Stability Studies. *2.6.1. Stability of Polysorbate-80-Coated and Apo-E-Functionalized Polysorbate-80-Coated Nanoparticles in SGF and SIF.* The effect of simulated biological fluids on particle size, PDI and percent entrapment efficiency (EE) is shown in Table 4. Significant changes in particle size, PDI and percent EE were observed in D2-NPs due to degradation of the surface coating. Minor differences in particle size, PDI and percent EE were observed in D3-NPs although the changes were insignificant due to the stability of coating in biological fluids. Moreover, it was observed that a percentage of surfactant coating remaining in SGF was 89.48 ± 6.4% for D3-NPs and 46.8 ± 5.3% for D2-NPs. A similar pattern was observed after 6 h of incubation in SIF, where the percentage of coating remaining for D3-NPs was found to be 86.54 ± 3.9% and 39.72 ± 5.4% for D2-NPs. These findings suggest that the percentage coating of polysorbate-80 remaining/protected was significantly more in D3-NPs than D2-NPs due to the stability of ApoE3 in gastrointestinal fluids.

2.6.2. Long-Term Stability Studies. The long stability study for nanocarrier delivery system is the major parameter to be considered and should thoroughly characterize its resuspendability. Long-term stability studies were performed as per the ICH guidelines for all formulations. The size, PDI, and zeta potential were evaluated for 3 months where the formulations were stable and did not show any significant change in comparison to initial sample (Table 5). Moreover, lyophilized polymeric nanoparticles showed less than 1 min for redispersion. Thus, the designed lyophilized formulations were found to be stable under various storage conditions.

2.7. Cell Culture Studies. *2.7.1. Cell Viability Assay.* The cytotoxicity of pure drug and nanoparticles were determined in the HepG2 and U-87 cells using the MTT cytotoxicity assay. The MTT assay measures cellular oxidative metabolic activity based on the activity of NAD(P)H-dependent dehydrogenase enzymes. The effect of various concentrations (50−500 μg/mL) of DNP on the viability of HepG2 (Supporting Information; Figure S4) and U-87 cells (Figure 4) were explored. The cell viability above ~85−90% in the concentration range 50−150 μg/mL, indicates the biocompatibility and nontoxicity of the polymeric nanoparticles. However, DNP showed 50−60% cell viability in HepG2 cells and 60−70% viable cells in U87 cell lines at the concentration range 300−500 μg/mL, whereas DNP polymeric nanoparticles showed slightly less cytotoxicity in both HepG2 and U-87 cells. These results indicate that the DNP polymeric nanoparticles are biocompatible in the concentration range 50−500 μg/mL and can further be utilized at slightly higher concentrations and thus are appropriate as drug carriers.

2.7.2. Cellular Uptake Studies. D3-NPs showed significantly higher uptake into the U87 cells (85.96 ± 1.86) in

Table 3. Drug Release Kinetic Modeling

	D1-NPs		D2-NPs		D3-NPs	
mathematical modeling	parameters	R^2	parameters	R^2	parameters	R^2
zero order	K_0: 1.08	0.8598	K_0: 1.11	0.8618	K_0: 0.58	0.8732
first order	K_1: 0.02	0.9147	K_1: 0.03	0.9130	K_1: 0.03	0.9476
Higuchi	K_H: 9.30	0.9350	K_H: 9.50	0.9326	K_H: 9.67	0.9550
Korsmeyer–Peppas	K_K: 30.27; n: 0.19	0.9735	K_K: 31.14; n: 0.19	0.9688	K_K: 26.87; n: 0.23	0.9871
Hixson–Crowell	K_{HC}: 0.007	0.8996	K_{HC}: 0.007	0.8998	K_{HC}: 0.008	0.9295

E

Table 4. Stability of Polymeric Nanoparticles in Simulated Gastric Fluids[a]

media	particle size (nm)				PDI				% EE			
	D2-NPs		D3-NPs		D2-NPs		D3-NPs		D2-NPs		D3-NPs	
	initial	final	initial	final	initial	final	initial	final	initial	final	initial	final
SGF pH 1.2	122.48 ± 2.09	95.12 ± 3.15	138.48 ± 2.94	141.88 ± 1.98	0.17 ± 0.03	0.33 ± 0.12	0.19 ± 0.06	0.22 ± 0.06	90.79 ± 2.91	64.15 ± 3.51	91.09 ± 1.98	88.29 ± 2.15
SIF pH 6.8	122.48 ± 2.09	86.23 ± 2.18	138.48 ± 2.94	143.18 ± 2.33	0.17 ± 0.03	0.45 ± 0.11	0.19 ± 0.06	0.21 ± 0.09	90.79 ± 2.91	59.18 ± 4.39	91.09 ± 1.98	86.18 ± 2.36

[a]Results were represented as mean ± SD, $n = 3$.

comparison to the pure drug (45.94 ± 2.65); this might be due to its nanosize and surface modifications for active targeting (Figure 4).

2.8. Pharmacokinetic Studies. The pharmacokinetic studies for pure drug, D1-NPs, D2-NPs, and D3-NPs were performed in wistar rats. Mean ± SD plasma concentration versus time profile for DNP solution and all the nanoparticles is shown in Figure 5. As per our previous report, a biexponential decline in the plasma concentration was observed for DNP, hence data was fitted in a two-compartmental model.[36] After oral administration of DNP and its formulations at a dose of 5 mg/kg, the drug was absorbed quickly and the peak plasma concentration was reached at approximately 0.7 h as represented in the Table 6. The D3-NPs exhibited ~6.1-fold ($p < 0.05$) increase in plasma elimination half-life ($t_{1/2\beta}$) than DNP solution, indicating slow distribution and elimination of the drug. The $AUC_{0-\infty}$ was found to be higher in D3-NPs (16532.19 ± 776.54 ng·h/ml) in comparison to other formulations and the pure drug, which indicated the long circulating ability of the formulation in plasma. The clearance (Cl) of D3-NPs showed ~13.38-fold ($p < 0.05$) decrease than the pure drug.

2.9. In Vitro in Vivo Correlation. Level-A (IVIVC) model was developed using the drug dissolution and in vivo data.[32] The IVIVC profile is essential for dosage form development and also for quality control point of view. IVIVC plot displaying correlation between plasma concentration (ng/mL) and fraction bioavailability versus time (h) of the formulations were determined (Supporting Information; Figure S5). Further, statistical parameters percent prediction errors for C_{max} and AUC were within the limits with a fold error less than 10 for the selected internal validation formulation (Supporting Information; Table S3).

2.10. Biodistribution Studies. Biodistribution studies were performed to identify the potential nanoformulation for the brain targeting efficiency after oral administration. DNP distribution pattern was quantified in all the major organs. The concentration time profiles of brain and liver are represented in Figure 6. It has been observed that the D3-NPs showed ~5.8-fold ($p < 0.05$) increase in the brain uptake and ~15.30-fold ($p < 0.05$) decrease in brain clearance in comparison with the pure drug (Table 7). Moreover D1-NPs and D2-NPs also exhibited the same trend but the highest brain uptake was observed in D3-NPs due to be presence of ApoE3 lipoprotein carrier. Moreover, D3-NPs has a strong affinity with LDL-R, binds specifically to these receptors, and enters the brain due to the presence of ApoE3 coating. Apart from this, ApoE3 has antioxidant and antiamyloidogenic activity which helps in rapid amyloid beta clearance.[37] DNP has been reported for hepatoxicity due to excess accumulation at liver, and D3-NPs showed significant decrease in the liver might be because of bypassing of liver and targeting moieties attached over the surface of nanoparticles.[32] On the other hand, significantly reduced concentrations were noticed in the other organs also (Supporting Information; S5). Moreover, these finding suggested that the biodistribution studies were complementary to the systemic pharmacokinetic data as well as cellular uptake studies by U-87 cells.

2.11. Pharmacodynamic Studies. To explore the pharmacodynamic efficacy of DNP, the AChE inhibitory activity was estimated in the brain samples (Figure 7). The data obtained from the pharmacodynamic studies are in correlation with the brain concentration levels, as the

Table 5. Long-Term Stability Studies[a]

	long-term stability studies (storage conditions 25 ± 2 °C, 60 ± 5% RH)								
	size (nm)			PDI			zeta potential (mV)		
sampling time	D1-NPs	D2-NPs	D3-NPs	D1-NPs	D2-NPs	D3-NPs	D1-NPs	D2-NPs	D3-NPs
initial	107.15 ± 1.48	124.26 ± 2.19	137.65 ± 2.69	0.24 ± 0.04	0.18 ± 0.02	0.21 ± 0.05	0.79 ± 0.26	−6.97 ± 0.99	−2.08 ± 0.36
after 1 month	110.25 ± 2.18	128.15 ± 1.98	141.32 ± 3.65	0.26 ± 0.06	0.21 ± 0.06	0.19 ± 0.07	0.81 ± 0.15	−7.04 ± 1.19	−2.22 ± 0.63
after 2 month	115.18 ± 3.18	131.09 ± 4.12	148.18 ± 2.65	0.25 ± 0.03	0.19 ± 0.04	0.22 ± 0.06	0.72 ± 0.33	−6.88 ± 0.98	−2.49 ± 0.54
after 3 month	114.09 ± 2.41	129.48 ± 3.29	145.17 ± 3.18	0.26 ± 0.04	0.21 ± 0.03	0.23 ± 0.08	0.80 ± 0.19	−7.07 ± 0.89	−2.95 ± 0.71

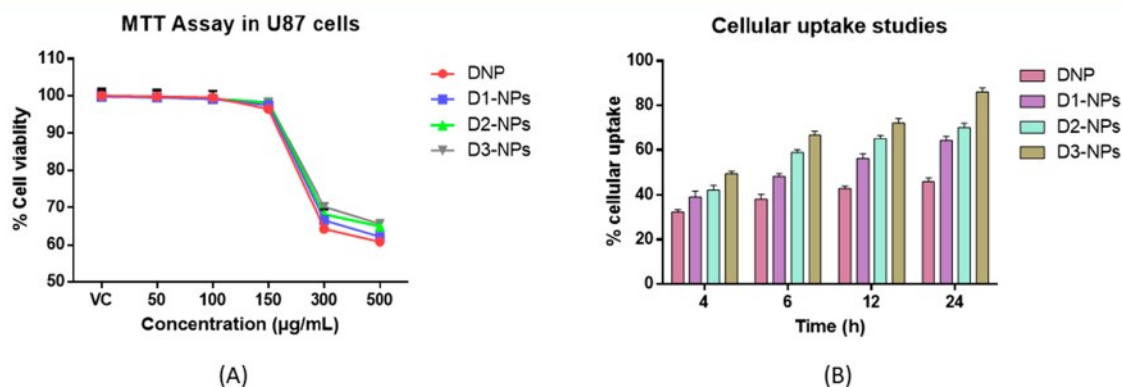[a]Results were represented as mean ± SD, n = 3.

Figure 4. Cell culture studies of nanoparticles in (A) MTT assay in U87 cells. (B) Cellular uptake studies in U87 cells.
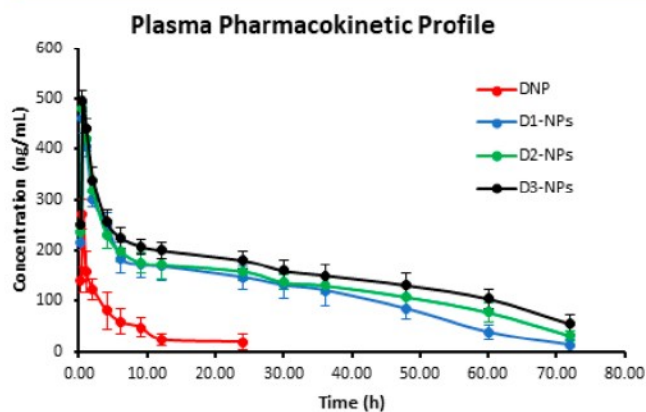

Figure 5. Plasma concentration−time profile for DNP (mean ± SD, n = 4).

maximum inhibitory activity was achieved in 1 h itself; thereafter, a decrease in activity was observed. Moreover, in D3-NPs the AChE inhibitory activity was found to be significantly high in comparison with the pure drug. The maximal inhibitory action was observed at 1 h, which clearly indicates the pharmacological action of DNP and in agreement with the tissue distribution data. Therefore, the data obtained in this study was found to be reliable and can be utilized for pharmacokinetic−pharmacodynamic modeling.

3. CONCLUSION

In summary, we have fabricated ApoE3 coated polymeric nanoparticles using QbD approach for enhancing the brain uptake by oral route. Brain permeation of neuroactive agents is the major challenge. The in vitro data in simulated gastric fluids suggested that polysorbate 80 coating was found to be stable in ApoE3-coated nanoparticles and the concentration of polysorbate 80 remaining on the surface was in agreement with

Table 6. Pharmacokinetic Parameters of DNP after Per Oral (5 mg/kg) Administration in Rats

| parameter	units	pure drug	D1-NPs	D2-NPs	D3-NPs
T_{max}	h	0.48 ± 0.01	0.70 ± 0.03	0.69 ± 0.02	0.69 ± 0.02
C_{max}	ng/mL	237.76 ± 56.18	446.46 ± 89.15	466.16 ± 72.29	484.61 ± 86.49
$T_{1/2\alpha}$	h	0.28 ± 0.01	0.51 ± 0.02	0.67 ± 0.06	0.68 ± 0.05
$T_{1/2\beta}$	h	6.30 ± 0.91	28.83 ± 2.19	39.39 ± 3.48	44.79 ± 3.18
K_{01}	1/h	2.69 ± 0.06	2.42 ± 0.09	2.84 ± 0.05	2.99 ± 0.15
K_{10}	1/h	0.43 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.04 ± 0.01
K_{12}	1/h	1.50 ± 032	0.87 ± 0.19	0.67 ± 0.09	0.63 ± 0.06
K_{21}	1/h	0.63 ± 0.04	0.42 ± 0.05	0.33 ± 0.04	0.36 ± 0.03
AUC	h × ng/mL	1235.57 ± 298.48	10203.48 ± 315.15	13097.33 ± 395.14	16532.19 ± 386.12
CL	mL/h/kg	4046.71 ± 198.15	490.03 ± 95.11	381.76 ± 86.28	302.44 ± 54.48
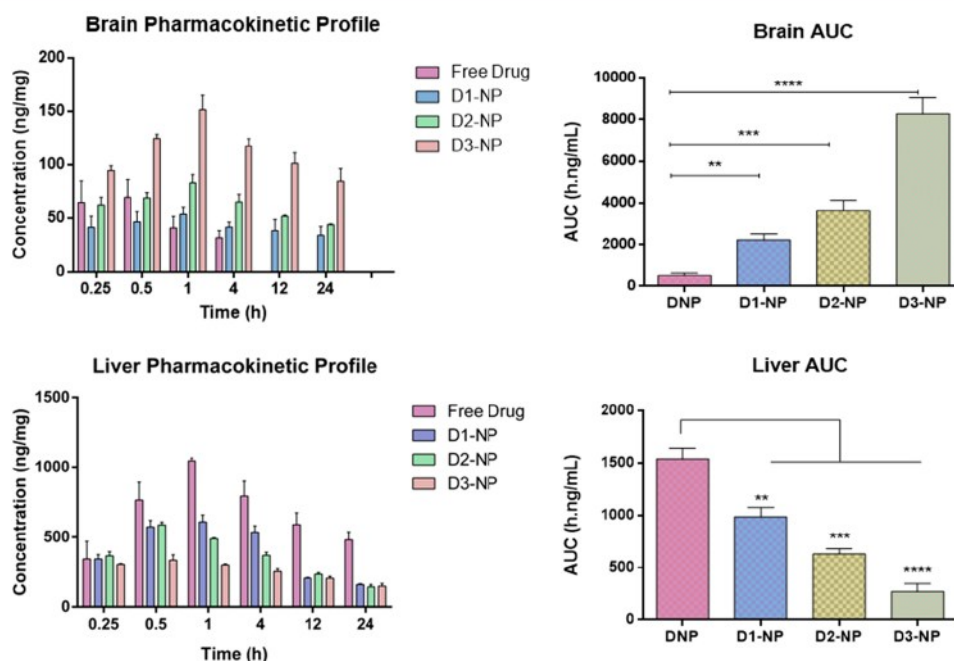
Figure 6. Brain and liver concentration−time profile for DNP (mean ± SD, $n = 3$), * indicates $p < 0.05$, when compared with the pure drug.

Table 7. Brain Pharmacokinetic Parameters of DNP after Per Oral (5 mg/kg) Administration in Rats

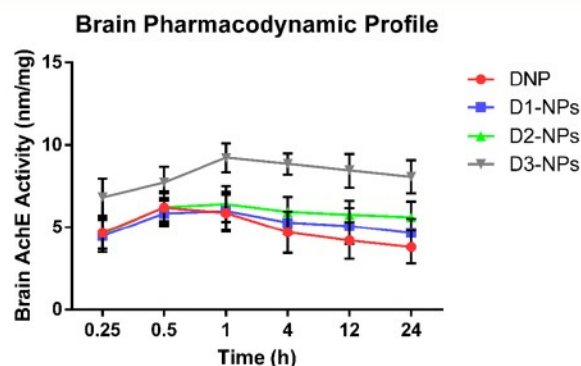| parameters	units	pure drug	D1-NPs	D2-NPs	D3-NPs
T_{max}	h	0.50 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
C_{max}	ng/mg	69.82 ± 16.35	53.90 ± 21.59	83.34 ± 26.94	151.51 ± 34.18
MRT	h	11.72 ± 2.98	40.42 ± 7.26	52.20 ± 9.54	67.54 ± 8.66
AUC	h × ng/mL	541.01 ± 86.45	2226.63 ± 136.59	3629.89 ± 269.89	8274.35 ± 298.45
CL	mL/h/kg	9241.91 ± 120.69	2245.54 ± 109.85	1377.45 ± 70.98	604.28 ± 55.32

Figure 7. Brain pharmacodynamic data for DNP (mean ± SD, $n = 3$).

the initial coating amount. Moreover, level A correlation was established using in vitro and in vivo data. ApoE3-coated polymeric nanoparticles have improved pharmacokinetic profile and exhibited significant increase in the brain levels of DNP, which concludes the improvement in the brain uptake of DNP. From the pharmacodynamic data it is evident that the AChE activity was significant with the level of DNP in the brain.

4. METHODS

4.1. Chemicals. DNP and loratadine were kind gift samples of Vasudha Pharma Chem Limited (Hyderabad, India). HPLC grade solvents acetonitrile, methanol and dimethylformamide, dichloromethane, hexane, and isopropyl alcohol were procured from Merck Millipore (MA, U.S.A.). Coomassie brilliant blue G dye was procured from Himedia (Mumbai, India). Ammonium formate, methoxy poly(ethylene glycol) (Mn, 5000), tin(II) 2-ethylhexanoate, ε-caprolactone, trehalose, polysorbate 80, pepsin, acetyl thiocholine, ApoE3, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and methyl-thiazolyldiphenyl-tetrazolium bromide (MTT) were acquired from Sigma-Aldrich (St. Louis, MO, U.S.A.). Polystyrene standards were procured from Waters (U.S.A.). Solid-phase cartridges (Clearnert PEP-3 SPE) were procured from Agela Technologies (Wilmington, U.S.A.). Ultrapure water was acquired from Milli-Q system (Merck Millipore, MA, U.S.A.). Dialysis tubing (3.5 kDa) was procured from Thermo Fischer scientific (Rockford, U.S.A.). Fetal bovine serum (FBS), dulbecco's modified Eagle's medium high glucose (DMEM), and penicillin−streptomycin solution were purchased from Gibco Life Technologies (U.S.A.). Dried toluene, triethylamine, cobalt nitrate hexahydrate, ammonium thiocyanate, bovine serum albumin, sodium chloride, monobasic potassium phosphate, sodium hydroxide, pancreatin, sodium phosphate, benzyl alcohol, and diethyl ether were procured from SISCO research laboratories (SRL, India).

4.2. Synthesis of Amphiphilic Diblock Polymer. Methoxy polyethylene glycol (Mn ∼ 5000 Da, 1 g, 0.2 mmol) was dissolved in dried toluene. ε-caprolactone (131.41 equiv, 3.0 g, 26.28 mmol) was added dropwise to the above solution in a Schlenk tube, and later tin(II) 2-ethylhexanoate (10 mol % of initiator, 15 μL) was mixed and the reaction was processed under nitrogen atmosphere for about 24 h at 110 °C.[32,33] The reaction mixture was further concentrated by using a rotary evaporator, and the product was dissolved in dichloromethane and precipitated two times by chilled methanol and third time with chilled diethyl ether. The polymer was properly dried, evaluated, and characterized by ^{1}H NMR (Bruker, Germany) and by gel permeation chromatography (GPC) (Waters, U.S.A., Styragel HR column). The polymeric sample was dissolved in

dimethylformamide at a concentration of 5 mg/mL and filtered using 0.4 μ syringe filter. The average molecular weight of the polymer was calculated by GPC equipped with Waters 2414 RI detector.

4.3. Formulation of Polymeric Nanoparticles. Polymeric nanoparticles were prepared by nanoprecipitation method.[34,38] In brief, 12.5 mg of polymer, 5 mg of DNP, and 1.6 μL of triethylamine (equal molar of donepezil hydrochloride) were dissolved in 1.8 mL of acetonitrile (organic phase) followed by the dropwise addition to 5 mL of Milli-Q water (aqueous phase) under stirring for 20 min. The resulting nanodispersion was dialyzed with distilled water for 8 h using dialysis tubing (MWCO, 3.5 k Da) to remove unentrapped drug. Water was changed twice during the course of dialysis. With the help of dynamic light scattering (DLS) method mean particle size, the polydispersity index (PDI) and zeta potential of nanoparticles were measured. Morphology of the polymeric nanoparticles was evaluated by field emission scanning electron microscopy (FE-SEM) (Apreo LoVac, FEI, U.S.A.) and transmission electron microscope (HR-TEM) (Technai G2, 200 kV, FEI, U.S.A.). Loading efficiency and entrapment efficiency of DNP in nanoparticles was measured by using the following formula.[34]

$$\text{Loading efficiency (\%)} = \frac{\text{Weight of DNP in polymeric nanoparticles}}{\text{Weight of polymeric nanoparticles}} \times 100$$

$$\text{Entrapment efficiency (\%)} = \frac{\text{Amount of DNP in polymeric nanoparticles}}{\text{Amount of DNP taken in prepartion of polymeric nanoparticles}} \times 100$$

4.3.1. Optimization of Polymeric Nanoparticles by Central Composite Design. A central composite design (CCD) was utilized to obtain the minimum number of experiments required to optimize the DNP loaded polymeric nanoparticles using Design-Expert (Version 8.0.7.1, Stat-Ease Inc., Minneapolis, MN, U.S.A.). On the basis of preliminary batches, the drug/polymer ratio (A, w/w) was varied from 0.1 to 0.9 and solvent/nonsolvent ratio (B, v/v) was varied from 0.02 to 0.48, opted as independent variables. As proposed by the design, a total of 13 runs were executed for particle size (Y_1), polydispersity index (Y_2) and percent drug loading (Y_3) as the response (dependent) variables (Table 1). Five center points were included to evaluate the reproducibility of design. This design gives information about the interaction effects of independent variables and its formulation characteristics. Statistical model for each response was selected based on the highest order polynomial where the additional terms are significant and the model is not aliased. Analysis of variance for all the responses were executed and the best fit model was statistically selected based on various parameters like multiple correlation coefficient (R^2), adjusted, and predicted correlation coefficients, standard deviation, and coefficient of variance. Graphically, 3D response surface plots were generated by software. The level of significance was considered at p-value <0.05. The optimized nanoformulation (D1-NPs) was prepared as per the predictor profile for maximum desirability to attain the targeted goal within the design space. The effect of each independent variable on responses was analyzed using CCD. Moreover, the model optimization was executed on the basis of attaining the lowest particle size and PDI with higher percent drug loading.

The optimized formulation with 5% trehalose as cryoprotectant was transferred into a 15 mL glass vial, frozen at −80 °C, and then lyophilized using Freeze-Dry System (LabConco) for 48 h. The lyophilized nanoformulation was then reconstituted by adding Milli-Q water followed by gentle agitation. The dispersed nanoformulation was evaluated for redispersibility index (S_f/S_i, ratio between particle size after and before lyophilization); if the value is lower than two, then the selected cryoprotective system was found to be efficient. Further the residual organic solvent content (acetonitrile and triethylamine) in the lyophilized nanoformulation was estimated by headspace gas chromatography.

4.4. Surface Coating of Optimized Nanoparticles by Polysorbate-80. Surface coating of optimized nanoparticles by polysorbate-80 was performed to enhance the brain uptake (D2-NPs).[23,39] In brief, freeze-dried nanoparticles were dispersed in water containing 1% of polysorbate-80 (20 mg/mL of nanoparticles). This dispersion was further bath sonicated for 10 min for complete dispersion of nanoparticles and then incubated at room temperature for 30 min. The quantification of surface coating was performed as per reported methods by colorimetric method using ammonium thiocyanate and cobalt nitrate hexahydrate.[23]

4.5. ApoE3 Conjugation with Coated Nanoparticles. The ApoE3 (20 μg/mL) was finally adsorbed to the lyophilized surface coated nanoparticles by stirring for 1 h at room temperature and further bath sonicated for 10 min (D3-NP).[37] Quantification of ApoE3 conjugation efficiency was determined as per previously reported methods by bradford assay using coomassie brilliant blue G dye.[37,40]

4.6. Protein Binding Assay. Polymeric nanoparticles were evaluated for its protein binding efficiency using Biuret assay as per reported methods.[41] Briefly, 10 mg of polymeric nanoparticles was incubated with 10 mL of 4% bovine serum albumin (BSA) and placed in an orbital shaking incubator for 24 h at 120 rpm. The nanoparticular suspension was centrifuged at 15 000 rpm, and the biuret reagent (4 mL) was added to the supernatant (1 mL) and incubated for 30 min in the orbital shaker at 120 rpm. Further, the supernatant was analyzed by UV spectrophotometry (Shimadzu UV-1800) at 540 nm for unabsorbed protein content. The standard calibration curve for BSA was plotted in the range of 500−2500 μg/mL which is used for quantification.[34]

4.7. In Vitro Drug Release Studies. The in vitro release for all the formulations viz, D1-NPs, D2-NPs, and D3-NPs was investigated in phosphate buffered saline (PBS, 0.1 M) pH 7.4 under sink conditions using dialysis membrane method. Briefly, pure drug solution and all the formulations (equivalent to 1 mg of DNP, $n = 3$) were sealed in a dialysis bag (MWCO 3.5 kDa) and dialyzed against 100 mL of PBS (pH 7.4). The samples were maintained at 37 °C in orbital shaking incubator at 120 rpm. At selected time intervals (0.25, 0.5, 1, 2, 4, 6, 9, 12, 18, 24, 36, 48, 72, and 96 h), 1 mL aliquots of the medium were withdrawn followed by replacement of same volume with the fresh release medium. All the samples were filtered and analyzed by using in-house developed and validated high-pressure liquid chromatographic method (HPLC).[36] In brief, Shimadzu HPLC system (Kyoto, Japan) operated by LC solution software (version 1.24 SP1) were utilized to analyze the samples. The analysis was carried out in Waters, Nova-Pak C18 column (3.9 × 150 mm, 4 μm) with a mobile phase consisting of acetonitrile and ammonium formate (pH 5.4; 0.01M) (60:40% v/v) in isocratic mode at a flow rate of 1 mL/min at 268 nm. Under these conditions, DNP was eluted at a retention time of 3.9 min and a calibration curve was plotted in the range of 0.1−50 μg/mL. The modeling of release data was evaluated with different kinetic models like zero order, first order, Higuchi, and Korsmeyer−Peppas to determine the mechanism of drug release. Best fit model and release mechanism were identified based on highest regression values of correlation coefficients for release data.

4.8. Stability Studies. *4.8.1. Stability of Polysorbate-80 Coated and ApoE3-Conjugated Polysorbate-80-Coated Nanoparticles in Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF).* Stability in SGF and SIF is one of the major concerns which may alter the in vivo parameters of the nanoformulations. The stability studies of D2-NPs and D3-NPs in SGF and SIF were carried out as per a previously reported method.[23] In brief, 200 μL of D2-NPs and D3-NPs were incubated with 1800 μL of respective fluids for 2 h in SGF while 6 h in SIF and the effect on particle size, PDI, percent EE, and the intactness of surface coating were determined.

4.8.2. Long-Term Stability of Lyophilized Formulation. The long-term stability study for all the formulations were performed as per the International Council for Harmonization (ICH, Q1A, R2).[34,42] Lyophilized polymeric nanoparticles were placed in the stability chamber at 25 ± 2 °C, 60 ± 5% RH for 3 months. All formulations

were monitored for its size, PDI and zeta potential after 1, 2, and 3 months.

4.9. Cell Culture Studies. *4.9.1. Cell Viability Assay.* The human liver hepatocellular carcinoma cells (HepG2) and human glioblastoma cell line (U87) were procured from National Centre for Cell Science (NCCS), Pune, India. Cells were cultured in T-25 cm^2 tissue culture flasks with DMEM supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and with 10% FBS. Cells were maintained at 37 °C in humidified 5% CO2 incubator. (Esco Incubators, India).

The cell viability of pure drug, D1-NPS, D2-NPs, and D3-NPs were measured by using MTT cytotoxicity assay on both cell lines. In brief, the cells were subcultured into sterile 96-well plate at a density of 5 × 10^3 cells per well. After incubation of the cultures for 24 h to allow the cells to reach 70−80% confluence, cells were treated with varying concentration at a range of 50−500 μg/mL of DNP and incubated for 24 hours. Further, the media were removed and replaced with fresh media (150 μL) containing 0.5 mg/mL MTT dye and incubated for 4 h at 37 °C. The media was removed and 150 μL of DMSO solution was added to each well. The absorbance was measured at 570 nm with reference wavelength at 630 nm using Tecan Infinite M200 microplate reader (Tecan, Switzerland). Percentage of cell viability was calculated as follows

$$\text{Cell Viability (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

4.9.2. Cellular Uptake Studies. To understand the cellular uptake of the developed formulations, human glioblastoma cell line (U87) was selected. Cultured cells were incubated with the pure drug, D1-NPs, D2-NPs, and D3-NPs at a concentration of 150 μg/mL ($n = 4$) for selected time intervals (4, 6, 12, and 24 h) and the untreated cells were kept as a control group. Further, media was withdrawn and centrifuged at 1500 to remove any cell or debris. DNP was extracted as per our previously developed HPLC method.[36] Briefly, 50 μL of loratadine (internal standard) and 150 μL of 10 mM ammonium formate buffer will be added to the media and followed by solid phase extraction. Further, samples were analyzed by using HPLC.

4.10. Pharmacokinetic Studies. Animal studies were performed on male wistar rats procured from central animal facility, department of pharmacy, BITS-Pilani, Pilani campus, India. Animals were kept in polyacrylic cages and maintained under controlled conditions (12 h light/dark cycle, 22 ± 1 °C with relative humidity of 60%). Animals were fed with normal pellet diet and purified water ad libitum. All the experiments were performed as per IAEC guidelines of BITS-Pilani, Pilani campus, India (protocol number IAEC/RES/22/05/Rev-1/23/25). Animals were randomly divided into four groups (each group consists of 4 animals) viz. the pure drug was dissolved in 0.9% saline, and D1-NPs, D2-NPs, and D3-NPs were administered orally at a dose of 5 mg/kg. Blood samples were collected in heparinized polypropylene disposable tubes from the retro-orbital plexus at predetermined time intervals. Plasma was separated by centrifuging the blood samples at 6000 rpm for 15 min and stored at −80 ± 10 °C until analysis.

The plasma samples were analyzed by in-house developed and validated HPLC method by using loratadine as an internal standard with solid phase extraction technique.[36] As per our previous reports, DNP follows two compartmental analysis, and different pharmacokinetic parameters such as drug concentration, area under curve (AUC$_{t-24}$, AUC$_{t-inf}$), half-life ($t_{1/2}$), volume of distribution (V_d), mean residence time (MRT), clearance (Cl), and elimination rate constant were estimated accordingly by Phoenix WinNolin Certera (Pharsight, U.S.A; version:8.0).[36,43]

4.11. In Vitro in Vivo Correlation (IVIVC). The IVIVC was explored using Level-A correlation to establish correlation between in vitro dissolution and in vivo concentration of D1-NPs, D2-NPs, and D3-NPs. The model-independent approach viz. numerical deconvolution was performed.[32] The numerical deconvolution involved the utility of a weighing fraction, unit impulse response (UIR) for which pharmacokinetics of single i.v. bolus dose of pure drug was employed.[36] Regression analysis data was used to validate the

correlation between the fraction of in vivo drug release and in vitro drug release. Analysis was executed using Phoenix WinNonlin 8.0 with an add-on IVIVC toolkit (CertaraTM, U.S.A.) software. As per FDA guidelines, the mean absolute percent prediction error (% PE) for the predicted C_{max} and AUC should not exceed more than 10%.

