Supplementary Information of Manuscript entitled ""Inflammation reduction potential of Nanostructured Lipid Carriers encapsulated with Rat's Bone Marrow cells' lysate."

Matrials and Methods:

Animals and their maintenance

In this study, healthy *Sprague dawley* rats were used, which were housed under the standard living environmental state, *i.e.,* with the provision of a 12-hour light/12-hour dark schedule, with unrestricted availability of water and standard rat food. All experimental procedures were in compliance with the Guide for the care and use of laboratory animals by the USA National Institutes of Health (NIH publication number 85-23).

Cell Culture: Isolation, harvesting, and culturing of BMSCs

The method of Sharif et al. (2007) was followed; the BMSCs were isolated from the Sprague dawley rats' tibia and femur after flushing their bone marrow. Suspension of isolated cells was centrifuged and the pellet was re-suspended in high glucose Dulbecco's modified Eagle's medium (DMEM) and plated in 25 cm2 culture flasks. 1% of streptomycin, 3.7 g/L of sodium bicarbonate (NaHCO3), 20% of fetal bovine serum (FBS), and 0.1% of penicillin-G were added in the cultured medium. Freshly isolated cells were kept in an incubator at 37°C with 5% CO2. Unattached cells were removed after incubation for 24 hours. The used medium of cells was changed every two days. To change the medium, the old medium was discarded and cells were carefully washed with phosphate-buffered saline (PBS) (3 times). Fresh medium was added. When cells reached 70-80% confluency, the cells were subcultured. For subculturing, the cells were washed with PBS and were detached using 0.05% trypsin-EDTA. The detached cells were centrifuged at 2000rpm for 5 minutes. The pellet was re-suspended in medium. The cells were kept and grown till passage 2.

Preparation of BMSCs lysate

BMSCs in the 2^{nd} passage was used to prepare cell lysate. BMSCs lysate was prepared by the osmotic breakdown of these BMSCs in a sterile centrifuge conical tube using deionized water. At passage 2, the fibroblast like stromal cells were detached using 0.05% trypsin EDTA. The detached cells were centrifuged at 2000rpm for 5 minutes. The pellet was re-suspended in deionized water for 30 min to allow osmotic rupture of cells. After three cycles of freeze-thaw at the temperature of -80 degrees, it was centrifuged at 1000g , and the supernatant was collected as BMSCs lysate. Absence of viable cells were checked via trypan blue staining of lysate.

Preparation of Nanostructured Lipid Carrier (NLC) and NLC loaded BMSCs lysate

NLC was prepared by a nanotemplate engineering technique used by Kim *et al.* (19), making minor changes Arshad *et al*. (20). The mixture, Span 60 (0.6mg), Miglyol (1mL), Tween 80 (3mL), Polyethylene glycol (PEG-400) (6mL), and stearic acid (2mg), was liquefied at 70°C in a pre-heated water bath. The hot sterile water (10mL) for injection was supplemented with the prepared mixture. To form NLC, the mixture was continuously stirred at 70°C for a duration of 40 minutes at 700 rpm. The obtained nanoemulsion was incubated with demineralized water (5mL) and BMSCs lysate (5mg/mL) for 5 mins to form the lysate-loaded NLC. In order to solidify the lipid core, the temperature of NLC was reduced to 4°C. To remove large particles, NLC was filtered using a 0.45µm PVDF filter. Blank and loaded NLC were stored at 4°C to use in subsequent experiments.

Particle size analysis & zeta-potential

The size analysis was carried out by using the Zetasizer Nano (ZS-90, Malvern Instruments, Malvern, UK). Samples were put inside the Zetasizer cuvettes, and readings were taken for polydispersity index (PDI) and size analysis. Aliquots of 10 µL of samples were diluted in 990 µL of PBS (pH 6.8, 10 mM) with 137 mM sodium chloride (NaCl) for measuring the size of particles while samples were diluted in distilled water (DW) for measurement of zeta potential.

Scanning electron microscopy (SEM)

The micromorphological analysis of NLC and NLC loaded BMSCs lysate was done using the scanning electron microscope (machine: JSM-7400F). After dilution and sonication, the samples were analyzed at 25°C. After that, the samples were placed on the grid. The dried samples were observed under the microscope.

