Nicotine Impaired Bone Histomorphometric Parameters And Bone Remodeling Biomarkers In Sprague-Dawley Male Rats

H Hermizi[1], O Faizah[1], S Ima Nirwana[2], S Ahmad Nazrun[2], DA Luke[3] and M Norazlina*[2]

[1]Department of Anatomy & [2]Department of Pharmacology, Faculty of Medicine, and [3]Department of Clinical Oral Biology, Faculty of Dentistry, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia. Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia.
**Corresponding email: azlina@medic.ukm.my*

ABSTRACT

The effects of nicotine administration on structural and cellular parameters of bone histomorphometry, cotinine, and biomarkers of bone remodeling were studied in twenty-one Sprague-Dawley male rats. Rats aged three months and weighing between 250-300 g were divided into three groups. Group 1 was the baseline group, which was sacrificed without treatment. The other 2 groups were the control group and the nicotine group. The nicotine group was treated with nicotine 7 mg/kg body weight and the C group was treated with normal saline only. Treatment was given by intraperitoneal injection, six days a week for a period of 4 months. Histomorphometric analysis was done on the metaphyseal region of the trabecular bone of the left femur by using an image analyzer. Biochemical analysis was done using ELISA-test kit to compare the serum cotinine, osteocalcin and pyridinoline (PYD) levels between pretreatment and after 4 months treatment in the control and nicotine groups. Histomorphometric analysis revealed that nicotine significantly decreased the trabecular bone volume (BV/TV) and the osteoblast surface (Ob.S/BS), and increased the osteoclast surface (Oc.S/BS) and the eroded surface (ES/BS) compared to the baseline and control groups. In addition, biochemical analysis showed that nicotine treatment for 4 months significantly decreased the osteocalcin (bone formation marker) levels while the cotinine and PYD (bone resorption marker) levels were increased as compared to pretreatment. We concluded that treatment with nicotine 7 mg/kg for 4 months exerted negative a effect on the trabecular bone histomorphometric parameters and bone remodeling biomarkers.

KEYWORDS: nicotine, bone histomorphometry, bone remodeling marker, male rats

INTRODUCTION

Cigarette smoke contains high concentrations of oxidants and free radicals (Church & Pryor 1985, Pryor & Stone 1993) and is well known as one of the exogenous factors of oxidative stress in human populations (Block *et al.,* 2002). Nicotine, one of more than 4700 compounds found in unfiltered mainstream tobacco smoke (Hoffmann *et al.,* 2001) can stimulate angiogenesis and atherosclerosis, promote tumor growth (Heeshen *et al.,* 2001) and osteoporosis (Braulik & Jarab 1993). Approximately 85% of nicotine is rapidly transformed to the main metabolite, cotinine (Benowitz *et al.,* 1983). The half-life period of cotinine is about 18 to 20 hours compared with only 1 to 2 hours for nicotine (Benowitz & Jacob 1993). As a consequence cotinine has become the principal biomarker of nicotine exposure in tobacco research paradigms (Ziegler *et al.,* 2004).

Animal experiments have shown that nicotine induced oxidative stress in both *in vitro* and in vivo (Wetscher *et al.,* 1994, Kalpana & Menon, 2004). Crowley-Weber and coworker (2003) reported that nicotine increased oxidative stress, activated nuclear transcription factor-kB (NF-

kB), induced apoptosis and increased genotoxic stress in the tissues of smokers. NF-kB-signaling pathways are important for bone homeostasis, in particular for osteoclast differentiation (Jimi & Ghosh 2005). Recently, oxidizing agent (Ferric nitrilotriacetate, Fe-NTA) was found to effect bone metabolism by suppressing bone growth and increasing bone resorbing cytokines (interleukin-1, interleukin-6) (Ahmad *et al.,* 2004; Ahmad *et al.,* 2005).

A number of investigations have shown that nicotine might have a direct effect on bone metabolism by influencing the bone remodeling process. Nicotine suppressed cellular proliferation in UMR 106-01 rat osteoblastic osteosarcoma cells (Fang *et al.,* 1991) and suppressed osteogenesis through a decrease in alkaline phosphatase (ALPase) and type I collagen production by osteoblasts (Tanaka *et al.,* 2005). In contrast, nicotine appears to stimulate osteoclasts resorption in a porcine marrow cell model (Henemyre *et al.,* 2003) and increased bone resorption by induction of the bone resorbing cytokine, interleukin-1 (Norazlina *et al.,* 2004). These studies suggested that nicotine was not only a direct inhibitor of osteoblast differentiation but also a regulator of osteoclast activity.

Biochemical markers of bone remodeling can be measured in the serum or urine and reflect the various processes involved in bone metabolism by detecting the activity of osteoblasts and osteoclasts (Weisman & Matkovic, 2005). Osteoblasts synthesise type I collagen, growth factors and non-collagenous proteins. Osteocalcin is a bone specific non-collagenous protein which is widely accepted as an indicator of osteoblast activity (Risteli & Risteli, 1993) and known as a sensitive and specific marker of bone formation (Dogan & Posaci, 2002). Whereas, osteoclasts resorb bone and collagen type I. When collagen is degraded, pyridinolines and telopeptides are released into extracellular fluids and subsequently transported in to blood and urine (Watts, 1999). Pyridinoline (PYD) and deoxypyridinoline (DPD) are collagen crosslinks that are mainly found in bone and known as the most promising markers of bone resorption (Dogan & Posaci, 1993).

Bone histomorphometric analysis has been used extensively to study the morphological changes in cancellous bone. This method allows the study of bone at the tissue or cell level to enable cellular measurements at the intermediary level of bone organization. Besides that, biochemical markers of bone formation and resorption are relatively sensitive indicators of bone turnover but do not reflect the changes in bone mass and enable assessment of bone structure (Compston & Croucher, 1991). A combination of bone histomorphometry and biochemical analysis therefore will provide a powerful tool to assess the changes in the bone morphology and metabolism, and also to evaluate bone cellular activities.

In this present study, nicotine was used to induce oxidative stress in rats and the effects on bone were measured with structural and cellular bone histomorphometric, and serum biochemical markers of bone remodeling analysis. We hope it will provide a better understanding of the adverse effects of nicotine on bone turnover and the negative impact of smoking on bone health.

MATERIALS AND METHODS

Animals and treatment

Twenty-one male Sprague-Dawley rats, about 3 months old and weighing 250-300 gram were obtained from the Animal Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia. The rats were kept two and three per cage under 12 hour natural light-dark cycles. Rats were allowed to acclimatize for one week before the treatment was started, and were given tap water ad libitum and normal rat chow obtained from Gold Coin (Port Klang, Malaysia). The rats were randomly assigned into 3 groups with 7 rats in each group. Group 1 was the baseline group, which was sacrificed without treatment. The other 2 groups were the control group and the nicotine group. The nicotine group was treated with nicotine 7 mg/kg body weight and the control group was treated with normal saline only. Nicotine and normal saline solutions were injected intraperitoneally.

The rats' body weights were measured weekly. Treatment period was 4 months. This study was approved by Universiti Kebangsaan Malaysia Animals Ethics Committee (UKMAEC : FAR/2002/IMA/23-JULY/076).