4.12. Biodistribution Studies. To investigate, the biodistribution of the pure drug, D1-NPs, D2-NPs, and D3-NPs was administered to wistar rats orally at a dose of 5 mg/kg, respectively. Animals were anaesthetized and sacrificed at 0.25, 0.5, 1, 4, 12, 24 h (three rats/time point) by cervical dislocation. All the organs (brain, heart, lungs, kidney, spleen, and liver) were collected promptly and thoroughly rinsed in ice-cold saline to remove blood and other content and blotted dry with filter paper. Further, the samples were prepared and analyzed as per our previously reported method.[36]

4.13. Pharmacodynamic Studies. The level of AChE is the major biological marker for AD.[44] AChE inhibitors have a tendency to restore the level of acetylcholine, and DNP enhances the level of acetyl choline in the brain by breakdown of AChE in synaptic cleft. The AChE activity in brain homogenates were carried out as per our reported method.[36,45] In brief, 50 μL of brain homogenate sample, 500 μL of 10 mM sodium phosphate buffer (pH 8), 50 μL of 100 μM acetylthiocholine iodide, and 50 μL of DTNB (Ellman reagent) were mixed and incubated for 5 min. The absorbance was measured at 412 nm using UV−visible spectrophotometer. Protein content in brain homogenates was measured by the method of Lowry et al., using BSA (1 mg/mL) as a standard.[46] Results were calculated using molar extinction coefficient of chromophore (1.36 × 104 M^{-1} cm^{-1}) and expressed as nanomoles per milligram protein. Maximal AChE inhibition time required for DNP and its formulations were estimated in brain homogenates.[36]

4.14. Statistical Analysis. The data is expressed as mean ± standard deviation (SD) and all experiments were independently repeated at least three times. Statistical analysis was assessed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison tests calculated using GraphPad prism (v 6.0) with p-values <0.05.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.9b00343.

> Characterization of polymer including ^{1}HNMR and GPC; QbD optimization of nanoformulation; residual organic content estimation in the optimized nanoformulation; MTT assay in HepG2 cell lines; IVIVC model statistical validation; biodistribution data analysis (PDF)

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K.V.K. designed, performed, and wrote the manuscript. G.W. helped in pharmacokinetic data analysis. A.A. and G.S. helped in the discussion of results and drafting the manuscript. N.K. and R.K. helped in performing cell culture studies. S.K.D. and R.N.S. conceived and approved final version of the manuscript.

Notes
The authors declare no competing financial interest.

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Herbal Medicines in Neurodegenerative Disorders: An Evolutionary Approach through Novel Drug Delivery System

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ABSTRACT: Neurodegenerative disorders are one of the most debilitating, chronic, and often irreversible disorders of the brain and have gained a widespread attention of researchers. Since current therapeutics seem to be inadequate treatment of these disorders, scientists are exploring their options with herbal drugs. This review discusses herbal constituents with the potential to treat neurological disorders. These herbal drugs, when combined with a novel delivery system, provide added advantages to nanocarrier systems. The review also presents research on different herbals through liposomes, polymeric nanoparticles, and solid lipid nanoparticles. The data are collected from various online databases, including PubMed, ScienceDirect, references from relevant review articles, and other official publications. Broad search term criteria are followed for searches so that all pertinent information is included.

KEY WORDS: Alzheimer's disease, herbal drugs, oxidative stress, mitochondrial dysfunction, novel delivery system

ABBREVIATIONS: AD, Alzheimer's disease; **BDNF**, brain-derived neurotrophic factor; **GSK**, glycogen synthase kinase; **HD**, Huntington's disease; **HSP**, heat shock proteins; **NP**, nanoparticles; **PD**, Parkinson's disease; **PEG**, polyethylene glycol; **PLA**, polylactic acid; **PLGA**, poly(lactic-co-glycolic acid)

I. INTRODUCTION

Neurodegenerative disorders are a collective terminology comprising disorders that significantly affect the central nervous system.[1] Disorders that can be included in the category of neurodegenerative disorders are Alzheimer's disease, Parkinson's disease (PD), Huntington's disease, spinal muscular atrophy, and spinocerebellar ataxia.

All these diseases are well characterized by progressive neuronal damage leading to dementia, and they are commonly found in people older than 65 years.[2–4]

According to the latest Diagnostic and Statistical Manual of Mental Disorders (DSM), dementia is characterized by such symptoms as defective memory, speech, judgment, learning, logical reasoning, language, planning, and a difficulty in performing day-to-day activities.

Based on reports from the US Food and Drug Administration (FDA), there are more than 5 million people suffering from Alzheimer's disease in the United States alone and estimated that there are currently 46.8 million people suffering from dementia, globally. Despite the painstaking efforts of scientists all over the world to find treatments for Alzheimer's disease, the FDA has managed to approve only five drugs: tacrine, donepezil, galantamine, rivastigmine, and the latest approved drug, memantine, in 2003.[5,6]

According to reports made by the Parkinson's Disease Foundation, more than 10 million people are suffering from PD globally. In addition, the number of people diagnosed at an age less than 50 years is less than 4%. In the United States, there are more than 60,000 people diagnosed with Parkinson's disease every year.[7,8]

Drugs approved by the FDA for treating neurodegenerative disorders often cause many undesirable side effects and poor pharmacokinetics parameters.[9,10] For this reason, the focus has been

shifted to find herbal medicines that can be used to treat neurodegenerative disorders.

Some common pathological processes of neurodegenerative disorders are oxidative stress, mitochondrial dysfunction, and protein aggregation.[11,12] These diseases also show evidence of oxidative damage, ion and metal dyshomeostasis, aberrant signal transduction, and mitochondrial dysfunction, which leads to cell death. Oxidative stress is the process of unregulated production of reactive oxygen species, such as hydrogen peroxide, superoxide, nitric oxide, and highly reactive hydroxyl radicals, which finally increases the metal content in cells and leads to neuronal cell death.

II. METHODOLOGY

An extensive literature search was conducted on recent developments of herbal therapies used to treat neurodegenerative disorders. Various search engines were explored, such as Google Scholar (http://scholar.google.com), and database searches were also carried out. Databases included PubMed (http://www.ncbi.nlm.nih.gov/pubmed), Medline (https://www.medline.com), Scopus (http://www.scopus.com), and ScienceDirect (http://www.sciencedirect.com). This review investigates the herbal constituents that have the potential to treat neurological disorders. These herbal drugs, when combined with a novel delivery system, provide added advantages of nanocarrier systems. The review also reveals research done on different herbal compounds combined with liposomes, polymeric nanoparticles, and solid lipid nanoparticles.

III. HERBAL MEDICINES IN NEURO-DEGENERATIVE DISORDERS

Medicinal plants have been known for their therapeutic properties since the Vedic Period. Because of their low toxicity, herbal medicines are attracting the attention of scientists. The previoiusly mentioned problems associated with current therapeutics, scientists are now emphasizing novel drug delivery systems of herbal medicines for the treatment of neurodegenerative disorders.[13,14] Use of herbal medicines are well explored in other conditions such as those affecting the eye, some of which have used targeted drug delivery systems.[15–17]

Important herbal medicines known for their abilities to ameliorate the symptoms of neurodegenerative disorders are discussed in the next sections.

A. Curcumin

Curcumin, a polyphenolic antioxidant found in turmeric (*Curcuma longa*) has been widely studied for its multifarious properties in different disorders including Alzheimer's disease.[18] Curcumin is known to bind β-pleated sheets (on the positive charge on amino acids) and modulates aggregation of Aβ-pleated sheets because of diketone bridges in its structure. Curcumin also acts via heat shock proteins (HSPs) pathway. HSPs are molecular chaperones that are involved in protecting cells from toxic oligomers of huntingtin, α-synuclein, tau, and Aβ by degrading them. Curcumin activates HSP 70, HSP 90, HSP 60, and HSP 40, and in turn, helps to reduce aggregation of misfolded Aβ proteins. Data also suggest that in an AD model, curcumin nanoparticles promote neurogenesis by promoting BDNF and by regulating GSK 3β through Wnt/β-catenin pathways.[19] Figure 1 shows the molecular targets of curcumin.

B. Centella

Centella asiatica: The extract of centella was shown to prevent cognitive deficits in an intracerebroventricular streptozotocin model of AD in rats. It is believed that an antioxidant mechanism is involved. Asiatic acid, the main constituent of *C. asiatica* has been shown to reduce hydrogen peroxide–induced cell death and lower levels of intracellular free radicals. Asiatic acid has beneficial properties for treating dementia. It also protects cultured cortical neurons from excitotoxicity induced by glutamate activation. The memory enhancing activity of *C. asiatica* may be due to the acetylcholinesterase inhibiting activity of asiatic acid and betulinic acid, which may be a potential leads for the symptomatic treatment of AD.[20] Figure 1 shows the different molecular targets of centella.
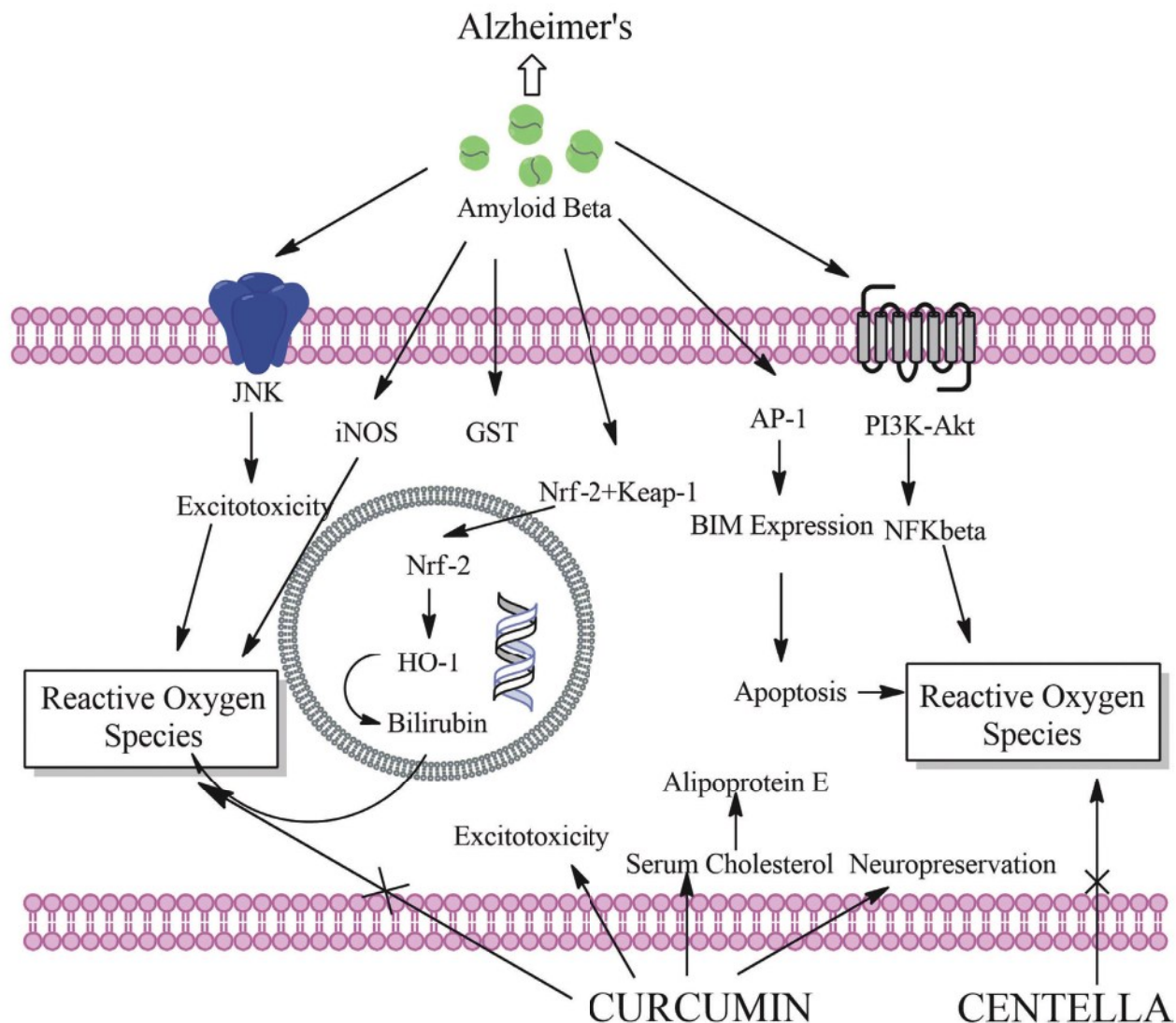
FIG. 1: The molecular targets of curcumin and centella (Nrf-2, nuclear factor–like 2; Ho-1, heme oxygenase; GST, glutathione S-transferase; BIM, pro-apoptotic protein)

C. Ashwagandha

Withania somnifera: Scientists have been investigating the properties of *Withania* plants as a treatment for Alzheimer's disease. The study has lead to a clinical trial for withanamide. The hypothesis put forward by scientists was that accumulation of β-amyloid peptides is responsible for damaging cells in the hippocampal region of the frontal lobe of the brain. Withanamides are supposed to have the property of neutralizing the effects of β-amyloid peptides.

Withanamides are also known for scavenging free radicals that are generated during the initial stages of Alzheimer's disease. They have been found to be effective in the treatment of PD, AD, and HD. A methanol/ethanol extract (3:1) of *Withania* was useful against Aβ induced neurotoxicity.[21]

D. Chinese Herbs

Two Chinese medicines, yi-gan san (YGS) and chaihu-jia-longgu-muli tan (CLMT), were reported

to reduce symptoms of Huntington's disease (HD) in four patients.[22] In earlier studies, YGS was also tested in clinical trials and shown to be effective in treating dementia.[23]

Shenwu capsule and GEPT (a combination of five Chinese active herbal components) showed anti-HD properties by decreasing amyloid cytotoxicity and the level of Aβ, respectively, and are presently at different phases of clinical trials.[23]

E. Brahmi

Bacopa monnieri: It contains bacoside A, bacoside B, betulinic acid, stigmasterol, stigmastanol, D-mannitol, β-sitosterol. Bacoposides have been shown to be effective against AD by inhibiting acetylcholinesterase activity.[24]

F. Shankhpushpi

Convolvulus pluricaulis: It contains convoline, convolidine, convolvine, confoline, convosine, kaempferol, and the steroid phytosterol. Generally, learning and memory functions are associated with the hippocampal region of the brain, which is affected in Alzheimer's disease. This plant extract acts through acetylcholinesterase reduction in a dose-dependent manner, alleviating the symptoms of Alzheimer's disease.[24]

G. Huperzia

Huperzia serrata: It consists of lycoposerramine H, serratidine, obscurumine A, 11α-O-acetyl-lycopodine, huperzine A, huperzine B, huperzinine, lycodine. It is especially used to treat Alzheimer's disease.[24] The extract is known to be the powerful inhibitor of acetylcholinesterase.

H. Ginseng

Panax ginseng: It consists of ginsenosides, or saponins, 20(S)-protopanaxadiol (PPD) and 20(*S*)-protopanaxatriol. It helps to reduce the aggregation of amyloid plaques and interferes with hyperphosphorylation of tau protein. Thus, it can be used in the symptomatic treatment of Alzheimer's disease,

Parkinson's disease, and Huntington's disease.[24] In light of Alzheimer's disease, an open-label study was conducted to find out whether ginseng is clinically beneficial to Alzheimer's patients. Based on results of cognitive performance tests (Mini-Mental State Examination and Alzheimer's Disease Assessment Scale) after administering ginseng at 4.5 g/day for 12 weeks, scores were found to increase, indicating progressive improvement in cognitive ability. On another hand, after discontinuation of ginseng treatment for 12 weeks, scores became normal and corresponded to the control group.[25]

I. Euphorbia

Euphorbia denticulata: This plant contains one of the important classes of triterpenoids. It contains polyphenolic compounds, such as catechin, epicatechin, gallic acid, *p*-hydroxybenzoic acid, and rosmarinic acid. A methanolic extract of flowers and leaves of *E. denticulata* was found to be a potent inhibitor of the enzymes acetylcholinesterase and butyrylcholinesterase, and it can be used in treating neurodegenerative disorders. It is also an antioxidant and a free radical scavenger.[26]

IV. POLYMERS USED IN NOVEL DELIVERY SYSTEMS FOR NEURODEGENERATIVE DISORDERS

A. Chitosan

Chitosan is a biodegradable and biocompatible polymer obtained from the polysaccharide chitin. Chitin is often found in the exoskeleton of fungi, crustaceans, and yeasts. Chemically, chitosan is composed of a linear chain of *N*-acetyl-D-glucosamine and D-glucosamine attached by β-1,4-glycosidic bond. It has the ability to form water-soluble salts. The molecular weight of chitosan is the deciding factor for encapsulation efficiency, with low molecular weight chitosan giving higher encapsulation efficiency. Low molecular weight chitosan is obtained from high molecular weight chitosan through depolymerization by adding sodium nitrate, strong acids such as hydrochloric acid, sulfuric acid, and phosphoric acid. To cross the blood-brain barrier

(BBB), chitosan requires surface modifications. The surface changes also help to stabilize nanocarriers in the altered blood composition found in Alzheimer's patients. Alkylated chitosan, sugar modified chitosan, quaternized chitosan, and PEGylated chitosan are some of the examples of modified chitosan. For Alzheimer's disease, *N*-trimethyl chitosan (TMC) bound to chitosan has been extensively studied for its stability in blood because of electrostatic interactions between negatively charged sialic acid residues on glycoprotein part on BBB and cationic TMC.[27]

B. Poly(Lactic-Co-Glycolic Acid)

Poly(lactic-co-glycolic acid) (PLGA) is approved by the FDA as a synthetic copolymer of lactic acid and glycolic acid residues that yield a biocompatible and biodegradable polymer. Aluminum isopropoxide, and tin-2-ethyl hexanoate are examples of catalysts used in the synthesis of PLGA. Depending on the molar ratio of lactide and glycolide, different grades of PLGA are produced. PLGA polymer is especially useful in nanoparticulate drug delivery systems. Quercetin loaded PLGA nanoparticles were formulated and evaluated for the treatment of Alzheimer's disease. PLGA nanoparticles of rivastigmine were also successfully formulated and evaluated.[27]

C. Polylactic Acid

Polylactic acid is yet another FDA approved polymer used for sustained release delivery of drugs. It is a hydrophobic polymer consisting of lactic acid monomers. It is available in two optical isomer forms: D-lactide and L-lactide. The racemic mixture is amorphous in nature with lower tensile strength than poly L-lactic acid. Apart from its property of biocompatibility, it also shows one striking feature. It can be molded for use in scaffolds, sutures, films, micelles, and nanoparticles. To impart a hydrophilic part to the polymer, PLA can be surface modified with PEG.[28]

D. Poly(Alkyl Cyanoacrylate)

Different derivatives of cyanoacrylates are being used as nanocarriers, including methyl, ethyl, isobutyl, and 2-octyl derivatives. Polyisobutylcyanoacryl-

ate derivatives are specifically used for targeted delivery within the brain. Scientists studied selegiline-functionalized PEGylated fluorescent poly(alkyl cyanoacrylate) nanoparticles and their interaction with amyloid Aβ peptide (1-42). Amyloid Aβ peptide inhibited fibrillogenesis and proved to be beneficial in tackling Alzheimer's disease. Different cyanoacetate derivatives were used: fluorescent rhodamine B cyanoacetate; methoxypolyethylene glycol cyanoacetate for stealthing; hexadecyl cyanoacetate as a hydrophobic core for nanoparticles; and selegiline-PEG-CA.[29]

V. NOVEL DRUG DELIVERY AND PHYTOCONSTITUENTS

A. Resveratrol

Conventional drug delivery systems such as dry powders are problematic because of their poor pharmacokinetic properties. Low aqueous solubility, first pass metabolism to a greater extent, and low stability towards light result in low bioavailability. Because of these problems, a need for nanoformulations of resveratrol is underscored.[30]

1. Polymeric Micelles

Resveratrol loaded polymeric micelles were formulated to assess the drug's ability to counteract β-amyloid toxicity in PC12 cells. Micelles were prepared from block copolymers, which tend to self-assemble above with critical micellar concentration (CMC). The particle size was not larger than 100 nm, with drug loading 20% w/w and encapsulation efficiency of 89%, an *in vitro* study was carried out with 35% initial burst for 8 h and then sustained release for 5 days. Cells were shown to be protective against β-amyloid toxicity when preincubated with resveratrol for 12 h. This was due to the drug's ability to reduce oxidative stress and caspase-3 activity.[31]

2. Polymeric Nanoparticles

Various biodegradable polymers are used to formulate nanoparticles. Poly(lactic acid), polycaprolactone, poly(ethylene glycol), and PLGA are some examples. Scientists formulated resveratrol loaded

methoxy-PEG-PCL nanoparticles (mPEG-PCL-NP). They found that loaded mPEG-PCL NP showed free radical scavenging activity compared to free resveratrol in aqueous solution. By formulating polymeric nanoparticles of resveratrol, it becomes possible to overcome some of the pharmacokinetic problems. Furthermore, scientists also formulated resveratrol loaded PEG-PLA NP and transferrin modified PEG-PLA NP. Transferrin receptors are abundantl on brain capillaries and useful for targeting delivery to the brain. Nanoparticles were found to be approximately 150 nm in diameter with drug loading 4.4% w/w and an encapsulation efficiency of 80%. An *in vitro* study showed sustained release for the first 24 h with not more than 10% of resveratrol released.[32] Figure 2 illustrates the general scheme of the method used to prepare polymeric nanoparticles.

B. Breviscapine

Breviscapine is a flavonoid from the Chinese medicinal herb *Erigeron breviscapus* (Compositae)

and known to have a neuroprotective role against ischemia. Multivesicular liposomes (MVL) were prepared for breviscapine (Bre) to prolong its action through a sustained release formulation. Liposomes were prepared by double emulsification process with phosphatidylcholine, phosphatidylglycerol, cholesterol, and triolein or tricaprylin. The mean residence time of MVL-Bre was found to be 16 times longer than Bre solution.[33]

C. Curcumin

Polymeric nanoparticles of curcumin were formulated using a PEG-PLA diblock copolymer and PVP as a stabilizer. With a multiinlet vortex mixer and flash nanoprecipitation, curcumin was successfully loaded into particles. Particles were found to be well below 100 nm in diameter. An *in vitro* study was performed on a Madin-Darby canine kidney cell monolayer for assessing permeability through the BBB. An *in vivo* test was done on Tg2576 mice (transgenic mice). Sixty mice were divided into
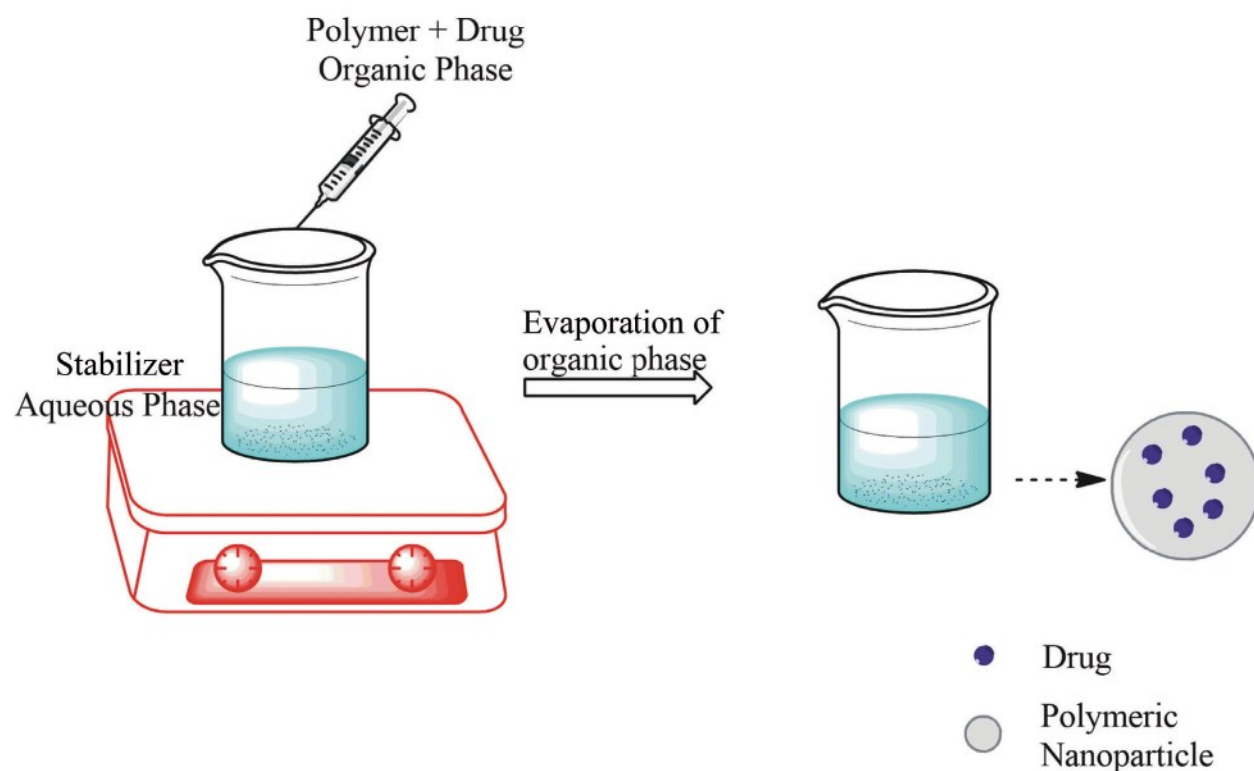

FIG. 2: Preparation method of polymeric nanoparticles using solvent evaporation or nanoprecipitation technique

three groups, each containing 20 mice, for treatment with nanocurcumin or curcumin, or control. The results showed that there was an appreciable increase in the concentration of curcumin in plasma when curcumin nanoparticles were administered compared to ordinary curcumin. Also, there was a sixfold increase in area under the curve and mean residence time of curcumin in the brain compared to ordinary curcumin.[34]

Curcumin magnetic nanoparticles were formulated with a magnetic resonance imaging (MRI) technique. It was revealed through study that there was no instance of any cytotoxicity in human neuroblastoma cells or Madin-Darby canine kidney cells.[34]

The tolerability and efficacy of curcumin administered through oral route was studied in a randomized, double-blind, placebo-controlled clinical trial. It lasted 24 weeks and 36 people with some symptoms of Alzheimer's disease were enrolled. Measurement of plasma levels of curcumin and its metabolites, as well as the performance of patients based on the Alzheimer's Disease Assessment Scale, was checked after 24 weeks. No clinically significant differences were found between treatment groups as far as efficacy and bioavailability were concerned.[35]

D. Rivastigmine

Rivastigmine is one of the drugs approved by the FDA for the treatment of Alzheimer's disease. It is a semisynthetic derivative of physostigmine, which is obtained from calabar beans. To date, different nanoformulations of rivastigmine have been examined, including solid lipid nanoparticles, polymeric nanoparticles, and liposomes.

1. Rivastigmine Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNs) contain lipids such as triglycerides, fatty acids, steroids, and waxes for the core part, and emulsifiers for stabilizing the formulation. The particles were in the range from 10 to 1000 nm in diameter. It is possible to incorporate both hydrophilic and hydrophobic drugs into SLNs. SLN uptake by the brain is supposed to take place through the interaction of endothelial cells at the BBB, and plasma proteins getting adsorbed on SLNs. In one study, scientists used GMS, compritol, and PA (Precirol) as lipids. Poloxamer-188 and tween 80 were used as surfactants. SLN of rivastigmine was formulated by high-speed homogenization. Different batches were prepared, and depending on the ratio of drug to lipid, and the quantity of surfactant used, particle size varied from 130 to 300 nm and encapsulation efficiency ranged from 40% to 80%.[36]

2. Rivastigmine Nanoparticles

Rivastigmine PLGA nanoparticles were formulated by the nanoprecipitation method. Particle size was around 135 nm in diameter, and entrapment efficiency was 75%. Another formulation of poly(butyl cyanoacrylate) (PBCA) NP loaded with rivastigmine was studied and evaluated with a particle size of around 145 nm and entrapment efficiency of 57%. Although an *in vitro* study showed that 30% and 45% of the drug was released for 72 hours from PLGA and PBCA NP, respectively.[37]

Herbal medicines and their respective novel formulations for neurodegenerative disorders are summarized in Table 1.

VI. CONCLUSION

Although a lot of research has focused on neurodegenerative disorders, cures are still elusive because of our limited understanding of the pathogenesis of neurological disorders. There are drugs available that can only be used for palliative care. Additionally, these drugs are often subjected to a number of side effects; therefore, there is growing interest in phytoconstituents. Chinese, Indian, and Arabic medicinal plants are screened for their phytoconstituents and reported to be an excellent alternative for drugs. Since the last decade, collaborative research on novel delivery systems for herbal drugs has focused on treatment of neurodegenerative disorders. This review summarizes the research work on herbal drugs through the formulation of polymeric nanoparticles, polymeric micelles, solid lipid nanoparticles, and liposomes.

TABLE 1: General overview of herbal medicines and their novel formulations

Sr. No.	Plant source	Novel System	Particle Size	Entrapment Efficiency	Reference
1	Resveratrol	Formulated polymeric micelles to assess drug's potential to protect against beta amyloid toxicity in PC12 cells. Formulation showed initial burst release of 35% within first 8 hours and sustained release over 5 days.	Not more than 100 nm	89%	38
2	Resveratrol	Formulation of mPEG-PCL nanoparticles to assess antioxidant properties of drug. Compared to free Res aqueous solution, Res NP showed more anti lipid peroxidation and radical scavenging property. Moreover, PCL degrades more slowly than other polymers like PEG, PLA,PLGA, etc., so find use in sustained release formulations	150 nm	80%	39
3	Rivastigmine	Formulation of solid-lipid nano-particles by homogenization and ultrasonication method. Optimization by 3^3 factorial design with factors as Drug: Lipid, surfactant conc, homogenization time	130–00 nm	40-80%	40
4	Rivastigmine	Formulation of PLGA nanoparticles by modified nanoprecipitation method. Optimization by 3^3 factorial design with factors like polymer conc, surfactant conc and volume of internal phase. *In vitro* release rate was found to be decreased when lactide content and mol wt of polymer increased	135 nm	75%	41
5	Rivastigmine	Formulation of PBCA nanoparticles by *in situ* anionic emulsion polymerization method. Optimization by 3^2 factorial design with surfactant conc. and monomer conc. as two factors. *In vitro* release rate was decreased as hydrolysis rate was decreased due to shielding of longer chains of hydrophobic alkyl groups against hydroxyl ions on ester group of polymers	145 nm	57%	42
6	Curcumin	Formulation of magnetic nanoparticles made of superparamagnetic iron oxide and coating with PEG-PLA block co-polymers. Microscopy study by TEM and AFM and structural study by XRD, TGA. No cytotoxicity was seen in MDCK cell lines	70–90 nm	60–90%	43
7	Curcumin	Formulation of PEG-PLA nanoparticles by flash nanoprecipitation method. Evaluation of formulation in Tg2576 mice model and *in vitro* as well	60–70 nm	96–97%	44

mPEG-PCL: methoxy-Poly(ethylene glycol)-Poly(caprolactone);NP: Nanoparticles; PCL: Poly(caprolactone); PEG: Poly(ethylene glycol); PLA: Poly(D,L-lactide); PLGA: Poly(lactic-co-glycolic acid); PBCA: Poly(butylcyanoacrylate); PEG-PLA: Poly(ethylene glycol)-Poly(D,L-lactide); TEM: Transmission electron microscope; AFM: Atomic force microscopy; XRD: X-ray powder diffraction; TGA: Thermogravimetric analysis.

VII. FUTURE DIRECTIONS

The burden of neurological disorders on people, especially among the aged population, is increasing at an alarming rate. To confront these disorders, scientists all over the world are trying their best to get the situation under control, be it through hormonal therapy, antioxidants and vitamin therapy, stem cell treatment, gene therapy, siRNA therapy, or herbal drugs. As far as Alzheimer's disease is concerned, only aducanumab, a human recombinant monoclonal antibody, is in phase III trial, whereas other NCEs failed during earlier trials. Herbal drugs delivered through novel nanocarriers prove to be a better option. As the potential of nanodrug delivery for neurological disorders has already been strengthened through extensive research, herbal drugs delivered through these novel systems may yield methods to combat neurodegenerative disorders in the future.

