

Original Article

Albumin conjugated lipid nanoemulsion for site specific delivery of rapamycin at inflammatory site of spinal cord injury

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Abstract: It was aimed to develop albumin decorated phospholipid nanoemulsion of rapamycin for improved specificity at spinal cord injury site after systemic administration. Nanoemulsion was prepared by hot homogenization and ultrasonic emulsification of phospholipids. Interface of nanoemulsion was stabilized using cholesterol and functionalized by albumin. Nanoemulsion was optimized on the basis of particle size and zeta potential with the help of one variable at a time concept. Finally optimized nanoemulsion (RN7) was conjugated with albumin (ARN7) and both were evaluated for drug content, entrapment efficiency, morphology, *in vitro* release, *in vitro* cytotoxicity (MTT assay), *in vitro* anti-inflammatory potential (LPS induced model) and *in vivo* potential (post SCI like conditions in rat). Nano sized droplets with spherical shape, smooth surface and positive charge showed controlled release of rapamycin when compared with rapamycin solution (RS). RN7 and ARN7 kept astrocytes viable when compared with RS at the same dose. This may be due to avoidance of instant availability of rapamycin inside astrocytes. ARN7 showed significantly higher potential in reducing LPS induced pro-inflammatory cytokines when compared with RN7. Rapamycin delivery through ARN7 was found to be more effective in treating *in vivo* post SCI like conditions (behavioral and histopathological changes) when compared with RN7 due to the presence of albumin over the surface. In conclusion, albumin functionalized, cholesterol stabilized, phospholipid nanoemulsion was developed successfully with controlled release of rapamycin, improved cytokine inhibition and improved *in vivo* efficacy that may offer safe and effective mean for the treatment of post SCI like conditions.

Keywords: Spinal cord injury, lipid nanoemulsion, rapamycin, albumin, systemic delivery

Introduction

There is strong need to investigate an efficient neuroprotective component for the treatment of spinal cord injury (SCI). Various drugs are being used for the treatment of post SCI inflammatory conditions however; they are inefficient due to several shortcomings associated with them [1]. Treatments available are becoming not as effective to manage such conditions as needed. This could be due to the rapid first pass effect, shorter elimination half-life, less oral bioavailability, slight aqueous solubility, less partition coefficient and many more. They used to face various limitations when administered through various routes. Most of the drugs are unable to cross the blood brain barrier, withstand inflammatory and acidic/basic environment and avoid first pass metabolism to reach the injury site like post SCI [2, 3].

One of such kind of pharmaceuticals is rapamycin which is a macrolide antibiotic and has been observed to show antifungal effects initially. Recently, it has been reported to show anti-tumor and immunosuppressant (specially used after transplantation of several organs) potential. It has also been reported to prevent degradation of nerve tissue after SCI on account of its anti-inflammatory effects. Its anti-inflammatory effect is due to the alleviation in the expression of MPO, IL-1 β and TNF- α [4]. Unfortunately, it is slightly soluble in water and prone to first pass metabolism. At the therapeutic dose when administered via i.v. route, it sometime shows sub-therapeutic effect [5]. Improving pharmacokinetic parameters and neurovascular availability of rapamycin could be the possible and suitable ways to enhance its efficacy. Encapsulating rapamycin in a site specific carrier may improve its distribution in the systemic cir-

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ulation by releasing it in the controlled manner and helping it accumulate at the desired site of action (specifically at SCI site) [3, 6].

The carrier of choice could be nanoemulsion that is meant to carry both hydrophilic and lipophilic molecules, sustain their release and improve their distribution [7]. Its nano size range facilitates rapid penetration however; lipidic membrane helps in efficient interaction with the biological environment. Charged nanoemulsions are developed to help improve interaction with the oppositely charged biological membrane [8]. Development of charged nanoemulsion is carried out majorly by emulsifying drug with the help of proteinaceous or lipoidic emulsifiers followed by coating with oppositely charged polyelectrolytes. Type of charge is chosen on the basis of the nature of charge available over the biological environment/membrane [7, 9]. Positive charge over nanoemulsion i.e. developed using phospholipid and cholesterol could be established using stearylamine. Cholesterol is used to stabilize the phospholipid interface [10].

