CHAPTER 7

SUMMARY AND CONCLUSIONS

"People do not like to think, if one thinks, one must reach conclusions. Conclusions are not always pleasant."

-Helen Keller

Summary and Conclusions

7.1 Summary

The current thesis, titled "Design, Development and Characterization of Drug Delivery System for Bioactive(s)," is focused on enhancing the efficacy of selected bioactive compounds through two innovative dosage forms: The Ready to Infuse (RTI) dosage form and liposomes. These approaches are tailored for specific medical indications, employing distinct routes of administration. In this investigation, the bio actives, oxytocin (OXT), vasopressin, and angiotensin have been chosen for their potential therapeutic benefits, with a particular emphasis on the development of RTI formulations. Furthermore, liposome technology is utilized to facilitate the delivery of OXT and vasopressin through the nasal route for targeting the brain directly. Apart from that, conjugation technology with OXT i.e., chemical modification was also explored.

RTI delivery system: The limitations of injections and lyophilized powder for injections which need to be diluted before administration limit the urgent need for a formulation that is ready for immediate use, enabling healthcare professionals to administer it directly to patients without delay. Additionally, the process of manufacturing injectable drugs that are sensitive to both oxygen and light introduces complexities when utilizing plastic infusion bags. These bags possess a semipermeable nature that can react with atmospheric oxygen during the manufacturing stages, which extends up until the final packing of the product. By acknowledging the challenges associated with dilution and stability, this research aimed to design, develop, and optimize highly effective RTI delivery systems for the bioactive peptides OXT, Vasopressin, and Angiotensin-II. By doing so, the study sought to resolve the various issues tied to these selected peptides. The adoption of the RTI method will facilitate the production of injections with lower concentrations of active ingredients, ultimately enhancing patient compliance and safety. Ensuring patient safety is paramount in this context, as the RTI approach uniquely minimizes the risk of dosing errors to zero while also significantly reducing the chances of cross-contamination.

The proposed "Ready to Infuse" dosage form is anticipated to be a ground breaking solution, characterized by a novel pharmaceutical strategy for administration, innovative formulations,

and unique dosage forms that are well-suited for intellectual property protection and commercialization.

Liposomes system:

The rapidly developing nanotechnology has provided new possibilities for transporting therapeutic drugs across the BBB to treat brain diseases. Considering the biocompatibility, safety, and complexity of the production process, liposomes are superior among all synthetic nanocarriers. Vesicles are aqueous core containing nanostructures enclosed by a lipid/polymer bilayer encapsulating an active ingredient or therapeutic molecule. Liposomal systems bridge gap between hydrophilic and hydrophobic heterogeneity in one drug delivery system. Either hydrophilic drug or lipophilic one can be encapsulated in vesicles, even both of them simultaneously. Liposomes can contain a large range of proteins, lipids, and nucleic acid species. Encapsulation of a drug into vesicles can markedly modify its plasma half-life, site, specificity and bioavailability. Therefore, liposomes are considered to be potential drug-delivery systems that can stably deliver their cargo into targeted cells.

Conjugation of AEEA with OXT: Presently, chemical modification is recognized as a powerful strategy for developing therapeutic peptide analogues with specifically targeted and desirable structures. Among the selected bio actives in this research, OXT has been identified as a model molecule, primarily due to its very short half-life in circulation (ranging from approximately 4 to 10 minutes). This research aimed to significantly enhance the retention time of OXT within the body while simultaneously improving its ability to permeate BBB. This objective was accomplished through the conjugation of 2-(2-(2-Aminoethoxy) ethoxy) acetic acid (AEEA) with one of the amino acids present in the OXT structure, thus aimed at enhancing its biopharmaceutical properties and therapeutic efficacy.

In a short stance, brief highlights of work underwent in thesis are presented here as under:

7.1.1 Analytical method development of oxytocin, vasopressin and angiotensin

The analytical framework for OXT has been discussed, highlighting the necessity for further validation and establishment of its measurements across various dosage forms for diverse studies. A novel and efficient Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method was successfully developed, adhering to the guidelines set forth by the

International Conference on Harmonization (ICH). This method was routinely employed to quantitatively estimate OXT during the research studies. The calibration curve achieved remarkable linearity, exhibiting an R^2 value of 0.9931 within a concentration range of 0.02 to 0.2 µg/mL. The limits of detection (LOD) and quantification (LOQ) for OXT were determined to be 0.00195 µg/mL and 0.00558 µg/mL, respectively. This suggests that the developed HPLC method is capable of effectively detecting and quantifying OXT even at remarkably low concentrations. The findings confirmed that the RP-HPLC method demonstrated high resolution, accuracy, and precision, making it suitable for the identification and quantification of OXT in any dosage form.

To ascertain the presence and concentration of vasopressin, significant efforts were made to develop an analytical technique based on Ultraviolet (UV) and HPLC methodologies. The HPLC analytical approach underwent a rigorous validation process, focusing on parameters such as linearity, accuracy, precision, LOD, and LOQ. Within the tested concentration range of 0.5 to 50 µg/mL, the calibration curves consistently displayed linearity with an impressive R^2 value of 0.999. For the assessment of percent accuracy, vasopressin was analysed at concentrations, viz., 5, 20.0, and 50.0 µg/mL. The data reflected that the analytical method exhibited a high degree of repeatability, indicated by the corresponding percent relative standard deviation (% RSD) values, which ranged from 0.78 to 0.97 for intra-day studies and from 0.88 to 1.19 for inter-day studies. All values fell well within the permissible RSD threshold of less than 2%, demonstrating that the developed method possessed adequate precision and repeatability for estimating vasopressin. Moreover, the LOD was determined to be 0.049 µg/mL, while the LOQ was found to be 0.147 µg/mL, affirming that even low concentrations of vasopressin could be reliably detected and quantified using this validated HPLC method.

In an additional effort to evaluate the presence and concentration of angiotensin, HPLC technique was employed. The analytical method for angiotensin was similarly validated for parameters such as linearity, accuracy, precision, LOD, and LOQ. Across the measured concentration range of 2 to 20 µg/mL, the calibration curves yielded a strong linearity, achieving an R^2 value of 0.9986. For accuracy estimation, angiotensin was assessed at drug concentrations including 2, 10.0, and 20.0 µg/mL. The results indicated that the analytical method demonstrated sufficient repeatability, as evidenced by the % RSD values ranging from

0.41 to 0.48 for intra-day studies and from 0.1 to 0.78 for inter-day studies. All obtained values remained comfortably within the acceptable limits of RSD less than 2%, reaffirming that the method showcased the necessary precision and repeatability for estimating angiotensin levels. The LOD for angiotensin was measured at 0.09 µg/mL, and the LOQ was established at 0.186 µg/mL, indicating that even low concentrations of angiotensin could be detected and quantified effectively with the selected HPLC method.

