Investigating effects of nutraceuticals on abilities of human diseased chondrocytes to form articular cartilage tissues \textit{in vitro}

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What is Articular Cartilage (AC)?

- Hyaline cartilage specialized connective tissue of moving joints
- It is a 2 to 4 mm thick
- Lowest density of cells of all tissues (2%)
- AC has no chondrogenic progenitor cells
- Its ECM is composed mainly of water (65-80%), collagen (60% dry weight) and proteoglycans (30% dry weight)
- Devoid of blood vessels, lymphatics and nerves
- Subject to harsh chemical environments (acidic, hypoxic, higher osmotic pressure)
- Functions as a lubricant and a load barrier
- Its multiphasic and heterogeneous in its structure (four zones)
Articular Cartilage Damage (Osteoarthritis (OA))

- Inability for intrinsic self-repair
- 30% of the population (ages 45 to 65 years) worldwide are affected by OA
- 80% of those age 65 and above will have evidence of OA
- In 2008, 63% of knee replacements were performed on women*

Risk factors

Mech. injury  Age  Diabetes  Infections  Hormones  Obesity  Heredity

* American Academy of orthopedic Surgeons
Osteoarthritis (OA)


Symptoms

In small joints, hard bony enlargements

Increased pain with cold temperatures

Joint pain

Disability
Current Management of OA

- Oral medications
- Injectables
- Topical medications
- Weight loss
- Devices and braces
- Physical therapy
- CAM remedies

By 2030, total knee replacement surgeries will increase by 673% (3.5 million surgeries)

Tissue Engineering of AC

Engineer an AC tissue that mimics the *in vivo* tissue in function, structure and properties and can be ultimately personalized and implanted in patients who suffer from OA with hopes to restore function.
Tissue Engineering of AC

Scaffold

Bioreactor – Micro-environment

Cells

Growth Factors

Functional Engineered Tissue
Our Long-Term Goal

Combine mechanical, chemical and physical stimuli to cells in unique bioreactors to engineer a functional AC tissue
Goal and Hypothesis

**Goal:** Investigate the effects of nutraceuticals on the abilities of osteoarthritic human chondrocytes (hACHs) extracted from donors of variable age and sex to form AC extracellular matrices (ECMs) and reduce inflammation.

**Hypothesis:** Addition of nutraceuticals to monolayer cultures will improve cellular abilities to form ECM and reduce inflammation in age and sex dependent manner.

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Nutraceuticals

- Parts of food that provide health benefits
- They have antioxidant and anti-inflammatory effects
- Taken by 47% of OA patients

- Gallic Acid
- Nuts
- Catechin Hydrate
- Green tea extract
- Alpha Tocopherol
- Vitamin E
- Ascorbic Acid
- Vitamin C
Cellular Isolation

- In collaboration with Dr. E. Tingstad at Inland Orthopedics, Pullman, WA, AC samples wasted during knee replacement surgeries were obtained from 25 donors.
- Upon receipt, AC tissues that are minimally inflamed were collected.
- Tissues were digested with collagenase II to extract cells.
- Cells were allowed to proliferate, achieve confluence, counted, and cryo preserved until used.
- 1 million cells per donor.
## Cellular Phenotypes

### Age and Weight  (Females only)

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>Age range (years)</td>
<td>50-69</td>
<td>70-80</td>
</tr>
<tr>
<td>Average weight (kg)</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>Average BMI (kg/m²)</td>
<td>34.06</td>
<td>31.43</td>
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### Sex

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Sex</td>
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<td>Female</td>
</tr>
<tr>
<td>N</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Average age (years)</td>
<td>66.2 ± 3.5</td>
<td>64.2 ± 3.1</td>
</tr>
</tbody>
</table>
**Methods**

**Protocols**
- Dose curves
- Viability
- Nitric Oxide (NO): day 1 and day 21
- mRNA quantified for main chondrogenic and inflammatory markers
- ECM formation quantified in terms of total collagen and GAG
Results

1. Viability-Sex Study

Viabilities of cultured chondrocytes at day 21 for both studies were statistically indifferent between groups as a function of nutraceutical treatments.
Results
2. Extracellular Matrix (ECM) Formation
Collagen Staining/Sex/Day 21

- Collagen is present in all treatments

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>Catechin</th>
<th>Gallic acid</th>
<th>α-tocopherol</th>
<th>Ascorbic acid</th>
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<tbody>
<tr>
<td>Females</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
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<tr>
<td>Males</td>
<td><img src="image6" alt="Image" /></td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
</tr>
</tbody>
</table>