Hypoalbuminemia and expression of albumin receptors are the key physiological changes which usually occur in inflammatory conditions. Albumin is biodegradable, obtainable, non-toxic and non-immunogenic when used internally and these merits make albumin an ideal system to use as ligand in drug carries for site specific targeting of drugs [10]. Therefore, albumin was used as a functional ligand of albumin receptors which might carry R to the specific inflammatory site of SCI. We hypothesized to prepare, characterize, and evaluate albumin coupled, positively charged, lipid nanoemulsion for site specific delivery of rapamycin to the inflammatory sites of SCI like condition. Albumin coupled, lipid nanoemulsion is expected to penetrate and interact with the biological membrane efficiently and releases R in a very controlled manner so as to avoid its instant or inadequate availability around there.

Material and methods

Materials

Materials used were purchased in China only. Rapamycin (R), phosphatidylcholine (EPC-80), 1-Ethyl-3-(3-dimethylamino) propyl carbodiimide (EDC), poly-L-lysine, trypsin, cytosine β -D-arabinofuranoside, dimethyl sulfoxide (DMSO), Coomassie brilliant blue, α -tocopherol acetate,

lipopolysaccharide (LPS) and bovine serum albumin (BSA) were from Sigma Aldrich Chemie; cholesterol, methanol, chloroform, acetonitrile, stearylamine (SA), EDTA and glycerol were from Merck chemicals; centriscort tubes was from Sartorius, Germany; dialysis membrane MWCO 12-14 KDa, carrageenan and buffer tablets were from Himedia, Pvt. Ltd.; Dulbecco's modified Eagle's medium was from Invitrogen; Cell Counting KIT-8 was from Dojindo Laboratories Inc.; Alcohol, Hematoxyline, Eosin, Dpx mount, and Xylene were from Thomas Baker Pvt. Ltd.; TNF- α and IL-1 β ELISA kit were from Thermo fisher scientific. Milli-Q water was used throughout the study. All other chemicals used were of reagent grade.

Animals

Female adult Sprague-Dawley rats (220-250 g) were procured from the Slac Laboratory Animal Co., Ltd. Shanghai and housed in a 12 h day and 12 h night environmental condition at room temperature. All surgical interventions and postoperative animal care were carried out in accordance with the guidelines of care and use of rats Institutional Animal Experimentation Ethics Committee (AEEC), First People's Hospital Shangqiu, China for Animal Care and Use.

UV method

A known concentration (10 μ g/ml) of R in DMSO/water (2:98, v/v) was scanned between 200 and 400 nm using UV-visible spectrophotometer (Shimadzu, Kyoto, Japan). The absorption maximum was found to be at 280 nm. Consequently, the stock of 1 mg/ml solution of R was prepared in DMSO/water (2:98, v/v). Various dilutions of R were prepared and the absorbances of the dilution were taken at 280 nm. The calibration curve was plotted between absorbance and concentration of the drug. Linearity was observed in the concentration range of 5-50 μ g/ml.

Preparation of lipid nanoemulsion of rapamycin, optimization and characterization

Preparation of the nanoemulsion was carried out by hot homogenization and ultrasonic emulsification method [10]. Drug (R), EPC-80, cholesterol, α -tocopherol acetate and SA were added to soybean oil, heated using the controlled temperature water bath at 70°C with gentle stirring until the system was clear. Gly-

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Table 1. Effect of cholesterol and SA concentration of different formulations parameters

Batches	Formula of prepared nanoemulsion (g)									Formulation parameters		
	Rapamycin	Soybean oil	EPC-80	α -tocopherol	Cholesterol	SA	Albumin	Glycerol	Water (ml)	Size (nm)	PDI	Zp (mV)
RN1	0.0025	1	0.12	0.025	0.0	0.0	0.0	0.225	10	197 ± 4.16	0.29 ± 0.03	-32.7 ± 0.7
RN2	0.0025	1	0.12	0.025	0.01	0.0	0.0	0.225	10	185 ± 11.9	0.23 ± 0.20	-32.8 ± 1.0
RN3	0.0025	1	0.12	0.025	0.02	0.0	0.0	0.225	10	169 ± 3.9	0.19 ± 0.16	-33.2 ± 0.4
RN4	0.0025	1	0.12	0.025	0.03	0.0	0.0	0.225	10	162 ± 5.4	0.157 ± 0.05	32.1 ± 0.28
RN5	0.0025	1	0.12	0.025	0.03	0.01	0.0	0.225	10	186 ± 4.5	0.376 ± 0.1	38.1 ± 5.20
RN6	0.0025	1	0.12	0.025	0.03	0.02	0.0	0.225	10	178 ± 6.6	0.292 ± 0.03	42.7 ± 3.03
RN7	0.0025	1	0.12	0.025	0.03	0.03	0.0	0.225	10	163 ± 1.7	0.168 ± 0.04	49.3 ± 6.65
ARN7	0.0025	1	0.12	0.025	0.03	0.03	0.01	0.225	10	198 ± 4.2	0.162 ± 0.01	28.8 ± 2.51

