Tocotrienols Regulate Bone Loss through Suppression on Osteoclast Differentiation and Activity: A Systematic Review

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Abstract: Background: There are accumulating studies reporting that vitamin E in general exhibits bone protective effects. This systematic review, however discusses the effects of a group of vitamin E isomers, tocotrienols in preventing bone loss through osteoclast differentiation and activity suppression.

Objective: This review is aimed to discuss the literature reporting the effects of tocotrienols on osteoclasts, the cells specialized for resorbing bone.

Results: Out of the total 22 studies from the literature search, only 11 of them were identified as relevant, which comprised of eight animal studies, two in vitro studies and only one combination of both. The in vivo studies indicated that tocotrienols improve the bone health and reduce bone loss via inhibition of osteoclast formation and resorption activity, which could be through regulation of RANKL and OPG expression as seen from their levels in the sera. This is well supported by data from the in vitro studies demonstrating the suppression of osteoclast formation and resorption activity following treatment with tocotrienol isomers.

Conclusion: Thus, tocotrienols are suggested to be potential antioxidants for prevention and treatment of bone-related diseases characterized by increased bone loss.

Keywords: Osteoclast, tocotrienols, bone loss, anti oxidants, osteoblasts, osteoclast.

1. INTRODUCTION

Bone is a dynamic organ that must continuously undergo remodelling, in which bone resorption by osteoclasts is coupled with bone formation carried out by osteoblasts [1]. Osteoclast is a cell originated from monocyte or macrophage precursor cells and its differentiation produces large multinucleated cells, responsible in resorbing bone matrices [2]. Physiologically, osteoclast number must be maintained in order to prevent excess of bone resorption that would result in pathological bone loss [3].

Bone loss is a serious public health concern in our global ageing population. In general, bone loss could be divided into two categories, which are systemic and local bone loss. A classic example of systemic bone loss is osteoporosis, a disease characterised by decrease in bone mass and density which leads to the increase in the bone fragility and hence susceptibility to bone fractures. Local bone loss diseases include rheumatoid arthritis and peri-implant osteolysis. These bone diseases share similarity in the pathological aspect, whereby there is excessive bone resorption due to imbalance in bone remodeling resulted from the increase in osteoclast formation and activity [4-6].

Receptor activator of nuclear factor-kappa B (RANK), its ligand (RANKL) and its decoy receptor osteoprotegerin (OPG), which have been discovered in the late 1990’s [7-11], play roles in bone remodeling [12]. The binding of RANKL to its receptor RANK stimulates osteoclast differentiation via various downstream signaling pathways such as NF-κB, AKT, extracellular signal-regulated kinase (ERK) c-JUN n-terminal kinases (JNK) and p38 MAP kinases (p38) [13]. The binding of RANKL to OPG, on the other hand, prevents RANKL from binding to RANK, thereby suppressing osteoclast differentiation and activation.

The expression and levels of RANKL and OPG have been reported to be altered in osteoporosis [14, 15], rheumatoid arthritis [16-18] and peri-implant osteolysis [19-21], and this may suggest that increased osteoclast formation and activity could be responsible for the bone loss observed in those diseases.

Oxidative stress, which is an aspect associated with osteoporosis [22-24], rheumatoid arthritis [25, 26] and peri-implant osteolysis [27, 28], is also a factor that could promote osteoclastogenesis. It has been long understood that the presence of free radicals increases the formation and activity of osteoclasts [29-32]. Free radicals or reactive oxygen spe-
cies are generated in the pathway that mediates RANKL-induced osteoclastogenesis [31-34]. This fact has made antioxidants as good candidates of therapeutic agents for treating bone loss, not only for their function in reducing oxidative stress in the pathological conditions but also for their potential in suppressing osteoclastogenesis [35-38].

Vitamin E, which is an example of antioxidants, has been found to be beneficial in treating bone loss probably attributed to its property of scavenging free radicals, leaving it to receive wide attention in bone research. Vitamin E is a lipidsoluble antioxidant, which prevents free radicals from attacking on membrane lipids. It protects the membrane by inhibiting the formation of oxidized phospholipids during lipid peroxidation, thus increasing the stability of the cell [39]. There are two forms of vitamin E, tocopherol, and tocotrienol, which can be further divided into α-, β-, γ- and δ- isomers for each. α-tocopherol (ATF) has the highest bioavailability as it is preferentially absorbed by the body and has been widely studied in bone loss study model [40].

There is accumulating number of studies and findings suggesting the potential of vitamin E supplementation in protecting bone health. It has been shown that, in rat model of postmenopausal osteoporosis, supplementation of palm vitamin E was able to prevent decrease of bone calcium content and maintain bone density through increase in bone formation [41]. Supplementation with high dose vitamin E was also found to improve the strength and density of the bones in aged rats [42]. With regards to the bone protective properties that vitamin E has, it is believed that tocotrienols exhibit more superior effects than tocopherols [30, 43].

While there have been systematic reviews [44, 45] evaluating the effectiveness of vitamin E in general for protecting bone, however with several reports describing ATF as damaging to the bone health [46, 47], it would be a great interest to assess the osteo-protective effects of tocotrienols only, particularly at the cellular level of the resorbing cells, osteoclasts. In addition, to the best of our knowledge, there is none reviewing specifically the effects of tocotrienols on osteoclast differentiation and its resorption. Therefore, the aim of this review is to discuss the literature reporting the effects of tocotrienol isomers supplementation specifically on osteoclasts.

