Reheating of soy oil is detrimental to bone metabolism in oestrogen deficient rats

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ABSTRACT

Introduction: The short-term and long-term effects of heated soy oil on bone metabolism in ovariectomised Sprague-Dawley rats were studied.

Methods: Three-month-old female rats, were divided into five groups: normal control (NC); ovariectomised control (OVXC); ovariectomised and fed rat chow with added fresh soybean oil (SOF) or once-heated soy oil (SOI) or five-times-heated soy oil (SO5). Short-term parameters measured after one month were serum interleukin-6 (IL-6) and osteocalcin. Long-term parameters measured after six months were the structural bone histomorphometrical parameters. Vitamin E content in the soy oil subjected to the different heating treatments were also measured.

Results: Rats in the SO5 group had higher levels of IL-6 after one month compared to the other four groups. Osteocalcin levels in the SOI and SO5 groups remained high after treatment, while those in the NC and SOF groups declined. After six months, bone mass declined in the SO5 group. Vitamin E assay in the oils showed that levels of alpha-tocopherol decreased after heating the oil once and five times, while levels of gamma- and delta-tocopherols only declined after heating five times.

Conclusion: Repeated heating of soy oil destroyed the tocopherols causing raised serum IL-6 and osteocalcin levels, leading to increased bone resorption and osteoporosis in the long term.

Keywords: bone metabolism, heated soy oil, oestrogen deficiency, soy oil, vitamin E

INTRODUCTION

In homes and restaurants, vegetable oils used for cooking are usually heated to very high temperatures, and may be reused several times to save costs. The recycling process involves repetitive heating of the oils. This practice may be bad for health.[1] Heated vegetable oils were found to have higher content of free radicals compared to fresh vegetable oils.[2] Reactive oxygen species present in the heated oils can impair bone metabolism due to lipid peroxidation if taken as part of the daily diet.[3] Therefore, the adverse effects of heated oils may increase the risk of developing osteoporosis, especially in post-menopausal women who lack oestrogen. As oestrogen inhibits bone resorption, oestrogen deficiency will increase bone loss.[4] Oestrogen down-regulates bone-resorbing cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6) and PGE2;[5] and up-regulates TGF-β, which is an inhibitor of bone resorption.[6] IL-1 is secreted by monocytes and macrophages which are activated by several factors, including oxidative stress.[7] IL-1 can then activate osteoblasts to secrete IL-6. IL-6 can increase the differentiation of osteoclasts from haematopoietic progenitors.[8] However, osteoblasts can also be activated by other factors, such as parathyroid hormone and tumour necrosis factor-α (TNF-α);[8] while osteoclasts may not need IL-6 for differentiation.[5]

Oxidative stress has been shown to be related to osteoporosis. Yee and Ima-Nirwana showed that iron nitrilotriacetate (Fe-NTA), an oxidising agent, reduced bone calcium content in young growing rats.[9] Fe-NTA was also found to increase IL-1 and IL-6 levels, as well as decrease trabecular bone volume and thickness. Mean osteoblast number was decreased, while mean osteoclast number was increased.[10,11] Mody et al[12] and Bai et al[13] showed that oxidative stress inhibit osteoblast differentiation. Soy oil is a common cooking oil used in several parts of the world. Soy oil is rich in polyunsaturated fatty acids and tocopherols. Previous studies have shown that alpha-tocopherol was effective in protecting rats against osteoporosis due to ovariectomy.[14] However, no studies were done to observe the adverse effects of repeated heating of soy oil on bone metabolism. Therefore, this study was done to determine the effects of fresh and heated soy oil on

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bone metabolism in ovariectomised female rats, i.e. a model of osteoporosis due to oestrogen deficiency. Short-term effects were assessed by measuring the levels of serum osteocalcin, a marker for bone formation; and IL-6, a cytokine involved in the process of bone resorption. Long-term effects were determined by studying bone histomorphometric parameters of bone structure.

