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Enhancement in brain uptake of Vitamin D_3 nanoemulsion for treatment of cerebral ischemia: Formulation, Gamma scintigraphy and efficacy study in transient middle cerebral artery occlusion rat models

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ABSTRACT

Aim: For the treatment of cerebral ischemia, Vitamin-D_3 loaded nanoemulsion were developed.

Method: Tween 20 and polyethylene glycol were choosen as surfactant/co-surfactant, while oleic acid as oil phase. The formulation was characterized for various *in-vitro* parameters. Targeting efficiency was investigated through radiometry, gamma scintigraphy and efficacy was studied in transient middle cerebral artery occlusion (MCAo) rat model.

Result: Vitamin D_3-Nanoemulsion showed a mean size range of 49.29±10.28 nm with polydispersity index 0.17±0.04 and zeta potential 13.77mV. Formulation was found stable during thermodynamic stability study and permeated within 180 min through sheep nasal mucosa (permeation coefficient 7.873±0.884 cm/h). Gamma scintigraphy and radiometry assay confirmed better percentage deposition (2.53±0.17 %) of ^{99m}Tc-Vitamin D_3-Nanoemulsion through nasal route compared to IV administered ^{99m}Tc-Vitamin D_3 solution (0.79±0.03%). Magnetic Resonance Imaging (MRI) of the ischemic model confirmed better efficacy of Vitamin D_3-Nanoemulsion.

Conclusion: This work demonstrated better permeation, deposition and efficacy of VitaminD_3-Nanoemulsion through intranasal route.

KEYWORDS: Gamma scintigraphy, Ischemia, MRI, Nanoemulsion, Vitamin D_3

Introduction

Cerebral ischemia is considered to be one of the major reasons for 85% of all cases of neurological disorders (Lakhan *et al.* 2009, Benjamin *et al*. 2017). Several reports have also shown that numerous brain-related disorder occurs at high altitude (Basnyat *et al*. 2001). Among those disorders, cerebral ischemia remains to be the most prevalent. People residing at higher altitudes for longer periods are more susceptible to ischemia, as hypobaric hypoxia can incite an increase in the concentration and viscosity of haemoglobin, leading to insufficient blood flow (Brand-williams *et al*. 1995). Cerebral ischemia develops from the obstruction of the blood vessels in the regions of brain, minimizing the supply of blood and oxygen to the brain cells. This leads to a condition called hypo perfusion which could induce cell death (Hossmann 1994, Garcia 1996). Many studies reported that the condition of hypo perfusion stimulates the production of free radicals and also shows increase in the level of pro-inflammatory cytokines. Specifically, there is an increase in a transcription factor called nuclear factor kappa-B (NF-κB), responsible for the death of nerve cells (Ridder *et al*. 2009).

NF-κB, a transcription protein complex majorly found in the neuro-system, plays an essential role in inflammatory reactions and in controlling the apoptosis and cell division processes. This factor involved in cerebral ischemia can get activated through several stimuli such as pro-inflammatory cytokines, reactive oxygen species, etc. (Mattson *et al*. 2001). It is normally found in the cytoplasm along with an inhibitory protein, IκB. Under normal conditions, IκB binds to the transcription complex and prevents its activation. But upon stimulation, IκB gets converted to IκB kinase via phosphorylation and the components of NFkB get migrated to the nucleus. This stimulates the production of cytokines and reactive oxygen species (ROS) augments, that disrupt the integrity of blood-brain barrier (BBB) and eventually promotes apoptosis of neuronal cells (Baldwin *et al*. 1996, Camandola *et al*. 2007, Ridder *et al*. 2009).

Over the past years, low levels of Vitamin D have been identified as a risk factor for cerebrovascular diseases. Reported studies imply evidentiary support that its lower level promotes ischemic stroke (Majumdar *et al*. 2015, Gupta *et al*. 2016, Lowe *et al*. 2017, Markisic *et al*. 2017, Wei and Kuang 2018). Other than being involved in bone metabolism, Vitamin D also plays an essential role in neurodevelopment, acting as a neuroprotectant (Gupta *et al*. 2016, Lowe *et al*. 2017, Zhou *et al*. 2018). As per several reports, Vitamin D is known to increase the levels of NF-κBin cytoplasm, inhibiting nuclear migration (Liu *et al*. 2018, Yu *et al*. 2019). Vitamin D receptors are directly involved in this

process as it expresses low IκB levels, leading to increased migration of NF-κB to the nucleus, making the cells without receptors more susceptible to inflammatory reactions (Cohen-Lahav *et al*. 2006, Liu *et al*. 2018). Vitamin D has significantly shown an increase in the levels of IκB, by stabilising the IκB-mRNAs and reducing the phosphorylation. Decreased levels of NF-κB result in the reduction of the pro-inflammatory factors. Therefore, targeting NF-kB can minimize ischemic damage (Brea *et al*. 2009).

