High-Fat Diet Augments Ovariectomy-Induced Bone Resorption in Rats

Dieta Rica en Grasas Aumenta la Resorción Ósea Inducida por Ovariectomía en Ratas

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SUMMARY: Menopause complications such as cardiovascular and bone diseases represent a major public health concern. We sought to determine whether a high-fat diet (HFD) can augment ovariectomy-induced bone resorption in a rat model of menopause possibly via the upregulation of the inflammatory biomarkers and dyslipidemia. Rats were either ovariectomized and fed a standard laboratory chow (model group) or were ovariectomized and fed with a HFD for 15 weeks before being sacrificed. Ovariectomy significantly ($p<0.05$) increased body weight, dyslipidemia, insulin resistance, pro-inflammatory cytokines tumor necrosis factor-a (TNF-α) and interleukin-6 (IL-6), and biomarker of bone resorption, nuclear factor-kB (NF-kB), which were augmented by feeding animals with a HFD. This was confirmed through immunohistochemical study, where ovariectomy induced expression of p65/NF-kB protein in tibia bone sections of the model group, which were augmented by HFD. HFD augments ovariectomy-induced bone resorption through increased inflammatory biomarkers and NF-kB in rats.

KEY WORDS: Ovariectomy; Menopause; Inflammation; NF-kB, Dyslipidemia; Rat model.

INTRODUCTION

Osteoporosis and obesity are two body composition disorders which are growing in high proportion in worldwide (Rosen & Bouxsein, 2006; de Oliveira *et al.*, 2011; Lobo *et al.*, 2014). Due to high body fat content in obesity, it is usually accompanied by abnormalities in insulin resistance (IR) (Raisz, 2005; Chan *et al.*, 2010). Human studies have shown that adipose tissue produce pro-inflammatory cytokines, such as TNF-α and IL-6. These cytokines are known to stimulate the proliferation and differentiation of osteoclasts and hence osteoporosis (Sang *et al.*, 2017).

To mimic the molecular pathogenesis of bone loss in postmenopausal osteoporosis in humans, the OVX rat model has been used to induce estrogen deficiency and bone loss. Estrogen deficiency has been found to be associated with increased proinflammatory cytokines (Fischer *et al.*, 2018). Increased cytokines (TNFα, IL-6) potentially enhances the transcription factor NF-kB has been proved to cause ovariectomy-induced osteoporosis. The transcription factor family, nuclear factor kB (NF-kB), consists of several factors capable of crossing the nuclear membrane, binding to specific promoters and regulating expression of numerous genes involved pathologic functions (Ting & Endy, 2002). The founding members of this family were NF-kB1 (p50), NF-kB2 (p52), RelA (p65), R elB, and Rel-c (Baldwin, 1996). These NF-kB subunits form homo and heterodimers through their N-terminal Rel homology domains that permit specific DNA binding (Hayden & Ghosh, 2004).

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The aim of the present work is to investigate that obesity augments bone resorption in ovariectomy-induce postmenopausal rat model via up regulation of and pro-inflammatory biomarkers and NF-kB.

MATERIAL AND METHOD

Animals. Twenty-four healthy female Wistar rats at ten weeks age and weighing 150-200 g were used for these studies. The rats were fed with a standard laboratory diet, given water and maintained under laboratory conditions of temperatures ranging 22±3 °C, with 12 hour light and 12-hour dark cycles. All experimental procedures involving the handling and treatment of animals were approved by the Ethical Committee of Kasr Al Aini Faculty of Medicine, Cairo University.

Experimental design. After a one week adaptation period, rats were randomly allocated into four groups (n= 6) as follows: The sham-operated control rats (Control), animals were subjected to all surgical procedures of ovariectomy except for removal of ovaries and fed with standard laboratory chow for 15 weeks. The sham-High fat diet group (HFD), were subjected to all surgical procedures of ovariectomy except for removal of ovaries. Two weeks later, they fed a high-fat diet for 15 weeks (Aragno *et al.*, 2009); the third group: Ovariectomized (OVX), rats were subjected to bilateral ovariectomy. Two weeks later, they fed with standard laboratory chow for 15 weeks. The fourth group: Ovariectomized-High fat diet (OVX-HFD), rats were subjected to bilateral ovariectomy. Two weeks later, they fed with high-fat diet for 15 weeks.