Nicotine

The nicotine used was nicotine hydrogen tartrate salt, which was purchased from Sigma (St. Louis, USA). Nicotine at a concentration of 7 mg/kg was prepared by mixing 0.07 gram of nicotine in 10 ml normal saline. A total of 0.1 ml/100g rat weight of the preparation was given intraperitoneally 6 days a week for 4 months.

Specimen collection

For biochemical analysis (control and nicotine groups), blood samples were collected before the start of treatment and after 4 months of treatment. Samples were obtained from the retro-orbital vessel after the rat had been anesthetized with diethyl ether. After 3 hours the blood was centrifuged at 1000 rpm for 10 minutes (IEC Centra-EC4R, USA) to obtain the serum and stored in freezer at the temperature of -70°C. For bone histomorphometric analysis (baseline, control and nicotine groups), the rats were anaesthetized with diethyl ether and sacrificed by cervical dislocation. The femurs were removed and the distal parts were kept in 70% ethanol.

Bone histomorphometry

The distal part of the rat femur was fixed with 70% ethanol and cut into 2 fractions for preparing undecalcified and decalcified samples. Undecalcified bone sample were processed and embedded in polymer methyl methacrylate (BDH, Poole, England) medium according to Difford (1974). The polymer block was sectioned at 9 µm thickness using a microtome (Leica RM2155, GmbH, Germany), stained with Von Kossa for structural parameters and Masson Goldner stain for osteoid measurements. The decalcified samples were decalcified with EDTA for 2 months and embedded in paraffin. The paraffin blocks were sectioned at 5 µm thickness with a microtome (Leica RM2135, GmbH, Germany) and stained with hematoxylin and eosin for identification and measurement of osteoblasts and osteoclasts. Nomenclature and symbols used were as described by the American Society of Bone Mineral Research (ASBMR) Histomorphometry Nomenclature Committee (Parfitt *et al.,* 1987).

The parameters of bone histomorphometry were measured using an image analyzer. For structural parameter measurements, analysis was done using VideoTest-Master software (VT, St.-Petersburg, Russia; LB Technology Supp. Sdn. Bhd., Kuala Lumpur, Malaysia). For static parameter measurements, analysis was done using software Pro-Plus 5.0 (Media Cybernatics, Silver Spring, MD, USA) and Weibel technique (Weibel *et al.,* 1966, Freere & Weibel, 1967). All analysis were done randomly at the metaphyseal region, 3 to 7 mm above the growth plate (Baldock *et al.,* 1998).

Biochemical markers of bone remodeling

Serum cotinine, osteocalcin and pyridinolines (PYD) were measured before and after treatment using an enzyme linked immunoassay (ELISA) and enzyme immunoassay (EIA) techniques. Kits used were Cotinine ELISA kit (DRG Instruments, GmbH, Germany), Rat-MID Osteocalcin ELISA kit (Nordic Bioscience Diagnostics A/S, Herlev, Denmark) and METRA Serum PYD EIA kit (Quidel Corporation, San Diego, CA, USA) and were measured using an ELISA

reader (VERSAmax, Sunnyvale, CA, USA).

Statistical analysis

Data analysis was performed using Statistical Package for Social Sciences (SPSS 12.0.1, Chicago, IL, USA) software. Normality test used was the Kolmogorov-Smirnov test. Statistical test used was ANOVA followed by Tukey's hsd for normally distributed data and Kruskal-Wallis and Mann-Whitney test for data that was not normally distributed. Comparison of data between before and after the treatment period for the same group was done using the paired Student's t-test. To compare data from the control group and nicotine group, unpaired Student's t-test was used. For all analyses, differences were considered significant at $P < 0.05$. The results were presented as mean values ± standard error of the mean (SEM).

RESULTS

Effects of nicotine on body weight

We observed a time-dependent gain in weight among both control and nicotine groups. Body weight increased steadily from week-0 to week-6. However, in the middle phase of the study beginning week-7 to week-16, the nicotine group had significantly lower body weight as compared to the control group (Figure 1).

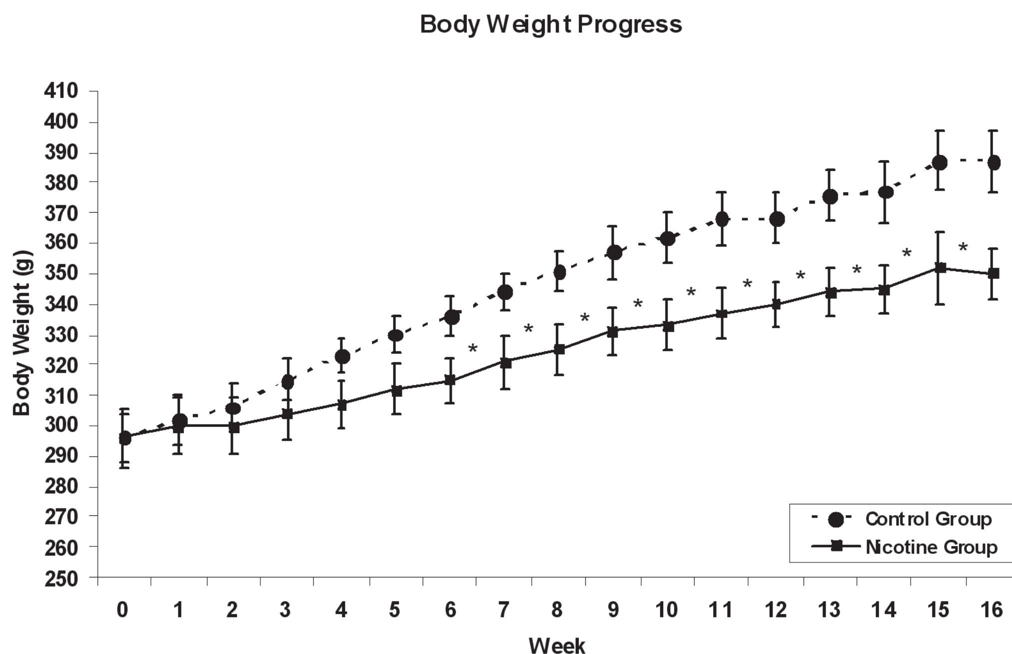

Figure 1. Mean body weight progress of control group and nicotine group (week-0 to week-16).
Control group : Normal saline 0.1 ml/100kg
Nicotine group : Nicotine 7 mg/kg
All values are expressed in Mean ± SEM, *$P < 0.05$ compared with control group