7.1.2 Development of RTI dosage

7.1.2.1 Development of RTI dosage form for oxytocin

Various preliminary variables for development of RTI formulation for OXT were evaluated to acquire a final formulation with assay ranging 90-110% and osmolarity range of 250-350 mOsmol/Kg. Therefore, different buffering agents (sodium acetate, tartaric acid, citric acid), amino acids (Glycine, L-methionine, L-arginine), metal ions ($MgCl_2$), osmogens (mannitol, sucrose, trehalose, lactose, dextrose, sodium chloride), stabilizer (HPβCD) and antioxidants (SMBS) were evaluated. The different compositions in PHC, AOB and TH82 bag were formulated and screened on the basis of their assay and osmolarity value. The assay was found to be below 90% in all trials taken in three different packaging materials. From initial trials, we concluded that PHC and AOB bag are better than TH82 bag. Hence, PHC and AOB bag were selected for further study for sequential screening of osmogenes, buffers and stabilizers

Further, formulations were prepared with sodium acetate and citrate buffers with various stabilizers and filled in two different packaging materials. The formulations which were formulated with lactose, sucrose and sodium chloride (with HPβCD) have shown assay value within the limits. Based on the results obtained, sodium acetate as buffer, sod chloride as osmogen, while HPβCD as stabilizer were found to be prominent compared to others. Further, the concentration of sodium acetate buffer and HPβCD were optimized. As sodium chloride is fixed at 0.9 mg/mL as optimum osmogen, sodium acetate and HPβCD were optimized from 0.01 and 0.5 mg/mL and 0.1 mg to 0.5 mg/mL respectively. On the basis of results, all the formulations prepared with different concentration of HPβCD and sod acetate combination have shown assay values within the limits in both bags at initial stage. Subsequently, HPβCD was also evaluated to find its role in the stability of OXT. The effect can be clearly seen in the formulation with and without HPβCD, where it increased the stability of OXT after 3M at

25°C/40%RH. The assay of OXT was found to be 95% in formulation without HPβCD while nearly 97 % in formulation with HPβCD after 3M at 25°C/40%RH. The assay of formulation remained same when concentration of HPβCD increased from 0.1 to 0.5 mg/mL. Therefore, we decided to select lower concentration i.e. 0.1 mg/mL for further study.

With the optimized composition, five formulations of different pH (5.0, 4.5, 4.0, 3.5 and 3.0) were prepared. Formulations were tested at initial stage and for 3M at 25°C/40%RH. After 3M, results were compared with their respective initial results. pH variation of formulation over the period changes the physical and chemical properties of OXT RTI formulation. Initially, there were no remarkable difference in assay values of all formulations, formulated at different pH. However, over the period of 3M at 25°C/40%RH, the assay value was decresed remarkably at pH 3.0 and 5.0. The formulation prepared at pH 4.0 had shown better stability after 3M among all tested formulations. Therefore, pH 4 was finalised for OXT RTI formulation. Further, autoclave study showed that OXT is thermolabile and cannot be autoclaved due to drastically decreased assay values compared to unautoclaved samples. Therefore, we decided for aseptic manufacturing process.

Freeze thaw study is important to find out the excursion storage condition during transport. RTI formulation of OXT were placed on stability storage at -20°C for 24 h. Samples were then transferred to 40°C / 75%RH for 24 h. Procedure was repeated for a total of 14 cycles. Samples were analysed for Critical quality attributes (CQAs), assay, pH and osmolality after 7 and 14 cycles respectively. There was no remarkable difference in the assay, pH value, and osmolality of the formulation after 7 and 14 days of study. This signifies the robustness and stability of developed formulation. Further, three formulations were prepared in presence of different gases viz air, nitrogen and oxygen. The formulations were filled and packed in respective environment. All the formulations were tested at initial stage and were kept on stability at 25°C/40%RH for 3M. After 3M, samples were withdrawn and analysed. The developed formulation was found to be stable in presence of nitrogen. However, formulations, that were prepared in presence of air and oxygen had shown prominent degradation with most significant degradation observed in formulation that was prepared in presence of oxygen.

Photostability testing was also undertaken to provide information necessary for manufacturing, handling, packaging, and labelling. The assay of OXT in RTI formulation was found reduced

when it was exposed to light. There was no effect on pH and osmolarity of formulation. Therefore, utmost care should be taken during manufacturing, handling and packaging of OXT RTI. Further, study was conducted to evaluate the suitability of material of construction (MOC) of manufacturing vessel (SS316L) for bulk solution manufacturing. The outcome of vessel compatibility study suggested that in all four conditions, the product was compatible with manufacturing vessel up to 24 h. After 24 h and till end of study, the assay of product was found in decreasing trend. The rate of degradation of OXT was seen higher in case of unfiltered and without nitrogen environment. From vessel compatibility study, we concluded that formulation can be held for 24 h in SS container without loss of potency of OXT in presence of N_2.

Filter membrane compatibility study was also evaluated for different available filter membranes for their physical and chemical compatibility with product. A study was conducted to evaluate the compatibility and adsorption of OXT by filter membrane used and selection. A bulk solution was prepared and passed through filter membranes (Nylon, PES, PVDF) separately and kept at room temperature (~20-25°C). Samples were withdrawn at Initial (0 h), 24 h, 48 h, and evaluated for drug product description, pH, assay and particulate matters. The outcome of filter compatibility study suggested that the product is most compatible with PES membrane up to 48 h. After 24 h and till end of study, assay of the product was found in decreasing trend with Nylon and PVDF filter.

The stability study of OXT RTI formulation (0.02 IU/mL was done at 5 ± 3 °C, 25°C/40%RH & 30°C/65%RH-6M. Obtained stability data was also processed with Minitab in order to evaluate in respect of time. RTI formulations were stored at 5 ± 3°C, 25°C/40%RH and 30°C/65%RH for stability studies, and tested for assay, pH, osmolality, PMT, % absorbance, % Transmittance, and sterility. The % of assay changed from 102.2±1.76 to 95.73±0.95 after 24 M at 5 ± 3 °C, while 93.13±1.01 after 12 M at 25°C/40%RH and 91.09±1.06 after 6M at 30°C/65%RH-6M. OXT showed non-significant change at 5 ± 3°C, while at 25°C/40%RH and 30°C/65%RH-6M, it showed changes in assay. Hence, the stability testing data indicated that RTI formulations of OXT stored at 5 ± 3°C were more stable.