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Recent Expansions on Cellular Models to Uncover the Scientific Barriers Towards Drug Development for Alzheimer's Disease

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Abstract

Globally, the central nervous system (CNS) disorders appear as the most critical pathological threat with no proper cure. Alzheimer's disease (AD) is one such condition frequently observed with the aged population and sometimes in youth too. Most of the research utilizes different animal models for in vivo study of AD pathophysiology and to investigate the potency of the newly developed therapy. These in vivo models undoubtably provide a powerful investigation tool to study human brain. Although, it sometime fails to mimic the exact environment and responses as the human brain owing to the distinctive genetic and anatomical features of human and rodent brain. In such condition, the in vitro cell model derived from patient specific cell or human cell lines can recapitulate the human brain environment. In addition, the frequent use of animals in research increases the cost of study and creates various ethical issues. Instead, the use of in vitro cellular models along with animal models can enhance the translational values of in vivo models and represent a better and effective mean to investigate the potency of therapeutics. This strategy also limits the excessive use of laboratory animal during the drug development process. Generally, the in vitro cell lines are cultured from AD rat brain endothelial cells, the rodent models, human astrocytes, human brain capillary endothelial cells, patient derived iPSCs (induced pluripotent stem cells) and also from the non-neuronal cells. During the literature review process, we observed that there are very few reviews available which describe the significance and characteristics of in vitro cell lines, for AD investigation. Thus, in the present review article, we have compiled the various in vitro cell lines used in AD investigation including HBMEC, BCECs, SHSY-5Y, hCMEC/D3, PC-2 cell line, bEND3 cells, HEK293, hNPCs, RBE4 cells, SK-N-MC, BMVECs, CALU-3, 7W CHO, iPSCs and cerebral organoids cell lines and different types of culture media such as SCM, EMEM, DMEM/F12, RPMI, EBM and 3D-cell culture.

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Graphical Abstract
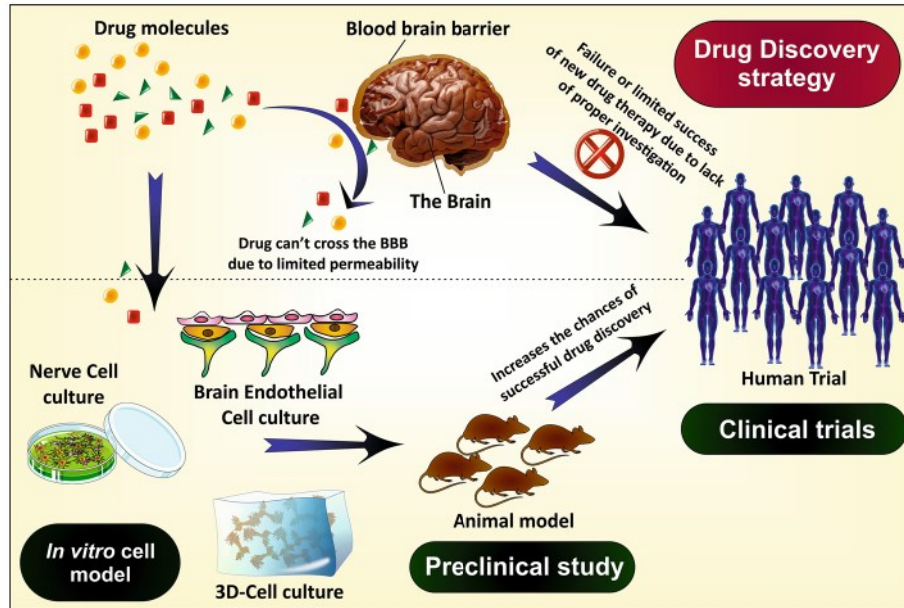
Keywords Alzheimer's disease · In vitro cell line · BCECs · HBMEC · SK-N-MC

Introduction

AD is a progressive neurodegenerative disorder, considered as a prime cause of dementia which affects the routine activity and daily life of the patient. The statistics show more than 5.4 million cases of dementia only in the USA in the year 2016 (Alzheimer's Disease Facts and Figures 2017; Agrawal et al. 2018a) from which AD accounts for about 50–75% of all. In 2016, globally 47 million people have dementia which was supposed to rise by three folds till 2050 (Martin Prince et al. 2016; Alladi et al. 2010; Anand et al. 2017). AD affects various functions of the brain like memory, cognition, behavior, and learning abilities, regulated through the entorhinal cortex, hippocampus, frontal, parietal, and temporal lobe of the brain (Kumar et al. 2015; Giacobini 1994; Wood et al. 2005). The formation of β-amyloid (Aβ) plaques and hyperphosphorylated τ-protein tangles are the pathological hallmarks of AD resulting in degeneration of neurons or affecting the neurotransmission (Agrawal et al. 2018a). Depending on the age of onset, AD is of two types: early-onset AD (EOAD) and late-onset AD (LOAD). EOAD is generally associated with mutations in presenilin and APP genes, while LOAD, mainly associated with the constant weakening and damage of nerve cells and brain function with aging (Szigeti and Doody 2011; Davis and Laroche 2003). LOAD is primarily characterized by amnesia, while EOAD, associated with visuospatial and motor dysfunction (Tellechea et al. 2018; Kupershmidt et al. 2012).

The brain remains the most vital organ of our body processing all the information, memory, coordination, voluntary and involuntary actions—the human brain composed of three prime regions including cerebrum, cerebellum, and medulla oblongata. AD primarily affects the cerebrum, further divided into occipital, temporal, parietal and frontal lobes (Arshavsky 2014). The loss of memory and cognitive functions are the primary features of AD which are due to neuronal degeneration in the temporal lobe (hippocampus) and frontal lobe, respectively (Agrawal et al. 2018b). In later stages, it affects another region of cerebrum responsible for vision (occipital lobe) and motor function (parietal lobe) (Arshavsky 2014; Hyman 1997).

Blood–Brain–Barrier (BBB) along with the vascular system acts as a shield to the central nervous system (CNS) preventing the entry of various harmful substances (Boursereau et al. 2015; Agrawal et al. 2017). Being highly lipophilic, it restricts the entry of hydrophilic molecules and allows the passage of specific lipophilic substances via passive diffusion. Although, it permits the transfer of some tiny hydrophilic particles through the small gaps of tight junctions (TJs) (Agrawal et al. 2017, 2018b). Small hydrophobic gases like O_2 and CO_2 can diffuse freely across the barrier. In addition, the paracellular pathway relies on the concentration gradient and permeability of the compounds, thus restricting macromolecules. The TJs are the essential characteristic feature of BBB which holds the brain capillary endothelial cells tightly to maintain the strength of the

BBB (Jiang et al. 2007). There are mainly three TJs proteins, claudins, occludins and junctional adhesion proteins which can use as biomarkers to examine the BBB integrity (Wolff et al. 2015). BBB primarily consists of five types of cells; astrocytes, endothelial cells, pericytes, neurons, and microglia collectively called the neurovascular unit. Endothelial cells act as the central basal membrane part of the BBB lining. Astrocytes release several chemicals that retain and increase the strength of the barrier. Usually, in the in vitro cell culture, astrocytes are co-cultured along with endothelial cells to increase the TJs complexity and transendothelial electrical resistance (TEER) value (Zhang et al. 2010). Pericytes are also crucial in maintaining the integrity of BBB. Their absence results to increase the permeability of BBB by inhibiting the expression of TJ proteins (Keaney and Campbell 2015). In all, the BBB creates a significant hurdle for a drug to reach the brain and this becomes a massive challenge for the researchers. Hence, a proper understanding of the BBB is essential to develop a potential AD therapy.

For the establishment of any new therapy, scientists need some in vivo tools like animal models or postmortem brain (preclinical study), to evaluate the efficacy of the therapy. These tools are presently very popular in scientific interventions. However, the success rate is abysmal as it is challenging to replicate the exact state of AD as in the human body because of physiological, pathological and genetic difference between human and the animal (Cundiff and Anderson 2011; Dohgu et al. 2004). In addition, it is difficult to arrange or to work on the human brain. Moreover, the in vivo animal studies are very expensive, complicated and have some ethical issues (if excessively done). Thus, to overcome these hurdles

nowadays in vitro studies are being carried out which mimic the condition of AD to a greater extent. The combination of both the in vivo and in vitro models offers a more promising way to investigate the disease pathophysiology, associated factors, disease progression and potency of the therapy. The advantages and limitations of the in vitro cell models used to study the human brain, neurodegenerative disorders, and screening of therapeutics are discussed in Table 1. In this review article, we have discussed the histopathological factors of AD and the in vitro cellular models used for understanding the disease initiation, progression and mechanism and in drug discovery and development process for AD.

Pathophysiology of AD

Amyloid Cascade Hypothesis and APP (Amyloid Precursor Protein) Processing

APP gene situated on chromosome 21 gets decoded to form APP. It is a transmembrane protein with a glycosylated N-terminal and a C-terminal projecting out and into the cell, respectively (Fig. 1). APP gets cleaved by three secretase enzymes, namely α, β and γ. N-terminal of the protein gets cleaved by α-secretase to form sAPPα (secreted APPα), leaving behind the C-terminal with 83-amino acids (Musardo and Marcello 2017). Similarly, β-secretase also act on the N-terminal forming sAPPβ and 99-AA C-terminal. The formed 99-AA C-terminal gets cleaved by γ-secretase at the transmembrane regions producing Aβ 40 (soluble) or 42 (insoluble) and C-terminal.

Table 1 Advantages and limitations of in vitro cell models used to investigate AD pathophysiology, disease progression, and drug screening

Advantages	Limitations
It provides a relatively simple, less invasive and economical method to recapitulate different parts of the human brain, nerve cells and BBB (Gumbleton and Audus 2001)	It just provides a primary tool to investigate the disease pathology and drug screening and give a supportive direction for further in vivo and clinical investigations (Arber et al. 2017)
It offers a potential means to investigate various drug diffusion process across the BBB including the paracellular and transcellular drug diffusion, active transport and metabolism (Zhang et al. 2014a)	The immortalized in vitro cell line mostly fails to recreate the perfect paracellular barrier for investigation of drug permeability (Gumbleton and Audus 2001)
It demonstrates the undefined interaction of cellular material and drug which cannot be studied by in silico techniques (Gumbleton and Audus 2001)	It cannot fully recapitulate the microarchitecture and all the cellular functions of the organ (Wang 2018)
The primary culture of BCEC more prominently resembles the in vivo cells (Gumbleton and Audus 2001)	Among all the available in vitro models, no one can correctly reiterate all the cellular mechanisms including cell interactions, toxicity, response to drug, metabolism, etc. (Ghallab 2013)
The amyloidogenic response of rodent model differs from the human brain in such condition the iPSC and hESC (human embryonic stem cell) derived BCEC and nerve cells more prominently recapitulate the AD brain condition (Carolindah et al. 2013; Sullivan and Young-Pearse 2017)	
The patient-derived iPSCs and 3D cerebral organoids offer a more precise way to recapitulate the human brain and disease progression, as it is originated from the human cell (Poon et al. 2017)	
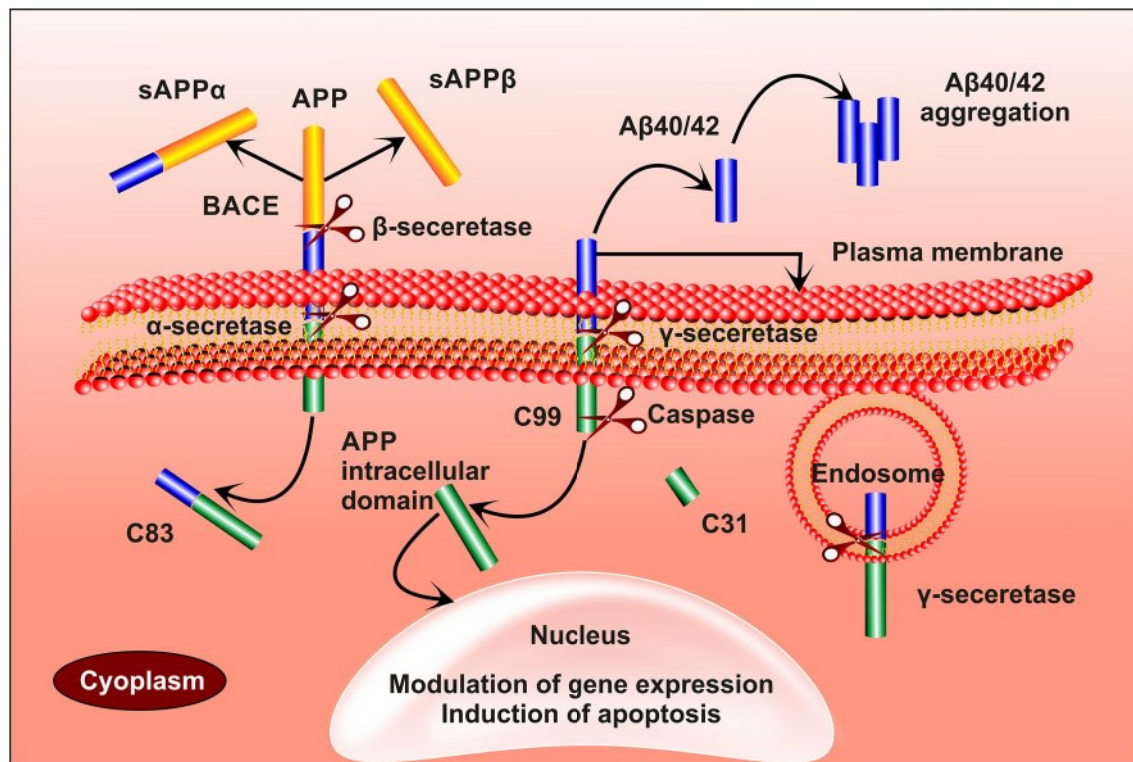
Fig. 1 α-secretase and β-secretase acts on APP to form sAPPα and sAPPβ leaving aside the C-83 and C-99 amino acid terminal aside, respectively. C-99 is acted upon by γ-secretase forming Aβ-40/42 and an internal domain which migrates to the nucleus causing gene expression. In some instances, C-99 is cleaved by caspases to form C-31, which is a neurotoxin

The remaining internal domain migrating to the nucleus regulates the expression of genes. Sometimes the 99-AA C-terminal gets cleaved by caspases resulting in the production of the neurotoxic peptide (31AA C-terminal) (Mattson 2004; Maloney and Bamburg 2007b). Aβ 40 is a soluble form of amyloid β peptide, cleared through the BBB. The insoluble Aβ42 forms small aggregates which further converted into the harmful plaques (Table 2). The Aβ aggregate causes an imbalance in calcium homeostasis and reactive oxygen species (ROS) (Bachmeier et al. 2011; Martins et al. 1991; Maloney and Bamburg 2007a). It is also found to alter the physiology of mitochondria by enlarging the size of mitochondrial transition pores and sometimes even causes the apoptosis of mitochondria (Kumar and Singh 2015).

Table 2 Etiological factors and enzyme activity responsible for AD progression

S. no.	Etiological factor	Protein	Enzyme	Proteolytic products	Behavior
1	Amyloid cascade hypothesis	APP	α-secretase	sAPPα	Promote neurite outgrowth and improves memory (Chow et al. 2010)
2			β-secretase	sAPPβ	Activate death receptor 6 (DR6), induce axonal disintegration (Kwak et al. 2006)
3			α and γ-secretase	Aβ17–40/42	Amyloid plaque formation (Wei et al. 2002)
4			β and γ-secretase	Aβ1–40/42, Aβ11–40/42	Amyloidogenic (Barritt and Viles 2015; Schmidt et al. 2009)
5			Caspase-3	C31	Neurotoxic (Zhang et al. 2011)
6	Tau Hyperphosphorylation	Tau protein	Caspase-3 and 7	N-terminal fragment	Inhibit ROS production, participate in intrinsic apoptosis (Brentnall et al. 2013)
7			Calpain	Multiple curtailed species	Production of neurotoxic tau fragment (Ferreira and Bigio 2011)

τ -Protein

τ-protein is a microtubule-associated protein which stabilizes the structure of nerve cell (Mandelkow and Mandelkow 2012). In AD there is an imbalance between kinases and phosphatases enzyme leading to the detachment of τ-protein with microtubule, thereby disturbing the structure of the cell which further promotes the aggregation of τ-protein (Kalra and Khan 2015; Blennow et al. 2001). This results in the formation of neurofibrillary tangles (a dead/collapsed neuron). Neurofibrillary tangles (NFT), the hallmarks of τ-protein pathogenesis in AD occupy the space present in the perinuclear cytoplasm of cortical pyramidal cells. NFTs, are formed by aggregation of paired helical filaments (PHFs) (Fig. 2; Table 2) (Wischik et al. 2014; Jouanne et al. 2017; Iqbal et al. 1994). The aggregates of τ-protein are notorious for declining the activity of proteasome leading to cell death by the atypical accumulation of proteins and also initiates subsequent cascade reactions. It also activates immune responses by activating microglial, and astrocytes thus favor the development of neuroinflammation (Laurent et al. 2018).

Cholinergic Hypothesis

The depletion of acetylcholine (ACh) is one of the primary cause of AD which results in loss of cognitive functions and memory impairment. The reduction in ACh level is the consequence of the gradual death of cholinergic neurons in basal forebrain and collapse of neurotransmission in the cerebral cortex and hippocampus (Martorana et al. 2010). The downfall of neurotransmission is due to the damage of cholinergic nerve synapse by Aβ oligomers, formed in the early stages of AD (Ferreira-Vieira et al. 2016).

Mitochondrial Cascade Hypothesis

This hypothesis mainly has three postulates first, the tolerability of individual mitochondrial DNA (mtDNA) over the produced ROS; second, the decrease in the mitochondrial functions is interrelated with aging and environmental factors and third, the changes in mitochondrial function with progressing AD (Swerdlow et al. 2014). These three factors affect the functioning of mitochondria by damaging mtDNA resulting in apoptosis or destructive mitochondrial electron transport chain (ETC). It increases the ROS levels, causing oxidative stress in plasma membrane inducing the soluble Aβ to adopt insoluble conformation (Swerdlow and Khan 2004).

Excitotoxicity

Glutamate, the primary excitatory neurotransmitter involved in the memory and learning functions that are affected in AD. Glutamate receptors are of two types: ionotropic (NMDA) and metabotropic receptors (G-coupled). Neurodegeneration generally saw in later stage due to the toxic
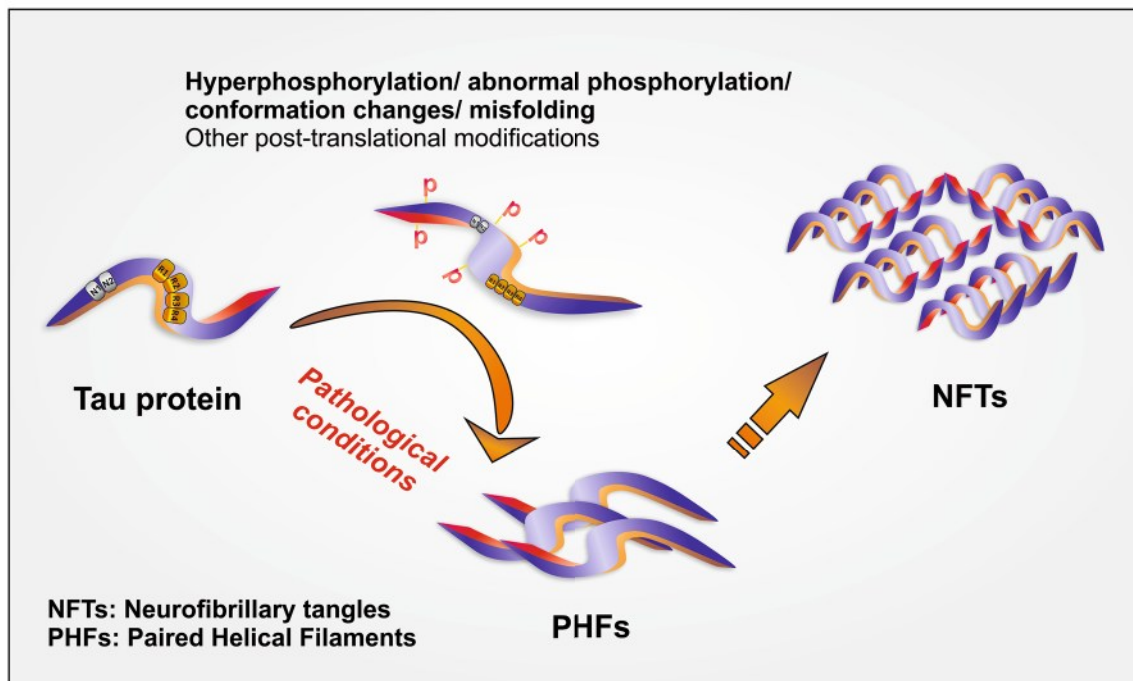
Fig. 2 Formation of neurofibrillary tangles of τ–protein (1) hyper-phosphorylation/ misfolding/conformational changes in τ protein leading to club formation forming paired helical filaments (PHFs). (2) PHFs aggregate to form neurofibrillary tangles (NFTs)

effects of glutamate mediated by Aβ. Aβ interferes with excitatory amino acid transport (EAAT) and NMDA receptor (NMDA NR2α) thereby, blocking the uptake of glutamine (Mehta et al. 2013). Thus, the excess glutamine present in the synaptic cleft activates the extra-synaptic NMDA receptors (NMDA, NR2β) thereby, reducing the intracellular release of calcium ion and leading to activation of pathogenic pathways like hyper-phosphorylation of τ-protein (Esposito et al. 2013).

Genetic Factors

Presenilin

Presenilin-1 (PS-1) and Presenilin-2 (PS-2) are the homologous proteins obtained from PSEN-1 gene present on chromosome-14 and PSEN-2 gene on chromosome-1, respectively. It plays a vital role in the formation and activity of the γ-secretase enzyme (Cacquevel et al. 2012). The mutations in PSEN-1 & 2 affects the functioning of PS-1 & 2 causing overexpression of γ-secretase resulting in producing abnormal quantities of Aβ. The mutations in PS-1 and PS-2 are mainly involved in LOAD and EOAD, respectively. Mutations in PS-1 alters the calcium channel in glutamatergic neurons (Salek et al. 2010).

ApoE

ApoE is an essential lipid carrier responsible for the transfer of lipid in the brain and also involves repairing injuries. ApoE exists in allelic forms such as E_2, E_3, and E_4, among these the E4 alleles, are more prone to develop AD. The $ApoE_4$ facilitates the formation of Aβ42 or assists the Aβ aggregation and also decreases the Aβ clearance rate (Liu et al. 2013). It affects the extracellular Aβ degradation by reducing the expression of insulin-degrading enzyme (IDE) in the brain (Kanekiyo et al. 2014) which may lead to neurodegeneration.

Other Associated Factors

Oxidative Responses

The production of ROS is common in cells, responsible for aging and related disorders. Our body has a proper defensive mechanism which minimizes the oxidative stress-induced diseases. In AD-like condition, the natural defense mechanism becomes poor and results in uncontrolled ROS generation (Butterfield et al. 2006; Zhu et al. 2005). The increased amounts of ROS induced overproduction of Aβ, increasing the expression of β and γ-secretases, destroying the mtDNA, and altering the metabolism of metals and lipids. The relation between the Aβ and ROS is vivid because the

overproduction of either of the one initiates the production of the other. These factors lead to the formation of Aβ aggregation, hyperphosphorylation of τ-protein, finally leading to apoptosis (Tonnies and Trushina 2017; Mark et al. 1996).

Immune and Inflammatory Mediators

In the brain, the immune and inflammatory mediators, produced in response to the neural infection or trauma. Astrocytes and microglial cells play a significant role in releasing the mediators. The activation of astrocytes releases the cytokines, proinflammatory mediators and cyclooxygenase which activates microglial cells (COX-1). The activated microglial cells may become phagocytic which removes infectious organisms or dead cell debris or may produce subsequent inflammatory mediators (Zhao et al. 2018). Some studies revealed that these cells abundant around the Aβ-aggregates/plaque. The released mediators may cause synaptic dysfunction, apoptosis or necrosis of neurons, astrocytes, and microglia (Morales et al. 2014).

Environmental Factors

Environmental factors contribute to about 30% risk of acquiring AD. It includes the exposure of various metals (copper, aluminium), pesticides (organophosphates), chemicals from industries (bisphenol A), air pollutants and antimicrobial agents (polychlorinated organic compounds). Each factor act in a different way for example copper stimulates the Aβ plaque formation, aluminum increases the oxidative stress, air pollutants like CO, NO, and SO_2 increases the production of ROS, antimicrobials alter the immune response, and pesticides cause neurotoxicity or change the cell signaling (Yegambaram et al. 2015).

Aging

Aging may promote the risk of AD through the mitochondrial pathway, where there is a decrease in the functioning of mitochondria production of ROS, leading to a decline in pathological conditions. However, the deterioration of mtDNA upon aging may lead to apoptosis. The target of rapamycin (TOR) regulates protein homeostasis which inhibits autophagy and translation of mRNA. Decreased TOR signaling was observed to reduce the formation of toxic aggregates and decline the progression of AD (Perry et al. 2000). However, cells with defective autophagy shown to accumulate aggregates of Aβ. It observed that upon aging signaling of insulin-like growth factor (IGF-1) decreases which leads to the formation of toxic aggregates and further causing cytotoxicity (Bishop et al. 2010).

Methodology

For the compilation of this review article, we have conducted an exhaustive literature survey, and the data is collected using the two very popular search engines, 'SCOPUS' and 'PUBMED.' For more precise search results, the keywords remain defined by Boolean operators. The data collection was done using various set of keywords including; (Alzheimer) AND (BBB) OR (cell line) OR (v cell model) OR (pathology) OR (Culture media) AND (BCECs) OR (HBMEC) OR (SHSY-5Y) OR (hCMEC/D3) OR (PC-2 cell) OR (bEnd3 cell) OR (hNPCs) OR (RBE4 cell) OR (SK-N-MC) OR (BMVECs) OR (CALU-3 cell) OR (7W CHO cell) OR (standard culture medium) OR (Eagle's minimal essential medium) OR (DMEM/F12) OR (RPMI media) OR (endothelial cell basal medium) OR (3D-cell culture) OR (patent). The data search takes place till 19th June 2018. At the same time no particular filter of the period, source, type of article was applied at the time of data collection. Finally, the collected data, segregated into different categories including the introduction of AD, pathology of AD, in vitro cell models and culture media, to finalize the structure of the article. The discussion section mainly based on the origin/source of the cell line, its isolation procedure, fundamental properties, culture media and environmental condition, merits, and demerits of the particular cell lineage. We have also tried to provide a graphical description and tabular data wherever possible; all the figures were prepared initially using COREL DRAW software.

In Vitro Cells

The development of effective therapy for the treatment of AD needs a complete and thorough understanding of the AD etiology. Immense research work has been already done and/or under progress to study the pathologies behind the disease including amyloidogenesis and neurodegeneration due to tau hyperphosphorylation. Unfortunately, the success rate is not satisfactory because of the limited availability of in vivo and in vitro models which reiterate the human brain anatomy and physiology (Arber et al. 2017).

In general, the animal models are popularly used to study the effect of drugs and human brain pathophysiology (Epis et al. 2010). However, they are unable to replicate the exact scenario like the human brain because of the physiological and genetical differences. The human brain can only be used to study the mechanism of progression of AD but, the main problem is that the brain is available just after post-mortem, that too, at the last stage of

AD which also decreases the scope of disease progression study. In this context, the in vitro cellular models remove such obstacles and provide an attractive method to study the etiology of neurodegenerative disorders including AD. Therefore, the use of in vitro cell models is increasing day by day to enhance the translational value of in vivo models (animal model) and provide a deep understanding of invaluable mechanistic insights of the disease (Poon et al. 2017). Different cellular models used in AD research are discussed below (Table 3).

Human Brain Microvascular Endothelial Cell (HBMEC)

The inadequate number of phenotypically relevant cellular models is the major obstacle to study the exact physiology and behavior of the BBB. Though, there are few cell lines which utilizes immortalized human BMECs and type II Madin-Darby canine kidney cells (MDCK). However, they are unable to mimic the true nature of BBB cells. The human brain microvascular endothelial cell (HBMEC's) have several physiological similarities with that of BBB cells in having several relevances like tight junction proteins (claudins, occludins, and junctional adhesion proteins), transporters and efflux pumps (Katt et al. 2016). These cells, obtained from tissue of the human, are cultured on collagen type I surface. The central theme in using collagen type 1 surface is that it adheres to the endothelial at a quicker rate than the remaining cells in the tissue (Navone et al. 2013; Chen et al. 2013). These cells generally derived from human immunopotent cells (hiPCs). This small modifications in the method of the culturing process developed by Lippmann et al. In this, initial differentiation of iPCs cells modification, done in UM/F-media, then selective maturation of cells in endothelial cell media (EC), later the matured cells are purified by sub-culturing the cells on the collagen IV and fibronectin-coated surfaces (Fig. 3). Thus, the formed hBMECs cells are used for study and in research (Alexandrov et al. 2015). C-reactive protein (CRP) is an inflammatory mediator released by vascular endothelial cells, used as a biomarker to identify the presence of vascular inflammation associated with the progression of AD. The potency of aluminum in expressing CRP was studied by Alexandrov et al. (2015) on hBMECs. Aluminum was found to be potent enough to express CRP by altering the physiology of vasculature even in nano-molar concentrations (Alexandrov et al. 2015). Li et al. (2015) also utilized these cells to study the cytoprotective nature of naturally occurring flavonoid quercetin (a well-established neuro-protective agent) over Aβ induced cytotoxicity on endothelial cells of BBB. Findings revealed that the quercetin relived the cytotoxicity induced by Aβ, by reducing the lactate dehydrogenase secretion and by relieving condensation of the nucleus (Li et al. 2015). The changes

Table 3 Specification of cell lines that make them useful as in the in vitro neuronal model, along with their isolation process, culture medium and uses

In vitro cell line	Cell specifications	Isolation process	Culture medium	Uses/studies conducted	Reference
HBMEC (Human brain microvascular endothelial cell)	Expresses tight junction protein transporters, and efflux pumps. TEER value 100 $\Omega.cm^2$ increases to 2000 $\Omega.cm^2$ when cultured with retinoic acid	Derived from hiPSCs or the human brain tissue	EMEM	Used to study CRP (C-reactive protein) level in human serum and its synthesis through brain endothelial cells. To study the effect of CRP level on AD progression. To study the nanomolar aluminum sulfate on CRP expression. It expresses tight junction proteins like claudin-5, occludin, ZO-1, and VD-cadherin	Alexandrov et al. (2015), Katt et al. (2016)
BCECs (brain capillary endothelial cell)	They are of animal origin, possess high TEER value	Isolated from microvessels of the brain of animals like a rat, mice or rabbit, etc. by dissection	DMEM/F12	Recapitulate BBB. To study the permeability properties and cytotoxicity of drugs and Aβ on BBB. To create protein factories by genetic modification. To study α, β and γ secretase activity in APP processing	Burkhart et al. (2015), Zandl-Lang et al. (2018)
hCMEC/D3 (human cerebro microvascular endothelial cell)	Tumorous cell lines. Expresses the protein transporters and the BBB receptors. Possess shallow TEER value	Derived from microvessels of the human temporal lobe. Later, immortalized by SV40 large T-antigen	EMEM	Used mainly in the studies related to cerebral endothelium. Mechanisms relating to Aβ and cytokines	Weksler et al. (2013), Biemans et al. (2017)
BMVECs (brain microvascular endothelial cells)	These cells exhibit similarities with that of BBB by possessing TJ-proteins etc	Isolated from the temporal cortex of the human brain. The obtained cells are converted into immortal cells by SV40 antigen	DMEM/F12	Used in the study of mechanisms that alter the integrity of BBB. To screen the passage of drug molecules across the BBB. Expression of junction protein such as occludin and claudin	McCarthy and Kosman (2015), Haorah et al. (2008)
bEnd3 (mouse brain endothelial cells)	The expresses most of the proteins like TJ and P-gp etc. as that of BBB. TEER value of these cells is very less but, can be overcome by co-culturing with astrocytes	Derived from brain endothelium of mouse. Immortalized by transfecting with T-antigen	DMEM/F12	To study the tight junction protein, stiffness, P-glyco-protein function and permeability of BBB. To study the inflammatory responses and Aβ clearance mechanisms	Qosa et al. (2016) Yang et al. (2017a), He et al. (2014)

Table 3 (continued)

