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SYSTEMATIC REVIEW ARTICLE

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

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ARTICLE HISTORY

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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-

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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 lipid-soluble 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

Table 1. Summary of studies included in this systematic review.

Study	*In vitro*	*In vivo*	Tocotrienol	Sample/ Subject/ Population	Methods	Results	Comment or Outcome
Study 1 Deng *et al.,* 2014		√	Polyethylene glycol (PEG-400) emulsified γ-tocotrienol (GTT)	C57BL/6 female mice (8 week-old, about 23-25g). 8 mice per treatment group. Mice were injected by γ-tocotrienol *via* subcutaneous injection.	1. C57BL/6 female mice were administered GTT (100 mg/kg body weight) with PEG-400 emulsion by subcutaneous injection. Mice for each treatment group were euthanized on day 3 and 14 after administration of emulsified GTT or olive oil. 2. GTT levels in different tissues (including in blood) were determined by HPLC with a fluorescence detector. 3. Analysis of gene expression in homogenized tissues: GTT of 100mg/kg dose was given to mice. After the induction by db-cAMP (100mg/kg), RANKL and OPG mRNA level from femur and spine were assessed on day 1, 3, 7 and 14 by real-time PCR. Dibutyryl-cAMP (db-cAMP) was used to upregulate RANKL and downregulate OPG mRNA expression level.	GTT predominantly accumulated in adipose tissue on both day 3 and 14. Increased of GTT level in heart and spleen (comparison between day 3 and 14). No significant difference in RANKL and OPG mRNA expression between control and GTT treatment group in both femur and spine across all time points assessed. GTT inhibited the up-regulation of RANKL mRNA expression and down-regulation of OPG mRNA expression following induction by db-cAMP.	Level of GTT in both bone tissues relatively stable over time in mice. In both femur and spine tissues, GTT 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) emulsified γ-tocotrienol (GTT)	C57BL/6 female mice (8-week old, about 23-25g). 8 mice for each group. Mice were either sham-operated or overiectomized (OVX) bilaterally.	1. Mice were given either emulsified GTT (100 mg/kg body weight) or excipient (olive oil emulsified with PEG-400) administered once per month for 3 months. Mevalonate (25 mg/kg body weight) was supplemented by oral gavage once daily for 3 months from the time of GTT first administered. There were also control groups of excipient or mevalonate alone. 2. Bone histomorphometric and μCT analysis: BMD of left femur were assessed by dual energy X-ray absorptiometry & intact left femur & partial spine for μCT analysis (for bone structural determination) using high-resolution μCT SkyScan 1176. Parameters of bone volume per total volume (BV/TV), mean trabecular thickness (Tb.Th), mean trabecular number (Tb.N.) & mean trabecular separation (Tb.Sp) were computed for structural histomorphometry. Parameters of static histomorphometry included osteoclast and osteoblast numbers obtained from TRAP-toluidine blue staining on undecalcified sections of proximal tibia bone. Dynamic histomorphometry included mineral apposition rate and bone formation rate measured through double fluoresecent calcein and alizarin complexone labeling on sections from tibial metaphysis. 3. Serum biochemical markers of bone metabolism: Determination of serum osteocalcin levels, biochemical marker of bone turnover & serum levels of carboxyterminal cross-linking telopeptide type I collagen (CTX-I) using ELISA. 4. Expression of osteogenic gene in femurs and in UAMS-32P *in vitro* (RANKL, OPG, Osterix, Runx2) was measured by real-time PCR, normalized to ribosomal protein S2 mRNA.	Bone density measurement Bone density of femur and spine showed supplementation of GTT significantly prevented decrease in OVX mice. Daily mevalonate supplementation blocked the bone protective effect of GTT Bone structural parameters GTT treatment significantly prevented the reduction of BV/TV, Tb.Th, Tb.N & the increase in Tb.Sp. Mevalonate reversed the effects of GTT. Static bone histomorphometric parameters GTT significantly decreased osteoclast numbers and increased osteoblast numbers in OVX mice, but this was reversed by mevalonate supplementation. Dynamic bone histomorphometric parameters GTT significantly increased mineral apposition rate and bone formation rate but these effects were inhibited by mevalonate supplementation. Serum level of biomarkers of bone metabolism GTT significantly increased serum osteocalcin level and decreased serum CTX-I level, and the effect was reversed by mevalonate supplementation. Osteogenic expression GTT significantly inhibited the OVX-induced increase of RANKL and blocked the OVX-induced decrease of OPG mRNA expression in femur. GTT significantly increased both Osterix and Runx2 mRNA expression in femur. Mevalonate reversed the modulation of GTT on the gene expression. *in vitro* The inhibition of GTT for RANKL expression stimulated by parathyroid hormone (PTH) was blocked by mevalonate. Increased osteoclast formation by PTH in co-culture was arrested by GTT, but this was reversed by mevalonate supplementation. GTT reduced FPP and GGPP (HMG-CoA reductase intermediates) levels in UAMS-32P, and this indicates that suppression of osteoclastogenesis in UAMS-32P by GTT is mediated by mevalonate pathway.	