METHODS

The soy oil used was purchased commercially (Yee Lee Edible Oils, Malaysia). The oil was either used fresh (unheated), heated once or heated five times. The heating process involved using 2,500 ml of the oil to fry 1 kg of *keropok lekor* (a local delicacy made from fish and flour) in a metal wok. The temperature of the heated oil reached about 180°C, and the cooking process lasted about ten minutes. To reheat the oil five times, the hot oil was cooled for five hours, then the whole frying process was repeated with a fresh batch of *keropok lekor* each time. Standard rat pellets (Gold Coin, Ipoh, Malaysia) were ground and mixed with the respective oils at 15% weight/weight. The pellets were reformed, dried in an oven at 70–90°C and used to feed the animals.

Female Sprague-Dawley rats aged three months and weighing between 180–250 g were obtained from the university animal facility. The first group was not ovariectomised and used as normal controls (NC). This group was fed the standard rat pellets without any addition of oils. The rest of the animals were ovariectomised and divided into four groups: (i) ovariectomised control group fed standard rat pellets (OVXC); (ii) ovariectomised and fed standard rat pellets with added fresh soy oil (OVX + SOF); (iii) ovariectomised and fed standard rat pellets with added once-heated soy oil (OVX + SO1); and (iv) ovariectomised and fed standard rat pellets with added five-times-heated soy oil (OVX + SO5). There were eight rats in each group. Dietary treatment was started two weeks post-ovariectomy and continued for six months. Serum IL-6 and osteocalcin were measured using the ELISA method (kits: BMS625 Medisystems Diagnostics GmbH, Austria and Rat-Mid™, Nordic Bioscience Diagnostics, Denmark, respectively). The reading was done using an ELISA microplate reader (VERSAmax, Molecular Devices Corporation, USA). The samples were taken before ovariectomy and after four weeks of dietary treatment.

After six months of treatment, the animals were sacrificed by cervical dislocation under anaesthesia. The left femur was dissected out and used for structural bone histomorphometric studies. The undecalcified samples were dehydrated and embedded in methyl methacrylate according to Difford J.[15] The block was sectioned at 8 μm with a microtome (Leica 2155, Leica Microsystems GmbH, Ernst-Leitz-Strasse, Wetzlar, Germany) and stained with the Von Kossa stain. The structural parameters were measured using a microscope (Olympus BH-2, Olympus America, Melville, NY, USA) attached to an image analyser (Quantimet 520, Cambridge Instruments, Woburn, MA, USA). The software programme used was VideoTest-Master (VideoTesT Ltd, St.Petersburg, Russia). All histomorphometric parameter measurements were performed at the metaphyseal region, which is located 3–7 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 structural parameters measured were bone volume/tissue volume (BV/TV), trabecular thickness (TbTh), trabecular number (TbN) and trabecular separation (TbSp). These parameters were defined according to the American Society of Bone Mineral Research Histomorphometry Nomenclature Committee.[16]

Vitamin E levels in soy oil were measured using high-performance liquid chromatography (Waters, Milford, USA) techniques. Six samples from different batches of the fresh, heated-once and heated-five-times soy oil were analysed. Statistical analyses were done using ANOVA and paired T-test for normally-distributed samples, and Kruskall-Wallis and Wilcoxon tests for data which were not normally distributed. The software programme used was the Statistical Package for Social Sciences version 12.0.1 (SPSS Inc, Chicago, IL, USA). This study was approved by the university's Research and Animal Ethics Committees. The approval reference number was FAR/2003/KAMSIAH/25-JANUARY/090.

RESULTS

The levels of alpha-tocopherol decreased steadily with heating, i.e. it decreased significantly after heating once, and decreased further after heating five times. The levels of gamma- and delta-tocopherols did not decrease after heating once. It only decreased significantly after heating for five times (Fig. 1). The serum IL-6 levels were higher in the rats fed with soy oil that was heated five times (SO5) compared to all the other groups. It was also higher compared to the pre-treatment levels in that group (Fig. 2). Serum osteocalcin levels declined significantly post-treatment in the NC and SOF groups. However, the treatment groups did not differ pre- or post-treatment (Fig. 3).