In vitro cell line	Cell specifications	Isolation process	Culture medium	Uses/studies conducted	Reference
RBE4 cells (Rat brain endothelial cells)	They exhibit cationic, carnitine, serotonin and P-gp transports similar to that of human BBB. Possess low TEER value. Unable to express γ-glutamyl transporter-1	Isolated from the endothelium of the rat's brain by enzymatic digestion of unwanted cells. Later on, immortalized by SV40 or T-antigen	DMEM/F12	Used to study the mechanisms that disturb the integrity of BBB the movement of substances across the membrane	Roux and Couraud (2005), He et al. (2014)
SHSY-5Y (human neuroblastoma cell)	Tumorous cells. Resembles mature cholinergic neurons upon differentiation	Isolated from neuroblastoma in the embryonic stages	EMEM, DMEM/F12, RPMI	To study the mechanisms of AD including Aβ, τ-protein and other pathways. To investigate the relationship between AD pathophysiology and cerebral glucose metabolism. To study oxidative stress, neurotoxicity and neurodegeneration process	Koriyama et al. (2015), Forster et al. (2016), Krishna et al. (2014)
SK-N-MC	These cells exhibit cholinergic properties. They were found to possess double-minute chromosome	Isolated from the metastatic supra-orbital human brain tumor	EMEM, DMEM/F12, RPMI	To study the efficacy of the anti-AD drug on Aβ insulted SK-N-MC model. To study AD pathology, Toxicity assessment	Z. Yang (2012), Kuo and Tsao (2017)
PC-2 cell line (pheochromocytoma cell line)	The differentiated forms resemble sympathetic ganglion neurons	Derived from the pheochromocytoma of the adrenal cortex of the rat	EMEM, DMEM/F12, RPMI	Used for neurite growth or differentiation into nerve like cell. Used the study of different pathways like Aβ, τ-protein and oxidative stress	Sun et al. (2018), Kudo et al. (2011)
hNPCs (human Neural Progenitor Cell)	Different types of CNS neuronal cells can be formed by these cells. They give a positive response to glutamine and GABA	Derived from hESCs and hiPSCs by TGF-β signal modulation pathway	EMEM	Used in the study of several pathophysiological processes of AD. Patient-derived cells can be used in studying the patient-specific genetic mechanisms responsible for AD. For drug screening	Kang et al. (2017), Tsai et al. (2015)
HEK293 (Human embryonic kidney cell 293)	Even though these cells are non-neuronal cells, they express neuro-filament subunits and Tau-protein	Derived from normal human embryonic kidney but are transfected with Adenovirus-5-DNA to convert them into immortal cells	DMEM/F12	To study of τ-protein phosphorylation and neurodegeneration	Gordon et al. (2013), Houck et al. (2016)

Table 3 (continued)

In vitro cell line	Cell specifications	Isolation process	Culture medium	Uses/studies conducted	Reference
7W CHO cell lines (7W Chinese Hamster ovary cell lines)	These cells express Aβ similar to that of neurons even though they are of non-neuronal origin	Derived by transfection of CHO cells with human APP751 genes	DMEM	Used to study the Aβ pathways relating to AD the effect of genes leading to AD	Spilman et al. (2014), Uemura et al. (2010)
CALU-3	These cells exhibit the properties similar to that of the nasal-brain barrier. They can express higher TEER values depending on the mode of culture	Isolated from adenocarcinoma of the serous epithelium of human lung	DMEM/F12	Used to study the mechanisms by which the drug reaches the brain when administering through the nasal cavity	Zheng et al. (2015), J. Fogh (1975), Srinivasan et al. (2015)
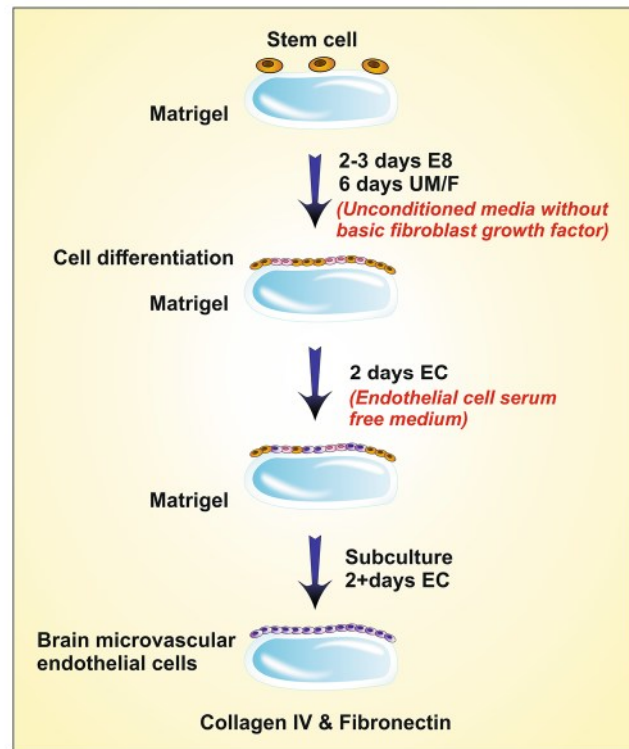
Fig. 3 Derivation of HBMEC from stem cells (1) Growing of stem cells on matrigel (2) differentiation of cells by growing in Unconditioned media without primary fibroblast growth factor (3) growing of cells in endothelial cell serum-free media (4) Cells were grown on collagen IV and fibronectin surface

in the characteristics of these cells upon co-culturing with astrocytes and in the presence of astrocyte-conditioned medium (ACM) remained studied by Kuo et al. (2011). They used hBMECs, hBMECs co-cultured with human astrocyte (hBMEC/HA) and hBMECs cultivated with 50% and 100% ACM to compare the resulting cells similarity with BBB. The finding revealed that the TEER values of these cells were in the order of hBMEC/HA > hBMECs cultivated with 100% ACM > hBMECs cultivated with 50% ACM > hBMECs and penetration property, expressed in exact reverse order. Thus, disclosing that the co-cultured cells are having higher similarity with BBB (Kuo and Lu 2011). In another study conducted by Moon et al. (2012) these cells were used to study the effect of rosiglitazone (thiazolidinedione a popular anti-diabetic) on the expression of a low-density lipoprotein receptor-related protein (LRP1) which is an Aβ efflux transporter in BBB. They concluded that rosiglitazone at 10 nM concentrations could upregulate the LRP1 expression, thereby clearing the Aβ (Moon et al. 2012).

These studies reveal that hBMECs, used as a potent in vitro BBB model for studying the brain homeostasis and effects of Aβ in altering the physiology and properties of

BBB along with the impact of secondary metabolic cascade reactions, etc. Though these cells were found to have many similarities with BBB obtaining these cell lines is very difficult as they remain obtained from human tissue, which is not readily available and has many complicated ethical problems. The trans-endothelial electrical resistance (TEER) value of cells derived from hIPCs was very less (approximately 100 $\Omega.cm^2$) when compared to that of human BBB (5900 $\Omega\ cm^2$) (Srinivasan et al. 2015). However, it can remain compensated by the addition of retinoic acid in the culture medium which increases the TEER value to (2000 $\Omega\ cm^2$) (Katt et al. 2016).

Brain Capillary Endothelial Cell (BCECs)

Brain capillary endothelial cells (BCECs) are the primary component of the Blood–Brain–Barrier (BBB) which in combination with astrocyte and pericytes assist the function of BBB. The development of a potential therapy for brain disorder needs a suitable in vitro model that represents the BBB. The in vitro BCECs cell line offers a promising tool to study the behavior of BBB in preliminary research investigations (Burkhart et al. 2015). The BCEC cells are isolated from micro-vessels of various animal models like a rat, mice or rabbit by dissection and collection of the forebrain, which is cultured by digesting the unwanted area (Fig. 4) (Huntley et al. 2014). Sometimes, it is co-cultured with the astrocytes and pericytes to make the BCECs mimic the BBB more prominently.

Zandl-Lang et al. (2018), studied the amyloid β (Aβ) clearance and amyloid precursor protein (APP) processing ability of simvastatin and ApoJ on the in vitro BCEC model. For the study, they utilized the primary BCEC, porcine BCEC and murine BCEC in vitro model which were isolated from the 3xTg AD mice model. The murine BCECs, when treated with hydrocortisone showed improved barrier properties (Zandl-Lang et al. 2018). Similarly, Candela et al. (2015) utilized the co-culture of pericytes with the
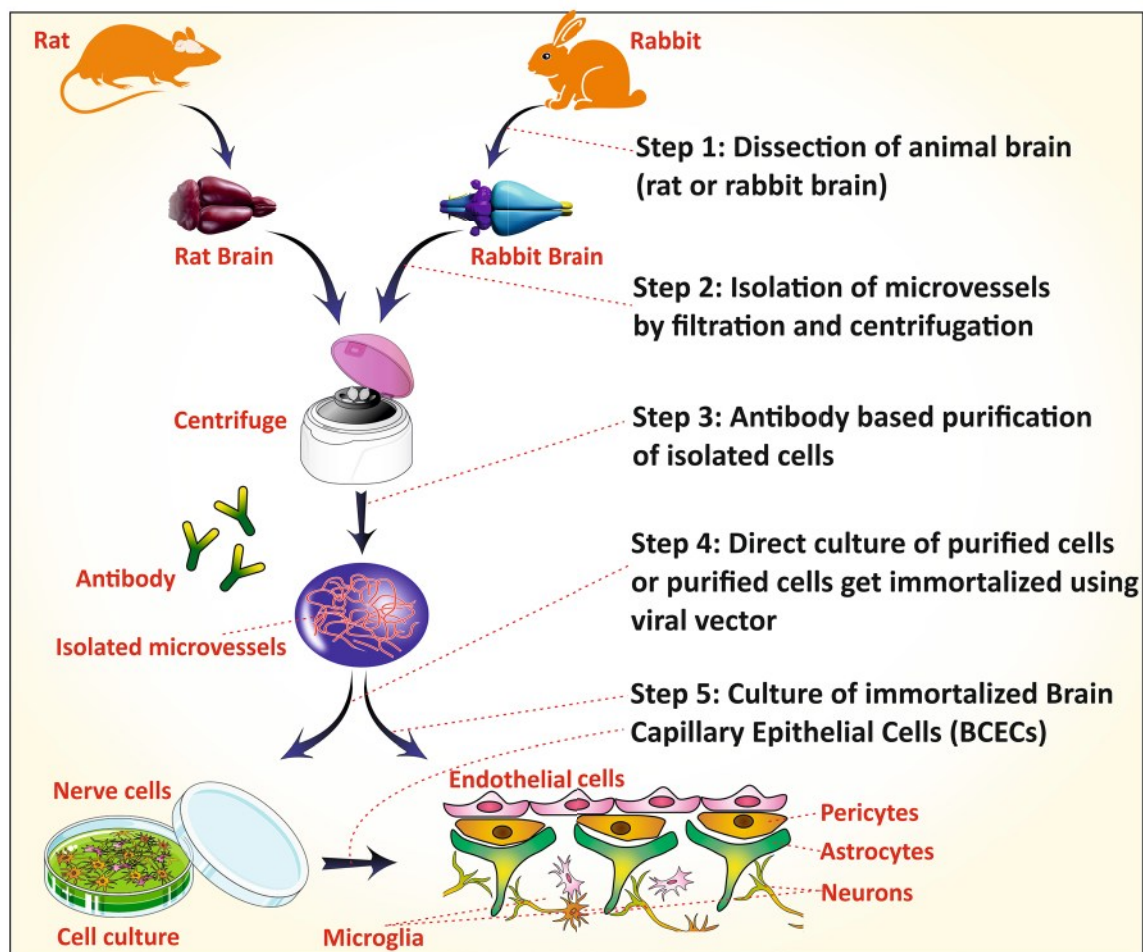
Fig. 4 Steps of BCECs culture from the animal brain. (1) Dissection of animals(rat/rabbit) brain and filtration or centrifugation to isolate microvessels. (2) Antibody-based purification (or) fluorescence-based cell shortening to get purified cells (3) Direct culturing of purified cells (or) purified cells are immortalized using viral vectors (4) Culturing of immortalized BCECs

bovine BCEC as in vitro BBB model to study the role of a low-density lipoprotein receptor-related peptide (LRP1) on the transcytosis and endocytosis behavior of BBB (Candela et al. 2015). Meng et al. (2015) did another critical study in 2015. They studied the cell uptake and cytotoxicity of the curcumin-loaded nano lipid carrier in the bovine BCECs model (Meng et al. 2015; Agrawal et al. 2018b). One more similar piece of work, included the study of BBB permeability of nerve growth factor (NGF) loaded rat monocyte. The drug transportation ability of rat monocyte was evaluated in vitro in the *BCEC* monolayer (Bottger et al. 2010). Such studies demonstrated the applicability of BCECs as the popular in vitro BBB model for studying the permeability, cytotoxicity and cellular uptake of the drug, bioactive or carrier systems. It is challenging to maintain the exact in vivo environment in the laboratory culture media. Thus, the isolated of BCECs may lose various essential characteristics of BBB (like low-passive permeability and high TEER) in the in vitro culture. The co-culture of BCECs with the astrocytes and the pericytes sometimes helps to retain the characteristic behavior of BBB (Burkhart et al. 2015).

hCMEC/D3

Human cerebro microvascular endothelial cell (hCMEC) being immortal human cell lines can proliferate in culture. They exhibit many similarities with that of human BBB by expressing most of the transporters and receptors, including tight junction proteins (TJPs), immune migration properties (Weksler et al. 2013). Co-culturing can increase the integrity of the cells with astrocytes (Daniels et al. 2013). These cell lines, derived from microvessels of the human temporal lobe. The isolate with cerebral microvessel endothelial cells (CECs). Later they are subsequently immortalized by transduction with the catalytic subunit of human telomerase reverse transcriptase using lentivirus as vector and then by SV40 large T-antigen followed by selective isolation of cells by restricting cloning dilution (Weksler et al. 2013). The negative effects of MicroRNA-155 on the function of BBB during neuro-inflammation was studied using these cell lines by Ramirez et al. (2014). The microRNA-155 was found to affect BBB in two ways. One by disassembling the inter-endothelial junctional complex (IJC) and integrin focal adhesion (FA) complexes, which may be due to the altered TJPs and the other way is by increasing permeability of BBB due to cytokines (Lopez-Ramirez et al. 2014). Pal et al. (2016) used these cells to study the histidine-rich Protein II produced by *Plasmodium falciparum*, in promoting the pathogenesis of brain in malarial condition. This protein was found to make the endothelial cells leaky by producing the inflammatory factors (Pal et al. 2016). Sun et al. (2018) studied the expression and processing of Aβ upon aging and the effect of angiotensin II using these cell lines. The

production of Aβ increases with aging due to an increase in β-secretase and reduced expression of α-secretase by Angiotensin II (Sun et al. 2018). Freese et al. (2014) used these cells to study their newly established cell-based bioassay for testing the ability of drugs to cross BBB with the help of Acitretin as a model drug (Freese et al. 2014). From the above studies, it can know that these cells, used as an in vitro model cell lines for BBB. These cell lines help in studying the cerebral-endothelial inflammations, mechanisms of Aβ production, cytokines, immune migration studies, and permeability. These cells despite having several advantages, unable to express the TJPs like occluding and claudin-5 in levels similar to that of primary cerebral endothelial cells (Weksler et al. 2013). They also have very less TEER value (30–50 Ω cm^2) when compared to normal human BBB (5900 Ω cm^2) which may be possibly due to the imperfect formation of tight junction (Weksler et al. 2013; Biemans et al. 2017). However, this problem can be solved by co-culturing the cells with astrocytes (Daniels et al. 2013).

BMVECs

Brain microvascular endothelial cells (BMVECs) are the essential constituents of BBB, and their characteristic morphology allows the development of two discrete surfaces; the blood-sided surface (apical membrane) and brain-sided surface (basal layer) (McCarthy and Kosman 2014). It also possesses astrocytes on the basolateral surface, providing additional feature to these cells and even in protecting the cells against harmful chemicals like ROS. They form TJs, which give the BBB's their integrity and regulates the movement of molecules across BBB (McCarthy and Kosman 2015). Thus, it is used to study the drug transport mechanism across the BBB, and the effect of altered physiological environment upon the integrity of the BBB. These cells, generally obtained from rat brain rather than human. The temporal-cortex is a part of epileptogenic foci of the brain, removed by the surgical process and is subjected to a series of isolation processes to obtain the required BMVECs cell lines (Haorah et al. 2008). Though these cell lines have shown the closest similarity with that of brain epithelium, they need more time for culturing and are prone to contamination, easily. Therefore, they are converted to immortalized cells as they are feasible to maintain and is economical. The conversion is mostly by the introduction of immortalized foreign genes using SV40 large T antigen as a vector (Navone et al. 2013). The neuroprotective action of saikosaponin C (SSc) obtained from the root of *Bupleurum falcatum* was screened on BMVCs cell lines derived from bovine by Lee et al. (2016). SSc showed the reduction of both the release of Aβ and the hyperphosphorylation of Tau (Lee et al. 2015). McCarthy et al. (2014) used co-culture cell line of BMVCs and C6 glioma cells (as an astrocyte cell line) to investigate

the pathways involved in the movement of iron across the BBB and effect of ceruloplasmin and hepcidin on the flow of iron. In general, the ceruloplasmin increased the efflux and hepcidin increased the internalization of iron in the brain (McCarthy and Kosman 2014). The neuro-inflammatory effect on BBB in HIV-1 encephalitis (HIVE) condition was studied using these cell lines by Ramirez et al. (2010a, b). In HIVE, there is an upregulation of CD40/CD40L mediated by JNK signaling, which in turn upregulates the expression of several adhesion molecules. Thereby, increasing the adhesion of monocyte to BBB and thus it stimulates their migration across BBB (Ramirez et al. 2010a). A similar study was conducted by Ramirez et al. (2010a, b) to study the effect of GSK-3β on inflammation of brain endothelial cells. GSK was known to stimulate the immune responses and inflammatory responses. Findings reported that the suppression of GSK would, in turn, suppress the secretion of proinflammatory mediators from inflamed cells, thereby, inhibiting the monocyte adhesion and migration by suppressing the expression of adhesion molecules (Ramirez et al. 2010b). These studies expose the use of BMVCs as in vitro BBB model and help to study the mechanisms that act to alter the integrity of BBB during neuronal diseased conditions and permeability of molecules across it. Though, as usually obtained from the human brain, due to ethical problems most of the time they are received from animals, which has its limitations like the difference in the biochemistry of cells. Even the culturing is time consuming, costly and loss of characteristics over the passage of generations. To overcome these limitations, these cells were transformed into immortal cells, even though they are more beneficial than healthy cells, they suffer from tumors of nature (Navone et al. 2013).

Mouse Brain Endothelial Cells (bEnd3)

Endothelial cells are the primary components of the BBB that lines the blood capillaries. bEnd3 cells, derived from the brain endothelial cells express the TJ proteins, transport proteins mainly P-glycoprotein (P-gp) an efflux transporter used in clearing the Aβ from the brain and also these cells can form tight polarized monolayer limiting the paracellular permeability mimicking the properties of BBB (Qosa et al. 2016). These cells are derived from the endothelium of mouse brain and immortalized by the introduction of polyomavirus middle T-antigen using NTKmT retrovirus as a vector (Andrews et al. 2010). The increased effectiveness of PLGA PEGylated memantine nano-particles in comparison with the free drug for treating AD was screened using these cell lines by Sanchez-Lopez et al. (2018). The PEGylated formulation was found to have sustained release profile, and it also decreased the frequency of administration (Sanchez-Lopez et al. 2018; Alexander et al. 2014b). The neuroprotective nature of rifampicin and caffeine was screened on these

cell lines by Qosa et al. (2012). Both these compounds were found to act in the same manner by upregulating LRP-1 and P-gp proteins, thereby increasing the clearance rate of Aβ from the brain (Qosa et al. 2012). Adiponectin is an adipokine known to control several metabolic functions and to decrease inflammation. Its role in controlling the apoptosis and expression of TJPs in brain endothelial cells was studied on these cell lines by Song et al. (2017). The activation of adiponectin inhibits the activation of NF-κB under Aβ toxicity, thereby inhibiting the release of pro-inflammatory cytokines. Hence, it protects the integrity of BBB by seizing the apoptosis/death of endothelial cells (Song et al. 2017; Gremmels et al. 2015). The murine brain endothelial cell line was utilized by Park et al. (2014) to understand the mechanism underlying the reduced expression of P-gp by Aβ. Usually, the expression of P-gp, induced by factors released from astrocytes that are in contact with endothelial cells. However, sometimes in the presence of Aβ astrocytes loose contact with endothelial cells via NF-κB signaling interacting through RAGE receptors. Therefore, the P-gp expression remains excluded in the presence of Aβ (Park et al. 2014). Above studies exposes the use of these cells as an in vitro model of BBB. It helps in studying the neurodegenerative mechanisms and their effect on BBB integrity like TJPs, permeability, inflammatory mechanisms and Aβ clearance rates from the brain. These cells, derived from mouse, so cannot replicate the exact conditions as that of human BBB. They cannot effectively differentiate the transcellular and para-cellular transport (Yang et al. 2017a). They also suffer from having very less TEER value (15 Ω cm^2) when compared to that of BBB which can be increased to about 250 Ω cm^2, when co-cultured with astrocytes (He et al. 2014).

Rat Brain Endothelial Cells (RBE4 cells)

RBE4 cells, used as an in vitro model for studying the characteristics of BBB. Being derived from a rat's brain, they possess some similarities with that of human BBB by exhibiting cationic, carnitine, serotonin and P-gp transporters expresses the majority of neuronal factors as it is of neuronal origin (Naassila et al. 1996; Kis et al. 1999). They also possess high TEER values as they are co-cultured with astrocytes. They are mainly used to study the transport mechanisms of drugs across the BBB (Roux and Couraud 2005). The primary cultures of RBE cells are derived from the endothelium of the rat's brain by enzymatic digestion, given by Molino et al. (2014) (Molino et al. 2014). The transduction of these primary cultures with an immortalizing gene (SV40 or adenovirus EIA or polyomavirus large T-antigen) using either plasmid DNA or retroviral vectors making the cells immortal. RBE4 cells are a result of co-culturing of primary RBE along with astrocytes, thus

making the cells responsive to astroglial factors (Roux and Couraud 2005). Fonseca et al. (2014), used these cells to study the mechanism by which Aβ dissuade proteostasis in brain endothelial cells. According to this, Aβ persuades the failure of the response mediated by endoplasmic reticulum stress adaptive unfolded protein and deregulating the ubiquitin–proteasome system, which declines the activity of proteasome along with accretion of ubiquitinated proteins and damaging the autophagic protein degradation pathways that end up in cell death (Fonseca et al. 2014). The feasibility of chitosan as a nano-carrier for anti-AD drugs to pass through BBB was examined using these cell lines by Sarvaiya and Agrawal 2015. Chitosan (cationic polysaccharide), used as a nano-carrier because of hemocompatibility and biocompatibility with their application in parenteral and nasal drug deliveries (Sarvaiya and Agrawal 2015; Alexander et al. 2014a, 2016a). The effect of Cilostazol on the functioning of BBB was studied using these cells by Horai et al. (2013). Cilostazol is a selective phosphodiesterase-3 inhibitor and seems to increase BBB's TEER value and decrease permeability (Horai et al. 2013). Durk et al. (2012) also utilized these cells to study the effect of 1α,25-dihydroxyvitamin-D3 on P-gp. 1α,25-dihydroxyvitamin-D3 upon binding to a Vit-D receptor on nucleus which upregulates the expression of P-gp (Durk et al. 2012). The above studies revealed the use of these cells as an in vitro model for BBB. It helps in studying the mechanisms that disturb the integrity of BBB, permeability across BBB and inflammatory responses. The limitation of this model is its high para-cellular permeability to small molecules. These cells are unable to generate compact monolayer and also has small barrier functions due to reduced expression of γ-glutamyl transporter-1 and alkaline phosphate activity (He et al. 2014). The TEER value is also decidedly less when compared to that of BBB when co-cultured with astrocytes and pericytes the TEER value increased but is not up to the mark (Roux and Couraud 2005).

Human Neuroblastoma Cell (SHSY-5Y)

Neuroblastoma cells are one of the widely employed cell lines as an in vitro model of AD. AD is characterized mainly by the depletion of acetylcholine, which is primarily due to the degradation of cholinergic neurons in the hippocampal region (Belle et al. 2004). Neuroblastoma cells, obtained in both undifferentiated and differentiated forms. Undifferentiated forms resemble mainly the immature cholinergic neuronal cells, and mature cholinergic cells, obtained upon differentiation (Kovalevich and Langford 2013). These similarities made the neuroblastoma cells very useful in studying the development, progression and drug discovery of AD. These cells are isolated from neuroblastoma a cancer of immature neuronal cells in the embryonic stage (Giri et al.

2018). These SHSY-5Y cells remain obtained after subcloning the parent line SK-N-SH. The resultant undifferentiated cells can remain differentiated into different neuronal phenotypes depending on the type of differentiating agent used. SH-SY5Y cells differentiate into cholinergic neuron phenotype upon treating with retinoic acid (RA). If phorbol esters are co-administered with retinoic acid, dopaminergic phenotype cells remain formed in higher percentages. If treated with a dibutyryl cyclic AMP, noradrenergic phenotype cells are formed predominantly (Kovalevich and Langford 2013; Elnagar et al. 2018). Koriyama et al. (2015) used these cells to study the glyceraldehyde (glycolysis inhibitor) as an inducer of AD. Glyceraldehyde induces AD-like pathophysiology by inducing production of GA-derived advanced glycation end products (GA-AGEs), cell apoptosis, and decreasing the concentration of Aβ but, increases τ-phosphorylation (Koriyama et al. 2015). The effect of anatabine on lowering the $A\beta_{1-40,42}$ levels was screened by Paris et al. (2011a, b) using these cell lines. Anatabine has shown a decrease in levels of $A\beta_{1-40,42}$ by inhibiting the transcription of β-secretase thus, affecting the β-cleavage of APP (Paris et al. 2011b). Petratos et al. (2008) also used these cell line to study the effect of Aβ on the neurite outgrowth from these cells and also to determine the initiation of downstream signaling and mechanisms that facilitate these effects. The neurite outgrowths are due to the phosphorylation of collapsin response mediator protein-2 (CRMP-2) where the extent of phosphorylation, regulated by Aβ induced an active form of RhoA (Petratos et al. 2008). These cells were also used by Avrahami et al. (2013) to study the inhibition of glycogen synthase kinase-3 (GSK-3) in ameliorating the pathology of Aβ and the mechanisms underlying that. The study gave positive results, by TOR receptor restoration and inhibiting the lysosomal acidification (Avrahami et al. 2013; Chonpathompikunlert et al. 2011). The above studies revealed that SH-SY5Y cells could be used as an in vitro neuronal model to study the different mechanisms other than well-known Aβ and τ-protein, which lay the path to AD, inflammatory cytokines, and NF-κB-signaling pathways. Despite having many advantages, they suffer from their drawbacks such as having cancerous cell nature like withstanding high-oxidative stress, expressing high levels of oxidative stress response genes in similar forms and high rates of glycolysis, which may alter the experimental outcomes (Forster et al. 2016; Krishna et al. 2014).

SK-N-MC

SK-N-MC, human neuroblastoma cell lines, are mainly cholinergic. The cholinergic nature is primarily due to the presence of α7-nicotinic receptors. These cell lines are influential in the study of AD, due to the reason that the mainly affected neurotransmitter in AD is acetylcholine (Gheysarzadeh and

Yazdanparast 2012). Besides the cholinergic property, they are immortal cells. Hence they are very feasible to culture and cost-effective (Kuo and Tsao 2017; Alexander et al. 2016b). These cells are obtained from a metastatic supra-orbital human brain tumor. The cancer tissue is minced by placing in the medium and then treating with 0.125% trypsin and 0.02% EDTA in phosphate buffer devoid of calcium and magnesium, within 2–3 days a cell suspension remains formed. The cell suspension is (Biedler et al. 1973; Pin et al. 2007). The efficacy of liposomes grafted with lacto-ferrin (Lf) in delivering the drug across BBB was studied on these cell lines by Kuo and Wang (2014). In their study, a neuroprotective agent NGF was loaded into liposomes and screened for their efficacy to pass through HBMECs and to protect these cells against β-amyloid toxicity (Kuo and Wang 2014; Agrawal et al. 2017). Kuo and Tsao 2017) led a similar study to screen the efficacy of quercetin-loaded liposomes, which are grafted with lactoferrin, RMP-7 and bradykinin analog to cross the HBMECs and to shield these cells against toxicity induced by Aβ (Kuo and Tsao 2017). Shaykhal-ishahi et al. (2010) studied the neuroprotective action of AA3E2 (triazine derivative) against Aβ induced toxicity, by co-incubating the drug with these cells. The AA3E2 found to attenuate the production of ROS, caspase-3 activation and also decreased the rate of apoptosis (Shaykhalishahi et al. 2010). The neuroprotective nature of 1,3-diaryl-2-propane-1-one derivatives against damage induced by H_2O_2 was screened using these cell lines by Bayati et al. (2011). The mentioned studies reveal the use of SK-N-MC cells as an in vitro neuronal model. They help in studying the mecha-nisms of disease development. They are of tumorous origin; hence they may sometimes express properties of tumors. The evidence of the presence of double-minute chromosomes makes them differ from the neurogenic nature. These prop-erties make them away from their usage as neuronal models (Biedler et al. 1973).

Pheochromocytoma Cell Line (PC-2 Cell Line)

Pheochromocytoma cell line (PC-2) cells, despite being the non-neuronal origin, are used in studies related to the nervous system. These cells are available in two forms as undifferentiated and differentiated cells (Lv et al. 2017). The undifferentiated cells are similar to immature neurons and express some neural characteristics like synthesis, storage, and secretion of some neurotransmitters such as dopamine (DA) and noradrenaline (NA). The differentiated PC12 cells phenotypically resemble sympathetic ganglion neurons despite being used in the study of AD via the Aβ pathway; these cells used for analytical estimation of other patho-logical factors like tau-phosphorylation and oxidative stress conditions (Wei-Li Wang et al. 2015). PC12 cells are com-monly derived from the pheochromocytoma of the adrenal

cortex of *Rattusnorvegicus* (Kudo et al. 2011). Initially, the isolated similar cell lines get differentiated into specified cell lines upon treating with nerve growth factor (NGF) (Wei-Li Wang et al. 2015). The effect of phenolic compounds obtained from *Pueraria lobate* in protecting the neuronal cells against Aβ-toxicity was screened by Choi et al. (2010) utilizing these cell lines (Choi et al. 2010). The antioxi-dant activity of ethanol extracts and other compounds from *Pyralo decorate* against H_2O_2-induced cytotoxicity by using these cell lines was screened by Yang et al. (2017a, b) (Yang et al. 2017b). The neuroprotective effect of selaginella using differentiated PC12 cell lines on cytotoxicity and apoptosis induced by L-glutamate remained screened by Wang et al. (2010). Selaginella possesses anti-oxidant and anti-apoptotic behavior and hence served as a promising neuroprotective agent. It is commonly isolated from *Saussurea pulvinate* (Wang et al. 2010). These cells were also used by Zhang et al. (2016a, b) to study the neuroprotective nature of lep-tin. Leptin is an endogenously produced adipocytokine; it decreases the levels of Aβ and phosphorylation of τ-protein by antagonizing the Wnt-signaling pathway (Zhang et al. 2016b). The above studies reveal the use of these cells as an in vitro neuronal model and mainly used to study the path-ways for AD pathophysiology and in screening the anti-AD drugs. PC12 cells are complicated to cultivate, time consum-ing and costly. The origin of these cells is also problematic in studies relating to human because as they are from rat, they are unable to mimic the exact scenario of human AD condition. They are also less sensitive to acetylcholine, but this problem remains solved by differentiation (Wei-Li Wang et al. 2015).

Human Neural Progenitor Cell (hNPCs)

Human neuronal progenitor cells (hNPCs) are used as in vitro neurological models to study the neurological dis-eases and also has the potential to treat neuronal diseases such as spinal cord injury or stroke (Fukusumi et al. 2016). These cells exhibit a positive response to glutamine, GABA and synapsin 1 representing the presence of glutamatergic and GABA receptors and also shows synaptic transmission with each other. These can also differentiate into various types of CNS cells (Kang et al. 2017). hNPCs, derived from multiple sources like human embryonic stem cells (hESCs), human-induced pluripotent stem cells (hiPSCs) and some-times from the dead human cortex (Fig. 5) (Brafman 2015; Schwartz et al. 2003; Hibaoui and Feki 2012). HiPSCs are mostly used for generating hNPCs. There are various meth-ods for producing these cells from hiPSCs. Fukusumi et al. (2016) used the embryoid body formation-based method (EBFM) and embryoid body formation method using dual SNAD inhibitors (dSMADi) (Fukusumi et al. 2016).

