Supplementary Materials

Nordihydroguaiaretic acid microparticles are effective in the treatment of osteoarthritis

Authors

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Materials

Chemicals, polymers and other biological actives: Poly (D, L-lactic-co-glycolic) acid (PLGA, 50:50) of different molecular weights from 10-15 kDa (AP041), 85-100 kDa (AP036), and 190-240 kDa (85:15) (739979) with carboxylic acid end groups were purchased from Akina (West Lafayette, Indiana, USA) and Sigma (St Louis, MO, USA) respectively. Poly (vinyl alcohol) (PVA, 87~89% hydrolyzed, Mw 13,000~23,000kDa) was purchased from Sigma (363170) (St Louis, MO, USA). NDGA (or Sirolimus, 99.5% purity) was procured from Sigma (74540) (Ward Hill, Massachusetts, USA). Insulin/ transferrin /selenium (ITS) purchased from Gibco (Carlsbad, California, USA) were used for micromass culture of human chondrocyte cell line (C28/I2). All other

chemicals were procured from Sigma Aldrich and Thermo Scientific as analytical grade and used as received. Surgical sutures (poly (glycolic acid) based absorbable sutures with ¾ reverse cutting-edge needle) were obtained from Dolphin sutures (Futura Surgicals, India).

Antibodies used: Rabbit Anti MMP-13 polyclonal antibody (Abcam, Cambridge, UK, ab39012), Rabbit Anti ADAMTS-5 polyclonal antibody (Abcam, Cambridge, UK, ab41037), Rabbit Anti p19ARF polyclonal antibody (Thermo Fischer, Waltham, Massachusetts, USA, PA1-30670), Rabbit Anti LC-3B polyclonal antibody (Thermo Fischer, Waltham, Massachusetts, USA, PA1-16930), Rabbit Anti LOX-15 polyclonal antibody (Thermo Fischer, Waltham, Massachusetts, USA, PA5-77049), Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Abcam, Cambridge, UK A11008), HRP conjugated goat anti-rabbit secondary antibody (Thermo Fischer, Waltham, Massachusetts, USA, 31460).

Mice: All the animal experiments were approved by the Institutional Animal Ethics committee (IAEC) (CAF/Ethics/612/2018 and CAF/Ethics/808/2020). IAEC guidelines were followed for the design, experimentation, and analysis of animal experiments. Mice were housed at the Central Animal Facility, Indian Institute of Science (IISc) in individually ventilated cages under monitored temperature and humidity with automated 12 h light-dark cycles. Mice were fed with standard laboratory food and water. Female wild-type (WT) mice of C57BL/6 strain (6-7 weeks) were used for intra-articular injection studies, and male wildtype (WT) mice of C57BL/6 strain (6-7 weeks) were used for OA studies.

Human cartilage samples: All the human *ex-vivo* experiments were approved by the Institutional Human Ethics committee (IHEC) (02/31.03.2020) and MS Ramaiah Medical College, Ethics Committee (MSRMC/EC/AP-06/01-2021). IHEC and MSRMC/EC guidelines were followed to design, experiment, and analysis of human experiments. After obtaining informed consent, human knee joint surfaces from the femur and tibia were obtained from patients undergoing total knee replacement due to end-stage OA from Ramaiah Medical College and Hospital. The information obtained by the analysis of the samples was always treated under confidentiality. The details about the volunteers used in the study are listed in **Table S2.**

Methods

Synthesis of PLGA microparticles: PLGA microparticles were synthesized from PLGA polymer of various molecular weights, using the single emulsion technique described earlier. Briefly, 100 mg PLGA of molecular weights: 10-15 kDa, 85-100 kDa and 190-240 kDa were dissolved in 2 mL of dichloromethane (DCM) with 1mg NDGA (NDGA-MP) or without NDGA (Blank-MP), and the homogenization was carried out in 1% polyvinyl alcohol (10 mL) at 12,000 RPM. This solution was added to 1% PVA (110 mL) and was allowed to stir continuously for 3–4 h to evaporate DCM completely. The solution was then centrifuged at 11,000 *g*, and the pellet was washed using deionized water twice to wash away the excess PVA. The microparticles were then resuspended in deionized water and rapidly frozen at −80 °C, followed by lyophilization. The lyophilized particles were used for *in vitro* and *in vivo* studies by making a suspension in 1x PBS according to required concentrations and were sterilized under UV for 20 mins. For determining the size of PLGA microparticles, 50 µg particles were dispersed in deionized water (1 mL) and sonicated briefly before analysis. Particle size distributions and zeta potential were determined using dynamic laser light scattering (Nano ZS ZetaSizer (Malvern Instruments, Worcestershire, UK)). For *in vivo* studies, we encapsulated 5 mg of NDGA in 85 – 100 kDa PLGA using the same method described above to deliver the therapeutic payload required for disease modification. The PLGA particles were loaded with Cy3 dye (50 µg) for imaging purposes while synthesising the particles.

Physiochemical characterization of the microparticle formulation

Encapsulation efficiency (EE): The amount of NDGA encapsulated in PLGA particles was estimated by plotting a standard curve. Different concentrations of NDGA were spiked in DMSO solution containing PLGA (10 mg mL^{-1}). The absorbance was read using SPL UV Max™ plate (33096) at 283 nm using a plate reader (Tecan™ Microplate Spectrophotometer). The NDGA-PLGA microparticles (10 mg mL^{-1}) were weighed and dissolved in DMSO, and the amount of NDGA encapsulated was determined from the standard curve. EE was calculated using the following equation:

$$Encapsulation\ Efficiency = \frac{Amount\ of\ NDGA\ encapsulated}{Total\ amount\ of\ NDGA\ added} * 100$$

Isolation of human articular chondrocytes from human knee joint surfaces: Knee joints excised during surgery were immediately placed in sterile containers with 1x PBS and immediately brought to the tissue culture facility. The human articular knee joint surfaces were briefly rinsed in 1x PBS (containing 1% penicillin-streptomycin) once before scraping the cartilage from the underlying bone surface into thin slices of 1-3 mm^3. These slices were placed in a 10 cm dish and were washed three times with 1x PBS. The slices were then incubated at 37° C for 30 minutes in DMEM-F12 media containing pronase (1mg mL^1). Slices were again rinsed with 1x PBS and were incubated at 37° C for 16- 18 h in DMEM-F12 media containing collagenase A solution (3mg mL^{-1}). After 16 – 18 h, the clumps were broken apart using a pipette, and the cells were pelleted down at 1000 *g* for 10 minutes. The cells were washed with 1x PBS thrice. Finally, the cells were resuspended in DMEM-F12 media, counted, and seeded at a density of $1x10^6$ cells mL^{-1}. The media was changed every two days, and the cells were subsequently sub-cultured and used for experiments within the first two passages. Transwell inserts were used in experiments in which the chondrocytes were treated with microparticles. The transwell setup was used to simulate the *in vivo* conditions wherein the chondrocytes lie embedded in the cartilage matrix, and the intra articularly injected particles do not come in direct contact with the microparticles.

WST assay: The human immortal chondrocyte cell line – C28/I2 (Merck) was used in this study. Cells were cultured in DMEM/F-12 (Ham) media (Invitrogen) containing 1 mM sodium pyruvate, 10 mM of HEPES, 140 mM of glucose. The media was supplemented with 10% fetal bovine serum (US origin, Sigma) and 1% antibiotic cocktail containing penicillin/streptomycin (Invitrogen) in an atmosphere of 5% CO2 and 37° C. Cell's metabolic activity was determined by Water Soluble Tetrazolium Salts -1 (WST) assay (Cayman Chemicals). Briefly, chondrocytes (C28/I2) were plated at 15,000 cells per well in a 96-well plate and allowed to adhere overnight. The next day, NDGA were added in increasing concentrations (30 µM, 10 µM, 3 µM, 1 µM, 200 nM, 40 nM, 800 pM), and the cells were incubated for 24 h or 48 h. After the incubation time, WST reagent was added, and the cells were incubated for four hours, followed by absorbance readings at 470nm.

