Intracellular ROS-scavenging nanozymes for the treatment of osteoarthritis

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INTRODUCTION: Nanozymes, antioxidant-mimicking nanoscale biomaterials, are an emerging therapeutic strategy in medical applications related to oxidative stress. These nanomaterials can be engineered to mimic endogenous enzymes, such as catalase or superoxide dismutase, or can directly scavenge excess reactive oxygen species (ROS) that contribute to the progression of oxidative stress. Oxidative stress plays a central role in the development of osteoarthritis, however it remains an elusive therapeutic target – conventional small molecule antioxidants and natural enzymes have been limited by poor retention and localization to the osteoarthritic (OA) joint. In contrast, nanozymes can be engineered with properties that confer better stability and bioavailability than existing antioxidant therapies[1]. One example includes manganese dioxide nanoparticles (MnO_2 NPs), which our group has engineered with properties favorable for cartilage localization and chondrocyte uptake, and consequently chondroprotection upon oxidative stress induction[2]. However, there is little understanding of the molecular mechanisms and compartment specific functions of MnO_2, or other nanozymes currently evaluated as potential OA therapies. The objective of this work was to evaluate the uptake mechanisms and intracellular localization of MnO_2 NPs in human OA chondrocytes and determine the compartment specific ROS scavenging activity of MnO_2 NPs.

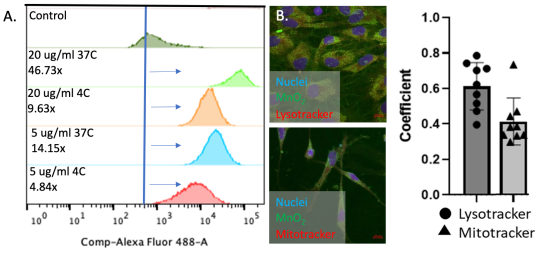

Fig 1. Figure 1. A) MnO2 uptake into human OA chondrocytes is temperature is dose dependent, but uptake is measurable at both 4C and 37C indicating both active and passive uptake mechanisms can be used. B) Following uptake, MnO_2 moderately colocalizes with both lysosomes and mitochondria.

METHODS: MnO_2 NPs were synthesized with cartilage targeting properties (cationic, <15 nm) following previously reported methods[2]. Human OA chondrocytes were isolated from patients undergoing total knee arthroplasty (n = 3 male and n = 3 female). Passive and active methods of NP uptake into human OA chondrocytes were determined via temperature dependent uptake studies (37°C and 4°C) at 5 and 20 ug/ml MnO_2. Intracellular localization of M nO_2 NPs was determined via confocal microscopy for colocalization with MitoTracker and LysoTracker fluorescent tags. To evaluate ROS scavenging activity and location, bovine chondrocytes were transfected with plasmids encoding HyPer7, an ultrasensitive genetically encoded hydrogen peroxide (H_2O_2) sensor, treated with MnO_2 NPs (5ug/ml and 20 ug/ml), and then washed and exposed to increasing levels of H_2O_2 over time[3]. Oxidation of the probe by H_2O_2 results in a shift in its excitation wavelength - the readout of HyPer7 is thus presented as ratio of fluorescence emission intensity a following excitation at the 2 respective wavelengths (488 and 405nm). HyPer7 plasmids contained localization signals targeting either the mitochondrial matrix, mitochondrial intermembrane space, or cytosol, were used to determine compartment specific H_2O_2 scavenging ability of the MnO_2 NPs. Statistical analysis was conducted on GraphPad PRISM 10.2 whereby error bars indicate standard deviations and statistical comparison of means were conducted in GraphPad *via* a Dunnett's test or one-way ANOVA with Tukey's multiple comparisons tests.

