ORIGINAL ARTICLE

Effects of Vitamin E on Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL) and Osteoprotegerin (OPG) in Rats Treated With Nicotine

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SUMMARY
Vitamin E is found to reverse the effects of nicotine on bone and this study aimed to determine its mechanism. Male Sprague Dawley rats were divided into four groups and treated for 3 months: Group 1 was the control group (RC). Groups 2 (N), 3 (N+TT) and 4 (N+ATF) received nicotine 7 mg/kg throughout the treatment period. In addition, groups 3 and 4 received tocotrienol 60 mg/kg and α-tocopherol 60 mg/kg respectively during months 2 and 3. Parameters measured were serum osteoprotegerin (OPG), serum receptor activator of nuclear factor kappa B ligand (RANKL), femoral and lumbar bone calcium content and body weight. Nicotine did not affect OPG or RANKL levels but reduced bone calcium content suggesting the calcium loss is not due to increase osteoclastogenesis. OPG was increased in N+ATF while RANKL was slightly increased in N+TT. Both vitamin E supplements restored bone calcium loss induced by nicotine. Nicotine impaired weight gain in all treatment groups starting week 4 however, N+TT group was comparable to RC from week 6 onwards. Bone protective effects of ATF, but not TT, may be partly due to inhibition of osteoclastogenesis.

KEY WORDS:
Nicotine, Vitamin E, Osteoprotegerin, RANKL ligand, Rats

INTRODUCTION
Bone is a specialized connective tissue which undergoes constant remodeling; composed of bone resorption and bone formation processes. It is possible to measure the remodeling processes by means of various biochemical markers.

Osteoprotegerin (OPG) is a protein which protects bone from being degraded. It is also known as osteoclast inhibitory factor (OCIF). It is secreted by osteoblasts and acts as a false receptor for receptor activator of nuclear factor kappa B ligand (RANKL) which leads to prevention of production and activation of osteoclasts. Osteoprotegerin has also been reported to stimulate apoptosis of osteoclasts.

RANKL, on the other hand, is a molecule produced by osteoblasts which has an important role in the differentiation of osteoclasts. Binding of RANKL to its receptor, RANK, stimulates osteoclastogenesis in vitro which requires the presence of macrophage-colony stimulating factor (M-CSF). Alterations in the ratio of RANKL to OPG may lead to several skeletal-related diseases.

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MATERIALS AND METHODS
Animals and treatment
Three-month-old male Sprague-Dawley rats obtained from the Laboratory Animal Resource Unit, Universiti Kebangsaan Malaysia, were randomly divided into four groups of eight rats each: (1) control group (RC), (2) nicotine-treated group at the dose of 7 mg/kg for 3 months (N), (3) group treated with nicotine (7 mg/kg) for 3 months and supplemented with tocotrienol mixture (60 mg/kg) during months two and three (N+TT) and (4) group treated with nicotine (7 mg/kg) for three months supplemented with α-tocopherol (60 mg/kg) during months two and three (N+ATF). Another group of eight rats were sacrificed untreated and acted as baseline control (BC).

The rats were kept four per cage under 12-hour natural light/dark cycles, given tap water ad libitum and weighed every week. All rats received normal rat chow obtained from Gold Coin (Port Klang, Selangor, Malaysia). The nicotine used was in the form of hydrogen tartrate salt which was purchased from Sigma Chemical Co. (St Louis, MO, USA). Alpha-tocopherol acetate was purchased from Sigma Chemical Co. (St Louis, MO, USA) while the Malaysian Palm Oil Board (Bangi, Selangor, Malaysia) supplied the tocotrienol mixture. Blood samples were obtained before the treatment commenced and at the end of the treatment period.

Nicotine has been shown to increase the production of inflammatory mediators and nicotine has been associated with pathogenesis of diseases by promoting proinflammatory mediators. On bone, nicotine induced bone loss and reduced bone mechanical strength in rats. In humans, smoking has been implicated in osteoporosis and is considered one of the risk factors for osteoporosis. Our previous studies showed that nicotine increased bone resorbing cytokines, interleukin 1 and 6 and impaired bone histomorphometrically. Interestingly, vitamin E, a naturally-occurring antioxidant, was able to prevent the increase of bone resorbing cytokines and reverse the damage on bone histomorphometry in nicotine-induced rats.