Trabecular BV/TV and TbTh was significantly lower in the OVXC group compared to the NC group. These parameters were higher in the SOF group compared to all the other groups, including the NC group. However, after heating the soy oil once (SO1), BV/TV and TbTh decreased until they no longer differ from those of NC, but are still higher than those
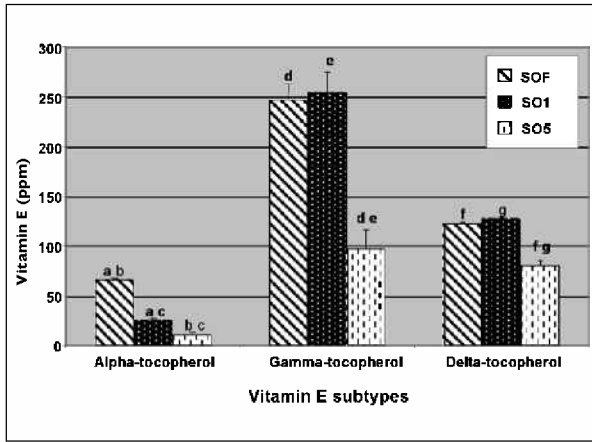
Fig. 1 Bar chart shows concentrations of vitamin E subtypes in soy oil.
Data labelled with the same letter indicates significant difference between treatment groups. * indicates significant difference between pre-treatment and post-treatment values for the same group. Data was presented as mean + SEM. Significant level was taken at $p < 0.05$.
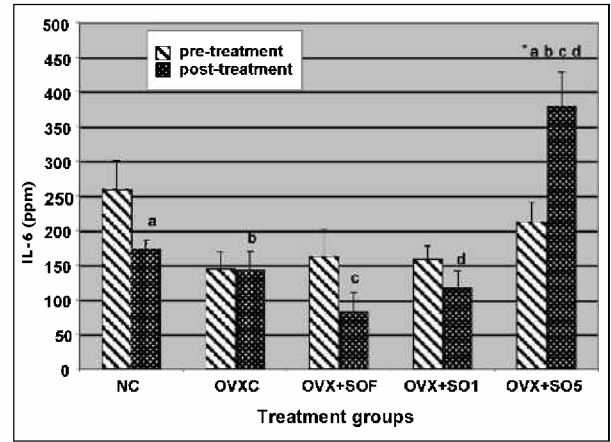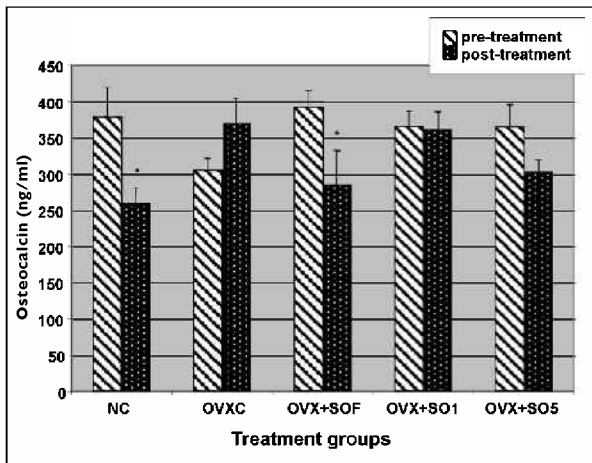

Fig. 2 Bar chart shows serum IL-6 concentrations in treatment groups.
Data labelled with the same letter indicates significant difference between treatment groups. * indicates significant difference between pre-treatment and post-treatment values for the same group. Data was presented as mean + SEM. Significant level was taken at $p < 0.05$.
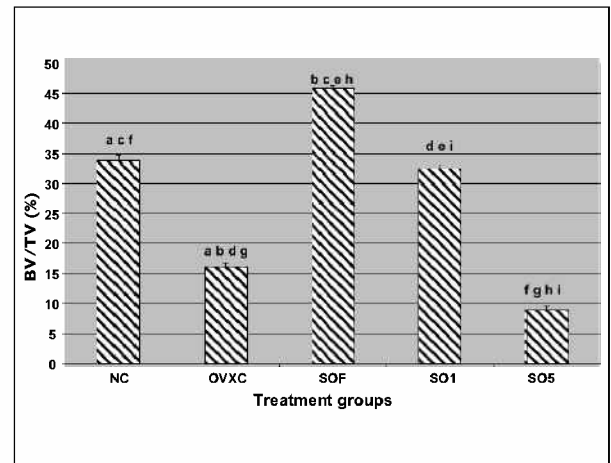

Fig. 3 Bar chart shows serum osteocalcin concentrations in treatment groups.
Data labelled with the same letter indicates significant difference between treatment groups. * indicates significant difference between pre-treatment and post-treatment values for the same group. Data was presented as mean + SEM. Significant level was taken at $p < 0.05$.