2. METHODS

2.1. Literature Review

A comprehensive review of relevant literature on the effects of tocotrienol(s) on osteoclasts differentiation and activity was conducted using SCOPUS and PUBMED MEDLINE search engine. The relevant medical science journals published between 1946 until March 2016 were identified with the keywords of osteoclast* AND tocotrienol*.

2.2. Selection of Research Articles

The eligibility criteria for this review were limited to original research articles, without language limitation. The study models should be represented by bone loss either in vivo or in vitro. Treatment included must be either any isomers of tocotrienols (α-, β-, δ- or γ-tocotrienol) or treatment with the mixture of tocotrienols in it.

2.3. Data Extraction

Firstly, the list of articles obtained from both search engines was gathered for screening process. Based on title screening, duplicates were removed. Papers were then screened via abstract following the inclusion criteria determined. The authors agreed to include a comprehensive reading on the full-text of the remaining articles prior to data extraction. In order to systematically assemble the data, there were several study characteristics extracted using data collection form, including the type of study, type of treatment, samples or subjects recruited, methods used, results obtained, and the comments and conclusion from the reviewers for each of the study. Any dissimilarity in thoughts throughout the screening and extraction processes was resolved through discussions between at least two reviewers (NFMR, NASI, EA).

3. RESULTS

3.1. Literature Search Results

A total of 36 articles were found from those two search engines mentioned. After pre-screening the titles of the articles, 14 were removed as duplicates. The abstracts of remaining 22 relevant articles were evaluated in line with the eligibility criteria stated. Only eligible 11 papers were proceeded with the full-text screening, meanwhile others were excluded from this review since they were not original research articles or studies associating tocotrienol with osteoclasts. During the final phase of screening, all 11 articles were selected for data synthesis and listed in Table 1. The selection process of potential articles is illustrated in Fig. (1).

3.2. Study Characteristics

Studies involved in this review were primary articles published between 2005 and 2014, which comprised of eight in vivo studies, two in vitro studies and a study that had both in vitro and in vivo models. Animals involved in those in vivo studies were rats and mice. Meanwhile, the type of cells used for the in vitro studies were human peripheral blood-derived CD14+ cells, CD14+ cell lines [48] and macrophage-derived from mouse bone marrow [49]. There were two studies that used co-culture of osteoblasts and osteoclasts. Ha and coworkers [49] used primary osteoblasts from newborn ICR mouse calvariae in their in vitro coculture system. The study done by Deng et al., [50] which also included in vivo work (Table 1), used UAMS-32P cell line as the osteoblasts in their co-culture system for providing RANKL for osteoclast differentiation.

The types of tocotrienol(s) used in each individual study are discussed in this review. Two in vivo studies [51, 52] used annatto bean-derived tocotrienols, which are comprised of 90% δ- and 10% γ-tocotrienol composition. These two studies compared the effect of annatto bean tocotrienols (with highlights placed on δ-tocotrienol as the main tocotrienol isomer) with lovastatin and testosterone enanthate. Four studies used palm oil-extract tocotrienols-rich fractions (γ-tocotrienol, GTT was highest in the content) sourced from Malaysian Palm Oil Berhad (MPOB) and Palm Oil Research Institute of Malaysia (PORIM). Both studies by Deng and coworkers [50, 53] used γ-tocotrienol isomers emulsified in
| Study | In vitro | In vivo | Tocotrienol | Sample/Subject/Population | Methods | Results | Comment or Outcome |
|-------|----------|---------|-------------|---------------------------|---------|---------|-------------------|
| Study 1 (Deng et al., 2014) | √ | | Polyethylene glycol (PEG-400) emulsi-fied γ-tocotrienol (GTT) | C57BL/6 female mice (8 weeks old, about 23-25g), 8 mice per treatment group. Mice were injected with γ-tocotrienol via subcutaneous injection. | | GGT predominantly accumulated in adipose tissue on both day 3 and 14. Increased of GGT level in heart and spleen (comparison between day 3 and 14). No significant difference in RANKL and OPG mRNA expression between control and GGT treatment group in both femur and spine across all time points assessed. GGT inhibited the up-regulation of RANKL mRNA expression and down-regulation of OPG mRNA expression following induction by db-cAMP. | Level of GGT in both bone tissues relatively stable over time in mice. In both femur and spine tissues, GGT significantly inhibits the increase in RANKL and decrease in OPG mRNA expression. |
| Study 2 (Deng et al., 2014) | √ | √ Co-culture of bone marrow osteoclast precursors with PTH-treated UAMS-32P | Polyethylene glycol (PEG-400) emulsi-fied γ-tocotrienol (GTT) | C57BL/6 female mice (8 weeks old, about 23-25g), 8 mice for each group. Mice were either sham-operated or osteoclast-terminated (OVX) bilaterally. | | Bone density measurement Bone density of femur and spine showed supplementation of GGT significantly prevented decrease in OVX mice. Daily mevalonate supplementation blocked the bone protective effect of GGT Bone structural parameters GGT treatment significantly prevented the reduction of BV/TV, Tb.Th, Tb.N & the increase in Tb.Sp. Mevalonate reversed the effects of GGT. Static bone histomorphometric parameters GGT significantly decreased osteoclast numbers and increased osteoblast numbers in OVX mice, but this was reversed by mevalonate supplementation. Dynamic bone histomorphometric parameters GGT significantly increased mineral apposition rate and bone formation rate but these effects were inhibited by mevalonate supplementation. Serum level of biomarkers of bone metabolism GGT significantly increased serum osteocalcin level and decreased serum CTX-I level, and the effect was reversed by mevalonate supplementation. Osteogenic expression GGT significantly inhibited the O VX-induced increase of RANKL and blocked the OVX-induced decrease of OPG mRNA expression in femur. GGT significantly increased both Osterix and Runx2 mRNA expression in femur. Mevalonate reversed the modulation of GGT on the gene expression. | GGT blocked OVX-induced bone loss. This effect could be seen from the increased bone density and structure, higher osteoclast number, modulated serum levels of bioclinical markers of bone metabolism and higher expression of osteogenic genes in bone. However, the protective effect of GGT can be overcome by daily supplementation of mevalonate. In vitro study indicated that GGT suppressed PTH-induced RANKL expression in UAMS-32P through mevalonate pathway. |