Senescence induction assay: Primary human articular chondrocytes (HACs) were plated in a 24 well plate with 15,000 cells per well in the untreated group and 30,000 cells per well for other treatment groups. The chondrocytes (C28/I2) were plated in a 24 well plate with 7,500 cells per well in the untreated group and 15,000 cells per well for other treatment groups to maintain cells under sub confluency until the end of the experiment. Cells are plated at different numbers to maintain similar cell confluency at the end of the 48-h experiment since senescent cells have a lower population doubling time than untreated cells.

A genotoxic stress agent- BrdU (200 µM) or oxidative stress agent- hydrogen peroxide (H_2O_2) (200 µM) was given to induce senescence. The primary treatment groups were vehicle-treated cells (DMSO), BrdU (200 µM) or H_2O_2 (200 µM) treated cells, BrdU or H_2O_2 along with free NDGA (3 µM) co-treated cells, BrdU or H_2O_2 along with NDGA-MP (equivalent to 3 µM) co-treated cells, BrdU or H_2O_2 along with Blank-MPs co-treated cells. Colorimetric SA-β Gal activity was used to stain for senescent cells as previously described[1]. After exposing the cells to these stress agents for 48 h, cells were washed thrice with 1x PBS and then fixed with a fixative solution containing 2% formaldehyde and 0.2% glutaraldehyde in 1x PBS for 10 mins. Following fixation, cells were incubated in SA-β Gal staining solution (1 mg mL^{-1} 5-Bromo-4-chloro-3- indolyl-beta-d-galactopyranoside (X-Gal), 1x citric acid/sodium phosphate buffer (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM sodium chloride, and 2 mM magnesium chloride) at 37° C overnight. The enzymatic reaction was stopped after 16 h, and cells were washed three times with 1x PBS. Five random brightfield images were taken per well.

To automatically score the senescent cells, we developed a custom-built macros algorithm to score the senescent cells based on their size and the intensity of SA-β Gal staining. Bright-field images were taken from each treatment group (vehicle (1x PBS) treated cells, H_2O_2 treated cells, H_2O_2 and NDGA/NDGA-MP treated cells, etc.). The macros algorithm was used to count the total number of senescent cells.

Macros program code used in ImageJ:

Senescence quantification:

```
run("8-bit");

setAutoThreshold("Default");

//run("Threshold...");

setThreshold(0, 50);

setThreshold(0, 50);

run("Analyze Particles...", "size=4.00-Infinity show=Outlines display exclude
clear summarize");
```

Autophagy quantification:

```
run("8-bit");

setAutoThreshold("Default dark");

//run("Threshold...");

setThreshold(95, 115);

run("Analyze Particles...", "exclude clear summarize");
```

NDGA-MP treatment in senescence induction assays (Micromass culture): The HACs were seeded as a 15 µL suspension in growth media in a 24-well plate at a density of 2.5 x 10^7 cells mL^{-1}. The cells were allowed to adhere to the well plate for 3 h, after which growth media was added. After 24 h, the growth media was changed to differentiation media containing supplements (Insulin/Transferrin/Selenium, TGF-β (10 ng mL^{-1}), and ascorbic acid). Micromasses were treated with BrdU (600 µM) or H_2O_2 (100 µM) and co-treated with free NDGA (3 µM) or NDGA-MP (final concentration 3 µM). After 48 h of incubation, the micro masses were fixed using 4% formaldehyde followed by Alcian blue staining at pH<1 to stain the sGAG overnight. After 16 - 18 h, micro masses were washed with deionized water to remove any non-specific stains, followed by Alcian blue stain extraction using 6M Guanidine HCl. The extracted Guanidine HCl's absorbance was read at 630 nm using a plate reader (Tecan™ Microplate Spectrophotometer) to quantify the sGAG present in the micro masses after various treatments. Similar experiments were carried out with an incubation time of up to 8 days with BrdU or H_2O_2 and BrdU or H_2O_2 and free NDGA or NDGA-MP for eight days. Media was changed every two days and replenished with respective treatments.

Immunocytochemistry: After 48 h of treatment, the HACs were fixed using 4% PFA followed by permeabilization using 0.05% Triton X-100. The cells were then blocked using 5% non-fat dry milk and incubated overnight at 4° C with rabbit anti-LC-3 B polyclonal antibody (0.5 µg mL^{-1}). After 16 – 18 h, the cells were washed and incubated with Alexa Fluor 488 goat anti-rabbit IgG (H+L) (1 µg mL^{-1}) for one hour, followed by nuclear staining using DAPI (300 nM) for 10 mins. These cells were then visualized using FITC (495/519 nm) and DAPI (358/451 nm) channel using IN Cell Analyzer 6000 (GE Life Sciences) at 40x magnification. The obtained images were quantified for the LC3B puncta per cell using a custom-built automated macros algorithm in ImageJ. For counting LC3B puncta, the FITC channel images' threshold was adjusted to pick up and count the individual puncta. The same threshold values were used to analyse all the images in the experiment.

Measurement of 15(S)-HETE metabolite by enzyme-linked immunosorbent assay (ELISA):
The chondrocytes (C28/I2) after treatment with H_2O_2 and the co treatments with NDGA as free drug or as MP formulation were lysed using cell lysis buffer, followed by incubating with 18 µM arachidonic acid at 37 °C for 45 min. The reaction was terminated by adding 0.2 M HCl. The production level of 15(S)-HETE in cells was measured using the 15(S)-HETE EIA Kit (Cayman Chemicals).

The residence time of PLGA MPs in mouse knee joint: Female WT C57BL/6 mice (25g) were used for this study. PLGA microparticles of molecular weight 85 kDa - 100 kDa with Cy7 dye were suspended in 1x PBS at a concentration of 20 mg mL^{-1}. Ten µL of this formulation was injected in mice's left knee joints by intra-articular injections on Day 0. The contralateral legs were used as control and were given an equivalent dose of free Cy7 dye. The mice (n=5 per group) were anesthetized by isoflurane before

transfer in the imaging system (IVIS® Spectrum In Vivo Imaging System), and the fluorescence signal was measured with a predetermined exposure time. Imaging (fluorescence) was performed on Day 0 (pre-and post-injection), 1, 5, 7, 14, 21, 30, and 35 using the IVIS. Radiance efficiency (p s^{-1} sr^{-1} μW^{-1}) within a region of interest (ROI) was quantified by the IVIS® Spectrum Living imaging software.

Studying the effect of NDGA-MP on PTOA mouse model

PTOA Mice model: Male C57BL/6 mice (starting weight of around 25 g) were used for this study. Following intraperitoneal injection of ketamine (80 mg kg^{-1}) and xylazine (10 mg kg^{-1}) as a cocktail in sterile 1x PBS. A medial parapatellar incision was made to expose the knee joint capsule. The patella was displaced laterally, followed by transection of the medial meniscotibial ligament using surgical blade number 11 and scalpel. Following irrigation of the operated site with saline, the capsule and skin were sutured separately using absorbable PGA sutures. Sham groups received only an incision to expose the medial collateral ligament, then sutured back like the other DMM-operated groups. Postoperatively, the mice were allowed to move freely in the cage. Each experimental group was evaluated by gross morphological examination for swelling, pain, or change in gait of the animal post-surgery and during the entire duration of the experiment.