Fig. 4 Bar chart shows bone volume/tissue volume in treatment groups.
Data labelled with the same letter indicates significant difference between treatment groups. * indicates significant difference between pre-treatment and post-treatment values for the same group. Data was presented as mean + SEM. Significant level was taken at $p < 0.05$.

of OVXC. The group fed soy oil that was heated five times (SO5) had lower trabecular BV/TV compared to all the other groups (Figs. 4 & 5). TbN did not differ after ovariectomy. However, the TbN in the SOF group was higher than all the other groups, including the NC group. Heating the oil once (SO1) and five times (SO5) reduced the TbN to lower than that of the NC and SOF groups (Fig. 6).

The distance between trabeculae (TbS) was increased after ovariectomy. However, giving fresh soy oil (SOF) reduced the TbS compared to all the other groups, including the NC group. Distance between the trabeculae

was increased in the SO1 group compared to the NC and SOF groups, but it was less than the OVXC and SO5 groups. The SO5 group had the largest distance between trabeculae compared to all the other groups (Fig. 7). Photomicrographs of the structural histomorphometric parameters are provided in Fig. 8.

DISCUSSION

Alpha-tocopherol in the soy oil was not stable and started to decline even after the first heating. On the other hand, the gamma- and delta-tocopherols were quite stable and did not decline after the first heating.
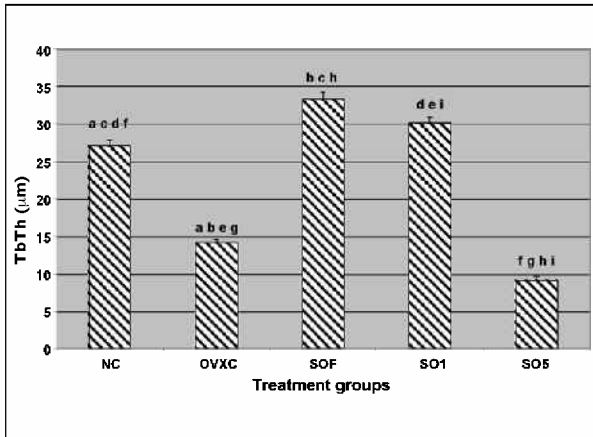
Fig. 5 Bar chart shows trabecular thickness in treatment groups.
Data labelled with the same letter indicates significant difference between treatment groups. * indicates significant difference between pre-treatment and post-treatment values for the same group. Data was presented as mean + SEM. Significant level was taken at p < 0.05.
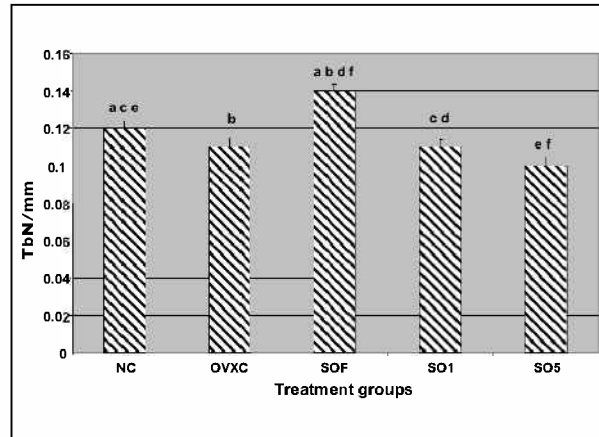