GTT blocked OVX-induced bone loss. This effect could be seen from the increased bone density and structure, higher osteoclast number, modulated serum levels of biochemical markers of bone metabolism and higher expression of osteogenic genes in bone. However, the protective effect of GTT can be overcome by daily supplementation of mevalonate. *In vitro* study indicated that GTT 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	√		α- tocotrienol (ATT), GTT and δ- tocotrienol (DTT) (0.01, 0.1 and 1mM)	Human blood-derived $CD14^+$ cells from three different donors 2 $CD14^+$ cell line for substrate resorption assay	1. Peripheral blood mononuclear cells (PBMCs) isolated from the buffy coats of blood by density gradient centrifugation. They were later further selected for $CD14^+$ subpopulation using microbeads. 2. 1 x 10^5 $CD14^+$ 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 (0.01-1.0mM) 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 α-, GTT and DTT (0.01-1.0 mM) were assessed *via* resorption assay. Dentin disc was stained with toluidine blue (0.1%) meanwhie 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.	Cell proliferation Comparing between different doses, there were significant differences in cell proliferation across all tocotrienol isomers tested. For GTT , cell proliferated more as the dose increased (0.01mM-1mM) For DTT, 1mM dose led to cell toxicity. 1mM of ATT gave higher cell proliferation than the lower concentrations used. Osteoclast formation In the presence of MCSF & RANKL, osteoclast formed within 2 weeks on dentin disc, 10 days on collagen and 3-4 days on calcium phosphate. However, there was no osteoclast formed on any substrate when $CD14^+$ cultured without RANKL. There was considerable variation in number of $TRAP^+$ osteoclast per field and in the total number of cells between donors. γ-tocotrienol reduced the formation of $TRAP^+$ osteoclast in dose-dependently. $TRAP^+$ osteoclast number observed was lower in all tocotrienol groups than in the tocopherols groups. Subtrate resorption by osteoclast Resorption pits 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 (0.01-1mM) in both cell lines tested.	Between isomers of tocotrienols, γ-tocotrienol appeared to give greatest inhibition $TRAP^+$ osteoclast formation and resorption activity. Higher dose of γ-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 counteract. Study on $CD14^+$ cell lines showed dose-dependent reduction of resorption with increasing dose of γ- and δ-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 ($4x10^4$ cells/well) were cultured with MCSF (30ng/ml) and RANKL (100ng/ml) for 4 days. Coculture system consisted of BMM cells ($3x10^5$ cells/well) and primary osteoblasts ($2x10^4$ 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 $1,25(OH)_2D_3$ (10nM) plus PGE_2 (100nM) for 24h to induce RANKL and OPG protein expression. 3. For resorption assay, mature osteoclasts were grown in coculture system on OAAS plates coated with carbonated calcium phosphate. The cells were pretreated with ATT (50μM) or ATF (50μM) 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 retrovrirally transduced using retroviral vectors pMX-IRES-EGFP. 6. Western blot analysis for phosphorylated ERK1/2, ERK, phosphorylated JNK1/2, JNK, phosphorylated p38, p38, phosphorylated IκBα, IκBα, NFATc1, c-Fos and β-actin were performed. 7. To detect protein complexes with nucleic 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 $1,25(OH)_2D_3$ plus PGE_2 -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. The inhibitory effect of ATT was not fully reversed by the exogenous addition of RANKL. Single-cell osteoclast culture Treatment with ATT but not ATF significantly supressed the osteoclast formation in dose-dependant manner. ATT effectively inhibited osteoclast formation at early stage (first 2 days in culture). Gene and protein expression following c-Fos and NFATc1 overexpression ATT suppressed both mRNA and protein expression of c-Fos and NFATc1 induced by RANKL. The suppression effect was reversed by the overexpression of NFATc1 or c-Fos. ATT inhibited RANKL-induced acute ERK activation without significantly affecting JNK and p38 pathways. ATT did not affect acute NF-κB activation but it suppressed RANKL-induced delayed NF-κB activation. Bone resorption assay ATT but not α-TP reduced the resorption pits formed by mature osteoclasts, without affecting the cell numbers.	ATT inhibited the formation of $TRAP^+$ osteoclast either indirectly by upregulating RANKL expression in osteoblast or directly inhibiting the early stage of octeoclastogenesis in osteoclast precursors cells. Gene and protein expression indicated that RANKL-induced MAPKs activation was inhibited by ATT treatment at early signaling pathway. Meanwhile NFκB activation was suppressed at delayed stage of activation. ATT also markedly suppressed osteoclast resorption activity.