The application of nanoemulsions has recently emerged as a successful drug delivery system. Nanoemulsions are a homogenous mixture of small particles dispersed in an aqueous medium (Li J *et al* 2018, Silva *et al*. 2011, McClements 2012). The nano-size implies to be the only reason that supports its stability for a longer period (Maali and Mosavian 2012). McClements (2012) has developed this delivery system using nanoemulsions for improving the bioavailability of biologically active compounds like Vitamin D to the targeted areas (Guttoff *et al*. 2015, Gupta *et al*. 2018). Evidence suggests that the intranasal delivery system has been the most effective in brain targeting, allowing the drug to cross the Blood-Brain Barrier via the nerves of the nasal cavity. The nanocarriers interact with the mucosal layer of the nose and release the drug, which is further taken up by the nerve cells and ultimately reaches to the brain (Bourganis *et al*. 2018, Alexander *et al*. 2019). The primary objective of this study is to utilize the applications of nanocarriers for the treatment of cerebral ischemia through successful brain delivery of Vitamin D. Additionally, the nose to brain targeting efficiency and therapeutic efficacy of prepared delivery systems in ischemic rat model will be examined.

Materials and Methods

Material

Vitamin D_3 and Oleic acid were purchased from Sigma-Aldrich (St. Louis, MO). Tween 20 and polyethylene glycol was purchased from Hi media Laboratory Pvt. Ltd. (Mumbai, India). Technetium-99m ($_{99m}Tc$) for radiolabeling was procured from Bhabha atomic research centre, Mumbai, India and stannous chloride ($SnCl_2$) was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used were of analytical grade. Double distilled water was used throughout the study to prepared all solutions and formulations.

Assessment of solubility of Vitamin D_3 in various oils, surfactants and co-surfactant

The solubility of Vitamin D_3 in different oils surfactant and co-surfactant were determined by the shake flask method (Patel *et al*. 2013). Briefly, an excess amount of Vitamin D_3 was dissolved in 1 mL of different oil, surfactant and co-surfactant in eppendorf. The solution was mixed well by periodic vortexing on vortex mixer (Remi, Mumbai, India) and followed by sonication on bath sonicator (PCI Analytics, Thane, India) till 24 h. The solution was then centrifuged (Remi, Mumbai, India) at 10,000 g for 10 min to settle undissolved drug followed by filtration of supernatant using membrane filter 0.45 µm (SS Filters, Mumbai, India) carefully. The amount of drug dissolved in each excipient was determined by UV-Vis spectrophotometer (Shimadzu UV-1700, Kyoto, Japan) at λ_{max} of 264 nm.

Pseudo-ternary phase diagram study

Vitamin D_3-Nanoemulsion was prepared using the method published in the previous report (Thakkar *et al*. 2015). On the basis of solubility oleic acid was selected as an oil phase. Tween 20 and polyethylene glycol were selected as surfactant and co-surfactant respectively. Phosphate buffer (pH 6.2) was used as aqueous media for the construction of phase diagram. Surfactant and co-surfactant (S_{mix}) were mixed in different ratio (1:1, 1:1.5, 1:2, 2:1 and 1.5:1). For each S_{mix} ratio, formulations were prepared by taking the different ratios of oil and S_{mix} phase (ranging from 1:9 to 9:1) to delineate the boundaries of phases precisely formed in the phase diagrams. Finally required quantity of aqueous phase was added slowly followed by vortexing (Remi, Mumbai, India) and sonication (PCI Analytics, Thane, India). Prepared formulations were observed for single-phase, transparency and flowability and marked on pseudo three-component phase diagram with representing oil, S_{mix} and aqueous phase on three-axis using CHEMIX- Ternary diagrams software. From each phase diagram constructed, the final formulation was selected from the nanoemulsion region for the addition of the drug. The selection criteria were: quantity of oil phase to dissolve one dose of drug, minimum S_{mix} concentration, stability during heat cooling cycle and freeze cycle.

Droplet Size, polydispersity index and zeta potential

The mean droplet size and polydispersity index of Vitamin D_3-Nanoemulsion were determined using dynamic light scattering Zetasizer 1000 HS (Malvern Instruments, Malvern, UK). Sample (0.1 mL) was dispersed in 50 mL distilled water. Droplet size was estimated at 25°C temperature and 90^o

scattering angle. The surface charge on the prepared nanoformulation was determined by Zetasizer using the same dilution.

Transmission electron microscopy

Morphology of Vitamin D_3-Nanoemulsion was determined using transmission electron microscopy (TOPCON 002B, Tokyo, Japan). The sample was diluted 10-times with water and the diluted drop was deposited on the holey copper grid followed by treatment with a drop of 2% (w/v) phosphotungstic acid. The sample was allowed to dry at room temperature after removing excess liquid using blotting paper sheets. Images were captured under high-voltage electricity of 200 kV.

***Ex-vivo* permeation study**

Ex-vivo permeation study of prepared Vitamin D_3-Nanoemulsion and Vitamin D_3 solution in oleic acid was done using Franz diffusion cell (Hanson Research Corporation, CA, USA) (Pangeni *et al*. 2014). Sheep nasal mucosa was obtained from a local slaughter house and used as a permeation layer. Nasal mucosa was immediately stored in phosphate buffer (pH 6.8) and used within 2 h of the experiment. The mucosa was mounted on the Franz cell (area: 1.77 cm^2) using nylon thread in the manner to keep the mucosal side towards the donor compartment. Both donor and receiver compartment was filled with phosphate buffer (pH 6.8) and allowed to equilibrate at 37.0±0.5^oC. After 15 min media of the donor compartment was replaced with either nanoemulsion or solution equivalent to Vitamin D_3. The media was stirred at 50 rpm and aliquots of 1 mL were withdrawal at a predetermined time interval up to 6 h. Sink condition was maintained by adding the equal quantity of fresh media in the receptor compartment. The drug concentration in samples was estimated using a UV-Visible spectrophotometer (Shimadzu UV-1700).