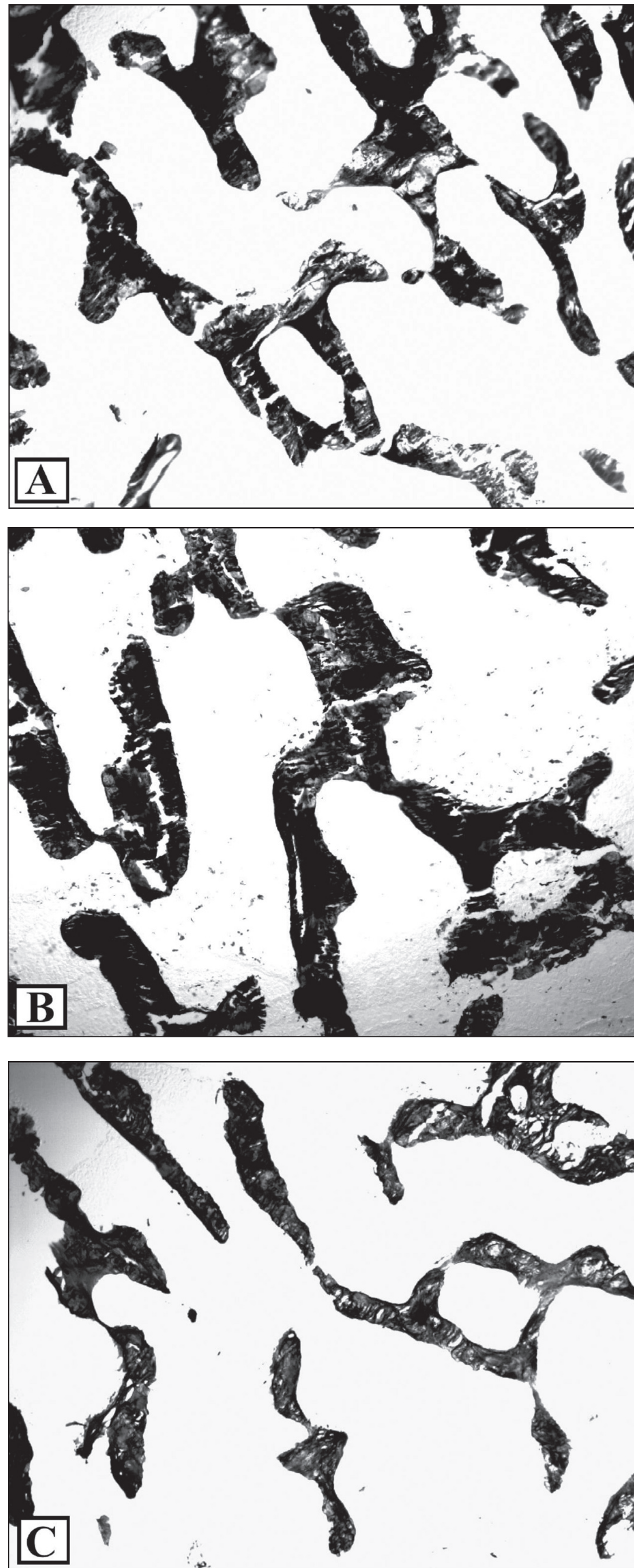

Figure 2. Photomicrographs of trabecular bone. Undecalcified section (100x magnification) shows trabecular bone (black) with white background stain with Von Kossa method in baseline (A), control (B) and nicotine (C) groups.

Bone histomorphometry

After 4 months of treatment, nicotine significantly reduced the BV/TV (24.5 ± 1.13, 30.8 ± 2.00, 31.3 ± 0.98 %, $P < 0.05$, respectively) and Ob.S/BS (43.5 ± 2.57, 56.3 ± 2.19, 57.7 ± 1.71 %, $P < 0.0005$, respectively), while caused an increased in the Oc.S/BS (25.2 ± 2.58, 15.9 ± 2.10, 18.5 ± 4.7 %, $P < 0.005$, respectively) and ES/BS (34.2 ± 0.60 , 23.0 ± 1.61 , 20.7 ± 0.27 %, $P < 0.0005$, respectively) compared with the baseline and control groups (Figures 2 and 5).

Nicotine treatment for 4 months caused a significant reduction in the Tb.N (5.5 ± 0.18, 6.7 ± 0.44, 6.0 ± 0.23 mm-1, $P < 0.05$, respectively) and an increase in the Tb.Sp (142.0 ± 5.72, 113.4 ± 10.05 µm, $P < 0.05$, respectively) compared to the baseline group. Nicotine treatment also decreased the Tb.Th (44.0 ± 1.45, 52.2 ± 1.40 µm, $P < 0.05$, respectively) as compared to the control group (Figures 2 and 5). There were no significant differences in the OS/BS and the OV/BV among all 3 groups (Figure 5). In addition, the control group showed no significant changes in all 9 bone histomorphometric parameters as compared to the baseline group (Figures. 2 and 5).

Biochemical markers of bone remodeling

Serum cotinine levels were significantly increased after 4 months treatment with nicotine as compared to pretreatment (3.2 ± 0.18, 56.1 ± 0.38 ng/ml, $P < 0.05$, respectively). However, no significant changes were observed in the control group between pretreatment and after 4 months treatment with normal saline. After 4 months of treatment, serum cotinine levels in the nicotine group were significantly higher than in the control group that was given normal saline (3.5 ± 0.29, 56.1 ± 0.38 ng/ml, $P < 0.05$, respectively) (Figure 6).

After 4 months of treatment with nicotine, there was a significant decrease in serum osteocalcin levels compared to pretreatment values (134.1 ± 9.75, 220.9 ± 34.71 ng/ml, $P < 0.05$, respectively). However, no significant changes were observed in osteocalcin levels for the control group between pretreatment and after 4 months treatment with normal saline. No significant differences were seen between the two groups (Figure 7).

In both control and nicotine groups, serum pyridinoline levels were increased after 4 months treatments as compared to pretreatment (3.7 ± 0.38, 2.5 ± 0.24; 5.2 ± 0.23, 2.0 ± 0.31 nmol/l, $P < 0.005$, respectively). After 4 months treatment, the serum PYD levels were significantly higher in the nicotine group as compared to control group (5.2 ± 0.23, 3.7 ± 0.38 nmol/l, $P < 0.05$, respectively) (Figure 8).

DISCUSSION

The effectiveness of nicotine (7 mg/kg/day) administration in this present study via intraperitoneal injection was confirmed by high levels of serum cotinine. The nicotine group had a significantly higher concentration of cotinine after 4 months compared the pretreatment levels and to the control group. The rats which were given nicotine had peak serum cotinine levels ranging from 3.32 ng/ml to 56.08 ng/ml, which was almost similar to the concentration of serum cotinine seen in humans who smoke between 10 to 20 cigarettes a day (61.59 ng/ml) using ELISA method (Ziegler *et al.,* 2004). Another study showed that active smokers usually have serum cotinine levels higher than 15 ng/ml (Rebagliato, 2002). Therefore, the 7 mg/kg body weight nicotine treatment used in this study is similar to the degree of smoke exposure experienced by humans who smoke moderately. In addition, the nicotine dose (7mg/kg) chosen in this study was the optimum dose to observe on bone based on our previous studies. Treatment with nicotine 7 mg/kg body weight was found to increase bone resorption by induction of the bone-resorbing cytokine, interleukin-1 (Norazlina *et al.,* 2004) and also reduced calcium content in left femur as compared to 3mg/kg and

