VITAMIN E ACTION ON BONE SIGNALING PATHWAYS, RANKL/RANK/OPG IN A RAT MODEL OF BREAST CANCER-INDUCED BONE PAIN

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Abstract

Cancer-induced bone pain is currently facing inadequate pain management due to unwanted side effects and relative ineffectiveness. The search for alternative therapy to alleviate pain and target a few mechanism pathways might improve survival in metastatic patients. Vitamin E which has been promoted as anti-inflammatory, anti-cancer, and anti-metastatic were chosen in this study to potentiate its capability in a cancer-induced bone pain rat model. Rats were randomly grouped into five groups, and a breast cancer cell line was induced into the left femur of four groups: Negative Control (NC), Alpha Tocopherol (ATF), Tocotrienol Rich Fraction (TRF) and Zoledronic Acid (ZA), whereas Sham group as healthy subjects induced with supplementary media. Pain assessment tests were carried out at four days intervals. The animals were sacrificed after 21 days following SPECT/CT imaging. Bone specimens were analyzed for ELISA and gene expression studies. The results showed that the animal model was successfully validated via the presence of abnormal uptake of the skeletal system. Pain assessment tests demonstrated that vitamin E, specifically TRF significantly alleviate pain compared to the NC group. Biomarker activity illustrated that the TRF supplement group was able to regulate the bone turnover activity comparable to the ZA treatment group. Gene expression studies signify the role of TRF supplement comparable to the ZA group in the ability to regulate osteoclastogenesis, osteoclast activation, and regulating the secretion of metastatic cancer cytokine. This finding addressed the beneficial potency of TRF compared to ATF as a therapeutic option in the management of cancer-induced bone pain.

Keywords: Vitamin E, Pain Alleviation, Cancer-induced Bone Pain, RANKL-RANK Pathway

Introduction

Breast cancer currently contributes to 11% of mortality cases within Malaysia, as mainly of the incidence commonly occurred in women. Furthermore, as high as 50% of the relapse cases of breast cancer patients were diagnosed with bone metastases. Metastasis occurs when primary cancer spreads from its origin. In this light, primary breast cancer has a distinct predilection to simultaneously metastasize to multiple bones such as the vertebrae, femurs and pelvic bone (1).

Breast cancer bone metastasis usually negatively impacts cancer patients' lives as they may experience limitations in daily activities due to severe pain, hypercalcemia, pathological fracture and anemia, all known as skeletal-related events (SREs) (2). During early stages, bone cancer pain can be intermittent, but then it rapidly progresses into continuous pain aggravated by a series of breakthrough pain. The pain experienced by patients is intractable as it is one of the most challenging pain conditions to be treated as it is related to weight bearing activities and physical movement (3). Once the chronic pain has been established, the condition will get worsen once mechanical allodynia develops (4). Mechanical allodynia happens when a normally non-painful activity and/or stimulation is perceived as painful where it can be intermittent but it advances promptly into continuous pain condition that is aggravated by a series of breakthrough pain (5). Cancer-induced bone pain is always associated with bone destruction caused by anomalous activation of osteoclasts (6), and the stimulation of osteoclasts is positively modulated by the receptor activator of nuclear factor kappa-B ligand/receptor activator of nuclear factor kappa-B (RANKL/RANK) – related signaling and negatively modulated by osteoprotegerin (OPG) (7). It is highly presumed that breast cancer cells release inflammatory mediators such as parathyroid hormone-related protein (PTHrP) and macrophage colony-stimulating factor (M-CSF) into the bone microenvironment leading to osteoclastogenesis and further promoting vicious cycle in bone destruction and bone cancer pain (8). Hence, bone pain signaling pathway could be a target for designing a new therapy as an therapeutic therapy for cancer-induced bone pain (9).

At present, bone pain related to breast cancer is currently incurable, and cancer pain management mainly focuses on improving the patient's life through palliative care (10), limiting cancer growth by chemotherapy (11) and also reducing SREs with bone-targeted therapies (12). Current clinical analgesics therapy such as bisphosphonate and nonsteroidal anti-inflammatory drugs (NSAIDS) provide temporary pain relief to half of the patients (13, 14). Understanding breast cancer metastasis mechanism to bones could probably prompt to the discovery of a novel therapeutic target for reducing and preventing bone lesions. This could provide better management for patients with bone cancer.

The antioxidant activities of vitamin E have been linked to pain management therapy and are actively linked to the pain signaling pathway (15, 16). Hence, using in-vivo animal models with breast cancer-induced bone pain might postulate the roles of vitamin E supplementation in managing pain caused by cancer. Vitamin E is a fat-soluble vitamin. It can be divided into two types: tocopherols and tocotrienols where each of the type has four distinct analogues (gamma, beta, alpha and delta) based on the locations of methyl groups on the chromanol ring (17). Tocopherols are the saturated forms of Vitamin E, whereas tocotrienols are the unsaturated forms, which can be characterized by three double bonds in the tails of tocotrienols (18). Both of the vitamin E are regularly found in edible plant oils such as rice bran, barley, coconut, palm oil and annatto oil in varying proportions (19). Vitamin E has antioxidant, anti-inflammatory and anti-carcinogenic properties and has the potential to be used as an adjuvant treatment for cancer (16, 20). Several *in-vitro* and *in-vivo* studies have examined the anti-metastatic properties of various types of vitamin E (21, 22). Vitamin E used in this study were alpha-tocopherol (ATF) and a tocotrienol-rich fraction (TRF), in which ATF is the most biologically active form of vitamin E (23) and TRF is known as the natural form of vitamin E (24).

Due to the vitamin E's antioxidant and anti-metastatic properties, it is relevant to characterise the bone pain signaling pathways in the rat model with breast cancer-induced bone pain. Thus, this paper focuses on the actions of vitamin E on bone pain signaling pathways in the rat model with breast cancer-induced bone pain.