Further, the permeation coefficient (p) was calculated by putting the data in following equation.

$$P = dq/dt/C_oA$$

Where dq/dt is the drug flux, C_o is the initial drug concentration in donor compartment and A is the surface area of the nasal mucosa.

Biodistribution and brain targeting study

Radiolabeling of Vitamin D_3

Radiolabeling of Vitamin D_3 was performed as per our previous publication using ^{99m}Tc-pertechnetate as labelling material (Sharma *et al*. 2018). Briefly, 5 mg drug was suspended in the labelling media containing mixture of 1 mL stannous chloride and 2% v/v ethanol aqueous solution, 2.5 mL sodium chloride (0.9% w/v), 2.5 mL sodium acetate (100 mg/mL) and ^{99m}Tc-Pertechnetate (of about 20 microcurie activity). The mixture was vortexed for about 10 min and the drug was separated by centrifugation followed by washing with sterile distilled water and freeze-dried.

Formulation of ^{99m}Tc labelled Vitamin D_3-Nanoemulsion

The complex of ^{99m}Tc-Vitamin D_3 was used to prepare drug-loaded nanoformulation using the same method discussed above. ^{99m}Tc-Vitamin D_3-Nanoemulsion complex was stored in lead pot till further use. For comparative study, a separate batch of conventional ^{99m}Tc-Vitamin D_3 formulation was prepared by dispersing required quantity of radiolabeled drug into sterile water for injection and administration through Intravenous route (IV) route.

Drug administration

Sprague Dawley rats (240–260 g, either gender) were obtained from the Animal Experimental Facility of Institute of Nuclear Medicine & Allied Sciences, New Delhi and were housed in polypropylene cages (38 x 23 x 10 cm) with not more than 4 animals per cage. The animals were maintained under standard laboratory conditions with natural dark-light cycle (14±60 min light; 10±60 in dark; mention temperature). They were allowed free access to standard dry rat diet (Aashirwad Industries, Chandigarh, India) and tap water *ad libitum*. All experimental procedures were reviewed and approved by the Institutional Animal Ethics Committee (536/IAEC/13).

Animals were divided into groups with 6 animal (n=6) in eachgroup. Animals were anesthetized by injecting sodium pentobarbital (9.1 mg/Kg) intraperitoneally, and anesthesia was maintained by injecting isoflurane when required. Subsequently, ^{99m}Tc-Vitamin D_3-Nanoemulsion (equivalent to 100 ng/kg Vitamin D_3, 0.01 mL) was administered in each nostril using low-density polyethylene attached tubing in micropipette whose internal diameter was 0.1 mm at the delivery site. Similarly, an oil solution of ^{99m}Tc-Vitamin D_3 (prepared by dissolving required quantity of 100 ng/kg Vitamin D_3 in

oleic acid) was administered (0.05 mL) through IV route into the animals tail vein. All the animals were positioned in supine during the course of study.

Radiometry biodistribution

Biodistribution study of ^{99m}Tc-Vitamin D_3-Nanoemulsion and ^{99m}Tc-Vitamin D_3 oil solution was performed using a radiometry method with little modifications (Md *et al*. 2013). The animals of each group were sacrificed at different time intervals (1, 2, 4 and 24 h), abdominal cavity was exposed by 3 cm ventral midline incision and different organs (brain, liver, kidney, spleen and heart) were collected. Organs were washed twice using saline and weight was recorded. Radioactivity quantification in every organ was done using well-type gamma scintillation counter (Capintec, New Jersey, USA). The following equation was used to measure the uptake of ^{99m}Tc labelled Vitamin D_3 in each organ.

% Radioactivity/g of organ = (Radioactivity count/weight of organ x total count injected) x100

Brain targeting by Gamma Scintigraphy

Gamma scintigraphy using dual head gamma camera (Symbia T2; Siemens, Erlangen, Germany) was performed to determine targeted delivery of ^{99m}Tc-Vitamin D_3-Nanoemulsion and conventional radiolabeled Vitamin D formulation in the brain. Gamma camera was equipped with 36 cm field of view and low energy parallel collimator. ^{99m}Tc-Vitamin D_3-Nanoemulsion and ^{99m}Tc-Vitamin D_3 formulations were administered to the rat through nasal and IV route respectively using same protocol as discussed in biodistribution study. Static images (60 sec/image) were captured under gamma camera (Siemens gamma camera) after 2 h of drug administration.