cerol was dissolved in MilliQ water and heated to 70°C separately. This aqueous phase was mixed with the oil phase at the same temperature and stirred for 5 minutes at 7000 rpm using high speed homogenizer (IKA t25 Digital Ultra Turex, Staufen, Germany) which resulted coarse emulsion (10 mL). That coarse emulsion was sonicated for about 10 minutes to get the nanoemulsion. Final composition of the ingredients in optimized batch (RN7) was taken in gram which is as follows; rapamycin (0.0025), soybean oil (1), EPC-80 (0.12), α -tocopherol acetate (0.025), cholesterol (0.030), SA (0.03), Glycerol (0.225) and water up to 10 ml. Nanoemulsion was optimized on the basis of one variable at time by observing the effects of different cholesterol concentration and SA concentration over nanoemulsion characteristics such as size, polydispersity index and zeta potential (**Table 1**). All these parameters were determined by DLS (Malvern, U. K.). Level of other ingredients was kept constant for all the batches as used in the optimized batch (RN7).

Attachment of albumin to the surface of emulsion droplets

Albumin was attached to RN7 (3 mL) surface by coupling reaction [10] and as a result ARN7 was prepared. RN7 was dispersed in phosphate buffered saline (PBS; pH 7.4) containing albumin (15 μ M), and EDC (183 μ M) and incubated for 2 h at RT. The unbound albumin was removed by loading of nanoemulsion on sephadex G-75 gel column which was equilibrated using PBS pH 7.4. ARN7 was also characterized for droplet size, polydispersity index, zeta potential, morphological characteristics and evaluated for drug content, entrapment efficiency, *in vitro* release, *in vitro* cell viability, anti-inflammatory potential and *in vivo* potential.

Characterization and evaluation of the developed formulation

Morphological characteristics of ARN7 were observed using transmission electron microscopy (TEM; JEOL, Tokyo, Japan) after clicking digital images at 6000 \times . Drug content and entrapment efficiencies of RN7 and ARN7 were determined using the procedure reported by Kandadi et al. in 2011 [11]. For drug content, about 1 ml of RN7 and ARN7 were diluted in the mixture of equal ratio of 10 mL chloroform/methanol and homogenized at 500 rpm for 15 min. About 1 ml sample was taken, dilute 10 times fold, absorbance was taken at 280 nm using UV-Vis spectrophotometer and R content was measure using standard curve equation for each formulation. Entrapment efficiency (EE) was determined by back calculation. For this purpose, the aqueous medium (2% DMSO) from RN7 and ARN7 was separated using centrifuge tubes i.e. assembled with filter membrane (20,000 Da MWCO) at the base of the sample receiver. RN7 and ARN7 were further centrifuged at 6500 rpm for 15 min (C24 cooling centrifuge, Remi) and the supernatant was pipette out, filtered and placed in the separated aqueous medium. The amount of R in the aqueous phase was estimated using UV-Vis spectrophotometer and entrapment efficiency was calculated by following equation:

$$EE = \frac{\text{Drug content} - \text{drug in aqueous medium}}{\text{Drug content}} \times 100$$

Physical stability study of ARN7 was assessed in terms of centrifugal stress (6000 rpm), thermal stress (-20 to 40°C), dilution stress (1000 times in water) and 6 month storage at room temperature (RT) using the previously reported procedure.

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In vitro release study

Cellulose membrane of 12 kDa MWCO was used to prepare the dialysis bag. The cumulative amount of drug release from the prepared formulations (RN7 and ARN7) and drug solution (RS) was observed in phosphate buffer (pH 7.4) containing 2% DMSO. Samples were collected at different time interval for the duration of 12 h. The amount of drug was estimated using the linear standard calibration curve plotted at the known concentrations of R at 280 nm using UV-Visible spectrophotometer [12].

Culture and subculture of astrocytes

Ventral mesencephalic cells were dissected from Sprague-Dawley rats. In brief, tissues were removed from fetuses and dissociated to single cells by protease treatment (2.5 mg/mL trypsin) and additional mechanical shearing. Cells were kept in Dulbecco's modified Eagle's medium supplemented with 10% FBS and seeded at a density of 5×10^5 cells/cm² on 24- or 6-well plates pre-coated with 0.01% poly-L-lysine. On the second day after initial seeding, cytosine β -D-arabinofuranoside (5-10 μ M) was added to suppress glial proliferation. After 2 days incubation fresh media was added to the culture. After seven day, cultures that rich in neuronal cells were used to obtain astrocytes. For this, the remnant cells were separated with trypsin-EDTA. After 4 passages, nearly pure astrocytes were obtained. For sub-culture, cells were re-suspended in the culture medium and sub-cultured in 12-well (0.4×10^6) or 6-well (1.0×10^6) plates for experiments. After 24 h the astrocyte co-cultures were used for further study [13].