This study was carried out to further determine the mechanisms of how nicotine damages the bone by measuring OPG and RANKL levels as well as bone calcium content. In addition, the effects of vitamin E (tocotrienol mixture and α-tocopherol) on the above parameters were also determined.
Nicotine (7 mg/kg) was prepared by mixing 0.07 g of nicotine in 10 ml normal saline. The vitamin E solution was prepared by mixing 3 g of the respective vitamin Es in 50 ml olive oil (Bertolli, Secaucus, NJ, USA). A total of 0.1 ml/100 g rat weight of the nicotine and vitamin E preparations were given respectively intraperitoneally and orally via oral gavage, six days a week. The control group received the vehicles i.e. normal saline, intraperitoneally, and olive oil, via oral gavage. Upon sacrifice of the rats, left femur and 4th lumbar vertebra bones were harvested for bone calcium content analysis.

**Bone biochemical markers**

Serum OPG levels were measured using ELISA kit Cat No BI-20602 while serum RANKL levels were determined using ELISA kit Cat No BI-20522. Both kits were purchased from Biomedica Medizinprodukte GmbH Co, Vienna, Austria.

**Bone calcium content**

For the measurement of bone calcium content, briefly, the femur and 4th lumbar bones were subjected to two phases of drying i.e. 100°C for 24 hours and 800°C for 12 hours. The bones were then dissolved in nitric acid and the solutions were diluted with lanthanum chloride before analysis was carried out using flame atomic absorption spectrophotometry (Analyst 100, Perkin ElmerTM Instruments, Wellesley, Massachusetts, USA) at 422.7 nm.

**Analyses of data**

All the data were found to be normally distributed by the Kolmogorov-Smirnov test. The data were then analysed using the one-way analysis of variance (ANOVA) and Tukey’s honestly significant difference test was selected as the post-hoc test. To compare data before and after treatment, the paired student's t-test was used. All the analysis was carried out using the Statistical Package for Social Sciences version 12.0 (SPSS Inc, Chicago, IL, USA) software.

This study was approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) with the approval number FAR/2003/IMA/12JUNE/095.

**RESULTS**

Serum OPG levels did not show any significant difference in nicotine-treated group as compared to the control group (Fig 1). Even though nicotine group showed a trend of lowering the OPG levels, significance level was not reached. Supplementing the rats with vitamin E (tocotrienol mixture and α-tocopherol) increased OPG levels but the values did not differ as compared to RC and N groups. However, OPG level in N+ATF group after the treatment was significantly different than before treatment.

Similar to OPG, RANKL levels were not different when compared between the RC and N groups (Fig 2). However, a trend of increasing RANKL levels could be observed in N group. No changes were observed in the vitamin E supplemented groups as compared to RC and N groups. However, N+TT group had a higher level of RANKL after treatment than before treatment.

Femur bone calcium content was reduced in nicotine treated group as compared to RC group (Fig 3). Supplementation of tocotrienol and tocopherol were able to reverse the effects and bone calcium content was increased back to normal levels and was higher compared to BC group. In addition, femur bone calcium content of the N+ATF was even higher than the BC group. No significant changes were observed in the 4th lumbar bone calcium content amongst the four treatment groups except that all four groups had a higher bone calcium content than the BC group (Fig 3).

The body weight of the rats showed an increasing trend from week 0 to week 12 for all groups (Fig 4). Starting from week 3, the RC group had a higher body weight than N+TT and N+ATF. At week 4, body weight of RC group was significantly higher than the other treatment groups. The trend persisted until the end of the treatment period except for N+TT which had comparable body weight to RC starting from week 6.

**DISCUSSION**

The dose of nicotine used in this study (7 mg/kg) was based on an earlier study i.e. the effective dose in causing an increase in bone resorbing cytokines and reducing bone calcium content. In this study, administration of nicotine for three months did not cause any changes in the OPG levels. We also observed that nicotine did not cause an increase in RANKL levels. OPG and RANKL are indicative of osteoclastogenesis inhibition and stimulation respectively. To the best of our knowledge there is paucity of reports on nicotine and OPG or RANKL. In an in vitro study, administration of nicotine and lipopolysaccharide into osteoblast cell culture caused an initial increase in OPG expression but decreased at later stage of culture.

Despite the lack of changes observed in OPG and RANKL levels, we observed bone calcium loss in the femur of the nicotine-treated group. The findings were consistent with a previous study which showed that nicotine caused a reduction in bone mineral content. However, in this study, fourth lumbar bone calcium content was not affected by nicotine treatment. Even though lumbar bones consisted of trabecular bone and was more prone to change compared to cortical bones, reduction in calcium content of the lumbar bones was not observed in this study which was in contrast to our previous study. This may be due to the fact that lumbar bones are smaller than femur bones, thereby containing less calcium. Therefore smaller changes would be more difficult to detect.