Middle cerebral artery occlusion (MCAo) model for cerebral ischemia

Animals were kept on fasting overnight before the initiation of the experiment. Animals were scrutinized and anesthetized by injecting sodium pentobarbital (9.1 mg/Kg) intraperitoneally. A midline incision was made in order to expose the external, internal and common carotid artery. The middle cerebral artery was occluded with the help of monofilament nylon thread (3.0, Ethicon, Jonson & Jonson) and was allowed to remain in that state for 2 h. This, in turn, occludes the artery and for reperfusion, it is gently retracted. Scoring in terms of the neurological deficit was done in control and treatment groups for a period of 24 h on a scale of 0-5. Scale 0 depicts no neurological deficits, scale 1 refers to the failure to extend left fore paw to its fullest extent, scale 2 refers to circling to the left,

scale 3 refers to paresis to the left, scale 4 refers to the inability to spontaneous walking and scale 5 refers to the mortality/death.

Magnetic resonance imaging (MRI) study: The imaging protocol was carried out on an animal MRI (7.0-tesla scanner, Bruker MRI GmbH Biospec 70/20 US). The instrument has a 72 mm transit polarized coil containing only ^{1}H with rat brain surface coil. The ^{1}H polarized coil containing the capabilities of receiving only. The ischemic region was identified and the diffusion-weighted images were acquired.

A 10% homogenate of brain tissue was prepared in ice-cold 0.1M phosphate buffer (pH 7.4) and levels of superoxide dismutase (SOD) (Marklund *et al*. 1974), malondialdehyde (MDA) (Ohkawa *et al*. 1979), glutathione peroxidase (GSH) (Ellman 1959) and catalase (CAT), were estimated using the standard protocol in earlier publication (Gupta *et al*. 2017, Punj *et al*. 2017).

Malondialdehyde level: Lipid peroxidation marker, MDA was measured as per the reported method (Ohkawa *et al*. 1979). For estimation of MDA, 0.1 mL processed tissues was added to 1.5 mL of 20% acetic acid, 1.5 mL of 0.8% thiobarbituric acid and 0.2 mL of 8.1% sodium dodecyl sulphate and then it was vortexed gently. The mixture was then heated on water bath at 100^{o}C for 60 min. After cooling, 5 mL of n-butanol-pyridine (15:1) and 1 mL of distilled water were added. Thereafter, the mixture was centrifuged at 4000 rpm for 10 min to separate the organic layer. The absorbance was read at 532 nm and the concentration was expressed in nM/mg of protein.

Statistical Analysis

The data collected was compiled in an Excel sheet and analysed using Statistical Package for the Social Sciences (SPSS) version 22 software. Analysed data was expressed as Mean value±Standard deviation. One way analysis of variance (ANOVA) was applied to determine the significant difference between the data using Sigmastat 2.03 software (SPSS, Inc, Chicago, IL). The level of significance for this study was 5% with a value of <0.05.

Result and Discussion

The decisive factor for the selection of different phases of thermodynamically stable nanoemulsion formulation was that all the ingredients should be pharmaceutically acceptable and fall under the category of generally regarded as safe. On the basis of solubility of Vitamin D_3, oleic acid was selected as oil phase. Tween 20 and polyethylene glycol were selected as surfactant and co-surfactant respectively. Regions of o/w nanoemulsion was determined using pseudo-ternary phase diagram which was prepared by plotting concentration of oleic acid, surfactant/co-surfactant and the water phase at three different axes (**Figure 1)**. In diagram unshaded regions represent for o/w nanoemulsion while the shaded region signifies coarse emulsion. Obtained pseudo-ternary phase diagram indicates that the nanoemulsion region gradually increased by increasing the S_{mix} ratio in the formulations. It may be due to the reason that higher concentration of co-surfactant (polyethylene glycol) in S_{mix} may accommodate a large volume of oil (oleic acid) for the formation of nanoemulsion. Obtained data was found to be in accordance to the finding of Shafiq et al. (2007), which demonstrated that the solubilization of a large volume of oil can be accomodate using mixture of co-surfactant and surfactant, compare to the condition when only surfactant is used. According to Lawrence et al. (2000), this may be attributed to the fact that the higher concentration of co-surfactant is essential to greater penetration of the oil phase in the hydrophobic region of the surfactant monomers thereby further decreasing the interfacial tension and formation of nanoemulsion (Lawrence *et al*. 2000, Shi *et al*. 2015). Further, from each S_{mix} group few formulations were selected on the basis of the quantity of oil phase to dissolve at least one dose of Vitamin D_3 with minimum concentration of S_{mix}. These formulations were further subjected to the thermodynamic stability tests such as freeze thaw cycle (in deep freezer at -20°C for 24 h) and heating-cooling cycle (six cycles between 4°C and 40°C for 48 h) to observe whether the formulations were showing any signs of phase separation, creaming or cracking with the variation of temperature'. The formulations which showed maximum stability (no phase separation, creaming or cracking) were returned to its original state quickly at room temperature and all instable formulations were excluded. Among the various S_{mix} and oil to S_{mix} ratios employed in the pseudo-ternary phase diagram study, nanoemulsion formulation with surfactant to co-surfactant at a ratio of 1:2 and oil to S_{mix} at a ratio of 1:5 which is thermodynamically stable was chosen as the product candidate for further studies.

