Alcohol metabolism negatively affects early ATDC5 chondrocyte differentiation independent of *NOX4* expression

Hardy Hang[1], Alexandra Denys[1], Martin J. J. Ronis[1]

[1]Department of Pharmacology and Experimental Therapeutics, LSU Health Sciences Center, New Orleans, LA

Background

- Alcohol (ethanol) abuse is a widely recognized risk factor in the development of several diseases, including skeletal disorders.
- Previous studies in our lab have shown that ethanol induces reactive oxygen species (ROS) production in osteogenic cells, which results in oxidative damage, skeletal dysfunction, and osteoporosis.
- In osteogenic cells, a major source of ethanol-mediated ROS is derived from NADPH oxidases (NOXs).
- Ethanol inhibits epiphyseal plate proliferation in longitudinal bones and affects chondrocyte function, which results in shorter bones.
- It remains uncertain if ROS produced by NOXs during alcohol metabolism result in impaired chondrocyte function and differentiation.

Objective

To investigate if chronic alcohol exposure in murine chondrocytes will affect chondrogenesis through the induction of *Nox* expression and subsequent ROS production.

Methods

Cell Culture

Murine ATDC5 pre-chondrocytes were seeded onto 6-well plates at 6,000 cells/cm^2 and grown for 3 days in ATDC5 media containing 5% FBS in DMEM/F12 (1:1) + 1X GlutaMAX and 1% penicillin/streptomycin (Gibco). After reaching confluence, the cells were induced to differentiate using ATDC5 media supplemented with 1X insulin/transferrin/selenium (Gibco), 10 mM β-glycerophosphate (Sigma), and 50 µM sodium L-ascorbate (Sigma) in the presence of either vehicle, 50 mM ethanol (EtOH), or 5 mM sodium acetate (Sigma) for 7 or 14 days. Media was replaced every 2-3 days.

Alcian Blue Staining

Cell monolayers were fixed using a solution containing 30% EtOH, 0.4% para-formaldehyde, and 4% acetic acid for 15 mins. Cells were rinsed with distilled water and stained with 0.05% Alcian blue-EtOH solution overnight at 37°C. Cells were rinsed with distilled water and imaged using the trans-illumination setting on the Amersham Imager 600 (GE Healthcare). Cell monolayers were then de-stained using 6 M guanidine hydrochloride (Sigma) overnight and OD_{595} values were measured using a UV-Vis spectrophotometer (Molecular Devices).

Total RNA Isolation and Analysis

Total RNA was isolated by harvesting cell monolayers in TRIzol and homogenizing using the TissueLyser II (Qiagen) for standard phenol-chloroform phase extraction. RNA concentrations were quantified using the Nanodrop ND-1000. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed using the PowerSYBR Green RNA-to-C_T 1-Step Kit (Applied Biosystems) with 12.5 ng RNA on the LightCycler 480 System (Roche). All target genes were normalized to *β-actin* and analyzed using the $2^{-\Delta Ct}$ method.

Results

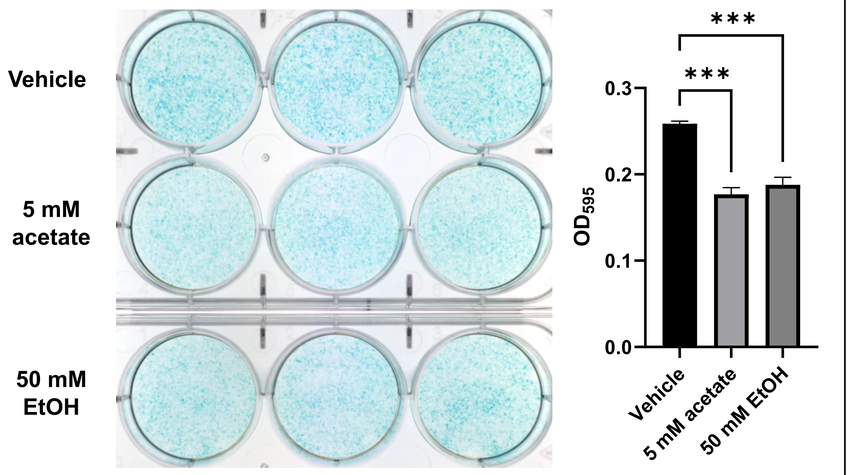

Figure 1. Cartilage formation is downregulated by ethanol and its end metabolite, acetate, in early ATDC5 chondrocytes.
Fixed ATDC5 cell monolayers (n = 3 per condition) were stained using Alcian blue in 7-day post-differentiated chondrocytes (left) and the absorbance values (OD_{595}) of the de-staining solutions were measured using spectrophotometric analysis (right). *** p < 0.001 vs. vehicle control.