Fig. 5 Derivation of hNPCs from different sources. HECs and iPSCs, derived from inner cell mass of the blastocyst and reprogrammed somatic cells are induced to differentiate into hNPCs by giving neuronal-specific stimuli. These hNPCs are further used to form mature neurons, glial cells, and other neural-related cells
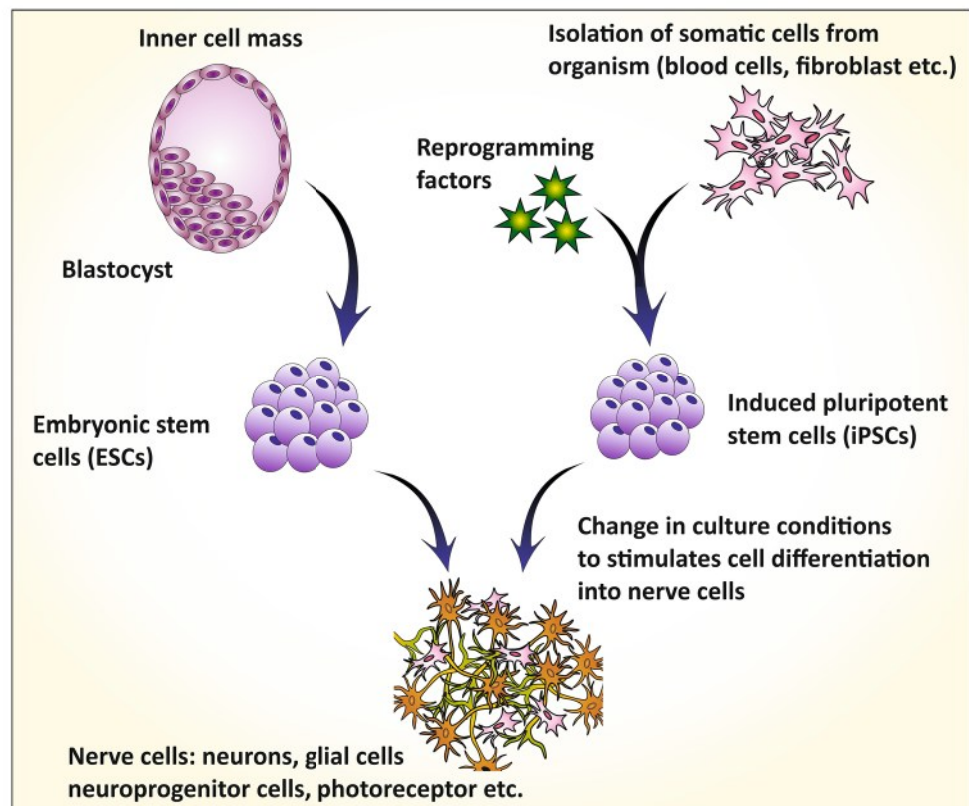
Brafman (2015) used serum-free differentiation method grounded on TGF-β signal modulation (Brafman 2015).

Kim et al. (2018) used these cells to study the underlying mechanisms relating to TNF-α in protecting the neurons under ischemic conditions. The procedure includes pretreating the cells with TNF-α and then exposed to ischemic conditions. They observed that the neuroprotective nature is due to the drastic increase in NF-κB signaling by TNF-α (Kim et al. 2018). Daugherty et al. (2017) used these cells to examine the effect of new and novel AD drug, targeting inflammation and fatty acid metabolism (Alexander et al. 2016a). The anti-AD drug, CAD-31 a derivative of J147 (neurotropic molecule) that acts in this unique pathway, where it increases the production of precursors like Acetyl-CoA and acylcarnitine's that enhance oxidation of fatty acids. Simultaneously, it inhibits the fatty acid synthesis by activating adenosine monophosphate-activated protein kinase (AMPK) (Daugherty et al. 2017). The neural progenitor proliferation by an herbal drug from *Acori tatarinowii* (AT) was screened using these cell lines by Mao et al. (2015). Neuro progenitor cells (NPCs) proliferation and self-renewal can be seen throughout the life but decreases upon aging and in some neurodegenerative disorders. The active constituents of AT are found to increase proliferation of NPCs by activating extracellular signal-regulated kinase (ERK) (Mao et al. 2015). These studies reveal the use of hNPCs as a potent in vitro neuronal model and assists in studying underlying pathophysiological mechanisms, cellular electrophysiology and protein biochemistry. hNPCs derived from specific patient helps in understanding the patient-specific genetic mechanisms underlying the pathophysiology. The disadvantage of this cell is that to date there is no clear description of the characteristic parameters of differentiated cells in different stages of development (Kang et al. 2017). Existing differentiating substrates used for discriminating hNPCs such as laminin (LN) are very costly and isolating the differentiated cells is also very difficult (Tsai et al. 2015).

Human Embryonic Kidney Cell 293 (HEK293)

HEK293 cells are non-neuronal cell lines, but it remains used in the studies relating to neurological diseases, for the reason that these cells exhibit several neurological factors like expressing neuro-filament subunits and other neuron-specific proteins (Gordon et al. 2013). They seem to be very useful in the study of tau-related neurological effects as they provide general stability, viability, and feasibility for transfection of tau protein (Houck et al. 2016). HEK 293 cells, derived from healthy human embryonic kidney (HEK) cells. These HEK cells, cropped with Adenovirus-5-DNA which makes these cells similar to immature neurons (Lin et al. 2014). These cells again derived into 293T, 293S, and 293SG, representing the behaviors like generating the stable

clone, adapting to suspension growth and cytotoxic lectin selection (Lin et al. 2014). In 2014, Zhang et al. (2014a, b) used the cells to study the paradoxical effects that occur due to inhibition of TrKA in AD. A cytotoxin is containing 31 amino acid chain (C31), produced by slicing the APP with caspase, mediated via TrKA signaling. Inhibition of TrKA reduces the production of C31 thus, reducing cytotoxicity (Zhang et al. 2014b). Chang et al. (2016) used these cells to examine the effect of *Glycyrrhiza inflata* aqueous extract in upregulating the unfolded protein response, which mediates the chaperones to decrease the misfolding of tau protein and production of ROS in AD cell models (Chang et al. 2016). He et al. (2010) used these cells. In understanding the new therapeutic targets for AD, reporting the discovery of γ-secretase-activating protein (GASP). GASP was seen to interact with γ-secretase and APP, increasing the production of Aβ. The inhibition of GASP, used as a novel promising therapy of AD, by decreasing Aβ without affecting any other essential function of γ-secretase (He et al. 2010). These cells were also utilized by Zhou et al. (2017) to study the systemic clearance and distribution in the brain of new anti-AD drugs. They reported the novel Carbazole-based cyanine compounds which inhibited the aggregation of Aβ (Zhou et al. 2017). The above studies suggest the use of these cells as an in the vitro neuronal model in studying the pathways leading to AD mainly related to τ-protein, cytotoxicity, and screening of anti-AD drugs. The main drawback of these cells is their non-neurological origin. Even though these cells express NF-M, NF-L, and α-internexin as major NF protein along with minor NF-H whose concentrations are similar to that of developing mammalian central neurons, indicating HEK293 cells similarity with neuronal stem cells, they cannot match with neurons or neurologically originated cell lines (Gordon et al. 2013).

7W Chinese Hamster Ovary Cell Lines (7W CHO Cell Lines)

These cells originated from the ovary of the Chinese hamster, which upon transfection with human APP751 express the APP protein, Aβ, and related proteins. They also show and act as a model to study the mechanisms relating to presenilin genes, upon transfecting PS1 and PS2 genes (Octave et al. 2000). When transfected with ApoE genes, they can express the mechanisms underlying ApoE and can act as a model for it (Irizarry et al. 2004). The primary cell line for 7W CHO cell line is Chinese hamster ovary (CHO), which upon transfection with human APP751 genes converts into 7W CHO (Paris et al 2014). The transfection is mediated by pCMV751 an expressing vector using lipofectin-facilitated transfection (Koo and Squazzo 1994). In addition, pACL29 vector applied for a transfecting PS1 gene into CHO. Anekonda and Quinn (2011) used these cells to screen Ca²⁺

channel blockers as a therapeutic approach for treating AD by taking isradipine as a model drug. Isradipine is a dihydropyridine Ca^{2+} channel blocker, which selectively binds to the L-type Ca^{2+} channel present on the somatodendritic and axons of the hippocampal and cortex regions. The blocking of these channels lowered the τ-burden and improved the brain autophagy functions (Anekonda and Quinn 2011). A similar type of study was conducted by Paris et al. (2011a, b) to screen the effect of several anti-hypertensive dihydropyridine Ca^{2+} channel blockers in reducing the levels of Aβ. Among the tested drugs, nilvadipine and amlodipine showed promising results (Paris et al. 2011a). Spilman et al. (2014) used these cells to study the effect of tropisetron in treating AD. Tropisetron binds to the ectodomain of APP and decreases the production of anti-tropic, neurite-retractive peptide Aβ, thus reverses AD condition (Spilman et al. 2014). D. Paris et al. (2014) studied the mechanism by which the spleen tyrosine kinase (Syk) helps in regulating Aβ production and tau hyperphosphorylation. Inhibition of Syk mimics and downregulates the production and increases the clearance of Aβ by decreasing the secretion of sAPPβ by stimulating Aβ clearance (Paris et al. 2014). It also plays an essential role in reducing τ-phosphorylation by acting through protein kinase A. These studies reveal the use of 7W CHO cell lines as an in vitro model for Alzheimer's in studying the mechanisms relating AD pathology and effect of different genes in AD. These cells, originated from hamster, have a physical and biochemical difference with that of the human. Koo and Squazzo (1994) showed the difference like Aβ produced from CHO cells with that of human-derived cell lines (Veeraraghavan et al. 2011).

CALU-3 Cell Lines

Nowadays, administration through the nasal route for targeting brain remains widely used. Through the nasal administration, the drug can directly enter the brain by passing through trigeminal and olfactory nerves or by absorbing through the nasal mucosa, avoiding BBB hindrance. However, the mucosa of the nasal cavity forms a nose–brain barrier restricting the conduction and bioavailability of drugs to the brain. These cell lines by mimicking nose–brain barrier help in studying the mechanisms of the passage of the drug through the nasal cavity to the brain (Zheng et al. 2015). These cells, initially derived by Fogh (1975), from adenocarcinoma of the serous epithelium of human lung (Fogh 1975). Zheng et al. (2015) used these cells in 2015 to screen the efficiency of the H102 peptide, a novel drug candidate administered through the intranasal route, for the treatment of AD. It is a β-sheet breaker, delivered in the form of liposomes (Zheng et al. 2015). A similar study for delivering the puerarin, an isoflavone glycoside extracted from *Radix puerariae lobatae* to the brain via intranasal administration,

conducted by Zhang et al. (2016a, b). These cells can be used as an in vitro model for nasal passages and are useful in understanding the mechanism of the passage of drug through the nasal membrane to brain administered via nasal route (Zhang et al. 2016a). These cells develop from the tumor cells. Hence they express the cancerous properties such as higher rates of oxidation. They also represent a wide range of TEER values from 100 to 2500Ω cm^2 depending on the type of culture and culture medium (Srinivasan et al. 2015).

Induced Pluripotent Stem Cells (iPSCs)

iPSCs are self-renewable cells that are able to differentiate into different types of cells including neuronal cell line and represent a promising tool for researchers to study the disease etiology, progression and drug efficacy (Haston and Finkbeiner 2016; Poon et al. 2017). iPSCs have the potential to proliferate and differentiate into different AD-relevant cells such as astrocytes, microglia, forebrain neurons, etc. which allows the study of individual neuronal cells, their functions and interdependency within the brain (Fig. 6) (Sullivan and Young-Pearse 2017; Zhang et al. 2014a). The iPSCs was first derived by Takahashi and Yamanaka (2006), they investigated that some factors present in oocyte cytoplasm possess an ability to reprogram the somatic cells into

the embryonic cell like a stage and thereby induced pluripotency to the somatic cells. Initially, they induced pluripotency to the adult mouse fibroblast by treating with Sox2, Oct3/4, Klf4 and c-Myc cytoplasmic factors (Takahashi and Yamanaka 2006; Mungenast et al. 2016). These four oocyte cytoplasmic factors are termed as 'Yamanaka factors.' Later in 2007, they reprogramed the human fibroblast cells with these Yamanaka factors into hiPSCs (human-induced pluripotent stem cells) (Takahashi et al. 2007). From the first iPSC discovery to date, the cell biology developed different methods to differentiate the iPSCs into specified human neuronal cells which recapitulate the AD-like condition (Sullivan and Young-Pearse 2017). For in vitro AD modeling, the iPSCs have been particularly differentiated into astrocytes (GFAP+) and neurons like βIII-tubulin and MAP2+ (Yagi et al. 2011; Israel et al. 2012). However, the neurons of cortical forebrain (TBR1+) and cholinergic nerves of basal forebrain (CHAT+), primarily associated with the AD etiology can be recreated by some special growth factor signals produced by additional media supplements (Duan et al. 2014).

In 2011, Yagi and team first demonstrated the application of iPSCs in AD modeling. They produced iPSCs from patients having a familial AD with mutated genes presenilin-1 (A264E) and presenilin-2 (N141I). The culture of AD cell line contains an excess of extracellular Aβ_{40-42} in the
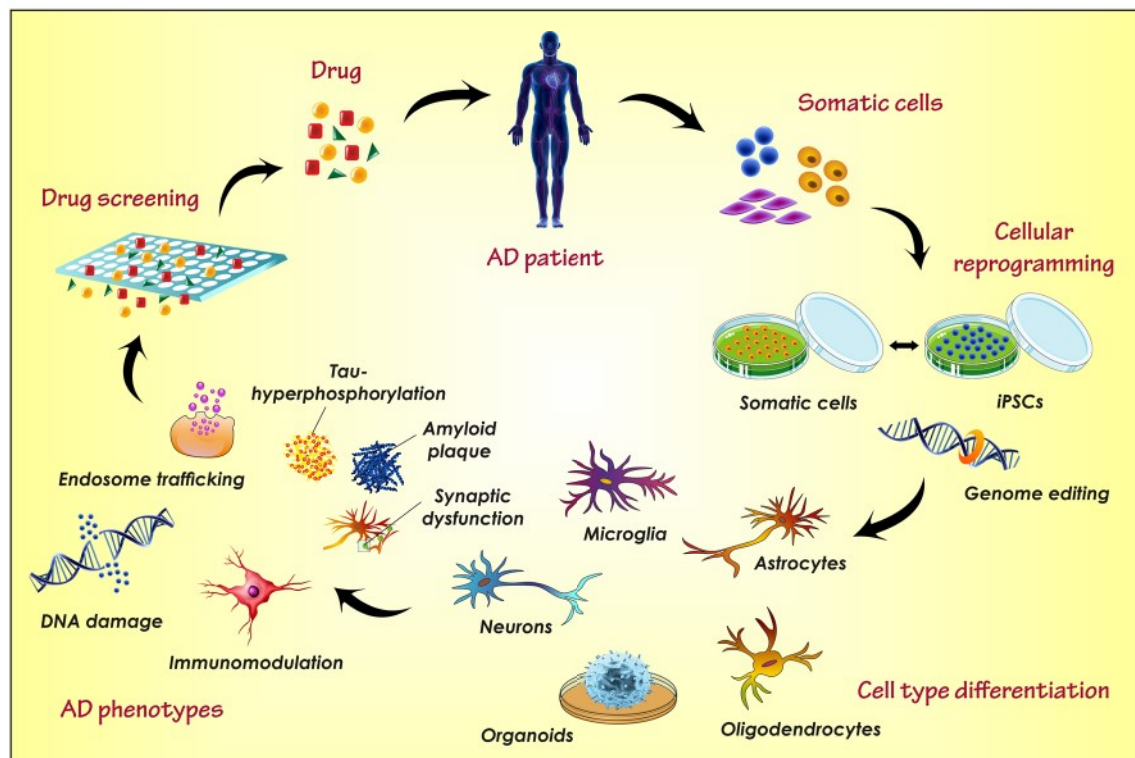
Fig. 6 The figure illustrates the reprogramming and culture of AD patient-derived somatic cells/nerve cells as iPSCs and its differentiation in different nerve cells, application in the investigation of AD pathophysiology and screening the drug efficacy [adapted and modified from (Mungenast et al. 2016)]

supernatant which is the prime component of AD pathology. While a significant reduction in the $A\beta_{40-42}$ ratio was observed when treated with a γ-secretase inhibitor (Yagi et al. 2011). Later, in 2014, Sproul et al. also produced iPSCs line with increased $A\beta_{42/40}$ ratio by culturing the nerve cells of AD patients with a mutation in the presenilin gene (Sproul et al. 2014). Further, Balez and co-workers (2016) had co-cultured the iPSC-derived nerve cells with the murine-activated microglial cells. They recognized the effect of oxidative stress and inflammation in AD progression and observed considerably higher apoptosis of iPSC-derived AD nerve cells than the familial and sporadic AD patients (Balez et al. 2016). Similarly, it is used to study various phenotypes of AD which facilitates the understanding of AD etiology and also assists the study of novel therapeutics. Such studies support the application of iPSCs as a promising tool for the investigation of AD pathology. However, its application is limited because the iPSCs cannot recapitulate all the pathological phenotypes of AD which may be due to the simple culture methods (Poon et al. 2017).

Cerebral Organoids and 3D Models

Neurofibrillary tangles and amyloid plaque formation is the primary factor of AD which further initiates or boosts the other phenotypes of AD-like inflammation, nerve cell death, synaptic and cholinergic dysfunctioning, etc. Despite the availability of numerous in vivo and in vitro models, the suitable experimental model which exactly recapitulate the essential features of AD nerve cells is still lacking (Gonzalez et al. 2018). In this sequence, the cerebral organoids and iPSCs represents a new and promising model for AD investigation. With the advancement of technologies, the scientists make it possible to develop a three-dimensional organ-like structure or artificial organs from the pluripotent stem cells (both the embryonic and induced pluripotent stem cells) known as organoids (Wang 2018; Clevers 2016). In the past few years, various protocols have been established to create the brain or cerebral organoids from the pluripotent stem cells (Dyer 2016; Di Lullo and Kriegstein 2017; Giandomenico and Lancaster 2017; Kadoshima et al. 2013; Lancaster and Knoblich 2014; Li et al. 2017; Sutcliffe and Lancaster 2017). Moreover, some studies claim that cerebral organoids can also be produced from fate-restricted neural stem cells (Clevers 2016; Monzel et al. 2017). These artificial brains or cerebral organoids represents a potential strategy to study the human brain anatomy, physiology, and etiology of neurological disorders.

The brain organoids can be produced by 3D-culture of PSCs or specified neurons. The application of 2D-cell culture is limited to the disease modeling of iPSCs or other single cell lines, particularly for investigation of various neurological disorders. The 3D culture or 3D models

overcome the limitations of 2D models by offering a more physiologically pertinent model of specified tissues, cells, cell-matrix or cell–cell connections (Clevers 2016). The brain organoids created from the 3D culture of neuroepithelium are not just more complex than the 2D models but it also able to recapitulate the different regions of the human brain, the synapse or connection between different regions and connection between various cellular components (Lancaster and Knoblich 2014; Paşca 2018).

The production of a suitable in vitro model for sporadic AD is always a challenge to the scientist because the neurons of in vitro culture are immature to recapitulate the exact scenario of AD brain. Thus, the 3D model and brain organoids offer a promising tool for the modeling of more complex etiological factors of AD including Aβ-aggregation and tau-phosphorylation (Lee et al. 2016; Choi et al. 2014). In 2016, Raja et al. utilized iPSCs of familial AD patients and introduced a scaffold-free cell culture method to create the brain organoids. This brain organoids successfully recapitulates the AD pathologies like the tau-pathies, Aβ aggregation, disease progression and also provide a good prototype for the development of novel therapeutics (Wang 2018; Yan et al. 2018).

The Technique of Cell Culture/Culture Medium Development

The cell culture media helps in the growth and development of cells which can be in the form of liquid or semi-solid. It remains used for maintaining proper growth, replication, and differentiation of cells. Thus, it is very crucial to select and keep in an appropriate medium for in vitro propagation of cells. A suitable culture medium has an appropriate energy source, essential amino acids and vitamins, growth regulators, attachment factors and sometimes differentiating agents.

Standard Culture Medium (SCM)

The standard culture medium is the essential media containing salt solutions, potassium lactate, casein digest and glucose for energy supplementation. Casein acts as a source of nitrogen and amino-acid (except cysteine) (Marshall and Kelsey 1960). As it is an essential medium, mostly it is supplemented with additives like a serum. This media, however, generally used for the culturing of mesenchymal stem cells, human-induced pluripotent stem cells and a few more cell types (Danielyan et al. 2014; Nieweg et al. 2015).

Eagle's Minimal Essential Medium (EMEM)

EMEM has mostly used to culture mammalian cells, and its name came from its developer Harry Eagle. It is pure, basal media containing balanced salt solutions, sodium pyruvate, eight vitamins and 12 kinds of non-essential amino acids. It is formulated to use with 5% CO_2. As a simple medium, it always supplemented with additives, or a high concentration of serum makes them suitable to grow mammalian cells (Yang 2012; Arora 2013). This medium is used to culture embryonic nerve cells, glial cells, and cell lines like SH-SY5Y cells, hBMECs, hCMECs, hNPCs, PC12 and SK-N-MC cells (Waheed Roomi et al. 2013; Aldick et al. 2007; Kiss et al. 2013; Ravichandran et al. 2011; Zheng et al. 2016).

Dulbecco's Modified Eagle Medium Nutrient Mixture F12 (DMEM/F12)

DMEM is the mostly employed cell medium in cultured neuronal cells and is the advanced version of EMEM containing double the amount of amino acids and quadruple the number of vitamins including sodium pyruvate, ferric nitrate, and additional amino acids. Depending on the concentration of glucose this medium occurs in two forms, low-glucose type (1000 g/L) for standard cell cultures and high glucose type (4500 g/L) for cultures containing tumors. These media remains mostly supplemented with 5–10% bovine serum. Same to that of EMEM this is medium is also formulated to use with 5% CO_2. DMEM/F12 is 1:1 mixture of DMEM and Ham's F-12 media (Yang 2012; Arora 2013). It can remain formulated as both serum and serum-free media. This media,

therefore, are used for culturing endothelium, mouse neuroblastoma, and cell lines like bEND3, BCECs HEK293, RBECs, SHSY-5Y, PC12, and Sk-N-MC cells (Hayakawa et al. 2014; Li et al. 2013; Veeraraghavan et al. 2011; Yang et al. 2017b; Park et al. 2014; Zhou et al. 2017).

RPMI Media

These media are developed at Roswell Park Memorial Institute thus the name RPMI. This media is an advanced version of McCOY's 5A media and has proper concentrations of phosphate and remains formulated for use with 5% CO_2. It is one of the utmost broadly employed media and remains used for culturing tumor cells, hybridoma cells and cell lines like PC12, SHSY-5Y and SK-N-MC cells, etc. (Yang 2012; Arora 2013; Wei-Li Wang 2015).

Endothelial Cell Basal Medium (EBM)

EBM is a widely used medium for culturing of endothelial cells. It consists of all the essential salts fetal, vitamins, amino acids and growth factors such as epidermal growth factor and other growth factors depending on the nature of endothelial cells (Labitzke and Friedl 2001). It remains supplemented with fetal calf serum (2.5–5%) or human serum based on the kind of cells. It also has an antibiotic like penicillin or gentamicin and an antifungals agent like amphotericin to protect the cells from contamination during the culturing of the cells (Luu et al. 2010). This media, further, are used in the culturing of HCMECs, umbilical cord vein cells, and hBMECs (Labitzke and Friedl 2001; Biemans et al. 2017; Alexandrov et al. 2015).

Fig. 7 3D cell culture model of an AD for screening AD drugs. Usually, in 2D culture models, testing the drugs for an AD in human (clinical phase) requires the confirmation of potency of the drug in development and screening phases followed by the preclinical stage (animal model). In 3D culture models, the testing of the drug on 3D-cultured cells is enough to carry out the study in clinical aspects as the 3D-cultured cells replicate the exact in vivo conditions. Thereby, 3D models are cost-effective and time-saving than 2D models in the development of new AD drugs
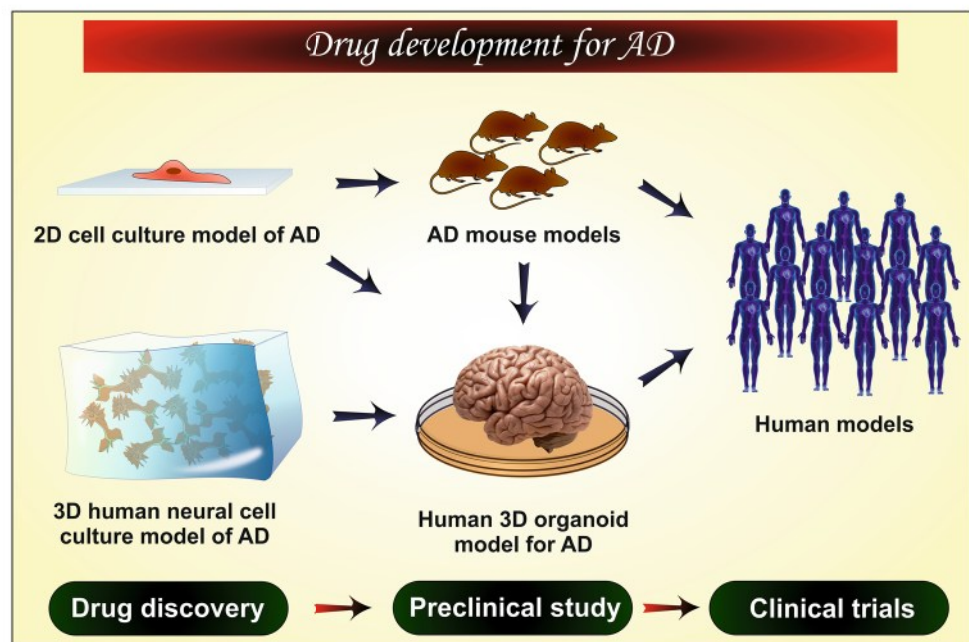
3D-Cell Culture

From decades, 2D culture models are widely used for cell-culturing, and it helped in drug discovery. However, the 2D model does not truly mimic the in vivo condition (Fig. 7) (Jorfi et al. 2018) whereas a 3D culture model offers a higher resemblance with in vivo condition. In Alzheimer's models, secreted Aβ should aggregate within the intercellular spaces. However, in 2D models, most of the secreted Aβ is diffused into the medium and is lost while changing the culture medium on a regular basis. Whereas, in the 3D model, the diffusion remains prevented into medium and there are high chances of forming aggregates as that of an AD, genuinely replicating the in vivo conditions. Different types of cells, most commonly HiPSCs and hNPCs, used in this culture, and the matrices commonly available for culturing are hydrogels, polysaccharide matrices, and matrigel. Among which matrigel remains more suitable for brain cells because of the presence of several proteins such as laminin, entactin, heparan sulfate proteoglycans and collagen (Choi et al. 2016).

The Significance of Cell Line Study

The cell line is a cell culture that is developed from a single cell, and therefore, daughter cells are phenotypically and genetically similar to that of the parent cell, until and unless they are prone to differentiation using differentiating agents. They are used in place of a primary cell and are vital in studying the physiology, mechanism, and progression of disease or disorder and are used in the drug development process. The in vitro cell model offers a promising tool to explore the complex organ of the human body like brain. It provides a supporting data to the researcher's assumption and enhances the translational value of the in vivo models. This method, in general, is practiced in culturing many cells like hair cells, bone, cartilage, and neuronal cells. Usually, it is accessible to culture fibroblast cells in comparison to epithelial cells as they are easy to grow, maintain and take less time. Immortalization can be carried out using large T antigen of Simian virus 40 (S40) or E6E7 gene of human papillomavirus 16. Proper selection of cell preparation methods and cell culture media plays a vital role in maintaining the quality and integrity of the desired cell line which in turn can mimic the in vivo environment. It helps in understanding the exact mechanisms underlying the disease condition. In vitro studies utilizing cell lines is more advantageous in the understanding AD, as well as it is ethically more appropriate in comparison to animal studies (A.B. Ulrich 2001; Kim et al. 2015).

Future Prospects

The drug discovery for brain disorder is very challenging because of the complex structure of the brain and limited experimental sources to study the specific brain environment and pathology of the disease. At the same time, the available in vitro and in vivo techniques and models are very costly, time taking and sometimes raises ethical issues (when it comes to sacrificing animals or human clinical trials). In this context, the in vitro cell models or artificial cell culture provide a more rapid and better means of evaluation for researchers to understand the more precise mechanism, responses, pathophysiology, drug permeation behavior, the toxicity profile of the new drugs/therapy on the brain cells. This will also minimize the need for laboratory animals for preliminary studies during drug development. The cell culture models jointly demonstrated the in vivo conditions and cellular responses which help the scientist to further conclude preclinical and clinical investigations. The artificial cell culture and 3D-cell culture are a more advanced form of the in vitro cell lines, which saves the time as well as the cost of the study and generates more precise results. Various new analytical techniques are currently under research which is supposed to effectively recreate the body environment in the laboratory. Such technologies assure the successful development of AD therapy in the very preliminary stage.

Conclusion

Some in vivo and in vitro models using non-neuronal human cells are being tried to recapitulate the exact scenario of AD, but none of them have succeeded. In vivo models utilizing rodents may not be the method of choice for carrying out AD studies as Aβ formation and accumulation behavior is physically and biochemically different in rodents in comparison to the humans. The most appropriate method is the use of human brain to study the exact mechanism of AD progression, but at the same time, the implementation of the human brain for the preliminary research is highly impractical and unethical. Henceforth, the scientists working for neurological disorders only utilize the postmortem brain. Such restrictions of analytical sources, i.e., the unavailability of suitable in vitro model for cellular estimation diminishes the proper understanding of disease progression which further reduces the chances of development of promising therapy for AD. In this context, the in vitro cell lines mimicking the brain microenvironment appears as a method of choice for understanding initiation, progression, and mechanism of AD. The human

neuronal cells, such as the hiPSCs and embryonic stem cells remain widely used as the most suitable in vitro cell model for AD investigations.

Moreover, a newly emerging 3D culture model is an advanced way out for in vitro study of AD. Various studies have proven that in comparison to the other nerve cell models, the 3D-cell culture recapitulates the exact scenario of the brain environment and can successfully recreate the extracellular Aβ aggregates. Hence, it facilitates the more appropriate estimation of drug efficacy and gives a precise direction to the researchers for the development of promising drug therapy. The future project in this regard, assure to produce an accurate in vitro cellular model to fulfill the new strategies adopted to treat AD, with a definite intention to serve humanity.

Future research can be focussed to produce a more accurate in vitro model which can be used for the development of newer drug-targeting AD, in turn, it can be a tremendous service to humanity.

Compliance with Ethical Standards

Ethical Approval No ethical issues involved.

Conflict of interest We declare we have no conflict of interest.

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Review article

Insulin mediated novel therapies for the treatment of Alzheimer's disease

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ABSTRACT

Alzheimer's disease, a progressive neurodegenerative disorder, is one of the leading causes of death in the USA, along with cancer and cardiac disorders. AD is characterized by various neurological factors like amyloid plaques, tau hyperphosphorylation, mitochondrial dysfunction, acetylcholine deficiency, etc. Together, impaired insulin signaling in the brain is also observed as essential factor to be considered in AD pathophysiology. Hence, currently researchers focused on studying the effect of brain insulin metabolism and relation of diabetes with AD. Based on the investigations, AD is also considered as type 3 or brain diabetes. Besides the traditional view of correlating AD with aging, a better understanding of various pathological factors and effects of other physical ailments is necessary to develop a promising therapeutic approach. There is a vast scope of studying the relation of systemic insulin level, insulin signaling, its neuroprotective potency and effect of diabetes on AD progression. The present work describes worldwide status of AD and its relation with diabetes mellitus and insulin metabolism; pathophysiology of AD; different metabolic pathways associating insulin metabolism with AD; insulin receptor and signaling in the brain; glucose metabolism; insulin resistance; and various preclinical and clinical studies reported insulin-based therapies to treat AD via systemic route and through direct intranasal delivery to the brain.

1. Introduction

Alzheimer's disease, a progressive neurodegenerative disease affecting approximately about 50 million people worldwide, which is supposed to affect around 75 million people by 2030, and 131.5 million by 2050 [1,2]. The American Alzheimer's Association states that about 5.7 million people are found to have Alzheimer's, with almost one person per 65 s generating AD.[1] The disease has become the 6th leading cause of death in America [3,4]. It leads to a progressive decline in cognitive functions and memory (declarative memory) along with behavioral and personality changes [5–7]. The main reason for neurocognition is the impaired activity of acetylcholine in the forebrain [1,8]. Also, impaired glucose metabolism and insulin activity add up to

cognitive impairment [8]. The familial AD is generally supposed to occur because of chromosomal mutation. In recent years, AD is seen to share a link with Diabetes and studies reveal that people who have diabetes are at high risk of acquiring AD [9]. Diabetes, in return, is found to cause neurodegeneration by impairment of normal cellular functions, cerebral blood supply, and neuronal structure due to aberrant insulin signaling and glucose metabolism. It causes hyperphosphorylation of tau protein, the structural unit of the microtubule, results in the formation of neurofibrillary tangles. At the same time, it also stimulates the aggregation of beta-amyloid peptide or $A\beta$ plaque formation which causes neurodegeneration and increases the risk of AD [10,11]. Due to the association of Ad with insulin impairment and abnormal glucose metabolism, some scientists also consider AD as 'type

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  [1] Alzheimer's disease.