Classification of study:

Preventive Dosing Study

Group 1- DMM operated animals with no treatment (7 mice)

Group 2- Surgical control receiving no treatment (Sham) (5 mice)

Group 3- DMM operated with free NDGA injection (4 µg) (7 mice)

Group 4- DMM operated with free NDGA injection (400 ng) (7 mice)

Group 5- DMM operated with NDGA-MP injection (200 µg particles containing the equivalent of 1 µg NDGA) (7 mice)

Group 6- DMM operated with NDGA-MP injection (200 µg particles containing the equivalent of 100 ng NDGA) (7 mice)

Group 7- DMM operated with Blank-MP injection (200 µg particles) (7 mice)

Curative two dose Study

Group 1 - DMM operated animals with no treatment (7 mice)

Group 2 - Surgical control receiving no treatment (Sham) (7 mice)

Group 3 - DMM operated with free NDGA 2 dose injections (4 µg) (7 mice)

Group 4 - DMM operated with NDGA-MP 2 dose injections (200 µg particles containing the equivalent of 1 µg NDGA) (7 mice)

Curative single dose Study

Group 1 - DMM operated animals with no treatment (9 mice)

Group 2 - Surgical control receiving no treatment (Sham) (9 mice)

Group 3 - DMM operated with free NDGA 1 dose injection (4 µg) (9 mice)

Group 4 - DMM operated with NDGA-MP 1 dose injections (200 µg particles containing the equivalent of 1 µg NDGA) (9 mice)

In the preventive study, IA injection was administered on Day 7 after DMM surgery, followed by injections on Day 24 and Day 42 with euthanasia at Day 63. The injection was administered as a two-dose regimen (Day 24 and Day 42) and single-dose regimen (Day 31) after DMM surgery in the curative studies, followed by euthanasia on Day 63.

Histopathology: The joints were fixed in 4% paraformaldehyde for 24 h. The joints were embedded in paraffin after decalcification in 5% formic acid for five days. Five-micron sections were used for further staining and analysis.

Safranin-O Fast green staining: Five-micron sections were stained with safranin-O-fast green staining. The slides were deparaffinized with xylene and gradient alcohol. After a wash using running tap water for 5 min, slides were stained with fresh Wiegert's iron hematoxylin for 10 min. Following a 5-min wash in running tap water, slides were soaked in 0.1% safranin-O (10 min) and 0.05% fast green (5 min) in this order. After dehydration with alcohol and xylene, the slides were stained with safranin-O-fast green staining and mounted using DPX mountant. The processed slides were graded according to the OARSI guidelines provided as

osteoarthritis cartilage histopathology assessment system. A veterinarian blinded to the study examined the histopathological images and provided the appropriate scores.

Hematoxylin and Eosin (H&E) staining: The tissue sections of 4 animals from each group from the preventive dosing study were sent to Vetlesions Veterinary Diagnostic Laboratory for hematoxylin and eosin (H&E) staining. For each animal, two sections were used for imaging, and the synovial membrane in the medial region of the joints was captured. Three images of the synovial membrane at different locations along the length were captured, and subsequently, their thickness was measured at these different locations using MagVision® software.

Immunohistochemical studies: Sections were processed similar to Safranin O staining until the alcohol step followed by protease induced epitope retrieval using 0.5% Trypsin and 1% Calcium chloride in 1x TBST buffer (pH 7.2), followed by blocking using non-fat dry milk (0.8%). The slides were rinsed in 1x TBST and incubated with MMP-13, ADAMTS-5, LC3B, 15-LOX, and p19ARF antibodies (1:300) diluted in blocking solution at 4^{o} C for 16 – 18 h. The slides were again washed in 1x TBST followed by incubation with HRP conjugated secondary antibody (1:150) diluted in blocking solution at room temperature for two hours. The slides were rinsed, and DAB (3,3'-Diaminobenzidine) substrate was added and incubated for one hour. The slides were then washed, dried, and covered using mounting media before brightfield imaging. The number of cells positive in each image was quantified using an automated macros program in ImageJ software.

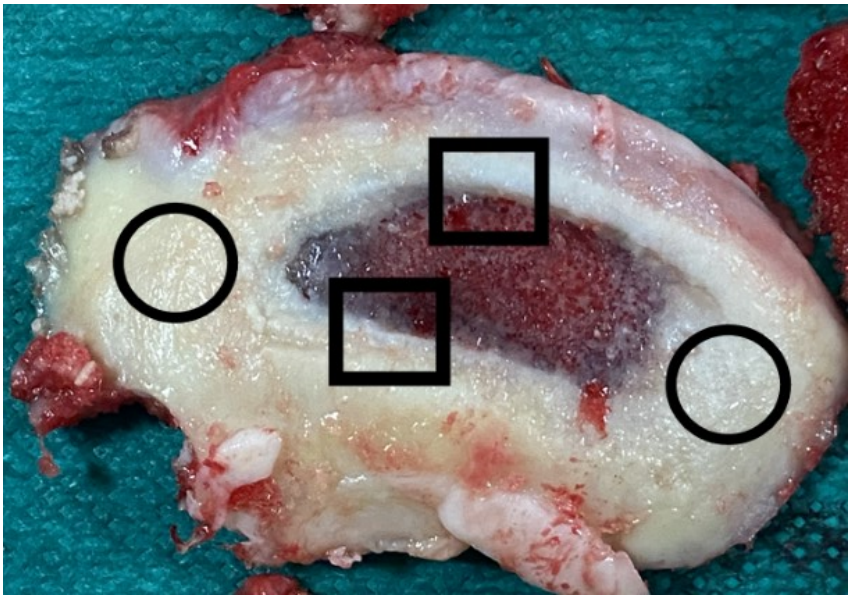
MicroCT experiments: Joints were extracted after euthanasia and scanned using Bruker SKYSCAN 1272. The joint was mounted in the instrument and 750 projections were acquired at source voltage 50 kV, current 200 µA, and voxel size 10 µm. Scans were evaluated and reconstructed using Scanco Medical evaluation software. 2D tomographs compatible with Scanco proprietary formats were generated by importing DICOMs from original scan data. The medial plateau was manually contoured in axial 2D slices, and two volumes of interest (VOI) were further defined for regional analysis: 1. subchondral bone and 2. medial marginal osteophytes. Subchondral bone VOIs were defined as the first axial slice that the medial tibial bone could be clearly distinguished (most proximal to the femur) and ended 2 slices prior to the proximal growth plate becoming visible. Outcome measures for subchondral bone analyses were subchondral bone volume and thickness. Osteophyte VOIs were defined as medial marginal tissue growths terminating before the posterior medial horn and manually contoured through the range established in the previous subchondral bone VOI. The primary outcome for osteophyte morphometrics was mineralized marginal osteophyte volume. Built-in Scanco evaluation scripts were used for all analyses and 3D reconstructions with a lower threshold of 105 (Gauss filter parameters: Sigma=1.2, Support=2).

Assessment of Pain: Joint nociception was evaluated using a Von Frey anesthesiometer (IITC, United States), which is used to assess mechanical sensitivity. Mice were placed on a polycarbonate mesh surface in an acrylic chamber in a temperature-controlled room and rested for 10 min before testing. The plantar surface of mice was vertically stimulated with anesthesiometer. The force required to provoke a paw-withdrawal reflex was recorded and measured in grams. The experiment was performed as three trials, and the average response was taken. A maximum force of 6 g was applied.

Alanine aminotransferase (ALT) measurement: Blood samples were collected from mice *via* the retroorbital route using hematocrit capillaries 24 hours post-dosing on day 42 of the preventive dosing study. Serum was isolated from the blood samples by centrifugation at 8000g, 5 minutes, 4°C. The resulting serum samples were then submitted to Vetlesions Veterinary Diagnostic Laboratory for analysis of alanine aminotransferase (ALT) levels.

Statistical analysis: All statistical analysis was performed using Graph Pad Prism 6. Data were presented as mean ± standard deviation. Non-linear regression (least square method) two-phase exponential decay curve was used to fit the NDGA release profile with constrains of maximum value as 100 and minimum value, 0. For the *in vivo* Cy7 PLGA MPs residence monitoring, a one-phase exponential decay curve was used to fit the residence times. Differences between groups were analyzed by t-test or one-way analysis of variance (ANOVA), and non-parametric data were analyzed using Mann Whitney U test or Kruskal Wallis test with Tukey's multiple comparison test, with $p < 0.05$ considered significant.