Enzyme-linked immunosorbent assay (ELISA)

Solid-phase sandwich ELISA was used to confirm the loading of BMSCs lysate in NLC. For this purpose, the levels of IL-6 and VEGF were measured in NLC, BMSCs lysate, and NLC loaded BMSCs lysate following the methodology of Wajid *et al.* (22). Briefly, anti-IL-6 and anti-VEGF antibodies (product line of Santa Cruz Biotechnology, USA) were coated on a 96-well plate (Corning, USA) at 4°C for 48 hours. After washing with 1X tris buffered saline with 0.1% tween 20 (TBS-T) (three times), 10% BSA was used as a blocking solution for 30 minutes. Following blocking, the 100µL of each sample was loaded for 18 hours. After this, the samples were removed and washed thrice with TBS-T. Post-washing step HRP conjugated donkey anti-rabbit secondary antibody (product line of Santa Cruz Biotechnology, USA) was incubated at 4°C for 12 hours. Following washing, 3,3',5,5;- Tetramethylbenzidine (TMB) (product line of

Invitrogen, USA) was incubated for 20 minutes. To stop the reaction, 0.18M H_2SO_4 was used, followed by taking absorbance at 450 nm.

Culturing of cell line

NIH 3T3 (mouse fibroblast) cell line was obtained from the Cell and Tissue Culture lab established at the research center known as "Center for Research in Molecular Medicine (CRiMM)". The cryogenic vials of NIH 3T3 cells were thawed and were cultured in DMEM-HG (enriched with $NaHCO_3$ (3.7 g/L), streptomycin (1%), FBS (10%) and penicillin (0.1%)) Cultured cells were placed in an incubator at 37°C with 5% CO_2. When NIH 3T3 cells attained 70-80% confluency, sub-culturing was performed. To subculture, cells were 1X PBS washed for three times and attached cells were detached using 0.05% trypsin-EDTA. The detached cells were centrifuged at 2000rpm for 5 minutes. The pellet was re-suspended in the medium.

In-vitro H_2O_2 injury model

In-vitro inflammation of the cells was administered with the help of H_2O_2. As a preliminary step, for the optimization of the *in-vitro* H_2O_2 injury model for inflammation, NIH 3T3 cells plated into 96-well microplate and 100µl of different concentrations, *i.e.,* 2mM, 4mM, 6mM, 8mM & 10mM of H_2O_2 prepared in phosphate-buffered saline and further diluted with the serum-free medium were applied to cells for two hours. After 2 hours, culture media was substituted with fresh plain (serum-free) media. Upon calculation of inhibitory concentration 50 (IC_{50}) of H_2O_2, this concentration was used for inducing *in-vitro* injury/inflammatory conditions in the subsequent experiments.

MTT cell viability assay

MTT cell viability/cytotoxicity assay was undertaken to evaluate the cytotoxicity of various concentrations, *i.e.,* 2mM, 4mM, 6mM, 8mM & 10mM of H_2O_2 and further calculations of IC_{50} of H_2O_2. This assay was also applied to detect the proliferative potential of various concentrations (500ug/µL, 1mg/mL, 2mg/mL, 3mg/mL) of two treatments, *i.e.,* NLC, BMSCs lysate and NLC loaded BMSCs lysate after injury with H_2O_2 and further calculations of standardized viability calculations (SVC). After H_2O_2 injury for 2 hours, 100µl of different treatments were given as mentioned above for 24 hours. Cells were PBS washed and exposed to serum-free DMEM (100 µL) and 5mg/mL 3-(4, 5-dimethylthiazol-2- yl) -2, 5-diphenyltetrazolium bromide (MTT) (Invitrogen Inc., USA) solution in PBS for 2 hours. After the formation of purple coloured formazan crystals, 10% of sodium dodecyl sulfate (SDS) (Invitrogen Inc., USA) solution in PBS was added to solubilize the crystal. Absorbance was taken at 570 nm. The percentage of cell viability was derived by using the following formula:

$$\%\ viability = (Sample\ OD\ at\ 570nm \div Control\ OD\ at\ 570nm) \times 100$$

For all subsequent experiments, the SVC value of each group was used.

