EFFECTS OF INDIVIDUAL TOCOTRIENOL ISOMERS ON BONE CELLS IN A 3D CELL CULTURE SYSTEM

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OSTEOPOROSIS

Disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk.
OSTEOPOROSIS IS A GLOBAL ISSUE

Annual fracture incidence:
Red: > 250/100,000
Orange: 150–250/100,000
Green: <150/100,000

Hip fracture rates for men and women combined in different countries of the world
OSTEOPOROSIS IS A FUTURE PROBLEM

Number of hip fractures projected to increase 3 to 4-fold worldwide

Total number of hip fractures worldwide projected to increase 3- to 4-fold in next 50 years

1950: 1.66 million
2050: 6.26 million

Estimated number of hip fractures (1000s)

Incidence of hip fracture of Malaysia in comparison with other Asian countries and US

Bone health status among Malay and Chinese men in the Klang Valley (2009-2012)

N = 818 Malay and Chinese men aged ≥ 20 years. Bone density measured by Calcaneal Quantitative Ultrasonometer (QUS)

<table>
<thead>
<tr>
<th>Bone health status</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>76.4</td>
</tr>
<tr>
<td>Osteopenia</td>
<td>19.9</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Findings:
- Bone health deteriorate with increased age
- 23.6% has subnormal bone health.

Chin et al. J Clin Densitom 2012
BONE REMODELING

Resorption
Osteoclasts remove bone mineral and matrix, creating an erosion cavity

Reversal
Mononuclear cells prepare bone surface for new osteoblasts to begin building bone

Formation
Osteoblasts synthesize a matrix to replace resorbed bone with new bone

Resting
A prolonged resting period follows until a new remodeling cycle begins

The risk of osteoporosis increases when the rate of bone resorption exceeds the rate of formation during the process of bone remodelling
Pathophysiology of osteoporosis

- Imbalance between bone formation and bone resorption

Bone resorption >>>> Bone formation = Bone loss
Risk factors for osteoporosis

- Age
- Family History
- Previous Fracture
- Menopause
- Testosterone deficiency in men
- Glucocorticoid
- Low BMI
- Sedentary lifestyle
- Malnutrition
- Cigarette smoking
- Alcohol
- Sedentary lifestyle

International Osteoporosis Foundation.
Vitamin E is found in plant oils (PALM OIL, annatto oil), green vegetables (spinach, broccoli) and nuts (almonds, hazelnuts) (Munne, 2002).

Tocotrienol and Osteoporosis

Effects of palm vitamin E mixture on bone histomorphometry in a glucocorticoid-induced osteoporotic rat model.

In vivo studies
### PALM VITAMIN E (PTT) MIXTURE

<table>
<thead>
<tr>
<th>PRODUCT DESCRIPTION</th>
<th>Gold-Tri.E™ 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>QUANTITY</td>
<td>1x1kg Aluminium canister</td>
</tr>
<tr>
<td>BATCH</td>
<td>SB11091950</td>
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</table>

<table>
<thead>
<tr>
<th>Total Tocopherol/ Tocotrienol Content</th>
<th>553.0 mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-alpha-tocopherol</td>
<td>124.4 mg/g</td>
</tr>
<tr>
<td>d-alpha-tocotrienol</td>
<td>147.5 mg/g</td>
</tr>
<tr>
<td>d-beta-tocotrienol</td>
<td>22.2 mg/g</td>
</tr>
<tr>
<td>d-gamma-tocotrienol</td>
<td>173.0 mg/g</td>
</tr>
<tr>
<td>d-delta-tocotrienol</td>
<td>85.9 mg/g</td>
</tr>
</tbody>
</table>
Male Sprague Dawley Rats
250-300g

SHAM
I/M vehicle (palm olein)
+ Vehicle palm olein
0.1ml/100 g
(oral gavage)

Adrenalectomy
n=20

ADRX + DEX
I/M Dexamethasone
120ug/kg/day
+ Vehicle olive oil
0.1ml/100g
(oral gavage)

ADRX + DEX + TT
I/M Dexamethasone
120ug/kg/day
+ Palm vitamin E
60mg/kg/day (oral gavage)