Materials and Methods

Ethics statement

Ethical approval for using animal models was obtained from Universiti Sains Malaysia (USM) Institutional Animal Care and Use Committee (USM IACUC) with the reference no: USM/IACUC/2017/(106)(851). The protocol was conducted strictly based on the guidelines outlined by USM IACUC. Animal work was carried out at Seksyen Penyelidikan Haiwan, Advanced Medical and Dental Institute, Universiti Sains Malaysia, under the supervision of an attending veterinarian.

Bone cancer pain model

Female Sprague Dawley (SD) rats weighing around 200-250g were randomly grouped into Sham, Negative Control (NC), ATF, TRF and Zoledronic Acid (ZA). ZA group act as a positive control group used to assess the outcome of the animal models by giving the current drug treatment used in clinical practice for metastatic bone diseases (25). A breast cancer cell line (MDA-MB-231) was directly implanted into the left distal femur of rats in NC, ATF, TRF and ZA groups, and the Sham group was implanted with a culture medium only. The cell line was cultured in a Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and prepared at $1x10^6$ cells/ml before injection. This study modified a surgical procedure from a previous study (26). Rats were deeply anaesthetised with ketamine (100 mg/kg) and xylazine (10 mg/kg) cocktail with a 1:1 ratio at a volume of 0.2 ml per 100g rats' weight. The left femur of each rat in the treatment group was exposed, and a hole was drilled via a dental round bur (size 2.35 mm) with Escort II Pro Dental Micro Motor. A hole was drilled for around 1 cm deep until the area of spongy bone was exposed, and a 10 µL injection volume of MDA-MB-231 cells was inserted using a Hamilton syringe. The injection site was then covered with bone wax, and all rats were subjected

to postsurgical care under ideal conditions of temperature, humidity and light, and they were fed with *ad libitum* pellet and filtered water. Iodine solutions were applied on the incision site, whereas 5% Baytril (Enrofloxacin) were injected subcutaneously following recovery for 3 days once daily to each individual rat. All rats were then caged separately to recover after surgery for 5 days.

Oral dosing

Supplementation started a day after surgery, where the rats were weighed and treated daily via oral gavage. Both Sham and NC were fed with olive oil daily. Moreover, ATF and TRF were diluted in olive oil (Bertolli) to obtain the dose of 60 mg/kg body weight daily. The powdered Zoledronic Acid was diluted with sterile distilled water to a selected dose of 5 µg/kg following a 7-day interval.

Pain assessment test

The pain assessment test was conducted in 4-day intervals, which included the Von Frey test, which is an assessment of paw withdrawal activity (Figure 1A), and the Hot Plate test (Figure 1B), which was adapted from previous studies (27, 28). In the Von Frey test, rats were acclimated to the cage for 10 minutes before the procedure. A single, unbending filament was pressed perpendicularly with an increasing force from 0, 1, 2, and 5 g against the hind paw of each individual. The 5 g force is set according to the highest threshold level the healthy rats can withstand during optimisation before conducting the study. In the Hot Plate test, rats were acclimated individually within the beaker for 10 minutes before the actual procedure. The hotplate temperature was set at 55°C, and the beaker with rats was placed on the Hot Plate for 60 seconds.

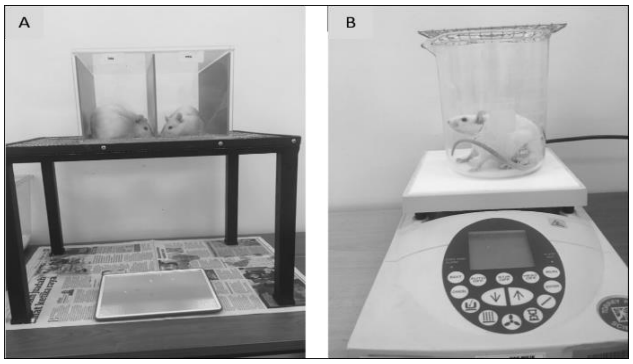

Figure 1: Acclimatization setup before procedure of pain assessment test; (A) Von Frey test (B) Hot Plate test

Following 20 days after surgery, the animals were injected with ±37 MBq of ^{99m} Tc-MDP via the tail vein and kept on hold for at least 2 hours before sedating the animals for imaging. The animals were placed on the table, and the emission scan was performed using SPECT/CT GE 670 (GE Healthcare, USA). SPECT acquisition was performed with 60 steps in steps and shoot mode, 20 seconds per step.

Enzyme-linked immunosorbent assay (ELISA)

Procollagen I N-Terminal Propetide (PINP) and Tartrate-resistant acid phosphatase 5b (TRACP-5b) were measured using Rat ELISA Kit by Elabscience to determine the bone formation and bone resorption activity, respectively, both according to the manufacturer's instruction. The protocols for bone sample collection were obtained and modified from a previous study by Schroeter *et al*., 2016 (29). Each rat's femur was dissected, soaked in saline before pulverised with mortar and pestle in the presence of liquid nitrogen. The bone powders were demineralised with 0.6 M HCl (5 ml/g) and incubated at room temperature 27°C overnight. The samples were then pelleted by centrifugation for 20 minutes at 7200 g. Pellet was then resuspended (5 ml/g) in 4 M guanidine hydrochloride (GuHCl) in 50 mM Tris (pH 7.4). Samples were incubated at 65°C on a heating block overnight and then centrifuged to collect the supernatant as samples for ELISA testing.