In vitro cell viability study

The viability of astrocytes in the presence of RS, RN7 and ARN7 was observed at various dose of R with the help of Cell Counting KIT-8. About 0.025 to 5% of R was incorporated in RS, RN7 and ARN7 and their toxicological profile on cultured astrocytes was observed. These dilutions were added to the cultured astrocytes incubated in CCK-8 solution at 37°C for 2 h in 5% CO₂ rich incubator. Absorbance was measured at the wavelength indicated in the user's manual of the kit and the number of astrocytes was correlated with optical density [2].

In vitro anti-inflammatory potential

Cellular treatments and quantification of TNF- α and IL-1 β : After reaching confluence, media of the astrocytes was replaced with serum-free DMEM, and incubated in the absence or presence of RN7 and ARN7 for an hour. Subsequently, 10 μ g/mL LPS was added in each group and incubated for 3 h at 37°C in an atmosphere with 5% of CO₂. In order to quantify the expressed pro-inflammatory cytokines in the astrocytes culture, culture medium was collected and the concentration of TNF- α and IL-1 β was determined using a rat TNF- α and IL-1 β ELISA kit following the manufacturer's instructions [13].

In vivo efficacy against SCI like condition

Sprague-Dawley female rats (220-250 g) were divided in to 3 groups of 10 each. Laboratory animals were kept under 12 h light and dark cycle at 22-25°C. Rats were anesthetized by pentobarbital sodium (30 mg/kg; IP) and placed on a hard base. Animals were underwent spinal cord T11 laminectomy as per the procedure reported by Song et al. 2015 and the wounds were closed aseptically. To prevent infection, intraperitoneal injections of ampicillin and gentamicin (100 and 12 mg/kg respectively) were administered daily followed by bladder massage two times a day [4, 14]. First group was kept untreated and in second, third and fourth group, respectively RS, RN7 and ARN7 i.e. equivalent to 1 mg/kg daily dose were administered intravenously. In injection schedule, three intraperitoneal injections i.e. first at 4 h after spinal cord contusion injury and rest 5 at an interval of 24 h were administered. During the study period, animals were assessed for behavioral changes and at 8th day the animals were sacrificed and injured spinal cord samples were collected for the assessment of the histological changes using transverse section.

Behavioral assessment

Sensory-motor functions recovery was assessed by grid and beam walking tests after producing SCI. Behavior of animals of SCI, RS, RN7 and ARN7 groups was observed after 2nd, 4th, 6th and 8th day of post dosing. This study was carried out as per the procedure reported by Ren et al. 2014 [2]. Data was expressed

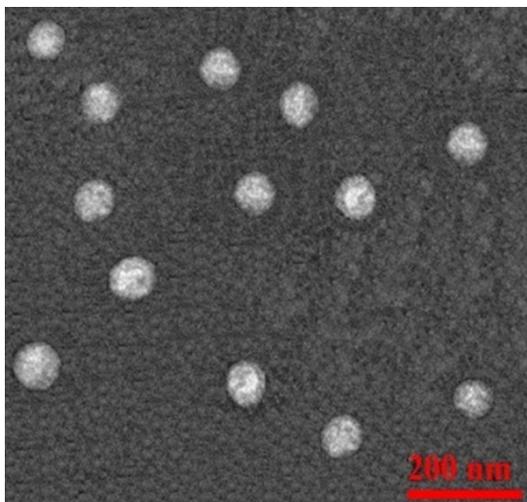


Figure 1. TEM image of ARN7.

Table 2. Change in size, PDI and Zp of ARN7 in 6 month period of stability study

	Size (nm)	PDI	Zp (mV)
Zero day	163.0 ± 5.5	0.169 ± 0.10	49.0 ± 3.4
2 months	172.3 ± 8.0	0.22 ± 0.07	45.2 ± 4.7
4 months	173.0 ± 4.3	0.22 ± 0.03	46.2 ± 4.3
6 months	178.0 ± 2.0	0.27 ± 0.05	43.3 ± 2.3

in the form of percentage of missteps. During beam walking, the total number of missteps of hindlimb was counted off the beam during animals crossing of the beam.