7.1.2.2 Development of RTI dosage form for vasopressin

In view of knowledge gathered during formulation development of OXT RTI and considering effect of concentration of different osmogens on osmolarity of formulation, various developmental trials were taken in order to stabilize Vasopressin in large volume aqueous based formulations. For this purpose, osmogens such as sodium chloride, mannitol, lactose, sucrose, trehalose and dextrose were evaluated. In initial screening, pH was kept 3.7 as per concentrate vasopressin data. All formulations were prepared with sodium acetate buffer (0.01mg/mL) with different osmogens and filled in two different packaging materials (PHC & AOB). The assay of formulations prepared with mannitol, dextrose sucrose, lactose, trehalose, and sodium chloride were found to be above 90% and within proposed specification (90-110%). But, assay in formulation containing sodium chloride as osmogen was found to be 100% in both bags (PHC & AOB), while others prepared by other osmogens were found to be below 98%. Further, to check the effect of HPβCD as stabilizer, same formulation with HPβCD was formulated in both bags and kept for 3M at 25°/40%RH. However, the effect of HPβCD was not significant compared to without HPβCD after 3M at 25°/40%RH. Based on the results, sodium chloride was selected as osmogen for further development.

For pH stability study, batches were formulated at pH 3.0, 3.3, 3.8, 4.3, 4.6 and 5.0, and kept for 3M at 25°C/40%RH. The decreasing assay for all pH formulations was observed after 3M, while other CQAs i.e., pH, osmolality, PMT, % of transmittance & absorbance was found unchanged. Over the period of 3M at 25°C/40%RH, the assay value was decresed remarkably at all pH except the formulation prepared at pH 3.8. Therefore, we decided to keep pH 3.8 in our formulation for further studies. In order to evaluate a suitable sterilization method, vasopressin RTI formulation was sterilized at different recommended sterilization conditions, and analysed thereafter. In all sterilization conditions, assay of vasopressin in RTI formulation was found remarkably reduced. However, no effect on pH and osmolarity of formulation was observed. Therefore, the process comprising sterile filtration, pre-sterilized container and aseptic processing was chosen for formulation.

To evaluate the photosensitivity of vasopressin RTI, the samples (in infusion bag, in final pack and a control sample) were placed in the photostability chamber and exposed to light of 20 million lux for 24 hr. After exposure, the samples were withdrawn and analysed. The assay of

vasopressin in RTI formulation was found slightly reduced when it was exposed to light. There was no effect on pH and osmolarity of formulation. Therefore, utmost care should be taken while manufacturing, handling and packaging of vasopressin formulation.

Vasopressin RTI was formulated after optimizing composition and process, and stability study was conducted as described in section of OXT RTI formulation. The stability study of vasopressin RTI formulation 1.887 µg/mL (1 IU/mL) was done at 5 ± 3 °C and 25°C/40%RH. A risk assessment was performed in order to identify the CQAs of drug product. Drug product quality attributes were assessed for likely impact on product safety and efficacy. The stability data was processed with statistical tool (Minitab) to find out appropriate statistical model and to extrapolate shelf-life of drug product while considering all major CQAs of formulation. The vasopressin assay was found non-significantly changed at 5 ± 3°C, while found significantly decreased at 25°C/40%RH.

Percentage assay of vasopressin was found to decrease on storage at 25°C/40%RH, which may be due to instability of peptide molecules at higher temperature. This effect was least in case of formulation stored at 5 ± 3 °C, which indicates that degradation can be controlled by storing formulation at 5 ± 3 °C. There were no remarkable differences in initial value of pH and osmolarity when compared with end values observed after 12M. Hence, the stability testing data indicated that RTI formulations of Vasopressin stored at 5 ± 3°C were more stable.

Further, the extrapolation and stability report were generated as per ICH Q1E, and assay was found shelf life determining or indicating as there was statistically significant change happened during stability in both storage conditions, which is critical CQA to determine over all shelf life. Shelf life of another CQAs like Osmolality (mOsm/kg) and pH were found to be more compared to assay in both storage conditions. But, based on the stability data prediction as per ICH Q1E, overall shelf life was found to be 16.5 months and 25.36 months for 25°C/40%RH and 5±3°C respectively. The shelf life of vasopressin RTI is greater at 5 ± 3°C. Thus, it can be concluded that even in the worst-case scenario, the stability of vasopressin RTI formulation in sodium chloride injection is expected to be more than 12M at 25°C/40%RH and 36M at 5±3°C for proposed specification. Peptides are available in small volume and stored at 5 ± 3°C, but our developed formulation is large volume, so we recommend 25°C/40%RH storage condition for better feasibility and ease of transportation.

7.1.2.3 Development of RTI dosage form for angiotensin

In view of knowledge gathered during formulation development of OXT RTI, and Vasopressin RTI and considering effect of concentration of different osmogens on osmolarity of formulation, various developmental trials were taken in order to stabilize Angiotensin-II in large volume aqueous formulations. For this purpose, osmogens such as sodium chloride, mannitol and dextrose were evaluated. All formulations were prepared with various osmogens and buffers, and filled in two different packaging materials (PHC & AOB). The assay of formulation prepared by mannitol in combination of sodium chloride was found to be 100% and within proposed specification (90-110%). However, for formulations prepared with other excipients, assay was not found to satisfactory at initial stage. Therefore, we decided to go with sodium chloride for further study.

For pH stability study, batches were formulated at pH 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5, and kept for 3M at 25°C/40%RH. The decreasing assay for all pH formulations was observed after 3M, while other CQAs i.e., pH, osmolality, PMT, % of transmittance & absorbance was found stable. The assay difference after 3M for formulation with pH 5.5 was least compared to others. Therefore, we decided to keep pH 5.5 in our formulation for further studies.

In order to evaluate a suitable sterilization method, Angiotensin-II RTI formulation was sterilized at different recommended sterilization conditions and analysed. In all sterilization conditions recommended, the assay of Angiotensin-II in RTI formulation was found reduced. There was no effect on pH and osmolarity of formulation. Therefore, the process comprising sterile filtration, pre-sterilized container and aseptic processing was found suitable for further use. To evaluate the photosensitivity of Angiotensin-II RTI, the samples (in infusion bag, in final pack and a control sample) were placed in the photostability chamber and exposed to light of 20 million lux. After exposure, the samples were withdrawn and analysed. The assay of Angiotensin-II in RTI formulation was found slightly reduced when it was exposed to light. There was no effect on pH and osmolarity of formulation. Therefore, utmost care should be taken to manufacturing, handling and packaging of formulation of Angiotensin-II.

The stability study of angiotensin RTI formulation (0.01 mg/mL) was done at 5 ± 3 °C and 25°C/40%RH, as per ICH Q1E. A risk assessment was performed in order to identify CQAs of drug product. Drug product quality attributes were assessed for likely impact on product

safety and efficacy. The stability data was processed with statistical tool (Minitab) to find out an appropriate statistical model and to extrapolate the shelf-life of drug product while considering all major CQAs of formulation. The optimal storage conditions for the formulation were evaluated by measuring the percentage of assay after specific intervals of 3, 6, 9, and 12 months. The percentage assay of Angiotensin-II was observed to decline when stored at 25°C/40%RH, possibly due to the instability of peptide molecules at elevated temperatures. This degradation was less pronounced in the formulation stored at 5 ± 3 °C, suggesting that maintaining this temperature can help control degradation. No significant differences were observed in the initial pH and osmolarity values when compared to those recorded after 12 months. Therefore, the stability testing results showed that RTI Angiotensin-II formulations kept at 5 ± 3 °C were more stable. Nonetheless, it is also possible to store them at temperatures not exceeding 25°C/40%RH for up to 12 months without significant drug loss.