Droplet size, polydispersity index and zeta potential

Mean droplet size and zeta potential of Vitamin D_3-Nanoemulsion were found to be 49.29±10.28 nm (Polydispersity index 0.17±0.04) and 13.77±2.77 mV respectively. **Higher and rapid transport of drug carriers is related to the droplet size for transcellular transport through olfactory neurons to brain.** The critical requirement to cross the blood-brain barrier is the droplet size which should be lesser than 100 nm (Musa *et al*. 2013). In this regard mean droplet size of Vitamin D_3-Nanoemulsion (9.29±1.28 nm) was found to be suitable for targeted brain delivery. The value of polydispersity index indicates the droplet size uniformity. The higher the value of the polydispersity index, the lower the droplet size uniformity (Abdou *et al*. 2017). In our finding polydispersity index values indicates narrow size distribution. Similarly, the physical stability of nanoemulsion can be correlated by zeta potential. Zeta potential should greater or lesser than +20 mV or -20 mV respectively to achieve stable nanoemulsion otherwise droplets may undergo Ostwald ripening which may lead to aggregation and instability (Araujo *et al*. 2011). The obtained value of zeta potential indicates that the prepared Vitamin D_3-Nanoemulsion possess physical stability which could be due to the repulsion of droplets. Previous reports also suggested that the presence of oleic acid may also lead to stability of nanoemulsion. Therefore, it can be concluded that the oleic acid in prepared formulation would be acting as stabilizer and preventing droplet aggregation (Tran *et al*. 2014).

Transmission electron microscopy

The Transmission electron microscopy (TEM) image of drug-loaded nanoemulsion is depicted in **Figure 2a**. The obtained TEM image signifies that the droplets were spherical in shape with low size variation. The image also indicates that the droplet size was in agreement with the result obtained from the measurement of droplet size using zetasizer.

***Ex-vivo* permeation study**

Ex-vivo profile of Vitamin D_3 from the nanoemulsion and solution is represented in **Figure 2b**. Data indicates that drug was rapidly permeated in the form of nanoemulsion through sheep nasal whereas the permeation rate was slower with drug solution. The approximately complete drug was permeated in the form of nanoemulsion at 180 min, whereas only 28.37±2.64% drug was permeated in form of solution at the same time point. Obtained data indicates that the drug permeation through nasal mucosa was significantly higher ($p<0.05$) in case of nanoemulsion formulation. Vitamin D_3-Nanoemulsion

exhibited higher permeation coefficient (7.873±0.884 cm/h) as compared to drug solution (0.084±0.007 cm/h) at 360 min time course of study. A possible reason for higher permeation in the case of Vitamin D_3-Nanoemulsion could have been attributed due to the smaller particle size which causes larger surface area and thus favours permeation through nasal mucosa. Because of smaller droplets, the drug in nanoemulsion transports easily through various endocytic pathways of sustentacular or neuronal cells in the olfactory membrane (Abdou *et al*. 2017). Additionally, the presence of surfactant may also have contributed to enhanced permeation of nanoemulsion droplets verifying the reports from the previous study (Pangeni *et al*. 2014). According to the theory surfactant and co-surfactant acting as solubilizing agent and permeation enhancer increases overall concentration of drugs in receiver compartment. Surfactants chemically interacts with constituents of biological layer to promote drug flux and thus drug availability. Surfactant exhibits their action by any of the methods like (a) biological layer denaturation (b) binding of biological layer protein (c) solubilisation or disorganization of intracellular lipid (d) penetration through epidermal lipid barrier (e) Interaction with living cells (Pangeni *et al*. 2014).

Biodistribution and brain targeting of ^{99m}Tc-Vitamin D_3-Nanoemulsion

Biodistribution of ^{99m}Tc-Vitamin D_3-Nanoemulsion and ^{99m}Tc-Vitamin D_3 solution was performed following intranasal and IV administration respectively on Sprague Dawley rats. Data of percentage radioactivity in different organs at different time intervals are depicted in **Figure 3a**. Data indicates that brain deposition of ^{99m}Tc-Vitamin D_3-Nanoemulsion through intranasal route was significantly higher at all-time points in compared to the condition when ^{99m}Tc-Vitamin D_3 solution administered through IV route. Percentage radioactivity (correspond to Vitamin D_3) was found to be maximum (2.53±0.17 %) at 4 h post-administration of ^{99m}Tc-Vitamin D_3-Nanoemulsion, while it was only 0.79±0.03 % for ^{99m}Tc-Vitamin D_3 solution administered through IV route at the same time. About four-fold increased in brain deposition of ^{99m}Tc-Vitamin D_3-Nanoemulsion confirm the superiority of intra nasal route. Moreover, a large amount of radioactivity was observed in other organs (liver, kidney, spleen and heart) through IV route which may be due to the rapid absorption and thus biodistribution. However, in all the cases decrease in the deposited radioactivity was noted at 24 h due to the elimination of radioactive complex from the body.

