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ORIGINAL ARTICLE

Beneficial effects of IVIG treatment on experimental-induced osteoporosis

Efectos beneficiosos del tratamiento con IVIG en la osteoporosis inducida experimentalmente

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Abstract

Objective: *Estrogen (E2) plays a significant role in postmenopausal osteoporosis, and its deficiency is related to chronic low-grade inflammation. Intravenous immunoglobulin (IVIG) is composed of immunoglobulins derived from the plasma of healthy donors. Numerous anti-inflammatory pathways are responsible for IVIG's anti-inflammatory action The aim of this study is to investigate the effects of IVIG on experimental-induced osteoporosis.* **Materials and methods:** *Forty adult female Wistar rats were included in the study. Thirty rats underwent bilateral dorsal ovariectomy. Rats were grouped as Group 1 (n = 10, ovariectomy and saline); Group 2 (n = 10, ovariectomy and E2); Group 3 (n = 10, ovariectomy and IVIG), and Control group (n = 10, no oophorectomy). Histopathological examination of bone tissue, and biochemical analysis for beta-catenin, plasma Tumor Necrosis Factor-α, IL-6, receptor activator of nuclear-κB ligand (RANKL), and osteoprotegerin (OPG) levels were made.* **Results:** *The IVIG group had increased trabecular number, area, and thickness with increased bone mineral density as well as decreased trabecular separation compared with the saline group. IVIG group had lower serum RANKL and higher serum OPG levels when compared with the saline group. The bone marrow beta-catenin level was significantly higher in the control and ovariectomy + IVIG groups.* **Conclusion:** *IVIG has beneficial effects on experimentally induced osteoporosis with a possible action on inflammation and RANKL-β-catenin pathway.*

Keywords: *β-catenin. IVIG. RANKL. Osteoporosis.*

Resumen

Objetivo: *El estrógeno juega un papel importante en la osteoporosis posmenopáusica y su deficiencia está relacionada con la inflamación crónica de bajo grado. La inmunoglobulina intravenosa (IGIV) está compuesta por inmunoglobulinas derivadas del plasma de donantes sanos. El objetivo de este estudio es investigar los efectos de IVIG en la osteoporosis inducida experimentalmente.* **Materiales y métodos:** *30 ratas se sometieron a ovariectomía dorsal bilateral. las ratas se agruparon como: Grupo 1 (n = 10, ovariectomía y solución salina); Grupo 2 (n = 10, ovariectomía y estrógeno); Grupo 3 (n = 10, ovariectomía e IVIG) y Grupo Control (n = 10, sin ovariectomía). Se realizó un examen histopatológico del tejido óseo y un análisis bioquímico de los niveles de beta-catenina, factor de necrosis tumoral α (TNF-α), IL-6, RANKL y osteoprotegerina (OPG) en plasma.* **Resultados:** *El grupo IVIG había aumentado el número, el área y el grosor trabecular con una mayor densidad mineral ósea, así como una menor separación trabecular en comparación con el grupo de solución salina. El nivel de beta-catenina en la médula ósea fue significativamente mayor en los grupos de control y de ovariectomía + IVIG.* **Conclusión:** *IVIG tiene efectos beneficiosos sobre la osteoporosis inducida experimentalmente con una posible acción sobre la inflamación y la vía RANKL-β-catenina.*

Palabras clave: *β-catenina. IVIG. RANKL. Osteoporosis.*

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Introduction

Uncoupling of bone resorption and production in the same bone region characterizes postmenopausal osteoporosis (PMO). Hormones and bone-derived substances in the bloodstream regulate osteoblastogenesis and osteoclastogenesis[1]. By promoting osteogenic differentiation and decreasing osteoclastogenesis, estrogen (E2) plays a significant role in this process. During PMO, the number of osteoclasts (OC) in the bone may increase by up to 70%, but bone formation may increase to a lower degree, remain steady, or decrease, depending on the stage of menopause[2]. Therefore, PMO is characterized by an increase in bone turnover and a remodeling balance shift toward resorption[3].

E2 has been demonstrated to interact with a number of immune cells, resulting in chronic low-grade pro-inflammation in individuals lacking in E2[4].

Chronic inflammatory disorders mediated by immune complexes are often associated with bone loss. These conditions also increase the uncoupling of osteoclast and osteoblast activities, resulting in excessive and pathologic bone resorption[5].