Rats underwent ovariectomy surgery as follow:
Anaesthesia: Ketamine 40 mg/kg mixed with xylazine 20 mg/kg was injected intraperitoneally. The abdominal hair was shaved abdominal wall was sterilized, and anterior midline abdominal was incised. Both ovaries were removed. The postoperative antibiotic was cephalosporine 20 mg/kg intramuscular, once/day for three days. The postoperative analgesia was Na Diclofenac 2 ml/rat twice/day for three days. The rats were allowed to 2 weeks of recovery before doing any additional procedures.

Preparation of blood and tissues for analysis: After eight weeks, fasting blood samples were collected under anaesthesia using 40 mg Kg-1 sodium thiopentone, i.p. Blood samples were taken through puncture of the aorta. Bones of the tibia are immediately removed for immunohistochemical staining for NF-kB /P65. Blood samples were anticoagulated using sodium citrate for plas-

ma isolation and kept at 4 °C for subsequent measurements. Sera were separated and stored at -80 °C for subsequent measurements of biochemical parameters.

Determination of serum levels of glucose, insulin, triglyceride, LDL, VLDL, HDL, MDA, TNF-α, IL-6, and NF-kB: Animals were sacrificed after 15 weeks, and blood levels of glucose and insulin were determined colourimetrically using a Randox reagent kit (Sigma-Aldrich) for glucose and ultra sensitive ELISA Kit (Crystal ChemInc., Spain) for insulin. Triglycerides (TG), low-density lipoprotein cholesterol (LDL), very low-density lipoprotein (VLDL), and high-density lipoprotein cholesterol (HDL) were measured using commercial kits supplied by SPINREACT, Spain, according to the manufacturer's instructions. ELISA kits for determination of serum levels of TNF-α, (ELISA kit BIOTANG INC, Cat. No. R6365, MA, USA), Interleukin-6 (IL-6) (ELISA kit BIOTANG INC, Cat. No. RB1829, MA, USA), NF-kB (ELISA kit Wuhan Fine Biotech Co., Ltd, Cat. No. C6-323, Wuhan, Hubei, China).

Calculation of Homeostatic model assessment (HOMA) index: HOMA of IR was calculated by using the following equation: HOMA = (fasting glucose (mmol L) × fasting insulin (mU L ÷ 22.5. A HOMA value > 1 means insulin sensitive (healthy) and above 1.9 means insulin resistance (Matthews *et al.*, 1985).

Immunohistochemical staining for NF-kB / P65: The staining was done according to Bancroft and Cook using a rabbit polyclonal antibody (NeoMarkers For Lab Vision Corporation, USA, catalogue number #RP-9034). P65 immunopositivity appeared as a nuclear and cytoplasmic brown colour in the affected cells (Arabaci *et al.*, 2010).

Bone histomorphometric studies: Data were obtained using "Leica Qwin 500 C" Image Analyzer Computer System Ltd. (Cambridge, England). Mean area per cent of P65 immunopositive cells was measured in all immunostained sections. From each section 10 non-overlapping fields were examined using an objective lens ×10 (=total magnification ×100) and the mean value for each slide was obtained.

Statistical analysis: The data were expressed as the mean ± standard deviation (SD). Data were processed and analyzed using Graph Pad Prism software (version 5). One-way ANOVA was done followed by Tukey's post hoc test. Pearson correlation statistical analysis was done for the detection of a probable significance between two different parameters. Results were considered significant if $p \leq 0.05$.

RESULTS

Ovariectomy and or high-fat diet augments increase body weight and dyslipidaemia: Ovariectomy alone neither increase body weight or cause dyslipidemia. HFD alone increased body weight and induced dyslipidemia where CHOL, TG, LDL and VLDL were significantly elevated, and HDL was decreased in comparison to the control (p<0.05). OVX and HFD group causes increased BWT and dyslipidemia in comparison to control, HFD, and OVX groups (p<0.05) (Figs. 1 A-D).

Ovariectomy augments high-fat-diet-induced insulin resistance: Ovariectomy alone did not cause increase resistance index(HOMA0 IR) (Table I). However, HFD alone induced IR. OVX and HFD group augment IR in comparison to control, HFD, and OVX groups (p<0.05).