In light of the stability data predictions as prescribed by ICH Q1E, the overall shelf life was calculated to be approximately 16.41 months when stored at 25°C with 40% relative humidity, and an impressive 34.485 months when kept at a controlled temperature of 5±3°C. The extended shelf life at the lower temperature is attributed to the managed degradation of the assay, which helps maintain its integrity over time. Hence, it can be confidently concluded that even under the most challenging conditions, the stability of any angiotensin II RTI formulation is expected to last beyond 15 months at 25°C/40% RH and reach up to 36 months at the more favourable storage condition of 5±3°C, according to the proposed specifications.

All the tested RTI formulation samples have shown grade '0' in microscopic evaluation showing no *in vitro* reactivity. Similarly, in MTT test, all the tested samples have shown no *in vitro* reactivity. This indicates the biocompatibility of selected packaging materials.

Further, hemolytic Toxicity Study was performed with all RTI formulation. All RTU formulations were engineered to be isotonic, meaning their osmotic pressure was meticulously balanced to match that of human blood plasma. This precise formulation prevents the osmotic stress that was observed in the plain drug solutions. The excipients used in these RTU formulations were carefully selected for their biological compatibility and were present at concentrations considered safe and non-toxic, ensuring that the final products had no adverse

effects on the structural integrity of red blood cell membranes. This confirms the safety of the RTU formulations for clinical intravenous administration.

7.1.3 Development of lipidic liposomes for nose to brain delivery

7.1.3.1 Formulation and characterization

The study was designed to use OXT-loaded liposomes and Vaso-loaded liposomes for targeting the brain *via* intranasal route. The optimized molar ratio of HSPC: MPEG (2000)-DSPE: Cholesterol for both liposomes was 4:1:1 The average particle size of OXT-liposomes (95.06±0.86 nm) and Vaso-liposomes (99.06±1.01) was smaller than 100 nm. Further, PDI of all liposomes was found to be <0.2, which suggests the homogeneity in their particle size distribution. The zeta potential of OXT-loaded liposomes and Vaso-loaded liposomes formulation was estimated to be -22.1±0.12 and -21.8±0.17 mV respectively, which is probably due to the decoration of MPEG on the outer side of liposomes. The percentage drug entrapment (PDE) of OXT-loaded liposomes and Vaso-loaded liposomes was estimated to be 64.03±0.54 and 62.59±0.39% respectively.

The ZP of liposomes during stability was from -22.1±0.12 to -23.4 ± 0.14 and -21.38 ± 0.24 at 5±3°C and 25±2°C/60±5%RH after 6M and 3M respectively. The particle size increased from 99.06±1.01 to 112.5 ± 4.54, 124.1 ± 4.54, at 5±3°C and 25±2°C/60±5%RH after 6M and 3M respectively. The PS increase at 25±2°C/60±5%RH was significantly more compared to 5±3°C after tested stability tenure. The PDI of liposomes almost remained same at both storage conditions throughout the stability. The % of drug assay was decreased from 99.32 ± 1.73 to 98.02 ± 1.12 at 5±3°C after 6M, and to 97.21 ± 1.23 at 25±2°C/60±5%RH after 3M. OXT which was loaded in liposomes was more stable at 5±3°C without loss of drug potency. Hence, we recommend 5±3°C storage condition to get maximum shelf life.

The ZP of NVs during stability was from -21.8±0.17 to -22.4 ± 0.84 and -24.8 ± 0.84 at 5±3°C and 25±2°C/60±5%RH after 6M and 3M respectively. The particle size increased from 99.06±1.01 to 112.5±4.54 nm & 124.1±4.54 nm at 5±3°C and 25±2°C/60±5%RH after 6M and 3M respectively. The PS increase at 25±2°C/60±5%RH was significantly more compared to 5±3°C after tested stability tenure. The PDI of NVs almost remained same at both storage conditions throughout the stability. The % of drug assay was decreased from 98.02±1.87 to 96.89±1.25 at 5±3°C after 6M, while to 96.81±1.78 at 25±2°C/60±5%RH after 3M. As

vasopressin in NVs were more stable at 5±3°C without loss of drug potency, we recommend 5±3°C storage condition to get maximum shelf life.

7.1.3.2 Evaluation on monocytic cells (THP-I)

The study was intended to assess the effect of OXT, vasopressin, OXT-liposomes and Vaso-liposomes on the transcriptional pattern of apoptotic and inflammatory immune makers (caspase-3, 8, 9, Akt1, NF-kβ, BCl, CD40, Bim, Bak, IL-6). The reduced expression of caspase-3 and 8 was observed in the hTHP-1 cells treated with all formulations of vasopressin and OXT. Caspase-9 expression on the contrary was seen higher with the treated groups. A reduction in expression of inflammatory markers NfKB and IL-6 in the cells treated with all formulations of vasopressin and OXT was observed. Vasopressin and OXT loaded liposomes treated cells saw a reduced expression of BCL-2, a regulator of cell death, as compared to the plain drug treatment. Interestingly, the co-stimulatory marker (CD40) that regulates the programmed death and pro-apoptotic gene Bak was seen reduced with all formulations treated hTHP-1 cells, whereas Bim expression was seen higher by the OXT formulation treated cells. Since cell death and inflammation are two interlinked phenomena, the relative mRNA expression of IL-6 was determined. OXT formulation treated hTHP-1 cells showed a highly significant reduction in the expression of pro-inflammatory marker, IL-6 as compared to the vasopressin and untreated control.

Further, the inactive transcription factor FOXO1 (phosphorylated FOXO1) and pro-apoptotic factor (Bim) work in tight regulation by the over-expression of co-stimulatory (CD40) mediating the signalling (PI3-Akt) pathway in hTHP-1 cells. Thus, using confocal microscopy, we examined the qualitative expression of FOXO1 in LPS-stimulated hTHP-1 cells. After one hour of LPS stimulation, our data revealed a minor variation in the qualitative expression of FOXO1 between LPS-stimulated and unstimulated cells. Additionally, for FOXO1, we estimated the fluorescence intensity of cells counted in different fields. Compared to the unstimulated control, our data demonstrated FOXO1 expression. The drug encapsulated in the liposomes showed better results compared to plain drug while OXT-AEEA complex showed the best result amongst all tested formulation due to better cell permeability.

