Research Paper

Neuroprotective Potential of Dimethyl Fumarate-loaded Polymeric Nanoparticles against Multiple Sclerosis

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The aim of the present study was to access the potential of dimethyl fumarate-loaded chitosan polymeric nanoparticles for the management of multiple sclerosis. Dimethyl fumarate-loaded chitosan nanoparticles were prepared by polyelectrolyte complex coaservation technique. The prepared nanoparticles were characterized and found to have an average particle size of 324 nm, zeta potential of –34.85 mV and a poly dispersity index of 0.367. The entrapment efficiency was found to be 65.36 % and the drug loading was 28 %. The formulation's *in vitro* drug release profile and stability parameters were also evaluated. Cumulative percent drug release was found to be 84 % up to 24 hours and the formulation was found to be stable at 28° for 90 days. *In vitro* neuroprotective effect of the nanoformulation was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on human neuroblast SH-SY5Y cells and the treated cells showed improved cell viability under hydrogen peroxide-induced cell apoptosis. *In vivo* cuprizone model for multiple sclerosis in rodents also confirmed these findings by showing a significant increase in locomotion score.

Key words: Multiple sclerosis, chitosan nanoparticles, cuprizone model, SH-SY5Y cells, bioavailability

The neurodegenerative process is a key step in the pathogenesis of multiple sclerosis (MS) with symptoms of permanent neurological disability and impairment[1,2]. Currently, most available MS therapeutic agents are immunomodulatory or immunosuppressive in nature[3-5] and effectively inhibit immune cell-driven inflammation thus reduce the relapse rate. But they do not control the predominant neurodegenerative processes that mainly occur later in the disease course[6]. Dimethyl fumarate (DMF) is a fumaric acid ester that has been used since 1959 for the treatment of psoriasis[7]. FDA has now approved DMF as a first line oral treatment for lowering relapse rates in MS[8-11]. DMF and its active metabolite monomethyl fumarate (MMF) have antioxidant property by activation of the transcription factor, nuclear factor-erythroid derived 2-factor pathways[12-14]. Oxidative stress is one of the key factors for neuron degeneration and pathogenesis of MS disease, which is evidenced within MS lesions, and also in experimental autoimmune encephalomyelitis, a mouse model of MS[15]. In last decade, nanotechnology has proved its effectiveness for the diagnosis and treatment of variety of immune-mediated diseases. In the present study, chitosan (CS) dextran sulphate (DS) nanoparticles loaded with DMF were prepared using the polyelectrolyte complex coaservation technique and the nanoparticles' zeta potential and average particle size were measured. CS is a biocompatible, biodegradable, low toxic, cationic polymer with mucoadhesive properties. Polymeric nanoparticulate drug delivery systems offer enhanced penetrating ability of molecules across mucosal surfaces, good systemic availability, targeted drug delivery, improved pharmacokinetic profile, better half-life and drug pay load. Now a days nanotechnology offers a promising results and effective platform for many industrial and medical fields[16,17]. The developed CS-DS nanoparticles were further evaluated *in vitro* in human neuroblastoma SH-SY5Y cells and in an established cuprizone animal model *in vivo*.

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MATERIALS AND METHODS

CS (degree of acetylation=80.45 %) and DS were procured from Chemsworth Chemicals, Surat. DMF was obtained from Alfa Aesar, a Johnson Matthey Company. Dimethyl sulphoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's modified Eagle medium, Ham's F12 (DMEM/HF12), fetal bovine serum (FBS), and cuprizone were obtained from Aakaar Biotechnology Pvt. Ltd., Lucknow. Methanol, glacial acetic acid and acetone were of suitable analytical grade. Double-distilled water was used in the preparation of solutions and dispersion of CS nanoparticles.

Preparation of various batches of CS-DS nanoparticles:

CS-DS nanoparticles were prepared using the polyelectrolyte complex coaservation method[18-20]. Solution of CS was prepared by dissolving measured quantity of CS in 2 % v/v acetic acid solution and DS solution was prepared by dissolving measured quantity of DS in double-distilled water. The ratio between the volumes of DS and CS solution was kept at 1:4. DS solution was added into CS solution dropwise with vigorous stirring. The concentration range of both the polymer solutions were kept in the range of 1 to 0.125 % w/v. CS-DS nanoparticle suspension thus formed was stabilized by the addition of 1 ml of Tween 80 and was vortexed at 1200 rpm for further 1 h. All the formulations were prepared at room temperature and in triplicate. Prepared nanoparticles from all the batches were separated by centrifugation at 12 000 rpm for about 1 h. The prepared nanoparticle batches were optimized on the basis of their particle size, polydispersity index (PDI) and zeta potential[21,22].