(Table 1) contd....
| Study | In vitro | In vivo | Tocotrienol | Sample/Subject/Population | Methods | Results | Comment or Outcome |
|-------|----------|---------|-------------|---------------------------|---------|---------|-------------------|
| Study 3 Brooks et al., 2011 | ✓ | ✓-tocotrienol (ATT) | Human blood-derived CD14+ cells from three different donors | 2×10^4 cell line for substrate resorption assay | 1. Peripheral blood mononuclear cells (PBMCs) isolated from theuffy coats of blood by density gradient centrifugation. They were later selected for CD14 subpopulation using microbeads. 2. 1×10^6 CD14+ OC precursor cells monocytes were seeded and cultured on dentin, collagen, or calcium phosphate-coated plates in presence of MCSF (25 ng/ml) and RANKL (50 ng/ml). Treatment with tocotrienols began from day 1. 3. Cell proliferation following treatment with ATT, GTT and DTT (0.01-1.0 mM) was assessed using MTS assay. The number of large multinucleated TRAP+ cells in each view field was also counted and compared. 4. Bone resorption activity of osteoclast formed following treatment with ATT. GTT and DTT (0.01-0.1 mM) were assessed via resorption assay. Dentin disc was stained with toluidine blue (0.1%) meanwhile calcium phosphate-coated slides were stained using von Kossa reagent. Resorption area was quantified using PC Image (Synoptics). For osteoclast formation, cells were fixed before staining for vitronectin receptor. | | Between isomers of tocotrienols, γ-tocotrienol appeared to give greater inhibition TRAP+ osteoclast formation and activity. Higher dose of β-tocotrienol gives greater inhibition on osteoclast formation and activity. When compared between tocotrienols and tocopherols, α-, γ- and δ-tocotrienols exhibited greater suppression on osteoclast formation than the tocopherols counterparts. Study on CD14+ cell lines showed dose-dependent reduction of resorption with increasing dose of γ- and β-tocotrienols. |
| Study 4 Ha et al., 2011 | ✓ | α-tocotrienol (ATT) | Osteoclasts: from mouse bone marrow-derived macrophage (BMM) Osteoblasts: from newborn ICR mouse calvariae | 1. Osteoclast culture: Mouse BMMs (4×10^5 cells/well) were cultured with MCSF (30ng/ml) and RANKL (100ng/ml) for 4 days. Coculture system consisted of BMM cells (3×10^5 cells/well) and primary osteoblasts (2×10^4 cells/well) for 6 days. At the end of culture, cells fixed and stained for TRAP. 2. Primary osteoblasts were pretreated with or without ATT and ATf then stimulated with IL-1 (10ng/ml) or 1,25(OH)2D3 (100nM) for 24h to induce RANKL and OPG protein expression. 3. For resorption assay, mature osteoclasts were grown in co-culture system on OAAS plates coated with carbonated calcium phosphate. The cells were pre-treated with ATT (50µM) or ATF (50µg) for 12 hours and further incubated in the presence of RANKL (100ng/ml). Cells removed and resorption pits were photographed after 24 hours. 4. Mature osteoclasts in the coculture system were purified by removing osteoblasts with 0.1% collagenase in order to assess osteoclast survival. 5. To overexpress c-Fos and constitutively active NFATc1, those genes were retrovirally transduced using retroviral vectors pMX-ires-EGFP. 6. Western blot analysis for phosphoErd2, ERK, phosphorylated JNK1/2, JNK, phosphorylated p38, p38, phosphorylated IκBα, IκBα, phosphorylated JNK1/2, JNK, phosphorylated p38, p38, phosphorylated IκBα, IκBα, NFATc1, c-Fos and IκBα were performed. 7. To detect protein complexes with nuclear acids, nuclear extraction and electrophoretic mobility shift assay (EMSA) were done. 8. Gene expression analysis of RANKL, OPG, c-Fos, NFATc1 and GAPDH were performed. | BMM cell-osteoblast coculture ATT but not ATf inhibited the formation of TRAP+ multinucleated osteoclasts in IL-1 or 1,25(OH)2D3 plus PGE2-induced BMM cell-osteoblast coculture through suppression of RANKL expression in the osteoblasts. Both ATT and ATf did not affect OPG expression in the osteoblasts. Substrate resorption by osteoclast Resorption pins were seen after 3 weeks on dentin and clear areas appeared after 4 days on calcium phosphate films, with extensive resorption after 6 days. At 1mM, GTT completely inhibited resorption and resorption was very low in the DTT group. Resorption was decreased with increasing dose of GTT and DTT (0.01-1.0mM) in both cell lines tested. | ATT inhibited the formation of TRAP+ osteoclast either indirectly by upregulating RANKL expression or osteoclast differentiation or directly inhibiting the early stage of osteoclastogenesis in osteoclast precursors cells. Gene and protein expression indicated that RANKL-induced MAPKs activation was inhibited by ATT at early signaling pathway. Meanwhile, NFκB activation was suppressed at delayed stage of activation. ATT also markedly suppressed osteoclast resorption activity. |
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Study 5: Norazlina et al., 2010

- **Tocotrienol mixture from Malaysian Palm Oil Board (MPOB)**
- 3-month-old male Sprague-Dawley rats
- Randomly divided into 4 groups, 8 rats per group:
  - A: control group
  - B: nicotine-treated group
  - C: nicotine-treated group supplemented with tocotrienol mixture at 60 mg/kg during week 1 and 30 mg (NT- assaulted for 6 days a week for 12 weeks.

