THE ROLE OF PALM TOCOTRIENOLS IN THE PREVENTION AND TREATMENT OF OSTEOPOROSIS: IN VIVO STUDIES

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INTRODUCTION

Oxygen-derived free radicals are involved in the formation and activation of osteoclasts [Garrett et al 1990], leading to an increase in bone resorption and bone loss.

Bone histomorphometric studies have shown that ferric nitrilotriacetate (FeNTA), an oxidising agent, increased osteoclast numbers [Ebina et al 1991] and impaired mineralisation [Takeuchi et al 1997].

Osteoporotic women had significantly higher plasma superoxide dismutase (SOD) enzyme activity and higher malondialdehyde (MDA) levels (Ozgocmen et al 2007).

A negative correlation was found between SOD and lumbar bone mineral density (BMD) levels ($r = -0.328; p = 0.021$) (Yalin et al 2005).

Increased osteoclastic activity and decreased osteoblastic activity may be associated with an imbalance between oxidant and antioxidant status in postmenopausal osteoporosis (Altindag et al 2008).
Can antioxidants, namely tocotrienol, prevent and treat osteoporosis?
PALM TOCOTRIENOLS

The most easily available source of tocotrienols in Malaysia is from palm oil. Palm cooking oil contains 178.33 ppm α-tocopherol, 188.50 ppm α-tocotrienol, 260.83 ppm γ-tocotrienol and 69.83 ppm δ-tocotrienol [Siti Khadijah, 2011].

Our studies mainly used tocotrienols extracted from palm oil in the form of tocotrienol mixtures as well as the pure γ-isomer.

We used the rat as our animal model since previous studies have shown that their bone anatomy, bone remodeling & response to treatment are similar to humans [Abe et al. 1993, Mosekilde, 1995].
OBJECTIVES

To determine the effects of palm oil-derived tocotrienols on various animal models of osteoporosis.

To determine the effects of palm oil-derived tocotrienols on normal bone.
METHODOLOGY.

Animals and treatment

Male and female Sprague-Dawley or Wistar rats were used. The age and body weights were according to the respective experiments.

Palm oil derived tocotrienols in the form of mixtures or single isomers obtained from Palm Oil Research Institute/Palm Oil Board, Carotech Inc and Golden Hope. The palm tocotrienol extract was diluted in olive oil (Bertolli Classico, Italy) to the desired concentration.

Parameters

Bone histomorphometry – left femur

Bone biomechanical strength test – right femur
Bone histomorphometric studies (Parfitt et al. 1987)
• Structural parameters
• Dynamic parameters
• Static/cellular parameters

Image Analyzer Pro-Plus (Media Cybernatics, Silver Spring, MD, USA)
Light/Fluorescent microscope (Nikon Eclipse 80i, Japan)
Weibel grid (Freere & Weibel 1967)
Margin for histomorphometry analysis (Metaphyseal area):

(3-7 mm from the growth plate, 1 mm lateral cortex. * Active changes & contain high trabecular bone)

(Baldock et al 1998)
### STRUCTURAL HISTOMORPHOMETRIC PARAMETERS

<table>
<thead>
<tr>
<th>Terminology</th>
<th>Symbol</th>
<th>Formula</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Bone Volume/Tissue Volume #</td>
<td>BV/TV</td>
<td>bone area/tissue area x 100</td>
<td>%</td>
</tr>
<tr>
<td>2. Trabecular Thickness *</td>
<td>TbTh</td>
<td>2 / (bone perimeter/bone area)</td>
<td>µm</td>
</tr>
<tr>
<td>3. Trabecular Number *</td>
<td>TbN</td>
<td>(BV/TV)/Tb.Th x 100</td>
<td>#/mm²</td>
</tr>
<tr>
<td>4. Trabecular Separation *</td>
<td>TbSp</td>
<td>(1000/TbN) – Tb.Th</td>
<td>µm</td>
</tr>
</tbody>
</table>

Fig. 9 µm undecalcified section showing the trabecular bone (black). Von Kossa stain. x 100 magnification