Post-hoc power analysis: To evaluate the study's ability to detect the observed reduction in OARSI score between the treatment and control groups, a post-hoc power analysis was done using G*Power software. The study included 7 mice in the free NDGA treated, NDGA-MPs treated, Blank MPs treated and untreated DMM group, and 5 in the sham group for the prophylactic study. The two-dose therapeutic study included 7 mice in each experimental group, while the single-dose therapeutic study had 9 mice. The significance level (α) was set at 0.05 for hypothesis testing. The observed effect size (Δ), indicated by a 60-75% reduction in OARSI scores, was noted in the NDGA-MPs treatment group compared to the DMM-operated group. The analysis revealed an average power of 0.96, indicating a 96% probability of detecting reduced hallmarks of OA and a lower OARSI score with the given sample size. Specifically, the prophylactic study, the two-dose therapeutic study, and the single-dose therapeutic study exhibited power levels of 1, 1, and 0.88, respectively, confirming that the study was adequately powered with a greater than 95% chance of detecting the observed effects between the treated and untreated groups.



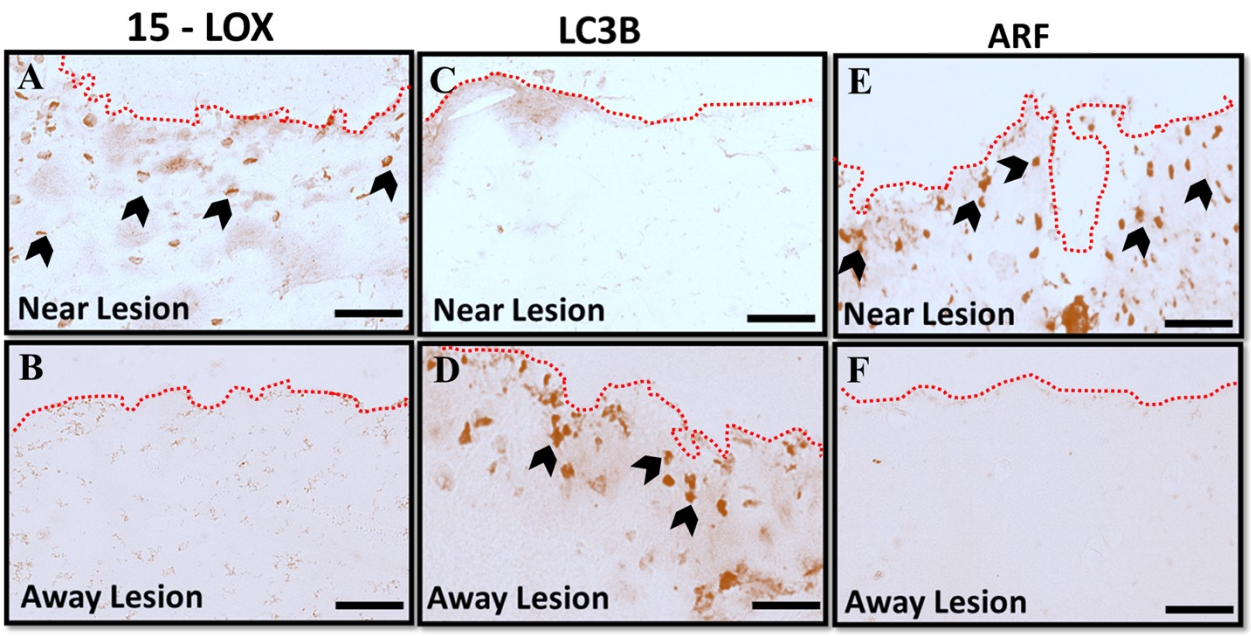


Fig. S1. Immunohistochemical studies of human osteoarthritic cartilage and characterization of NDGA microparticles. Top panel: Representative human tibial articular surface bearing lesions from OA patients. The rectangles represent the areas bearing OA lesions with involvement of subchondral bone, and the circles represent the non-lesioned areas used for IHC. **Bottom panel:** Human osteoarthritic cartilage sections stained with 15-LOX, **(A)** near the lesion, and **(B)** away from lesion. LC3B **(C)** near lesion and **(D)** away from lesion. ARF **(E)** near lesion and **(F)** away from lesion. The red dotted line denotes the articular cartilage surface. Black arrows specify the cells positive for the respective markers. The images represent data collected from three OA patients who underwent a total knee replacement. Scale bar, 25 μm.

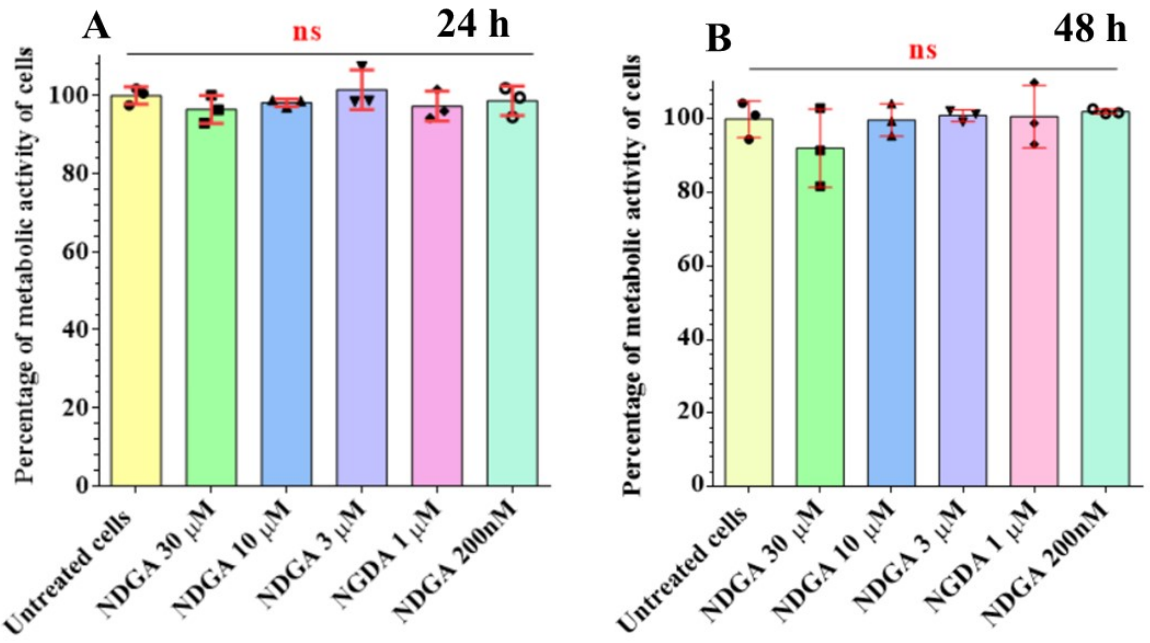


Fig. S2. Cytocompatibility of NDGA. Metabolic activity of chondrocytes treated with varying concentrations of NDGA for **(A)** 24 h and **(B)** 48 h.

