The Effects of Repeatedly Heated Frying Oil and High Cholesterol Diet on the Bone in Ovariectomised Rats

Nazrun AS[1], Chew CM[2], Norazlina M[1], Kamsiah J[1] & Ima Nirwana S[1]

[1] Department of Phamacology, Faculty of Medicine UKM, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia;
[2] Department of Biomedical Sciences, Faculty of Allied Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia

ABSTRACT

The use of repeatedly heated frying oils and intake of high cholesterol diet have been linked to bone damage. The aim of this study is to determine the combined effects of taking repeatedly heated frying oils (palm or soy oil) and high cholesterol diet on the dynamic histomorphometric parameters of bone. Ovariectomised rats were used as animal model of post-menopausal osteoporosis. After six months of treatment, Double-labeled Surface (dLS/BS), Mineralising surface (MS/BS) and Bone Formation Rate (BFR/BS) of ovariectomised rats (OvxC) were significantly reduced compared to the normal control group. Additions of fresh or once-heated palm or soy oil into high cholesterol diet seem to have improved the dynamic parameters towards the normal control values. However, when these oils were repeatedly heated, the protective effects were lost and the dynamic parameters except MS/BS dropped back towards the ovariectomised-control values.

INTRODUCTION

Vegetable oil is widely used for frying foods in households or food industries. The commonly used frying oils in the world are palm oil and soy oil (Oil World, 2002). Normally, frying oils are used repeatedly to fry foods to save cost until they have changed colour, smell, taste or consistency. During the process of frying, lipids especially polyunsaturated fatty acids (PUFA) undergo oxidation, hydrolysis and polymerisation which lead to generations of volatile and non-volatile degradation products (Cuesta, Sánchez-Muńiz & Varela, 1998; Dobarganes, Marquez-Ruiz & Velasco, 2000). The non-volatile products of degradation consisting of polymers and polar compounds remain in the oil (Dobarganes, Marquez-Ruiz & Velasco, 2000). There would also be an increase in the production of free radicals and polar compounds and compositional changes of free fatty acids content. Heated vegetable oils were found to have higher content of free radicals compared to fresh vegetable oils (Corocos et al., 1990). Approximately one-third of the dry weight of a deep-fried food can be made up of oil absorbed during the frying process (Mekhta & Swinburn, 2001). Therefore, the oil along with its degrada-

Correspondence author: Professor Dr Ima Nirwana Soelaiman, email address: imasoel@medic.ukm.my

tion products can enter the systemic circulation when the fried foods are consumed (Grootveld *et al.*, 1998). Ingestion of these degradation products has been linked to pathophysiological effects associated with oxidative stress (Estebauer, Schaur & Zollner, 1991). It has been linked to an increased risk of hypertension (Soriguer *et al.*, 2003), disturbance of the endothelial function (Williams *et al.*, 1999) and increased lipoprotein oxidation (Sutherland *et al.*, 2002).

It is suggested that consuming foods fried with repeatedly heated frying oil may affect bone metabolism as the oxidative stress condition may activate osteoclastic bone resorption *in vivo* and *in vitro* (Garret *et al.,* 1990). Oxidative stress has been shown to be related to osteoporosis. Yee and Ima-Nirwana (1998) showed that ferric nitrilotriacetate, an oxidising agent, reduced bone calcium content in young growing rats. Oral vitamin E given to these animals was able to protect them against the effects of ferric nitriloacetate. Mody *et al.* (2001) and Bai *et al.* (2004) showed that oxidative stress inhibits osteoblast differentiation.

The findings of this study may have implications for post-menopausal women. Post-menopausal women are at risk of hypercholesterolaemia as the protective effects of oestrogen on the lipid profile are lost with the onset of menopause (Folsom, McGoven & Nabulsi, 1996). Excess cholesterol has been linked not only to cardiovascular diseases, but studies have also suggested its implications in bone metabolism. Cholesterol is required for the differentiation of osteoclast, the cell responsible for bone resorption (Luegmayr *et al.*, 2004). Cholesterol may also stimulate the release of interleukin-1, a cytokine that promotes osteoclastic activity but inhibits osteoblastic activity (Sjogren *et al.*, 2002).