Reverse Transcription qPCR (RT-qPCR)

Each rat's femur was dissected, soaked, and stored immediately in RNA later Stabilisation solution. The RNA was then extracted from the femur by spin-column purification (GF-1 Total RNA Extraction Kit, Vivantis) following the manufacturer's instruction. The extracted RNA was then quantified and purified using UV Vis micro-volume spectrophotometry. Reverse transcription and cDNA synthesis using random primer was carried out per the manufacturer's instructions (One Script Plus cDNA Synthesis Kit, ABM). The forward and reverse primer sequence for each gene of interest is stated in Table 1. PCR amplification was performed in triplicate; of denaturation step at 95 °C for 2 minutes, with 40 cycles of amplification at 95 °C for 15 seconds and followed by annealing at 58 °C to 60 °C (depending on primer sequence) for 30 seconds using BlasTaq 2X qPCR Master Mix, ABM. The relative expression level of each gene was normalised according to the reference of β-actin and was calculated with the formula $2^{-\Delta\Delta CT}$ (2^-delta delta CT) to obtain fold change against Sham control (30).

Table 1: The sequence of forward and reverse primer

GENE	FORWARD PRIMER	REVERSE PRIMER
M-CSF	GACTTGGCTTGGGATGATTCT	GAGGGTCTGGCAGGTACTC
RANKL	CATCGGGTTCCCATAAAG	GAAGCAAATGTTGGCGTA
RANK	TGGTGTTCCTGCTCAGCTA	CCTCGTCGTCTGACCCAAA
OPG	TTGGCTGAGTGTTCTGGT	TTGGGAAAGTGGTATGCT
TGF-β	CCTGGAAAGGGGCTCAACAC	CAGTTCTTCGTGGAGCTGA
IL-6	CCACCAGGAACGAAAGTCAA	GGTTTGCCGAGTAGACCTCATA
PTHrP	TGGTGTTCCTGCTCAGCTA	CCTCGTCGTCTGACCCAAA
Beta actin	ATTGGCAATGAGCGGTTCCGC	CTCCTGCTTGCTGATCCACATC

Statistical analysis

All the data were shown in mean ± standard error of the mean (SEM). Statistical analysis was analysed using IBM Statistical Product and Service Solution (SPSS) software version 26. Data was run for normality to assess the normal distribution. Mixed ANOVA followed by post-hoc Tukey analysis was used for statistical comparison for the Pain Assessment test, and one-way ANOVA followed by post-hoc Tukey analysis was used for statistical comparison in ELISA. The statistical significance level was set at a value below 0.05.

Results

Pain alleviation activity

All rats showed a good health physical activity with no elements of postoperative pain or distress, and loss of weight was not recorded throughout the study. Mixed ANOVA was used to compare the mean differences between groups split into independent variables. In the Von Frey test (Figure 2A), the Sham group representing a healthy model showed the highest threshold value compared to other groups except for the ZA group. In contrast, the NC group reported the lowest threshold value compared to all groups displaying a condition of mechanical allodynia in the rat model with the untreated cancer-induced group. Results in both vitamin E groups demonstrated the most significant improvement in the threshold values compared to NC. Furthermore, there is no significant difference between the two, despite both showing lower threshold values compared to the Sham and ZA groups. There was no significant difference between the ZA and Sham groups suggesting the suitability of the control drug used in this animal model.

The Hot Plate test (Figure 2B) follows an almost similar pattern to the Von Frey test, in which the Sham and ZA groups have significantly higher threshold values than the NC group. As seen in Figure 2B, the NC group representing the untreated cancer-induced group displayed a significantly lower threshold value than all groups. The low value in the NC group indicates the pain response toward the thermal stimulation. Similar to the Von Frey test, both vitamin E supplementation in the Hot Plate test showed no significant difference. However, TRF supplementation displayed a wide variability throughout each time-point and showed significant differences from the Sham and ZA groups while showing no statistical difference from the NC group. In contrast to the TRF group, ATF demonstrated better performance with a higher threshold value than the ZA group. Within the ZA group, values in the Hot Plate test showed reduction compared to the Sham group, although no significant differences were found between the two, further confirming the role of ZA as a controlled drug in this animal model.

SPECT/CT analysis

The analysis of scan images was analysed using Amide, an open-source medical image analysis software. The SPECT/CT images displayed uptake of radiotracer in the bone, especially in the femur, spine and pelvic region. Figure 3 showed a representative image of each group following SPECT/CT scan where an increased radiotracer uptake was assessed as a hot spot or an uptake showing an abnormality in that particular region. The sham group showed no abnormal radiotracer uptake within the bone and retained normal bone uptake. On the contrary, the NC group displayed a higher frequency of abnormal bone uptake when compared to the other treatment groups. Both vitamin E treatment groups showed similar frequency of abnormal bone uptake when comparing the Vitamin E-supplemented groups. Moreover, both of the vitamin E groups reported high abnormal uptake within the spine region and femur bone compared to the pelvic bone. The frequency of abnormal bone uptake in the ZA group is lowest compared to other cancer-induced groups.