Preparation of DMF-loaded CS-DS nanoparticles:

Optimum concentrations of CS and DS solutions were prepared to synthesize DMF-loaded nanoparticles. DMF was dissolved in the solution of CS at a concentration of 1 mg/ml. DMF-loaded nanosuspension was separated by centrifugation and the obtained pellets were washed with distilled water, again redispersed in phosphate buffer (pH 6.8).

Characterization of DMF-loaded CS-DS nanoparticles, particle size, PDI and zeta potential:

DMF-loaded CS-DS nanoparticles were further characterized by determining their average particle size, zeta potential, PDI, entrapment efficiency (% EE) and *in vitro* % cumulative drug release. The average particle size, PDI and zeta potential were measured with a photon correlation spectroscopy Delsa Nano C (Beckman Coulter Counter, USA) particle size analyser. The samples of formulated CS-DS nanoparticles were placed in disposable cuvettes for measuring average particle size and zeta potential. The CS-DS nanoparticles were dispersed in an appropriate volume of high performance liquid chromatography (HPLC) grade water at 25°, at a detection angle of 90° for measurement of average size and PDI and an angle of 120° for measuring its zeta potential.

Percent EE:

The % EE was calculated as per previously reported procedures. DMF-loaded CS-DS nanoparticles were separated from the colloidal dispersion by ultracentrifugation at 12 000 rpm for 1 h. The DMF content in the supernatant was analysed using UV/Vis spectrophotometer at 208 nm. % EE was calculated using the formula give below[23,24]. % EE = (total amount of DMF–free DMF in supernatant)/total amount of DMF added×100.

In vitro drug release profile:

In vitro drug release from the DMF-loaded CS-DS nanoparticles was determined using the equilibrium dialysis process at 37±1°[25,26]. Accurate quantity of CS-DS nanoparticles (equivalent to 1 mg DMF) were suspended in 10 ml of phosphate buffer solution (PBS) pH 7.4 and this suspension was placed in a dialysis membrane bag and tied. This CS-DS nanoparticle-loaded dialysis membrane bag was then dipped in a beaker with 500 ml PBS, which was stirred at 50 rpm on a magnetic stirrer. Throughout the dissolution experiment, sink condition was maintained by withdrawing 5 ml of the aliquots at regular time intervals and replacing the same volume of fresh PBS after each withdrawal. To separate the polymeric CS-DS nanoparticles, the collected aliquots were than centrifuged at 12 000 rpm. The % cumulative DMF release profile was calculated by analysing the released DMF content with the help of UV/Vis spectrophotometer at 208 nm.

Cell culture and MTT assay:

In vitro neuroprotective potential of DMF-loaded CS-DS nanoparticles were determined using the MTT assay[27,28]. SH-SY5Y human neuroblastoma cells were seeded in a 96 well plate and maintained at a density of 5000 cells/well. These cells were allowed to grow in DMEM/HF12 medium containing 10 % v/v FBS and

antibiotic/actinomycin solution (1 % v/v) for 24 h. To study the neuroprotective effect of DMF-loaded CS-DS nanoparticles on SH-SY5Y neuroblastoma cells, hydrogen peroxide (H_2O_2) was used to induce cell apoptosis[29]. Cytotoxicity of H_2O_2 towards SH-SY5Y cells were tested with freshly prepared H_2O_2 solution at concentrations of 0, 50, 100, 200, 250 and 300 µM. H_2O_2 concentration which was able to produce 60 % cell death was selected as an optimum H_2O_2 concentration for further study.

Determination of neuroprotective activity of DMF-loaded nanoparticles:

To observe the neuroprotective potential of DMF-loaded CS-DS nanoparticles, cells were treated with different concentrations of DMF-loaded CS-DS nanoparticles (100, 50, 25, 12.5, 6.25, 3.125 µM) for 24 h. Thereafter, the treated cells were kept with an optimum H_2O_2 concentration (250 µM) to induce cell apoptosis and incubated for another 24 h followed by adding MTT solution (5 mg/ml in PBS) in each well. The plates were allowed to stand for further 4 h and formazan crystals formed inside the cells were than solubilized in DMSO. The absorbance of resulting solution was measured at 570 nm using a microplate reader[30,31].