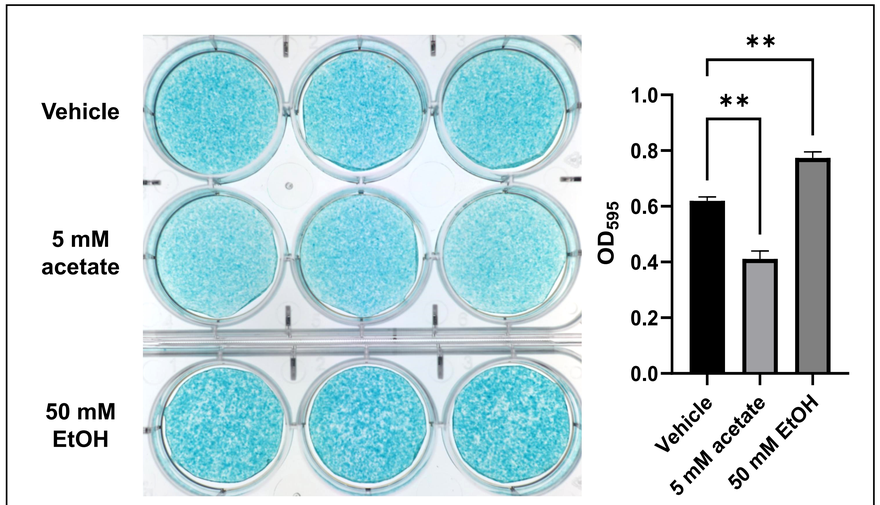

Figure 2. Cartilage formation is inhibited by chronic acetate treatment whereas it is upregulated by ethanol treatment in late ATDC5 chondrocytes.
Fixed ATDC5 cell monolayers (n = 3 per condition) were stained using Alcian blue in 14-day post-differentiated chondrocytes (left) and the absorbance values (OD_{595}) of the de-staining solutions were measured using spectrophotometric analysis (right). ** p < 0.01 vs. vehicle control.

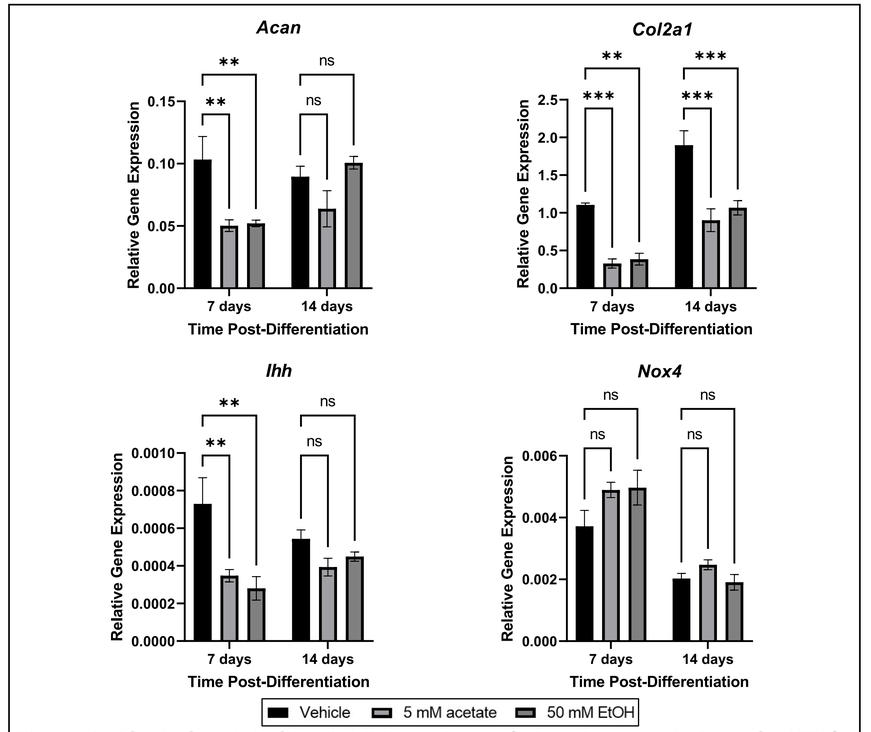

Figure 3. Alcohol metabolites inhibit markers of chondrogenesis in early ATDC5 chondrocytes independent of *Nox4* expression, but only *Col2a1* is downregulated in late chondrocytes.
Gene expression was analyzed in treated ATDC5 chondrocytes (n = 3 per condition) and assessed for the chondrogenesis markers, *Acan*, *Col2a1*, and *Ihh*, and an inducer of ROS formation, *Nox4*. Expression levels were normalized to *β-actin*. ** p < 0.01, *** p < 0.001 vs. vehicle control.

Conclusions

- In early 7-day chondrocytes, Alcian blue staining demonstrates decreased cartilage formation with ethanol and acetate treatments, and gene expression analyses similarly show that both treatments decrease expression of the chondrogenesis markers.
- In late 14-day chondrocytes, Alcian blue staining shows increased cartilage deposition with chronic ethanol treatment whereas acetate treatment diminishes cartilage deposition compared to the vehicle control. In addition, gene expression results show that that ethanol and acetate do not significantly affect expression of chondrogenesis markers except for *Col2a1*.
- In both early and mature chondrocyte cultures, *Nox4* mRNA was not significantly altered with either ethanol or acetate treatment.
- Overall, these results indicate that ethanol and acetate cause differential impairments in chondrocyte differentiation and function in a time-dependent manner independent of *Nox4* expression.

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