Palm oil and palm-oil derived vitamin E were shown to protect bones against osteoporosis in orchidectomised male rats (Ima-Nirwana *et al.*, 2000). The vitamin E content of palm oil mainly consists of tocotrienols, while the main vitamin E in soy oil is tocopherols (Goh, Choo & Ong, 1985). Tocotrienols have better antioxidant capacity than tocopherols (Serbinova *et al.*, 1991, Kamat *et al.*, 1997) and this may contribute to the better resistance to oxidative changes. Previous studies have also shown that palm oil-derived tocotrienols were comparable to α-tocopherol in protecting rats against osteoporosis due to ovariectomy (Norazlina *et al.,* 2000). Palm oil-derived vitamin E was also able to improve bone metabolism and survival rate of thyrotoxic rats (Ima-Nirwana *et al.*, 1999).

This study was aimed at examining the effects of feeding repeatedly heated frying oils (soy oil and palm oil) and high cholesterol diet to ovariectomised rats before measuring the bone histomorphometric parameters. Ovariectomised rat is an accepted model for postmenopausal bone loss (Kalu, 1991; Wroski & Yen, 1991).

MATERIALS AND METHODS

Groups of animals

Sixty-four female Sprague-Dawley rats aged 3 months old (200-250 g) were obtained from the University Animal House. The rats were divided randomly into 8 groups with 8 rats in each group. Rats in the first group were not ovariectomised and served as the normal control group (NC). Rats in the second group were ovariectomised and given high cholesterol diet (OvxC), while animals in the rest of the groups were ovariectomised and given high cholesterol diet with fresh soy oil (SOF), fresh palm oil (PO), oils heated once (SO1, PO1) or oils heated five times (SO5, PO5). The rats were treated daily for 6 months. This study was approved by the University Research and Animal Ethics Committee.

Ovariectomy procedure

All the rats except the control group were ovariectomised after being anaesthesised with intraperitoneal injection of ketamine hydrochloride and xylazine at doses of 50 and 10 mg/kg body weight respectively. Bilateral ovariectomies were performed from a dorsal approach. There was no sham-operated group as the treatment period was long that the acute surgical stress would not affect the parameters measured. The rat bones were fluorescence-labeled by injecting the rats twice with calcein at nine and two days before sacrifice. At the end of the study, the rats were anaesthetised with ether and killed by cervical dislocation before the femurs were harvested. Success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of the uterine horns. Food intakes were measured daily while body weights were measured weekly.

Method of oil heating

The soy oil (VeSoya, Yee Lee Edible Oil Industries, Perak, Malaysia) or palm oil (Buruh, Lam Soon Industries, Selangor, Malaysia) were not heated (SOF, POF) or heated according to modified methods of Owu *et al*. (1998). Briefly, the oils were heated in a stainless steel pan for 10 minutes at 180°C. The oils were left to cool for five hours. This would be the once-heated soy oil or palm oil (SO1, PO1). In order to prepare oils that were heated five times, the procedure was repeated four more times. Fresh or heated (once or five times) oils were mixed with high cholesterol diet (rat chow with 2% cholesterol) at a weight ratio of 15:100 and were fed to the rats daily for six months. The oil-to-high cholesterol diet ratio represents the average amount of daily oil intake in humans (Owu, Osim & Ebong, 1998).

Bone preparation

The distal portion of the femur samples were embedded in methyl methacrylate according to Difford (1974) and sectioned at 10 mm with a microtome (Leica, Wetzlar, Germany). Fluorescence microscope and an image analyser (Nikon Eclipse 80i, Japan) were used to measure the dynamic histomorphometric parameter at the metaphyseal region, which is located 3 to 7 mm from the lowest point of the growth plate and 1 mm from the lateral cortex. This secondary spongiosa area is rich in trabecular bone.

The dynamic parameters measured were double-labeled surface (dLS/BS, %), mineralising surface (MS/BS, %) and bone formation rate (BFR/BS, $mm^3/mm^2/$day). All the parameters were measured according to the American Society of Bone Mineral Research Histomorphometry Nomenclature Committee 1987 (Parfitt *et al.*, 1987).

Data analysis

The results were expressed as mean values ± SD. Data analysis was performed using SPSS for Windows software (SPSS Inc., version 12.0.1). Statistical test used was ANOVA followed by Tukey's HSD (Honestly Significantly Different) for normally distributed data and Kruskal-Wallis and Mann-Whitney test for data that is not normally distributed. Changes were considered significant for *p* values less than 0.05.