***In vivo* studies using cuprizone model for MS:**

In vivo experiments were carried out as per the protocol approved by the Institutional Animal Ethical Committee. Wistar rats (150-200 g) were randomly selected from the institutional animal house and placed at 22±2° temperature and 12 h light/dark cycles. The animals were further divided into 3 groups (n=6). To induce toxic demyelination in rodents, a dose of cuprizone was used between 0.4 to 2 % as reported in previous studies[32-34]. In the present research work, 1 % cuprizone dose was given after mixing it with powdered rat chow for a period of 4 w to develop toxic demyelination. The treatment group received DMF-loaded CS-DS nanoparticles at a dose equivalent to 50 mg/kg of DMF orally, from the very first day of study along with cuprizone.

Locomotor activity:

Spontaneous locomotor activity of rodents was studied with the help of actophotometer. Wistar rats from above 3 groups (n=6) were placed in actophotometer individually and their basal motor behaviour were recorded and compared.

Motor coordination (rotarod test):

The rotarod test equipment consists of a drum having diameter 7.0 cm attached with a rod. This rod was rotated with 20 revolutions/min. Rats (n=6) from each group were placed on the rotating rod and the number of falls from the rotating rod was counted individually for 5 min[35,36].

Open field test:

Open field test apparatus consisted of a plywood having dimension 72×72×42 cm, and was open from both the sides. The floor of plywood was equally divided into 25 squares. Wistar rats (n=6) from each group were kept at one end of the open field apparatus and number of squares crossed by individual rat in 3 min was counted.

***In vivo* pharmacokinetic study:**

Rats weighing around 200 g and 5-6 w old were randomly selected for oral pharmacokinetic experiments. These rats were fed with standard laboratory pellet diet and pure water *ad libitum*. The rats were divided into two groups (n=6) and first group was treated with pure DMF (50 mg/kg) and the second group was treated with CS-DS nanoformulations with a dose equivalent to 50 mg/kg pure DMF. At predetermined time intervals 0.5 ml blood samples were withdrawn from the retro orbital plexus and mixed with heparin solution to prevent clotting. Plasma was separated by centrifugation from the collected blood samples. The content of both DMF and its active metabolite MMF were determined using reversed-phase-HPLC[37-39].

Stability studies:

Stability study of CS-DS nanosuspension loaded with DMF was carried out to ensure its future commercial viability. The nanodispersion was packed in amber colored glass bottles and were stored at 2-8°, ambient condition and at (28±4°) for 90 d. At regular time intervals (1, 30, 60 and 90 d) samples from the stored formulations were withdrawn and their average particle size and residual drug content was analysed. The results of the stability study were subjected to analysis of variance to determine statistical significance ($p<0.05$).

RESULTS AND DISCUSSION

CS and DS are polymers having opposite charges are the basic reason for cross linking. The charge ratios per mole of both the polymers were calculated and effect of this variable on average particle size and

zeta potential was studied. At a particular weight ratio and calculated charge ratio of both the polymers, total 5 batches of nanoparticles were formulated. The results of z-average, PDI and zeta potential of all the prepared batches are listed in the Table 1. CS and DS both the polymers having opposite charges were used for preparation of polymeric nanoparticles and these particles were stabilized by Tween 80. Ionotropic gelation method was used to fabricate CS-DS nanoparticles. Effect of polymer concentration on particle size was studied and it was observed that as ratio of CS polymer in nanoformulation increases, particle size decreases. In general very small particle size was found with very low ratio of CS:DS. Charge ratio also effects significantly the z-average and zeta potential of formulation[40]. It was observed that as charge ratio increases z-average of particle and zeta potential value also increases.

In the present study, CS-DS nanoparticles prepared were characterized and the values of average particle size, PDI and z-average of DMF-loaded CS-DS nanoparticles were found to be 324 nm, <0.367 and -34.85 mV, respectively. The % entrapment efficiency of formulation was calculated to be 65.36 % and the drug loading was found to be 28 %. Better entrapment efficiency with a good drug load confirms a good drug carrying capacity of nanoformulation, which is one of most desirable characteristics of CS-DS nanoparticles. TEM microphotograph (fig. 1) also confirmed that the particles are smooth and spherical with non-aggregation[41]. Zeta potential value predicts the kinetic stability of colloidal dispersions, which is an indication of balance between attractive and repulsive forces of suspended particles. Colloidal suspensions with zeta potential values less than –30 mV and more than +30 mV are generally considered as stable. Thus the present colloidal dispersion with a zeta potential value of –34.85 mV also possessed a good kinetic stability.