- Serum levels of OPG and RANKL: Tocotrienol-induced bone loss by restoring the changes in bone static histomorphometric parameters, including increased osteoblasts and osteoblasts following ovariectomy.

- Bone histology treatment prevented osteoporotic-deficiency-induced bone loss by restores the changes induced bone loss in osteoblastic bone volume markers.

Study 6: Chin et al., 2014

- **Annatto tocotrienol (99% δ- and 10% γ- tocotrienol)**
- Male Sprague-Dawley rats (3 months old, 250-300g body weight)
- Randomly divided into 5 groups:
  - A: baseline (BL)
  - B: SH (sham)-treated group
  - C: Annatto tocotrienol-treated (AnTT) - testosterone enanthate-treated (TE).

- Treatment TT and ATF did not result in any improvement.

Study 7: Muhammad et al., 2012

- **Pure tocotrienols mixture (37.2% δ- and 39.1% γ- and 22.6% α-tocotrienol) from Palm Oil Research Institute of Malaysia (PORIM)**
- Female Wistar rats (1 month old, 200-250g body weight)
- Randomly divided into 5 groups:
  - A: baseline (BL)
  - B: SH (sham)-treated group
  - C: Tocotrienol-treated group (OVX + FTT) - α-tocopherol-treated group (OVX + ATF)

- Supplementation with ATF significantly increased serum RANKL levels following nicotine treatment.

(Table 1) contd....
| Study          | In vitro | In vitro | Tocotrienol | Sample/ Subject/ Population | Methods | Results | Comment or Outcome |
|---------------|----------|----------|-------------|----------------------------|---------|---------|-------------------|
| Study 8       |          |          | δ- and γ-tocotrienol (90% δ- and 10% γ-tocotrienol) | 48 female Sprague-Dawley rats (3 months old, 200-250g weight) |         | Serum bone biochemical markers level. | Treatment with δ-tocotrienol improved bone, even in the estrogen-deficient model. Treatment with lovastatin alone did not reduce estrogen-induced bone loss. Combination treatment of tocotrienol and lovastatin significantly further enhanced the effect of only δ-tocotrienol on osteoclast surface and osteoid volume. |
| Mehat et al., |          |          | δ- and γ-tocotrienol (90% δ- and 10% γ-tocotrienol) | 32 Sprague-Dawley male rats (3 months old, 200-250g weight) |         | Static histomorphometric parameters GTT and DTT treatment significantly reduced N.Oc ad OV/BV, meanwhile statistically increased N.Ob, OS/BS and OV/BV in normal rats. | Treatment with either GTT or DTT significantly affected all static histomorphometric parameters studied, including decreased osteoclast number. Treatment with ATF also significantly reduced osteoclast number in normal rats. GTT had more superior effect than DTT and ATF in all static histomorphometric parameters studied, including osteoclast number. |

At 6 months of study, the rats were divided into 6 groups: -Baseline (BL) -Sham (SH) -α-Tocotrienol treated group (OVX + TT) -δ-Tocotrienol treated group (OVX + LOV) -Lovastatin-treated group (OVX + LV) -Control group: supplemented with vehicle olive oil via oral gavage. The rats were divided into 4 groups: -Control group: supplemented with vehicle olive oil via oral gavage -ATF: rats were given ATF 60mg/kg body weight daily via oral gavage -GTT: rats were given GTT 60mg/kg body weight daily via oral gavage. -DTT: rats were given DTT 60mg/kg body weight daily via oral gavage. Treatments administered via oral gavage were for 4 months. The static histomorphometric parameters involved were osteoclast surface/bone surface (OBS/BS), osteoclast surface/bone surface (OxS/BS), osteoid surface/bone surface (ES/BS), osteoid surface/bone surface (OS/BS) and osteoid volume/bone volume (OV/BV). The dynamic histomorphometric parameters were single-labelled surface/bone surface (dLS/BS), double-labelled surface/bone surface (dLS/BS) and mineralization surface/bone surface (MS/BS), mineral apposition rate (MAR) and bone formation rate/bone surface (BFR/BS). Measurement at the metaphysical region, which rich in trabecular bone.

The rats were divided into 6 groups: -Baseline (BL) -Sham (SH) -α-Vitamin E tocotrienol treated group (OVX + TT) -δ-Tocotrienol treated group (OVX + LOV) -Lovastatin-treated group (OVX + LV) -Control group: supplemented with vehicle olive oil via oral gavage. The rats were divided into 4 groups: -Control group: supplemented with vehicle olive oil via oral gavage -ATF: rats were given ATF 60mg/kg body weight daily via oral gavage -GTT: rats were given GTT 60mg/kg body weight daily via oral gavage -DTT: rats were given DTT 60mg/kg body weight daily via oral gavage. Treatments administered via oral gavage were for 8 weeks. Serum was extracted from the blood before and after treatment. The levels of bone biochemical markers osteocalcin and C-terminal telopeptide of type 1 collagen (CTX) in serum was measured via ELISA.