Histopathological analysis

After 8th day of the treatment, rats were sacrificed humanly after anaesthetizing. The spinal cords were opened immediately post sacrifice and the mice were perfused with 4% paraformaldehyde in PBS transcardially. Spinal cords were removed carefully and fixed using fixative at 4°C for 6-8 h. Samples were stored in 30% sucrose solution in PBS (pH 7.4) for 12 h. About 3 cm part of the spinal cords with injury epicenters and implanted material were dissected out. After embedding in optimal cutting temperature compound, samples were cut into 10 µm sections with the help of cryostat. Sections were stained using H&E dye and observed with the help of a light microscope. Histopathological damages were analyzed on the basis of neuronal vacuolation, inflammatory cell infiltration, and hemorrhage [15].

Statistical analysis

Statistical analysis was carried out using two-tailed, Student's t-test (*P* values of <0.05 were considered significantly different).

Results

Optimization of nanoemulsion

Based on one variable at a time, nanoemulsion was optimized for its particle size and surface charge. Cholesterol and SA were the variable and based on their different concentration, seven batches of nanoemulsion were prepared. Positive charge was desired for the better interaction of nanoemulsion with the biological environment of inflammatory site and smaller particle size of uniform distribution could make the hypothesis a reality. The average droplet size was observed to be varied in range of 162-197 nm with lower PDI values i.e. below 0.376. Zeta potential was observed to be in the range of -32.7 to -33.2 after varying cholesterol and 32-42.7 mV after varying SA during nanoemulsion optimization (**Table 1**).

Effect of varying cholesterol concentration over nanoemulsion characteristics portrayed, decrease in average droplets size of the emulsion from 185 ± 11.9 to 162 ± 5.4 by increase in cholesterol concentration however, zeta potential did not change. Effect of varying SA concentration over nanoemulsion had shown no considerable change in the droplets size upon increasing SA concentration, whereas Zp varied significantly i.e. from 38.1 ± 5.20 to 49.3 ± 6.65. PDI was observed to be remained below 0.376 in all the batches. Finally, RN7 was selected as the optimum batch which coupled with albumin (ARN7) for site specific therapeutic delivery of R at the inflammatory site. ARN7 were evaluated on the basis of pharmaceutical and pharmacological parameters for better therapeutic effect.

Pharmaceutical characteristics of the developed formulations

Droplet size and zeta potential of ARN7 have changed significantly when compared with that of RN7 (**Table 1**). R content in RN7 and ARN7 was observed to be 97.7 ± 3.17 and 92.1 ± 6.28 respectively however; their entrapment efficiencies were observed to be 79 ± 2.0 and

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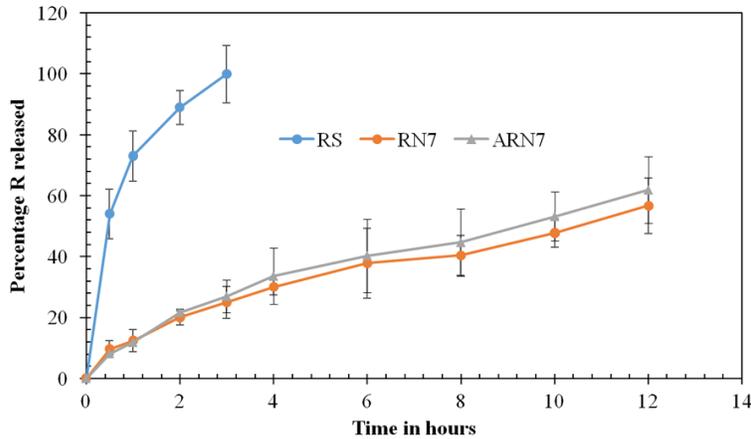


Figure 2. Illustration of *in vitro* release of R from RS, RN7 and ARN7.

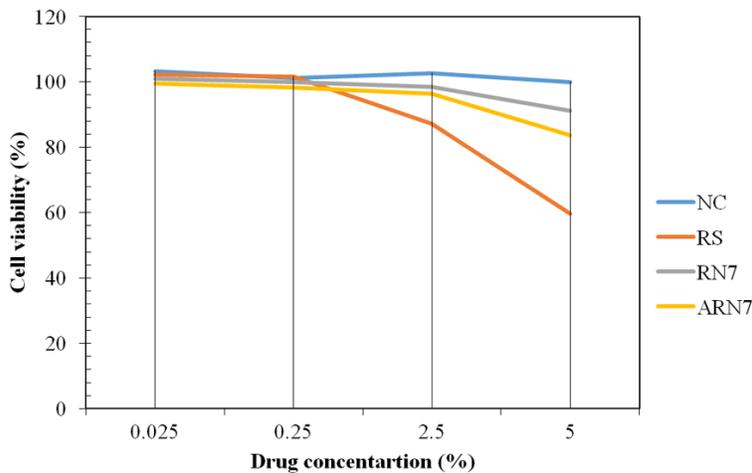


Figure 3. *In vitro* cell viability study to assess cytotoxic potential of different concentration of R encapsulated in RS, RN7 and ARN7.

