

Influences of High-dose Gentamicin Exposure on Human Articular Cartilage

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Introduction: Recently in Japan, a therapy known as Continuous Local Antibiotic Perfusion (CLAP) is increasingly used in cases of musculoskeletal infections [1,2,3]. This therapy perfuses the lesion with high concentration of gentamicin (GM) (1200µg/ml) over time [1,2]. Lately, its application on native joint infections were also reported although chondrotoxicity profiles on human cartilage of GM on mentioned concentration is poorly characterized and further studies are needed. [3,4] The objective of this study is to determine a safe concentration range of GM to be used in CLAP therapy also deepen understanding on GM chondrotoxicity profile.

Methods: Chondrocyte Isolation & Culture: Articular cartilage of the femoral condyle following total knee replacement (n = 6) is firstly cleaned with PBS and then minced into small pieces, followed with digestion with collagenase-2 overnight. Cells were then suspended in Dulbecco's Modified Eagle Medium (DMEM) medium (Sigma-Aldrich) containing L-arginine, 10% fetal bovine serum, and various antibiotics. Only cells from the second passage (P2) were used in this study. Various concentrations of GM (0 – 10000 µg/ml) are then added to the culture medium.

Cartilage Explant Culture: Articular cartilage explants were extracted using a 5 mm biopsy punch under sterile conditions from femoral condyle following total knee replacement (n=7). Explants were cultured in DMEM containing 10% fetal bovine serum and ascorbic acid. Various concentrations of GM (0 – 10000 µg/ml) were then added to the culture medium. Culture medium is then changed every 3 days, and cultures are maintained for 7 days.

Cell Viability Assay: For chondrocyte, cell viability on day 4,7, and 14 was then measured using cell counting kit 8 (Dojindo) on absorbance of 450 nm. Recovery capability was also measured by exposing chondrocytes to same concentration groups for 7 and 14 days of period before resuspended in growth medium without GM for 14 days.

Apoptosis Assay: Chondrocytes were exposed to GM ranging from 10 to 2000µg/ml plus 10000µg/ml. 48 hours following GM administration, TUNEL staining with the Apo-direct apoptosis Kit (Phoenix Flow Systems) was done as manufacturer protocols, and DAPI was used as a counterstain. Cells were then examined under a fluorescence microscope (Keyence). The apoptosis rate was calculated by TUNEL-positive cells within the overall DAPI-stained cells.

Safranin-O Staining: Explants are fixed using 4% formaldehyde and then decalcified for 14 days. Following decalcification, it is then mounted in paraffin and sliced with 6 µm thickness. Following the section, safranin-O staining was performed, and the Mankin score was taken for each explant for assessment of cartilage quality.

Glycosaminoglycan Quantification: Explant culture medium was collected during medium change. Glycosaminoglycan is then quantified using the dimethyl methylene blue assay from the glycosaminoglycan assay kit (Chondrex) as described within the manufacturer protocol. The result is normalized to explant wet weight.

Results: Cell Viability Assay: Cell viability was maintained for 14 days at a GM concentration of 100 µg/ml or less. Meanwhile, exposure to 2000 µg/ml of GM significantly decreased that from days 7 to 14, and that on exposure of 3000 µg/ml and 4000µg/ml was decreased significantly on day 14. (Fig. 1A). Cell viability at 0 and 10 µg/ml was significantly increased after 14 days resuspension following GM removal after 7 days of exposure, and 0, 10, and 100 µg/ml after 14 days of exposure, respectively. Meanwhile, at concentrations of 2000 µg/ml and above, significantly decreased cell viability was noted on both time points. (Fig. 2A, 2B)

Apoptosis Rate: Representative images of 100 µg/ml and 1000 µg/ml concentrations were attached (Fig. 1B). TUNEL/DAPI ratio is significantly increased on concentration of 1000 µg/ml and above. (Fig. 1B)

Safranin-O Staining: Mankin score is significantly increased after a concentration of 1000 µg/ml after 7 days of exposure. (Fig. 3A)

Glycosaminoglycan Quantification: Average glycosaminoglycan content on the medium is significantly increased after 7 days of exposure in groups of 2000 µg/ml and beyond. (Fig. 3B)