RESULTS: MnO_2 NPs were visualized within human and bovine chondrocytes. Uptake was dose dependent, with increasing mean fluorescent intensity from 5 to 20 ug/mL, without impacting viability. While uptake was greater at 37°C compared with 4°C, there was measurable uptake at both temperatures, suggesting that MnO_2 utilized both active and passive routes of uptake (Fig 1A). There is no significant difference in MnO_2 NP uptake between male and female donors, based on mean fluorescent intensity. Intracellularly, MnO_2 colocalized to mitochondria and lysosomes, indicated by 0.6 and 0.4 Pearson correlation coefficients, respectively (Fig 1B). Results from the HyPer7 probes (Hyper 7 ratio) showed that MnO_2 NPs reduced H_2O_2 levels in all three cellular compartments evaluated, with dose dependence noted in the mitochondrial matrix and intermembrane space. The thiol reducing agent dithiothreitol (DTT), was used to reverse the HyPer7 oxidation, acting as a positive control to confirm cell response returns to baseline.

DISCUSSION: MnO_2 NPs have a unique ability to enter chondrocytes and scavenge ROS in multiple cellular compartments. MnO_2 NPs enter chondrocytes through both passive and active transport mechanisms, rendering them available for localization to the cytosol and mitochondria. Colocalization and function of MnO_2 within the mitochondria may be driven by the electrostatic interaction between the cationic MnO_2 and the negatively charged mitochondrial matrix. Mitochondria are responsible for generating a significant amount of ROS within the cell and colocalization of MnO_2 may support endogenous antioxidant function, such as MnSOD (superoxide dismutase), or directly scavenging H_2O_2 produced by the mitochondria. This is the first mechanistic analysis of the redox activity of MnO_2 NPs in chondrocytes. The limited molecular tools to measure ROS has impeded the progress in antioxidant therapies. The HyPer7 probes have been introduced into the field 17 years ago and have been used with other cell types, but this is the first instance of their use in chondrocytes, further expanding their application potential. MnO_2 scavenging is dose dependent, with a lower HyPer7 ratio in cells treated with 20 ug/ml MnO_2 before the addition of exogenous H_2O_2 in all compartments except for the cytosol. This may indicate that there is a saturation effect of MnO_2 on H_2O_2 in the cytosol. Comparatively, scavenging H_2O_2 in the mitochondria may support overall cellular function and redox homeostasis, as suggested in our previous reports of the downstream effects of MnO_2 NPs. Advancing our understanding of how these nanomaterials deliver a therapeutic response is important for continuing to find treatments for stopping or slowing OA progression.

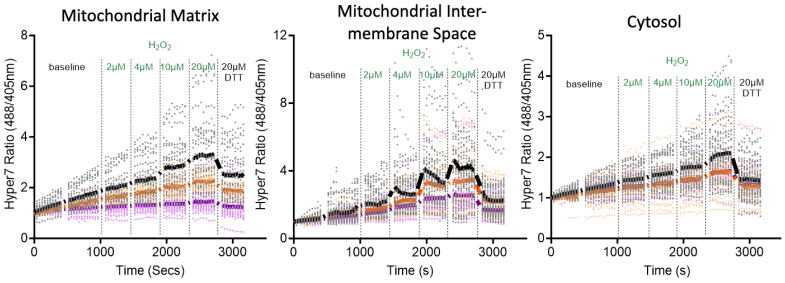

Fig 2. MnO_2 scavenges H_2O_2 from the A) mitochondrial matrix, B) intermembrane space, C) and cytosol in a dose dependent manner. The HyPer7 ratio is decreased in the presence of MnO_2 at all timepoints following the addition of exogenous H_2O_2.

SIGNIFICANCE/CLINICAL RELEVANCE:
Nanozymes such as MnO_2 are emerging technologies for effectively targeting oxidative in the treatment of numerous diseases including OA. This study is the first to interrogate the uptake and ROS scavenging mechanisms of MnO_2 NPs in chondrocytes, which will be important for further development and translation of this promising therapeutic strategy.

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REFERENCES: [1] Aldrich, J., et al., 2023 [2] Kumar, S., et al. 2019 [3] Pak, V., 2020