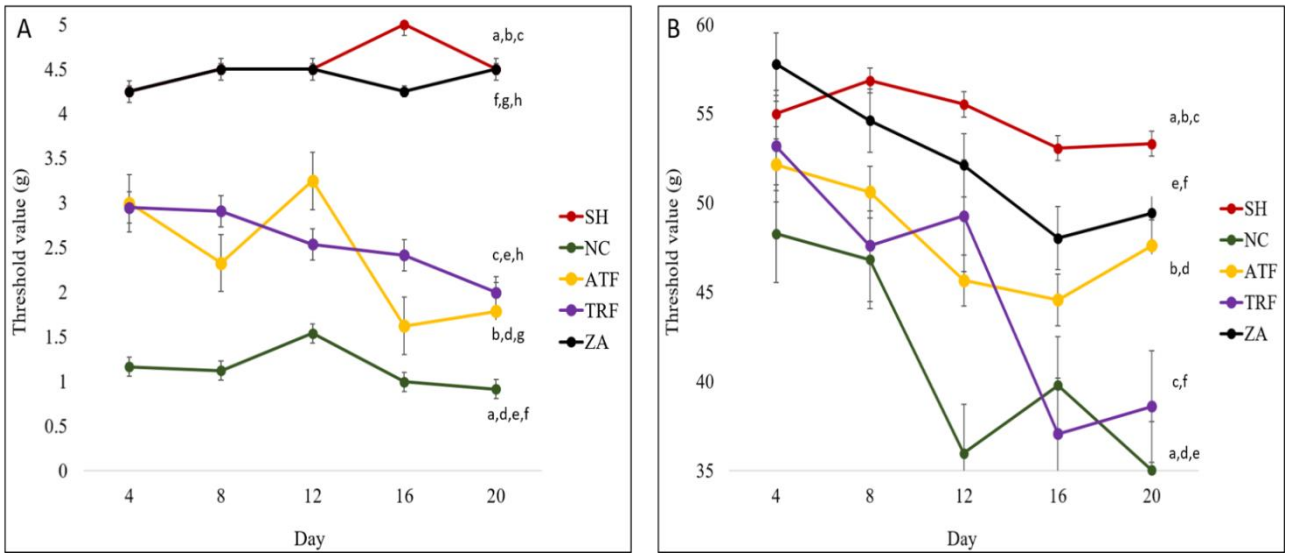

Figure 2: Withdrawal responses threshold of weight burden (g) to Von Frey filament [A] and withdrawal responses threshold (s) to Hot Plate [B] following intraosseous injection of MDA-MB-231 breast cancer cells or supplementary media (Sham group). n=8 per group. Data are presented in means ± SEM. Same alphabet indicates significant difference at $P < 0.05$.

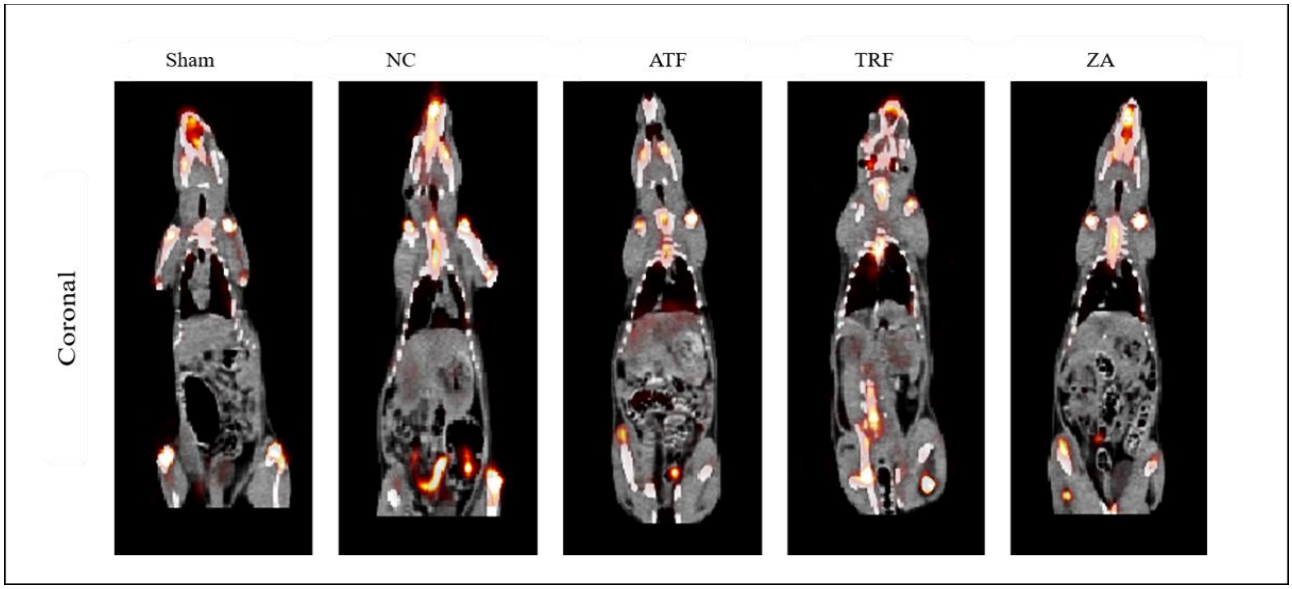

Figure 3: Representative coronal views of SPECT/CT images at 2 hours after intravenous injection of 99m Tc-MDP in in-vivo bio-distribution of radioactivity uptake in SD rats induced with MDA-MB-231 and supplementary media (Sham only). Results were expressed via uptake seen in each rat

Bone turnover activity

The healthy model represented by the Sham group showed significantly lower PINP concentration (Figure 4a) and TRACP 5b concentration (Figure 4b) compared to NC and both vitamin E groups. In the NC group, the PINP concentration and TRACP 5b concentration were significantly higher than in other groups suggesting high bone turnover activity within the bone microenvironment. However, both ATF and TRF treatments were reported to be significant with both Sham and ZA groups in PINP biomarkers, while in TRACP 5b biomarkers, both treatments did not demonstrate a significant difference between Sham and ZA groups. Comparing the biomarkers level of PINP and TRACP 5b in the ZA control group, both showed significant differences when compared to the NC group, whereas no significant difference was reported between Sham and ZA in TRACP 5b biomarker.