HFD augments inflammation induced by ovariectomy: HFD and ovariectomy alone significantly (p<0.05) increased TNF-α (Fig. 2A), IL-6 and NF-kB. Combined OVX and HFD significantly increased TNF-α, IL-6 (Fig. 2B) and NF-kB(Fig 2C) in comparison to control, HFD, and ovariectomized rats (p<0.05).

Dyslipidemia is also positively correlated with increased NF-kB where, Figure 3A showed that there is a positive correlation between LDL vs NF-kB (r= 0.86, p≤ 0.0001), Figure 3B showed that there is a negative correlation between HDL vs NF-kB (r= - 0.71, p≤ 0.0001).

HFD augments ovariectomy-induced nuclear p65 as a parameter for NF-kB activation: We investigated the effect of HFD and ovariectomy on nuclear p65 as a parameter for NF-kB activation using the immunohistochemistry approach. Immunohistochemistry images obtained from the tissues of a longitudinal section of rat tibia compact bone of the control group (Fig. 4A) showed negative immunostaining reaction in the osteoclast for anti-p65, where area % of p65 26.38 + 2.48) (Fig. 4 E). Immunohistochemistry image represents tibial sections of rats fed on HFD showed a significant increase of area % of p65 (31.17 + 1.60), (Fig. 4E) in comparison to control. Figure 5 B displays brown discolouration detected in the osteoclasts indicating positive
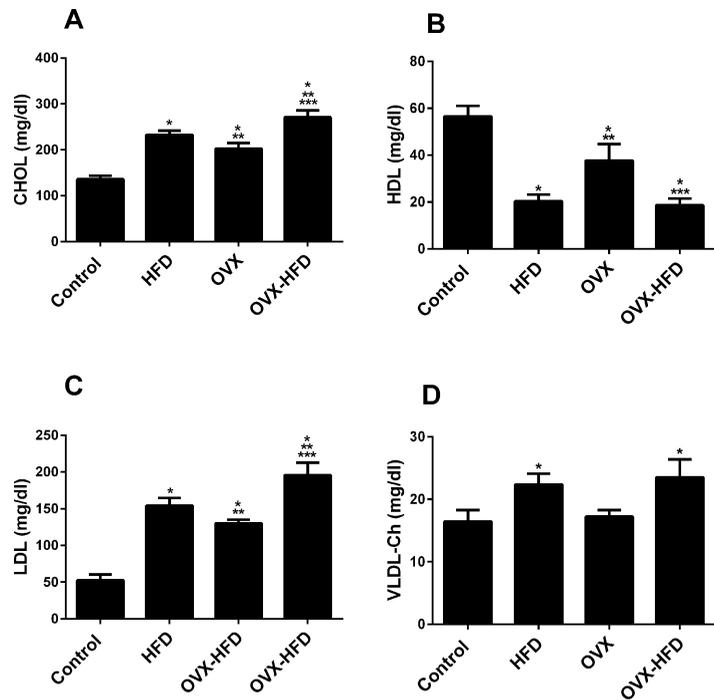

Fig. 1. High-fat diet and ovariectomy causes increased cholesterol (CHOL), low-density lipoprotein-cholesterol (LDL), very low-density lipoprotein-cholesterol (VLDL), and decreased high-density lipoprotein-cholesterol (HDL). After 15 weeks, fasting blood levels of CHO (A), HDL (B), LDL (C), and VLDL (D) were measured in 4 groups of rats: Control, HFD, ovariectomized (OVX), and (OVX and HFD). Results represent the mean (±SD); n=6 for each group. Experiments were performed in triplicate. *p<0.05 versus control, **p<0.05 versus HFD, ***p<0.05 versus ovariectomized group.
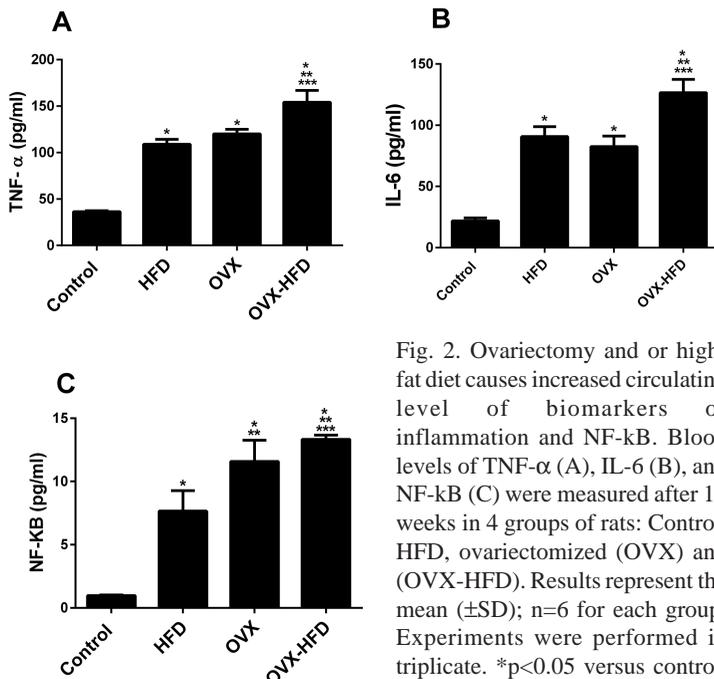