2 weeks

2 months

Sacrifice

Femoral bone - Histomorphometry
STRUCTURAL HISTOMORPHOMETRY - IMAGES

Von Kossa stain at 50x magnification
STRUCTURAL HISTOMORPHOMETRY – QUANTITATIVE DATA

Bone Volume/Tissue Volume (BV/TV)

- **SHAM**
- ADRX+Dex
- Adrx+Dex+TT

Tb.Th (nm)

- **Group**

**Trabecular Thickness (Tb.Th)**

- **Sham**
- ADRX+Dex
- Adrx+Dex+TT

**Trabecular Number (Tb.N)**

- **Group**

**Trabecular Separation (Tb.Sp)**

- **Group**

a: significant difference from SHAM, p<0.05
b: significant difference from ADRX+DEX, p<0.05
CONCLUSION: IN VIVO STUDIES

• Conclusion:
  • Palm vitamin E mixture prevents glucocorticoid-induced osteoporosis.

• Research question:
  • Which one of the individual tocotrienol (α, β, δ and γ) and α-tocopherol isomers has the most effective antiosteoporotic and anabolic effects?
EFFECTS OF INDIVIDUAL TOCOTRIENOL ISOMERS ON BONE CELLS IN A 3-DIMENSIONAL CELL CULTURE SYSTEM.

In vitro studies

- **Objectives:**
  1. To determine the anabolic effects of individual α, β, δ, γ-tocotrienol and α-tocopherol isomers on osteoblasts using bovine bone scaffold
  2. To compare the antiosteoporotic and anabolic effects of the individual tocotrienol (α, β, δ and γ) and α-tocopherol isomers using a 3-dimensional osteoblast/osteoclast co-culture system
3-DIMENSIONAL OSTEOBLAST/OSTEOCLAST CO-CULTURE SYSTEM

- Culture of bone cells on native bone scaffold.
- Mimic human skeletal microenvironment.
- Advantage over the 2-dimensional cell culture. Bone is 3-dimensional structure.
METHODOLOGY

PHASE 1
TO DETERMINE THE ANABOLIC EFFECTS OF INDIVIDUAL α, β, γ, δ-TOCOTRIENOL AND α-TOCOPHEROL ISOMERS ON OSTEOBLASTS CULTURE USING BOVINE BONE SCAFFOLD

1) 3-Dimensional osteoblast culture on bone scaffold
2) Testing Individual tocotrienol (α, β, δ and γ) and α-tocopherol isomers using hFOB 1.19 on 3-D osteoblast culture on bone scaffold

PHASE 2
TO DETERMINE THE ANABOLIC EFFECTS OF α, β, γ, δ-TOCOTRIENOL AND α-TOCOPHEROL ISOMERS ON OSTEOBLAST/OSTEOCLAST CO-CULTURE ON BONE SCAFFOLD

1) 3-Dimensional osteoblast/osteoclast co-culture on bone scaffold
2) Testing of individual tocotrienol (α, β, δ and γ) and α-tocopherol isomers on 3-D osteoblast/osteoclast co-culture on bone scaffold
PREPARATION OF BONE SCAFFOLD

DEMINERALISATION PROCESS

CUT INTO SMALL PIECES (10mm x 10mm x 15mm)
3-D OSTEOBLAST CULTURE AND TESTING OF ISOMERS

PHASE 1

Culture for 3 days until 90% confluency, then transfer to scaffold

Addition of isomers (100 nM/ml)