(Table 1) contd….

Study	*In vitro*	*In vivo*	Tocotrienol	Sample/ Subject/ Population	Methods	Results	Comment or Outcome
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 for each group. Nicotine and vitamin E (either tocotrienols mixture or ATF) were given intraperitoneally and orally *via* gavage for 6 days a week for 12 weeks.	1. The rats divided into 4 groups: a) control group b) nicotine-treated group at 7mg/kg for 3 months (N) c) nicotine-treated group and supplemented with tocotrienol mixture at 60mg/kg during month 2 and 3(N+TT) d) nicotine-treated group and supplemented with ATF at 60mg/kg during month 2 and 3(N+ATF) 2. Serum OPG and RANKL levels in blood before the treatment commenced and at the end of treatment were measured using ELISA. 3. Femur and 4th lumbar bones were analyzed using flame atomic absorption spectrophometry for assessing bone calcium content.	Serum levels of OPG and RANKL Nicotine did not significantly change the serum levels of OPG and RANKL. Only treatment with ATF significantly increased serum OPG levels Meanwhile only treatment with tocotrienols mixture significantly increased serum RANKL levels. Bone calcium content Femur bone calcium content decreased significantly following nicotine treatment. Supplementation of TT and ATF were able to restore the femur bone calcium content. There was no change in bone calcium content in the lumbar following treatment with nicotine, tocotrienols mixture and ATF. Reduction on weight gain Treatment with nicotine slowed down the weight gain over the 12 weeks period, statistically significant starting from week 4. Treatment with TT and ATF did not result in any improvement.	Supplementation with ATF significantly increased serum OPG levels following nicotine treatment. Supplementation with tocotrienols mixture significantly increased serum RANKL levels following nicotine treatment. Supplementation of ATF and TT had reversed the nicotine-induced reduction in bone calcium content. There appeared trend of reduction in serum OPG level and increase in serum RANKL level after 12 weeks of age across all groups. Nicotine treatment also appeared to reduce serum OPG level, however only the big standard deviations left the difference between the control and nicotine-treated groups to be non-statistically significant. The data appeared to suggest that tocotrienols mixture also increased serum OPG level, however the change was not significant due big standard deviation in the tocotrienols-treated group.
Study 6 Chin *et al.,* 2014		√	Annato tocotrienol (90% δ- and 10% γ-tocotrienol)	40 male Sprague-Dawley rats (3 months old, 250-300g body weight) The rats were randomly divided into 5 groups: -Baseline (BL) -Sham (SH) -orchidectomized (ORX) -annato tocotrienol-treated (AnTT) - testosterone enanthate-treated (TE). 8 rats for each group	1. The rats were randomized into 5 groups: a) BL: sacrificed at the beginning of study b) SH: undergone same surgical procedure but testes were not removed c) ORX: Testes were removed d) AnTT: orchidectomized and given 60 mg/kg daily *via* oral gavage e) TE: orchidectomized and given 7 mg/kg weekly *via* intramuscular injection 2. Serum were extracted at the beginning and end of treatment to determine the levels of N-terminal propeptide of type I procollagen (PINP) and osteoclast-derived tartrate-resistant acid phosphatase form 5b (TRACP 5b) using enzyme-linked immunosorbent assay (ELISA). 