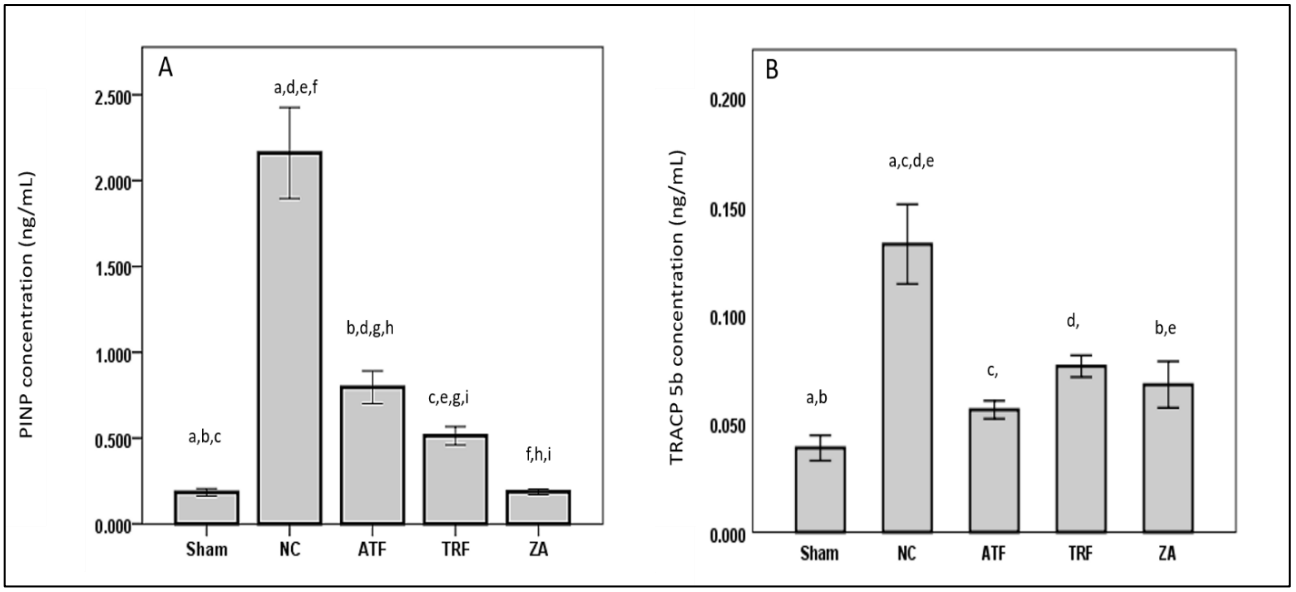

Figure 4: PINP concentration (pg/ml) (A) and TRACP 5b concentration (ng/ml) (B) in bone samples from SD rats of each group (n = 8). Data presented as mean ±SEM. Same alphabet indicates significant different between groups

Gene expression analysis

Fold change in log2 of the relative gene expression of RANKL, RANK, OPG, PTHrP, TGF-β, IL-6 and M-CFS is displayed in Figure 5, which shows different treatment groups demonstrating contrasting expression levels of the genes of interest. NC group demonstrated an upregulation for all genes of interest except RANK. In addition, the upregulated gene of interest in the NC group showed an almost equivalent value of fold change except for IL-6, where the gene expression was nearly 4-fold higher compared to M-CFS, RANKL, OPG, TGF-β and PTHrP. In contrast, all of the genes of interest in the ATF group demonstrated an upregulation expression with an almost equivalent value of fold change. Compared to the ATF group, TRF demonstrated a contrasting trend between both vitamin E-supplemented groups, where OPG, IL-6, PTHrP and RANK showed a downregulation expression, whereas M-CFS, RANKL and TGF-β showed an upregulation expression. On the other hand, the ZA treatment group showed an upregulation expression in RANKL, OPG and IL-6, while M-CSF, TGF-β, PTHrP and RANK demonstrated a downregulation expression.

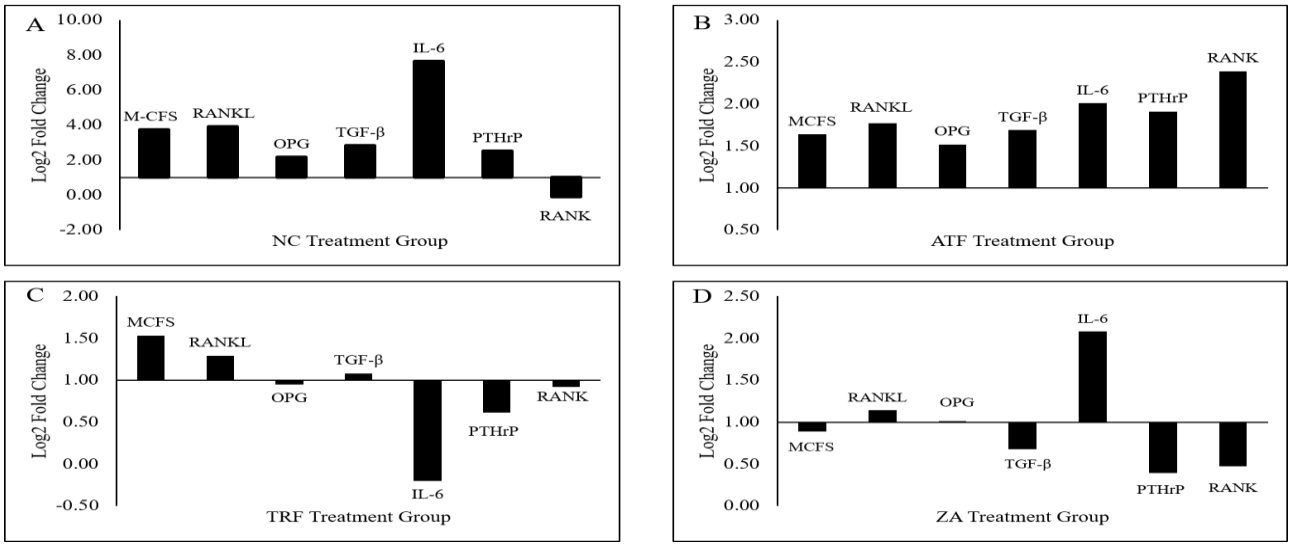

Figure 5: RT-qPCR analysis of fold change expression level against Sham control of mRNA target gene; RANKL, RANK, OPG, PTHRP, IL-6, TGF-β and M-CSF in femur bones (n=3) after induction of breast cancer cell line, MDA-MB-231 in NC group (A), ATF group (B), TRF group (C) and ZA group (D). Fold change of less than 1 imply down-regulated expression and fold change more than 1 imply up-regulated expression. Data were expressed in Log2.