RESULTS

Food intake and body weights

There was no significant difference of food intake or body weight between the groups throughout the study (p>0.05) (Table 1).

Table 1. Body weight and food intake for all the study groups

Groups	Initial body weight (g)	Final body weight (g)	Intake per rat (g/day)
NC	222.50 ± 12.61	301.50 ± 32.91	13.01 ± 1.70
OVXC	211.17 ± 7.12	324.67 ± 12.06	13.69 ± 1.24
POF	209.17 ± 14.56	333.50 ± 25.61	12.10 ± 0.35
PO1	213.50 ± 6.86	334.33 ± 19.85	11.80 ± 0.36
PO5	211.17 ± 11.23	339.17 ± 34.10	11.90 ± 0.98
SOF	225.33 ± 16.21	318.50 ± 46.80	11.07 ± 0.23
SO1	214.33 ± 16.60	305.00 ± 16.04	11.29 ± 0.62
SO5	210.33 ± 8.04	332.50 ± 31.35	12.29 ± 1.13

Keys:

NC   : Normal control
OvxC : Ovariectomy control + high cholesterol diet
SOF : Ovariectomy + high cholesterol diet + fresh soy oil
SO1 : Ovariectomy + high cholesterol diet + soy oil heated once
SO5 : Ovariectomy + high cholesterol diet + soy oil heated 5 times
POF : Ovariectomy + high cholesterol diet + fresh palm oil
PO1 : Ovariectomy + high cholesterol diet + palm oil heated once
PO5 : Ovariectomy + high cholesterol diet + palm oil heated 5 times

Values are expressed as mean ± SD

Bone histomorphometry

After six months, as expected, double-labeled surface (dLS/BS), mineralising surface (MS/BS) and bone formation rate (BFR/BS) of ovariectomied rats fed with high cholesterol diet (OvxC) were significantly reduced compared to NC group (Figure 1, Figure 2, Figure 3). SOF, POF, SO1 and PO1 groups have higher dLS/BS compared to OvxC group. However, the dLS/BS of SOF and SO1 groups were lower than the NC group. As for the SO5 and PO5 groups, the dLS/BS was not different from the OvxC group. Therefore, additions of fresh or once-heated soy oil only partially reversed but fresh or once-heated palm oil completely reversed the effects of ovariectomy and high cholesterol diet on dLS/BS. Additions of five-times-heated palm or soy oil have no effect on dLS/BS of the ovariectomised rats.

All the groups have higher MS/BS compared to OvxC group but lower MS/BS compared to NC group. This means that addition of fresh, once-heated or five-times-heated palm or soy oil was able to partially reverse the effects of ovariectomy and high cholesterol diet on MS/BS.