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### Study I  
**Hermizi et al., 2009**

| Study | Tocotrienol or Tocopherol | Methods | Results |
|-------|--------------------------|---------|---------|
|       | in vitro                 |         |         |
|       | Palm oil tocotrienol     | ELISA   | No significant change in all histomorphometric parameters between control and baseline group. |

#### Sample/Subject/Population

- **Population**
  - 49 young adult Sprague-Dawley male rats (3-month old, 250-300g weight)
- **Baseline** (BL) control group
  - nicotine group (N)
- **Nicotine cessation** (NC) tocotrienol enhanced fraction (TEF) - Δ-tocopherol (ATF)

#### Methods

- **Sample Treatment**
  - 7 rats for each group

#### Results

- **Histomorphometry**
  - All vitamin E treated groups showed increase in BV/TV, MAR and BFR/BS.
  - Both TEF and GTT treatment also increased Tb.Th and Ob.S/B.
  - Treatment with TEF or GTT significantly reduced Oc.S/B and Oc.S/BS.
  - Treatment with ATF or tocotrienols gave more superior effect than ATF.

- **Bone Resorption**
  - Tocotrienols (TEF and GTT) significantly reversed the negative effects of nicotine and improved trabecular bones’ cellular properties in animals.
  - Tocotrienols suppressed bone resorption by significantly reduced osteoclast number and eroded surfaces over bone surfaces ratio. For some of the histomorphometric parameters such as eroded surfaces over bone surfaces ratio, tocotrienols gave more superior effect than ATF.

- **Bone Formation**
  - The dynamic histomorphometric parameters were measured in single-labeled surface/bone surface (sLS/BS), mineral apposition rate (MAR), and bone formation rate/bone surface ratio (BFR/BS).

#### Study II  
**Ahmad et al., 2005**

| Study | Study I: Blood serum weekly | Study II: Food and feeding for 3 weeks | Study III: Food and feeding for 3 weeks |
|-------|-----------------------------|----------------------------------------|----------------------------------------|
|       | Athar al (100雄性Wistar rats) | male Wistar rats (4 weeks old, 90-120g weight) | male Wistar rats (4 weeks old, 90-120g weight) |
|       | PPAR-α and PPAR-γ knockout, 55.2% Δ-tocotrienol and 14.1% Δ-tocotrienol | male Wistar rats (4 weeks old, 90-120g weight) | male Wistar rats (4 weeks old, 90-120g weight) |

#### Methods

- **ELISA**
  - Treatment with tocotrienols, but not ATF, at 60mg/kg and 100mg/kg doses significantly prevented the increase in FeNTA-induced IL-6 level. Treatment with any dose of ATF did affect the serum level of IL-1. Treatment with TEF or tocotrienols at 30mg/kg, 60mg/kg and 100mg/kg significantly prevented the increase in FeNTA-induced IL-6 level.

#### Results

- **Histomorphometric Analysis**
  - Injection of FeNTA significantly reduced Ocn/B and BFR/BS.
  - Injection of FeNTA also increased Ocn and EBS; however treatment with tocotrienols reduced only EBS. Treatment with either ATF or PTT at 100mg/kg did not affect osteoclast number.

- **Bone Resorption**
  - Tocotrienols (TEF and GTT) significantly reversed the negative effects of nicotine and improved trabecular bones’ cellular properties in animals. Tocotrienols suppressed bone resorption by significantly reduced osteoclast number and eroded surfaces over bone surfaces ratio. For some of the histomorphometric parameters such as eroded surfaces over bone surfaces ratio, tocotrienols gave more superior effect than ATF.
polyethylene glycol PEG-400. The work of Mehat et al. (2010) is another *in vivo* work that studied the effect of individual tocotrienol isomers. All *in vitro* studies covered in this systematic review used individual tocotrienol isomers. There were six studies that compared the effects of tocotrienols with ATF (all from Sigma Aldrich). While Ha et al. (2011) directly compared α-tocotrienol (ATT) and ATF, Brooks et al. (2011) is the only group that compared tocotrienol isomers with tocopherols other than ATF. The summary of the study characteristics is shown in (Table 1).

For *in vivo* studies, Sprague-Dawley rats were used as the most common animal model. Wistar rats were used in two studies [30, 54]. Deng and colleagues, on the other hand, used C57BL/6 female mice in both of their studies [50, 53]. The highest number of animals recruited were 132 male rats [30] and other studies used between 32 to 49 rats. There was a study that did not mention the total number of animals involved [53]. Out of those nine *in vivo* studies reviewed here, six of them used animal models representing osteoporosis, a pathologic bone loss associated with increased in osteoclasts number and activity.

Majority of the *in vivo* studies covered in this systematic review assessed the effect of tocotrienol(s) on the osteoclast formation based on the static histomorphometric parameters osteoclasts number (OcN) per mm$^2$ and percentage of osteoclast surface over bone surface (OcS/BS). Meanwhile the assessment on the osteoclast bone resorbing function was based on the percentage of eroded surface over bone surface (ES/BS) reported [30, 51, 52, 55, 56]. Two fully *in vivo* studies carried out by Deng et al. [53] and Norazlina et al., [57] measured the RANKL and OPG mRNA expression in tissues and their protein level in serum, respectively. In Norazlina et al. (2010), the serum levels of RANKL and OPG were measured using enzyme-linked immunosorbent assay (ELISA), meanwhile, in Deng et al. [53], RANKL and OPG gene expression levels in the mice femur and spine were measured via real-time quantitative polymerase chain reaction (qPCR).