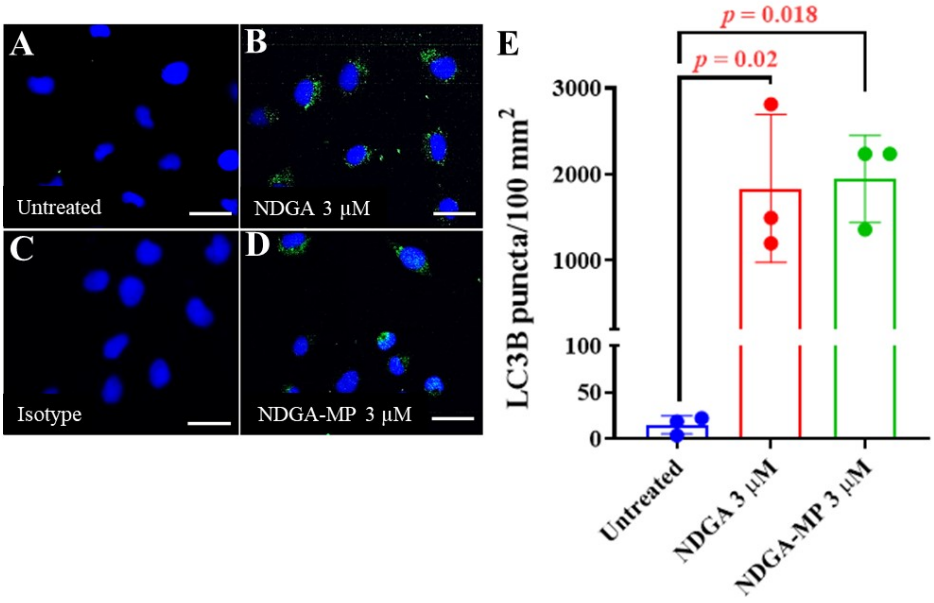


Fig. S3. NDGA-MP induce autophagy in chondrocytes. Fluorescence microscopy images of C28/I2 cells stained with DAPI and LC3B after (**A**) no treatment, **(B)** free NDGA (3 µM), **(C)** isotype antibody, and **(D)** NDGA-MP (3 µM NDGA) treatment. **(E)** Quantification of LC3B puncta per 100 mm^2 using ImageJ software (n = 3 per group). Data in graphs represent the mean ± s.d. and *p* values were determined by one-way analysis of variance (ANOVA) and Tukey's post hoc tests. *p*-value < 0.05 was considered significant. *ns* - nonsignificant; Scale bar, 20 µm

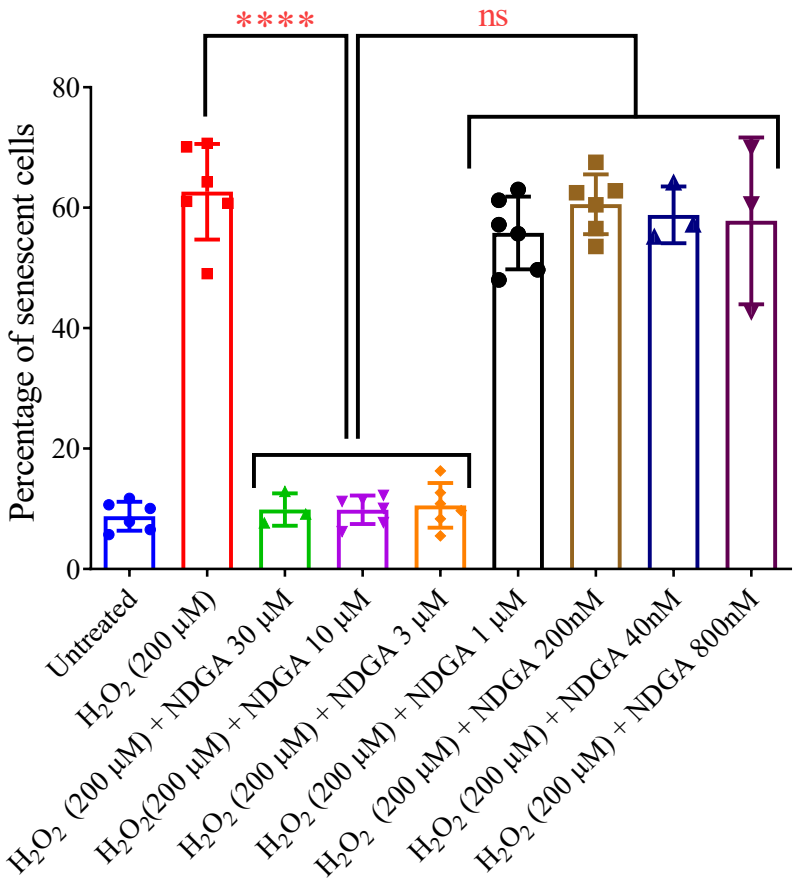


Fig. S4. Dose titration of NDGA in preventing senescence in chondrocytes (C28/I2) under oxidative stress. Percentage of senescent cells under oxidative (H_2O_2) stress condition (n = 3 per group). Data in the graph represent the mean ±, s.d. and *p* values were determined by one-way analysis of variance (ANOVA) and Tukey's post hoc tests. *p*-value < 0.05 was considered significant. *ns* - non-significant.

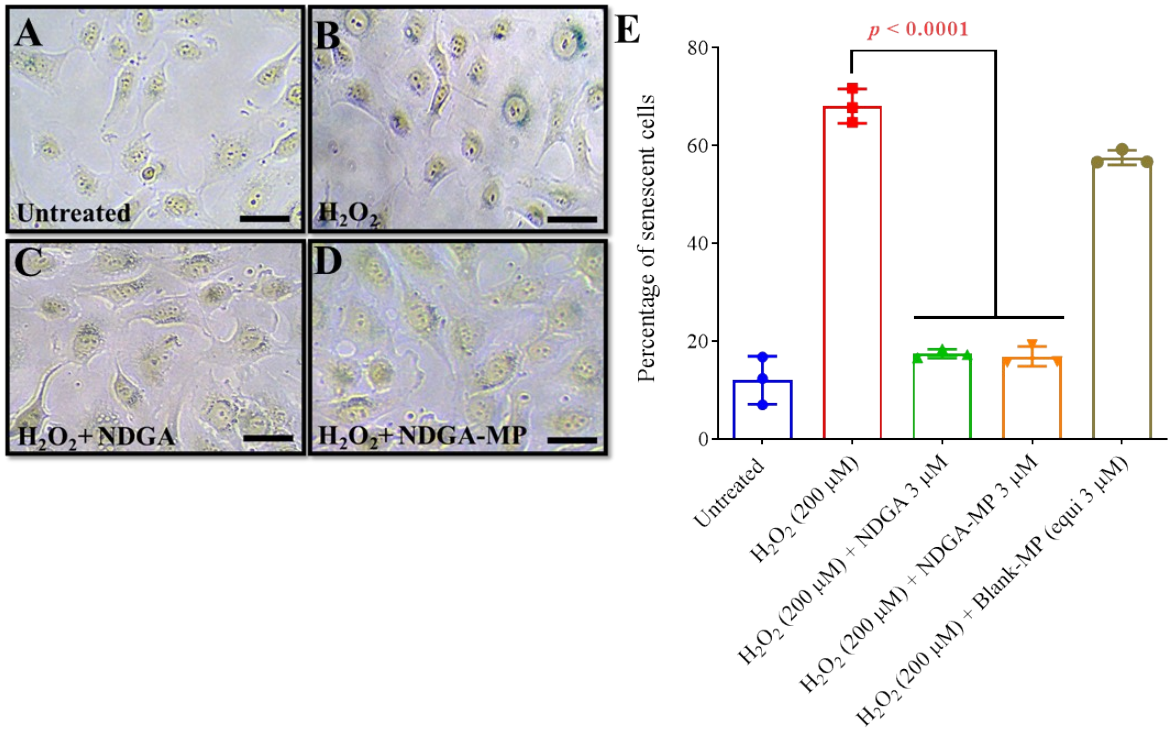


Fig. S5. NDGA-MP prevent senescence in chondrocytes (C28/I2) under oxidative stress. SA-β Gal-stained images of C28/I2 cells exposed to **(A)** no treatment, **(B)** oxidative (H_2O_2) stress, **(C)** oxidative (H_2O_2) stress along with free NDGA (3 µM) treatment and, **(D)** oxidative (H_2O_2) stress along with NDGA-MP (3 µM) treatment. **(E)** Percentage of senescent cells under oxidative (H_2O_2) stress condition (n = 5 per group). Data in the graph represent the mean ±, s.d. and *p* values were determined by one-way analysis of variance (ANOVA) and Tukey's post hoc tests. *p*-value < 0.05 was considered significant. Blank-MP-Blank Microparticles, NDGA-MP- NDGA loaded Microparticles. *ns* - non significant; Scale bar, 40 µm.