RANKL/OPG ratio

The RANKL/OPG ratio indicates the bone resorption activity that happen inside the bone microenvironment, in which a higher RANKL indicates a higher osteoclast activity which consequently leads to abnormal activity of bone resorption. A lower value reflects better remodelling activity within the bone. Based on Table 2, the Sham group showed the lowest RANKL/OPG ratio, whereas the NC group reported a substantially higher ratio compared to other groups. It is interesting to note that ATF supplemented group had a comparable value to the ZA group and demonstrated a lower RANKL/OPG ratio. In contrast, the TRF group had a slightly higher ratio than the ATF group. The ZA group reported a better RANKL/OPG ratio than the cancer-induced groups.

Table 2: RANKL/OPG ratio of each treatment group

	RANKL	OPG	RATIO
Sham	0.00	0.00	0
NC	3.91	2.16	1.81
ATF	1.77	1.52	1.16
TRF	1.29	0.95	1.36
ZA	1.14	1.01	1.13

Discussion

The breast cancer bone pain model in this study was evaluated based on the effect of vitamin E, ATF and TRF supplementation and its related activity in pain stimuli and the bone microenvironment following cancer invasion directly into the bone. The Sham group undergoes surgical intervention without cancer implantation to represent a healthy model, whereas the NC group are representative of the untreated cancer invasion model. ATF and TRF were the treatments to be evaluated against the bone cancer pain model. The clinical control drug model was represented by ZA, the current clinical drug used for bone pain-related diseases. Zoledronic Acid was chosen as a controlled drug in this study as bisphosphonates. Studies have found that it positively affects bone metastasis and is a preferred option for pain management in bone metastasis (31).

Supplementation of olive oil as the vehicle in both Sham and NC groups is due to its low content of vitamin E (1 mg per 100 mg) (32) and low toxicity towards rodents in general (33). The dose for both vitamin E at 60 mg/kg was generally considered safe in bone studies and reported no toxicity to animal health (34). Published data has reported olive oil's beneficial potency in anti-cancer activity in in-vitro studies (35, 36) and preclinical studies (37). However, the dose given in this study was too minimal compared to the published finding in in-vitro studies, and the duration of treatment in this study was shorter compared to the preclinical studies. In addition, the NC group that received a daily dose of olive oil showed high activity of abnormal radiotracer uptake within the bone region compared to other treatment groups. Changes in body weight were recorded every week, and no significant weight drops were recorded, displaying no distress condition and no post-operational pain in the animal, suggesting the suitability of the surgical model (38). Two types of stimuli, mechanical and thermal, were introduced in the pain assessment test. Both of these stimuli serve different purposes for pain assessment tests.

In mechanical stimuli, the nociceptors respond to intense pressure, while in thermal stimuli, the nociceptors respond to extreme hot or cold temperatures (>45°C or <5°C) (39). The underlying mechanism of mechanical stimuli is believed to be neuropathic pain, whereas thermal stimuli is a serotogenic response. Thus, it is important to identify the pain mechanism in cancer-induced bone pain following the Vitamin E treatment. As pain cannot be directly measured from rodents, different sensory targets should be introduced to identify the analgesic mechanism of vitamin E treatment. Both stimuli also have different features where specific sensory neurons have different sensitivities to various stimulations that release different neurotransmitters (40). The Sham group representing healthy individuals had the highest threshold value in both Von Frey and Hot Plate tests, comparable to other studies (41). In contrast, the NC group has the significantly lowest threshold among all the groups in both thermal and mechanical stimuli.

Mechanical allodynia is a condition that occurs when one experiences pain from non-painful situations caused by a damaged nerve (42). Rats in both Sham and ZA groups can withstand higher threshold levels, which is a justification for this condition. Tumours can directly induce structural damage to tissue, especially sensory neurons in bone. This will activate the sensory fibres. At the same time, cancer cells can directly activate sensory fibres (43), subsequently causing a unique pain. Pain signaling from peripheral tissue is initiated by tissue stimulation of primary afferent sensory fibres in which the bone has a rich sensory fibre and thus might stimulate the nerves in the system (42).

Both ATF and TRF-supplemented groups in the Von Frey tests showed a significant difference from the NC group demonstrating the ability of both ATF and TRF supplements to alleviate pain in the rat model with cancer-induced bone pain. On the contrary, in the Hot Plate test, only ATF treatment has no significant difference, with Sham and ZA groups displaying the ability of the supplementation to be on par with the current treatment drug and with healthy animal groups. Vitamin E has been associated with other studies linked with pain management and has been cited for its analgesic properties (20). However, tocotrienol treatment within this study showed no significant difference with the NC group, demonstrated a contravene data with previous studies that evidently linked tocotrienol with analgesic activity and anti-inflammatory properties (44, 45). Results from the Hot Plate test showed a more varied date and value between the treatment groups compared to the Von Frey test, which probably caused by the application of the subject

towards the stimuli. In Von Frey test, the hind paw was the only area that was evoked to the stimuli. In comparison, during the Hot Plate Test, whole rat's body, which include the tail, was fixed within the beaker. Spatial summation is a term where there is an increase in pain perception if larger base areas of pain stimulation are evoked (39). In addition, thermal stimuli which influenced serotonergic response might show fewer sensitivities toward vitamin E, which are known as peripherally acting analgesics (46). Thermal stimuli test was done in this study to evaluate the pain sensitivity of the animal model and its behaviour following thermal pain application.

ZA group in both tests showed no significant difference from the Sham group, suggesting that the control drug treatment, Zoledronic Acid, can reduce pain and may strengthen the bone. The bisphosphonate reduces the disintegration of bone during osteoclast activity and act as specific blockers during secretion of cancer cells' growth factor (47). It is the commonly used bisphosphonate in treating bone cancer pain due to its efficiency of pain relief activity and also provides a simple and nontoxic results (2).