For *in vitro* studies, in single cell culture of osteoclasts, the number of osteoclasts formed following treatment with tocotrienol(s) was assessed based on positive staining for tartrate-resistant acid phosphatase (TRAP) [49] or dual immunostaining for vibronectin and actin [48]. On the other hand, the impact of tocotrienol(s) on the osteoclast bone resorption activity was assessed using osteoclast resorption assay either on dentin disc or carbonated calcium phosphate plate coating. As for the co-culture system by Ha et al. (2011), only osteoclast formation was assessed using TRAP staining. Brooks et al. (2011) also conducted TRAP staining in determining the optimum dose for tocotrienol isomers.
besides MTS assay for evaluating toxicity of vitamin E used based on cell proliferation.

Study conducted by Deng et al., (2014) [50] is the only study covered in this review that had combination of both in vivo and in vitro models. The in vivo model used was ovariectomized (OVX) C57BL/6 female mice, in which number of osteoclasts as one of the histomorphometric parameters, was assessed following the supplementation with PEG-400-emulsified GTT. In their in vitro work on co-culture of bone marrow osteoclast precursors with parathyroid hormone (PTH)-treated UAMS-32P osteoblast cell line, as the level of RANKL mRNA expression following treatment with PEG-400-emulsified GTT was also assessed.

3.3. Effects of Tocotrienols on Number of Osteoclasts, Osteoclast Surfaces and Bone Eroded Surface In Vivo

Out of those nine in vivo studies, seven assessed osteoclast formation through static histomorphometric parameters either OcS per mm² [30, 50, 56] or the percentage of OcS/BS [51, 52, 55]. In studies that used orchidectomy (ORX) [51] and OVX rat models [50, 52, 54], it was found that treatment with tocotrienols blocked the increase in OcS/BS and OcN. Treatment with tocotrienols also significantly reduced OcS/BS in rat model of nicotine-induced osteoporosis [55]. Similarly, treatment with tocotrienols for two and four months significantly reduced OcN in normal and oxidative stress model rats [30, 56]. Comparison between ATF and tocotrienols-treated groups found tocotrienol had greater effect in reducing OcS [55] and OcN [30, 56].

As for the impact of tocotrienol(s) in reducing bone erosion as indicator of osteoclast bone resorption activity in vivo, all studies which assessed ES/BS [30, 51, 52, 55, 56] indicated that treatment with tocotrienols significantly reduced this histomorphometric parameter in their corresponding animal models following supplementation of tocotrienols. In comparison to the ATF-treated rats, supplementation of tocotrienols appeared to result in greater reduction in ES/BS [30, 55, 56].

3.4. Effects of Tocotrienols on the Formation and Activity of Osteoclasts In Vitro

Technically, only the in vitro studies allowed us to assess the direct effects of tocotrienol(s) on osteoclast formation and activity. In Brooks et al., (2011) comparison between α, δ and γ-tocotrienol isomers showed that treatment with γ-tocotrienol appeared to be the most potent in suppressing osteoclast differentiation and activity in PBMC-derived osteoclast culture.

All those tocotrienol isomers suppressed (exception to ATT that gave no significant reduction) the formation of TRAP-positive multinucleated cells, but not the tocopherol counterparts. Increasing dose of GTT only appeared to give greater inhibition of osteoclast formation [48]. Data by Ha et al. (2011) indicated that treatment with ATT significantly inhibited the formation of TRAP-positive multinucleated cells derived from mouse bone marrow-derived macrophages (BMMs), moreover, in a dose-dependent manner.

In Ha et al., (2011), treatment with ATT but not ATF reduced formation of TRAP-positive osteoclast directly by inhibiting early stage of osteoclastogenesis. Furthermore, protein expression in RANKL-induced osteoclastogenesis showed that ATT affected mitogen-activated protein kinase (MAPK) activation at early signaling pathway and nuclear factor kappa B (NFκB) activation at a later stage.

In Brooks et al. (2011), following the treatment with GTT and 6-tocotrienol (DTT), there appeared to be a trend of greater inhibition effect on osteoclast resorption activity with increasing dose of the tocotrienols. At 1mM, GTT completely inhibited osteoclast resorption activity [48]. While in Brooks et al. (2011) 10 to 1000μM of ATT appeared to give no significant reduction in the resorption activity of the PBMC-derived osteoclasts, work on osteoclast culture from mouse BMM by Ha and colleagues (2011) demonstrated that 50μM of ATT significantly inhibited resorption pits. Treatment with ATF, on the other hand, did not inhibit resorption activity of the osteoclasts [49].

3.5. Effects of Tocotrienols through Modulation on Osteoblasts and Expression of RANKL and OPG

The formation and resorption activity of osteoclasts could also be regulated through modulation on the expression of RANKL and OPG by osteoblasts [7, 58]. The main purposes of why Ha et al. (2011) [49] and Deng et al. (2014) [50] used co-culture system of osteoclast and osteoblast in vitro was to assess the indirect effect of tocotrienols on the osteoclasts through the modulation on osteoblasts and expression of RANKL and OPG. Ha and coworkers (2011) reported that besides the direct inhibitory effect on osteoclastogenesis, pre-treatment with ATT but not ATF also indirectly reduced formation of TRAP-positive osteoclast via suppression on RANKL expression from the primary osteoblasts in the culture. This data is supported by another in vitro co-culture system by Deng et al. (2014) [50], which demonstrated that GTT could reduce RANKL mRNA expression from osteoblast cell line UAMS-32P.