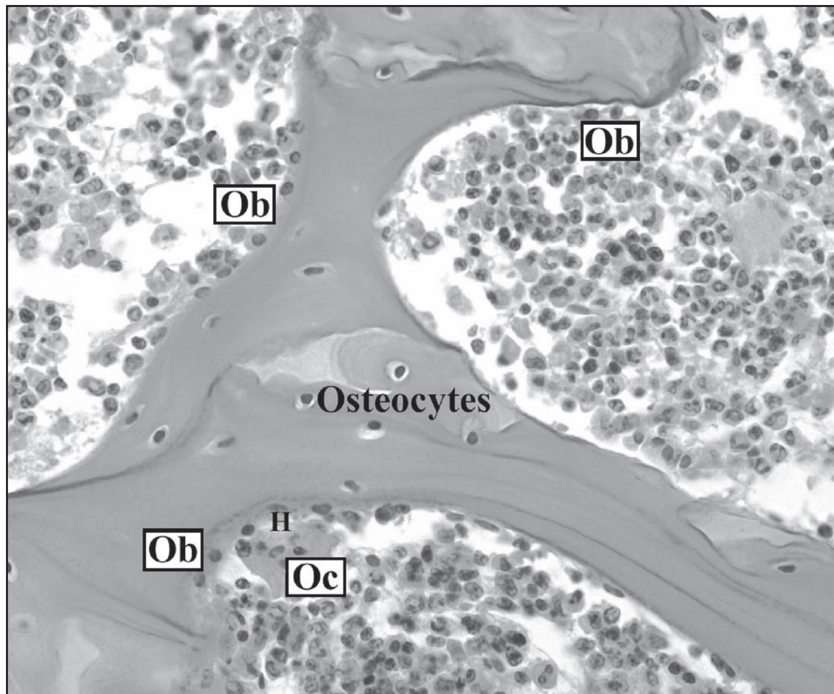

Figure 3. Decalcified section (400x magnification) showing the bone cells; osteoblasts (Ob) and osteoclast (Oc) which lying on the trabecular bone surface. Osteoblasts lay down on the organic components of bone matrix; before mineralization occurs, the organic matrix is known as osteoid. Osteoblasts have abundant basophilic cytoplasm, a large Golgi apparatus and a pale stained nucleus with a prominent nucleolus, they entrapped in the bone and become osteocytes. Multinucleated osteoclast is shown adjacent to the bone tissue which being resorbed or called as Howship's lacunae (H). The aspect of the osteoclast in apposition to the bone is characterized by fine microvilli which form a ruffled border. Hematoxylin & Eosin stain.

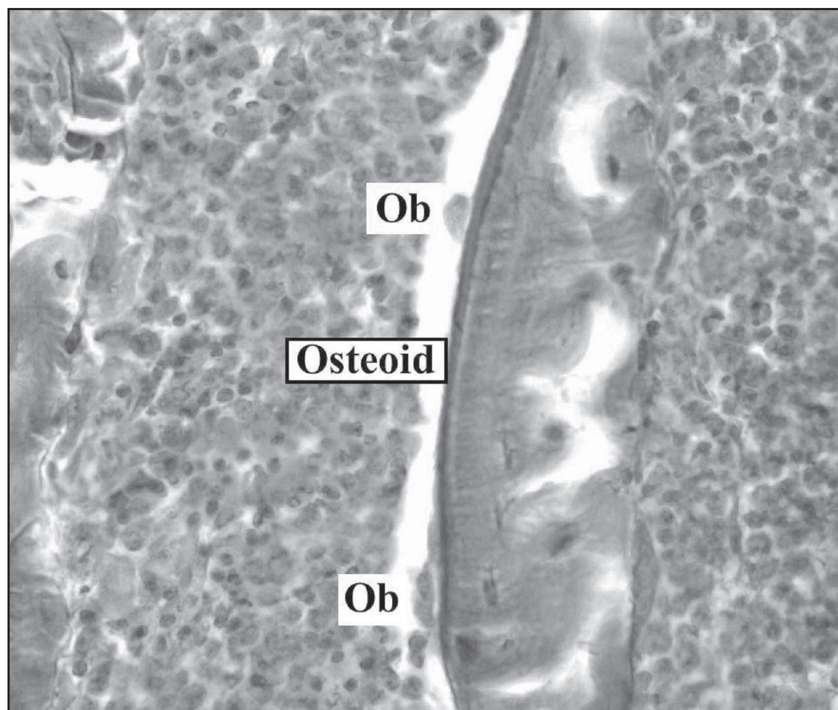

Figure 4. Osteoid clearly demonstrated in undecalccified section which appears as red stained zone between a layer of active osteoblasts (Ob) and the mineralized zone (400 x magnification). Masson Goldner stain.

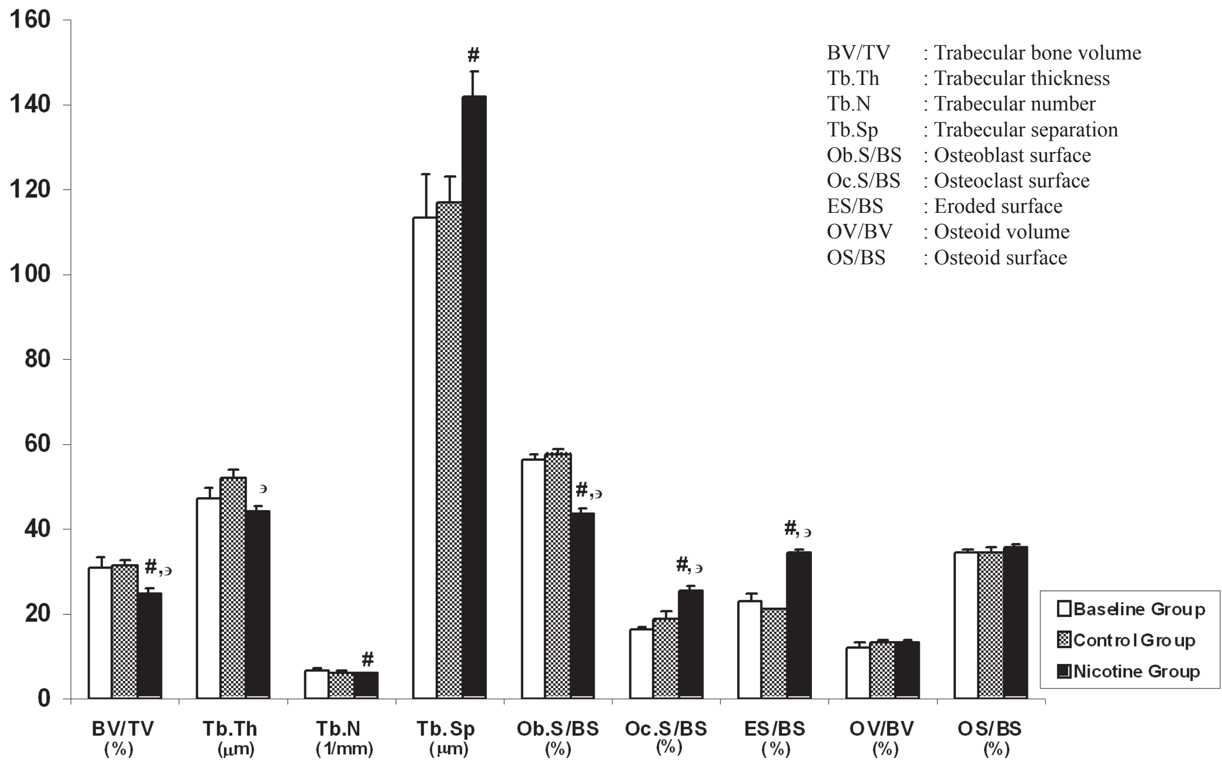

Figure 5. Mean of bone histomorphometric parameters in baseline, control, and nicotine groups.
All values are expressed as mean ± SEM