The SOF, POF, SO1 and PO1 groups have higher BFR/BS compared to OvxC group but have lower BFR/BS compared to NC group. The BFR parameter for SO5 and PO5 groups was not different from the OvxC group. Therefore, additions of fresh or once-heated palm or soy oil seem to have partially reversed the effects of ovariectomy and high cholesterol diet on BFR/BS. Additions of five-times-heated palm or soy oil to high cholesterol diet have no effect on BFR/BS of the ovariectomised rat.
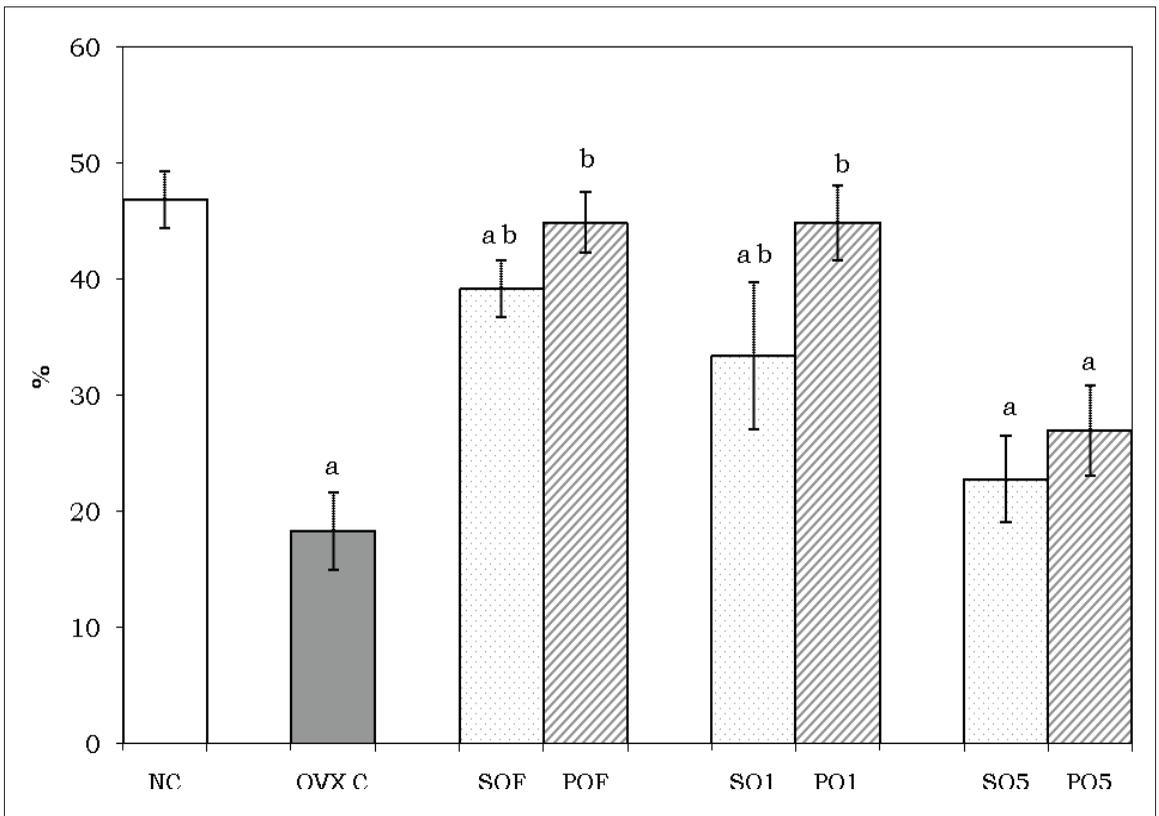
Figure 1. Effects of heated oils and high cholesterol diet on double-labelled surface

a = significantly different to NC
b = significantly different to OvxC (p<0.05)

Keys:

NC   : Normal control
OvxC   : Ovariectomy control + high cholesterol diet
SOF   : Ovariectomy + high cholesterol diet + fresh soy oil
SO1   : Ovariectomy + high cholesterol diet + soy oil heated once
SO5   : Ovariectomy + high cholesterol diet + soy oil heated 5 times
POF   : Ovariectomy + high cholesterol diet + fresh palm oil
PO1   : Ovariectomy + high cholesterol diet + palm oil heated once
PO5   : Ovariectomy + high cholesterol diet + palm oil heated 5 times