Examination on the expression of RANKL and OPG in femurs from mice model of postmenopausal osteoporosis showed higher RANKL and lower OPG mRNA level [50]. Treatment with GTT reduced RANKL and increased OPG mRNA expression in the femurs. Different outcomes of results were observed from a study looking at levels of serum OPG and RANKL following supplementation of tocotrienols mixture. This study on an in vivo model of nicotine-induced osteoporosis found an increase in serum RANKL level, but no significant change in OPG level following supplementation with tocotrienol mixture [57]. On the other hand, treatment with ATF increased serum OPG level [57].

4. DISCUSSION

Osteoclastogenesis involves several stages, starting from the precursor cells, which differentiate into mature osteoclasts [59]. Knowledge on the regulation of osteoclast differentiation is important in providing insights on suitable therapeutic targets for intervening bone loss diseases like osteoporosis. The results obtained throughout this systematic review generally demonstrate that tocotrienols, which are examples of antioxidants, had inhibitory effects on osteoclast forma-
tion and activity in both physiological and pathological states. In pathological state, the imbalanced osteoblast and osteoclast activities are associated with increased level of reactive oxygen species (ROS) [60, 61]. As antioxidants, tocotrienols are good therapeutical agents for protecting bone from oxidative stress due to its ability to donate hydrogen atom from hydroxyl group on its chromanol ring to ROS and free radicals [43]. While tocotrienols appear to exhibit inhibitory effect on osteoclasts as indicated from this study, they give protective effects on osteoblasts, the bone forming cells. Nizar et al. (2012) found that low concentrations of GTT supplementation could protect osteoblasts from hydrogen peroxide (H₂O₂)-induced oxidative stress and apoptosis [62, 63]. These data of protective effect of tocotrienols on osteoblasts are well supported by a later in vivo study that demonstrated treatment with annatto tocotrienol decreased orchidectomy-induced reduction in osteoblast surface [51].

Across various models representing different diseases of bone loss in vivo (ranging from ones induced by postmenopausal osteoporosis, testosterone-deficient, oxidative stress and nicotine-induced), tocotrienols appeared to exhibit positive impacts in reducing osteolysis accompanied by decrease in the number of osteoclasts. In the majority of the in vivo studies reviewed here, the main technique that allows us to evaluate the effects of tocotrienols on osteoclasts in vivo is static histomorphometry, particularly on parameters like OcS/BS, OcN/BS and ES/BS. Generally the results from all histomorphometric studies reviewed in this systematic review [30, 50-52, 54-56] indicated that there were reductions in those histomorphometric parameters following tocotrienol supplementation. There is a concern on the accuracy of histomorphometric parameters osteoclast number and surface to be used for assessing modulation on osteoclasts in vivo. Histomorphometric parameters osteoclast number and surface are commonly counted and measured based on morphology, as it appeared in all reviewed in vivo studies. Findings from Ballanti and coworkers (1997) found that osteoclast number and surface in histomorphometric parameters measured based on the cell morphology alone could be inaccurate and underestimated as much as 50-60% compared to the histomorphometric counts of osteoclast stained for TRAP [64].

Beside histomorphometry, other assessable indicators for osteoclast formation and activity in vivo are the expression and serum level of osteoclastogenesis-associated cytokines RANKL and OPG. It is well known that RANKL and OPG are the primary determinants of osteoclast differentiation and activity. RANKL is a member of tumor necrosis factor (TNF) family that is expressed on activated T-cells, osteoblast/stromal cells and chondrocytes, meanwhile OPG is a decoy receptor for RANKL, which is secreted by osteoblasts following activation.

In a rat model of nicotine-induced bone loss, it was quite surprising to find that treatment with tocotrienols mixture for 12 weeks increased serum RANKL level, but did not significantly change OPG level [57]. At a closer look on the data of the study, treatment with tocotrienols mixture seemed to increase the serum OPG level, however the significant statistical difference was denied by large standard error of mean in the group of tocotrienols-treated rats, which may be attributed to technical errors and number of replicates. Hence there is no conclusive discussion that can be drawn from the findings of this study [57].

There is also a possibility that nicotine modulates bone resorption through regulation of osteoclastogenesis OPG and RANKL expression on bone tissues, not in serum. In another study by Deng et al. (2014) [53], following supplementation with GTT on mice (100mg/kg body weight), it was reported that the tocotrienol isomer inhibited the increase in RANKL and decrease in OPG mRNA expression level on bone tissues (spine and femurs) induced by db-cAMP, a cell permeable analogue of cAMP. Earlier, it had been described that the level of GTT in those bone tissues as stable over of two-week duration [53].

The mechanism by which tocotrienols modulate osteoclast formation and activity indirectly through regulation of RANKL and OPG expression in osteoblasts was also explored in vitro co-culture system. It has been well known that osteoblasts may also play role in modulating osteoclast differentiation and activity through regulation of RANKL and OPG expression. In a co-culture system of mouse cells, Ha and coworkers (2011) [49] found that 50μM ATT, but not ATF, downregulated RANKL mRNA expression while elevated mRNA expression of OPG. Quite similarly, finding from another co-culture study by Deng et al., (2014) [50] also found parathyroid hormone (PTH)-induced increase of RANKL mRNA expression was inhibited by treatment with 10μM GTT. It was interestingly to note that, even the addition of exogenous RANKL following treatment with ATT could not fully restore the number of osteoclasts formed in the co-culture system [49]. This data prompted Ha and colleagues (2011) to investigate if ATT could directly inhibit osteoclast formation and activity.