Fig. 6 Bar chart shows trabecular number in treatment groups.
Data labelled with the same letter indicates significant difference between treatment groups. * indicates significant difference between pre-treatment and post-treatment values for the same group. Data was presented as mean + SEM. Significant level was taken at p < 0.05.
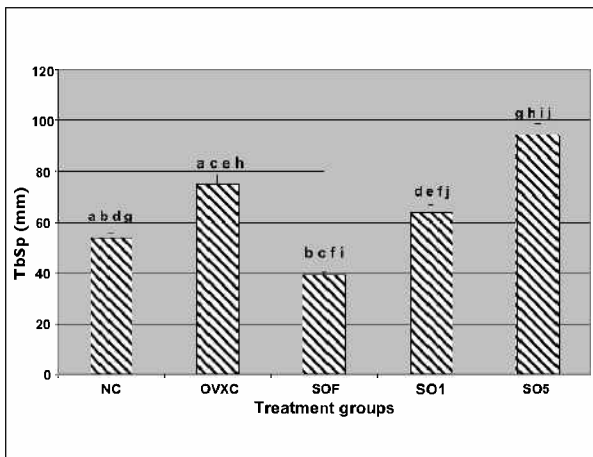

Fig. 7 Bar chart shows trabecular separation in treatment groups.
Data labelled with the same letter indicates significant difference between treatment groups. * indicates significant difference between pre-treatment and post-treatment values for the same group. Data was presented as mean + SEM. Significant level was taken at p < 0.05.

Significant reduction in the gamma- and delta-tocopherol levels were only observed after the fifth heating process. Other researchers have reported a deterioration in alpha-tocopherol content after repeated heating of soy oil.[17] This is consistent with other reports which state that there was an increased content of free radicals in heated cooking oils.[2] Soy oil contains a large amount of polyunsaturated fatty acids (PUFA), i.e. about 63.3% of the total fatty acid content.[18] PUFAs are easily oxidised, producing free radicals as by-products. Therefore, the decline in tocopherol levels could be due both to heat degradation as well as

to being used up to detoxify the free radicals produced during heating.

In the study, ovariectomy as well as addition of soy oil heated once, did not affect IL-6 levels, while multiple heating of soy oil was found to increase the levels of IL-6. Heated vegetable oils produced free radicals.[19] Vegetable oils with high polyunsaturated fatty acid content tend to produce large quantities of free radicals, such as hydroperoxides and cyclic hydroxylesters after heating.[20,21] These free radicals can activate osteoclasts by increasing the levels of bone-resorbing cytokines such as IL-1, IL-6 and TNF-α.[22,23] Therefore, the higher levels of IL-6 in the SO5 group could be due to the increased production of free radicals due to repeated heating.

Osteocalcin is a marker for bone formation activity by osteoblasts.[24] The serum osteocalcin declined after four weeks in the NC group. This may be because the rats are growing, and bone turnover is reduced as the animals grow older and overall growth slows down. The serum osteocalcin was not decreased in the OVXC group, indicating that the high level of bone turnover persisted. This is expected since ovariectomy resulted in oestrogen deficiency. Oestrogen functions to down-regulate bone-resorbing cytokines, such as IL-1 and IL-6.[25,26] Lack of oestrogen will increase bone resorption, leading to a compensatory increase in bone formation. In the ovariectomised animals given the fresh soy oil, serum osteocalcin was also found to be lower after treatment. This suggests that fresh soy oil was able to suppress the increased bone turnover due to oestrogen deficiency. However, in the animals given once-heated and five-times-heated soy oil, no reduction was seen
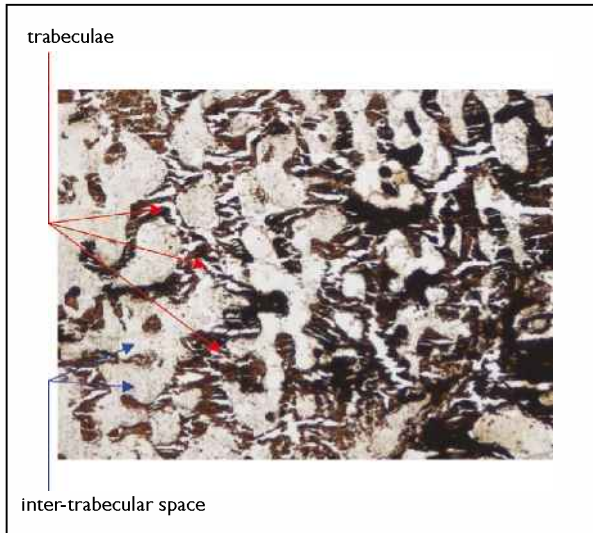
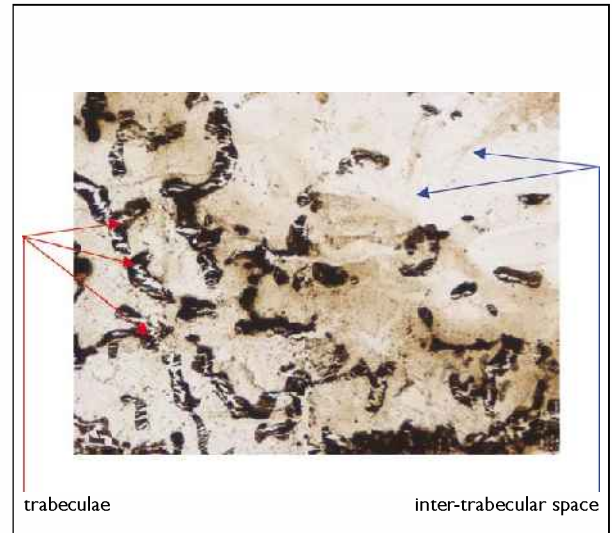**Fig. 8a** Photomicrograph of undecalcified bone. Normal control (NC) (Von Kossa stain, ×4).