TABLE 1: FORMULATION AND CHARACTERISATION OF VARIOUS BATCHES OD CS-DS NANOPARTCLES

Batch No	Weight ratio of CS:DS	Charge ratio of Polymers	Particle Size±SD (nm)	PDI±SD	Zeta potential ±SD (mV)
1	5:5	1.12	723±35	0.847±0.08	24.12±1.2
2	5:2.5	0.57	940±43	0.763±0.02	32.24±0.8
3	5:7.5	1.45	514±21	0.542±0. 03	-19.15±1.5
4	5:10	2.25	364±15	0.412±0.07	-28.4±2.2
5	5:20	4.47	320±8	0.367±0.03	-35.23±2.4

CS is chitosan, DS is dextran sulphate, PDI is polydispersity index, SD is standard deviation for n=3 observation

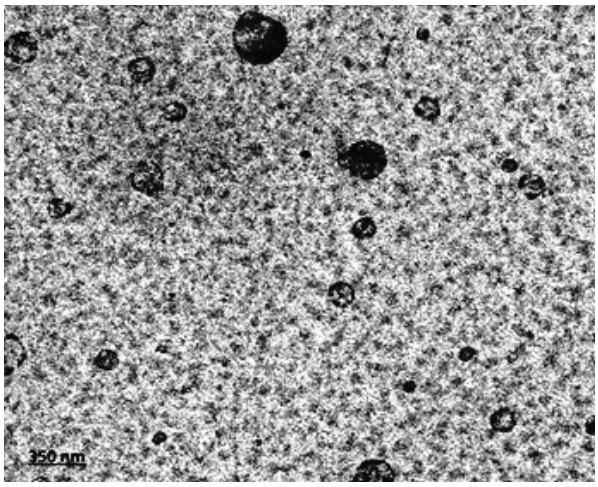

Fig. 1: TEM microphotograph of CS-DS nanoparticles

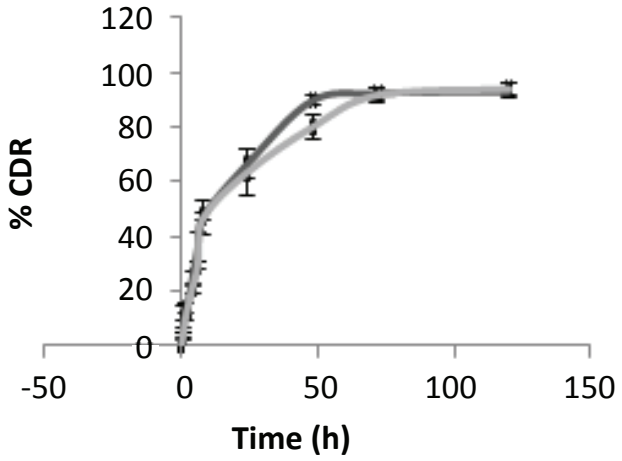

Fig. 2*: In vitro* drug release profile
(—) Avg (HCl), (—) Avg (PBS), CDR: cumulative drug release

In vitro drug release profile confirmed a sustained release pattern with a % cumulative drug release of 84 % over a period of 24 h, which is represented in fig. 2.

It was clearly evident from the MTT assay that on SH-SY5Y neuroblastoma cells, cytotoxicity increased with increase in H_2O_2 concentration (Table 2). The H_2O_2 dose used was 250 µM, which showed 60 % cell death. Treatment of SH-SY5Ycells with DMF-loaded CS-DS nanoparticles impacted the viability of these cells significantly. There was a significant increase in % cell viability among treated cells as compared to the cells treated with H_2O_2 alone (fig. 3). The % cell viability was increased at different concentrations of pure DMF and DMF-loaded CS-DS nanoparticles. A dose equivalent to 12.5 µM had shown maximum cell viability of 85.21 %. The DMF-loaded colloidal formulation demonstrated neuroprotective potential in H_2O_2-induced SH-SY5Y cell apoptosis. The results of study were analysed and compared by means of

ANOVA ($p<0.05$) and it was found that the cell viability was significantly increased.

Cuprizone is a copper chelator and this agent was used to induce toxic demyelination in rodents[41]. The motor behavioural scores, grasping ability and forelimb strength were found to be significantly higher in cuprizone model of Wistar rats after treatment with DMF-loaded CS-DS nanoformulations (Table 3). Decreased number of falls from rotarod also confirms a better grasping ability among treated groups of rats. Similar results were recorded in open field test as treated rats were crossed significantly more number of squares as compared to diseased groups as shown in fig. 4. *In vivo* study results were evaluated by ANOVA ($p<0.05$), which confirms the significant effectiveness of DMF-loaded CS-DS nanoformulation in cuprizone model of Wistar rat for MS.