Findings from the biodistribution study are in good agreement with that previously reported for biodistribution of astaxanthin and risperidone (Chandra *et al*. 2016, Kumar *et al*. 2008). The result of

biodistribution study was well explained by previous reports (Illum 2003, Vyas*et al*. 2005). Low brain uptake of ^{99m}Tc-Vitamin D_3 solution was due to the blood-brain barrier which is highly selective semi permeable border circulating blood and brain cells. This barrier allows the passage of some essential molecules into brain cells through passive diffusion. Olfactory passage through trigeminal nerve may allow the transport of molecules of limited size range. Our finding of high brain uptake of ^{99m}Tc-Vitamin D_3-Nanoemulsion through nasal route may be due the size specific passage of nano emulsion from olfactory route. It was also observed that time required for maximum brain deposition of radioactivity was less (2 h) in intranasal route while it was 4h in case of IV route. The reason behind above finding may be the transport of ^{99m}Tc-Vitamin D_3-Nanoemulsion into brain through the gap between trigeminal nerve of olfactory route whereas IV administration of ^{99m}Tc-Vitamin D_3 first needs to absorbed systemically and later brain deposition with least quantity.

Visualization of brain targeting of ^{99m}Tc-Vitamin D_3-Nanoemulsion and ^{99m}Tc-Vitamin D_3 solution through intranasal and IV route was performed using gamma scintigraphy 2 h post-drug administration. Obtained gamma scintigraphic images (**Figure 3b**) clearly shows highest brain deposition of ^{99m}Tc-Vitamin D_3 when delivered as Nanoemulsion formulation. In addition to this, ^{99m}Tc-Vitamin D_3 nanoformulation was found least distributed in peripheral tissue and other organs. Whereas in case of IV administration of ^{99m}Tc-Vitamin D_3 radioactive complex was distributed in peripheral organs (maximum uptake by liver followed by kidney, spleen and heart). As expected, very low amount of ^{99m}Tc-Vitamin D_3 was available in the brain through IV route. Observation of non-invasive, real-time gamma images were found in line confirming the finding of biodistribution study using radiometry which confirmed transport of nano-sized droplets through olfactory route resulting an increase in the drug concentration into the brain.

Efficacy Study of Vitamin D_3-Nanoemulsion

While performing MCAo model, out of total of 18 rats, only 12 rats were analysed for MRI and antioxidant assay. 4 rats died and 2 rats were excluded from the study. The exclusion criteria were excessive bleeding while performing surgery, exceeding operation time, or no infraction present. The neurological deficit scoring was also performed and we noted that there is minimal evidence of neurological deficits in vitamin D_3 nanoemulsion group. The MRI studies assessed that the ipsilateral hemisphere apparent diffusion coefficient (ADC) value was significantly lower than the normal contra lateral hemisphere at 30 min post-reperfusion. This implicates the restricted cellular movement of

water and/or progression of tissue injury in middle cerebral artery region and thereby developing ischemic conditions. We noted down the significant difference in ADC (right/left) ratio between groups, vitamin D_3 conventional and vitamin D_3 nanoemulsion. This prediction is based on one way ANOVA and we investigated significant improvement in vitamin D_3 nanoemulsion treated-group with ADC ratio considered near to 1. MRI images also predict the increase in signal intensity of ipsilateral hemisphere as compared to contra lateral hemisphere at 30 min post-reperfusion.

The antioxidant assay was performed to quantify the oxidant stress in the ischemic region. There was a significant difference in the brain MDA and GSH levels among both the groups. One way ANOVA with tukey post hoc analysis predicts a significant increase in MDA ($p<0.01$) and significant ($p<0.05$) decrease in GSH level in vitamin D_3 nanoemulsion treated group. Similarly the SOD and catalase content also improved in case of nanoemulsion group. During oxidative stress, an imbalance between the antioxidant enzyme defence system and ROS production occurs that gives rise to ischemic or cerebral infraction state. The findings are in agreement with previous reports of Milanlioglu *et al*. 2015 which stated that the poor neurological status was associated with elevated serum MDA level and decreased SOD, GSH and catalase activities. The pre-treatment with nanoemulsion is recommended at higher altitudes to prevent ischemia to develop. This hypothesis was in concordance to the findings of study in which MRI images predict the imbalance in water and ion homeostasis and further aggravates due to reperfusion (**Figure 4**).

Conclusion

In this study nanoemulsion formulation has been prepared for effective delivery of vitamin D into brain. The present study confirm prepared nanoemulsion formulation was of desired size range and thermodynamically stable. The gamma scintigraphy study confirmed that prepared delivery system allowed the maximum availability of radiolabelled drug in brain. Application of vitamin D_3 nanoemulsion highlights the clinical importance of formulation as a neuroprotectant in the cerebral ischemic MCAo model. It provides pre-treatment effect and this may be attributed due to its antioxidant properties. Further studies are warranted to explore the role of Vitamin D_3 as prophylaxis or therapeutic drug formulation to prevent or treat cerebral ischemia and to take this approach to the clinical level.