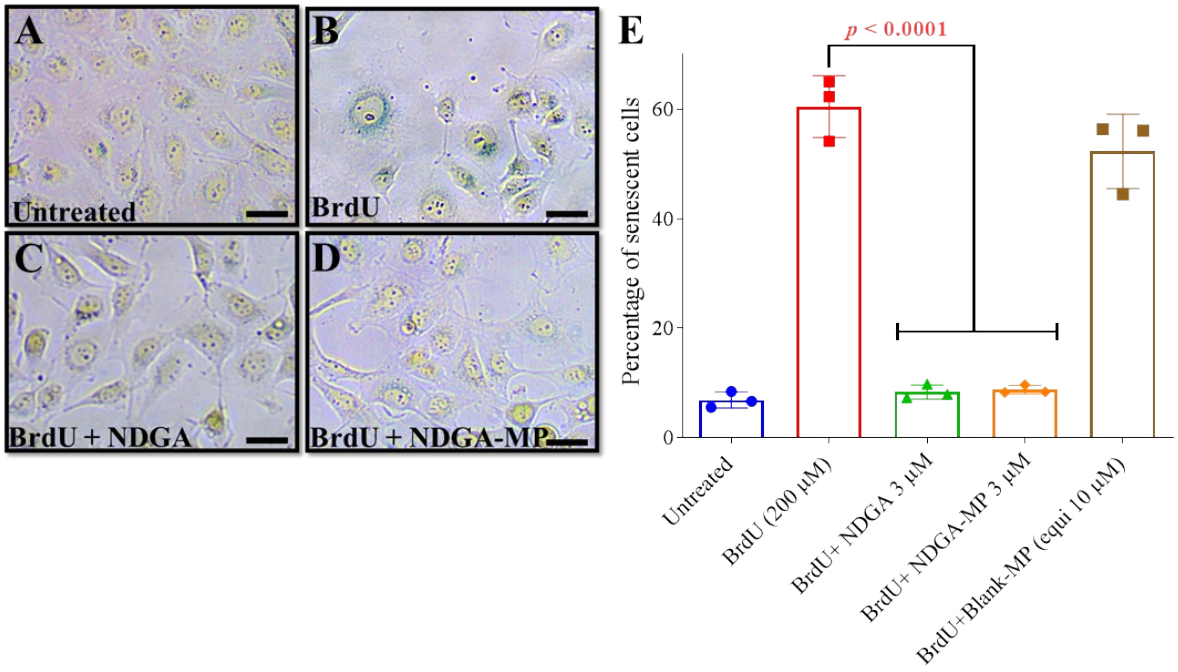


Fig. S6. NDGA-MP prevent senescence in chondrocytes (C28/I2) under genotoxic stress. SA-β Gal-stained images of C28/I2 cells exposed to **(A)** no treatment, **(B)** genotoxic (BrdU) stress, **(C)** genotoxic (BrdU) stress along with free NDGA (3 µM) treatment and, **(D)** genotoxic (BrdU) stress along with NDGA-MP (3 µM) treatment. **(E)** Percentage of senescent cells under genotoxic (BrdU) stress condition, (n = 3 per group). Data in the graph represent the mean ±, s.d. and *p* values were determined by one-way analysis of variance (ANOVA) and Tukey's post hoc tests. *p*-value < 0.05 was considered significant. Blank-MP-Blank Microparticles, NDGA-MP- NDGA loaded Microparticles. *ns* - non significant; Scale bar, 40 µm.

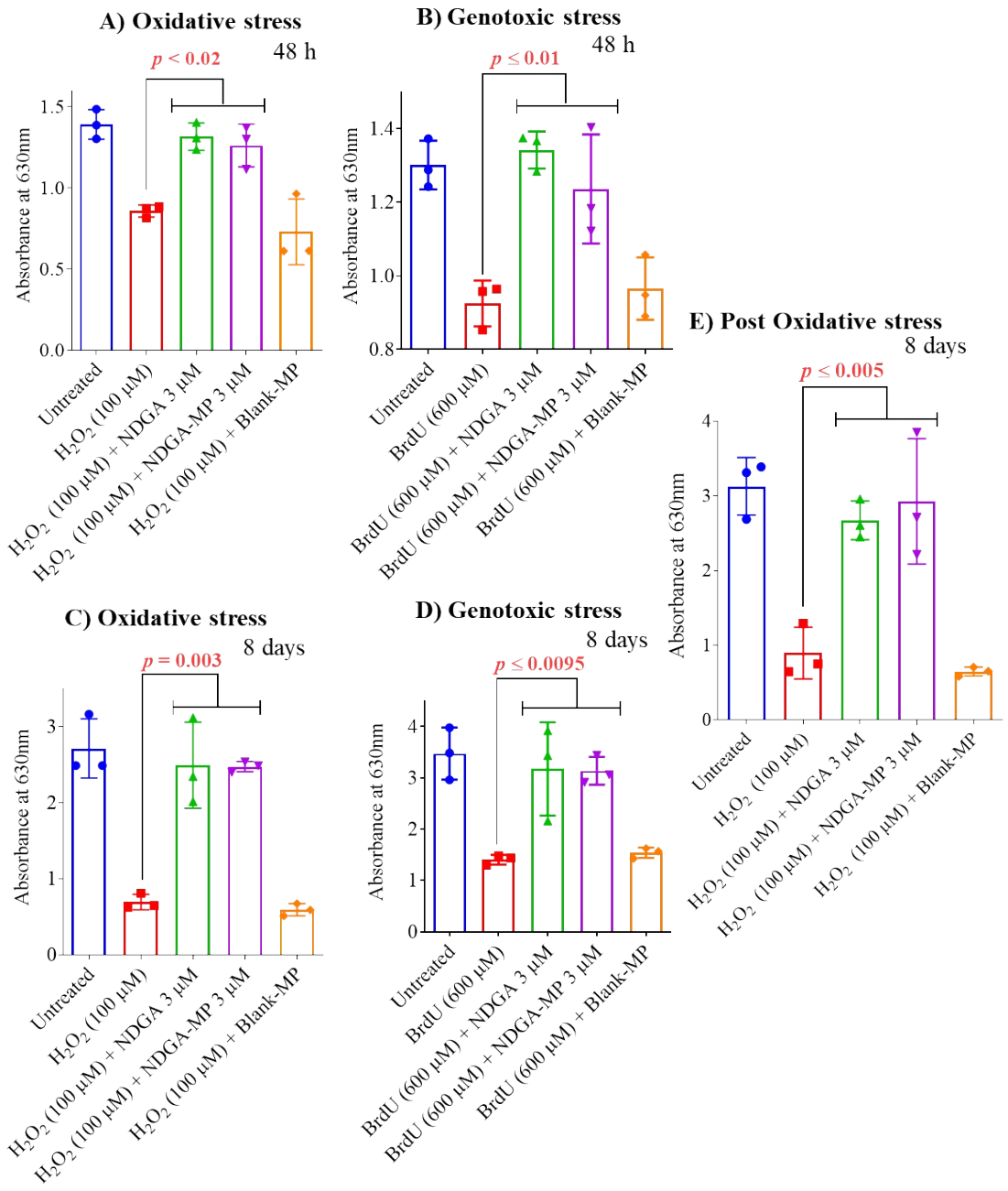


Fig. S7. NDGA-MP prevent loss of sGAG in micromass cultures exposed to genotoxic and oxidative stresses. sGAG production from C28/I2 micromass culture after treatment with various particle and drug formulations under **(A)** oxidative (H_2O_2) stress for 48 h (n = 3), **(B)** genotoxic (BrdU) stress for 48 h (n = 3), **(C)** oxidative (H_2O_2) stress for 8 days (n = 3), **(D)** genotoxic (BrdU) stress for 8 days (n = 3), and **(E)** Post oxidative stress treatment. Data in graphs represent the mean ±, s.d. and *p* values were determined by one-way analysis of variance (ANOVA) and Tukey's post hoc. *p*-value < 0.05 was considered significant. Blank-MP - Blank Microparticles, NDGA-MP - NDGA loaded Microparticles. ****p<0.0001, ***p<0.001, *ns* - non significant.