3 diabetes'. Clinical presence of dementia may also confirm AD which shows memory and cognitive worsening [12].

Aβ[2] plaque and neurofibrillary tangles formed due to hyperphosphorylation of τ-protein are the hallmarks of AD. Histopathological findings of the AD after autopsy were found to have neuronal losses, amyloid aggregates, fibrillary tangles alongside amyloid angiopathy [10,13,14]. Manipulation in the second chromosome that codes for the APP[3] gene brings about plaques on APP build up. Mutation in the presenilin 1 or 2 gene forms insoluble beta-amyloid 40 and 42, which aggregate plaques. Mutation in the tau protein because of their phosphorylation aggregates tau. While in ApoE[4] mutation, mainly, the E4 allele shows an increased risk of AD development by enhancing the oxidative stress and reducing the beta-amyloid clearance [15,16]. Neuronal loss in the superficial cortex region is a significant feature of AD [17].

Glucose is the primary fuel to the brain, whose deficiency may lead to coma, seizures and create permanent damage to the brain. Glucose reaches the brain via the CBF[5] through the endothelial cells, BBB[6] and finally to the glial cells via facilitated diffusion. Glucose is carried into the glial cells and neurons by the GLUT[7] carriers. Though insulin modifies the glucose metabolism in CNS,[8] it is not the primary mechanism in the brain for the neuronal glucose uptake. But, insulin influences activities like neuronal growth and survival, its synaptic plasticity, neurotransmitter release, tubulin activity, improvement in cognition, memory, learning and so on [18,19].

Till 1950, no evidence illustrated the role of insulin in glucose uptake. But later on, in-vitro studies proved that few areas of the brain are insulin sensitive [20]. The brain receives insulin from the systemic circulation which is then transported to CSF[9] across the BBB via a receptor-mediated process [21,22]. Insulin is also synthesized by the brain itself through the De-novo process [20,23]. Apart from regulating the glucose metabolism in the brain, insulin also plays a role in the growth and survival of neurons, triggering signal transduction cascades that take part in long-term memory consolidation, modulating the synaptic activities of receptors like GABA[10] and glutamate [12,24,25]. Hence, it is to be proved that insulin is essential for brain activity and its deficiency may lead to neurodegenerative disorders like AD (Fig. 1).

Recent findings have suggested that a long-term diabetic patient has more chances of acquiring AD. It is because insulin deprivation in the brain causes memory and cognitive impairments. Also, free radical production owing to oxidative stress causes vascular damage that impairs the nerve function. Brain cells that are exposed to amyloid bodies also produce free radical [26]. Thus, treating patients with insulin will help in managing AD. However, the long-term effects of insulin treatment for an AD may cause insulin resistance. Also, regular insulin treatment will produce a hypoglycemic effect in diabetic patients [27]. Thus, intranasal insulin therapy may be of a good choice for treating the same. This article aims to present the relation of AD, insulin therapy and diabetes. It covers many relevant studies from 2000 to 2018 demonstrating the application of insulin and its analogs or sensitizers for the treatment of AD. The use of intranasal insulin is also explained in detail [28].

2. Pathophysiology of AD

The AD is of two types, namely EOAD,[11] which occurs mainly before 65 years of age and LOAD[12] or sAD,[13] which is age-related and happens after 65 years of age. Only 1 to 6% of the total AD is considered to be early onset. EOAD is generally FAD,[14] occurring mainly due to the aggregation of Aβ plaques, hyperphosphorylation of tau proteins and mutations in presenilin genes (autosomally dominant). On the other hand, LOAD is primarily due to disturbances in the neurotransmission and ApoE [29]. Because of the competitive binding of Aβ to the insulin receptor, there occurs inhibition in the insulin transduction pathway in EOAD, whereas it is receptor desensitization in the case of LOAD [30,31]. LOAD lacks the clearance of Aβ plaques and shows more impairment in verbal memory than non-verbal. However, there is controversy about the same. The frontal-parietal lobe of the brain is supposed to be associated with EOAD, while it is the temporal lobe action with a proneness for the left hemisphere that is associated with LOAD [32,33]. The pathways for the putative mechanisms underlying EOAD and LOAD are as follows:

2.1. Cholinergic hypothesis

During LOAD, there is nearly 75% loss of cholinergic neurons, which hence leads to memory loss and cognitive impairment [34]. It is because of the degeneration of basal forebrain cells that help in the formation of the hippocampus [8]. Also, because of the reduction in acetyl CoA due to the diminishing activity of PDH,[15] Ach synthesis is said to drop down [24]. Impairment in the utilization of glucose because of IRs[16] is also reported to impair Ach synthesis. Therefore, treatment for an AD with ACh[17] agonist and AChEI[18] shows the significant impact in reversing memory impairment by increasing glucose uptake and insulin level in the brain and hence, increase ACh concentration. But the long-term cure was not effective with AChEI's like Rivastigmine, donepezil, and galantamine. Also, not everyone responded to AChEIs. Therefore, this hypothesis is not much in focus when AD is considered [35].

2.2. Amyloid cascade hypothesis

Amyloid beta is formed by the cleavage of a large precursor protein called APP, which is created by coding the APP gene which is present on chromosome-21. APP is a transmembrane protein with a large extracellular glycosylated N-terminal and short intracellular C-terminal [36]. During cellular metabolic processes, the APP can be cleaved by three different types of enzymes named (i) α-secretase, (ii) β-secretase and (iii) γ-secretase. In normal physiological conditions, the breakdown of APP takes place by α-secretase enzyme which forms soluble APPα, can be easily cleared from the brain during regular metabolic processes [37]. On the other hand, when abnormally cleaved by β and γ-secretase, there is an imbalance in the production and clearance of Aβ proteins, which gets aggravated into soluble oligomers. This later coalesces into insoluble fibrils and forms large, toxic aggregate termed as senile plaques [5].

In normal physiological conditions, the α-secretase pathway giving rise to APPα is the primary path, and the BACE-1[19] channel is considered as the minor path to cleave APP. α-secretase produce unprocessed APP and CTF[20]-α which goes into the endocytic vesicles while BACE-1, an aspartyl protease, produces CTF-β [38,39]. The α-secretase pathway is known as the non-amyloidogenic path, i.e., it does

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[2] β-amyloid peptide.
[3] Amyloid precursor protein.
[4] Apolipoprotein-E.
[5] Cerebral blood flow.
[6] Blood-brain barrier.
[7] Glucose transport proteins.
[8] Central nervous system.
[9] Cerebrospinal fluid.
[10] Gamma amino butyric acid.
[11] Early-onset Alzheimer's disease.

[12] Late-onset Alzheimer's disease.
[13] Sporadic AD.
[14] Familial AD.
[15] Pyruvate dehydrogenase.
[16] Insulin receptors.
[17] Acetylcholine.
[18] Acetylcholine esterase inhibitors.
[19] β-Site APP-cleaving enzyme-1.
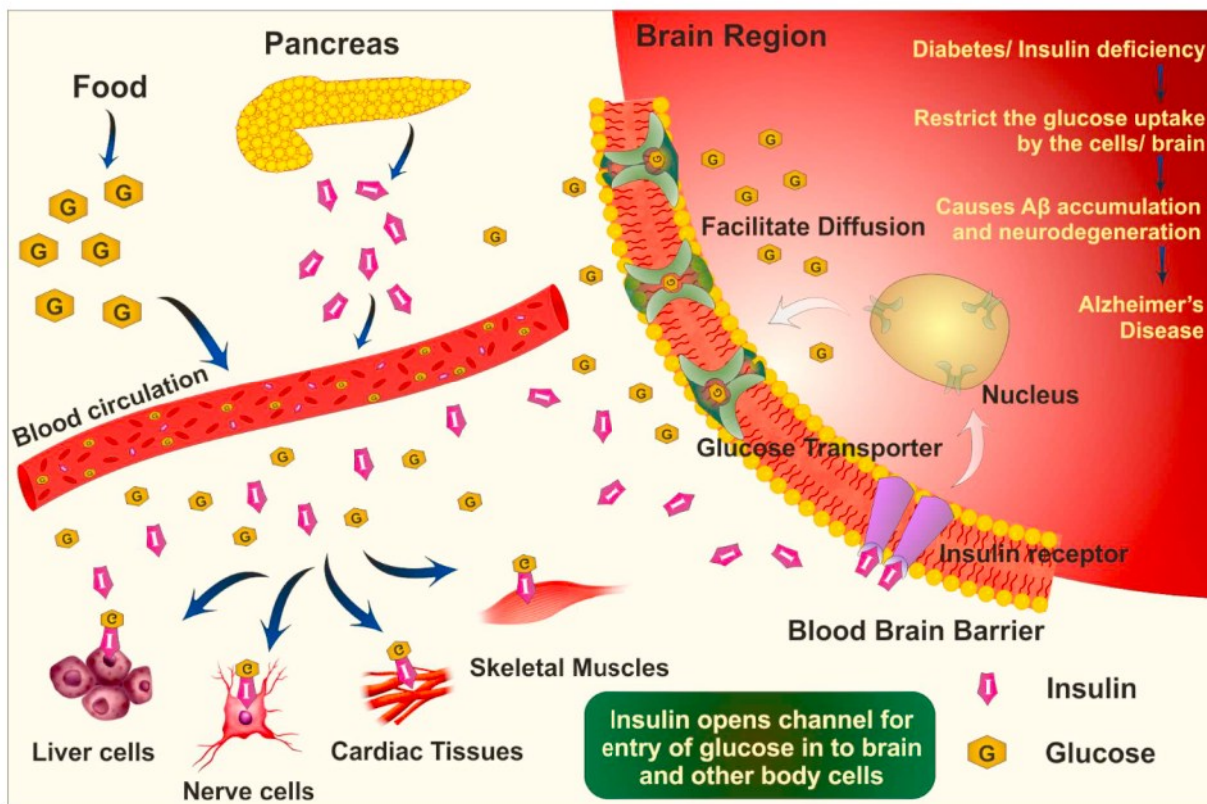[20] Carboxyterminal fragments of APP.

Fig. 1. Represents the glucose transportation pathway via GLUT to the brain. On reaching the blood circulation, the glucose transporters take up the glucose once the insulin channels open up.
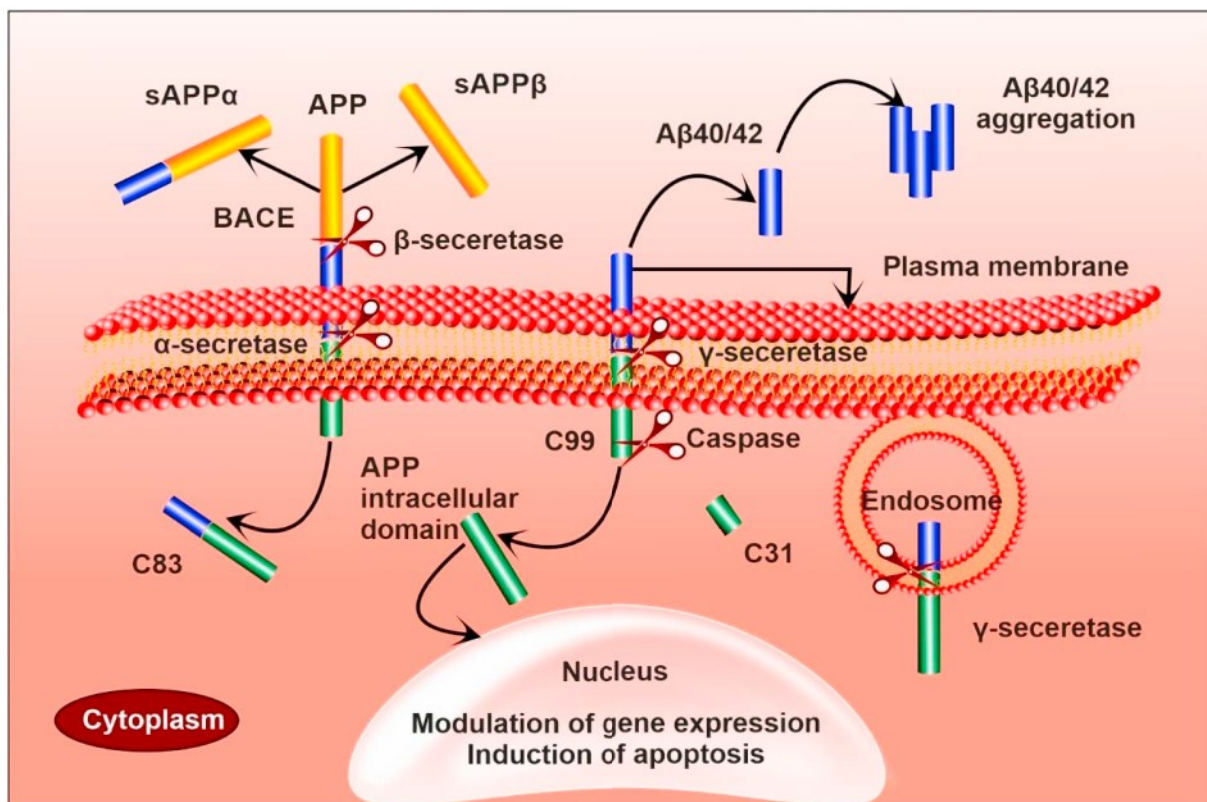

Fig. 2. Represents the Formation of Aβ-cleavage by α-secretase on N-terminal side results in the formation of secreted APPα (sAPPα), leaving the C-terminal with 88-amino acids (non-amyloidogenic pathway). Cleavage of the APP by β-secretase on N-terminal results in the formation of sAPPβ and going the C-terminal with 99 amino acids. Subsequent cleavage of the C-99 terminal by γ-secretase results in the accumulation of Aβ$_{40/42}$ (Aβ$_{40}$ soluble form and Aβ-42 insoluble form), leaving an internal C-terminal domain (amyloid pathway) (adapted with permission from [42]).

not produce toxic amyloid β fragments which further leads to the formation of amyloid plaque (Fig. 2).

The β and γ degradation of APP results in the formation of various Aβ derivatives among which $A\beta_{40-42}$ are found highly toxic and form more toxic senile plaque [40]. The $A\beta_{42}$ oligomers that are produced in both the nerve cells and astrocytes and disturb the calcium homeostasis of the nerve cell and increase oxidative stress in the brain. It also induces tau hyperphosphorylation, causing abnormalities like enlargement or opening of mitochondrial transition pores and sometimes apoptosis in mitochondria and increases the levels of reactive oxygen species [41].

2.3. Tau (τ) pathway

τ-Protein stabilizes the neuronal microtubules. During AD pathology, tau proteins detach from the microtubules. This causes axonal transport defects. Hyperphosphorylation of tau protein disturbs the regular assembly of microtubule which causes misfolding of neurons and results in the formation of NFT[21] [11]. It also causes an imbalance in various cellular enzymes which further int necessary cellular functions [43]. When GSK-3[22] is activated, tau proteins are hyperphosphorylated, therefore showing less binding affinity to the microtubules. On the other side, by regularizing different cellular metabolic processes insulin reduces the hyperphosphorylation of tau and thus, the neurotoxicity [24,44]. Insulin and IGF-1[23] have found to inhibit abnormal tau hyperphosphorylation via Akt[24]-induced inactivation of GSK-3[25] and thus strengthen the microtubule structure [45]. Also, hyperphosphorylated tau proteins get aggregated into the NFT as they cannot be transported across the axons. Therefore, they worsen the pathological condition by promoting oxidative stress, which further exacerbates the disease by leading to necrosis, mitochondrial dysfunction, and apoptosis [24]. The pre-fibrillar tau can also form aggregates (soluble oligomers or insoluble granules) that contribute to neurodegeneration by producing disconnections in the synapse and neuronal death [46]. Tau mutation alone was found to lead to dementia rather than AD, unlike the mutated APP. Two models are used in proposing this hypothesis (Fig. 3). The series model states that an increase in Aβ leads to an increase in tau phosphorylation which further increases the severities of AD. The dual pathway says that both Aβ and hyperphosphorylated tau lead to a neuronal loss in the AD [43,47].

2.4. Mutations in presenilin genes

Presenilin-1 (obtained from PSEN-1[26] gene on chromosome-14) and Presenilin-2 (obtained from PSEN-2 gene on chromosome-1) are homologous protein, with nearly 67% sequential identity [48]. They form a high molecular secretase complex (γ-secretase) which produces Aβ from APP cleavage process [29]. Presenilin regulates proteolytic activities of the γ-secretase enzyme. So, a mutation in them affects the functioning of γ-secretase, causing partial digestion of Aβ, therefore resulting in producing abnormally large quantities of longer Aβ during EOAD (PSEN-1, the longest) [39,49]. 121 types of mutations are detected until now because of PSEN-1 and 13 because of PSEN-2. It is postulated that both APP and PSEN-1 cause neuronal death alongside the amyloid deposition because of their intracellular signaling mechanism that is supposed to induce abnormal cell cycles [50]. It is also supported by the complex protein networks that interact with APP and presenilin during this process. Deletion of PSEN 1 and 2 cause a

shortfall of memory, synaptic functions, learning, and neuronal death because of the interaction on PSEN-1 with antiapoptotic membranes of the BCl-2[27] family (Fig. 4) [29,46].

2.5. ApoE pathway

ApoE is the primary cholesterol carrier in the brain that is formed by astrocytes, present along with ApoJ and $ApoA_1$ [34,51]. ApoE acts as a lipid transporter and repairs injuries in the brain apart from their support during synaptogenesis. They mediate lipoprotein transport in the brain via carriers like LRP-1,[28] which are involved in Aβ clearance from the brain. Thus, plaque buildup can also be because of decreased LRP-1 in the AD condition [52]. ApoE has 3-alleles-E_2, E_3, and E_4. E_4 carriers have increased the risk of developing AD (2–4 times with one E_4 allele and 12 times with two E_4 alleles) either by increasing the formation of $A\beta_{42}$, aggregation of formed Aβ or decreased Aβ clearance [52,53]. E_2 is protective and optimistically related to cognition on aging, along with enhancing the glucose uptake and glycolysis in the brain [52–54]. Interactions between ApoE and Aβ changes the β sheet configuration of the amyloids and the enhanced neuritic plaques because of ApoE allele increases the chances of acquiring AD by accelerating the formation of plaques [55]. ApoE4 also alters the expression of IDE[29] in the brain which affects the degradation of extracellular Aβ [56,57].

2.6. Neuroinflammation and oxidative stress

The phenomenon of chronic accumulation is extreme in AD which includes microglial activation along with prominent oxidative damage. The excessive production of microglial pro-inflammatory mediators can lead to neurotoxicity [58]. Hence it has been demonstrated that neuroinflammation that increases on aging leads to the promotion of neurological disorders including AD. Generation of free radicals that arise from neurotoxic trace elements such as mercury, copper, aluminum, lead, etc. has been observed in oxidative stress hypothesis. The initiating factors may be oxidative stress, mitochondrial damage, inflammation along with potentially the excess production or decreased clearance of Aβ peptides and aggregates that are toxic in nature. Since these initiating factors lead to promotion of production of Aβ and hyperphosphorylation of tau, a destructive cycle is begun. This is also because the pathological conditions lead to an increase in inflammation and oxidative stress. Due to the occurrence of microtubule-associated protein tau (MAP-tau) and Aβ in pathology of AD, studies focused on treatment mechanisms intended to decrease the development of these two major pathological proteins have grown in the last three decades [59,60]. Besides, to reduce the neuroinflammation and oxidative stress, memantine-alpha lipoic acid conjugate has been synthesized and reported for its promising therapeutic efficacy over Alzheimer induced rats [61]. But studies of such nature have still not yielded a US FDA-approved therapeutic alternative.

3. Association of diabetes with Alzheimer's disease

In due course of time, researchers have established connectivity between insulin activity, Alzheimer's, glucose metabolism and memory impairment. Diabetes is found to produce mild cognitive impairment, and it is proved that patients with insulin resistance (type-2 diabetes) [57,62] and impaired brain energy utilization are highly prone to be affected by the AD [63]. Insulin metabolism is abnormal in AD mice model (3xTg-AD mice), i.e., AD model was hyperinsulinaemic and hyperglycemic (hippocampus in particular), with low CSF-insulin level

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[21] Neurofibrillary tangles.
[22] Glycogen synthase kinase-3.
[23] Insulin growth factor.
[24] Protein kinase B.
[25] Glycogen synthase kinase 3β.
[26] Presenilin.

[27] B-cell lymphoma-2.
[28] Low-density lipoprotein receptor-related protein.
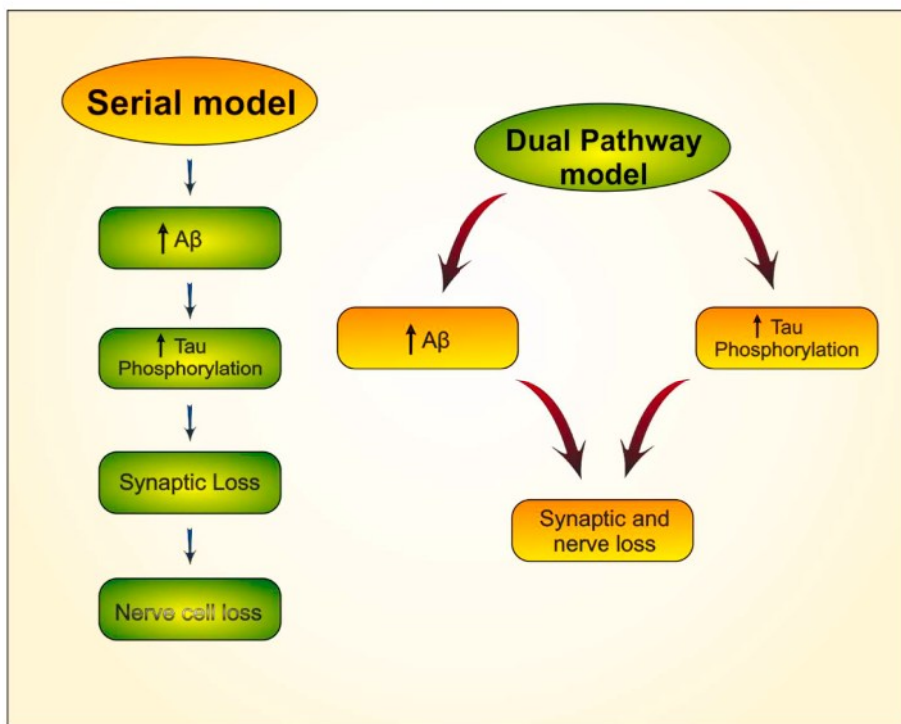[29] Insulin-degrading enzyme.

Fig. 3. Represents the neuronal loss pathway because of Aβ and tau pathway in the AD. The serial model states that the rise in Aβ levels increases the tau phosphorylation that leads to AD. The dual-path says that both Aβ and hyperphosphorylated tau lead to a neuronal loss in the AD.
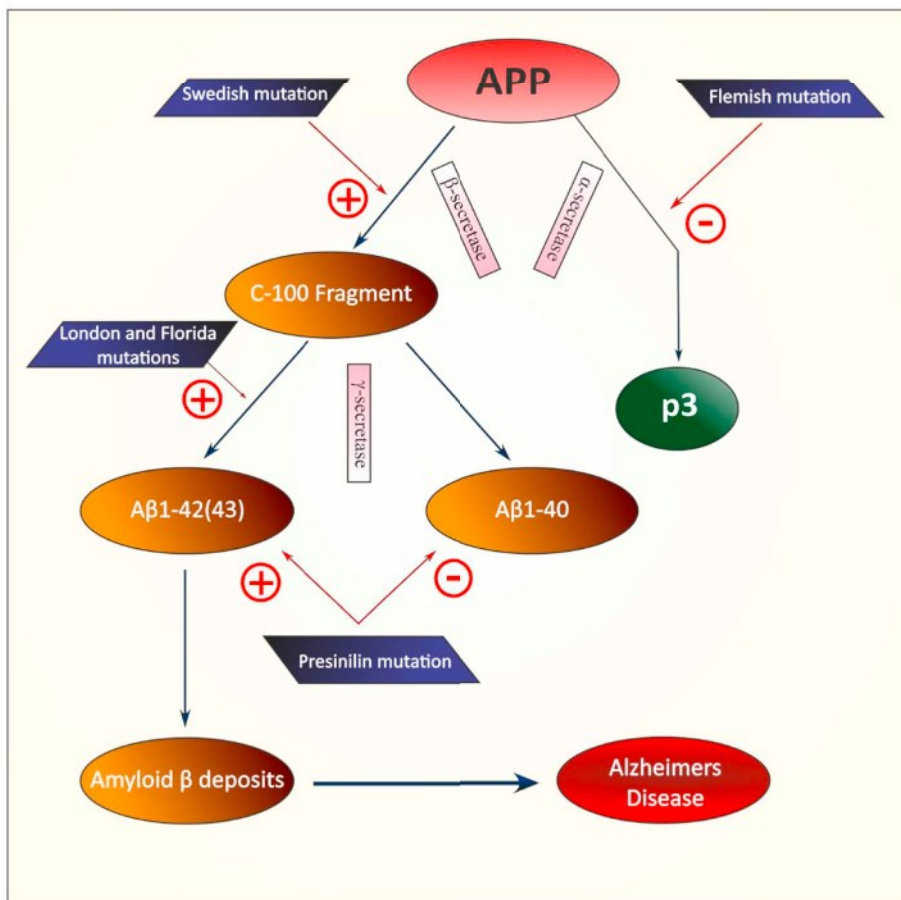

Fig. 4. This diagram represents the formation of Aβ deposits by presenilin mutation in the AD. The APP gene is cleaved by β secretase followed by γ secretase to give rise to Aβ-40 and 42, which give rise to plaque deposits. The presenilin mutation gives rise to Aβ-42 that in turn gives rise to the same process.

and low insulin-mediated glucose disposal [64]. Such physiological condition resembles type-2 diabetic patients. Biomarkers of type-2 DM[30] like glucose intolerance and insulin resistance in animal models suggests that there cause of the disease may have a similar pathway [65]. The AGE[31] formation in diabetes aggregates the plaques that are formed during AD [66]. Also, patients with type-2 DM have a cognitive impairment, like in AD [64], and that is why the AD is nowadays named as 'type 3 diabetes' or 'brain-specific form of diabetes' [18,67].

As discussed earlier, both insulin and glucose are essential for normal brain functioning. Impairment in glucose homeostasis will lead to impaired brain activities such as memory and cognition. It further increases the risk of AD because DM attenuates the APP metabolism and induces the formation of the plaques by IDE. The first evidence of insulin resistance in an Alzheimer's brain was studied by Frolich et al. in 1999 found that lower tyrosine kinase activity led to an increase in brain IR density. This impaired the insulin signal-transducing cascade [24]. During insulin resistance, the brain is leading to hyperinsulinemic conditions, where insulin sensitivity is reduced. This peripherally increased insulin levels in CSF further modifies various AD-associated factors like abnormal removal of Aβ plaques and increased tau phosphorylation, which ultimately leads to neurodegeneration by triggering the cellular cascade and finally cause AD. Chronic peripheral hyperinsulinemia leads to decreased insulin transport across the BBB, altering the insulin signaling in the brain and finally showing the anti-apoptotic effect. Administering insulin peripherally at specific doses to the brain may revert this effect and enhances the cognitive function of the brain of AD patients [18,68]. Also, various drugs used to treat type-2 DM, anti-oxidants, nutritional supplements, vitamins, polyphenols, and insulin derivatives also found effective for the above-said problems (Fig. 5) [18,69]. Clinical investigations in animal model demonstrated systemic administration of exogenous insulin may also result in memory deficit and neural dysfunction due to hypoglycemia, occurs if, the insulin is not administered with some glucose to keep the euglycemic condition [8]. On the other hand, some studies have proven that the induced hyperinsulinemia to uphold the euglycemia can boost the memory of AD as well as the normal brain [70,71]. Apart from reduced glucose metabolism in temporal and parietal regions of the brain, there is a loss of declarative memory which is due to hippocampal atrophy. Impairment in the glucose metabolism in regions like the hippocampus, temporal gyri (superior and middle), cingulate gyrus (reduced metabolism in these) and frontal lobes (increased metabolism) cause hippocampal atrophy. Thus, we can conclude that cerebral glucose metabolism is playing a major role in the progression of AD [8].

It was found that the early stages of the AD will face a reduction in cerebral glucose utilization by 45% and CBF by 20%. As the disease progresses, the CBF rate reduces to nearly 65%. Liu et al., in 2009 suggested that the GLUT receptors (isoforms 1 and 3) were responsible for neuronal glucose uptake. They were diminishing in due course of disease progression, which interrelated with decreasing O-glc-N-Acylation (O-linked *N*-acetylglucosamine moieties attached to the nucleus, cytoplasmic and mitochondrial proteins) and tau hyperphosphorylation apart from the reduced activity of the mitochondrial energy-related protein, that included PDH and other enzymes [72]. Hence, it affects energy production from the available glucose in the brain [24]. Initially, the administration of glucose reversed the blockade of the cholinergic system in rodents. This modulated the release of Ach in the forebrain. It was also proved that the administration of dose-dependent glucose or saccharin that produced acute hyperglycemia had improved recall memory and recognition. Thus, it was evident that severe hyperglycemia facilitates memory enhancement via glucoregulatory efficiency in patients with very mild to mild AD, but not chronic hyperglycemia [8]. But, elevating glucose levels in the

plasma also invokes insulin to be secreted, whose levels eventually rise in the plasma because of the same. The study showed that memory enhancement is attributed to the insulin rise in plasma, rather than that by glucose. To support this, Watson et al., in 2003, conducted separate studies on AD patients under two conditions: initially under normal plasma glucose levels and elevated plasma insulin levels and vice versa. They confirmed that an increase in endogenous insulin level was responsible for the memory enhancement activity, and not glucose [73]. Rivera et al. in 2005 suggested that there was a progressive decrease in mRNA levels of insulin, IGF-1 and (insulin growth factor) and its receptors [12]. Also, AD patients had more fasting plasma insulin levels and less of them in CSF. Patients with increased Aβ showed that insulin clearance is decreased and this provoked elevation of Aβ in the plasma [8].

4. Pathways associating AD and insulin activity

4.1. Insulin impairment in brain

IGF signaling pathways bring about plasticity in synapsis for cognitive functions. When insulin binds with the insulin receptors, it brings about a cascade of reactions such as activation of PI3K[32] in the cell [63,74]. PI3K helps in engaging Akt to the plasma membrane, which gets activated by phosphorylation [75]. Akt plays a major role in multiple cellular processes like metabolism of glucose and inhibition of GSK-3β.[33] GSK-3β polymerizes the microtubule by phosphorylation of τ-protein. Impairment of insulin signaling leads to increased activation of GSK-3β which causes hyperphosphorylation of τ-protein (Fig. 6). The pathological dysfunction of PI3K and Akt-I pathway boost the AD-associated factors. Similarly, type-2 DM reduces IR-1 levels and thus, increases oxidative stress more than impaired insulin/IGF-1 signaling, that ultimately leads to neuronal apoptosis and mitochondrial dysfunction [12,76].

4.2. IDE pathway

The foremost role of IDE[34] is the degradation of insulin and is also the major thiol metalloprotease used for degradation of extracellular Aβ [78], alongside Neprilysin. Gene encoding IDE is located on the 10q chromosome in human beings [64,79]. Under physiological conditions, IGE is found in CSF [80]. Some shorter peptides with low molecular weights of about 3 to 10 kDa like insulin, IGF-1 and 2 and amylin act as a substrate for IDE, where they share a comparable secondary structure and amyloidogenic character. Thus, IDE played an important role in clearing the plaque deposits. Insulin binds to IDE instantly. IDE overexpression in cell cultures increases the insulin degradation rate [81]. Mutated IDE genetically causes diabetes, which in turn reduces insulin degradation in rats (because of increased insulin levels) and causing type-2 DM. In DM, IDE degrades the amyloid bodies that are composed of amylin. Because IDE has a high affinity for insulin than Aβ, IDE is seized from degrading Aβ in hyperglycemia condition because of the demand for degradation of excess insulin and therefore worsening the condition of the AD (Fig. 7) [81,82].

4.3. AGE pathway

AGEs[35] are proteins or peptides, produced as an outcome of reaction between free amino acids of proteins and sugars (Milliard's reaction) [85]. AGEs usually mount-up in the cells upon normal aging, but a substantial rise in their levels is associated with diabetes mellitus [86].