Immunocytochemistry

Immunocytochemistry was also carried out following the procedure explained by Wajid *et al.* (24) on NIH 3T3 cells of different treatment groups. Briefly, 4% paraformaldehyde (PFA) was used to fix untreated and treated NIH 3T3 cells followed by blocking with BSA (5%). Incubation in primary antibodies (product line of Santa Cruz Biotechnology, USA), rabbit polyclonal anti-p53, anti-Annexin V and anti-VEGF was done for 1.5 hours. After washing with TBS-T (3 times), incubation in secondary antibody; tetramethylrhodamine (TRITC) tagged donkey anti-rabbit (Santa Cruz Biotechnology, USA) was done for 1.5 hours. Post-incubation with secondary antibody, Fluorescent dye 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI, 1µg/mL) was used to stain nuclei for 15 minutes. The stained NIH3T3 cells were observed under Floid cell imaging station.

Antioxidants enzyme evaluation

Antioxidants, ascorbate peroxidase (APX), glutathione reductase (GR), and superoxide dismutase (SOD) assays were performed in the pre-treated and post-treated medium according to the method explained by Shamim and Rehman (25).

Ascorbate Peroxidase (APX) Assay

The APX assay was also performed on all groups of NIH 3T3 cells in a 96-well plate. APX reaction mixture contains 2.5mM ascorbate, 25mM potassium dihydrogen phosphate (KH_2PO_4) buffer (pH 7.0) and 75mM H_2O_2. Used medium of different cells group was added to make 1mL reaction mixture. It was kept in light for 3 minutes and optical density (OD) was taken at 290 nm.

Glutathione Reductase (GR) Assay

GR assay was performed in a 96-well plate with a reaction mixture of 200µLin each well for all NIH 3T3 cells groups. GR reaction mixture consisted of 10mM oxidized glutathione, 20mM KH2PO4 buffer (pH 7.5) and 40mM EDTA. Secretome was added to this mixture. 20mM NADPH was added and absorbance was measured at 340 nm after 3 minutes.

Superoxide Dismutase (SOD) Assay

SOD assay was also estimated in 96-well plate. The 1.5mL mixture was prepared by addition of 13mM methionine, 2.25mM nitro-blue tetrazolium chloride (NBT), 100mM KH2PO4 buffer (pH 7.8), 60µM riboflavin and 0.1mM EDTA. Secretome was added to this mixture. It was given 10 minutes of light exposure. OD was taken at 560 nm.

Carrageenan-induced rat paw oedema

To check the effect of lysate on reducing inflammation/oedema, carrageenan was injected into the hind-paw to induce oedema following the procedures by Winter *et al.* (26). Rats were divided into seven groups, as shown in table 2. Briefly, to induce oedema in rats, all experimental groups except normal and vehicle control groups were injected with 1U of newly prepared solution of 1% (w/v) carrageenan (Sigma) into the sub-plantar area of the rat's left rear paw. The volume of the rear paw of all experimental groups was measured before carrageenan injection and then at subsequent hours 1, 2, 3, 6, and 24 after carrageenan injection.

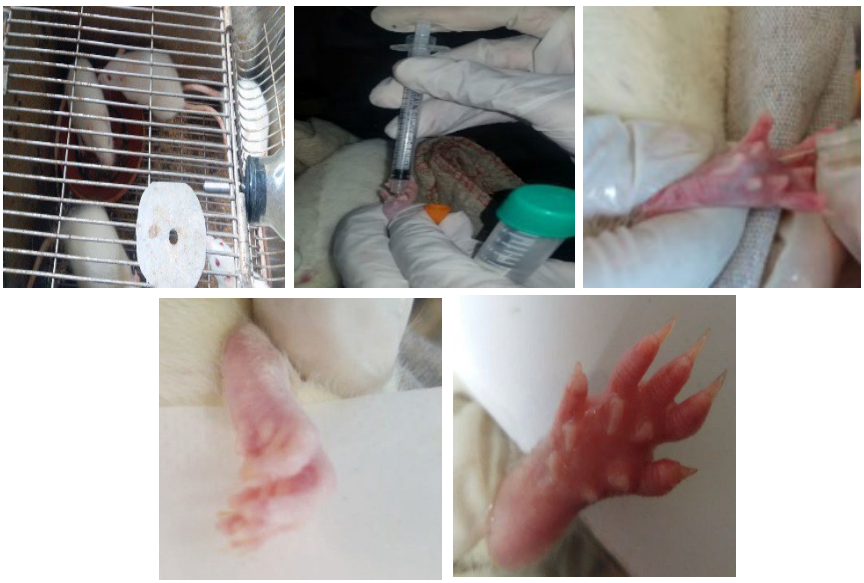


Figure 1: Photographic images of the *in-vivo* inflammatory assay experimental process.