Previously, nicotine was found to stimulate osteoclast differentiation and cause resorption of calcium phosphate. One of our earlier studies had shown that nicotine increased bone resorbing cytokines i.e. interleukin 1 and 6 as well as increasing pyridinoline levels, a marker for bone resorption. The above findings suggested that nicotine induces bone loss via other mechanisms and not through stimulation of osteoclastogenesis.

Supplementation of α-tocopherol to the nicotine-treated rats caused an increase in OPG levels. Since nicotine did not affect OPG levels, the changes observed upon α-tocopherol supplementation may be due to the supplementation itself. Although there has been no report on the effects of vitamin E on OPG levels, other antioxidants have been found to affect OPG levels and its mRNA expression. Increase in OPG levels implied reduction in bone resorption activity which
Fig. 1: Effects of nicotine administration and vitamin E supplementation on serum OPG levels.

Fig. 2: Effects of nicotine administration and vitamin E supplementation on serum RANKL levels.

Fig. 3: Effects of nicotine administration and vitamin E supplementation on femoral and lumbar bone calcium content.

Fig. 4: Effects of nicotine administration and vitamin E supplementation on body weight.

Guidelines to Figures 1-3
BC - baseline control
RC - rat chow
N - nicotine 7 mg/kg body weight
N+TT - nicotine 7 mg/kg body weight (1 month) followed by tocotrienol 60 mg/kg body weight (2 months)
N+ATF - nicotine 7 mg/kg body weight (1 month) followed by \( \alpha \)-tocopherol 60 mg/kg body weight (2 months)

* indicates significant difference to week 0 (p<0.05)
Groups which share the same alphabet indicates significant difference (p<0.05)

Guidelines to Figure 4
BC - baseline control
RC - rat chow
N - nicotine 7 mg/kg body weight
N+TT - nicotine 7 mg/kg body weight (1 month) followed by tocotrienol 60 mg/kg body weight (2 months)
N+ATF - nicotine 7 mg/kg body weight (1 month) followed by \( \alpha \)-tocopherol 60 mg/kg body weight (2 months)
a, b and c indicate significant difference between RC and N, RC and N+ATF and RC and N+TT respectively (p<0.05)
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would then lead to prevention of bone loss, hence the reversal of bone calcium loss seen in this group. This effect was not observed in the N+TT group.

The N+TT group did not show any change in OPG levels. Instead the RANKL levels were higher than before treatment. Again, since nicotine did not affect RANKL levels, we suggest that the tocotrienol mixture itself caused the increase in RANKL levels. To date, no reports have been published on the effects of vitamin E on RANKL. However, one study using α-lipoic acid, an antioxidant, reported an up-regulation of RANKL production by α-lipoic acid27. In that study, the researchers observed that even though RANKL was increased, osteoclastogenesis and bone resorption were reduced which led them to suggest that α-lipoic acid inhibited osteoclastogenesis by inhibiting RANKL signals and not by modulating RANKL production.

Similar arguments may be applied in the present study. Even though we found that RANKL was increased in N+TT group, the group was able to prevent bone calcium loss due to nicotine. We have also shown that tocotrienol, especially the gamma isomer, prevent adverse effects of nicotine in terms of biochemical markers (unpublished data) and histomorphometric parameters28. In other studies, tocotrienol was able to prevent bone loss in orchidectomized rats29 and maintain bone mineral density in ovariectomized rats30. This further suggested that the effects of tocotrienol in reversing nicotine-induced bone loss was not via the modification of OPG or RANKL activities. Other mechanisms might be responsible for the effects of tocotrienol.

In terms of body weight, this study is consistent with previous study which found reduction in body weight of rats after administration of nicotine31. It was suggested that nicotine decreased food intake in rats exposed to nicotine is due to involvement of perifornical hypothalamus through the action of catecholaminergic neurons32. Vitamin E, on the other hand, may play a role in maintaining body mass. Our previous study showed that rats fed with vitamin E deficient diet had a declining body weight as compared to the control rats33. In the present study, we observed that nicotine-treated rats supplemented with tocotrienol mixture were able to reverse the effects of nicotine on weight gain.

In conclusion, nicotine induced bone resorption without affecting OPG or RANKL levels. Nicotine also caused growth retardation. Both tocotrienol and α-tocopherol, via separate mechanisms, were able to restore bone calcium content which was lost due to nicotine treatment. However, tocotrienol supplementation, but not α-tocopherol, improved growth in nicotine-treated rats.

REFERENCES