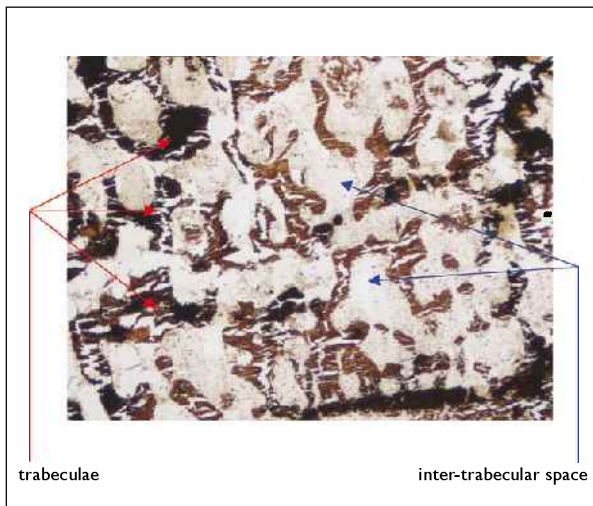**Fig. 8b** Photomicrograph of undecalcified bone. Ovariectomised control (OVXC) (Von Kossa stain, ×4).

Fig. 8c Photomicrograph of undecalcified bone. Ovariectomised + fresh soy oil diet (OVX + SOF) (Von Kossa stain, ×4).
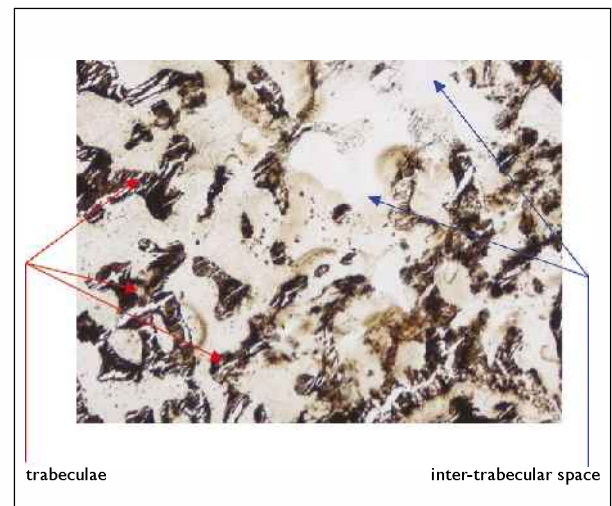

Fig. 8d Photomicrograph of undecalcified bone. Ovariectomised + soy oil heated-once diet (OVX + SO1) (Von Kossa stain, ×4).