Intravenous immunoglobulin (IVIG) is a medication composed of immunoglobulins derived from the plasma of thousands of healthy donors. It has been shown to be beneficial in the treatment of a number of autoimmune and chronic inflammatory illnesses. Fc gamma receptors, which bind immunoglobulin G Fc component, and CD209 are two receptors that transmit IVIG signals (also known as DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-Grabbing Non-integrin)[6,7]. Numerous well-established anti-inflammatory pathways are responsible for IVIG's anti-inflammatory action. However, the precise mechanisms behind the immunomodulatory and anti-inflammatory effects of IVIG therapy remain unknown.

Despite extensive studies of the beneficial effects of IVIG on inflammatory diseases, the effects of IVIG on osteoclastogenesis and osteoblastic functions in a sub-inflammatory state like post-menopausal osteoporosis are not known.

Regarding the inflammatory condition in post-menopausal osteoporosis, the aim of this study is to determine the effect of IVIG on bone structure and the markers of osteoblast, osteoclast, and osteocyte functions.

Materials and methods

Animals

In this study, 40 female Wistar albino mature rats at weighing 200-250 g, were used. The present study was approved by the Animal Ethics Committee (Science University, Ethical number: 03220201). The rats used in the experiment were obtained from the Experimental Animal Laboratory of Science University. Rats were fed ad libitum and housed impairs in steel cages having a temperature-controlled environment (22 ± 2°C) with 12-h light/dark cycles.

Experimental protocol

In the present study, 40 adult female Wistar rats were used. Thirty rats underwent bilateral dorsal ovariectomy. Ten rats did not undergo oophorectomy. For the surgical procedure, rats were anesthetized by intraperitoneal injection of a combination of ketamine hydrochloride at a dose of 50 mg/kg and 10 mg/kg xylazine hydrochloric.

Rats were kept at the postmenopausal period for 3 weeks and were ovariectomy rats divided into three groups. Normal control group rats have no ovariectomy and no any therapy. Group 1 (n = 10, ovariectomy and saline) rats were given 1 mL/kg/day saline (0.9% NaCl) by intraperitoneally; Group 2 (n = 10, ovariectomy and E2) rats were given 17-beta-estradiol (E2) 0.5 mg/kg dissolved in sesame oil daily orally by gavage; and Group 3 (n = 10, ovariectomy and IVIG) rats were given IVIG 250 mg/kg/day by intraperitoneally. All treatments were given for 12 weeks.

Twelve weeks later, bone mineral density (BMD) of experimental animals under ketamin anesthesia (50 mg/kg) was measured by Hologic QDR-4500A (DEXA Scan) and a "small animal" program. Measurements were taken with high resolution in two different regions: the left extremity proximal femoral diaphysis and the lumbar vertebrae. Blood samples were collected by cardiac puncture for biochemical analysis and the removal of femurs was performed for histopathological and biochemical examination.

Histopathological examination of bone tissue

For histological and immunohistochemical studies, all animals were anesthetized by an i.p. of ketamin (40 mg/kg, (40 mg/kg, Alfamine®, Ege Vet, Alfasan International B.V., Holland)/xylazine (4 mg/kg, Alfazyne®, Ege Vet, Alfasan International B.V., Holland) and perfused with 200 mL of 4% formaldehyde in 0.1 M phosphate-buffer saline (PBS).

Following the perfusion procedure, the left femurs of the animals were dissected and kept at room temperature in a 10% formaldehyde fixative for 24 h for histomorphometric analysis. Following fixation, specimens

were placed in 10% formic acid. After decalcification was completed within 28 days, they were taken into routine light microscope follow-up. From the prepared paraffin blocks, transverse sections were obtained in 3-micron thicknesses with a Leica MR 2145 microtome. For morphometric analysis, hematoxylenen-eosin dyed preparations were used[8].

Morphometric analyses

For each animal, five cross-sections were obtained, from the left hind extremity proximal femoral metaphysis in the paraffine blocks serially, for morphometric analyses. Sections were stained with hematoxylin-eosin, and 20× zoomed digital pictures were taken by an Olympus microscope. To measure trabecular count, trabecular thickness, trabecular area, and trabecular separation, the semi-automatic digital system UTHSCSA Image Tool for Windows Version 1.28 was used. Trabecular measurements were performed at 0.46 mm proximal of the epiphysis plaque and at equal distances from both sides of the cortex in femur preparations. The lengths were calculated as pixels using the program (1 pixel=128 × 10-8 mm)[8]. All measurements were implemented in accordance with the article by Parfitt et al.[9]

Morphometric measurements

For trabecular thickness (μm), measurements were taken at a minimum of 50 different points for every trabecula, and measurements continued to be taken until the mean values became constant. The trabecular count was obtained by counting all trabeculae and each trabecula parallel to each other at 0.46 mm distal to the epiphysis plaque at equal distances from both sides of the cortex. The trabecular area (mm^2) was calculated by determining the borders of the trabeculae in the region where the trabecular count was determined. Cortical thickness (μm) was calculated by mean values of fifty measurements from 3-micron sections in digital pictures of each preparation. Osteoblast and osteoclast counts were calculated in hematoxylene-eosin dyed preparations with 40× objective zoomed digital pictures using an image analysis program and counting cells around trabeculae 0.5 mm under the epiphysis plaque[8].