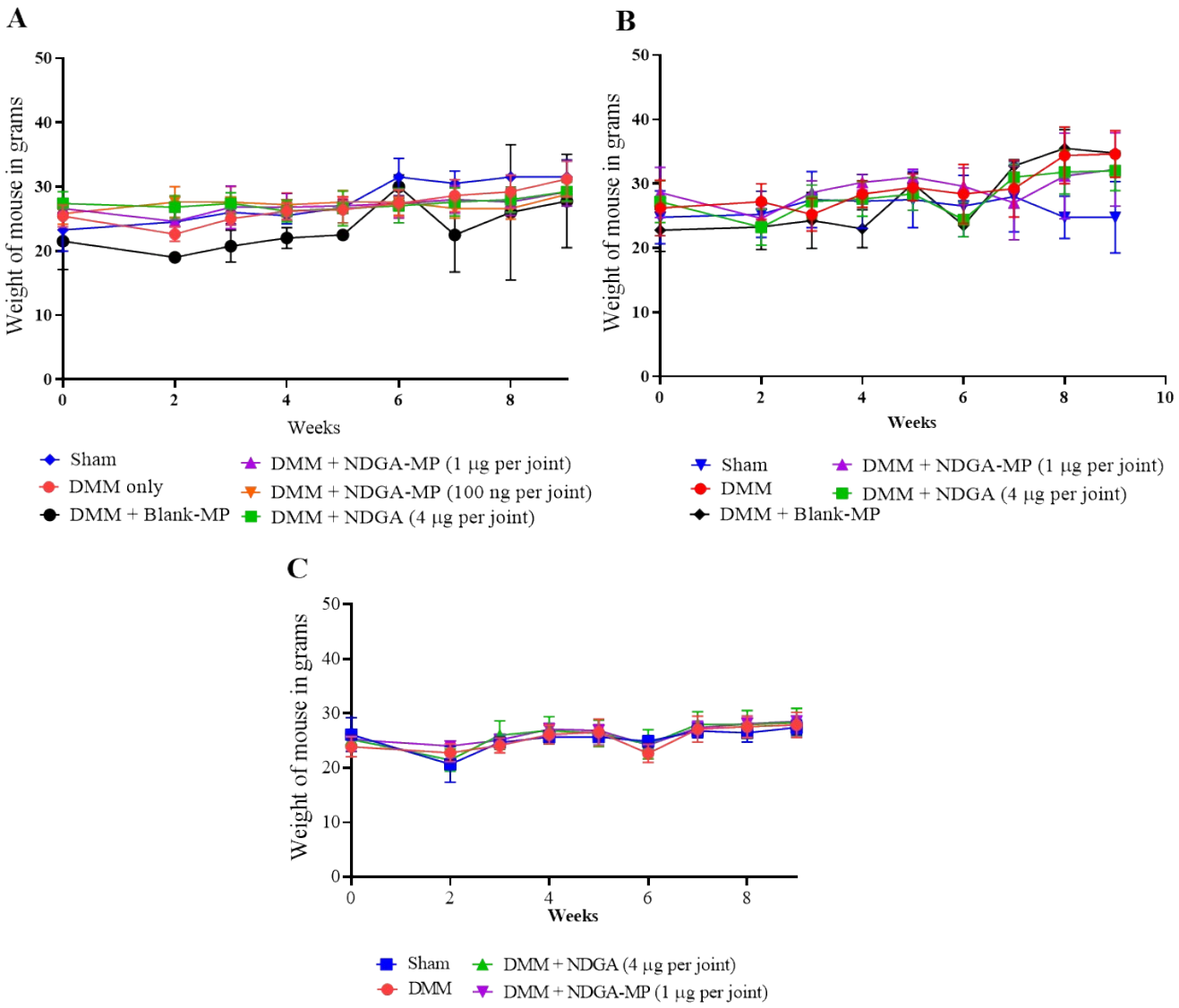


Fig. S8. Mice treated with NDGA as a Free Drug and as NDGA-MP do not cause toxicity-related loss of body weight. (A) Plot showing the weight of mice for the time after receiving different treatments under preventive regimen. (**B**) Plot showing the weight of mice for the time after receiving different treatments under a curative two-dose regimen. (**C**) Plot showing the weight of mice for the time after receiving different treatments under a curative single-dose regimen.

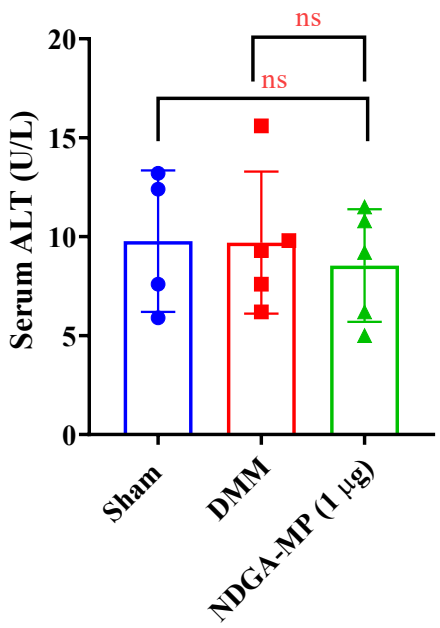


Fig. S9. Serum Alanine Aminotransferase (ALT) assay: Serum ALT assay shows no hepatotoxicity associated with NDGA-MP following intra-articular administration under the preventive dosing regimen. Mice received three injections during this regimen on Days 7, 24, and 42, and serum was analyzed from blood drawn on Day 43 ($n \geq 4$). ns = non-significant.