Values are expressed as mean ± SD. p<0.05 is considered to be significance.
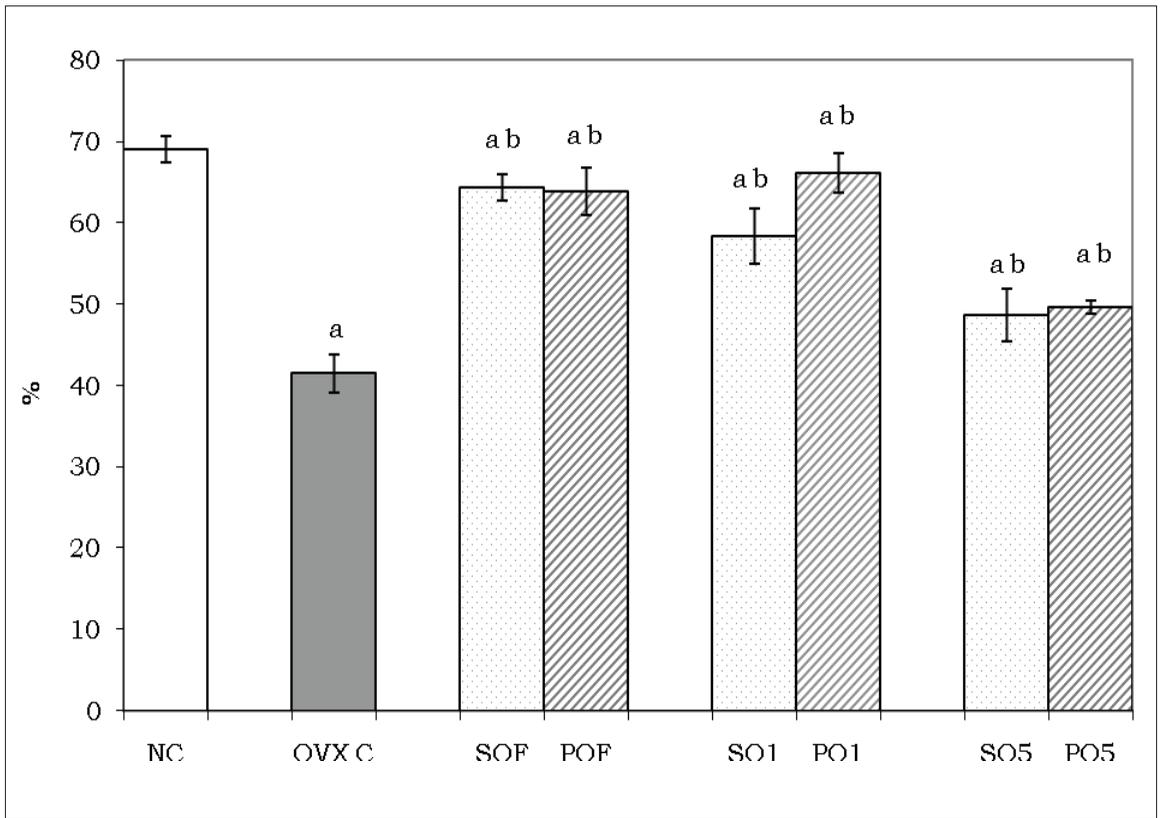
Figure 2. Effects of heated oils and high cholesterol diet on mineralising surface

a = significantly different to NC
b = significantly different to OvxC ($p < 0.05$)

Keys:

NC   : Normal control
OvxC : Ovariectomy control + high cholesterol diet
SOF   : Ovariectomy + high cholesterol diet + fresh soy oil
SO1   : Ovariectomy + high cholesterol diet + soy oil heated once
SO5   : Ovariectomy + high cholesterol diet + soy oil heated 5 times
POF   : Ovariectomy + high cholesterol diet + fresh palm oil
PO1   : Ovariectomy + high cholesterol diet + palm oil heated once
PO5   : Ovariectomy + high cholesterol diet + palm oil heated 5 times

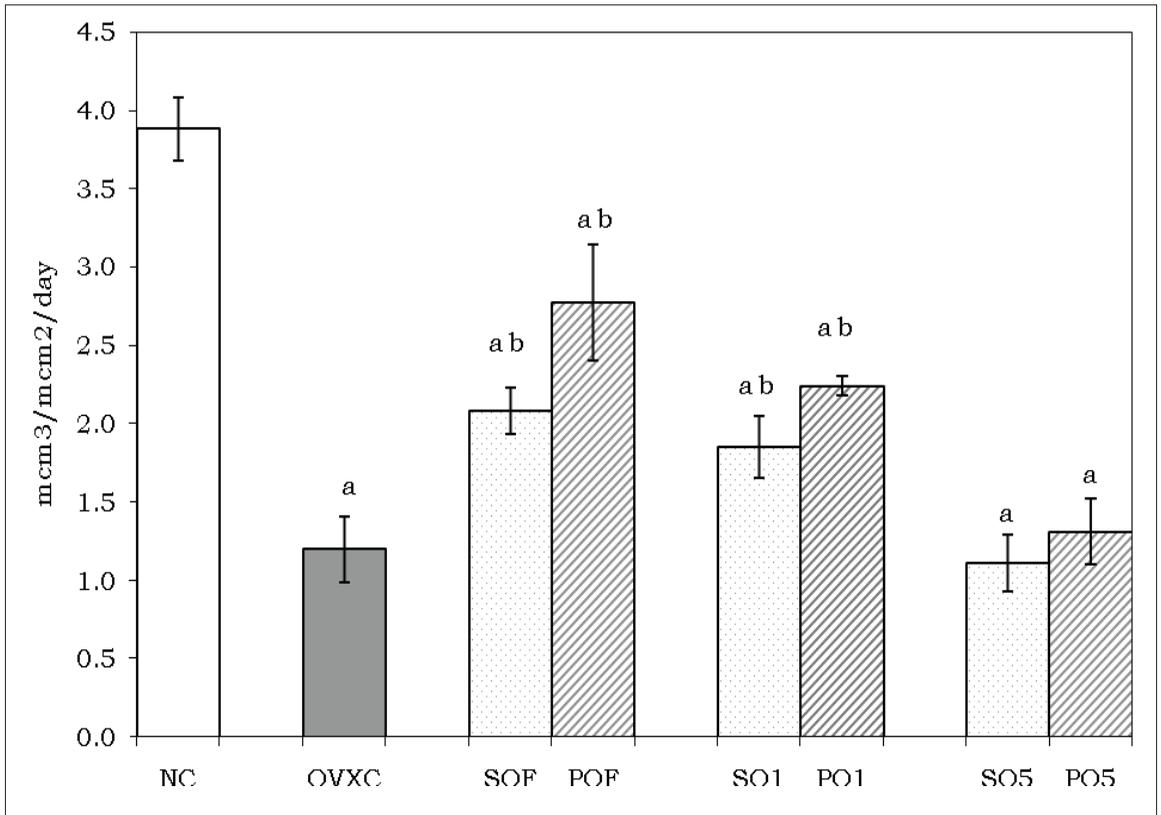Values are expressed as mean ± SD. $p < 0.05$ is considered to be significance.