71.6 ± 6.4% respectively. TEM analysis portrayed spherical shape, smooth surface, uniform size ARN7 droplets (**Figure 1**). Stability of ARN7 was observed for 6 month in terms of particle size, PDI and zeta potential. These parameters were found to be consistent for the whole study period (**Table 2**). Changes in the parameters were observed to be non-significantly different (below 4%). ARN7 was observed to be physically stable (no phase separation) at 600 rpm, -20 to 40°C and 1000 times dilution in water.

In vitro drug release

In vitro R release study from RN7 and ARN7 was carried out against RS for 12 h. The cumulative percentage drug release was plotted

against time in **Figure 2**. The cumulative percentage drug release from solution in 2 hours was observed to be 89.00 ± 5.60 however; RN7 and ARN7 released 56.7 ± 9.2 and 61.9 ± 11.2% respectively in 12 hours.

Cytotoxic effect of RS, RN7 and ARN7 on cultured astrocytes

Effect of various concentrations of R in RS, RN7 and ARN7 on viability of astrocytes was assessed using CCK-8 assay. No cytotoxicity was observed in all cases at 0.025 to 2.5% concentration of R however at 5% concentration, RS showed 60% viability however, viability showed by RN7 and ARN7 were observed to be 91 and 84% respectively (**Figure 3**). RS was found to be more toxic than that of RN7 and ARN7 at the same concentration. No significant change in the viability of the cells was observed in the case of vehicle control. At the therapeutic dose of R, no toxicity was observed in RS, RN7 and ARN7.

Anti-inflammatory potential of RN7 and ARN7

Result of ELISA based quantification of IL-1β and TNF-α portrayed significant alleviation by RN7 and ARN7 than that of the LPS controlled group (**Figure 4**). ARN7 was found to be more effective in the alleviation of cytokines level when compared with that of RN7.

Behavioral changes

The results of grid and beam walking tests at different time points (2, 4, 6, 8th day after injury) are illustrated in **Figure 5**. In the beam walking test, ARN7 treated groups performed considerably better than that of RN7 and RS at all time points. In the grid walking test also the performance of ARN7 treated animal was significantly better than the RN7 and RS at all time points. ARN7 treatment has improved the functional outcomes of the animals only in 8 days.

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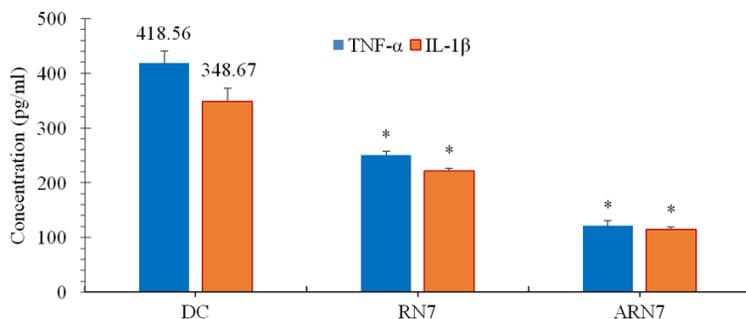


Figure 4. Comparison of level of expression of TNF- α and IL-1 β in RN7 and ARN7 treated groups.