Bone marrow biochemical analysis for beta-catenin

The material obtained was homogenized with a glass homogenizer in 5 volumes of PBS that was 5 times the volume of the obtained tissue (pH 7.4) and centrifuged at 5.000 g for 15 minutes. Beta-catenin the bone marrow supernatants was measured using commercially available rat enzyme-linked immunosorbent assay (ELISA) kits.

Plasma TNF-α, IL-6, RANKL, and OPG levels were measured using commercially available ELISA kits.

Results

The mean trabecular numbers (number/mm^2) were significantly higher in the control group compared with ovariectomy + saline, ovariectomy + E2, and ovariectomy + IVIG groups (respectively; $p < 0.01$, $p < 0.01$ and $p < 0.05$). The mean trabecular number was also significantly higher in the ovariectomy + IVIG group compared with ovariectomy + saline, and ovariectomy + E2 groups (respectively, $p < 0.01$ and $p < 0.05$). Trabecular area (μm^2) was significantly higher in the control group compared with ovariectomy + saline, ovariectomy + E2, and ovariectomy + IVIG groups (respectively; $p < 0.001$, $p < 0.001$ and $p < 0.05$). It was also significantly higher in the ovariectomy + IVIG group compared with ovariectomy + saline and ovariectomy + E2 groups (respectively; $p < 0.001$ and $p < 0.05$). Trabecular separation was significantly lower in control and IVIG groups compared with ovariectomy + saline and ovariectomy + E2 groups (respectively; $p < 0.001$, and $p < 0.001$; $p < 0.05$ and $p < 0.001$). The mean trabecular thickness (μm) was significantly higher in control group compared with with ovariectomy + saline and ovariectomy + E2 groups (respectively; $p < 0.01$ and $p < 0.01$). Similarly, it was found to be higher in the IVIG group compared with ovariectomy + saline and ovariectomy + E2 groups (respectively; $p < 0.01$ and $p < 0.05$). Femoral BMD (g/cm^2) was significantly higher in the control and IVIG groups compared with the saline group ($p < 0.01$ and $p < 0.05$). The mean values of lomber vertebra BMD (g/cm^2) were found to be significantly higher in the control and IVIG groups compared with saline group ($p < 0.01$ and $p < 0.05$). The data are presented at table 1 (Figs. 1 and 2).

The mean value of plasma TNF-α level (pg/mL) was significantly lower in the control group compared with ovariectomy + saline, ovariectomy + E2, and ovariectomy + IVIG groups (respectively; $p < 0.001$, $p < 0.001$, and $p < 0.01$). The mean plasma IL-6 level was significantly lower in the control group compared with ovariectomy + saline, ovariectomy + E2, and ovariectomy + IVIG groups (respectively; $p < 0.001$, $p < 0.001$, and $p < 0.05$). The IVIG group had a significantly lower

Table 1. Comparison of bone morphometric analyses and measurements between groups

Analysis results	Normal control (n = 10)	Ovariectomy + saline (n = 10)	Ovariectomy + E2 (n = 10)	Ovariectomy + IVIG (n = 10)
Trabecular number (number/mm^2)	12.21 ± 1.03	7.25 ± 0.9*	9.37 ± 1.2†	10.8 ± 0.7†
Trabecular area (μm^2)	24345.5 ± 2854.2	11457.8 ± 1021.2‡	17365.2 ± 985.7†	20215.3 ± 854.1§
Trabecular separation (μm)	121.3 ± 10.9	244.6 ± 12.8‡	198.5 ± 9.9§	155.8 ± 11.3§
Trabecular thickness (μm)	305.3 ± 24.9	168.2 ± 19.5*	202.1 ± 17.6†	288.5 ± 13.01§
Femoral BMD (g/cm^2)	0.48 ± 0.11	0.29 ± 0.08*	0.32 ± 0.15	0.35 ± 0.07†
Lomber vertebra BMD (g/cm^2)	0.30 ± 0.09	0.18 ± 0.12*	0.20 ± 0.05	0.22 ± 0.04†

*p < 0.05.
†p < 0.01.
‡p < 0.001 (different from the control group).
§p < 0.001 (different from ovariectomy and saline group).
Results were presented as mean ± SEM. Statistical analyses were performed by one-way ANOVA test.