Bone scintigraphy is the most frequently performed among radionuclide bone imaging as it plays a vital role in tumour staging and management (48). Radionuclide works by detecting areas of increased or decreased bone turnover activity, and the present work provides the potential for detecting bone metastases (49). A commonly used radionuclide for bone scintigraphy is 99mTc-MDP via SPECT/CT scan, which was generally used to localise bone metastases or any abnormal activity within the bone, such as lesions and hypercalcemia (50). Hypercalcemia is usually a result of high calcium levels caused by the secretion of cancer cells' growth factors that stimulate calcium production within the bone, which in turn alters the calcium homeostasis activity in the bone (51). Localisation of radionuclide to sites of abnormal bone activity is due to the coordination of phosphate group to calcium deposition in hydroxyapatite of bone (52). The ability of radionuclide in SPECT/CT imaging to characterise many bone pathologic conditions has made it useful for screening. Several studies have also been published on bone abnormalities using animal models in SPECT/CT imaging (53).

Figure 3 shows high abnormal uptake in cancer-induced groups within the spine and pelvic bone region, specifically NC and vitamin E groups. Vitamin E-supplemented groups showed a similar trend to the NC group with high uptake, although the role of vitamin E as anti-metastatic properties in both in-vivo and in-vitro studies has been widely published (54). High abnormal uptake within both vitamin E treatment groups could be inferred to be high bone turnover activity within the bone microenvironment. Although vitamin E has been reported as an anti-cancer agent, the high bone turnover activity could be presented during early treatment, which might vary if the treatment was prolonged (55). Interestingly, the results from the group with abnormal uptakes in both vitamin E treatments justified that vitamin E has potency in pain alleviation activity. This is evident in the results of the pain assessment tests, which showed that vitamin E treatment, specifically in the ATF group, has a significantly lower threshold value than the NC group. Bisphosphonate has been documented to inhibit the development of osteoclast progression and differentiation (56). In this study, we found that following SPECT/CT imaging, the ZA group showed less uptake frequency than other cancer-induced groups. Abnormal uptake within the spine and pelvic region was generally inferred as evidence of bone metastases and abnormal bone turnover activity, such as calcium deposition and spike elevation of osteoclast (52) within bone caused by cancer cell activity (11).

The accumulation of radionuclide 99mTc-MDP can then be linked to bone resorption and bone formation biomarkers. Bone formation markers are derived from osteoblast activity during differentiation, in which PINP is derived from pro-collagen cleavage at N-terminal from osteoblast synthesis during the bone formation process (57). Conversely, bone resorption markers are formed during the resorption phase as a by-product of osteoclasts following osteoclast activation, such as TRACP 5b (58). During bone metastases, enzyme levels of PINP and TRACP 5b were reported to be significantly high and are frequently related to skeletal lesions due to high bone turnover activity within the bone microenvironment (59).

Based on Figure 4, both PINP and TRACP 5b in the Sham group reported significantly low concentration levels compared to cancer-induced groups, demonstrating the normal concentration of PINP and TRACP 5b biomarkers for healthy individuals. An increase of enzyme concentration in the NC group within both PINP and TRACP 5b biomarkers demonstrated the aberrant activity of osteoblast and osteoclast, thus reflecting high bone turnover and resorptive activity within the bone (60). The results indicated that supplementation of both vitamin E significantly reduced the concentration level of PINP and TRACP 5b compared to the NC group. Furthermore, TRACP 5b biomarker concentration in both vitamin E-supplemented groups was statistically insignificant. In this regard, the ZA group displays the potent action of anti-resorptive properties of ATF and TRF. These findings are in line with results published by other studies (61, 62). Both ATF and TRF-supplemented groups showed no significant difference in TRACP 5b with the Sham group, and few studies on vitamin E towards bone healing and bone metastasis reported similar observations (63, 64). ZA treatment in the cancer-induced rat model significantly reduced PINP and TRACP 5b concentrations compared to the NC group. Zoledronic acid has demonstrated the ability to prevent aberrant bone turnover activity in the skeletal environment (65).

Many studies discussed the relationship between the RANKL/RANK/OPG pathway being associated with bone

metastasis and bone pain (66, 67), which is also related to other gene expression pathways such as M-CFS, TGF-β, IL-6 and PTHrP, which completed the vicious cycle of bone metastasis. This cycle induces bone turnover activity resulting in complex pain mechanisms. Breast cancer cells regulate the osteoblast and osteoclast activity within the bone microenvironment which then indirectly promote osteoclastogenesis and then releases more M-CSF and promotes osteoblast to secrete more RANKL into bone (68). Our study demonstrated that both vitamin E-supplemented groups showed an upregulation of both M-CFS and RANKL, but both supplemented groups showed a notable decrease in expression when compared with NC group demonstrating the potential of tocotrienol and tocopherol in regulating osteoclastogenesis. The role of vitamin E in bone-related diseases such as the action of osteoclast inhibition and bone resorption reduction activity has been widely published in previous studies (69, 70). However, the relationship between bone and cancer remains undefined in the literature. Comparing vitamin E with the ZA group demonstrated a comparable activity where the ZA group showed an upregulated RANKL gene expression similar to both vitamin E, although M-CSF expression in the ZA group was downregulated.