The oral pharmacokinetic parameters of pure DMF and DMF-loaded CS-DS nanoparticles were calculated and tabulated in Table 4. CS-DS-loaded nanoparticles showed an improved pharmacokinetic profile and the pharmacokinetic values of C_{max}, T_{max}, and area under the curve were found to be significantly higher for DMF-loaded CS-DS nanoparticles, which indicated a faster onset of action and a long absorption phase of the present polymeric colloidal particles. The half-life was also found to be increased 4 folds in the present nanocolloids providing a higher systemic bioavailability.

TABLE 2: EFFECT OF H_2O_2 CONCENTRATION ON % CELL VIABILITY

H_2O_2 (μM)	% Cell viability
0	100
50	73.2
100	63
150	66.3
200	51.2
250	40.5
300	31.9

SD is standard deviation for n=3 observation

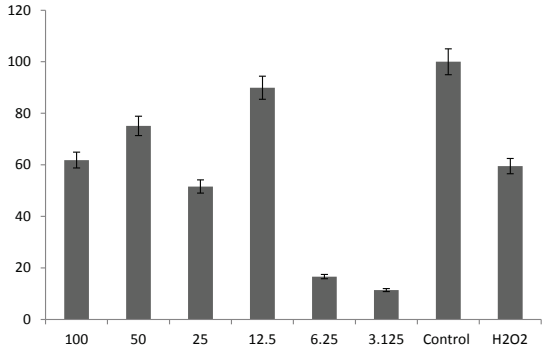

Fig. 3: Effect on DMF concentration on % cell viability (■) Cell viability

TABLE 3: LOCOMOTOR SCORE AND FALL OFF TIME

Group	Locomotor score±SD	Time to fall from rotating rod (seconds±SD)
Control	195.12±11.24	112.34±8.12
Diseased	107±12.36	56±10.45
Treatment	164±2.45	82±11.87

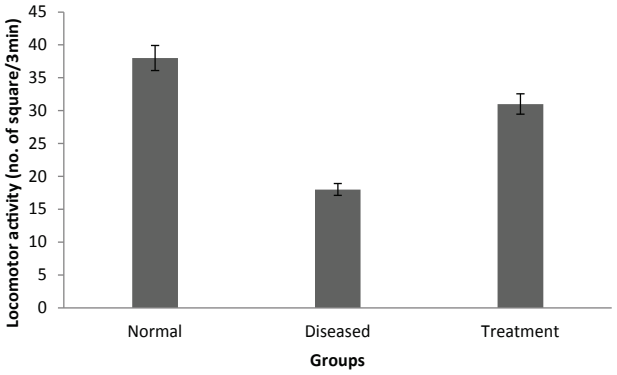

Fig. 4: Locomotor activity score

TABLE 4: PHARMACOKINETIC PARAMETERS

Parameters (unit)	Pure DMF	DMF-loaded CS DS nanoparticles
C_{max} (ng/ml)	1054.51	1290
T_{max} (h)	1.58	4
K_E (h^{-1})	0.187	0.0940
K_a (h^{-1})	1.28	5.80
$t_{1/2}$ (h)	3.5	7.37
$[AUC]_0^\infty$ (ng/ml.h)	7398.84	18528.5
$[AUMC]_0^\infty$ (ng/ml.h^2)	94249.66	335625.24
MRT (h)	12.74	18.114

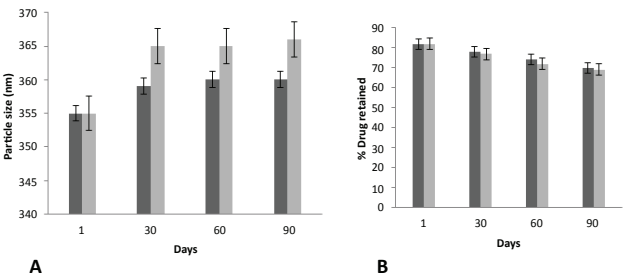

Fig. 5: Effect of storage time on (A) particle size and (B) % drug retained
(■) 2-8°, (■) 28±4°

The stability study results of nanoparticles are depicted in the fig. 5A and B. There was almost negligible changes in the parameters of z-average and % drug content with storage at 28° for 90 d. From the result of stability study it could be noted that the present formulation is stable on storage. The neuroprotective

ability of developed polymeric nanoparticles was studied on SH-SY5Y cells and was further evaluated to access its pharmacodynamic potential using cuprizone model of Wistar rats and the formulation has proved its neuroprotective potential.

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Conflicts of interest:

The authors declare that there is no conflict of interest.

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