Figure 3. Effects of heated oils and high cholesterol diet on bone formation rate

a = significantly different to NC
b = significantly different to OvxC ($p < 0.05$)

Keys:

NC   : Normal control
OvxC : Ovariectomy control + high cholesterol diet
SOF   : Ovariectomy + high cholesterol diet + fresh soy oil
SO1   : Ovariectomy + high cholesterol diet + soy oil heated once
SO5   : Ovariectomy + high cholesterol diet + soy oil heated 5 times
POF   : Ovariectomy + high cholesterol diet + fresh palm oil
PO1   : Ovariectomy + high cholesterol diet + palm oil heated once
PO5   : Ovariectomy + high cholesterol diet + palm oil heated 5 times

Values are expressed as mean ± SD. $p < 0.05$ is considered to be significance.

DISCUSSIONS

In this preliminary study, dynamic parameters of bone histomorphometry were used to evaluate the effects of combining repeatedly heated oils with high cholesterol diet on the bone of ovariectomised rats. As expected, ovariectomised rats fed with high cholesterol diet have reduced dynamic parameters compared to normal control group. Additions of fresh oils were able to protect the bone from these negative effects. In fact, fresh or once-heated palm oil was able to fully restore dLS/BS.

The protection from the negative effects of ovariectomy and high cholesterol diet on bone by the fresh or once-heated oils may be contributed to their vitamin E content. Palm oil is rich in tocotrienol while soy oil is rich in tocopherol (Goh, Choo & Ong, 1985). Several studies have shown the beneficial effects of vitamin E on bone. Vitamin E prevented the deleterious effects of skeletal unloading on bone mass and strength (Smith *et al.*, 2005). Vitamin E also improved material and structural bone properties in aged rats (Arjmandi *et al.*, 2002). Our studies have shown that palm vitamin E was able to prevent bone loss from FeNTA toxicity (Ahmad *et al.*, 2005), hyperthyroidism (Ima-Nirwana *et al.*, 1999), ovariectomy (Norazlina *et al.*, 2000) and orchidectomy (Ima-Nirwana *et al.*, 1998).

Fresh or once-heated frying oils may be beneficial to the dynamic parameters, but when they were repeatedly heated, the protective effects for dLS/BS and BFR/BS were lost. Vitamin E, which effectively protects fatty acids in the oil from oxidation, deteriorates after each frying episode (Andrikopoulus *et al.*, 2002). Rats fed on a diet containing 15% oxidised frying oil had significantly lower a-tocopherol concentrations in plasma and most tissues than rats fed on a diet containing similar levels of fresh soybean oil (Liu & Huang 1995). Therefore, repeated heating of fry-

ing oils destroys the vitamin E content and exposes the fatty acids to oxidation.