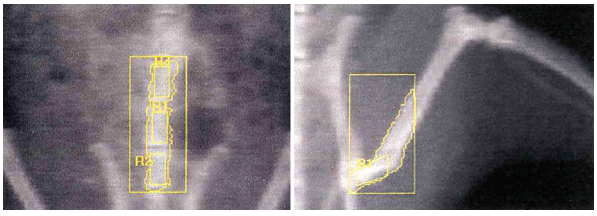

Figure 1. *Rat DEXA scan.*

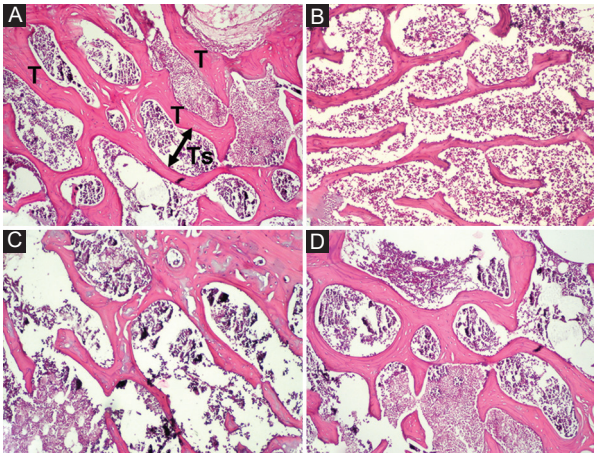

Figure 2. *Bone trabeculae (Tb) of rat femur with H&E stain ×20.* **A:** *normal control group.* **B:** *ovariectomized group trabecular thinning and widening of trabecular space 15 weeks after ovariectomy.* **C**: *ovariectomized and estradiol (E2) group restoration of the trabecular thickness.* **D:** *ovariectomized and IVIG group restoration of the trabecular thickness. T: trabeculae; Ts: trabecular separation.*

level of plasma IL-6 compared with the ovariectomy + saline group (p < 0.01). The mean plasma RANKL level was significantly lower in the control group compared with ovariectomy + saline, ovariectomy + E2, and ovariectomy + IVIG groups (respectively; p < 0.001, p < 0.001, and p < 0.01). It was also significantly lower in ovariectomy + IVIG group and ovariectomy + E2 group compared with ovariectomy + saline group (p < 0.01 and p < 0.05). The mean plasma OPG level was significantly higher in the control group compared with ovariectomy + saline, ovariectomy + E2, and ovariectomy + IVIG groups (p < 0.01). It was also significantly higher in the ovariectomy + IVIG group and ovariectomy + E2 group compared with the ovariectomy + saline group (p < 0.01 and p < 0.05). The mean levels of bone marrow beta-catenin level (pg/mg protein) were significantly higher in control and ovariectomy + IVIG groups compared with ovariectomy + saline and ovariectomy + E2 groups (respectively; p < 0.01, and p < 0.01; p < 0.01 and p < 0.01) (Table 2).

Discussion

In the current study, the administration of IVIG was shown to have favorable benefits, including an increase in trabecular number, trabecular area, trabecular thickness, femoral BMD, and lomber vertebra BMD, and a decrease in trabecular separation. In the current investigation, it was shown that IVIG is equally efficient as E2 in treating experimentally induced osteoporosis. Moreover, IVIG therapy has been demonstrated to be better in various osteoporosis-related parameters.

Osteoporosis is the most frequent metabolic bone disease, affecting 50% of women and 30% of men in their sixth and seventh decades of life[10]. Osteoporosis is characterized by uncoupled bone resorption, which results in a loss of bone mass. Continuous bone remodeling is

Table 2. Comparison of immune parameters, RANKL, OPG, and bone marrow beta-catenin between groups

Parameters	Normal control (n = 10)	Ovariectomy + saline (n = 10)	Ovariectomy + E2 (n = 10)	Ovariectomy + IVIG (n = 10)
Plasma TNF-α level (pg/mL)	30.1 ± 9.5	97.4 ± 14.8*	65.6 ± 12.9†	55.7 ± 9.8‡
Plasma IL-6 level (pg/mL)	405.5 ± 28.5	644.1 ± 11.3*	542.7 ± 8.8†	487.5 ± 13.5‡
Plasma RANKL level (pg/mL)	60.3 ± 2.24	145.4 ± 4.95§	114.1 ± 5.67†	108.03 ± 6.4†
Plasma OPG level (pg/mL)	20.5 ± 1.09	9.8 ± 1.1*	14.3 ± 0.8†	13.2 ± 1.5†
Bone Marrow Beta-catenin level (pg/mg protein)	21.4 ± 5.5	11.6 ± 2.9*	19.5 ± 1.5†	25.8 ± 2.3‡

*p < 0.05.
†p < 0.01.
‡p < 0.001 (different from ovariectomy and saline group).
§p < 0.001 (different from the control group).
Results were presented as mean ± SEM. Statistical analyses were performed by one-way ANOVA test.

a coordinated procedure for repairing microfractures and maintaining bone mass.