I. SCANNING ELECTRON MICROSCOPE (7 days)

II. HISTOMORPHOMETRY (28 days)

Media and treatment were replaced every 2-3 days for 28 days
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>OB (OSTEOPOROTIC BONE)</td>
<td>Decalcified bone scaffold with &gt; 350 µm porosity</td>
</tr>
<tr>
<td>NC (NEGATIVE CONTROL)</td>
<td>Osteoblast culture on decalcified bone scaffold with NO addition of vitamin E isomers</td>
</tr>
<tr>
<td>PC (POSITIVE CONTROL)</td>
<td>Undecalcified native bone (Cancellous bone)</td>
</tr>
<tr>
<td>AP (ALPHA TOCOPHEROL)</td>
<td>Decalcified bone scaffold treated with alpha tocopherol</td>
</tr>
<tr>
<td>AT (ALPHA TOCOTRIENOL)</td>
<td>Decalcified bone scaffold treated with alpha tocotrienol</td>
</tr>
<tr>
<td>BT (BETA TOCOTRIENOL)</td>
<td>Decalcified bone scaffold treated with beta tocotrienol</td>
</tr>
<tr>
<td>GT (GAMMA TOCOTRIENOL)</td>
<td>Decalcified bone scaffold treated with gamma tocotrienol</td>
</tr>
<tr>
<td>DT (DELTATOCOTRIENOL)</td>
<td>Decalcified bone scaffold treated with delta tocotrienol</td>
</tr>
</tbody>
</table>
• RESULTS: PHASE 1
Concentration of isomer = 100 nM (determined earlier, unpublished data)

Porosity

Clumping of cell
Cells cover up most of the surface of the scaffold.

Clumping of cell
Cells attached at certain area on the surface of the scaffold.

Clumping of cell
Cells attached at most area on the surface of the scaffold.

X300 magnification

Scanning electron microscopy (SEM) observations of hF.OB 1.19 growth within bone scaffolds. SEM images taken on day 7 showing that the cells had successfully adhered to the bone scaffolds.
STRUCTURAL HISTOMORPHOMETRY: IMAGES (DAY 28)

Von Kossa stain at 40X magnification
STRUCTURAL HISTOMORPHOMETRY: QUANTITATIVE DATA

BONE VOLUME / TOTAL VOLUME

TRABECULAR THICKNESS

Trabecular number

Trabecular separation
CONCLUSION: PHASE 1

• Tocotrienol isomers, especially the gamma- and delta-tocotrienol isomers, improve bone microstructure in a 3-D osteoblast culture system.
3-D OB/OC CO-CULTURE AND TESTING OF ISOMERS

**DYNAMIC OSTEOBLAST CULTURE**

1x10⁶ hF.OB 1.19

Remove the agarose mold and transfer scaffold coated with complexes of cell and fibrin to 6 well plate.

Complete base medium

Fibrin

Agarose mold

Bone scaffold

Osteoblast with fibrin gel

Culture for 3 days

Incubate at 37°C, 5% CO₂

**DYNAMIC OSTEOBLAST/OSTEOCLAST CO-CULTURE**

Transfer OB & fibrin to agarose mold.

Ob-scaffold complex

At day 3, PBMCs were added to dynamic culture of osteoblast

Ratio (1 OC:2 OB)

Addition of isomers (100 nM/ml)

Analysis of scaffold

MICROCT

HISTOMORPHOMETRY

MECHANICAL STRENGTH

PHASE 2
• PHASE 2: RESULTS
SUCCESSFUL ESTABLISHMENT OF CO-CULTURE

<table>
<thead>
<tr>
<th>RATIO</th>
<th>OB &amp; OC CO-CULTURE (NO STAINING)</th>
<th>OC-TRAP (TRAP STAINING)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1OC: 2 OB</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Presence of TRAP+ cells (osteoclast-like cells stained dark brown) and osteoblasts co-exist in the culture.

The picture was taken in a 2D culture for illustration purpose

(40X Magnification)
Bone scaffold was scanned in air using SKYSCAN 1076 high resolution mCT scanner (GE Medical Systems) at 28mm voxel resolution, at 75kV and 75mA
MICROCT: IMAGES
**MICROCT: QUANTITATIVE DATA**

**Total Bone Volume**

![Graph showing Total Bone Volume](Graph_1.png)

- OB
- NC
- PC
- AP
- AT
- BT
- GT
- DT

**Total Bone Porosity**

![Graph showing Total Bone Porosity](Graph_2.png)