3. The rats were sacrificed and distal left femurs were harvested for static histomorphometric analysis. Parameters assessed were osteoblast surface (Ob/BS), osteoclast surface (OcS/BS), eroded surface (ES/BS), osteoid surface (OS/BS) & osteoid volume (OV/BV).	ELISA There was decrease in PINP and TRACP 5b level after treatment in all groups studied, but the drops were.non-significant when compared between groups. Histomorphometry analysis There were significant changes in bone static histomorphometric parameters following orchidectomy. Treatment with AnTT and TE blocked the orchidectomy-induced increase in OcS/BS, ES/BS and decrease in ObS/BS, OS/BS and OV/BV. There was no significant difference in bone static histomorphometric parameters between AnTT and TE groups.	Annato tocotrienol treatment prevented testosterone deficiency-induced bone loss by restoring the changes in all bone static histomorphometric parameters, including increased OcS/BS and ES/BS following orchidectomy. Testosterone deficiency and treatment with annato tocotrienol did not result in signficant change on serum bone turnover markers.
Study 7 Muhammad *et al.,* 2012		√	Pure tocotrienols mixture (37.2% α-, 39.1% γ- and 22.6% δ- tocotrienol) from Palm Oil Research Institute of Malaysia (PORIM)	40 female Wistar rats (3 month old, 200-250g body weight) The rats were randomly divided into 5 groups: -Baseline (BL) -Sham (SH) -ovariectomized (OVX) rats -Pure tocotrienol-treated group (OVX + PTT) -α-tocopherol- treated group (OVX + ATF) 8 rats for each group	1. Rats were randomly divided into 5 groups: a) BL: sacrificed at the beginning of study b) SH: rats were sham-operated and given olive oil (vehicle) c) OVX rats d) OVX + PTT: Rats were given 60 mg/kg body weight daily *via* oral gavage e) OVX + ATF: Rats were given 60 mg/kg body weight daily *via* oral gavage 2. Left femurs of the rats were harvested and stained with Von Kossa for histomorphometric analysis. The structural histomorphometry parameters involved were trabecular bone volume volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N.) & mean trabecular separation (Tb.S). 3. Cellular histomorphometry parameters were measured from harvested decalcified right femoral bones. The parameters were osteoclast surface (Oc.S) and osteoblast surface (Ob.S).	Body weight OVX rats had significant weight gain compared to SH and treated groups. Histomorphometry analysis There were significant decreases in BV/TV and Tb.N and increase in Tb.S in OVX rats compared to the other groups. There was no significant change in Tb.Th in all groups. Treatment with both ATF and tocotrienols mixture prevented the decrease in BV/TV and Tb.N., and increase in Tb.S. following ovariectomy. For cellular parameters, there were significant increases in Oc.S and Ob.S in OVX rats compared to other groups. Treatment with both ATF and the tocotrienols mixture prevented the increase in Oc.S in ovariactomised rats. Treatment with both vitamin E in OVX rats did not give significant change on the increase in Ob.S following ovariectomy. Bone histology Treatment with either tocotrienols mixture and ATF restored ovariactomized-induced bone loss.	Treatment with either ATF or PTT restored ovariactomized-induced bone loss through blocking the drop in BV/TV and Tb.N., and reducing the increase in Tb.S and Oc.S. It is quite interesting to note that ovariectomy resulted increase in Ob.S, however this finding was left unexplained.