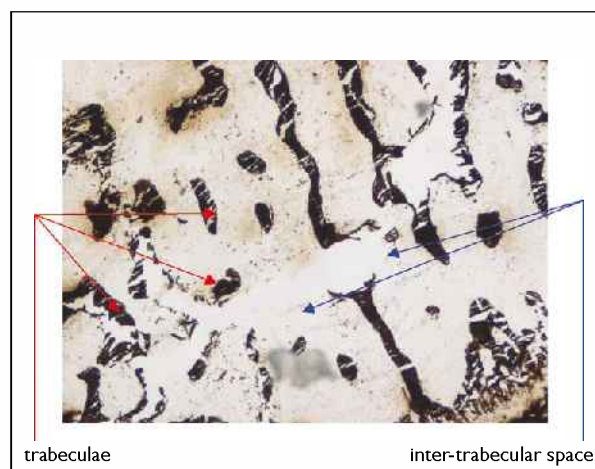**Fig. 8e** Photomicrograph of undecalcified bone. Ovariectomised + soy oil heated-five-times (OVX + SO5) (Von Kossa stain, ×4).

in the serum osteocalcin levels after treatment. This suggests that heating soy oil removed the protection that was seen in the fresh oil and in the normal oestrogen-sufficient animals. This further implies the antioxidant role of the tocopherols in the fresh soy oil which could confer similar bone protection to oestrogen, which is also an antioxidant.[22]

In this study, ovariectomy reduced BV/TV and TbTh, as well as increased the average distance between trabeculae. This is in agreement with Gal-Moscovici et al who found that removal of the ovaries, which resulted in oestrogen deficiency, decreased total bone volume and bone formation rate, and increased osteoclastic bone resorption.[27] Part of the antiresorptive action of oestrogen is mediated by the inhibition of osteoblastic production of IL-6.[28] In our study, we found that there was no increase in IL-6 one month after ovariectomy. However, this could be because the duration of study was not long enough, or because our model is an in vivo rat model, whereas Kassem et al used cell culture methods which could detect smaller changes in IL-6 production by osteoblasts.[28]

In this study, fresh soy oil (SOF) was shown to protect the rat bone against the bone-depleting effects of ovariectomy (OVXC). However, the additional surprising result was that it can also improve bone histomorphometric parameters in normal, non-ovariectomised rats fed with standard rat pellets (NC). This may be due to the high content of tocopherols in fresh soy oil (Fig. 1). Apart from the tocopherols, fresh soy oil also contains other micronutrients such as isoflavones and phytooestrogens which are effective antioxidants. When the soy oil was heated once (SO1), the bone parameters were still improved compared to the OVXC rats, but did not differ significantly from the normal controls (NC). Thus, once-heated soy oil was still effective in protecting the bone against the effects of oestrogen deficiency. However, once the soy oil was heated five times (SO5), the bone parameters deteriorated to a level that is worse than that of the OVXC group. This suggested that multiple heating of soy oil was detrimental to bone health, especially to the vulnerable bones of oestrogen-deficient rats. These findings were consistent with the serum IL-6 findings, which showed a significantly higher level of IL-6 in the SO5 group compared to all the other groups. These findings were also consistent with that of serum osteocalcin, where the osteocalcin levels were maintained at ovariectomy levels in both the SO1 and SO5 groups. However, in the SOF group, the osteocalcin declined post-treatment similar to that seen in the NC group, indicating that the fresh soy oil was able to prevent the changes in bone turnover seen due to ovariectomy. And all these changes were most probably due to the rapid decline in levels of all the

three tocopherol isomers in soy oil seen after heating it five times.

In conclusion, once-heated soy oil has no detrimental effects on bone metabolism in oestrogen deficient ovariectomised rats. On the other hand, soy oil that was repeatedly heated induced adverse changes in serum IL-6 and osteocalcin which was associated with structural bone loss in the long term. Therefore it is advisable for oestrogen-deficient post-menopausal women not to reheat or recycle their soy oil as this will destroy the tocopherol antioxidants, thus compromising bone health and further aggravating osteoporosis. However, fresh soy oil improved bone structure even in normal, non-ovariectomised rats, suggesting future use of soy tocopherols for prevention and treatment of osteoporosis.

ACKNOWLEDGEMENTS

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