7.1.3.3 *In vitro* evaluation on SH-SY5Y

The *in vitro* cell viability studies on SH-SY5Y were done by MTT assay. OXT and vasopressin alone and encapsulated into liposomes showed good biocompatibility and 85-90% cells were viable after 72 hr. Further, SH-SY5Y cell uptake study of coumarin-6 (C-6) co-encapsulated liposomes (C-6, C-6+OXT and C-6+AEEA-Oxytocin) formulations was carried out to determine internalization efficiency of liposomes. Liposomes showed greater uptake in SH-SY5Y. The results obtained with cell uptake study demonstrated remarkably higher internalization of OXT-liposomes and vasopressin-liposomes after 3h incubation.

The comprehensive study examining cell uptake of C6-OXT-liposomes, specifically the C6-Vaso-liposomes, was conducted using SH-SY5Y cells to evaluate the effectiveness with which these liposomes are internalized by the cells. The results from this meticulous cell uptake evaluation revealed a strikingly higher internal localization of both OXT-liposomes and vasopressin-liposomes following an incubation period of 3 h. These findings serve to support the hypothesis regarding the cellular uptake of liposomes, as indicated by the persistent and vibrant fluorescence signals detected after 3 h of incubation with the C-6 co-encapsulated liposomes. The effect of the OXT, vasopressin, OXT-loaded liposomes and vasopressin-loaded liposomes on the SH-SY5Y cells was evaluated by observing cellular morphology at higher concentration (100µg/mL). After the treatment with pure drugs and drug loaded liposomes for 48 h, the alterations in the morphology of cells were not detected under a phase contrast microscope. In SH-SY5Y cells treated with formulation, we could not see any changes in characteristic features of apoptosis, such as disruption of a cell wall, shrinkage of the cell, and decrease in the number of living cells, compared to control group.

Apoptosis was qualitatively characterized in SH-SY5Y cells after treatment with pure OXT and vasopressin, OXT loaded liposomes and vasopressin loaded liposomes by DAPI and dual (AO/EB) staining technique. Normal SH-SY5Y cells showed intact nuclei and exhibit modest DAPI fluorescence in SH-SY5Y cells treated with OXT, vasopressin, OXT loaded liposomes and vasopressin loaded liposomes. No morphological alterations of nuclei associated with apoptosis were seen, including nuclear condensation, enhanced brightness, and nuclear crinkle, as further corroborated by AO/EB staining. Cells in the control group remained viable and

exhibited green fluorescence in a circular arrangement, with the nucleus evenly distributed at the centre.

7.1.3.4 Evaluation in AD induced animal model

7.1.3.4.1 Oxytocin loaded liposomes

The Morris water maze test clearly depicted about breakthrough enhancement in efficacy of liposome OXT when delivered via intranasal route. The average mean time incurred for mice to reach on hidden platform was 37.16±6.75 seconds. Similarly, average time incurred for SCP mice to reach on hidden platform was 58.66±1.18 seconds. Mice administered with OXT via intranasal route were able to reach hidden platform in 45.58±4.45 seconds i.e., 1.23 and 0.78 times of placebo and SCP treated group. Similarly, mice administered with OXT via i.v. route was able to reach hidden platform in 42.73±8.30 seconds i.e., 1.15 and 0.73 times of placebo and SCP treated group. However, mice administered with liposome loaded OXT via intranasal route were able to reach hidden platform in 28.36±8.56 seconds i.e., 0.76 and 0.48 times of placebo and SCP treated group. Similarly, mice administered with liposome loaded OXT via intravenous route were able to reach hidden platform in 51.45±6.25 seconds i.e., 1.38 and 0.88 times of placebo and SCP treated group. To confirm molecular effectiveness of treatment. Gene expression studies of brain homogenate samples were studied for AIF-1, BDNF, GFAP, S100B analysis.

To validate the molecular efficacy of the proposed treatment, comprehensive gene expression studies were conducted using brain homogenates, focusing on key biomarkers including AIF-1, BDNF, GFAP, and S100B. These studies were performed on samples from animals subjected to the Morris Water Maze (MWM**)** test, comparing traditional oxytocin (OXT) delivery methods (intranasal and intravenous) with liposomal-OXT administered intranasally. Quantitative PCR analysis revealed that intranasal liposomal-OXT significantly modulated the expression of genes associated with neuroinflammation and neuroprotection. Specifically:

- AIF-1, a marker of neurodegeneration derived from microglia and macrophages, showed reduced expression in the liposomal-OXT (IN) group, indicating decreased neuronal cell death.

- S100B, a calcium-binding protein secreted during astroglial injury, was downregulated in the liposomal-OXT (IN) group, suggesting reduced neuroinflammation.
- GFAP, an astrocytic marker linked to neuroinflammation and elevated in Alzheimer's disease (AD), was markedly reduced in the liposomal-OXT (IN) group compared to other treatments, aligning closely with levels in the naïve control group.
- BDNF, a neurotrophic factor essential for neuronal survival and synaptic plasticity, was significantly upregulated in the liposomal-OXT (IN) group, matching expression levels seen in healthy controls. This suggests restoration of cholinergic and antioxidant signaling disrupted in AD. Expression patterns across treatment groups followed the order:

- BDNF: SCP < OXT (IV) < Lipo-OXT (IV) < OXT (IN) < Lipo-OXT (IN) ≈ Naïve
- GFAP, S100B, AIF-1: SCP > OXT (IV) > Lipo-OXT (IV) > OXT (IN) > Lipo-OXT (IN) ≈ Naïve

These findings collectively demonstrate that intranasal liposomal-OXT offers superior therapeutic potential by closely mimicking gene expression profiles of healthy brains, effectively reducing neuroinflammation and enhancing neuroprotection in AD models.

Oxidative stress markers such as MDA, nitrite, and H_2O_2 are elevated in Alzheimer's disease (AD), indicating lipid peroxidation and impaired antioxidant defense. MDA levels reflect oxidative damage, while nitrite is a by-product of nitric oxide metabolism, both of which increase in AD. Conversely, GSH (glutathione), a key antioxidant, is reduced in AD, with lower levels correlating to higher susceptibility.

Brain homogenates from naïve mice, scopolamine (SCP)-treated AD models, and mice treated with free or liposomal OXT via intravenous (IV) and intranasal (IN) routes were analyzed. SCP-treated mice showed significantly elevated levels of MDA, nitrite, and H_2O_2 compared to controls. IV administration of OXT and liposomal-OXT also resulted in elevated oxidative markers, though to a lesser extent. In contrast, intranasal liposomal-OXT markedly reduced these markers, closely resembling levels in the naïve group.

The order of oxidative stress marker levels was: SCP > OXT (IV) > Lipo-OXT (IV) > OXT (IN) > Lipo-OXT (IN) ≈ Naïve

This reduction indicates that intranasal liposomal-OXT is more effective in mitigating AD-related oxidative stress and maintaining therapeutic concentrations in the brain.