Fig. 2. Ovariectomy and or high-fat diet causes increased circulating level of biomarkers of inflammation and NF-kB. Blood levels of TNF-α (A), IL-6 (B), and NF-kB (C) were measured after 15 weeks in 4 groups of rats: Control, HFD, ovariectomized (OVX) and (OVX-HFD). Results represent the mean (±SD); n=6 for each group. Experiments were performed in triplicate. *p<0.05 versus control, **p<0.05 versus HFD, ***p<0.05 versus ovariectomized group.
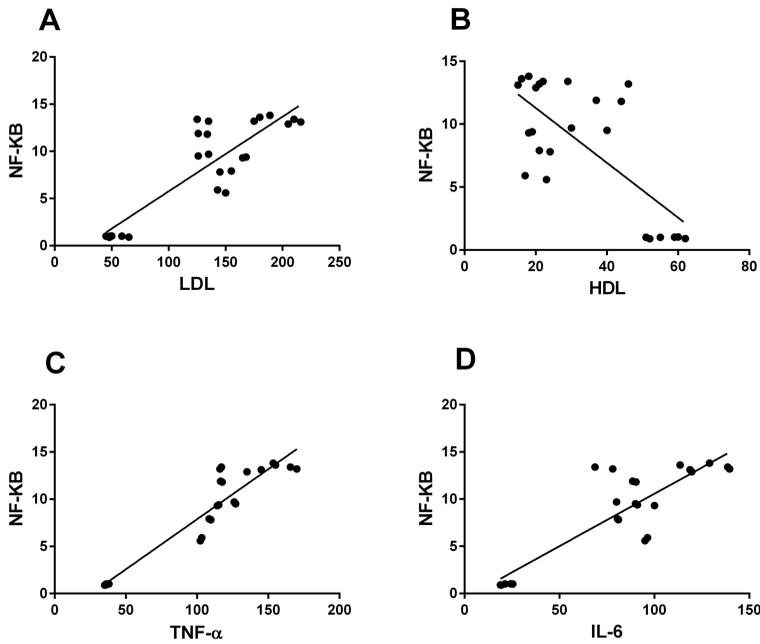
Fig. 3. Correlation between dyslipedimia and NF-kB and between inflammatory biomarkers and NF-kB. (A) showed that there is a positive correlation between LDL vs NF-kB (r= 0.86, p≤ 0.0001). (B) showed that there is negative correlation between HDL vs NF-kB (r= - 0.71, p≤ 0.0001). This figure showed that there is positive correlation between inflammatory biomarkers and NF-kB. (C, D) showed that there is a positive correlation between TNF-α vs NF-kB (r= 0.94, p≤ 0.0001) and IL-6 vs NF-kB (r= 0. 87, p≤ 0.0001).
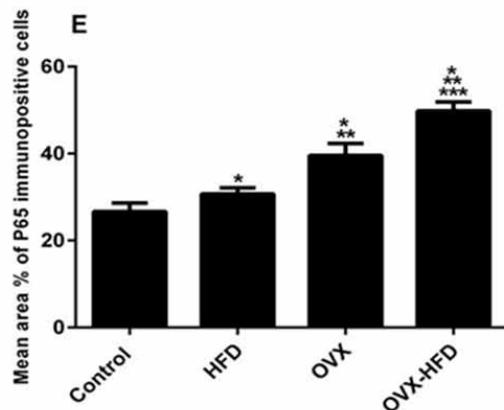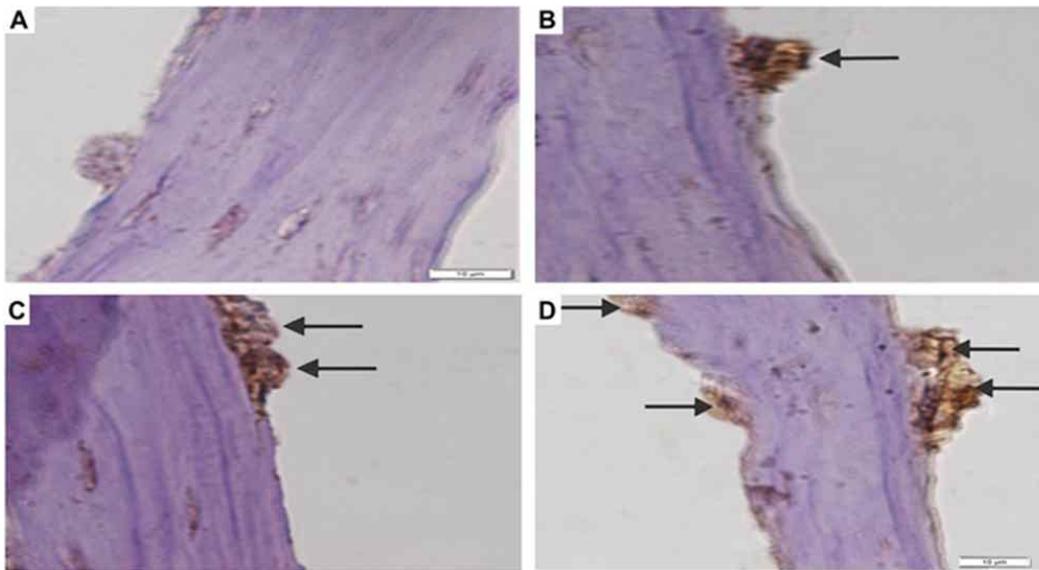