Conflict of interest: "No potential conflict of interest was reported by the authors''

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Figure Captions

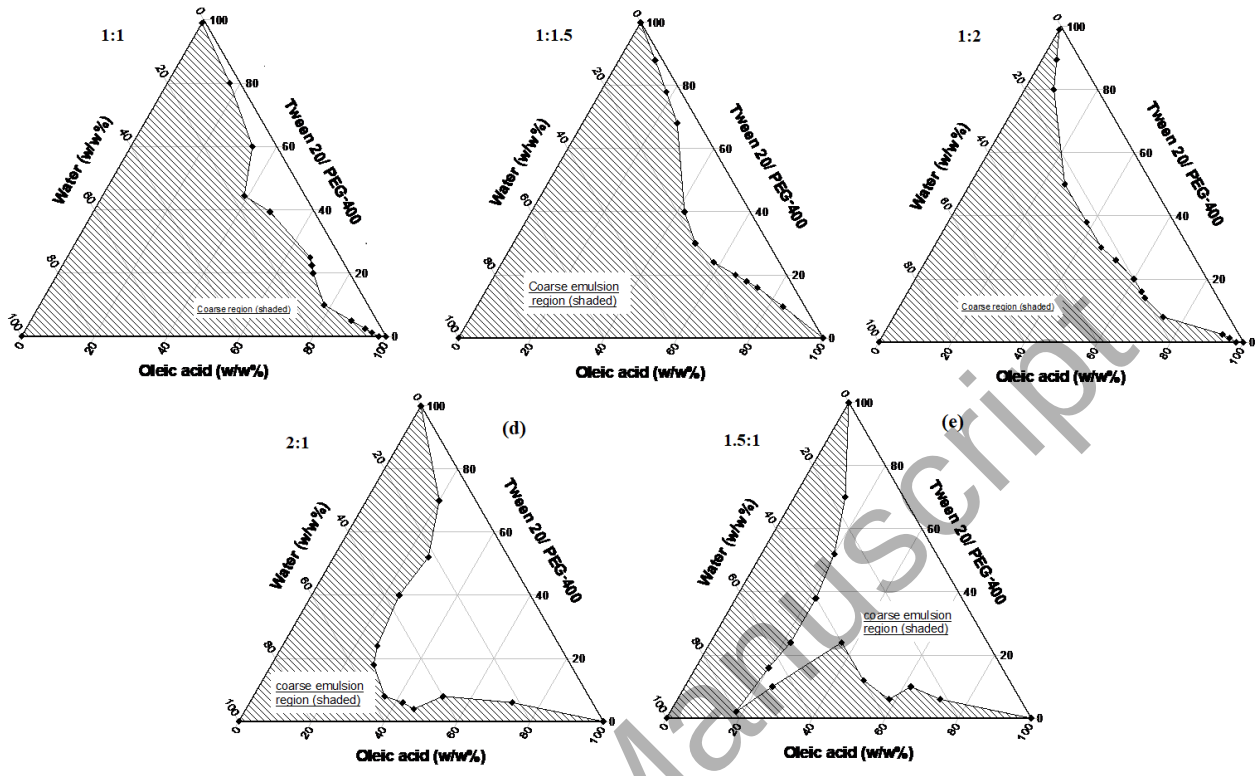

Figure 1: Pseudo-ternary phase diagram prepared using the oleic acid, the surfactant/co-surfactant and the water phase as three axes.

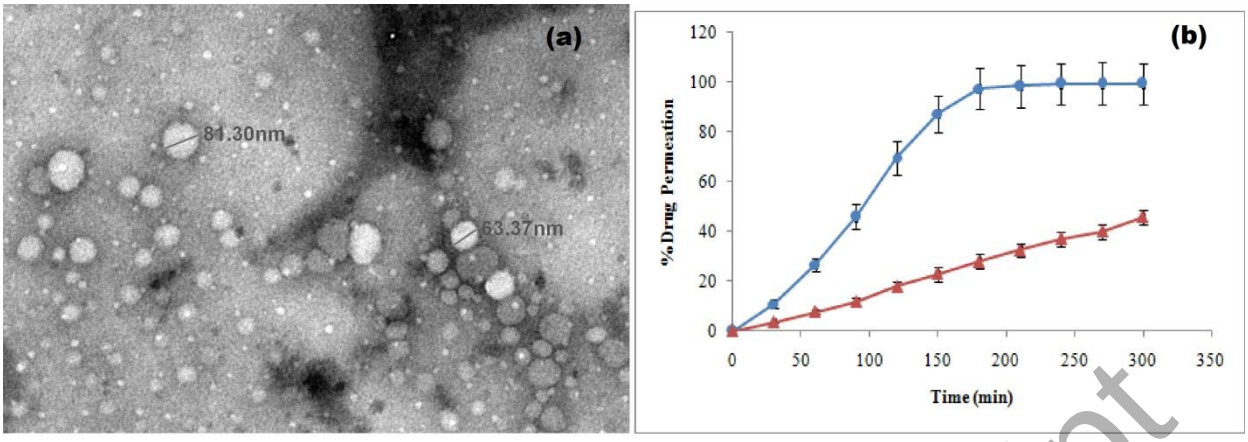

Figure 2: TEM image of drug loaded vitamin D_3 nanoemulsion (a). *Ex-vivo* permeation profile of Vitamin D_3 from the nanoemulsion (●) and drug solution (▲)(n =3)