**Values are in mean ± SEM**

- a p< 0.05 as compared to Osteoporotic Bone (OB),
- b p< 0.05 as compared to Negative Control (NC),
- c p< 0.05 as compared to Positive Control (PC),
- d p< 0.05 as compared to alpha-tocopherol (AP)
- e p< 0.05 as compared to alpha-tocotrienol (AT)
- f p< 0.05 as compared to beta-tocotrienol (BT)
- g p< 0.05 as compared to gamma-tocotrienol (GT)
STRUCTURAL BONE HISTOMORPHOMETRY: IMAGES

Von Kossa stain: 40X MAGNIFICATION
BONE HISTOMORPHOMETRY: QUANTITATIVE DATA

**Bone Volume Fraction**

<table>
<thead>
<tr>
<th>Vitamin E (Isomers)</th>
<th>OB</th>
<th>NC</th>
<th>PC</th>
<th>AP</th>
<th>AT</th>
<th>BT</th>
<th>GT</th>
<th>DT</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV %</td>
<td>250</td>
<td></td>
<td></td>
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**Trabecular Thickness**

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<tbody>
<tr>
<td>TB.TH (MM)</td>
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**Trabecular Number**

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<th>OB</th>
<th>NC</th>
<th>PC</th>
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<th>AT</th>
<th>BT</th>
<th>GT</th>
<th>DT</th>
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<tr>
<td>TB. N1/MM</td>
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**Trabecular Separation**

<table>
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<tbody>
<tr>
<td>TB.S (MM)</td>
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- a p< 0.05 as compared to Osteoporotic Bone (OB),
- b p< 0.05 as compared to Negative Control (NC),
- c p< 0.05 as compared to Positive Control (PC),
- d p< 0.05 as compared to alpha-tocopherol (AP),
- e p< 0.05 as compared to beta-tocotrienol (BT),
- f p< 0.05 as compared to gamma-tocotrienol (GT)

Values are in mean ± SEM
Compression Test was performed using Shimadzu Universal Testing Machine (Model: AGS-500NX) with load capacity of 400N and Maximum Speed : 10 mm/min
MECHANICAL STRENGTH

Bone Strength

![Bar chart showing mechanical strength comparison across different groups with statistical significance indicators: a p< 0.05 as compared to Osteoporotic Bone (OB); b p< 0.05 as compared to Negative Control (NC); c p< 0.05 as compared to Positive Control (PC); d p< 0.05 as compared to alpha-tocotrienol (AT); e p< 0.05 as compared to beta-tocotrienol (BT); f p< 0.05 as compared to gamma-tocotrienol (GT); Values are in mean ± SEM]
CONCLUSION: PHASE 2

- Gamma- and delta-tocotrienol isomers improve bone microstructure and strength in a 3-D osteoblast/osteoclast co-culture system.

- Delta-tocotrienol appear more effective in improving bone strength compared to the other vitamin E isomers.
Overall conclusion

• The gamma- and delta-tocotrienol isomers were effective in improving bone microstructure and strength in a 3-D co-culture system on bone scaffold. This system was designed to mimic the human physiological skeletal microenvironment.

• These results are in general agreement with our previous *in vivo* studies. However, more parameters are needed to come to a stronger conclusion. More *in vitro* studies are currently ongoing.

• Clinical studies are needed to confirm the findings in humans.
Tocotrienol

↑ Bone structure
Indicated by histomorphometry and µCT

↑ Bone calcium/phosphate
Indicated by Von Kossa staining

↑ Geometric properties of the bone

↑ Material properties of the bone

↑ Mechanical strength of the bone
Indicated by biomechanical strength test

Bone protected!
Thank you

• UKM and PPUKRM for the opportunity, facilities and research grants LAUREATE-2013-003 and FF-349-2011

• AMERICAN-RIVER NUTRITION for the gift of the tocotrienol isomers
RESEARCH TEAM

Front: Dr. Elvy Suhana Mohd. Ramli, Prof. Dr. Ima Nirwana Soelaiman, Puan Nur Farhana Mohd Fozi
Back: En. James Jam Jolly, Assoc. Prof. Dr. Chua Kien Hui, Dr. Chin Kok Yong, Dr. Ekram Alias