Beside Ha et al. (2011), there is another in vitro study that reported the direct effects of tocotrienols on osteoclasts carried out by Brooks and coworkers (2011). Brooks et al. (2011) [48] studied the effects on osteoclast formation and resorption by individual α-, γ- and δ-isomers of both tocotrienols and tocopherols, meanwhile Ha and colleagues (2011) focused on ATT and ATF only. Based on the comparison between tocotrienol isomers in Brooks et al. (2011), in which GTT and DTT appeared to be the most potent isomers, it may be suggested that tocotrienols extracted from annatto beans (containing 90% DTT and 10% GTT) could potentially become an ideal source of tocotrienols for suppressing bone loss.

It is quite interesting to compare differences in findings on ATT and ATF between these studies by Ha et al. (2011) and Brooks et al. (2011). Despite of differences in the cell types used, the dose of MCSF and RANKL applied and number of days of cell culture, both studies showed that ATT, at the high doses of 50 and 100μM, significantly reduced osteoclast resorption activity, but not on the formation of osteoclasts.

Throughout this review, there are five studies comparing the effect tocotrienols and tocopherols and suggested that tocotrienols are more superior to tocopherols in reducing bone resorption [30, 48, 49, 55, 56]. While the in vitro studies [48, 49] indicated that tocotrienols (but not ATF) sup-
pressed only the bone resorbing activity of the osteoclast, evidence from the in vivo studies [30, 55, 56], however, suggested that treatment with tocotrienols were also better than ATF in reducing numbers of osteoclast formed, as seen from the histomorphometric parameters osteoclast number and surface.

Even though the reduction of osteoclast surface in ovariec-tomized rats following treatment with either tocotrienols or ATF mixture was observed in Muhammad et al. (2012), statistical analysis indicated that there was no significant difference between the tocotrienol and ATF-treated groups [54]. On the other hand, the unconvincing data from Norazlinia et al. (2010), which indicated that ATF increased serum OPG and tocotrienols mixture increased serum RANKL, is the only evidence which may suggest ATF works better than tocotrienols in suppressing bone resorption, although there is a room for argument that serum levels of RANKL and OPG may not necessarily reflect the rate of osteoclast formation and activity as well as bone resorption.

Generally, it appeared that tocotrienols exhibit more superior health beneficial effects than ATF, including in bone which is highlighted and supported by the work by Ahmad et al. (2005) [30]. In the context of regulation specifically on osteoclasts, there is a study carried out by Fujita and co-workers (2012) that reported ATF indeed promoted bone loss by enhancing more osteoclastogenesis. In the study, Fujita et al. (2012) reported that treatment of ATF, but not other isoforms of tocopherols, increased cell differentiation of murine bone marrow-derived osteoclasts through the activation of DC-STAMP, which mediated the pre-osteoclast cells fusion. This induction of DC-STAMP following treatment with ATF, which eventually led to osteoclast fusion, was mediated by the activation of microphthalmia-associated transcription factor (MITF) and p38α [47]. Earlier, it had been found that mice genetically deficient in α-tocopherol transfer protein developed osteopetrosis (increased bone mass) [47]. This study provides further support for annatto bean-derived tocotrienols as an ideal therapeutic agent for reducing bone loss through down-modulation of osteoclasts.

It is quite interesting to note that recently, there has been evidence that senescent cells could play role in bone loss, as characterized by the lower bone erosion in vivo and increased osteoclast formation in vitro [65]. An earlier study found that tocotrienols, not only delay cellular senescence, but also are able to rejuvenate senescent primary cells [66]. Together, this could explain how tocotrienols reduce bone loss and inhibit osteoclast differentiation.

STRENGTH AND LIMITATION OF REVIEW

Findings from animal and in vitro studies covered in this review generally had shown the potential of tocotrienols in suppressing the osteoclasts, either or both of directly and indirectly. This could suggest supplementation of tocotrienols as an effective strategy in preventing and treating bone loss. Findings from both in vivo and in vitro studies, which support each other, further provide merit to conclude that tocotrienols could reduce and treat bone through suppression on osteoclasts. This review is important, as there has been no systematic review discussing the effects of tocotrienols specifically on osteoclasts.

There are several limitations identified throughout this review. Despite of having both in vitro and in vivo studies reviewed here, there is no human study included. The inclusion of human studies in any future systematic review could help a lot in giving more conclusive view on the effect of tocotrienols, nevertheless this is ethically impossible when looking at the suppression at the cell level, which is osteoclast in this context. The aspect of different sets of tocotrienol isomers used between studies is also another limitation to this review since direct comparisons between isomers were difficult to be made. Meanwhile in studies that used tocotrienol extracts or mixtures, there were issues of two main sources for tocotrienol extracts, which are annatto beans and palm oil, and different compositions of tocotrienol isomers between extracts or mixtures. Variations and differences in the effects seen between studies may be attributed to distinct sources of extract used.

RECOMMENDATIONS

Using standard tocotrienols mixture (containing fixed composition of tocotrienol isomers) in future studies will greatly help us in making comparison on data between studies. Modulation on osteoclast formation and resorption activity should become an important aspect that should be looked at in any future research on bone loss as it could be a good strategy for treating the diseases.

CONCLUSION

In summary, nearly all studies reviewed here demonstrated evidence that tocotrienols have inhibitory effects on osteoclasts, the bone resoring cells. In in vivo studies, all histomorphometric data showed reduction in osteoclast formation and activity following treatment with tocotrienols. The in vitro data indicated that tocotrienols affected osteoclasts directly in reducing the bone resorbing activity and indirectly down-modulate osteoclast formation through regulation of RANKL and OPG expression by osteoblasts. Therefore, outcomes of this review suggest tocotrienols could become a high potential anti-bone resorptive agent.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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