(Parfitt et al. 1987)
DYNAMIC HISTOMORPHOMETRIC PARAMETERS

<table>
<thead>
<tr>
<th>Terminology</th>
<th>Symbol</th>
<th>Formula</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Single Labeled Surface/Bone Surface #</td>
<td>sLS/BS</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>2. Double Labeled Surface/Bone Surface#</td>
<td>dLS/BS</td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>3. Bone Formation Rate/Bone Surface *</td>
<td>BFR/BS</td>
<td>{\frac{(dLS + \frac{1}{2} sLS)}{BS}} \times MAR</td>
<td>\mu m^2/day</td>
</tr>
<tr>
<td>4. Mineral Appositional Rate #</td>
<td>MAR</td>
<td></td>
<td>\mu m/day</td>
</tr>
<tr>
<td>5. Mineralised Surface/Bone Surface *</td>
<td>MS/BS</td>
<td>\frac{(dLS + \frac{1}{2} sLS)}{BS}</td>
<td>\mu m^2/day</td>
</tr>
</tbody>
</table>

(Parfitt et al. 1987)

Fig. Calcein labels along trabecula demonstrating the dLS, sLS and MAR (inter-label distance) using fluorescence microscope in an undecalcified section without staining. (x 200 magnification).
<table>
<thead>
<tr>
<th>Terminology</th>
<th>Symbol</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Mean Osteoclast Number #</td>
<td>N.Oc</td>
<td>#/mm²</td>
</tr>
<tr>
<td>2. Mean Osteoblast Number #</td>
<td>N.Ob</td>
<td>#/mm²</td>
</tr>
<tr>
<td>3. Eroded Surface/Bone Surface #</td>
<td>ES/BS</td>
<td>%</td>
</tr>
<tr>
<td>4. Osteoid Volume/Bone Volume #</td>
<td>OV/BV</td>
<td>%</td>
</tr>
<tr>
<td>5. Osteoid Surface/Bone Surface #</td>
<td>OS/BS</td>
<td>%</td>
</tr>
</tbody>
</table>

(Parfitt et al. 1987)
BIOMECHANICAL STRENGTH TEST

3-point configuration

(Helfrich et al. 2003)
BIOMECHANICAL STRENGTH TEST

Instron Machine Model 5560 Series (Instron® Co., USA)
Bluehill® 2 Software (Instron® Co., USA)
3-point bending test

Rat femur
<table>
<thead>
<tr>
<th>Parameters</th>
<th>EXTRINSIC (external bone structure)</th>
<th>INTRINSIC (internal bone structure)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Displacement [mm]</td>
<td>Stress [MPa]</td>
<td></td>
</tr>
<tr>
<td>Load [N]</td>
<td>Strain [%]</td>
<td></td>
</tr>
<tr>
<td>Stiffness [N/mm]</td>
<td>Viscoelasticity [MPa]</td>
<td>(Helfrich et al. 2003)</td>
</tr>
</tbody>
</table>
(A) Load-displacement curve, measures extrinsic properties of bone.

(B) Stress-strain curve, measures intrinsic properties of bone.
STUDY 1. ANTIOXIDATIVE EFFECTS OF PALM TOCOTRIENOLS IN BONE

Can palm tocotrienol prevent osteoporotic changes in rats exposed to ferric-nitrilotriacetate, an oxidising agent?

Parameter

Bone histomorphometry.

Thiobarbituric-acid reactive substances (lipid peroxidation by-product) in left femur by spectrophotometry.

(Nazrun et al 2005, Maniam et al 2008)
RESULTS – structural bone histomorphometry

RESULTS – structural bone histomorphometry

Figure 2: Structural Parameters of Bone Histomorphometry
a: significantly different from K group, b: significantly different from S group
c: significantly different from F group, d: significantly different from F+A group
Data is presented as mean ± s.e.m. Significance is taken at p < 0.05

RESULTS - TBARS levels in left femur

Fig. 1. Impact of α-tocopherol (ATF) and palm tocotrienol (TT) supplementation on lipid peroxidation in the femur of adult male rats. Values marked by the same letters indicate significant difference between groups at P < 0.05; *P < 0.05, significantly different from control group. TBARS, thiobarbituric acid-reactive substances.

Palm tocotrienol protected the bone of rats from osteoporotic changes caused by ferric-nitrilotriacetate, an oxidising agent (Nazrun et al. 2005).