GSH levels, inversely related to oxidative stress, followed the order: SCP < OXT (IV) < Lipo-OXT (IV) < OXT (IN) < Lipo-OXT (IN) < Naïve

Higher GSH levels in the intranasal liposomal-OXT group suggest enhanced antioxidant protection and better brain bioavailability compared to IV administration.

Together, the cognitive, gene expression, and biochemical findings confirm the superior efficacy of intranasal liposomal-OXT over plain OXT, demonstrating its ability to sustain therapeutic levels in the brain and effectively target AD pathology.

Further, the comparative difference in the effectiveness of the formulation suggested in the current study is indicated by the histological evaluation of hippocampal observations. The higher the neurons density, more effective was treatment. The density of neurons was found to be higher in naïve group followed by OXT loaded liposome formulation delivered via intranasal route, OXT delivered via intranasal route, OXT delivered via intravenous route, OXT loaded liposome formulation delivered via intravenous route and SCP treated group.

7.1.3.4.2 Vasopressin loaded liposomes

The Morris water maze test for vasopressin loaded liposomes clearly depicted about breakthrough enhancement in efficacy of liposome vasopressin when delivered via intranasal route. Liposome vasopressin when delivered via intranasal route exhibited 1.31 times efficacy when same formulation was administered via intravenous route. Similar results were observed for Vasopressin when delivered *via* intranasal route exhibited 1.30 times efficacy when same formulation administered *via* intravenous route. The lowest efficacy of vasopressin administered via intravenous route might be owing to possible degradation of vasopressin in nasal medium. The promising effect in case of liposome vasopressin when administered via intranasal route might be due to proper encapsulation of vasopressin in lipidic vesicles that prevent the physiological attack of nasal environment.

Gene expression analysis was conducted to evaluate the neuroprotective efficacy of vasopressin administered via different routes and formulations. Expression levels of key biomarkers BDNF, AIF-1, GFAP, and S100B were quantified across treatment groups,

including scopolamine-induced (SCP) models, naïve controls, and animals receiving free or liposomal vasopressin via intravenous (IV) and intranasal (IN) routes. Results demonstrated that BDNF expression increased progressively from SCP to liposomal-IN treatment, closely approximating naïve levels, indicating enhanced neurotrophic support. Conversely, AIF-1, GFAP, and S100B, markers of neuroinflammation and glial activation, showed a decreasing trend from SCP to liposomal-IN treatment, suggesting attenuation of neurodegenerative processes. These findings underscore the significance of administration route and formulation in modulating gene expression. Notably, intranasal delivery of liposomal vasopressin exhibited superior neuroprotective effects, supporting its potential for targeted nose-to-brain therapy in neurodegenerative conditions.

Biochemical profiling of brain homogenates was conducted across six groups: naïve, scopolamine (SCP)-treated, free vasopressin (administered intravenously and intranasally), and liposomal vasopressin (administered via both routes). SCP treatment significantly elevated oxidative stress markers—malondialdehyde (MDA), nitrite, and hydrogen peroxide (H_2O_2)—while reducing levels of glutathione (GSH), a key antioxidant.

Among all treatment groups, intranasal liposomal vasopressin demonstrated the most effective reduction in oxidative markers and the highest restoration of GSH levels, closely resembling the naïve control. The observed trends for oxidative stress markers were: SCP > Vaso (IV) > Lipo-Vaso (IV) > Vaso (IN) > Lipo-Vaso (IN) ≈ Naïve, while GSH levels followed the reverse order: SCP < Vaso (IV) < Lipo-Vaso (IV) < Vaso (IN) < Lipo-Vaso (IN) < Naïve.

These findings highlight the enhanced neuroprotective potential of intranasal liposomal vasopressin**,** supporting its application for targeted brain delivery in neurodegenerative conditions such as Alzheimer's disease.

7.1.4 AEEA-Oxytocin Conjugates for nose to brain delivery

7.1.4.1 Synthesis and Characterization of AEEA-Oxytocin

The AEEA-OXT was synthesised by solid phase sequential amino acid method which involved Manufacturing of AEEA-OXT-Stage-H-(01-10)-Resin followed by synthesis of AEEA-OXT. Following an initial transformation, the crude product was solubilized and subjected to a

cyclization reaction using iodine solution and subsequent purification to isolate final compound. The final step involved desalting of solution which achieved by introducing a 50% acetonitrile solution, containing 1% acetic acid in water, effectively removing any remaining salts and impurities. Ultimately, the purified solution was lyophilized, resulting in a dry form of AEEA-OXT.

The conjugation of AEEA with OXT was investigated using FT-IR spectroscopy in order to determine the C-C bond formation confirmation in AEEA-OXT. Spectral output was recorded by the transmittance as a function of wave number. Additional -C-O- stretching band (1050-1150 cm^{-1}) was observed compared to plain OXT IR spectra which confirmed the conjugation between OXT and AEEA in AEEA-OXT molecule.

Further conjugation of AEEA-OXT was confirmed by NMR. Twelve number of proton signal was found to be between 0.98-0.99 that represents the proton present in primary aliphatic carbon which confirms the isoleucine/leucine amino acid. Similarly, chemical shift from 1.19 to 1.61 and 1.62-3.77 represents number of protons present in aliphatic tertiary and secondary carbon respectively. Hence, 2 protons in tertiary and 22 protons in secondary carbon confirmed isoleucine/leucine and CH_2 for all amino acid respectively. Chemical shift from 3.81 to 4.88 represented the 8 protons present in chiral carbon, and 6.85 to 7.21 represented the 4 protons present in chiral carbon of tyrosine. Overall, the presence of protons confirmed the structure of amino acids of OXT. 2-(2-(2-Aminoethoxy) ethoxy) acetic acid was successfully conjugated with OXT which was confirmed by NMR spectroscopy.

2-(2-(2-Aminoethoxy) ethoxy) acetic acid was successfully conjugated with OXT and further confirmed by Mass spectroscopy. The total mass of AEEA-OXT was calculated 1151.509 that confirmed the conjugation of OXT with AEEA.

7.1.4.2 Evaluation on SH-SY5Y neuro cells

The cell viability study was conducted in SH-SY5Y cells employing MTT assay to investigate the biocompatibility of OXT and AEEA-OXT towards tested cells. OXT and AEEA-OXT, in 1µg/mL, 10 µg/ml and 100µg/ml concentration, incubated with SH-SY5Y neuro cells for 24, 48 and 72h was calculated. We found above 85% cell viability during increasing concentration at given time points (24hr, 48hr and 72hr) with OXT and AEEA-OXT. The conjugation of peptide with AEEA exhibited similar neuro protective potential compared to that seen with

plain OXT. In conclusion, we did not find significant reduction of cell viability during increasing concentration at incubated time points with OXT and AEEA-OXT. The outcome of current study suggests that OXT retained its compatibility on neuro cells even after being conjugated with AEEA and not have any impact on its neuro protective potential.