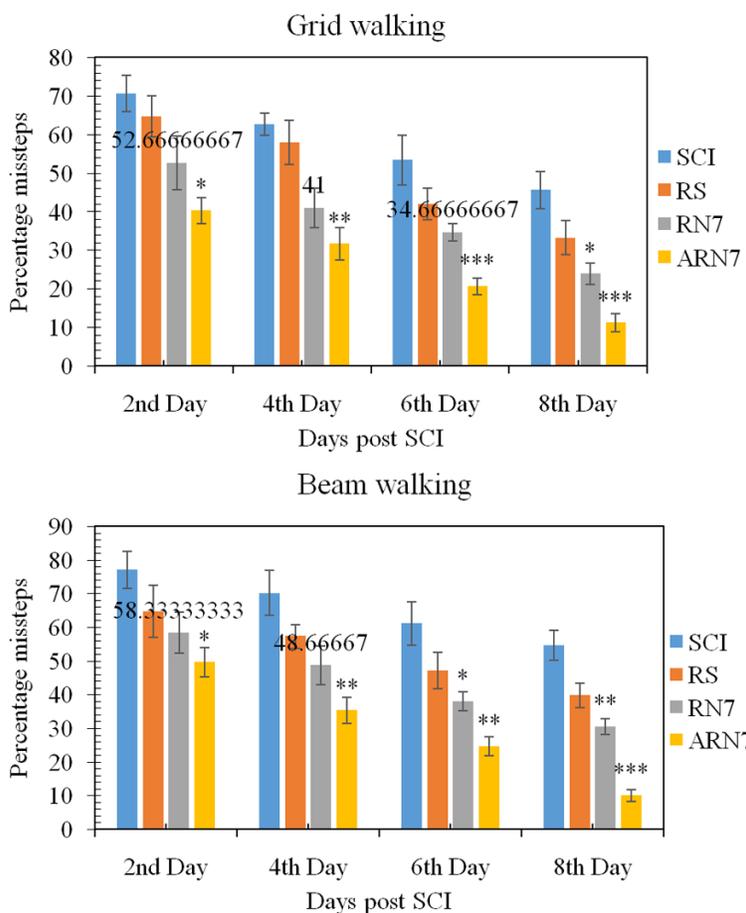


Figure 5. Modulation of behavior of rats in terms of Grid walking and Beam walking after administering RS, RN7 and ARN7 in spinal cord injured rat.

Histopathological modification

Rats were humanly killed at 8th day of the study period. RS, RN7 and ARN7 were administered daily once a day and examined for histological improvement as compared to SCI control rats

using transvers sections. SCI control group consisted intense inflammatory cell infiltration, hemorrhage and larger pseudocyst. In ARN7 group the integrity of the spinal cord was reestablished efficiently (**Figure 6**). Noteworthy reduction in the volume of the pseudocyst, intensity of the inflammatory cell infiltration and hemorrhage was observed after the administration of ARN7 as compared to SCI control group however, RN7 was observed to be less efficacious than that of ARN7.

Discussion

Inadequate aqueous solubility and frequent first pass metabolism of rapamycin restrict its delivery through oral route [5]. However, its higher biological half-life (63 h) suggested its proficient utility by systemic route of administration. As it is a potent anti-inflammatory molecule, its use in the case of secondary inflammation of spinal cord injury is well documented [4]. By ensuring its maximum availability at the injury site, its efficacy could be enhanced. Due to the condition like hypoalbuminemia, inflammatory tissue exhibits extravasation and passive accumulation of albumin. As a result, albumin receptors i.e. responsible for receptor-mediated endocytosis for albumin are expressed at such sites [10]. Albumin decorated lipid based nanoemulsion was hypothesized to be helpful in availing therapeutic concentration of R at SCI site and as a result, therapeutic efficacy of R could be enhanced. Biodegradable, lipid based ARN7 showed various potential advantages over RN7 and RS systemic delivery such as site specificity and lesser toxicity. The thera-

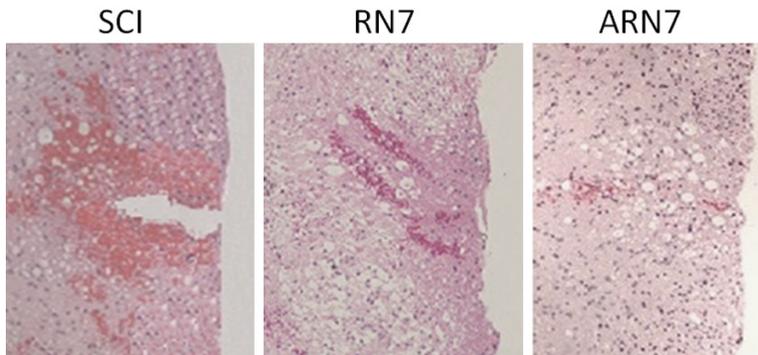


Figure 6. Histopathological images of SCI group and RN7 and ARN7 treated groups. Rats were sacrificed after eight days and the images of the H&E stained transverse section of SCI were taken at 10× magnification.

peutic effectiveness of R has been improved by virtue of its targeted and sustained delivery.