As a decoy receptor for RANKL, OPG, a cytokine secreted by osteoblast, inhibits the osteoclastogenesis pathway and prevents further bone resorption. Thus RANKL/OPG ratio provides the basis for bone resorption activities where a lower score ratio signifies lower bone resorption activities. Our study showed that OPG expression was upregulated in NC, ATF and ZA groups, whereas the TRF group demonstrated a downregulated expression of OPG. However, based on the RANKL/OPG ratio, both vitamin E-supplemented groups showed a comparable low ratio score as the ZA treatment group with the ATF group showed a lower value than the TRF group. Generally, bone turnover activity is represented by the homeostasis of the RANKL/OPG ratio, where breast cancer cells stimulate RANKL expression and osteoblast secrete OPG in the bone metastasis environment. Few studies have reported higher expression levels of OPG in bone metastasis and ER- breast cancer subtypes (71, 72). In addition, a study by Ryser et al. (2012) published that the OPG expression corresponded to the RANKL expression in order to counter the excessive bone resorption activity in tumour diseases (73).

Following osteoclastogenesis in bone turnover activity, active osteoclast secretes TGF-β, and subsequently stimulates metastatic cancer cells to secrete PTHrP that interferes with the RANKL/OPG pathway (11). Cancer cells activity within the bone affects the role of TGF-β, which leads the cytokine by promoting and facilitating the growth progression and invasion of the metastatic cancer cells (74). Our research showed that the TGF-β expression was upregulated by almost two-fold in cancer-induced groups with exception in bisphosphonate treatment group. Zoledronic acid has been proved to modulate the TGF-β expression (75). Previous studies have reported that higher TGF-β expression is linked with the tumour presence and activity and also a characteristic of advanced stages in cancer (76, 77). Both vitamin E-supplemented groups showed a decrease in the expression level of TGF-β compared to the NC group, where the ATF group exhibited higher fold change compared to TRF. In contrast to published findings, tocopherol has been widely used in cancer studies and showed potency in inhibiting breast cancer cells via functional knockout of TGF-β signaling (78).

PTHrP is claimed to enhance the progression of metastatic cancer cells, specifically within the bone microenvironment (79). Our finding in this research showed that the upregulation of PTHrP expression in both NC and ATF groups illustrated tumour presence and high metastatic activity (80). In contrast, the TRF group exhibited downregulation of PTHrP expression comparable to the ZA treatment group. Inhibitory activity of tocotrienol could result from the anti-cancer properties of vitamin E (16), which might modulate PTHrP signaling (81). Treatment with Zoledronic acid has been extensively used in the clinical setting to treat hypercalcemia attributed to the upregulation of PTHrP expression (82).

Another factor released by metastatic cancer cells is IL-6, an inflammatory cytokine capable of activating the osteoclastogenesis pathway (83). Higher upregulation of IL-6 expression within the NC group when compared to other groups demonstrated a higher presence of metastatic cancer cells. Previous studies have reported significant upregulation of IL-6 in TNBC subtypes in the clinical setting (84),(85). Although the ATF group exhibited upregulation of IL-6 expression, the fold change value is comparable to the ZA treatment group. On the other hand, it is worth mentioning that the tocotrienol treatment exhibited downregulated expression of IL-6 when compared to other cancer-induced groups. Vitamin E, specifically tocotrienol, has been widely published for its role as reactive oxygen species scavenger which probably resulted in decreasing of inflammatory cytokines (16, 22) and restoring the functional physiology condition of inflammation homeostasis activity within the body (86). Consistent with published findings, vitamin E demonstrated an inhibitory effect on inflammatory cytokine and reduced the level of IL-6 expression (87). In addition, supplementation of tocotrienol demonstrated potency better than the ZA control group, where a previous study showed that Zoledronic acid was positively associated with a reduction in IL-6 expression (88).

Higher bone resorption rates linked to extensive bone cell loss lead to dysregulation of bone cell production and survival (73). The activity of the metastatic cells within the bone convinced by the higher TGF-β and PTHrP expression within the NC group demonstrated predomination of tumour in bone, leading to undeveloped bone cells and imbalance activity in the

production of osteoclast precursor (89). Downregulated expression of RANK in the NC group might relatively cause by a higher tumour burden, which is consistent with a previous study that claimed that the RANK-knockout model exhibited osteoporotic characteristics (90). Our study also agrees with a previous literature by Owen et al. (2013) which he suggests that the downregulated RANK expression are commonly linked to the high activity of bone metastasis in deceased patients (91). In contrast, other published data reported contradicting finding where they claimed that a lower RANK expression will produce a better prognosis towards patients (67, 92). However, in vitamin E treatment groups and bisphosphonate treatment group, our finding demonstrated a reduced expression of PTHrP and IL-6 when compared to NC groups which probably caused by low presence of metastatic cancer. Then, an upregulated RANK expression might illustrate a poor prognosis with high osteoclast activity, as published by other study (93). The ATF group exhibited upregulation of RANK mRNA expression compared to the TRF group, which showed a downregulated expression. Downregulation of RANK is associated with inhibited osteoclastogenesis and prevention of RANKL-RANK binding activity (93). The potency of tocotrienol when compared to the tocopherol has been justified in the literature and it is postulated that the potency was modulated due to the high antioxidant activity and able to distribute tissue efficiently (16, 94), which makes it the most probable agent for potential effective therapeutics for the management of pathological bone diseases comparable to bisphosphonate. Other than the treatment in bone pathological fracture, bisphosphonate is also capable in reducing the osteoclast activation via inhibition of RANKL-RANK binding (95) which is demonstrated by the downregulated of RANK expression.

Conclusion

In conclusion, this study demonstrates that both vitamin E supplementations showed potential therapeutic effect in pain alleviation activity. Specifically, TRF also capable in regulating bone turnover activity by reducing excessive bone resorption activity and bone formation activity as effective as Zoledronic acid. Gene expression studies also demonstrated that TRF supplementation was more potent in regulating the osteoclastogenesis and preventing excessive bone resorption. It is also interesting to note that TRF supplementation might inhibited the growth of metastatic cancer cells based on this finding. As conclusion, although both vitamin E treatment have potency on pain alleviation activity, TRF showed a superior activity on a molecular level in bone biomarker and bone signaling pathways.

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Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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