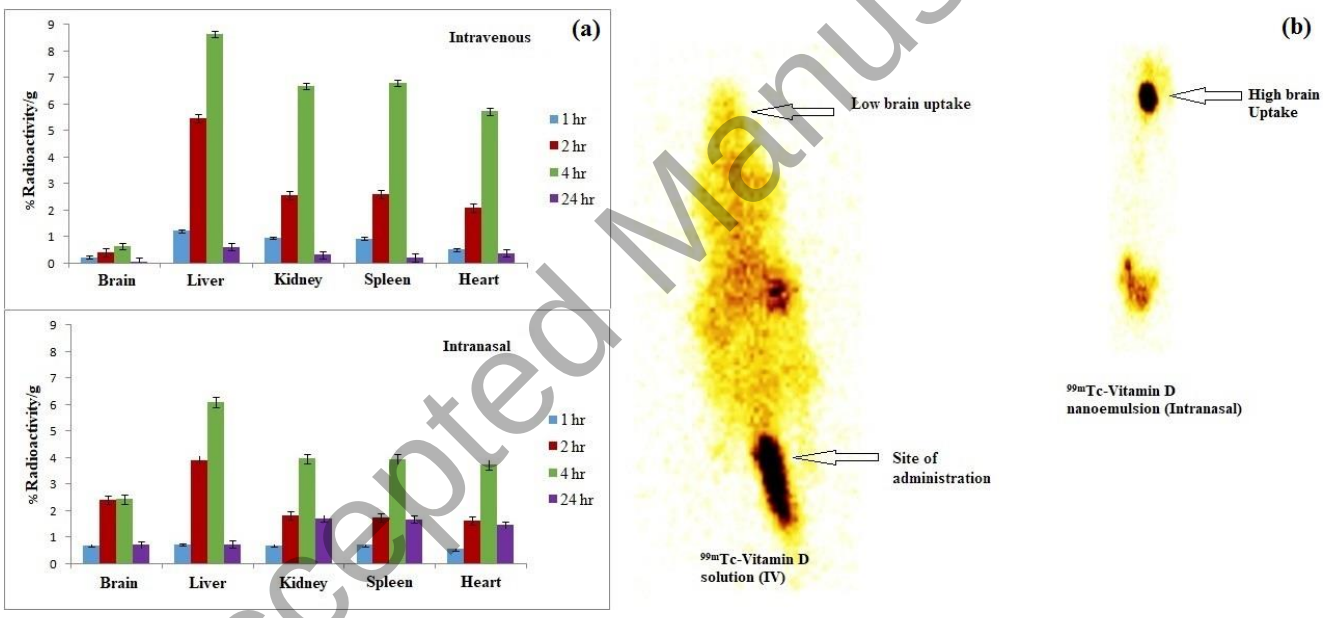

Figure 3: Data of percentage radioactivity of ^{99m}Tc labelled Vitamin D_3-Nanoemulsion in different organs at different time intervals when administered through intravenous route and intranasal route (a) (n=3). Gamma Scintigraphic Images of brain targeting of ^{99m}Tc labelled Vitamin D_3-Nanoemulsion and ^{99m}Tc-Vitamin D_3 solution at 2 h post-drug administration through intranasal and IV route respectively.

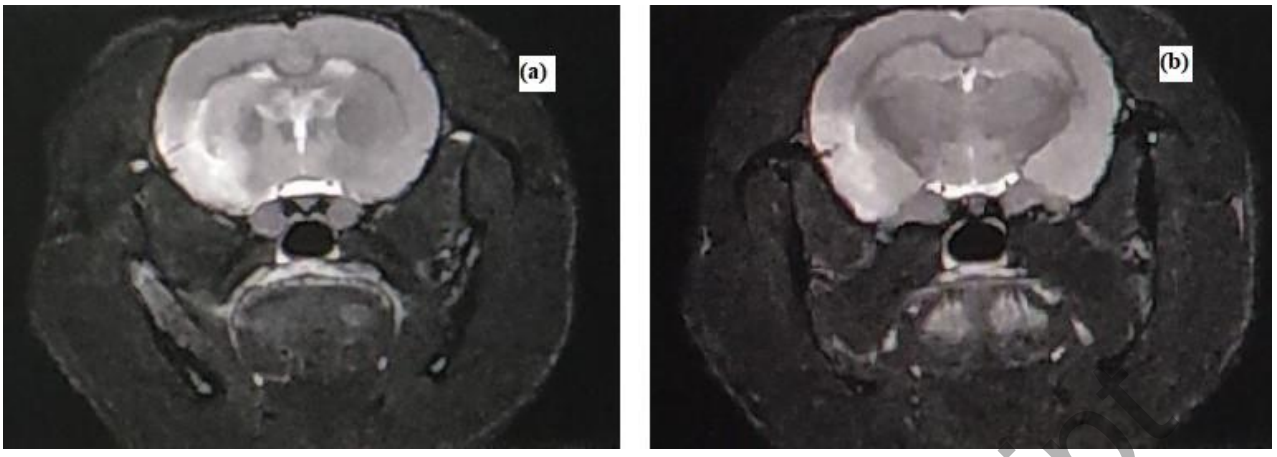

Figure 4: MRI scans of MCAo rat model after the treatment with ^{99m}Tc-Vitamin D_3 solution (a) and ^{99m}Tc Vitamin D_3-Nanoemulsion (b). Ipsilateral hemisphere apparent diffusion coefficient (ADC) value was significantly lower than the normal contra lateral hemisphere at 30 min post-reperfusion.