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[30] Diabetes mellitus.
[31] Advanced glycation end product.

[32] Phosphatidylinositol 3-kinase.
[33] Glycogen synthase kinase 3β.
[34] Insulin degrading enzyme.
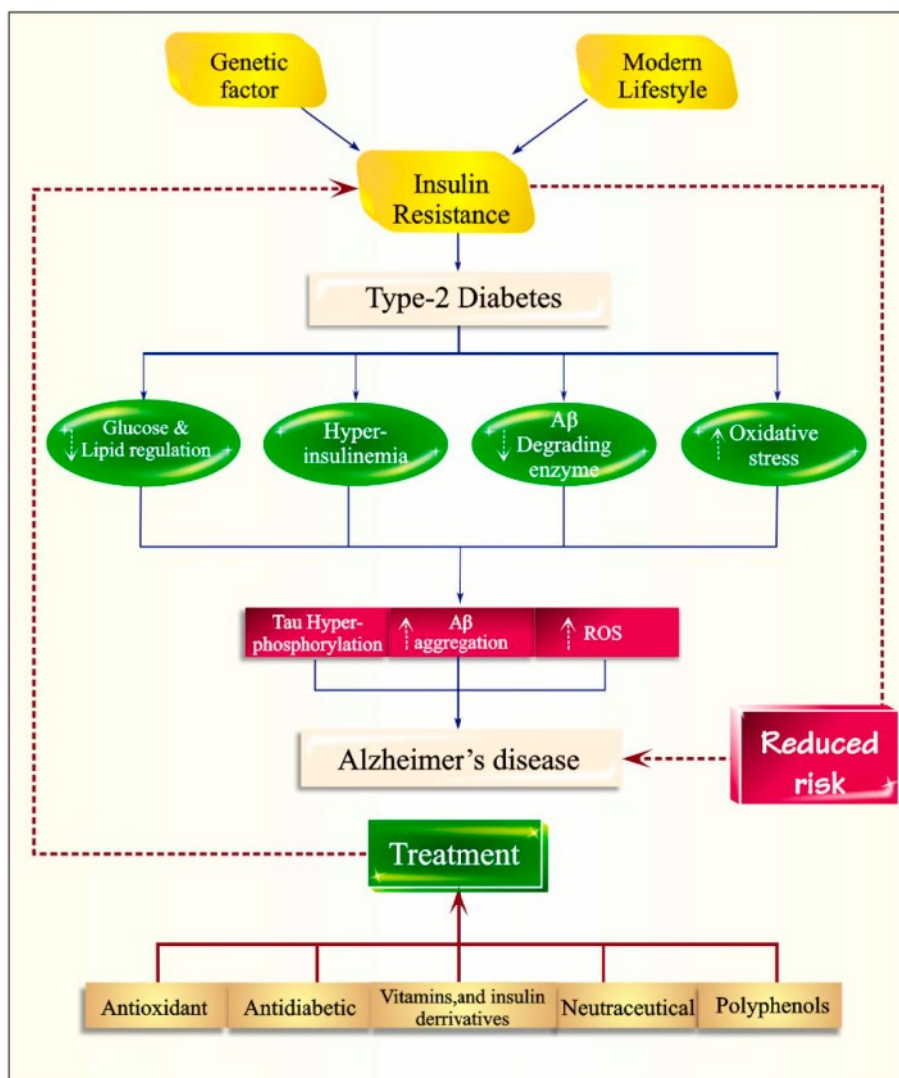[35] Advanced glycation end product.

Fig. 5. Effect of insulin resistance and type-2 diabetes on brain function which may further increase the risk of Alzheimer's disease. Various drugs, nutritional supplements and other therapies that treat the insulin resistance may also reduce the risk of AD.

RAGE,[36] the receptor of AGEs is found in the hippocampus and neurons which is responsible for oxidative stress-induced neurotoxicity by facilitating ROS[37] production. This promotes the aggregation of Aβ by regulating the APP pathway, finally forming neurotoxins [85,87]. Immunohistochemical analysis has found that the postmortem samples that were affected by both DM and AD had higher levels of AGE, Aβ plaques, and high RAGE levels. The plaque aggregation follows a nucleation-dependent polymerization mechanism that is accelerated by AGE mediated cross-linking. Also, they are involved in the glycation of τ-protein, resulting in the formation of paired helical filaments [88,89]. Through RAGE-mediated GSK-3 activation, AGE induces toxicity via tau hyperphosphorylation (Fig. 8) [87].

5. Insulin receptor and signaling in the brain

Insulin follows a receptor-mediated transport pathway by inhibiting gluconeogenesis in the liver. It inhibits the programmed neuronal death (apoptosis) by in-vitro action of protein kinase B which in vivo metabolizes amyloid precursors in the brain and thus removes the amyloid bodies from the brain. This is the regular function of insulin in the

brain; any alteration in this mechanism would lead to neurodegenerative disorders [90]. This IR impairment cascade occurs both in type-2 DM and sAD, indicating that both are interconnected [30].

IRs are present all over the brain in a specific pattern. IRs are abundant in the olfactory bulbs, hypothalamus, hippocampus, and cerebral cortex. Unlike the peripheral organs, it does not experience downregulation upon exposure to high insulin concentration in the CNS [30]. IR and IGF-1R belong to the tyrosine kinase family, where autophosphorylation of them in the presence of insulin will give rise to plasma insulin because of less CSF. Thus, there is impairment in insulin metabolism because insulin reaches the brain via CSF [91–93]. Intranasal insulin administration can rapidly increase the CSF concentration of the same, leaving the blood glucose unaltered. This helps in enhancing the working memory [94–96]. Because of GSK activation, tau protein will get hyperphosphorylated [46]. When insulin is given, GSK activity is decreased. Thus the amyloid bodies are cleared [90]. Therefore, the knocking down of IR or IGF-1R would result in amyloid aggregates formation [97,98].

The major functions of insulin or IGF receptor signaling include neuronal development, continued existence, differentiation, relocation, gene expression, energy metabolism, protein synthesis, cytoskeletal assemblage, synapse formation, neurotransmitter action, and plasticity. Thus, impairment in the receptor or insulin will alter the integrity of the

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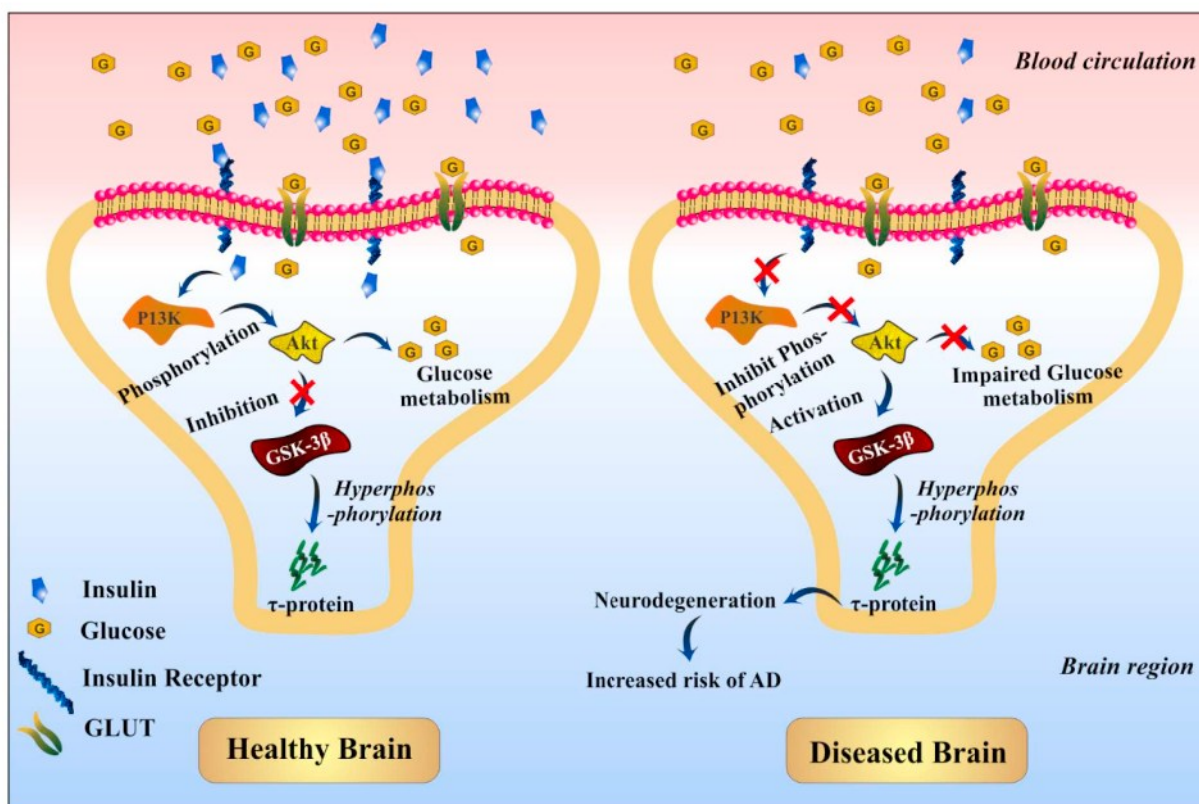[36] Receptor of advanced glycation end products.
[37] Reactive oxygen species.

Fig. 6. τ-Hyperphosphorylation and induction of AD due to insulin impairment. In a healthy brain, insulin regulates various cellular functions in the brain like metabolism of glucose and inhibition of GSK-3β. Impairment of insulin signaling causes hyperphosphorylation of τ-protein which further causes neurodegeneration.

central nervous system [46].

5.1. Glucose metabolism

Glucose metabolism is the primary energy source that is derived from the intestinal absorption of the digested dietary carbohydrates, glycogenolysis and gluconeogenesis [99]. Initially, glucose gets facilitatively transported across the plasma membrane by the GLUT down the concentration gradient. GLUT 3 has the highest transport rate when compared to astrocytic GLUT1. Though the brain weighs approximately 2% of the whole-body weight, it consumes 20% of the total energy. Energy is largely consumed for the generation of information processing and neuronal computation. Glucose metabolism also provides energy and acts as a precursor for neurotransmitter biosynthesis in the brain [100]. HK[38] that is present on the mitochondria converts glucose to G6P[39] by phosphorylation. This process later continues into the glycolytic pathway finally forming pyruvate, NADH,[40] and ATP[41] in mitochondria or moves into PPP[42] where it generates NADPH[43] to manage the oxidative stress. The glycolytic pathway produces two net ATPs for one glucose molecule, and TCA[44] cycle gives 30 ATPs [101,102]. PET scans have found that synaptic losses and dysfunctions (hypometabolism) tend to reduce neuronal energy demand in an AD-affected individual that results in reduced energy production [103,104].

Glycolytic flux and oxidation of pyruvate in the brain are stimulated by insulin parallel to its effect in the non-nervous system. PIP3K[45] regulates insulin function, activates protein kinase B and thus, inhibits the GSK-3 controlled amyloid-β synthesis [105]. Group of researchers has worked on AD female mice models proving that impairment in glucose metabolism is due to improper brain insulin signaling that lead to amyloid aggregation in the brain. Impairment of glucose metabolism can also be because of the increased levels of GLUT3 and 4 [106], leading to abnormal brain glucose transportation [107,108]. O-glycosylation also regulates glucose metabolism. When their levels go down, there may be chances of impairment in glucose metabolism. This forms neurofibrillary tangles and amyloid aggregation [109,110].

In EOAD, Aβ will competitively bind to IR. It reduces the binding of insulin to the receptors. This disturbance causes reduced receptor autophosphorylation by ATP, leading to reduced tyrosine kinase receptor activity. This ultimately leads to disruption in the receptor effect via IRS-1. It will cause a drastic reduction in glucose metabolism, with the CBF and cerebral oxygen metabolism being normal. This deficiency in glucose metabolism/reduced ATP production is maintained by brain substrates like glucoplastic amino acids (glutamate), which help in meeting the brain's energy demand. But glutamate's utilization during ATP formation will produce neurotoxic ammonia, which inhibits the mitochondrial dehydrogenases which takes part in the energy-producing chain. In EOAD, because of the competitive binding of Aβ to the insulin receptor, there is inhibition in the insulin transduction pathway, whereas receptor desensitization occurs in LOAD. Age, along with the NFTs from tau hyperphosphorylation can also be a factor inducing impaired glucose metabolism [111]. It is because of hypometabolism [112,113] (bilateral temporal and parietal hypometabolism in mild to severe AD [114]) in the cerebral cortices; there are more energy demand and insulin sensitivity, reduced activity of glycolytic and

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[38] Hexokinase.
[39] Glucose-6-phosphate.
[40] Nicotinamide adenine dehydrogenase.
[41] Adenosine tri-phosphate.
[42] Pentose phosphate pathway.
[43] Nicotinamide adenine dehydrogenase phosphate.
[44] Tricarboxylic acid.

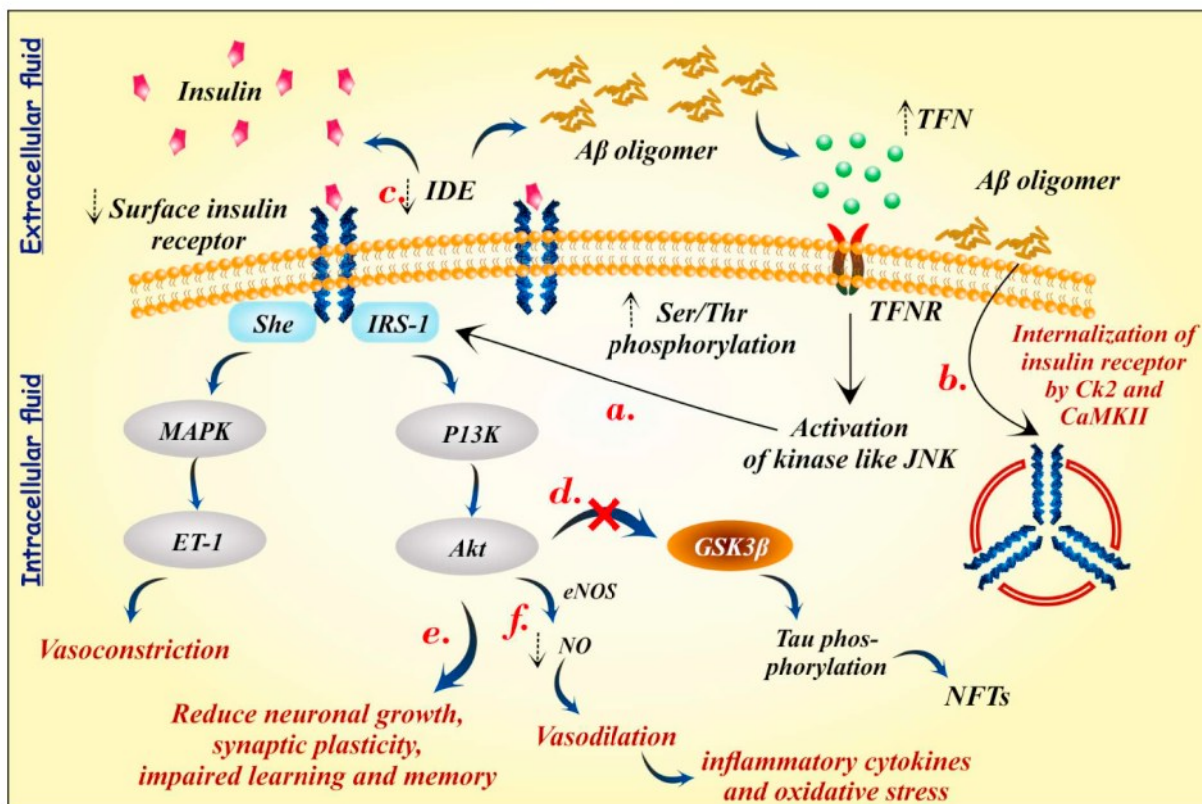[45] Phosphoinositide 3-kinases.

Fig. 7. Abnormal insulin signaling and impaired cellular functions in AD brain. In normal physiological conditions, the interaction of insulin with surface insulin receptors activates IRS-1 (insulin receptor substrate-1) by phosphorylation. This further activates P13K and Akt and hence regulates various cellular functions like neuronal growth, nerve survival, synaptic plasticity, and learning and cognitive function of the brain. The activation of insulin receptors causes both vasoconstriction and vasodilation. In normal physiological conditions, both the processes are kept in balance to fulfill the metabolic needs of brain tissues. While in the AD brain, the accumulation of Aβ oligomer brings a variety of neurodestructive reactions including (a) it increases the TNF (tumor necrosis factor) content in extracellular fluid. This TNF interacts with TNFR (tumor necrosis factor receptor) and results in the activation of various stress kinase-like JNK (c-Jun N-terminal kinase) inside the nerve cells. This stress kinase increases serine phosphorylation of IRS-1 from surface insulin receptor and further reduces the availability of surface insulin receptors and thus, hindered insulin-mediated metabolic functions of nerve cells; (b) Aβ peptide cause internalization of IRs via CK2 (casein kinase 2) and CaMKII (Ca2+/ calmodulin-dependent kinase II) and removes the IR from cell surface; (C) further, the insulin resistance due to lower surface expression of IRs, reduces the extracellular IDE level [83] and so, diminishes the Aβ degradation; (d) on the other hand, the disturbance in brain insulin signaling boosts the activity of GSK-3β [84] which further cause hyperphosphorylation of tau protein and produce NFTs (neurofibrillary tangles); (e) the interruption in IRs function deceases the NO (nitric oxide) production and so reduces cerebral blood flow which increases inflammatory cytokines and oxidative stress; (f) Moreover, the abnormal insulin signaling result in impaired nerve growth, synaptic plasticity and cognitive function of brain like memory and learning, etc.

dehydrogenating enzymes (like pyruvate dehydrogenase) that minimizes the acetyl CoA transferase activity. It disturbs not only the ATP cascade but also the intercellular cholesterol formation, which decreases the cholesterol levels in the membranes of the brain and CSF [115]. ApoE4 carrier showed a decline in glucose transport across BBB without a change in GLUT-1 expression, and not GLUT-3 (that is mainly present in neurons). This resulted in the reduction of cerebral glucose metabolism [54].

5.2. Brain insulin resistance

Insulin resistance can be caused by many factors like glucotoxicity, lipotoxicity, inflammation, metabolic (mitochondrial and ER[46]) stress [116]. In peripheral metabolic disorders like type-2 DM, insulin resistance occurs because of long-lasting metabolic stress, thus impairing the energy-producing ability of the cells. This condition is also seen in Alzheimer's affected individuals. Insulin receptors during AD reduce significantly, and a few of them become resistant [63]. About half of the older US population (> 60 years) shows this type of resistance. This phenomenon alters the brain insulin uptake, which decreases the long-

term insulin levels, thus causing AD. The resistance of IR can be because of less insulin concentration in the brain, less insulin activity or deviation in the chronic peripheral levels of insulin [117,118]. How-so-ever, the pathway underlying this must be studied so that new targets can be identified.

Recent studies have correlated peripheral insulin resistance and neuro-pathogenic mechanisms that are triggered by Aβ and tau phosphorylation. High levels of tau in CSF and toxicity produced by accumulated plaques are among the causes of insulin resistance [111,112]. Insulin resistance causes decreased signaling caused due to PIP3K, Akt and increased GSK hyper-activation. This gives rise to tau hyperphosphorylation [119].

It is recently confirmed that TNF-α[47] signaling aberration activates stress kinases like JNK[48] that phosphorylates IRS-1[49] (inhibitory) at a serine residue (IRS-1-pSer[50]) in the neurons of the hippocampus. Thus, blocks the insulin signaling downstream and causing insulin resistance. Post-mortems of AD brains suggested the elevated IRS-1-pSer levels, thus showing IR removal from the cell surface, confirming that insulin

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[46] Endoplasmic reticulum.

[47] Tumor necrotic factor-α.
[48] c-Jun N-terminal kinases.
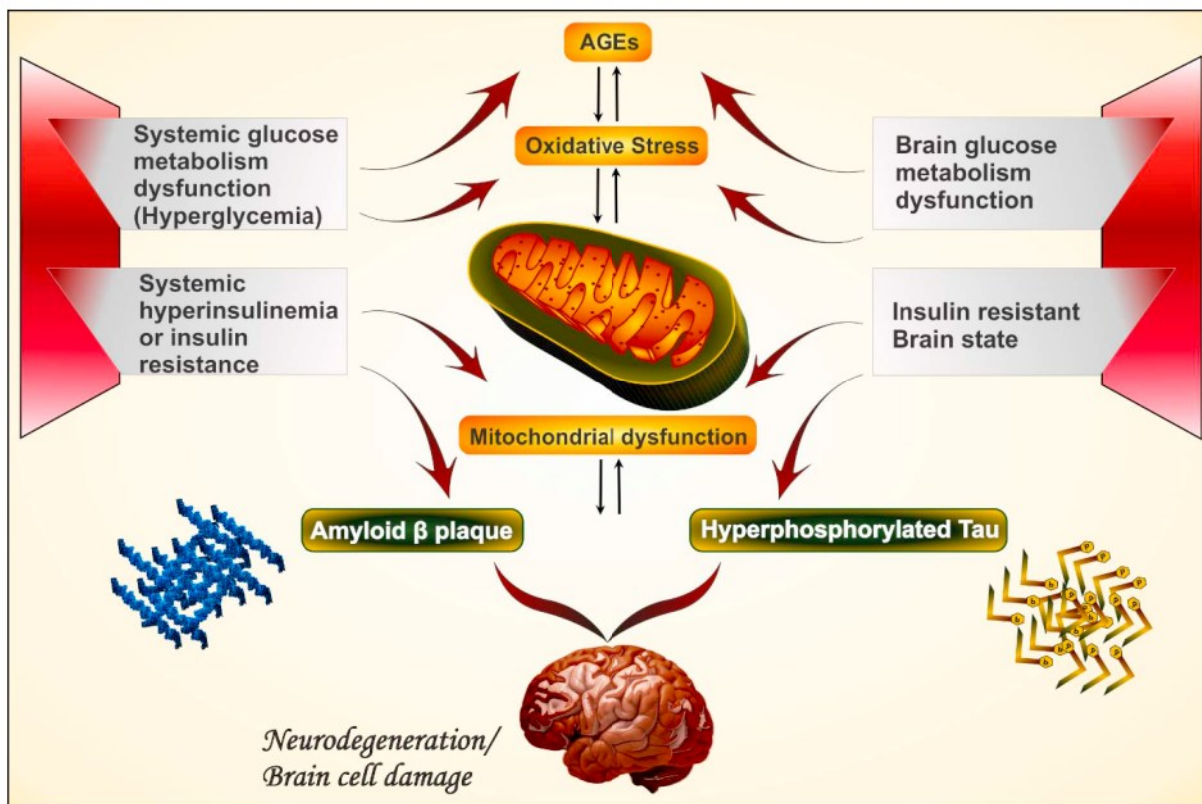[49] Insulin receptor substrate 1.
[50] Serine residue.

Fig. 8. Schematic representation of the AGE pathway. Both Aβ plaques and tau hyperphosphorylated proteins build up stress in the mitochondria leading to the formation of reactive oxygen species. The process is said to be two ways where either of them leads to neurodegeneration in the brain.

blocks IR downregulation and IRS-1-pSer that is induced by Aβ. Because of elevated pSer levels, feedback-dependent Akt inhibition takes place in the peripheral insulin resistance [67,120].

Aβ deregulates NMDA subtype glutamate receptors (NMDARs), whose abnormality, along with insulin resistance can generate ROS. The ROS further activates stress kinases (like GSK-3) and leads to neuronal dysfunction and insulin resistance [116]. Another possible mechanism is the excessive activation of NMDAR alongside triggered Ca^{2+} lead to glutamate-induced neuronal alterations, finally causing insulin signal impairment. This impairment may trigger the APP to produce amyloid plaques or aggregates. Thus, when insulin is administered as a neuroprotectant [121], it clears off the excess plaques, and insulin-mediated synaptic protection enhances cognition in AD-affected individuals [67].

6. Insulin therapy to treat neurodegeneration

6.1. Insulin therapy across BBB

As discussed earlier, it was initially doubtful if insulin could reach the brain by crossing BBB [122]. Also, a molecule that is identical to the ones produced by the β cells of Langerhans along with specific IR[51] and insulin was found to be well distributed throughout the CNS (highly in hippocampal region, cerebral cortex, cerebellum and olfactory bulbs), that affect functions like memory, cognition, feeding, reproduction and some more. IRs are expressed in glial cells as the peripheral type and in neurons as brain-specific type (concentrated highly in the synaptic end region, even though present throughout the neuronal processes and body) [18,123]. The mature brain receives insulin from the periphery that later gets transported from the CSF across BBB by an active

transport mechanism. Insulin is also synthesized by the brain itself through a De-novo process [23,124,125]. Various preclinical studies of insulin therapy across BBB for the treatment of neurodegenerative disorders are given in Table 1.

6.2. Intranasal insulin therapy

Studies suggested that intranasal insulins can boost the brain as well as whole-body insulin sensitivity by regulating hypothalamic activity [126]. The intranasal route directly delivers the insulin to the brain through the neural pathway [2] without the interference of BBB which further increases the availability of insulin in the brain region thus facilitates insulin-mediated cellular function [126,127]. At the same time, high vasculature of the nasal cavity also enables the entry of the drug into general circulation which makes it available in the peripheral region [128,129]. However, their safety and efficacy may be of the question [130,131]. Intranasal insulin is said to raise the insulin level in CNS only, and not in the plasma. Researchers stated that intranasal insulin enhanced memory and learning in patients who had decorative memory without this treatment [132,133]. Insulin enters CSF rapidly through olfactory pathways like bulk flow transport along with trigeminal transport mechanism bypassing the blood-brain-barrier [134]. Upon entering the brain, the protein is said to rapidly distribute within the brain because of the convective transport that occurs within the perivascular space of cerebral [135]. Additionally, perineural space that interacts with CSF allows the distribution of intranasally administered drugs throughout the brain [136]. Also, this serves another purpose of advantage that hypoglycemic effects on administering systemic Insulin to non-diabetic elders can be avoided [137]. A pilot study was done by Craft and co-workers (2012) in patients with AD and MCD[52]

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[51] Insulin receptors.

[52] Mild cognitive disorder.

Table 1
Insulin based preclinical approaches for the treatment of the neurodegenerative disorder (AD).

Drug and delivery approach	Dose of insulin	Animal model	DM related factors	AD-related factors	Targeted mechanism	Outcome	References
Subcutaneous insulin	0.3 U/24 h	• 9 weeks Swiss Webster mice • STZ induced type-1 DM mice	• Insulin deficiency • Decreased insulin sensitivity • Inhibit activation of SIRs by inhibiting phosphorylation of IRs	• Reduced IDE in CSF • Higher GSK activity • Tau hyperphosphorylation • Learning impairment	• Increase IRs phosphorylation • Reduce GSK activity • Decrease tau phosphorylation	• Reduce cognitive impairment • Improved learning and spatial memory was observed in Brane's maze • Revert diabetes-induced behavioral changes	[144]
Liraglutide and lixisenatide (GLP-1 analog)	1 or 10 nmol/kg/day intraperitoneal dose of lixisenatide and 2.5 or 5 nmol/kg/day of liraglutide	DM and AD induced APP/PS1 mice	• Lower GLP-1 level • Impaired insulin signaling	• Aβ aggregation stage • Increased inflammatory cytokines • Higher ROS	• Stimulate GLP-1 • Triggered insulin release in body • Improved insulin signaling	• Reduced oxidative stress • Decrease neural apoptosis • Neuroprotective effect • Suppress inflammation • Improved cognition • Decreased Aβ plaque after 2-month treatment • Lixisenatide found more effective in lower dose than liraglutide	[148,152]
Intravenous human insulin	1 U/kg IV dose	3xTg-AD mouse	• Glucose intolerance • Insulin resistance	• Amyloid β aggregation	• Rapid clearance of Aβ aggregates from the brain	• Remove Aβ plaque in 1–4 h of administration • Reduce the symptoms of AD • Enhanced peripheral insulin and hypoglycemia are the side effects	[64]
Oral insulin with amphipathic CPP[a]	10 U/kg	7 weeks old male ddY mice	• High serum glucose level • Lower Insulin content • Impaired insulin signaling	• Aβ aggregation • Memory impairment	• Reduce brain glucose • Improves insulin availability in the brain region • Increases insulin signaling	• CPP improve insulin permeation and protect it from enzymatic degradation • Reduce brain glucose level	[156]
IN insulin loaded microemulsion	1 U/kg	Diabetes-induced New Zealand white rabbits	Higher serum glucose level	–	By-passes BBB and increase insulin content in the brain	• Reduced glucose level by 30–40% • Microemulsion increases nasal permeability of insulin • Higher bioavailability than the subcutaneous insulin	[157]
IV and IN I[125] tagged IGF-1	143 µg/kg body weight	Male Sprague Dawley	• Glucose deprivation	• Impaired CNS functions • Reduced cellular and behavioral responses	• Target IGF-1 to the brain via olfactory path, bypassing the BBB • Activate IGF-1 signaling pathway • Reduce tau phosphorylation • Promote nerve cell survival	• Higher drug targeting to different regions of the brain • Activate insulin signaling pathway in the brain • Non-invasive and direct route to deliver neurotropic factor to the brain	[158]
IN insulin with CPP	2 U IN injection	ICV-STZ induced rat	• Insulin resistance	• Lower IDE • Impaired memory and brain function • Aβ aggregation • Tau hyperphosphorylation	• By-passes BBB and directly deliver insulin to the brain via olfactory and trigeminal nerve pathway • Reduced amyloid aggregation • Reduce tau hyperphosphorylation	• CPP enhance insulin permeation to the brain • Higher insulin in olfactory bulb and different another region of brain • Memory improvement upon 6-month treatment	[159]
IN-insulin with bovine serum albumin	0.112%inj/g	CD-1 and SAMP8 mice	• High serum glucose level • Lower Insulin content • Impaired insulin signaling	• Insulin receptor inhibition • Aβ aggregation • Tau hyperphosphorylation	• Reduce Ca^{2+} dependent hyperpolarization • Increased insulin signaling	• Improved behavioral response • Coadministration with Bovine serum albumin increases insulin concentration in brain • Improved memory	[136]

Table 1 (*continued*)

Drug and delivery approach	Dose of insulin	DM related factors	AD-related factors	Animal model	Targeted mechanism	Outcome	References
IN ^{125}I-insulin	4,00,000 cpm/μl	· High serum glucose level · Impaired insulin signaling	· Aβ aggregation · Tau hyperphosphorylation	Male CD-1 mice and 12-month old SAMP8 mice	· Inhibit PI3K · PKC inhibition · Increased insulin uptake	· Improve learning and memory functions of the brain · Reduced oxidative stress	[161]

a Cell-penetrating peptide.

demonstrated that insulin offers a promising therapeutic agent to enhance the memory and cognitive function of the brain and treat AD [133]. It is also stated that ApoE4 carriers worsen the situation of the AD, by reducing verbal memory improvement even after treatment with intranasal insulin [133,138]. Intranasal insulin therapy reduces the food intake without affecting the hunger and also enhanced access to CSF in comparison to systemic insulin therapy [139–141]. This probably maybe because of the increase in ATP levels on the administration of insulin, thus reducing the calorie intake of the body, hence simultaneously treating obesity with insulin deprivation in the brain [142]. Intranasal insulin has found to subchronically elevate the CNS insulin levels, showing less Amyloid-beta circulation, no BP or any secretory activity change or no harm as it is short-acting, unlike peripherally acting insulins [133,143].

6.3. Pre-clinical studies

Various preclinical investigations describing the role of insulin and insulin derivatives in treatment of AD via direct IN route and across BBB are discussed below and in Table 1.

Jolivalt et al., in 2008 conducted a study for 9 weeks on insulin-deficient type-1 diabetic mice (STZ-induced) that showed learning impairment and certain insulin-deficient AD-associated characters like reduction in IDE, increased GSK activity and decreased insulin receptor sensitivity. They proved that the insulin signaling pathway was impaired because of tau hyperphosphorylation and it got restored after the subcutaneous treatment of insulin (0.3 U/24 h) in the type-1 DM mice model (Swiss Webster). This does not seem in the case of type-2 diabetic model (db/db) treated in a similar way. The authors stated that it was because of the less diabetes induction time and variation in the type of animal model. For the behavioral tests to evaluate spatial and learning memory, Brane's maze was used and not the Morris water maze test. This was because the Swiss Webster mice are in general not found to show effective results in the latter. Also, cold and stress would lead to more of tau phosphorylation in the animal. Here, the insulin treatment increased insulin receptor phosphorylation and decreased GSK activity along with partial prevention of cognitive impairment and decreased tau phosphorylation. Thus, it was proved that diabetes-induced behavioral changes could be reversed by treating with Insulin [144,145].