(Table 1) contd….

Study	*In vitro*	*In vivo*	Tocotrienol	Sample/ Subject/ Population	Methods	Results	Comment or Outcome
Study 8 Abdul-Majeed *et al.,* 2012		√	Annato tocotrienol (90% δ- and 10% γ-tocotrienol)	48 female Sprague-Dawley rats (3 months old, 200-250g weight) The rats were divided into 6 groups: -Baseline (BL) -Sham (SH) -ovariectomized control groups (OVXC) rats -Lovastatin-treated group (OVX + LOV) -δ-tocotrienol treated group (OVX + TT) 8 rats for each group	1. Rats were divided into 6 groups: a) BL: sacrificed at the beginning of study b) SH: sham-operated for surgical stress stimulation c) OVXC: ovariectomised rats which received no other treatment d) OVX + LOV: ovariectomised rats were given lovastatin 11 mg/kg body weight daily *via* oral gavage e) OVX + TT: ovariectomised rats were given δ-tocotrienol 60 mg/kg body weight daily *via* oral gavage f) OVX + TT + LOV: ovariectomised rats were given δ-tocotrienol 60 mg/kg and lovastatin11mg/kg body weight daily *via* oral gavage Treatments administered *via* oral gavage were for 8 weeks. 2. 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. 3. Left femurs of the rats were harvested for histomorphometric analysis. The static histomorphometric parameters involved were osteoblast surface/bone surface (ObS/BS), osteoclast surface/bone surface (OcS/BS), eroded surface/ bone surface (ES/BS), osteoid surface/bone surface (OS/BS) and osteoid volume/bone volume (OV/BV).	Serum bone biochemical markers level Ovariectomy signficantly reduced serum osteocalcin and treatment with lovastatin alone could not restore the serum osteocalcin level.Treatment wih tocotrienol or combination of tocotrienol and lovastatin appeared able to restore serum osteocalcin level, Ovariectomy signficantly increased serum CTX. Treatment with lovastatin alone, unlike treatment wih tocotrienol or combination of tocotrienol and lovastatin, could not bring down the serum CTX level. Histomorphometric analysis Ovariectomy also significantly reduced Ob.S/BS, OS/BS and OV/BV. Treatment with could not restore the serum osteocalcin level.Treatment wih tocotrienol or combination of tocotrienol and lovastatin, but not lovastatin alone, appeared able to restore the reduction in those static histomorphometric parameters Ob.S/BS, OS/BS and OV/BV following ovariectomy. Opposite trends were observed for static histomorphometric parameters OcS/BS and ES/BS, whereby ovariectomy increased those parameters. Treatment wih tocotrienol or combination of tocotrienol and lovastatin, but not lovastatin alone, restored the ovariectomy-resulted change in OcS/BS and ES/BS. Combination treatment of tocotrienol and lovastatin significantly enhanced decrease in OcS/BS and increase in OV/BV when compared to treatment with tocotrienol alone.	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.
Study 9 Mehat *et al.,* 2010		√	δ- and γ-tocotrienol	32 Sprague-Dawley male rats (3 months old, 200-250g weight) The rats were divided into 4 groups: -Control group -ATF-treated -GTT-treated -DTT-treated 8 rats per group	1. 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. 2. The static histomorphometric parameters involved were osteoclast number (N.Oc), osteoblast number (N.Ob), eroded surface/ bone surface (ES/BS), osteoid surface/bone surface (OS/BS) and osteoid volume/bone volume (OV/BV). The dynamic histomorphmetric parameters were single-labeled surface/bone surface (sLS/BS), double-labeled surface/bone surface (dLS/BS), mineralizing surface/bone surface (MS/BS), mineral apposition rate (MAR) and bone formation rate/bone surface (BFR/BS). Measurement at the metaphyseal region, which rich in trabecular bone.	Static histomorphometric parameters GTT and DTT treatment significantly reduced N.Oc ad ES/BS, meanwhile statistically increased N.Ob, OS/BS and OV/BV in normal rats. ATF also significantly decreased N.Oc and ES/BS; and increased N.Ob and OS/BS. However, treatment with ATF in normal rats did not affect OV/BV. There was no significant difference in N.Ob, ES/BS, OV/BV and OS/BS between ATF and DTT treatment. Comparing between all vitamin E isomers studies, treatment with GTT had the greatest impact on N.Ob, OV/BV and OS/BS, N.Oc and ES/BS in normal rats. Dynamic histomorphometric parameters All vitamin E treatments significantly suppressed sLS/BS and increased dLS/BS and BFR/BS in normal rats. Following treatment with GTT and DTT in normal rats, MAR and MS/BS were improved significantly. Meanwhile, treatment with ATF did not affect both MAR and MS/BS.	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.