Fig. 4. Immunostaining reaction in the osteoclast for anti-P65 (Anti-P65) showed a marked an increase in a longitudinal section of rat tibia compact bone of obese postmenopausal rat model. (A) A photomicrograph of a longitudinal section of rat tibia compact bone from control group showing negative immunostaining reaction in the osteoclast for anti-P65 (Anti-P65 ×1000). (B) A photomicrograph of a longitudinal section of rat tibia compact bone from HFD group showing brown discolouration detected in the osteoclast (arrow) indicating positive immunoreactivity for anti-P65 (Anti-P65 ×1000). (C) A photomicrograph of a longitudinal section of rat tibia compact bone of the ovariectomized group showing positive immune-reactivity for anti-P65 in the osteoclasts (arrows). (Anti-P65 ×1000). (D). A photomicrograph of a longitudinal section of rat tibia compact bone of OVX-HFD group showing positive immune-reactivity for anti-P65 in the osteoclasts (arrows). (Anti-P65 ×1000).

Table I. Effects of high fat diet (HFD), overiectomy (OX) and OVX and HFA on body weight, triglycerides (TG), HOMA(IR) levels in all experimental groups of rats. Values are expressed as Mean ± SD for 6 rats in each group. Values were considered significantly different at P < 0.05. a: Significant in comparison to control; b:Significant in comparison to high fat diet (HFD) ; c: Significant in comparison to overiectomized group.

	Control	HFD	OVX	OVX + HFD
Body Weight (gm)	184.67 ± 3.88	200.50 ± 6.745[a]	198.17 ± 5.776	229.17 ± 1.169[abc]
TG mg/dl	79 ± 15.6	113.50 ± 10.29[a]	83.167 ± 12.83	124.50 ± 5.92[abc]
HOMA(IR)	2.097 + 0.22	4.837 + 0.73[a]	3.37 + 0.19	10.23 + 1.65[abc]

immunoreactivity for anti-p65. Figure 4 E showed a significant increase in area % of p65 (39.50 + 2.43) of ovariectomized rats in comparison to control and HFD groups, where Figure 4 C displays photomicrograph of a longitudinal section of rat tibia compact bone showing positive immunoreactivity for anti-P65 in the osteoclasts. Administration of HFD to ovariectomized rats (Fig. 4) causes a significant increase in area % of p65 (49.50 + 2.95) in comparison to control, HFD and ovariectomized groups. Figure 4E displays photomicrograph that showed positive immune-reactivity for anti-P65 in the osteoclasts of a longitudinal section of the rat tibia compact bone of OVX-HFD group.