Whenever there are high levels of glucose after meal consumption, the incretin effect occurs. Here, the body is prone to release more insulin, which is removed later by the DPP-4[53] enzyme. This is a normal process when people do not have diabetes. In patients with DM-2, this process is attenuated because of the reduction in active GLP-1[54] levels that leads to beta-cell apoptosis [146]. GLP-1, being a growth hormone, protects the brain from stress due to oxidation, decreases the apoptosis process and induces the neurite outgrowth [147]. They also instigate the multiplication of progenitor cells in the brain, thus treating AD [148,149]. GLP-1 (hormone Glucagon-like peptide-1) analogs like liraglutide and lixisenatide have been found to show improved insulin signaling in diabetes and AD induced APP/PS1 mice. McClean et al., in 2014, conducted this study for 10 weeks showing that daily intraperitoneal dose of 1 or 10 nmol/kg of lixisenatide was effective than 2.5 or 5 nmol/kg dose of liraglutide. The animals in the Aβ aggregation stage were treated with the drugs, and the responses were noted. Lixisenatide showed neuroprotective effects in a mouse model of the AD, showing a reduction in Congo red positive plaques and suppressed inflammation caused by microglial activation (by 33%). Lixisenatide, after 2 months of treatment showed improved cognition, LTP[55] [148], decrease in plaques and total APP levels. Liraglutide, after 60 days of treatment showed restorative effects in patients with LOAD.

[53] Dipeptidyl peptidase-4.
[54] Glucagon-like peptide.
[55] Long-term potentiation of synaptic transmission.

Lixisenatide, a substitute as a neuroprotective are found to improve memory formation and enhancement of synaptic plasticity in animals [150,151]. When compared to the other GLP-1 analog, lixisenatide showed greater bioavailability at lower doses due to its greater $t_{1/2}$. However, the drugs showed no memory effects on acute injections. This proved that the acute LTP effect does not improve memory. But after 10 weeks, the mice were able to perform the recognition of object task, proving that even the lowest of the doses showed memory improvement, demonstrating an improved synapse and neuronal plasticity. The memory task showed no difference in memory enhancement with an increase in the dose. Also, higher doses induce GLP desensitization. But this was not confirmed in this study. Also, when all doses showed Aβ plaque reduction, only the lowest dose of lixisenatide showed a response to the inflammatory reaction that is associated with the AD. This proved that higher doses that were used to treat DM were very high to treat AD. However, a clinical trial is conducted to show the effects of these drugs on human beings to treat AD [152].

Krishna et al. in 2019 have performed a pre-clinical study with ApoE3 conjugated polymeric nanoparticles derived from diblock copolymer methoxy poly(ethylene glycol)–polycaprolactone (mPEG[56] − PCL[57]) have been reported to boost the delivery of donepezil to the brain [153–155].

Vandal et al. in 2014 conducted a study on 3xTg-AD mouse models which were highly intolerant to peripheral glucose and showed the symptoms of amyloid aggregates, which induced diabetes in the mouse. Further, the high-fat diet worsened the disease condition along with insulin resistance. One acute IV dose of 1 unit/kg human insulin or 1 g/kg glucose rapidly cleared Aβ from the brain in the span of 1 to 4 h in vivo. This increased the Aβ levels in the plasma, stating that insulin reverses back the effects caused due to AD by clearing the plaques rapidly from the brain. However, hypoglycemia and enhanced levels of peripheral insulin were the side effects of this study [64].

In vivo testing of oral insulin along with amphipathic cell-penetrating peptides were tested in mice by Nielsen et al. in 2014. The reports suggested that the enzymatic degradation and the permeation problems of insulin along the GIT[58] were overcome by the use of D-penetrin. Penetrin is an enzyme resistant peptide, protecting insulin from enzymatic degradation and it enhances the half-life of insulin to triple from the normal because of the non-covalent intermolecular attraction between them. However, the degradation of D-penetrin is also faster relatively [156].

Sintov et al. in 2012 suggested that the poor systemic absorption of the insulin aqueous solution was overcome when they entered the brain via the olfactory pathway. However, they also proved that fluorescent-labeled w/o microemulsion was twice more effective in targeting the brain of rats. With enhanced viscosity and at a lower concentration, they are found to be effectively targeting the brain without any intended drainage through other routes. But, when compared to IV,[59] they showed a decline in the concentration reaching the brain after 3 doses. This was because the higher concentration proteins were enzymatically degraded in the olfactory bulb region and there was found to be more systemic loading through three IV doses, yielding more brain delivery of the drug via the pre-saturated blood-brain-barrier [143,157].

Thorne et al. in 2004 conducted a study on male Sprague Dawley rats to find out the effects of IV and IN[60] administration of I[125] tagged IGF-1. The IN drug administered rats showed the presence of the drug in CNS within 30 min, which quickly traveled to different parts of the brain and SC[61] [158].

Kamei et al. in 2015 worked on IN administration of penetrin, cell-penetrating peptide along with insulin, to overcome delivery efficiency issues via the general routes. Out of L and D types that were used, D-penetrin showed more effective brain delivery with less systemic exposure of insulin, proving that they could be of high value in delivering proteins like insulin to the brain [159]. When 2 U of intranasal injection was administered in ICV[62]-STZ[63] injected rats, the researchers found that the animals showed improvement in their memory after 6 weeks of treatment, with a decline in the formation of tau proteins. This may be beneficial in treating AD [160].

Rhea et al. in 2017 conducted a study to identify the IN-insulin transport and its brain distribution in healthy CD-1 mice and SAMP8[64] (older SAMP8 mice that are AD-like phenotypes and younger SAMP8 that are predisposed to develop this phenotype) for enhancing the memory. It was found that the pharmacokinetics of IN insulin distribution was similar in all three types, suggesting that animal strain or AD-like phenotype was found not to alter the IN uptake of insulin. It was found that insulin was transported within 5 min throughout the brain and was present even after 1 h. It was also told that the mechanism of transport here did not involve bulk flow in CSF, but rapid distribution through perivascular transport. Initially, insulin was concentrated in the olfactory region, and by 60 min, it was found high in the hypothalamus (where there are more insulin receptors) and less in the pons. When albumin was intranasally injected with insulin, it enhanced the brain uptake of the latter unaltering the relative distribution pattern because albumin and insulin are said to bind together, thereby extending the bioavailability of insulin [136].

Salameh et al. in 2015 conducted a study on CD-1 mice to observe the effect of insulin on learning and memory functions. 1 μl of 4,00,000 cpm/μl of ^{125}I-insulin was applied on a cribriform plate by the nasal route gets quickly distributed throughout the brain in varying degrees via a saturable process. The saturation is stimulated by inhibiting PKC.[65] The author stated that rapid ^{125}I-insulin distribution throughout the brain involved the perivascular space of the cerebral blood vessels. The highest levels of insulin uptake were found in this way because of the presence of more amounts of insulin proteins and receptors in the olfactory region. Less than 3% of the administered IN insulin was found in serum. Thus chances of mice getting affected by hyperinsulinemia were less. The researchers found a direct method to inhibit the distribution pathway of the administered insulin so that the exact mechanism of insulin transport to the brain via the olfactory pathway can be elucidated. Surprisingly, inhibitors like phenylarsine oxide affect N-type of calcium channel and phosphatidylinositol 4-kinase increased insulin uptake. It also inhibited insulin-stimulated glucose transport, unaffecting the insulin binding or the insulin receptor activity associated with a tyrosine kinase. Other agents like verapamil, Monensin, filipin, histamine, and phoinositide 3-kinase inhibitor LY294002[66] also showed an increase in ^{125}I-insulin uptake, except for lidocaine that blocked the action potential conduction by the closure of the voltage-gated sodium channels, and PMA,[67] which was a PKC activator that stimulated fluid phase transcytosis. This was because the latter two were PKC activators and the others were their inhibitors, opposite of the phenomenon that takes place in the muscles. To verify this activity, the study used a few more PKC inhibitors like bisindolylmaleimide-I, staurosporine, and bryostatin-I, also showed the same activity as the other PKC inhibitors. Also, to determine if enough insulin could reach the brain, SAMP8 mice that naturally develop memory and

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[56] Poly(ethylene glycol).
[57] Poly(caprolactone).
[58] Gastro intestinal track.
[59] Intravascular.
[60] Intranasal.
[61] Subcutaneous.

[62] Intra cerebro ventricular.
[63] Streptozocin.
[64] Senescence-accelerated prone mouse.
[65] Protein kinase C.
[66] Phoinositide 3-kinase inhibitor.
[67] Phorbol myristate acetate.

Table 2
Various clinical data concerning the insulin-based therapy for treatment of AD.

Drug	Dose	Type of study	Period of study	Clinical trial model	Targeted mechanism	Outcome	Downside	References
Metformin	1 g twice a day	Pilot randomized Placebo-controlled clinical trial	365 days	55–90 years obese/over-weight, diabetes untreated hyperinsulinemic AMDI patients	• Decrease peripheral insulin • Remove Aβ plaque • Improve insulin sensitivity	• Memory enhancement by Bushcke selective reminding test • Reduce inflammation • Prevent obesity, hypertension and diabetes	• Only 10% participant able to tolerate the actual dose of drug • GI intolerance in different extent • Need further investigation	[164]
Rosiglitazone	2, 4 and 8 mg	Randomized/placebo, double-blind, parallel-group study	24 weeks	ApoE positive patients with mild AD, no diabetes	The neurotoxic effect due to ApoE4	• Significant improvement in ADAS-cognitive ApoE negative patient • increased mitochondrial glucose utilization • Enhanced cognition	No improvement in ApoE4 positive patient	[167]
Pioglitazone	15 to 30 mg daily dose	Randomized, open-controlled Pilot study	6 months	Patients with AD and amnestic MCI with type 2 Diabetes Mellitus, treated with oral hypoglycemic medicine	Reduced beta-amyloid accumulation	Improved cognition	Mild peripheral edema	[172]
Insulin	4 × 40 IU/d	Double blind, between-subject comparison	8 weeks	36 healthy human volunteers	–	• Improves memory and mood • Reduce anger • Improve self-confidence	No side effect	[176]
Insulin aspart	4 × 40 IU/d	Double-blind, between-subject comparison	8 weeks	36 healthy men	Boost brain insulin signaling	Improves memory	No side effect	[175]
Insulin	20 and 40 IU	Randomized, double-blind, placebo-controlled trial	120 days	Adults with amnestic mild cognitive impairment (64) and mild to moderate AD (40)	• Reduced beta-amyloid accumulation • Reduce tau phosphorylation	20 IU insulin is more effective in delayed memory enhancement (story recall score and dementia severity rating score)	No severe adverse effect	[133]
Insulin	20 or 40 IU intranasal insulin	Randomized trial	4 months	AD/AMCI patients	ApoE genotype modulation	• Memory and cognitive improvements • Poor memory recall in ApoE positive female patients	–	[28]
Long-acting intranasal insulin detemir	20 and 40 IU insulin	Pilot trial	3 weeks	AD/MCI adults	• Increasing the hypothalamic and cerebrocortical insulin signaling • ApoE modulation • reducing the ratio of tau-$P_{181}/A\beta_{42}$	• memory improvement in ApoE4 positive patients • Highly insulin-resistant patients were also seen to show positive effects with 40 IU dose	Reduced effectiveness with time	[177–179]
Glulisine	20 IU	Double-blinded, acute, placebo-controlled randomized clinical study	2 weeks	65–85 years old adult with mild to moderate AD	ApoE induced memory impairment	• Drug well tolerated • Serum insulin level dropped • No change in blood glucose level	No significant effect on memory and cognition	[180]

learning defects, cholinergic defects decreased CSF reabsorption, brain oxidative stress, etc. were given a dose before the training period (not > 7 days before training). They affected both retention and acquisition of the task, which confirmed that insulin could improve cognition for a minimum of 24 h after their administration, and not > 168 h. 5-minute post-training insulin showed enhanced memory consolidation, but not 24 h of post-training. Object recognition also showed that memory enhancement needs insulin at that specific time of activity. However, administration of chronic insulin had no such effects, when compared to the unit intranasal dose of insulin. This is, therefore, proving that IN uptake of insulin can alter memory and cognition in AD models [161].

6.4. Clinical studies

Various clinical studies describing the role of insulin and insulin derivatives in the treatment of AD via direct IN route and across BBB are discussed below and in Table 2.

Metformin, an insulin sensitizer, was found to show increased intra and extracellular levels of beta-amyloid because of the phosphorylation of activated AMP[68]-protein kinases [162]. This is just the opposite of what insulin does to a diabetic patient with AD. But it was found that combining metformin with either insulin or any of the thiazolidinediones produces a synergistic effect with the anti-diabetic drugs and helps to regularize the insulin activity thus facilitates AD therapy [163]. However, only metformin could be harmful. Luchsinger et al. in 2016 conducted a 365 days long pilot, placebo-controlled, randomized (stratified and blocked) clinical trial in hyperinsulinemic AMDI[69] patients who were 55–90 years obese/over-weight and untreated for diabetes. The study used twice a day dose of 1 g metformin showed positive effects on memory-enhancing by Bushcke SRT[70] and not cognition. Metformin was found acting not directly in the brain, but by decreasing the peripheral insulin levels, thus clearing the amyloid plaques in the brain. It also reduced inflammation and coagulation and prevented metabolic syndrome like obesity, hypertension, diabetes, etc. The highest dose of the drug showed total recall in the SRT among the ApoE negative patients who had the highest baseline insulin during fasting conditions. The drawback of this clinical trial was that 7.5% of the participants were not able to tolerate the drug while 15% of them tolerated 500 mg/day dose, 35% tolerating 1000 mg/day dose, 32.5% tolerating 1500 mg/day dose and 10% tolerating the actual dose. GI intolerance was the only side effect found in the intolerant population. The study further needs to be conducted on different subgroups based on age, ApoE status and glycemic level of the patients. A larger trial should be conducted to showcase the cognitive safety and effectiveness of the drug [164].

As discussed earlier, ApoE alleles are known to be one of the major risk factors engendering AD [165]. A hypothesis on ApoE suggests that they enter the cytoplasm, to bind with the fragmented lipid-binding area, where mitochondrial interactions occur [166]. ApoE positive patients were said to show no cognitive improvement with Rosiglitazone in the study conducted by Risner et al. in 2006. Among the ApoE alleles, E4, in particular, damaged the mitochondria and showed neurotoxic effects. Thus, ApoE positive patients treated with rosiglitazone showed a decline in the disease [167]. A group of scientists suggested that PPARg[71] agonists, rosiglitazone modifies the mitochondrial function, thus increases the mitochondrial glucose utilization which further enhances cognition. On the other hand, the ApoE4 antagonist damaged the mitochondria and impaired glucose metabolism in CNS [168,169]. But this study lacked in further specifying the response of each of the

genotypes to rosiglitazone. Also, the relationship between ApoE, cognition, and rosiglitazone could not be deduced through this trial [167]. In a phase-3 clinical trial for patients with mild AD and no type-2 diabetes, rosiglitazone showed no efficacy for secondary prevention of cognitive decline. It may be because of cerebrovascular effects shown by the drug [164,170].

PPAR-γ agonist Thiazolidinediones that are good insulin sensitizers showed neuroprotective effects by decreasing the beta-amyloid plaques [171]. A study conducted by Hanyu et al. in 2009 in patients with AD and amnestic MCI[72] who were associated with type-2 diabetes mellitus showed that the patients treated with pioglitazone showed improved cognition at the end of 6 months of treatment. It was also seen that few people had edema and CHF[73] when treated with pioglitazone [172]. Further animal models treated with the same drug also proved the reduction of beta-amyloid accumulation [173]. However, the reason behind this is exactly not known. Study at a larger level may tell us the exact results of the drug being used for treating AD and MDI associated with DM [170]. In the 20th annual ASENT[74] meet, John et al. in 2018 presented the details of clinical trial results of rosiglitazone. It was found that the 3rd phase failed to statistically demonstrate the cognitive and functional effects of the drug in treating AD. This was because of its poor BBB penetration, high rates of metabolism and limited PPAR gamma target expression in the brain. Thus, T3D-959,[75] PPAR delta-activating compound (agonist), is in IND 2nd phase, for the treatment of the same. The drug acts as a dual agonist, activating PPAR gamma, providing an additive/synergistic effect in amending non-functional brain glucose/lipid metabolism. They show good BBB penetration, with a good therapeutic index. The principle of the Phase-2a clinical study was to demonstrate if T3D-959 could bring desired changes in cerebral glucose metabolism, which could indicate possibilities for cognitive improvements [174].

A group of scientists had proved that rapid-acting analog ASP-I[76] showed better verbal recall memory when compared to the normal RH-I[77] when both were given for 60 days (8 weeks). They also established that intranasal insulin would alter the hippocampal neuronal processes, thus treating AD by improving memory [175].

Benedict et al. in 2004 suggested that with enhancing the declarative memory, intranasal insulin was also found to improve mood by boosting self-confidence and controlling anger after the 8-week course of intranasal insulin [176].

A 120-day randomized, single, double-blinded, placebo-controlled trial that was used to infer the effects of insulin (20 and 40 IU with placebo) in treating memory and cognition in people with MCI and mild-moderate AD. The study proved that the use of 20 IU insulin to be better in treating memory-related delayed story recall as it showed optimal levels of the drug. Anything more or less than the optimal concentration was not found to be effective in memory enhancement (40 IU was more than the optimal value). However, multicentric large clinical trials must be done to prove its effectiveness [133].

Reger et al. in 2006 carried out a clinical study on AD/AMCI[78] patients. They were treated with placebo, 20 or 40 IU intranasal insulin for 4 months. Cognitive improvement was indicated by PET data that showed ^{18}F fluorodeoxyglucose in the parietotemporal, frontal, precuneus, and cuneus regions of the CNS following IN administration of insulin in comparison to placebo controls. Studies proved that a unit dose of IN insulin acutely improved memory in memory-impaired elderly with AD or MCI. Memory and cognitive improvements were also

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[68] Adenosine mono-phosphate.
[69] Amnestic mild cognitive impairment.
[70] Selective reminding test.
[71] Peroxisome proliferator-activated receptor.

[72] Mild cognitive impairment.
[73] Congestive heart failure.
[74] American Society for Nondestructive Testing.
[75] A dual PPAR-δ/PPAR γ nuclear receptor agonist.
[76] Insulin aspart.
[77] Regular human insulin.
[78] Amnestic mild cognitive impairment.

significant with multiple doses in patients with AD and MCI without a change in their plasma insulin or glucose levels. But in female and ApoE positives, there was a poor recall of memory, which also was the case with rapid-acting insulin. The study also found out that E4 patients who did not show improvement in this dose would show activity at lower doses of insulin, which was previously confirmed by Craft et al. in 2003 [70]. So, the difference between insulin transport to the CNS and ApoE status must be studied, which the study lacked. It may be possible due to decreased insulin-mediated glucose disposal in E4 negative patients. Two patients suffered nose bleeding and nose soreness that lasted for about a few minutes after 40 IU dose and needleless syringe insertion into the nasal cavity, respectively [28].

Claxton et al. in 2014 conducted a trial on IN administration of regular insulin that had lesser $t_{1/2}$ than insulin detemir, which is a long-lasting insulin analog because of the enhanced and reversible self-association of them with albumin, where AD/MCI patients were tested for improving cognition or everyday activity. Because detemir is lipophilic, a higher concentration reaches CSF. So, there is a delay in the absorption of the drug because of a higher percent of reversible albumin binding. This reduces the risks of hypoglycemia. But some controversies have shown that they don't cross BBB, so IN or ICV route have administration may be useful to tackle this problem. Also, because of its prolonged effect and higher CNS penetration, the safety and efficacy of IN detemir are of main concern. Among placebo, 20 IU insulin detemir and 40 IU insulin detemir that was given for 3 weeks, the latter showed good memory improvement in ApoE4 positive patients than the non-carriers. Highly insulin-resistant patients were also seen to show positive effects with 40 IU insulin detemir alongside an improvement in episodic, verbal and visuospatial working memory. Detemir shows greater central activity when compared to regular insulin by increasing the hypothalamic and cerebrocortical insulin signaling. All these collectively concluded that a daily dose of 40 IU insulin detemir is safe and enhanced memory and cognition in AD/MCI adults [177]. Claxton et al. in 2017 worked on finding out whether 40 IU detemir and 40 IU regular insulin would be safe on a long-term basis. So, they conducted a study for 4 months. The study suggested that detemir's efficacy decreased on a long-term basis by an unknown mechanism, whereas regular insulin showed beneficiary effects on memory enhancement. Long-acting insulin may produce chronic hyperinsulinemia or desensitize the insulin receptors when given on a long-term basis, thus giving rise to insulin resistance. Detemir's self-association with albumin will go down with time, thus producing the decreased effect. Regular insulin showed more memory enhancement effects by reducing the ratio of tau-P_{181}/Aβ_{42} along with the rapid clearance of the drug. However, the albumin-bound detemir was not able to cross the channels because of their increased molecular weight due to binding and thus showed a decline of its effectiveness over a period of time [178].

To find out the effect of sex and ApoE status on the insulin activity in AD or MCI patients, Claxton et al. in 2013 conducted a study, where 104 patients were prone to receive placebo, 20 IU or 40 IU of IN insulin for a period of 4 months with the help of an intranasal device. The studies suggested that women showed no cognitive improvement with 40 IU insulin dose showing hippocampal degeneration, but both the genders showed positive response towards delayed storytelling with 20 IU dose. With 40 IU dose, only ApoE4 non-carrying men and ApoE positive women showed definite signs of improvement. But ApoE positive women and men had no improvement or declination in the cognitive functions with 40 IU dose. Women have better whole-body insulin clearance than men, which suggests that they regulate more effective insulin metabolism in the peripheral organs. This is to say that even though women are more prone to obesity, because of this clearance activity, they have fewer chances of acquiring impaired glucose tolerance and low risks of diabetes when compared to men. Thus, men are prone to cognitive impairments, and higher doses of insulin may be useful in treatment [179].

Rosenbloom et al. in 2014 conducted a double-blinded, acute, placebo-controlled randomized clinical study to check the activity of RA[79] glulisine over the regular ones for verbal memory improvement in ApoE4 carriers with mild to moderate AD. The effect of acute IN insulin on ApoE4 carriers is attenuated. Glulisine that lacks zinc avoids olfactory toxicity, therefore being safe. Here though RAs have better activity than regular insulin, its unit dose was shown to not improve cognitive activities in ApoE carriers with mild to moderate AD, like regular insulin. Thus, it proves that ApoE status on insulin responsiveness holds the main part in regulating AD. But the mechanism underlying this is not explained in the current article. It was found that postmortem AD brains that were ApoE positive showed less cingulated cytochrome B4, thus losing the mitochondrial integrity that does not respond to the insulin that is supplemented [180,181].

7. Application of insulin based novel therapies

Anti-insulin receptor antibodies loaded liposomes have shown promising therapeutic efficacy in pre-clinical studies conducted by Boado et al. in 2006. In this research work, the HIRMAb[80] is a molecular Trojan horse for the transportation of protein drugs in the brain. The murine HIRMAb is also employed to deliver non-viral genetic material through the BBB. Herein the plasmid DNA has been encapsulated in the internal core of 100 nm liposomes with the liposomal surface being conjugated with the HIRMAb. Increased delivery of protein and/or non-viral gene therapeutics through the human BBB is attained via the available humanized HIRMAb [182].

A study was performed by Ulbrich, K et al. in 2010 concluded that by targeting the insulin receptor in the brain by coupling loperamide loaded nanoparticles with insulin or an anti-insulin receptor monoclonal antibody (29B4) improved brain delivery. HSA[81] nanoparticles loaded with loperamide with covalently attached insulin or the 29B4 antibodies led to prominent induction of antinociceptive actions in the tail-flick test on intravenous administration in ICR (CD-1) mice. This confirmed that insulin or the antibodies covalently attached to the HSA nanoparticles showed the ability to transfer the drug through the impermeable BBB [183].

Carmustine-loaded solid lipid nanoparticles were grafted with 83–14 monoclonal antibody and were utilized for the brain-targeting delivery. The results revealed promising targeting efficacy to the brain. The designed SLNs[82] were decorated with 83–14 MAb (83–14 MAb/BCNU-SLNs) intended to target the brain. 83–14 MAb/BCNU-SLNs were used in the incubation of HBMECs[83] to observe the interaction occurring among the lipidic nanoparticles and insulin receptors that were expressed. Besides, the transport of these nanocarriers in the brain via the BBB was improved in in-vitro conditions on increased percentage of the weight of Tween 80. The antibodies on the nanoparticles led to stimulation of endocytosis by HBMECs via insulin receptors further improving the permeation of the drug through the BBB [184].

8. Limitations

All these experimental aspects have shown to treat the disease with Insulin and its analogs/sensitizers via long-term exposure of the same. In a particular study, insulin administered via the intranasal route can lead to unwanted systemic toxic effects such as increased blood pressure and hypoglycemia [185]. Another possible adverse reaction is excessive delivery leading to higher local CNS levels. The higher levels

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[79] Rapid-acting.
[80] Humanized anti-human insulin receptor antibody.
[81] Human serum albumin.
[82] Solid lipid nanoparticles.
[83] Human brain-microvascular endothelial cells.

of insulin can lead to injury and the promotion of degeneration of tissue. This necessitates the optimization of dosing rates and subsequent levels to be achieved on insulin delivery. Also, insulin displays growth-promoting characteristics with extended use may lead to multiplication and proliferation of neoplasms. In another clinical study, repeated administration of insulin in a repeated manner each 10 min for 2 h, resulted in a quick and rapid increase in systolic, diastolic and mean arterial blood pressure. In an acute setting, this phenomenon could be risky for hypertensive patients. Further elaborate studies in human beings need to be done to prove the effectiveness and safety of the above-said methodologies for their long-term usage. The future may hopefully find these therapeutics in the market for treating AD by enhancing memory and cognition in the affected patients [24,73].

9. Future prospects and conclusion

The hypotheses that are considered to be a cause of the treatment must be blocked at any stage that does not further allow worsening the disease. Large molecular weight compounds like proteins and peptides find it difficult to reach the CNS through the GI route to show its therapeutic activity because of some of the physiological barriers like BBB, enzymatic degradation, clearance, binding effects, etc. However, IV can be the next route of choice. But that may lead to hypoglycemic effects in the patients. But in IN route, the drug bypasses the systemic circulation and BBB, directly enters the CSF, which rapidly gets absorbed into different parts of the brain and SC. Researchers are working on the use of different carriers and drug delivery systems that can help the drug reach the brain with good efficiency and lesser side effects like hypoglycemia. While acute treatments have shown safety and effectiveness, chronic treatment may cause desensitization in the insulin signaling pathway. However, all of them are successfully acting in preclinical or clinical stages (pilot studies). None of them have been approved to date to treat AD. Hopefully, the mere future may find them in treating AD.

10. Author perspective

Global improvement in AD commonness has a great socioeconomic effect, not only in terms of the patient's health but also the quality of life and economic burden on the family too. This demands a proper understanding of pathophysiology and other associated factors along with the development of novel techniques for enhanced and cost-effective therapies. Previously, the AD was supposed to be the disease of old age and genetic deficiencies. The understanding of pathology further expanded to various neurological factors including the amyloid β aggregation or amyloid plaque formation, neurofibrillary tangles due to tau hyperphosphorylation, cholinergic insufficiencies, mitochondrial dysfunctioning, excitotoxicity, presenilin gene mutation, ApoE pathway and NMDA receptor dysfunctioning. Thereafter, studies demonstrated the relation of other systemic ailments on AD progression, among which diabetes and insulin metabolism is the most prevalent one. It is evident that the Aβ plaque formation and tau hyperphosphorylation also affect the function of peripheral tissues like pancreatic cells, responsible for regulating insulin production and glucose metabolism. Besides, Aβ plaque and tau hyperphosphorylation also hindered brain insulin uptake by provoking brain insulin resistance. Such alterations account for glucose deprivation in the brain and periphery and worsen the neurodegeneration. Owing to such regions, AD is considered as 'brain associated diabetes' or 'Type 3 diabetes'.

Impaired insulin signaling is one of the primary risk factors associated with the pathophysiology of both the AD and type 2 diabetes mellitus. Various preclinical and clinical investigations suggested the moieties claimed to improve the insulin level and regularize brain insulin signaling also considerably enhance the cognitive function of brain. Also, the origin of insulin is from the peripheral organ thus regularization of systemic insulin level also has some effect on brain

metabolic function. The shared clinical and pathological trails of AD and diabetes mellitus raise the possibility of exploring the impact of neuroprotective agents on diabetes and antidiabetic drugs on the management of neurodegenerative disorder. Based on this notion, human insulin, insulin analogs and various antidiabetic drugs were widely studied for their effect on AD.

Although, till now, there is no promising cure is available for the treatment of diabetes associated AD. However, various successful studies exploring the impact of human insulin, insulin analog like insulin aspartate, herbal bioactives and synthetic moieties like metformin, liraglutide, rosiglitazone, pioglitazone, etc. on memory enhancement, insulin signaling and diabetes management are at preclinical or clinical stages. The studies focused on regularizing the neurodegeneration, insulin signaling path, glucose deprivations in the brain and peripheral organs, reducing the effect of insulin-degrading enzymes, delivering the drug across the BBB and brain targeting of bioactives to enhance bioavailability in the brain region. To achieve this, various advanced strategies including the development of nanocarrier system, various insulin derivatives, targeted delivery system by modification with brain targeting peptides like CPP, GLUT, etc. and intranasal administration (as an alternative route to directly approach the brain by bypassing the BBB) have been investigated. Most of the clinical trials also shown positive response which when approved further for clinical application supposed to create a new milestone in the treatment of AD.

Abbreviations

AD	Alzheimer's disease
Aβ	β-amyloid
Aβ$_{42}$	amyloid-β 42 fragments
ACh	acetylcholine
AChEI	acetylcholine esterase inhibitors
AGE	advanced glycation end product
Akt	protein kinase B
AMCI	amnestic mild cognitive impairment
AMDI	amnestic mild cognitive impairment
AMP	adenosine mono-phosphate
AMPK	AMP-protein kinases
APP	amyloid precursor protein
ApoE	apolipoprotein-E
ASP-I	analog insulin aspart
ASENT	American Society for Nondestructive Testing
ATP	adenosine tri-phosphate
BACE-1	β-site APP-cleaving enzyme 1
BBB	blood-brain barrier
BCl-2	B-cell lymphoma-2
BP	blood pressure
CBF	cerebral blood flow
CHF	congestive heart failure
CNS	central nervous system
CSF	cerebro spinal fluid
CTF	carboxy terminal fragments of APP
DM	diabetes mellitus
DPP-4	dipeptidyl peptidase-4
EOAD	early-onset Alzheimer's disease
ER	endoplasmic reticulum
FAD	familial AD
G6P	glucose-6-phosphate
GI	gastro intestinal
GLP	glucagon-like peptide
GLUT	glucose transport proteins
GSK-3β	glycogen synthase kinase 3β
HAS	human albumin serum
HBMECs	human brain-microvascular endothelial cells
HIRMAb	humanized anti-human Insulin Receptor antibody
HK	hexokinase

ICV	intracerebroventricular injection
IDE	insulin-degrading enzyme
IGF	insulin growth factor
IN	intranasal
IRs	insulin receptors
IV	intravenous
JNK	c-Jun N-terminal kinases
LOAD	late-onset Alzheimer's disease
LRP-1	low-density lipoprotein receptor-related protein
LTP	long-term potentiation of synaptic transmission
LY294002	phoinositide 3-kinase inhibitor
MAb	monoclonal antibody
MAP-tau	microtubule associated protein tau
MCI	mild cognitive impairment
NADH	nicotinamide adenine dehydrogenase
NADPH	nicotinamide adenine dehydrogenase phosphate
NFT	neurofibrillary tangles
NMDA	N-methyl-D-aspartic acid
NMDAR	NMDA subtype glutamate receptors
PDH	pyruvate dehydrogenase
PEG	poly(ethylene glycol)
PCL	poly(caprolactone)
PKC	protein kinase C
PMA	phorbol myristate acetate
PSEN-1	presenilin-1
PSEN-2	presenilin-2
PI3K	phosphatidylinositol 3-kinase
PPAR	peroxisome proliferator-activated receptor
PPP	pentose phosphate pathway
pSer	serine residue
PS1	presenilin 1
RAGE	receptor of advanced glycation end products
ROS	reactive oxygen species
SRT	selective reminding test
SC	subcutaneous
SAMP8	senescence-accelerated prone mouse
sAD	sporadic AD
SLN	solid lipid nanoparticles
STZ	streptozocin
T3D-959	dual PPAR-δ/PPAR γ nuclear receptor agonist
TCA	tricarboxylic acid
TNF-α	tumor necrotic factor-α

Declaration of competing interest

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