(Table 1) contd….

Study	*In vitro*	*In vivo*	Tocotrienol	Sample/ Subject/ Population	Methods	Results	Comment or Outcome
Study 10 Hermizi *et al.,* 2009		√	Tocotrienol-enhanced fraction (TEF) consisted of 43% α-tocotrienol, 31% γ-tocotrienol and 14% δ- tocotrienol 90% γ-tocotrienol (GTT)	49 young adult Sprague-Dawley male rats (3 month old, 250-300g weight) The rats were divided into 7 groups: -baseline (BL) -control group -nicotine group (N) -nicotine cessation (NC) -tocotrienol enhanced fraction (TEF) -γ-tocotrienol (GTT) -α- tocopherol (ATF) 7 rats for each group	1. Rats were divided into 7 groups: -BL: rats were killed at commencement of study -control group: rats were given normal saline for 4 months -N: rats were given nicotine 7mg/kg body weight daily for 2 months -NC: rats were given nicotine 7mg/kg body weight daily for 2 months and normal saline for the next 2 months -TEF: rats were given nicotine 7mg/kg for 2 months and TEF 60mg/kg body weight daily for the next 2 months -GTT: rats were given 7mg/kg nicotine for 2 months and 60mg/kg GTT for the next 2 months -ATF: rats were given 7mg/kg nicotine for 2 months and 60mg/kg ATF for the next 2 months. Nicotine and saline were injected intraperitoneally, while vitamin E or olive oil was given *via* oral gavage. 2. The structural histomorphometry parameters included trabecular bone volume over tissue volume (BV/TV), trabecular thickness (Tb.Th) & trabecular number (Tb.N.) 3. Cellular histomorphometry parameters were osteoclast surface (Oc.S/BS) and eroded surface (ES/BS). 4. The dynamic histomorphmetric parameters measured were single-labeled surface/bone surface (sLS/BS), mineral apposition rate (MAR) and bone formation rate/bone surface (BFR/BS).	No significant change in all histo-morphometric parameters between control and baseline group. Nicotine treatment decreased BV/TV, MAR and BFR/BS but increased Oc.S/BS and ES/BS significantly. 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/BS. Treatment with TEF or GTT signifi-cantly reduced Oc.S/BS and sLS/BS. Treatment with TEF or GTT, also significantly reduced ES/BS more than ATF. There was no significant change in ES/BS between ATF group and BL group.	Tocotrienols (TEF and GTT) signifi-cantly 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.
Study 11 Ahmad *et al.,* 2005		√	Palm tocotrienol mixture (PTT) from Palm Oil Research Institute of Malaysia (PORIM) consisted of 30.7% α-tocotrienol, 55.2% γ-tocotrienol and 14.1% δ- tocotrienol	Study I: 100 male Wistar rats (4 weeks old, 90-120g weight) The rats were divided into 10 groups: -saline control -ferric nitrilotriacetate (FeNTA) control -FeNTA + ATF10 -FeNTA + ATF30 -FeNTA +ATF60 -FeNTA +ATF100 -FeNTA + PTT10 -FeNTA + PTT30 -FeNTA + PTT60 -FeNTA + PTT100 10 rats for each group Study II: 32 male Wistar rats (4 weeks old, 90-120g weight) The rats were divided into 4 groups: -saline control -FeNTA control -FeNTA + ATF100 -FeNTA + PTT100	1. Rats were divided into groups as following: Study I: -saline control: rats were given 