Results:

To induce *in-vitro* inflammation in NIH 3T3 cells via H_2O_2, the inhibitory concentration (IC_{50}) value of H_2O_2 was assessed via MTT assay as a preliminary viability assay. NIH 3T3 cells received H_2O_2 in various concentrations 0 to 10mM for about 2 hours. As the concentration of H_2O_2 increased, the viability of NIH 3T3 cells reduced (see below Figure). As in below Figure, it was found that exposure of 6.628 mM H_2O_2 for two hours causes' 50% death of NIH 3T3 cells. So, this dose of H_2O_2 was used in further experiments to induce *in-vitro* injury and evoke inflammation *in-vitro*.

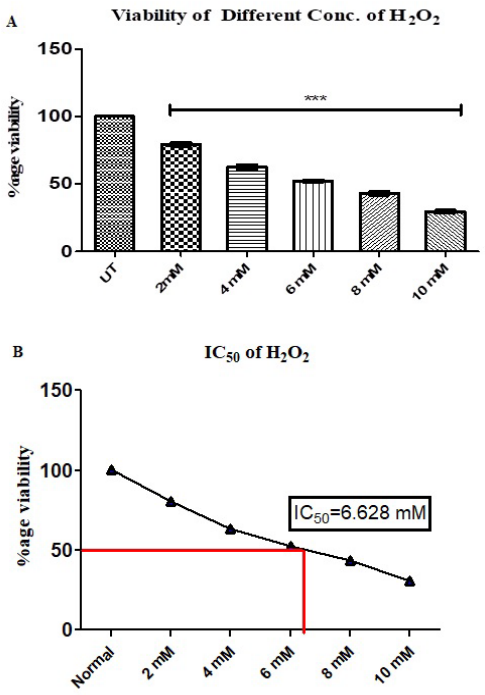


Figure: Cytotoxicity analysis and IC_{50} values of H_2O_2 on NIH 3T3 Cells via MTT assay. (A) Represents cytotoxicity of various concentrations (2mM, 4mM, 6mM, 8mM, 10mM) of H_2O_2. Data shown as mean ± standard error mean (SEM) where ***$P < 0.001$ (as established by using one-way ANOVA) (B) shows Inhibitory concentration 50 (IC_{50}) value of H_2O_2 on NIH 3T3 cells.