The quantitative cell uptake study of OXT and AEEA-OXT was carried out in SH-SY5Y cells to determine the internal localization proficiency. The data obtained of cell uptake evaluation study confirmed remarkably higher internal localization of OXT and AEEA-OXT after 3 h incubation. Maximum cell uptake was estimated to be 0.4 mg/mL that confirms the concentration dependent cellular uptake. Time-dependent cellular internalization of OXT and AEEA-OXT was demonstrated in the cell uptake investigation, and increasing the incubation period from 1 to 2 h significantly enhanced the amount of OXT that was taken up by the cells. Significant changes in cell uptake over time were observed when the incubation period was extended from 2 h to 3 h. In conclusion, time and concentration dependable cellular uptake was found for OXT and AEEA-OXT.

The effect of the OXT and AEEA-OXT on the SH-SY5Y cells was further evaluated by observing cellular morphology. After the treatment with pure OXT and its conjugation with AEEA (AEEA-OXT) for 48 h, the morphology of cells remained same as control and not detected any change compared to control under a phase contrast microscope. The comprehensive study examining qualitative uptake of C-6, C6+OXT and C-6+AEEA-OXT, was conducted using SH-SY5Y cells to evaluate the effectiveness of conjugation in internalization by the cells. The results from this meticulous cell uptake evaluation revealed a slightly higher internal localization of AEEA-OXT following an incubation period of three h. The findings serve to support the hypothesis regarding the cellular uptake of AEEA-OXT, as indicated by the persistent and vibrant fluorescence signals detected after three h of incubation with the C-6 + AEEA- OXT.

There was almost same emission of fluorescence of 100 µL/mL of OXT and AEEA-OXT in the reaction of generated ROS compared to control cells, which was indirectly correlated to biocompatibility and no effect on apoptosis cascade activation of the cells treated with pure drug formulations. The ROS analysis outcome suggested that OXT retained its biocompatibility on neuro cells even after being conjugated with OXT. AEEA- OXT also

showed the similar neuro protective potential as seen with plain OXT. Apoptosis was qualitatively characterized in SH-SY5Y cells after treatment with OXT and AEEA-OXT by DAPI and dual (AO/EB) staining technique. Normal SH-SY5Y cells have intact cell nuclei and were stained with faint fluorescence of DAPI. Cells in the control group were also alive and emitted green fluorescence in a circular pattern, and the nucleus was uniformly dispersed across the centre.

7.1.4.3 Evaluation of AEEA- OXT on monocytic cells (THP-I)

The study was intended to assess the effect of OXT and AEEA-OXT on the transcriptional pattern of inflammatory immune and apoptotic makers (NF-kβ, IL-6 Akt1, BCl, CD40, Bim, Bak, caspase-3, 8, 9). We observed a decrease in the levels of caspase-3 and caspase-8 in the hTHP-1 cells that were treated with OXT and AEEA-OXT. Caspase-9 expression on the contrary was seen higher with the treated group. The expression levels of the pro-apoptotic markers did not achieve statistical significance. It was noted that there was a decrease in the expression of the inflammatory markers NfKB and IL-6 in cells treated with all OXT and AEEA-OXT. Remarkably, the co-stimulatory markers (CD40) that regulates the programmed cell death and pro-apoptotic gene Bak was seen reduced with all formulations incubated with hTHP-1 cells, whereas Bim expression was seen higher in the cells which were treated with OXT formulations. OXT and AEEA-OXT treated hTHP-1 cells showed remarkable reduction in the expression of pro-inflammatory marker IL-6, as compared to the control.

The inactive transcription factor FOXO1 (phosphorylated FOXO1) work in tight regulation by the over-expression of co-stimulatory (CD40) mediating the signalling (PI3-Akt) pathway in hTHP-1 cells. Thus, using confocal microscopy, we examined the qualitative expression of FOXO1 in LPS-stimulated hTHP-1 cells. Following one hour of LPS stimulation, our results showed a significant difference in the qualitative expression of FOXO1 when comparing LPS-stimulated cells to those that were unstimulated. Additionally, for FOXO1, we estimated the fluorescence intensity of cells counted in different fields. Compared to the unstimulated control, our data demonstrated evident FOXO1 expression. The OXT and AEEA-OXT showed better results compared to LPS stimulated cells.

7.1.4.4 Neurological effects in experimental animals

Morris water maze (MWM) test was evaluated to compare the free OXT and AEEA-OXT administered via i.n. route against positive control (Naïve) and negative control group (SCP only). The mean time incurred for mice to reach on hidden platform was 37.16±6.75 seconds. Similarly, average time incurred for SCP mice to reach on hidden platform was 58.66±1.18 seconds. Mice administered with OXT via intranasal route were able to reach hidden platform in 39.58±4.45 seconds i.e., 1.07 and 0.67 times of placebo and SCP treated group. Mice administered with OXT conjugate administered *via* the intranasal route were able to reach hidden platform in 26.45±4.23 seconds i.e., 0.72 and 0.45 times of placebo and SCP treated group. This study clearly depicted about breakthrough enhancement in efficacy of OXT conjugate administered via the intranasal route. The memory of mice treated with AEEA-OXT *via* i.n was not only regained but also improved, and mice were found able to reach the hidden platform compared to placebo group.

Gene expression studies of OXT and AEEA-OXT treated animal brain homogenate samples were studied for AIF-1, S100B, BDNF, and GFAP analysis. The higher relative expression of BDNF, GFAP and S100b in AEEA-OXT administered through IN route and OXT given intranasally and intravenously treated group. A remarkable elevation in the expression of these markers was seen in the control (without AD) group. Whereas the experimental induced AD mice receiving intranasal treatment with the AEEA-OXT showed the increased expression of BDNF to that with the untouched control. Whereas BDNF expression was seen lower with other formulation and route of administration. More precisely, BDNF expression usually lower in AD patients in comparison to normal patients. Since the BDNF expression indicates the antioxidant and cholinergic transmission. Similar, pattern was observed in case of BDNF expression of AEEA-OXT and naïve mice brains. This means that AEEA-OXT is able to regulate BDNF expression in similar pattern to naive group. The pattern of BDNF expression was in order of AEEA-OXT (IN) = Naïve > OXT (IN) > OXT (IV) > SCP. The lower BDNF expression simulates to literature report of AD patients and higher BDNF expression simulates to literature report of normal patients. BDNF expression of AEEA-OXT group also simulates to naïve group that clearly indicates the efficiency of route and conjugated system.