Studies have shown that if vitamin E can be replenished or added, the frying oil's ability to withstand thermal oxidative changes is restored. Addition of vitamin E to frying oil was found to render polyunsaturated fatty acids more resistant to oxidation (Grootveld *et al.*, 1998). Supplementation of vitamin E to olive oil was found to increase the stability of this oil under pro-oxidant conditions, and its intake was found to decrease the oxidative damage generated by adriamycin in rats (Quiles *et al.*, 1999).

In conclusion, intake of repeatedly heated frying oils and high cholesterol diet had negative effects on the bone of ovariectomised rats. This may be contributed partly by the destruction of vitamin E when the frying oils were heated. The implication of the finding for humans is that perhaps post-menopausal women should avoid taking repeatedly heated frying oils and a high cholesterol diet at the same time as these may place them at increased risk of osteoporosis. However, the role of selenium as an anti-cancer agent requires more studies.

REFERENCES

Ahmad NS, Khalid BAK, DA Luke & Ima-Nirwana S (2005). Tocotrienol offers better protection than tocopherol from free radical-induced damage of

rat bone. *Clin Exp Pharm Physiol* 32:761-770.

Andrikopoulus NK, Dedoussis GV, Falirea A, Kalogeropoulos N & Hatzinikola HS (2002). Deterioration of natural antioxidant species of the vegetable edible oils during the domestic deep-frying and pan-frying of potatoes. *Int J Food Sci Nutr* 53:351-363.

Arjmandi B, Juma S, Beharka A, Bapna M, Akhter M & Meydani S (2002). Vitamin E improves bone quality in the aged but not in young adult male mice. *J Nutr Biochem* 13:543-549.

Bai XS, Lu D, Bai J, Zheng H, Ke ZY, Li XM & Luo SQ (2004). Oxidative stress inhibits osteoblastic differentiation of bone cells by ERK and NF-kB. *Biochem Biophys Res Comm* 314(1):197-207.

Corcos BP, Di Feice M, Gentili V, Tangliamonte B & Tomassi G (1990). Influence of dietary thermally oxidised soybean oil on oxidative status of rats of different ages. *Ann Nutr Metab* 34(4):221-231.

Cuesta C, Sánchez-Muñiz FJ & Varela G (1988). Nutritive value of frying fats. In: *Frying of Food.* Varela G, Bender AE & Morton ID (eds). United Kingdom: Ellis Horwood Ltd. Chichester. Pp. 112–128.

Difford J (1974). A simplified method for the preparation of the methyl methacrylate embedding medium for undecalcified bone. *Med Lab Tech* 31: 79-81.

Dobarganes MC, Marquez-Ruiz G & Velasco J (2000). Interactions between fat and food during deep frying. *Eur J Lipid Sci Technol* 102:521–528.

Esterbauer H, Schaur RJ & Zollner H (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* 11:81–128

Folsom AR, McGoven PG & Nabulsi AA (1996). Changes in plasma lipid and lipoprotein associated with starting and stopping post-menopausal hormone replacement therapy. *Am Heart J* 132:952-958.

Garrett IR, Boyce BF, Oreffo RO, 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(3):632-639.

Goh SH, Choo YM & Ong SH (1985). Minor constituents of palm oil. *J Am Oil Chem Soc* 62: 237-240.

Grootveld M, Atherton MD, Sheerin AN, Hawkes J, Blake DR, Richens TE, Silwood CJ, Lynch E & Claxson AW (1998). *In vivo* absorption, metabolism, and urinary excretion of alpha, beta-unsaturated aldehydes in experimental animals. Relevance to the development of cardiovascular diseases by the dietary ingestion of thermally stressed polyunsaturate-rich culinary oils. *J Clin Invest* 101:1210-1218.

Ima-Nirwana S, Norazlina, M & Khalid BAK (1998). Pattern of bone mineral density in growing male and female rats after gonadectomy. *J Asean Fed Endoc Soc* 16:21-26.

Ima-Nirwana S, Norazlina M, Abd. Gapor MT & Khalid BAK (2000). Palm vitamin E prevents osteoporosis in orchidectomised growing male rats. *Nat Prod Sci* 6(4):155-160.

Ima-Nirwana S, Kiftiah A, Sariza T, Abd. Gapor MT & Khalid BAK (1999). Palm vitamin E improves bone metabolism and survival rate in thyrotoxic rats**.** *Gen Pharmacol* 32:621-626.