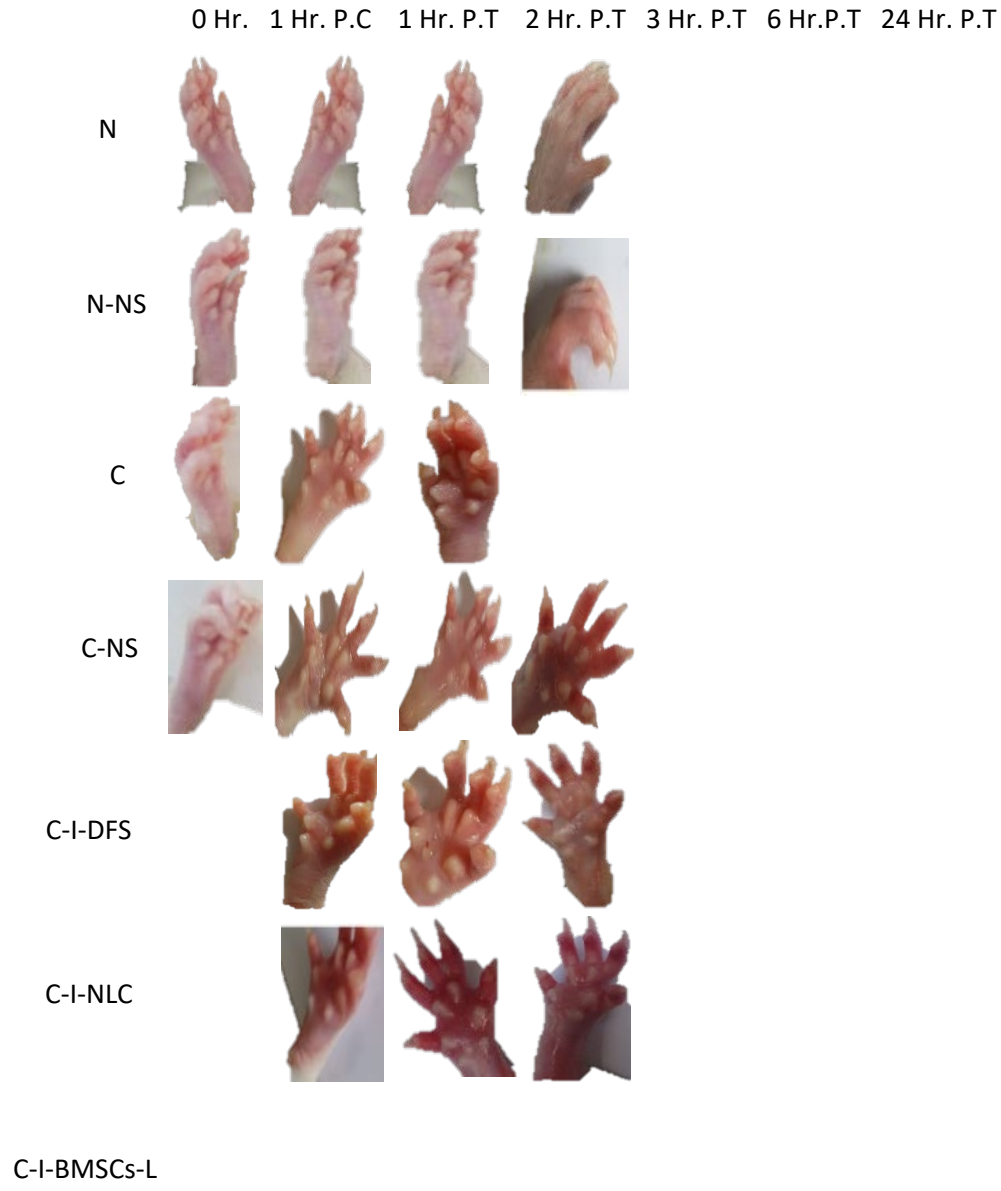
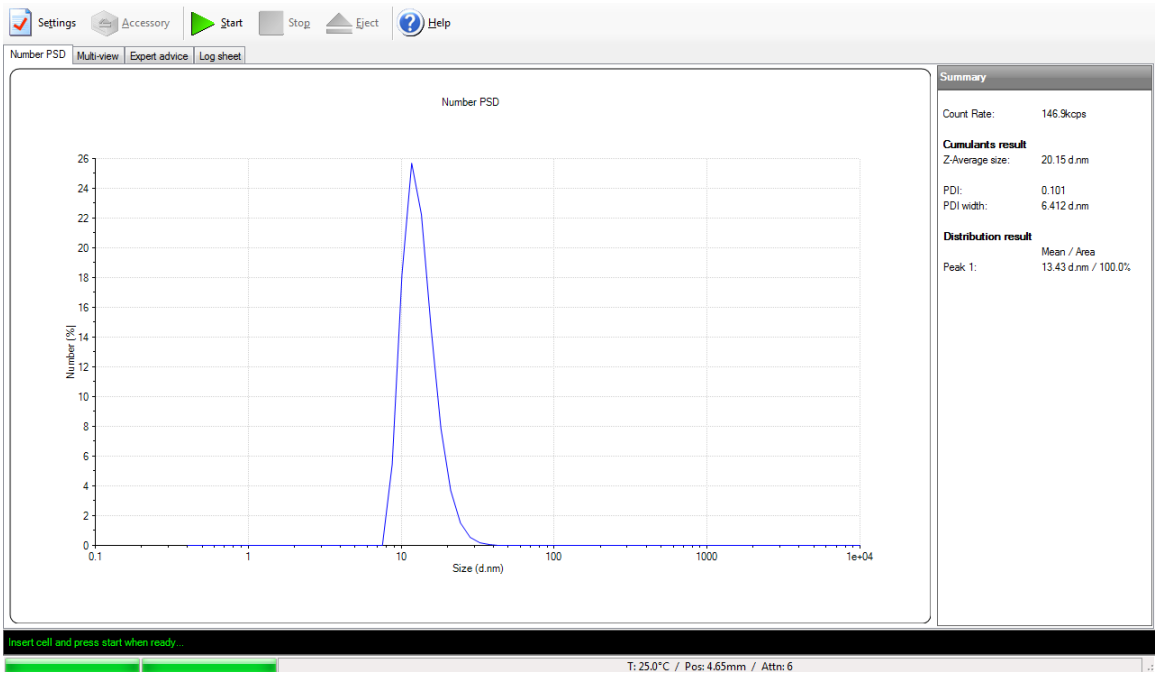