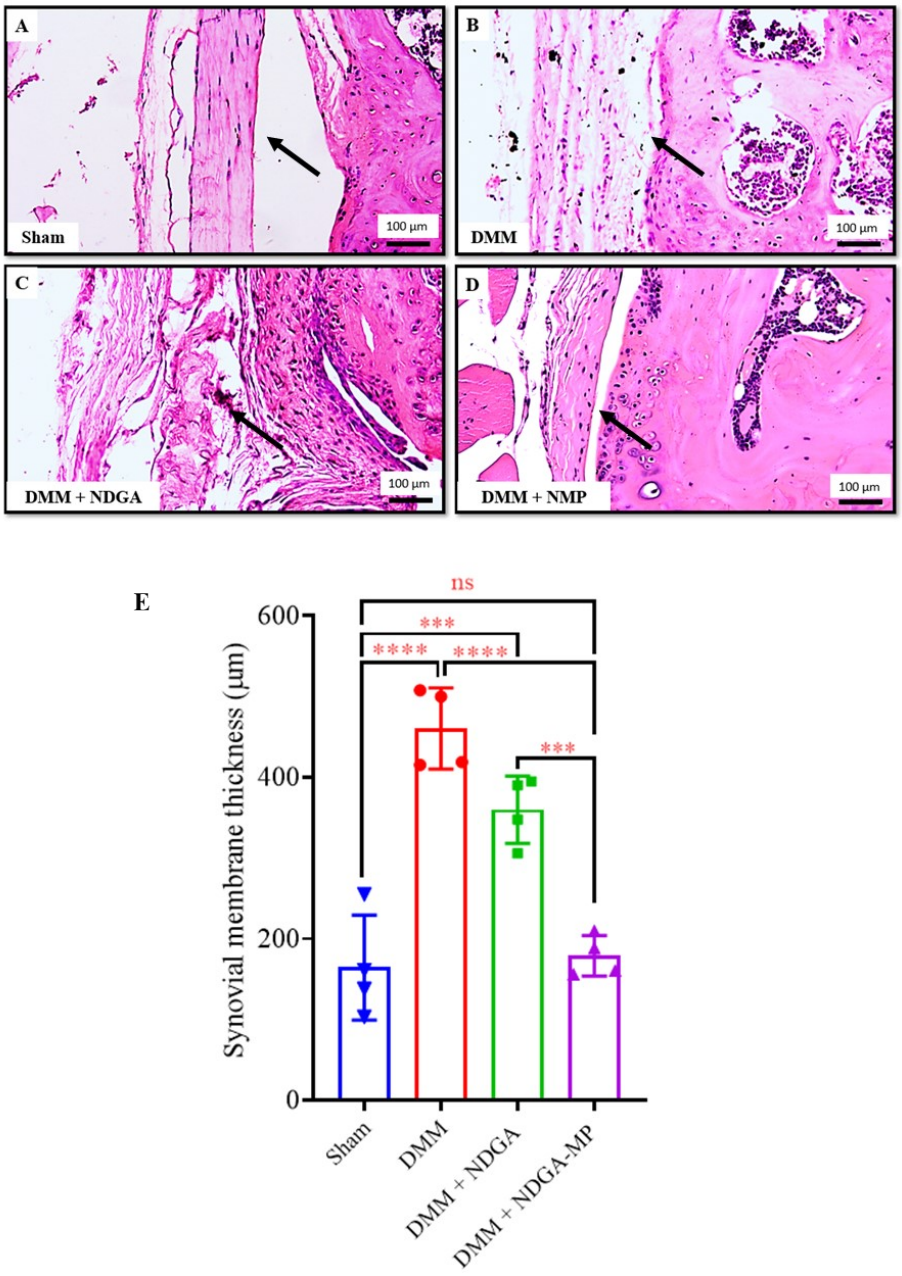


Fig.S10. NDGA-MP does not produce synovial inflammation in mice OA knee joints. Histological analysis of synovial inflammation in knee joints of DMM-operated and NDGA/NMP-treated mice using hematoxylin and eosin staining under a preventive dosing regimen. Representative images are shown for (A) Sham, (B) DMM, (C) DMM + NDGA, and (D) DMM + NMP groups. (E) Synovial membrane thickness of mice knee joints treated with a preventive dosing regimen 9 weeks post DMM surgery (n = 4) The black arrow points to the synovial lining. Data in graphs represent the mean ±, s.d. and *p* values were determined by one-way analysis of variance (ANOVA) and Tukey's post hoc. *p*-value < 0.05 was considered significant. NMP - NDGA loaded Microparticles. ****$p<0.0001$, ***$p<0.001$, *ns* - nonsignificant.

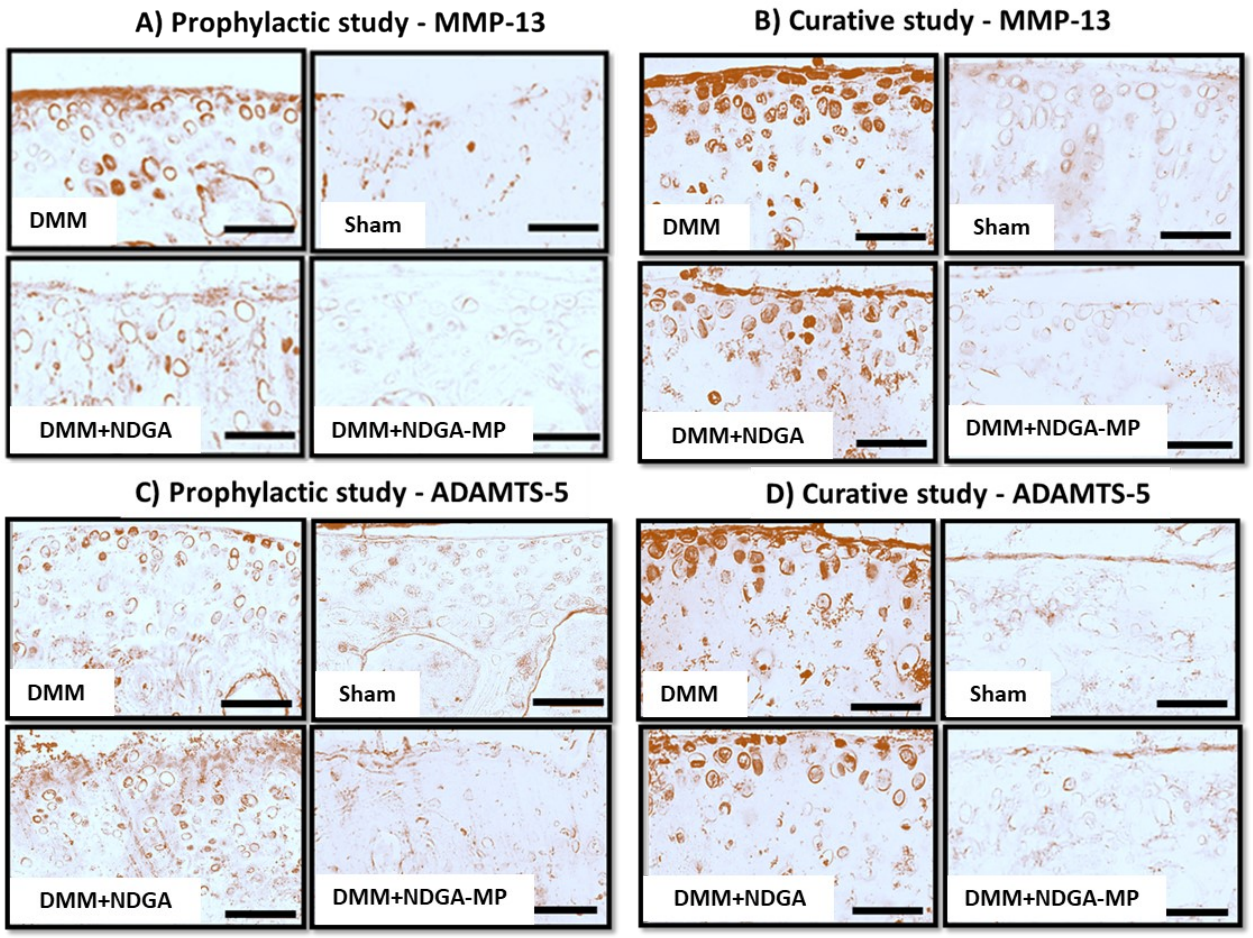


Fig. S11. NDGA-MP in prophylactic and curative regimens reduced inflammation markers associated with OA in surgically induced murine OA models. Immunohistochemical staining for MMP-13 in **(A)** Prophylactic study, **(B)** Curative study. Immunohistochemical staining for ADAMTS-5 in **(C)** Prophylactic study, **(D)** Curative study. DMM-Destabilization of Medial Meniscus model, Blank-MP - Blank Microparticles, and NDGA-MP – NDGA Microparticles. Scale bar, 50µm.

Table S1: PLGA microparticles of different molecular weights and PLA: PGA ratios, their respective sizes, and their NDGA encapsulation efficiency.

Molecular weight of PLGA (Ratio of PLA: PGA)	Size - DLS [nm]	NDGA encapsulation efficiency [%]
10 kDa - 15 kDa (50:50)	939.8 ± 335.3	8.98 ± 1.316
85 kDa - 100 kDa (50:50)	1139 ± 407.6	8.84 ± 0.565
190 kDa –240 kDa (85:15)	1118 ± 335.3	6.22 ± 0.745

Table S2: Details of volunteers who donated the knee joint samples following total knee arthroplasty.

Volunteer ID	Age/Gender	Primary Diagnosis
RMCH_001	48/M	Osteoarthritis of knee
RMCH_002	68/F	Osteoarthritis of knee
RMCH_003	61/F	Osteoarthritis of knee

References

[1] F. Debacq-Chainiaux, J.D. Erusalimsky, J. Campisi, O. Toussaint, Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and in vivo, Nature protocols 4(12) (2009) 1798-806.