Increased GFAP levels were correlated with increased amyloid β levels and declined cognition. This GFAP is present in astrocytes. GFAP primarily marks its elevated expression both in serum and CSF of AD patients. The similar fashion was observed in our studies also. GFAP levels were in order of SCP > OXT (IV) > OXT (IN) > AEEA-OXT (IN) = naïve. The decreased expression in CSF of AEEA-OXT group and naïve group clearly indicates that IN route and conjugation of OXT with AEEA showed efficacy and similarity in pattern to naïve group. OXT administered via IV route in not efficient much to diminish the SCP treatment. S100b is a neuroprotective factor i.e., governed by neuroinflammation and its expression tends to upregulate in AD patients and it is expressed lower in normal patients. Similar pattern was observed in current research. The levels of S100b was found to be in order of SCP > OXT (IV) > OXT (IN) > AEEA-OXT (IN) > Naïve group. This order clearly signifies the efficacy of conjugated OXT when administered via IN route. While OXT administered via IN route had higher expression this signifies diminished amount of OXT was delivered to brain and thus unable to treat.

AIF expression was correlated with neuronal cell death in cortex and hippocampal area of brain. The AIF expression was seen reduced with conjugated OXT administered intranasally similar to control. AIF expression was in order of SCP > OXT IV) > OXT (IN) > AEEA-OXT (IN) > Naïve. This is probably due to the protective sheath for OXT due to conjugation to protect OXT from physiological attacks of nasal environment and administration via IN route that clearly defines the efficacy of route and conjugation from above studies.

The brain homogenate biochemical studies were done for naiv̈e, SCP-treated, free OXT solution (i.n) and AEEA-OXT (i.n). MDA levels tends to rise in AD patients due to formation of peroxidation in brain and serum samples. MDA expression directly influences the oxidative stress in AD. Similarly, nitrite activity seems to be in similar fashion in AD patients as nitric oxide is one of the by-products. GSH expression is inversely proportion to oxidative stress. More GSH means patient is less prone to AD and lower the GSH patient is more prone to AD. H_2O_2 degrading activity is directly proportional to ROS. More the presence of H_2O_2 signifies the presence of oxidative stress due to inability of physiological system of brain to degrade it. And more accumulation leads to neuronal damage and neuroinflammation.

The prepared brain homogenate from naiv̈e, mice with experimental mice (by the SCP treatment), mice receiving free OXT through both intravenous and intranasal routes and intranasally treatment with AEEA-OXT were studied.

The SCP treatment showed higher secretion of MDA (lipid peroxidation biomarker), nitrite and peroxide activity levels and significantly comparative to naïve control group. However, OXT administered via IV route also showed the higher MDA, nitrite and peroxide activity levels in comparison to OXT administered via IN route in form of plain and AEEA-OXT. Conjugated AEEA-OXT upon IN administration reduced the MDA, nitrite and peroxide activity levels similar to naïve control. Summarizing, levels of MDA, nitrite and peroxide activity were in order of: SCP > OXT (IV) > OXT (IN) > AEEA-OXT (IN) ≥ Naïve group.

The reduced levels in treated group signifies that, OXT administered in conjugation and via IN route is more effective to deduce AD related biochemical parameters. Apart from that conjugated OXT tends to be present in therapeutic concentrations in brain when compared to with plain OXT administered via both IN and IV route.

The antioxidant GSH levels were in order of: SCP < OXT (IV) < OXT (IN) < AEEA-OXT (IN) < Naïve group. The higher level in conjugated OXT signifies that more OXT is able to reach in brain and tends to sustain in biological environment of brain in therapeutic levels. Apart from that, plain OXT injected through intravenous and intranasal route showed the protective effect by controlling GSH activity but not in significance to AEEA-OXT group.

The histological assessment of hippocampal observations indicates the relative differences in the effectiveness of the formulation suggested in the present study. The density of neurons was found to be higher in naïve group followed by AEEA-OXT delivered via i.n. route, OXT delivered via i.n. route, SCP treated group indicating the neuroprotective efficacy of AEEA-OXT.

7.2 Conclusions

The present research endeavour was initiated to thoroughly investigate and resolve the existing gaps and shortcomings in injectable dosage forms, especially in case of peptide therapeutics, with a particular focus on enhancing therapeutic outcomes through the utilization of a carrier approach. This initiative aimed to effectively reach and target the specific site of action

required for optimal therapeutic intervention. To achieve this goal, the studies were diligently planned and executed in a systematic manner, ensuring that all crucial aspects and dimensions of the research were comprehensively covered. Below, the key outcomes derived from this extensive planning are articulated in detail.

The field of analytics was recognized as a fundamental requirement for measuring and determining the quantitative levels of drugs in a variety of investigative studies. Therefore, robust analytical methods were developed for OXT, vasopressin, and angiotensin. Furthermore, an analysis of the challenges encountered during the dilution process, along with the related stability concerns associated with ready-to-infuse dosage forms, has led to the successful design, development, and optimization of tailored RTI (ready-to-infuse) delivery systems for OXT, vasopressin, and angiotensin-II. These innovative systems were developed specifically to tackle the unique challenges posed by the selected peptides. The RTI formulation technique developed represents a ground breaking combination of composition and process, aimed at producing injections of sensitive molecules that contain low concentrations of active ingredients. This advancement not only enhances patient adherence to treatment protocols but also significantly improves safety outcomes. The paramount importance of safety in RTI systems is underscored by their distinctive characteristics that minimize administration errors and reduce the risk of cross-contamination.

In the next segment, the focus shifts to the promising area of nose-to-brain delivery for OXT and vasopressin. Innovative formulation strategies employed during this phase of the research utilized novel components, including phospholipids and strategically designed carriers, to significantly enhance the permeation of these drugs into the brain. This methodology is particularly noteworthy as it incorporates lipid-based liposomes specifically targeting conditions such as Alzheimer's disease. A thorough review of the background literature reveals that while a considerable volume of work has already been accomplished in this research area, there remains a pressing need for further advancement to fulfil the unmet expectations related to the two specified drugs, OXT and vasopressin. Consequently, after setting varied and diverse objectives across different research dimensions, the studies were executed in a systematic fashion.

In addition, the extended research incorporated focused efforts to address the identified deficiencies in OXT delivery through the application of a conjugation strategy aimed explicitly at nose-to-brain delivery. This innovative conjugation approach was designed to enhance the efficiency of the drug's permeation into the brain, thereby improving its therapeutic potential.

Conclusively, we can assert with confidence that the developed RTI formulation, alongside the innovative liposome and synthesized AEEA-OXT-based delivery systems, hold the promise of transforming the behaviour of these molecules by manipulating their diverse physicochemical and biological properties through strategic design methods. Such advancements are poised to enhance the transport characteristics of the drugs, which will ultimately translate into improved efficacy across various investigational models, with safety being concurrently emphasized. However, there is a clear demand for further in-depth studies, particularly focusing on two significant aspects: the industrial considerations regarding process scale-up and the establishment of standardization parameters paired with clinically oriented research. Collectively, the findings of this work, augmented by such studies, have the potential to provide substantive value to the therapeutic arsenal available to practicing clinicians.