Baseline group
Control group : Normal saline 0.1 ml/100g + Olive oil 0.1 ml/100g
Nicotine group : Nicotine 7mg/kg + Olive oil 0.1 ml/100kg

* $p < 0.05$ when compared with baseline group
э $p < 0.05$ when compared with control group

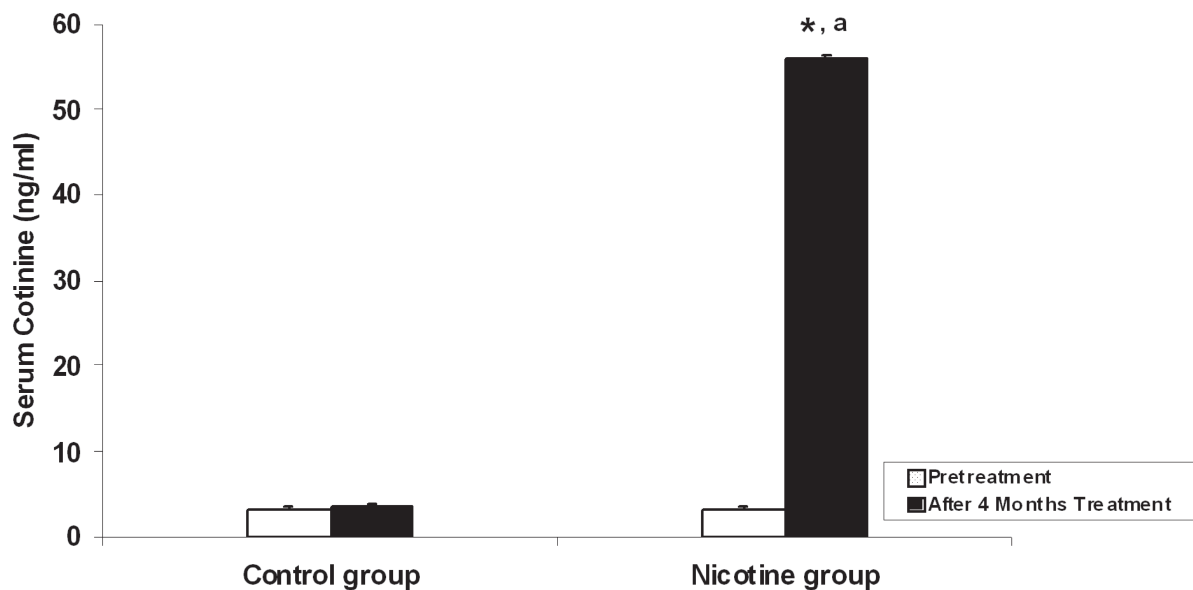

Figure 6. Mean of serum cotinine level before and after treatment in control group and nicotine group.

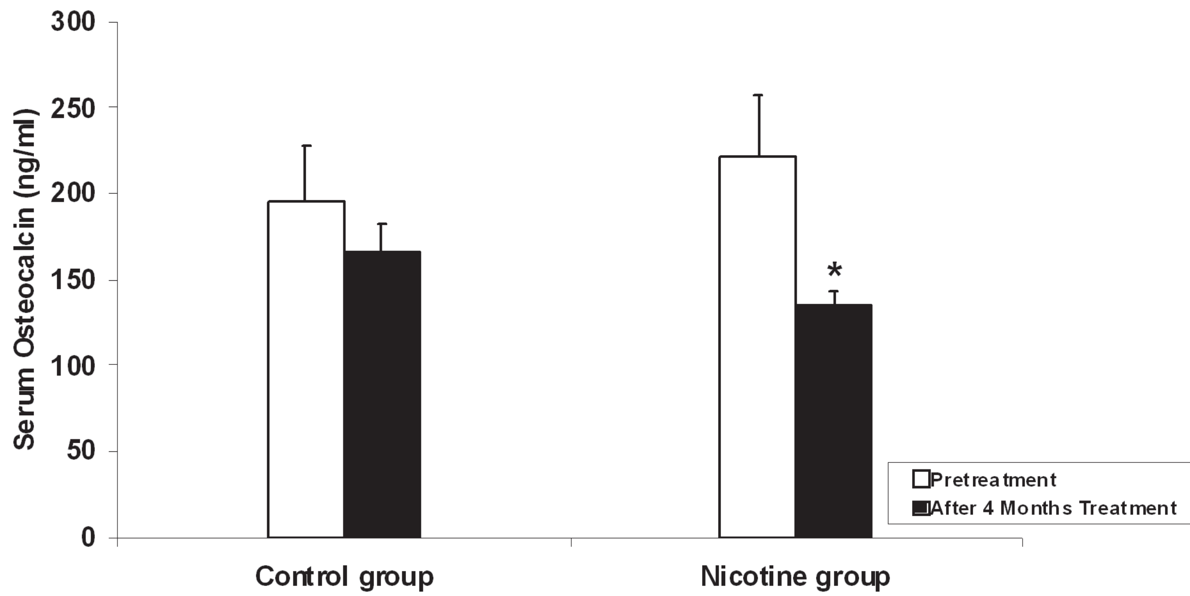

Figure 7. Mean of serum osteocalcin level before and after treatment in control group and nicotine group.

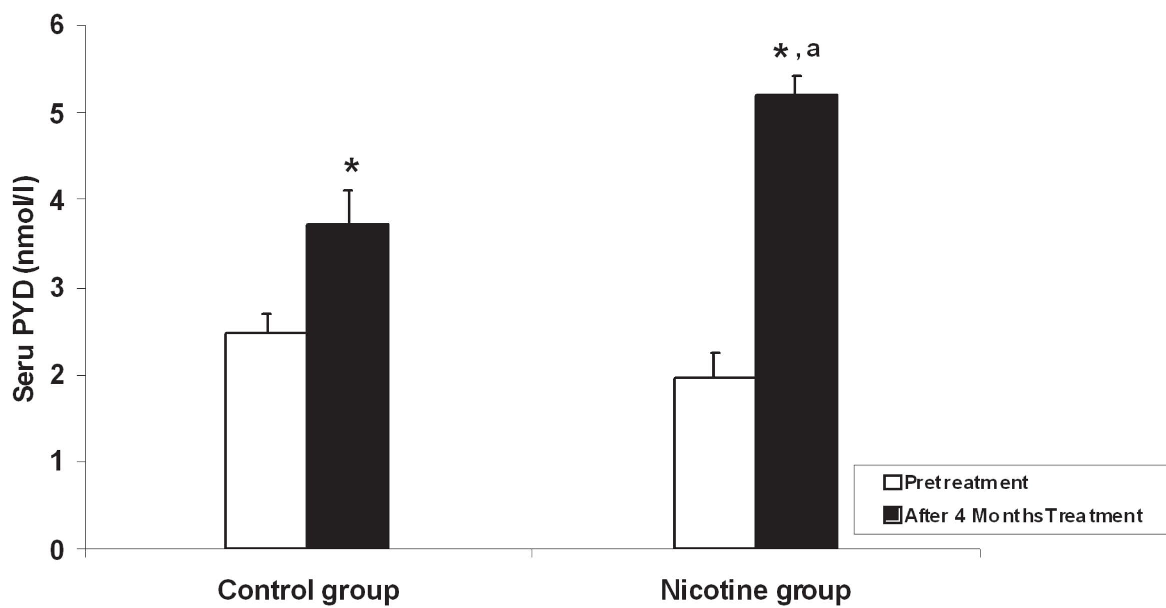

Figure 8. Mean of serum pyridinoline (PYD) level before and after treatment in control group and nicotine group.
Keys to Figures 6-8 :

Control group : Normal saline 0.1 ml/100g + Olive oil 0.1 ml/100g
Nicotine group : Nicotine 7mg/kg + Olive oil 0.1 ml/100kg

All values are expressed as mean ± SEM

[*] $p < 0.05$ compared with pretreatment in similar treatment group
[a] $p < 0.05$ compared with after 4 months treatment in control group

5mg/kg body weight (Ima-Nirwana *et al.,* 2006).