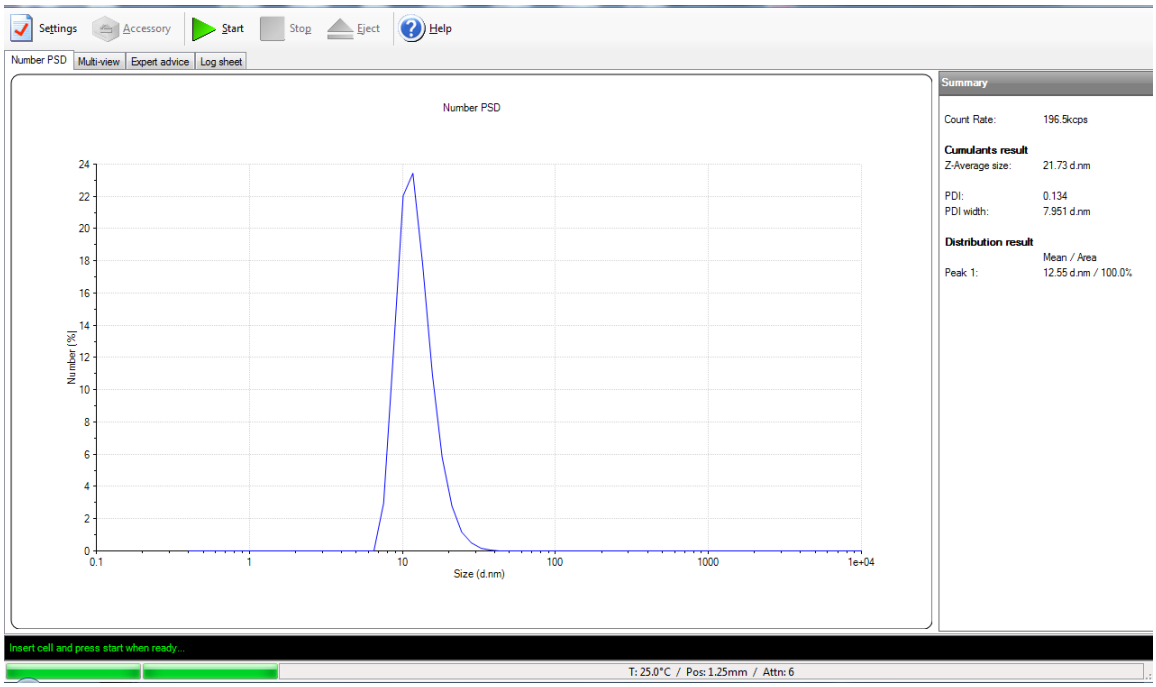
Figure: Inhibition of inflammation at different time-intervals in the carrageenan-induced rat paw oedema model. Effect of different treatment groups, *i.e.,* normal (N), normal rat paw injected with normal saline (N-NS), carrageenan injected group (C), carrageenan injected with normal saline group (C-NS), carrageenan injected with diclofenac sodium group (C-I-DFS), carrageenan injected with nanostructured lipid carrier group (C-I-NLC), carrageenan injected with bone marrow-derived mesenchymal stromal cells (BMSCs) lysate group (C-I-BMSCs-L)

and carrageenan injected with NLC loaded BMSCs-lysate group (C-I-NLC-BMSCs-L); on hind paw oedema at various hours 0, 1 2, 3, 6 & 24 hours). Where P.C stands for post-carrageenan and P.T is post-treatment.

Size Distribution Curve for NLC



Size Distribution Curve for NLC loaded BMSCs lysate



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