Discussion: GM exposure affected articular cartilage negatively. This effect is evidenced by the increased Mankin scores in explant cultures and the release of glycosaminoglycan in the medium after exposure to a high dose. These findings are consistent with prior reports of GM chondrotoxicity after 7 days of exposure to 5000 µg/ml. [5] Cell viability and recovery capability of chondrocyte were reduced around clinical dose of CLAP therapy (1200 µg/ml). Apoptosis was significantly higher in the high-concentration group, explained by the greater rate of TUNEL-positive cells. While short-term exposure may be tolerated, prolonged high-concentration GM exposure can compromise chondrocyte function and viability, consistent with dose- and time-dependent chondrotoxicity. Although CLAP therapy can be beneficial, it might affect articular cartilage on prolonged use of the therapy. Our in-vitro static immersion model differs from perfusion delivery method used clinically in CLAP and thus limits direct extrapolation to clinical practice.

SIGNIFICANCE: The current study demonstrates that GM negatively affect cartilage viability in a concentration- and time-dependent manner. Dose of 1200µg/ml is probably safe for short duration however possible negative effects will be seen for long duration use.

Reference: [1] Maruo A, *et al.* J Orthop Surg. 2021. [2] Oe K, *et al.* J Orthop Case Rep. 2021.[3] Himeno D, *et al.* J Orthop Sci. 2022.[4] Post H K, *et al.* Kans J Med. 2023. [5] Mayr *et al.* Antibiotics. 2023

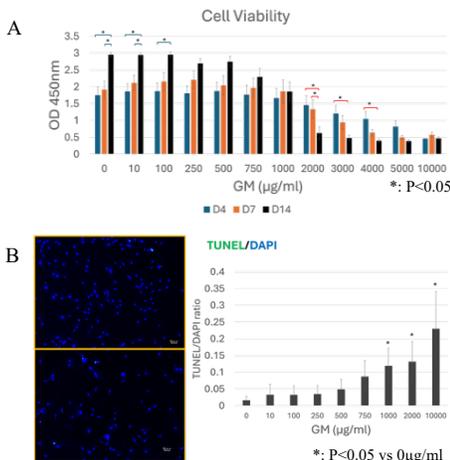


Fig 1 A: graphic showing cells viability across timepoints and concentration **B:** graph of apoptosis rate and representative figure from concentration of 100 and 1000 µg/ml of GM concentration

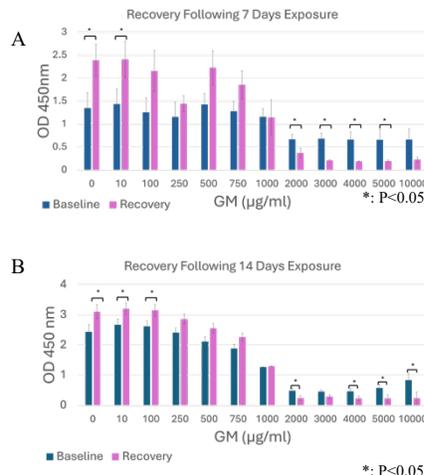


Fig 2 A: graphic showing 14 days recovery following 7 days GM exposure **B:** following 14 days GM exposure

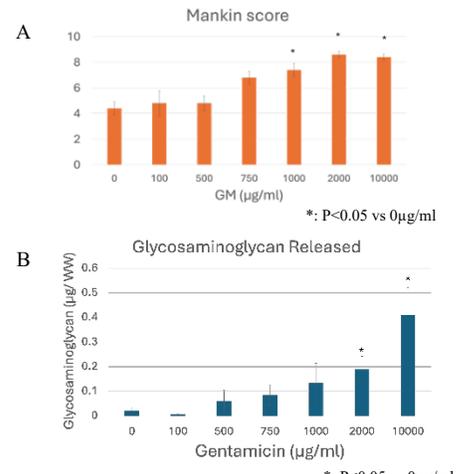


Fig 3 A: Mankin Score result **B:** graphic showing released glycosaminoglycan