2mg/kg saline -FeNTA control: rats were given 2mg/kg FeNTA -FeNTA + ATF10: rats were given 2mg/kg FeNTA and 10mg/kg ATF -FeNTA + ATF30: rats were given 2mg/kg FeNTA and 30mg/kg ATF -FeNTA +ATF60: rats were given 2mg/kg FeNTA and 60mg/kg ATF -FeNTA +ATF100: rats were given 2mg/kg FeNTA and 100mg/kg ATF -FeNTA + PTT10: rats were given 2mg/kg FeNTA and 10mg/kg PTT -FeNTA + PTT30: rats were given 2mg/kg FeNTA and 30mg/kg PTT -FeNTA + PTT60: rats were given 2mg/kg FeNTA and 60mg/kg PTT -FeNTA + PTT100: rats were given 2mg/kg FeNTA and 100mg/kg PTT Study II: -saline control: rats were given 2mg/kg saline -FeNTA: rats were given 2mg/kg FeNTA -FeNTA + ATF100: rats were given 2mg/kg FeNTA and 100mg/kg ATF -FeNTA + PTT100: rats were given 2mg/kg FeNTA and 100mg/kg PTT Saline or FeNTA was injected intraperitoneally and ATF or PTT was given orally *via* oral gavage. 2. Study I: Blood serum weekly (over 3 weeks of treatment) were obtained to measure IL-1, IL-6 and osteocalcin level, meanwhile urine were collected for de-oxypyridinoline cross-link (DPD) using ELISA technique. 3. Study II: After 8 weeks of treatment, the femur were harvested for histomorphometric analysis purposes. The structural parameters were trabecular bone volume (BV/TV), trabeculat thickness (TbTh) and trabecular number (TbN) The static histomorphometric parameters involved were mean osteoclast number (OcN), mean osteoblast number (ObN) and eroded surface/ bone surface (ES/BS). The dynamic histomorphmetric parameters were single-labeled surface/bone surface (sLS/BS), double-labeled surface/bone surface (dLS/BS), mean apposition rate (MAR) and bone formation rate/bone surface ratio (BFR/BS).	ELISA Treatment with tocotrienols, but not ATF, at 60mg/kg and 100mg/kg doses significantly prevented the increase in FeNTA-induced IL-1 level. Treatment with any dose of ATF did affect the serum level of IL-1. Treatment with ATF or tocotrienols at 30mg/kg, 60mg/kg and 100mg/kg significantly prevented the increase in FeNTA-induced IL-6 level. There were drops of serum level of osteocalcin after 3 weeks of treatment in the control group and both groups treated with 100mg/kg of ATF or tocotrienols. There was no difference in serum osteocalcin level between groups. Treatment with any dose of to-cotrienols significantly restored urine DPD values induced by FeNTA. Meanwhile, ATF only reduced DPD values at higher doses (60 and 100 mg/kg). Histomorphometric analysis Injection of FeNTA significantly reduced ObN, BFR/BS, BV/TV and TbTh, however these could be restored by treatment with 100mg/kg tocotrienols. Injection of FeNTA also increased OcN and ES/BS, however treatment with tocotrienols reduced only ES/BS. Treatment with either ATF or PTT at 100mg/kg did not affect osteoclast number.	Treatment with palm oil tocotrienol mixture prevented free radical-induced bone loss through reducing bone-resorbing cytokines IL-1, IL-6, serum osteocalcin, DPD values and eroded surface over bone surface. However, tocotrienol treatment did not affect osteoclast number.