20 images, quantified individually by Matlab for color density, averaged and an image that is close to the average was selected as the representative image to show.
## GAG Staining/Age-Weight/All Females

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
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<th>Gallic acid</th>
<th>α-tocopherol</th>
<th>Ascorbic acid</th>
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</thead>
<tbody>
<tr>
<td>50s-60s</td>
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<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
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<tr>
<td>70s-80s</td>
<td><img src="image6.png" alt="Image" /></td>
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</table>

- Overall and for all treatments, GAGs are present

Toluidine Blue stain
Colorimetric ECM Content-Sex Study

- Male/Female Collagen = 2.35 fold with G highest (3.27 fold)
- Use of AA in females resulted in more GAG compared to NC
Results

3. Nitric Oxide Content-Sex Study

- NO dropped at day 21 for both groups
- NO for females at day 21 were on average 1.12 fold higher than males
- AA reduced NO the most (insignificant)
- Nutraceuticals were similar to NC
Results

4. mRNA Gene Expressions

- **Chondrogenic markers**: Col II, Col XI and ACAN
  - Collagen (COL), Aggrecan (ACAN)

- **Hypertrophic or osteogenic marker**: Col X

- **Regulatory markers**: BMP2 (**degradation**), FOXO1 (**autophagy**), and Sox 9
  - BMP2-Bone Morphogenetic Protein-2, Forkhead-Box O1 (FOXO1), sex-determining region Y (SRY) box-9 (SOX9)

- **Inflammatory markers**: NOS2 (**inflammation**), MMP13 (**degradation**), and TNFAIP6 (**anti-inflammatory**)
  - Nitric oxide synthase 2 (NOS2), matrix metalloproteinase 13 (MMP13), and tumor necrosis factor alpha induced protein 6 (TNFAIP6)
Inflammatory Markers (NOS2)-Age and Weight Study

- Group 1: Younger and heavier
- Group 2: Older and leaner

Group 1/group 2-NOS (15-fold), with no differences between nutraceuticals for either group.

* P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, and **** P ≤ 0.0001
Discussion-Age and Weight Study

Nutraceuticals used did not show any significant effects from NC for reduction of inflammation, promotion of ECM formation or protecting against hypertrophy and dedifferentiation.
Take Home Messages-Age and Weight Study

• Chondrocytes of elder and leaner females (group 2) showed less inflammation (BMP2, MMP13, NOS2) and more or less similar phenotype (COLII, ACAN) compared to younger and more obese females (group 1) which is counterintuitive.

• Chondrocytes of group 1 responded better to nutraceuticals compared to group 2 by expressing genes for anti-inflammatory proteins (TNFAIP6 and FOXO1).

• Our findings stress the importance of weight management to help combat the progression and worsening of OA.

• Use of nutraceuticals reduced NO for both groups
## Discussion-Sex Study

<table>
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<th>C</th>
<th>G</th>
<th>Alpha</th>
<th>AA</th>
<th>Male</th>
<th>C</th>
<th>G</th>
<th>Alpha</th>
<th>AA</th>
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</tbody>
</table>

- Values measured at day 21 were normalized to NC quantified at day 21
- Up-pointing arrows represent values greater than 1 compared to NC and opposite is true for down-pointing arrows
- * Represents at least $P \leq 0.05$ compared to NC
Take Home Messages-Sex Study

• Use of nutraceuticals reduced the inflammatory environment.
• In females, G reduced inflammation and Alpha improved ECM formation.
• In males, Alpha outperformed other nutraceuticals in inflammation reduction and in enhancing ECM formation.
• Finally, our findings show that chondrocytes’ inflammatory and chondrogenic responses to nutraceuticals’ treatment are sex-dependent.
Highlights from both Studies

• Feeding cells for 21 days decreased NO in all treatments for both studies

• All cells were viable over 21 days of culture. Nutraceuticals were not toxic to cells

• Nutraceuticals did not outperform NC when it came to NO reduction, especially in the sex experiment
Future Recommendations and Directions

• Increase number of experimental repeats for statistical significance
• Investigate responses from individualized donors
• Investigate other phenotypes
• Culture cells in 3D matrices
• Study the effects of diabetes, obesity, acidity, and hypoxia on chondrogenesis
• How does artificially induced inflammation to chondrocytes isolated from healthy tissues compare to inflamed chondrocytes isolated from diseased patients?
Acknowledgements

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Thank you

Questions?