DISCUSSION

The obese-ovariectomized rat model we used in our study represented a standard model for obese and postmenopausal osteoporosis. Our results showed total body weight, insulin resistance, serum TG, cholesterol, LDL and VLDL were increased, while HDL was decreased in the HFD-OVX groups in comparison to the other three groups. Additionally, there is also significant increase of biomarker of p65 in the osteoclasts of the tibial shaft which indicate an increased bone loss in the obese-ovariectomized rats ,which is in accordance with previous studies which observed a reduction of bone mass density in obese menopausal women (Raska *et al.*, 2017).

Our obese ovariectomized group indicated that dietary lipids might enhance the inflammatory signaling pathway to increased production of inflammatory mediators (TNF-α, IL-6) associated with activation of NF-kB. We also showed a positive correlation between dyslipidemia and proinflammatory markers and also showed a positive correlation between proinflammatory biomarkers and NF-kB.

Elevated biomarkers of inflammation (TNF-α, IL-6) showed in our results may cause stimulation of osteoclasts with increased bone resorption through activation of NF-kB.

There are two primary mechanisms which may promote NF-kB activation in differentiated osteoblasts (Mao *et al.*, 2016). First, estrogen receptor (ER) has been found to directly inhibit NF-kB transcription in a ligand-dependent fashion by interacting with NF-kB. Since ER is expressed in osteoblasts, estrogen may negatively regulate NF-kB activities under the physiological conditions. However, during the pathogenesis of osteoporosis, this negative regulation may be diminished due to lack of estrogen, resulting in the elevation of basal NF-kB activities in the osteoclasts as indicated with increased in % area of its subunit P65/ NF-kB in our ovariectomized-HFD model. This was also confirmed with the immunohistochemical study as indicated with positive P65 immunostaining in the ovariectomized and HFD model. Second, the pro-inflammatory cytokines, including TNF-α, IL-1, IL-6 and IL-7, are highly expressed by T cells and other cells in osteoporosis (Weitzmann & Pacifici, 2006; Mao *et al.*). These cytokines can potently stimulate NF-kB activities in osteoclasts. Previously, most studies focused on how pro-inflammatory cytokines activate NF-kB to induce osteoclast formation and activation (Zhu *et al.*, 2016). However, it has long been known that the pro-inflammatory cytokines also inhibit osteoblast differentiation and bone formation in osteoporosis, arthritis, periodontitis and multiple myeloma (Jimi *et al.*, 2004; Pearse, 2006).

CONCLUSION

The summation of findings showed that our results suggested that obesity augments ovariectomy-induced biomarkers of proinflammatory cytokines (TNF-α, IL-6) that activate NF-kB, thereby increase bone resorption.

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RESUMEN: Las complicaciones de la menopausia, como las enfermedades cardiovasculares y óseas, representan un importante problema de salud pública. Intentamos determinar si una dieta alta en grasas (HFD) puede aumentar la resorción ósea inducida por ovariectomía en un modelo de menopausia en ratas, a través de la regulación positiva de los biomarcadores inflamatorios y la dislipidemia. Las ratas fueron ovariectomizadas y alimentadas con una comida estándar de laboratorio (grupo modelo) o fueron ovariectomizadas y alimentadas con un HFD durante 15 semanas antes de ser sacrificadas. La ovariectomía aumentó significativamente (p <0,05) el peso corporal, dislipidemia, resistencia a la insulina, citocinas proinflamatorias, factor de necrosis tumoral a (TNF-α) e interleucina-6 (IL-6), y el biomarcador de resorción ósea, factor nuclear-kB (NF-kB), que se aumentaron alimentando animales con un HFD. Esto se confirmó a través del estudio inmunohistoquímico, donde la ovariectomía indujo la expresión de la proteína p65 / NF-kB en secciones de hueso de tibia del grupo modelo, que fueron aumentadas por HFD. HFD aumenta la resorción ósea inducida por ovariectomía a través del aumento de biomarcadores inflamatorios y NF-kB en ratas.

PALABRAS CLAVE: Ovariectomía; Menopausia, Inflamación; NF-kB; Dislipidemia; Modelo de rata.

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