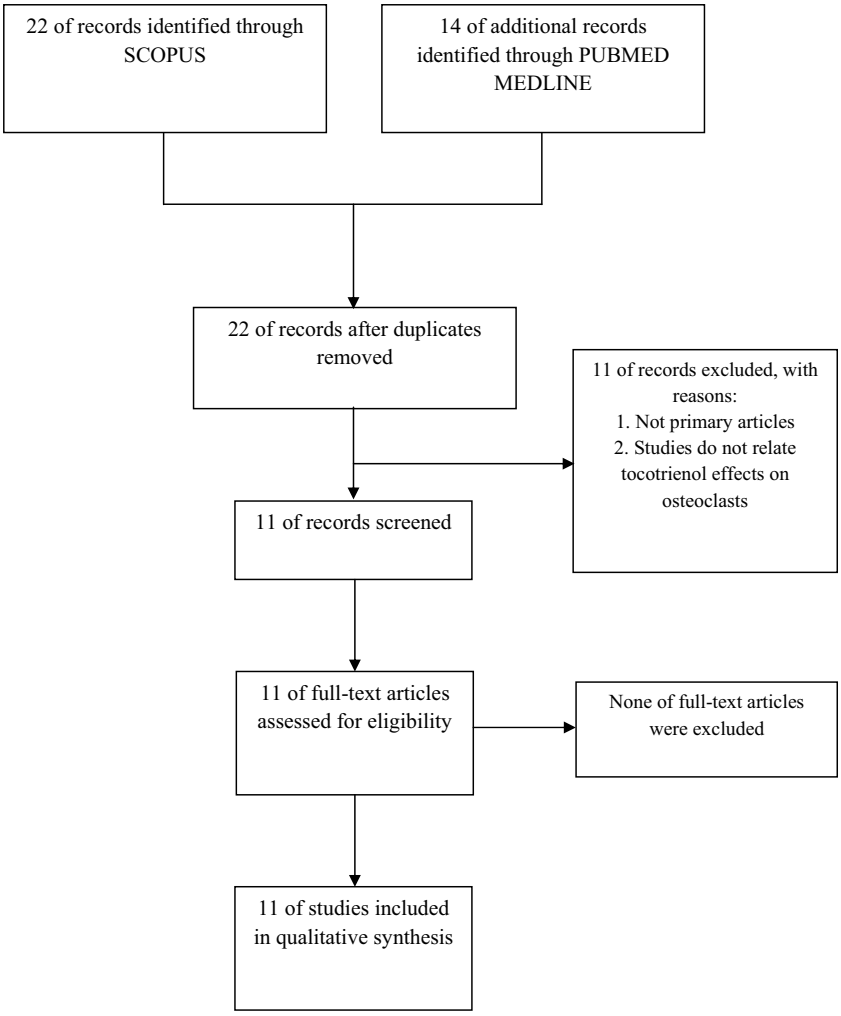

Fig. (1). Flow chart showing the selection process of the articles in this review.

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 ovariactomized (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 OcN per mm^2 [30, 50, 56] or the percentage of OcS/BS [51, 52, 55]. In studies that used orchidectomized (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 tocotrienols 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 δ-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 osteoblassts 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 co-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 protectitive effects on osteoblasts, the bone forming cells. Nizar *et al.* (2012) found that low concentrations of GTT supplementation could protect osteoblasts from hydrogen peroxide (H_2O_2)-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 orchidectomized-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 ovariectomized 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 Norazlina *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 coworkers (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 resorbing 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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