Formula of the nanoemulsion prepared by hot mixing and ultrasonication was soybean oil (10 wt%), EPC-80 emulsifier (1.2% w/v), SA (positive charge inducer), α -tocopherol acetate and glycerol (2.25%) to maintain isotonicity for intravenous administration. Owing to a weak emulsifier, phospholipids are used in the presence of cholesterol that enhances the elasticity of the prepared nanoemulsion due to a more rigid interface. Cholesterol decreases the size and makes the membrane of the nanoemulsion rigid. SA was used in the formulation by virtue of its long chain fatty acid and amino group, on account which it helps in the attachment and maintenance of the intactness albumin over lipoidal emulsion globules. Additionally, it also helps in developing positive surface charge over the droplets [10].

Cholesterol has stabilized the interface developed by the lipoidal emulsifier and helped to get the spherical shape and smooth surface of RN7. On account of which, entrapment efficiency of the prepared nanoemulsion was observed to be quite high. Lesser value of PDI shows the uniform size distribution which might help in efficient absorption through the biological membrane. The electrical charge on the RN4 droplets was negative (due to the presence of oleic acid) and it was changed to positive (due to the presence of SA) when RN4 was converted to RN7. The cationic charges over RN7 might ensure its efficient interaction with the negatively charged biological surface. Coupling of albumin with RN7 showed significant increase in droplet size and decrease in

surface charge. This might be due to the attachment of an additional molecule over the surface and negative charge over albumin molecule respectively. Negative charge has reduced the overall magnitude of the positive charge over RN7.

Sustained release of the R from RN7 could be attributed to the diffusion of drug from the droplets. Burst release of R from RS could be attributed to the sudden availability of R to the dissolution medium.

ARN7 release pattern was observed somewhat similar to RN7. This ensures that the integrity of the RN7 is not being changed after albumin coupling. RN7 and ARN7 may ensure long term availability of R in the systemic circulation which may help decrease the progression of secondary injury and accumulate ARN7 at the desired site.

Nanoemulsion RN7 and ARN7 showed ability in reducing dose dependent cytotoxicity produced by RS which might attributes to the sustained release and consistent diffusion of well-balanced quantity of R into the media. RN7 and ARN7 avoided the instant delivery of the drug to the cells [16]. Cytotoxicity reducing capability of nanoemulsion portrayed that these are safe even in *in vivo* conditions. Coupling of albumin over nanoemulsion did not affect the *in vitro* toxicity potential of RN7 significantly.

In vitro anti-inflammatory potential of ARN7 was observed to be significant higher as it decreased IL-1 β and TNF- α level significantly as compared to RN7. This may be due to higher selectivity of the ARN7 with the cells as compared to RN7. Potential of RN7 is based on diffusion however, potential of ARN7 against inflammatory mediators depends both on diffusion in to the media and selectivity with the albumin receptor i.e. present on astrocytes.

ARN7 has performed better in *in vivo* conditions when compared with RN7 and RS. It was observed that, sustained delivery of R from RN7 had significantly improved the efficacy of R in the improvement of functional outcomes (restored histological changes, decreased les-

sional volume, improved behavior of animals) of SCI injured rats but the efficacy of R was observed to enhanced tremendously when it was delivered in the form of ARN7 i.e. albumin decorated RN7. This was attributed to the site specificity of ARN7. Hypoalbuminemia and expression of albumin receptors are the key physiological changes which usually occur in inflammatory conditions. This has worked as the driving force to draw more and more ARN7 at the secondary inflammation site of SCI. ARN7 got in contact with the biological environment (pH 7.2) and positive charge over the its surface had helped it to interact with the injured site. Lipid and cholesterol interface had helped them to interact with the cellular bilayer of the biological membrane and release of R. Development of albumin decorated ARN7 offered an alternative for the treatment of SCI like conditions. That not only decreases the sessional volume but also promotes the proliferation of axons and growth of blood vessel.

Conclusion

Present study highlights the usefulness of albumin decorated lipid nanoemulsion in the effective delivery of R at the specific site of spinal cord injury. Lipid based nanoemulsion of R was prepared successfully. Albumin was used as ligand and decorated on the surface of lipid nanoemulsion using EDC resulting in ARN7. ARN7 is observed to be more selective for the LPS induced astrocytes as compared to RN7 due to the presence of coupled albumin. *In vivo* Intravenous administration of ARN7 portrayed higher therapeutic availability of R in granuloma fluid than that of RN7 and RS. This is also attributed to the presence of albumin and positive charge over RN7 droplets. ARN7 could be a cost effective, safe, efficacious and clinically pertinent option to available dosage forms for the potential delivery of RM at SCI site specifically.

Disclosure of conflict of interest

None.

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