Palm tocotrienol reduced the levels of lipid peroxidation by-product (thiobarbituric acid-reactive substances, TBARS) in the rat femur. (Maniam et al. 2008)

Therefore the bone protective effects of palm tocotrienol is most probably due to its antioxidant properties.
STUDY 2: EFFECTS OF PALM TOCOTRIENOLS ON BONE IN OESTROGEN-DEFICIENT OSTEOPOROSIS

CAN PALM TOCOTRIENOL PROTECT AGAINST OESTROGEN DEFICIENT OSTEOPOROSIS?

Methodology
Ovariectomised female rats ~ animal model of postmenopausal osteoporosis.
Bone histomorphometry

RESULTS – structural histomorphometry

![Images of histomorphometry results](image)

**Figure 5:** Photomicrographs of distal femur metaphyses from ovariectomized (a) and sham-operated (b) rats, as well as ovariectomized rats treated with alpha-tocopherol (c) and tocotrienol (d). Undecalcified histological bone sections stained with Von Kossa. Trabecular bone appear dark by Von Kossa staining. Loss of trabecular bone is apparent in (a) while treatment with either forms of Vitamin E prevented bone loss in ovariectomized rats ((c) and (d)). Light microscopy at magnification ×40.

RESULTS – structural histomorphometry

Figure 1: Mean percentage of bone volume to tissue volume. *Indicates significant difference from ovariectomized group \((P < 0.05)\). Data are mean ± SEM. OVX: ovariectomy; OVX + ATF: ovariectomy treated with alpha-tocopherol; OVX + PTT: ovariectomy treated with pure tocotrienol.

Figure 3: Trabecular separation in different groups of rats. *Indicates significant difference from ovariectomized group \((P < 0.05)\). Data are mean ± SEM. OVX: ovariectomy; OVX + ATF: ovariectomy treated with alpha-tocopherol; OVX + PTT: ovariectomy treated with pure tocotrienol.

Figure 2: Trabecular number in different groups of rats. *Indicates significant difference from ovariectomized group \((P < 0.05)\). Data are mean ± SEM. OVX: ovariectomy; OVX + ATF: ovariectomy treated with alpha-tocopherol; OVX + PTT: ovariectomy treated with pure tocotrienol.

Figure 4: Trabecular thickness in different groups of rats. OVX + PTT: ovariectomy + pure tocotrienol. Data are mean ± SEM. OVX: ovariectomy; OVX + ATF: ovariectomy treated with alpha-tocopherol; OVX + PTT: ovariectomy treated with pure tocotrienol.

Conclusion:

Tocotrienol was able to prevent osteoporotic changes in bone histomorphometry due to estrogen deficiency in ovariectomised rats (Norliza et al, 2012.).

FURTHER STUDIES

Bone formation rate was found to be higher in ovariectomised rats given palm tocotrienols supplementation compared to rats supplemented with calcium (Ima-Nirwana et al 2012) or oestrogen (Aktifanus et al 2012).

OVERALL CONCLUSION

Therefore tocotrienol has the potential to be used as supplements to prevent osteoporosis in peri-menopausal and post-menopausal women.
STUDY 3: EFFECTS OF PALM TOCOTRIENOLS ON NORMAL, NON-OSTEOPOROTIC BONE

Can tocotrienols improve bone structure and biomechanical strength in normal non-osteoporotic rats?

Methodology
Bone histomorphometry
Bone biomechanical strength test

(Shuid et al 2010).
RESULTS – structural bone histomorphometry

Fig. 12 Micrographs of undecalcified rat femur stained with Von Kossa (×50). The trabeculae are stained black. Grossly, GTT and ATF groups appear to have more trabeculae than the NC group; the GTT group has more trabeculae than the ATF group. NC vehicle, ATF 60 mg/kg α-tocopherol, GTT 60 mg/kg γ-tocotrienols

NC group
ATF group
GTT group
RESULTS – structural bone histomorphometry

Fig. 2 Trabecular bone volume. *Significant differences compared to NC group; # significant differences compared to ATF group (P < 0.05). Data are expressed as mean ± SEM. NC normal control group, ATF α-tocopherol group, GTT γ-tocotrienol group.

Fig. 3 Trabecular thickness. *Significant differences compared to NC group; # significant differences compared to ATF group (P < 0.05). Data are expressed as mean ± SEM. NC normal control group, ATF α-tocopherol group, GTT γ-tocotrienol group.