Results of structural bone histomorphometric analysis revealed that nicotine exerted negative effects on structural trabecular bone parameters by significantly reducing the BV/TV by 20% and the Tb.N by 17%, and increasing the Tb.Sp by 25% as compared to the baseline group. The nicotine group also had a 22% lower BV/TV, and a 21% higher Tb.Sp than the control group. The results of the cellular parameters were consistent with the structural parameters whereby nicotine administration significantly caused a reduction in the Ob.S/BS, and an increase in the Oc.S/BS and ES/BS as compared to both BC and C groups, respectively. The Oc.S/BS and Ob.S/BS are reliable histomorphometric indicator of osteoblast and osteoclast numbers (Parfitt *et al.,* 1987). These finding suggest that nicotine caused an increase in bone resorption.

The changes in trabecular bone structure and cellular bone contents in the nicotine group may be due to imbalance in the normal remodeling process. When rats were exposed nicotine, oxidative stress occur which resulted in increased free radical production (Wetscher *et al.,* 1995), and decreased antioxidant levels and antioxidants enzymes (Kalpana & Menon 2004). Nicotine induced osteoclast differentiation by activating NF-kB pathway (Crowley-Weber 2003, Jimi & Ghosh 2005). Nicotine was also shown to stimulate bone resorption (Henemyre *et al.,* 2003). Osteoclasts are activated by free radicals (Garrette *et al.,* 1990) and osteoclast itself can also produce free radicals to resorb bone (Key *et al.,* 1990). This will lead to the formation of many resorption pits or eroded bone surfaces. Decreased osteoblasts number and activity also occurred, which may be due to suppression of osteogenesis and osteoblast function (Tanaka *et al.,* 2005). Thus the rate of bone formation is insufficient to keep up with resorption. Excessive bone resorption can cause deterioration in trabecular bone microarchitecture leading to bone loss. Our findings suggest that the changes in trabecular bone structure in the nicotine group may due to excessive osteoclastogenesis and inadequate osteoblastogenesis.

In this study, the OS/BS and OV/BV were not significantly different in the nicotine-treated group as compared to baseline and control groups. Based on these results, nicotine treatment appeared not to affect the mineralization of the osteoid matrix. Osteoid is the unmineralized organic matrix secreted by osteoblasts and is present in small quantities in normal conditions, and is usually regarded as representing an initial stage in the formation of calcified bone tissue (Raina 1972). Previous human and animal studies demonstrated that osteoid can be found in osteoporotic and normal bone as well as in osteomalacia (Matsushima *et al.,* 2001, Matsushima *et al.,* 2003; Iwaniec *et al.,* 2002). However, the osteoid volume and the osteoid surface were markedly increased in osteomalacia than in normal and osteoporotic bone (Malcom 2002). Thus nicotine appears to cause osteoporosis rather than osteomalacia in the rat bone.

No significant differences were found in all structural and cellular bone histomorphometric parameters between baseline and control groups. These results were consistent with Wronski and coworkers (1989) who showed that bone histomorphometric parameters were expected to be constant between seven and ten months of age in untreated rats, and only decreased to approximately 10% by 540 days (21 months of age). Therefore, many authors (Fung *et al.,* 1998, 1999, Iwaniec *et al.,* 2000, 2001, 2002) did not include the baseline control group in their bone histomorphometric studies.

Although many studies found that nicotine caused adverse effects on bone (Riesenfeld 1985; Ramp *et al.,* 1991; Braulik & Jarab, 1993; Henemyre *et al.,* 2003; Norazlina *et al.,* 2004; Tanaka *et al.,* 2005), but several authors showed that nicotine did not cause any adverse effects on bone density, bone strength, bone turnover or cancellous bone histomorphometry on female rats (Syversen *et al.,* 1999; Fung *et al.,* 1998, 1999; Iwaniec *et al.,* 2000, 2001, 2002; Akhter *et al.,* 2003). This may be due to differences in species, gender, dosage, treatment period and method of nicotine administration. An *in vivo* study by Riesenfeld (1985), reported that female rats tolerated nicotine better than male rats and human study also found that self-administration of nicotine per

se may be less robust in women and women are less sensitive than male to some effects of nicotine (Perkins *et al.,* 1999). Therefore, it may be better to use a male model rather than a female rat model to study effects of nicotine on bone.

Bone formation activity, as measured by serum osteocalcin levels, was significantly decreased by 39% after 4 months treatment with nicotine as compared to pretreatment. This result is similar to the bone histomorphometric analysis which showed a marked reduction in the Ob.S/BS after nicotine treatment. Consistent with this study, earlier research done by de Vernejoul and colleagues (1983) found that smoking caused a detrimental effect on bone formation by impairment of osteoblast activity. Recently, Liu and coworkers (2001; 2003) reported that cigarette smoke may affect bone progenitor cells directly and can interfere with the ability of bone cells to participate in repair and remodeling events. The sequential expression of type I collagen, alkaline phosphatase, osteocalcin and deposition of calcium are known as markers of osteoblastic differentiation (Yuhara *et al.,* 1999) and nicotine administration had also been reported to inhibit collagen synthesis and alkaline phosphatase activity in osteoblast-like cells (Ramp *et al.,* 1991). Thus we suggest that reduction in osteoblast number and activity in the present study was responsible for the decreased production of osteocalcin.

Bone resorption activity as measured by serum pyridinolline (PYD) levels, was significantly increased after 4 months treatment as compared to pretreatment in both the control and nicotine groups. The increase in PYD levels in the control group was expected owing to the increase in bone turnover as the rat matures. In the nicotine group, nicotine treatment caused a 39% higher bone turnover rate (higher PYD levels) as compared to control group after 4 months treatment (5.2 ± 0.23; 3.7 ± 0.38, P = 0.007).

PYD and DPD are the collagen cross-links present in bone and also in nonskeletal tissues such as cartilage, aorta, tendons and ligaments. Urinary excretion of these collagen cross-links have been shown to be closely related to the bone resorption process (Fujimoto *et al.,* 1983). In the case of bone resorption, markers have generally been measured in the urine but this assay have some limitations. Their clinical application is hampered by pronounced analytical and biological variability, and the need for concurrent measurement of urinary creatinine excretion (Allen 2003). In addition, it was reported that PYD is not metabolized or absorbed from the diet (Colwell *et al.,* 1993) and assay of serum PYD is a potential marker for bone resorption (Hatta *et al.,* 1995). Since measurements in serum circumvent some of the limitations of urinary measurement and reflect bone resorption to the same extent as urinary indices (Woitge *et al.,* 1999), their use potentially improves the assessment of skeletal disorder. Thus serum PYD was used in this study as a bone resorption marker.