Kalu DN (1991). The ovariectomized rat model of postmenopausal bone loss. *Bone Min* 15:175-192

Kamat JP, Sarma HD, Devasagayam TPA, Nesaretnam K & Basiron Y (1997). Tocotrienols from palm oil as effective inhibitors of protein oxidation and lipis peroxidation in rat liver microsomes. *Mol Cell Biochem* 170:131-138.

Liu JF & Huang CJ (1995). Tissue -tocopherol retention in male rats is compromised by feeding diets containing oxidized frying oils. *J Nutr* 125:3071-3080.

Luegmayr E, Glantschnig H & Wesolowski GA *et al.* (2004). Osteoclast formation, survival and morphology are highly dependent on exogenous cholesterol/lipo-proteins. *Cell Death Diff* 1:108–118.

Mekhta U & Swinburn B (2001). A review of factors affecting fat adsorption in hot chips. *Crit Rev Food Sci Nutr* 41: 133-154.

Mody N, Parhami F, Saraflan TA & Demer LL (2001). Oxidative stress modulates osteoblastic differentiation of vascular and bone cells. *Free Radic Bio Med* 31:509-519.

Norazlina M, Ima-Nirwana S, Gapor MT & Khalid BAK (2000). Palm vitamin E is comparable to alpha-tocopherol in maintaining bone mineral density in ovariectomised female rats. *Exp Clin Endocrinol Diab* 108:305-310.

Oil World (2002). Oil World Annual 2002, ISTA; Mielke GmbH, Hamburg, Germany.

Owu DU, Osim EE & Ebong PE (1998). Serum liver enzymes profile of Wistar rats following chronic consumption of fresh or oxidized palm oil diets. *Act Trop* 69:65-73.

Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H & Meunier PJ (1987). Bone histomorphometry: standardisation of nomenclature, symbols and units. Report of the ASMBR Histomorphometry Nomenclature Committee. *J Bone Min Res* 2:595-610.

Quiles JL, Ramirez MC, Ibanez S, Alfonso J, Duthie CG, Huertas JR & Mataix J (1999). Vitamin E supplementation incereases the stability and the in vivo antioxidant capacity of refined olive oil. *Free Radic Res* 31:129-135.

Serbinova E, Kagan V, Han D & Packer L (1991). Free radical recycling and intramembrane mobility in the anti-oxidant properties of alpha-tocopherol and alpha tocotrienol. *Free Radic Biol Med* 10(5):263-275.

Sjögren U, Mukohyama H, Roth C, Sundqvist G and Lerner UH (2002). Bone-resorbing activity from cholesterol-exposed macrophages due to enhanced expression of Interleukin-1. *J Dent Res* 81(1):11-16.

Smith BJ, Lucas EA, Turner RT, Evans GL, Lerner MR, Brackett DJ, Stoecker BJ & Arimandi BH (2005). Vitamin E provides protection for bone in mature hindlimb unloaded male rats. *Calcif Tissue Int* 76:272-279.

Soriguer F, Rojo-Martinez G, Dobarganes MC, Garcia-Almeida JM, Esteva I,

Beltran M, Ruiz De Adana MS, Tinahones F, Gomez-Zumaquero JM, Garcia-Fuentes E & Gonzalez-Romero S (2003). Hypertension is related to the degradation of dietary frying oils. *Am J Clin Nutr* 78:1092-1097.

Sutherland WH, De Jong SA, Walker RJ & Williams SM (2002). Release of cholesterol from cell membranes to postprandial plasma from mildly hypercholesterolemic subjects: the effect of meals rich in olive and safflower oils. *Metabolism* 51:1306-1312.

Williams MJ, Sutherland WH, McCormick MP, de Jong SA., Walker RJ & Wilkins GT (1999). Impaired endothelial function following a meal rich in used cooking fat. *J Am Coll Cardiol* 33:1050-1055.

Wronski TJ & Yen CF (1991). The ovariectomized rat as an animal model for postmenopausal bone loss. *Cells Mat Suppl* 1:69-74.

Yee JK & Ima-Nirwana S (1998). Palm vitamin E protects against ferric nitrilotriacetate-induced impairment of bone calcification. *Asia Pacific J Pharmacol* 13:35-41.