Fig. 4 Trabecular number. *Significant differences compared to NC group; # significant differences compared to ATF group (P < 0.05). Data are expressed as mean ± SEM. NC normal control group, ATF α-tocopherol group, GTT γ-tocotrienol group.

Fig. 5 Trabecular separation. *Significant differences compared to NC group; # significant differences compared to ATF group (P < 0.05). Data are expressed as mean ± SEM. NC normal control group, ATF α-tocopherol group, GTT γ-tocotrienol group.
RESULTS – biomechanical strength test

Fig. 6 Load values. *Significant differences compared to NC group; \(^*\)significant differences compared to ATF group \((P < 0.05)\). Data are expressed as mean ± SEM. NC normal control group, ATF \(\alpha\)-tocopherol group, GTT \(\gamma\)-tocotrienol group

Fig. 7 Displacement. *Significant differences compared to NC group; \(^*\)significant differences compared to ATF group \((P < 0.05)\). Data are expressed as mean ± SEM. NC normal control group, ATF \(\alpha\)-tocopherol group, GTT \(\gamma\)-tocotrienol group

Fig. 8 Stress values. *Significant differences compared to NC group; \(^*\)significant differences compared to ATF group \((P < 0.05)\). Data are expressed as mean ± SEM. NC normal control group, ATF \(\alpha\)-tocopherol group, GTT \(\gamma\)-tocotrienol group

Fig. 9 Strain values. *Significant differences compared to NC group; \(^*\)significant differences compared to ATF group \((P < 0.05)\). Data are expressed as mean ± SEM. NC normal control group, ATF \(\alpha\)-tocopherol group, GTT \(\gamma\)-tocotrienol group
RESULTS – bone biomechanical strength test

RESULTS

Gamma tocotrienol improved bone structural histomorphometric parameters significantly more than the normal control group.

Gamma tocotrienol improved bone biomechanical strength parameters significantly more than the normal control group.

(Shuid et al 2012)
CONCLUSION

Gamma tocotrienol supplementation, improves bone structure, which contributed to stronger bone.

Therefore, tocotrienols has the potential to be used as an anabolic agent to treat osteoporosis or as bone supplements for young adults to achieve higher peak bone mass. This can prevent osteoporosis in later years.
OTHER STUDIES

Palm tocotrienols reversed the osteoporotic changes in rats given long term intraperitoneal nicotine (Hermizi et al 2009). Therefore palm tocotrienols maybe able to treat osteoporosis in chronic smokers.

(Winner of Gold Medal (Award of Merit) at the Invention and New Product Exposition (INPEX2008), Pittsburgh, Pennsylvania, USA, 2008).

Tocotrienols was found to improve the strength of fracture callous after fracture healing of osteoporotic bone in a rat model of post-menopausal osteoporosis (Sharlina et al 2012). Thus palm tocotrienols maybe used to accelerate osteoporotic fracture healing.
IS VITAMIN E BAD FOR BONES?


With the exception of α-tocopherol, none of the isoforms of vitamin E, including α-tocotrienol, which is 100-fold stronger in antioxidant activity than α-tocopherol, stimulated osteoclast fusion.

Moreover, except for α-tocopherol, none of the antioxidants tested, including ascorbic acid, which is the primary water-soluble antioxidant, stimulated osteoclast fusion.

Taken together, these results clearly show that, unlike other vitamin E isoforms and antioxidants, α-tocopherol specifically regulates osteoclast fusion independent of its antioxidant activity. (Fujita et al, 2012)

*Dose of α-tocopherol used was 600 mg/kg (10x our optimum effective dose)*
OVERALL CONCLUSIONS AND RECOMMENDATIONS

Our studies clearly showed that palm tocotrienols, especially the gamma tocotrienol isomer, has the potential to be further developed as an antosteoporotic agent. It has the efficacy to prevent and reverse osteoporosis due to various factors.

Our studies also suggest that palm tocotrienol supplementation to young adults may increase peak bone mass and strength, leading to reduced risk of osteoporosis in later life.

Specific and comprehensive toxicity studies should be carried out targeting the effective dose and duration of treatment from our studies, as well as the specific preparations and type of tocotrienol.

Clinical trials are needed to translate the effects of the animal studies onto humans. The effective dose and duration of treatment in humans need to be established.

Further studies to establish the molecular mechanism of action of tocotrienols on bone also need to be studied.
REFERENCES


REFERENCES – cont.