Body weight did not differ and almost at the same rate between control and nicotine group at the initiation stage of the study (week-0 to week-6). This situation showed that the rats were healthy and growing normally. However, in the middle phase of the study starting week-7 until the end of the study, nicotine-treated rats weighed between 6.7% (week-7) and 9.6% (week-16) lower than control rats. Earlier study by Grunberg and friends (1986) showed that nicotine administration decreased normal body weight gains in female rats. In contrast to the present study, the gender difference may be one of the reasons why no reduction in body weight was seen. Females were found to be more sensitive to the effects of nicotine on body weight as compared to the males (Grunberg *et al.,* 1986, 1987) and female sex hormones may be the reason for this. Faraday and coworkers (2005) stated that it is possible for estrus cycling to affect female responses to nicotine and stress by modifying feeding and corticosterone responses. In the current study, nicotine administration for 4 months did not affect the body weight gain of the rats but it seem to be significantly slower as compared to the control group. More significant differences might be seen if the treatment period is prolonged.

In conclusion, treatment with nicotine 7 mg/kg for 4 months was found to negatively affect

both bone histomorphometric and serum biochemical markers of bone remodeling. This is shown by deterioration in trabecular bone structure, increase in osteoclast number and bone resorption marker, and decrease in osteoblast number and bone formation marker. These effects may be due to increased oxidative stress induced by nicotine on bone.

ACKNOWLEDGEMENTS

This study was funded by the Ministry of Science, Technology and Innovation of Malaysia via research grant: IRPA/UKM 06-02-02-051-EA243. We also thank Mr Rafizul Mohd Yusoff, Mr Arizi Aziz, Mdm Hairi Ghazalli, Mdm Azizah Osman and Mr Faisal Ariffin for their technical assistance.

REFERENCES

Ahmad NS, Khalid BAK, Ima-Nirwana S (2004) Effects of vitamin E on interleukin-1 in ferric nitrilotriacetate treated rats. Malaysia J. Biochem. Biol. 9: 43-47.

Ahmad NS, Khalid BAK, Luke DA, Ima-Nirwana S (2005) Tocotrienol offers better protection than tocotrienol from free radical-induced damage of rat bone. Clin. Exp. Pharmaco. Physiol. 32:761-770.

Akhter MP, Iwaniec UT, Haynatzki GR, Fung YK, Cullen DM, Recker RP (2003) Effects of bone mass and strength in aged female rats. J. Orthop. Res. 21: 14-19.

Allen MJ (2003) Biochemical markers of bone metabolism in animals: uses and limitations. Vet. Clin. Pathol. 32:101-113.

Baldock PA, Morris HA, Need AG, Moore RJ, Durbridge TC (1998) Variation in the short-term changes in bone cell activity in three regions of the distal femur immediately following ovariectomy. J. Bone. Miner. Res. 13:1451-1457.

Benowitz NL, Kuyt F, Jacob P III, Jones RT, Osman AL (1983) Cotinine disposition and effects. Clin. Pharmacol. Ther. 34: 604-611.

Benowitz NL, Jacob P III (1993) Nicotine and cotinine elimination pharmacokinetics in smokers and nonsmokers. Clin. Pharmacol. Ther. 53:316-323.

Block G, Dietrich M, Norkus EP, Morrow JD, Hudes M, Caan B, Packer L (2002) Factors associated with oxidative stress in human populations. Am. J. Epidemiol. 156: 274-285.

Braulik PD, Jarab J (1993) The effect of chronic nicotine administration on bone mineral content in mice. Horm. Metab. Res. 25: 219-221.

Church DF, Pryor WA (1985) Free-radical chemistry of cigarette smoke and its toxicological implications. Environ. Health Perspect. 64: 111-126.

Colwell A, Russell RGG, Eastell R (1993) Factors affecting the assay of urinary 3-hydroxy pyridium crosslinks of collagen as markers of bone resorption. Eur. J. Clin. Investig. 23:341-349.

Compston JE, Croucher PI (1991) Histomorphometric analysis of trabecular bone remodelling in osteoporosis. Bone Miner. 14: 91-102.

Crowley-Weber CL, Dvorakova K, Crowley C, Berstein H, Berstein C, Garewal H, Payne CM (2003) Nicotine increases oxidative stress, activates NF-κB and GRP78, induces apoptosis and sensitizes cells to genotoxic/xenobiotic stresses by a multiple stress inducer, deoxycholate: relevance to colon carcinogenesis. Chemico-Biol. Interac. 145: 53-66.

De Vernejoul MC, Bielakoff J, Herve M, Gueris J, Hott M, Modrowski D, Kuntz D, Miravet L, Ryckewaert A (1983) Evidence for defective osteoblastic function. A role for alcohol and tobacco consumption in osteoporosis in middle-aged men. Clin. Orthop. Relat. Res. 179: 107-115.

Difford J (1974) A simplified method for the preparation of methyl methacrylate embedding

medium. Med. Lab. Tech. 31: 79-81.

Dogan E, Posaci C (2002) Monitoring hormone replacement therapy by biochemical markers of bone metabolism in menopausal women. Postgrad. Med. J. 78: 727-731.

Fang MA, Frost PJ, Iida-Klein A, Hahn TJ (1991) Effects of nicotine on cellular function in UMR 106-01 osteoblast-like cells. Bone. 12: 283-286.

Faraday MM, Blakeman KH, Grunberg NE (2005) Strain and sex alter effects of stress and nicotine on feeding, body weight, and HPA axis hormones. Pharmacol. Biochem. Behavior. 80: 577-589.

Freere RH, Weibel ER (1967) Stereologic techniques in microscopy. J. Royal Microsc. Soc. 87: 25-34.

Fujimoto D, Suzuki M, Uchiyama A, Miyamoto S, Inoue T (1983) Analysis of pyridinoline, a crosslinking compound of collagen fibers, in human urine. J. Biochem. 94: 1133-1136.

Fung YK, Mendlik MG, Haven MC, Akhter MP, Kimmel DB (1998) Short-term effects of nicotine on bone and calciotropic hormones in adult female rats. Pharmacol. Toxicol. 82: 243-249.

Fung YK, Iwaniec UT, Cullen DM, Akhter MP, Haven MC, Timmins P (1999) Long-term effects of nicotine on bone and calciotropic hormones in adult female rats. Pharmacol. Toxicol. 85: 181-187.

Garrett IR, Boyce BF, Oreffo ROC, Bonewald L, Poser J, Mundy GR (1990) Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone *in vitro* and in vivo. J. Clin. Invest. 85: 632-639.

Grunberg NE, Bowen DJ, Winders SE (1986) Effects of nicotine on body weight and food consumption in female rats. Psychopharmacol. (Berl). 90: 101-105.

Grunberg NE, Winders SE, Popp KA (1987) Sex differences in nicotine's effects on consummatory behavior and body weight in rats. Psychopharmacology (Berl). 91: 221-225.

Hata K, Miura M, Fukumoto S, Matsumoto T (1995) Assay of serum pyridinoline: a potential marker for bone resorption. Clinica Chimica Acta. 235: 221-227.

Heeshen C, Jang JJ, Weis M, Pathak A, Kaji S, Hu RS, Tsao PS, Johnson FL, Cooke JP (2001) Nicotine stimulates angiogenesis and promotes tumour growth and atherosclerosis. Nat. Med.7: 833-839.

Henemyre CL, Scales DK, Hokett SD, Cuenin MF, Peacock ME, Parker MH, Brewer PD, Chuang AH (2003) Nicotine stimulates osteoclast resorption in a porcine marrow cell model. J. Periodontol. 74: 1440-1446.

Hoffmann D, Hoffmann I El-Bayoumy K (2001) The Less Harmfull Cigarette: A controversial Issue. A Tribute to Ernst L. Wynder. Chem. Res. Toxicol. 14: 767-790.

Ima-Nirwana S, Cheng CT, Norazlina M (2006) Effects of nicotine on bone mineral density and calcium homeostasis in male Sprague-Dawley rats. Current Topic Pharmacol. (in press).

Iwaniec UT, Fung YK, Cullen DM, Akhter MP, Schmid M (2000) Effects of nicotine on bone and calciotropic hormones in growing female rats. Calcif. Tissue Int. 67: 68-74.

Iwaniec UT, Fung YK, Akhter MP, Haven MC, Nespor S, Haynatzki GR, Cullen DM (2001) Effects of nicotine on bone mass, turnover and strength in adult female rat. Calcif. Tissue Int. 68:358-364.

Iwaniec UT, Haynatzki GR, Fung YK, Akhter MP, Haven MC, Cullen DM (2002) Effects of nicotine on bone and calcified hormones in aged ovariectomized rats. J. Musculoskel. Neuron Interact. 2: 469-478.

Jimi E, Ghosh S (2005) Role of nuclear factor-kappaB in the immune system and bone. Immunol. Rev. 208: 80-87.

Kalpana C, Menon VP (2004) Protective effect of circumin on circulatory lipid peroxidation and antioxidant status during nicotine-induced toxicity. Toxicol. Mech. Methods. 14: 339-343.

Key LL, Ries WL, Taylor RG, Hays BD, Pitzer BL (1990) Oxygen derived free radicals in

osteoclasts: The location and specificity of nitroblue tetrazolium reaction. Bone. 11:115-119.

Liu XD, Zhu YK, Umino T, Spurzem JR, Romberger DJ, Wang EF, Reed E, Rennard SI (2001) Cigarette smoke inhibits osteogenic differentiation and proliferation of human osteoperogenitor cells in monolayer and three-dimensional collagen gel culture. J. Lab. Clin. Med. 137: 208-219.

Liu XD, Kohyama T, Kobayashi T, Abe S, Kim HJ, Reed EC, Rennard SI (2003) Cigarette smoke extract inhibits chemotaxis and collagen gel contraction mediated by human bone marrow osteoperogenitor cells and osteoblast-like cells. Osteoporos. Int. 14: 235-242.

Malcom AJ (2002) Metabolic bone disease. Cur. Diag. Patho. 8: 19-25.

Matsushima S, Hoshimoto M, Torii M, Ozaki K, Narama I (2001) Iron lactate-induced osteopenia in male Sprague-Dawley rats. Toxicol. Pathol. 29: 623-629

Matsushima S, Torii M, Ozaki K, Narama I (2003) Iron lactate-induced osteomalacia in association with osteoblast dynamics. Toxicol. Pathol. 31: 646-654.

Norazlina M, Nik-Farideh YMK, Arizi A, Faisal A, Ima-Nirwana S (2004) Effects of nicotine on bone resorbing cytokines in male rats. Int. Med. J. 3(2). www.e-imj.com/Vol3-No2/Vol-3-No2-B10htm.

Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR (1987) Bone histomorphometry: standardization of nomenclature, symbols and units. Report of the ASMBR histomorphometry nomenclature committee. J. Bone. Miner. Res. 2: 595-610.

Perkins KA, Donny E, Caggiula AR (1993) Sex differences in nicotine effects and self-administration: Review of human and animal evidence. Nicotine Tobacco Res. 1: 302-315.

Pryor WA, Stone K (1993) Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. Ann. N.Y. Acad. Sci. 28:12-27.

Raina V (1972) Normal osteoid tissue. J. Clin. Path. 25: 229-232.

Ramp WK, Lenz LG, Galvin RJ (1991) Nicotine inhibits collagen synthesis and alkaline phosphatase activity but stimulates DNA synthesis in osteoblast-like cells. Proc. Soc. Exp. Biol. Med. 197: 36-43.

Rebagliato M (2002) Validation of self reported smoking. J Epidemiol. Community Health. 56: 163-164.

Riesenfeld A (1985) Growth-depressing effects of alcohol and nicotine in two strains of rats. Acta. Anat. 122: 18-24.

Risteli L, Risteli J (1993) Biochemical markers of bone metabolism. Ann. Med. 25: 385-393.

Syversen U, Nordsletten L, Falch JA, Madsen JE, Nilsen OG, Waldum HL (1999) Effects of lifelong nicotine inhalation on bone mass and mechanical properties in female rats femurs. Calcif. Tissue Int. 65: 246-249.

Tanaka H, Tanabe N, Suzuki N, Shoji M, Torigoe H, Sugaya A, Motohashi M, Maeno M (2005) Nicotine affects mineralized nodule formation by the human osteosarcoma cell line Saos-2. Life Sciences. 77: 2273-2284

Watts NB (1999) Clinical utility of biochemical markers of bone remodeling. Clin. Chem. 45B: 1359-1368.

Weibel ER, Kistler GS, Scherle WF (1996) Practical stereological methods for morphometric cytology. J. Cell Biol. 30: 23-38.

Weisman SM, Matkovic V (2005) Potential use of biochemical markers of bone turn over for assessing the effect of calcium supplementation and predicting fracture risk. Clin. Therap. 27: 299-308.

Wetscher GJ, Bagchi M, Bagchi D, Perdikis G, Hinder PR, Glaser K, Hinder RA (1995) Free radical production in nicotine treated pancreatic tissue. Free Radic. Biol. Med.18: 877-882.

Woitge HW, Pcherstorfer M, Li Y, Keck AV, Horn E, Ziegler R, Seibel MJ (1999) Novel serum markers of bone resorption: clinical assessment and comparison with established urinary indices. J. Bone Miner. Res. 14: 792-801.

Wronski TJ, Dann LM, Scott KS, Cintron M (1989) Long-term effects of ovariectomy and aging on the rat skeleton. Calcif. Tissue Int. 45: 360-366.

Yuhara S, Kasagi S, Inoue E, Otsuka E, Hirose S, Hagiwara H (1999) Effects of nicotine on cultured cells suggest that it can influence the formation and resorption of bone. Eur. J. Pharmacol. 383: 387-393.

Ziegler UE, Kauczok J, Dietz UA, Reith HB, Schmidt K (2004) Clinical correlation between the consumption of nicotine and cotinine concentrations in urine and serum by competitive enzyme-linked immunosorbent assay. Pharmacology. 72: 254-259.