The two primary cell types involved in bone remodeling are bone-resorbing OC and bone-forming osteoblasts (OB). In the past 10 years, bone-embedded osteocytes have emerged as critical regulators. OC are multinucleated monocytic cells whose differentiation is controlled by the nuclear factor-kB receptor activator (RANK) and RANKL. Multiple signaling mechanisms, including WNT/b-catenin and BMP, govern OB formation from the mesenchymal stem cell lineage. During remodeling, the OC and OB form the bone remodeling unit[11].

E2 deficiency has been associated to a predictor of future osteoporosis in postmenopausal women for about eight decades. Despite extensive investigation, the mechanism behind E2 deficiency-induced accelerated bone resorption is unclear[12] Menopause and aging have been linked to reduced calcium absorption, deterioration of renal function, and changes in vitamin D metabolism[13].

In our study, TNF alpha and IL6 levels increased after oophorectomy. This elevation was greatest in the oopherectomy+ saline group and lower in the IVIG group.

The adaptive immune system has been shown in both human and animal models to have a critical role in the development of PMO. After detecting the influence of T-cell-derived cytokines on bone, Arron and Choi created the term osteoimmunology in 2000[14]. Proinflammatory cytokines produced by T cells, such as TNF and interleukin-17A, have proresorptive properties[15]. Chronic inflammation induced by an E2 deficiency may aggravate osteoporosis and lead to other complications[16].

Takayanagi et al. discovered that IFN-g generated by T cells may block RANKL signaling during OC formation[17]. Because Th1 cells produce a large amount of IFN-g, it was assumed that inflammatory bone loss was induced by Th1 cells.

IL-17A has been demonstrated to induce significant bone loss, especially in those with autoimmune disorders[17]. TNF has been shown to stimulate osteoclastogenesis by directly acting on OC[18]. Patients with rheumatoid arthritis, inflammatory bowel disease, and chronic lung disease are more likely to have osteoporosis[19]. An increase in local cytokines may accelerate bone resorption in these circumstances[20].

In the current study, the IVIG group had lower serum RANKL levels and higher serum OPG levels than the saline group.

RANKL is a cytokine that belongs to the TNF superfamily. The RANKL receptor, known as RANK, is strongly linked with CD40. It is well known that genetic deletion or mutation of RANKL causes severe osteoporosis, which is accompanied with a total deficiency on OC[21]. RANKL stimulation with RANK has a detrimental effect on OC development. TNF receptor-associated factors and kinases are activated when RANKL binds to RANK[22]. OPG is a RANKL receptor. While binding RANKL, OPG inhibits RANKL/RANK interaction and promotes bone resorption by blocking OC formation[23]. Experimental investigations have revealed that genetic deletion of OPG causes osteoporosis[24]. It is thought that an increase in RANKL and a concurrent reduction in OPG is a key risk factor for bone illnesses such as osteoporosis[25]. There have been two earlier studies that looked at the relationships between IVIG and RANKL. In addition to its immunosuppressive effects, Lee and colleagues revealed that IVIG directly suppressed osteoclastogenesis through a mechanism that included RANK signaling suppression[26]. Kim and colleagues discovered that IVIG reduced osteoclastogenesis when monocytes were cocultured with Th17 cells[27].

Although both studies demonstrated the effects of IVIG on RANKL in cell culture, they conceptually supported the positive benefits of IVIG in osteoporosis therapy.

In our research, bone marrow beta-catenin levels were higher in the IVIG group than in the saline group and Wnt signaling is widely known to have a function in bone remodeling and development. The Wnt/-catenin pathway is a key component of this signaling system[28]. As the quantity of -catenin grows, it translocates into the nucleus and activates the genes Lef1 and Tcf1[29]. Following this stimulation, osteoblastic differentiation and bone production ensue[30]. As a result, this route is crucial for bone. Furthermore, there has been therapeutic use of the Wnt pathway; sclerostin - which neutralizes antibodies[31]. These antibodies are currently accessible for clinical usage in the treatment of osteoporosis[32]. Nonetheless, these antibody medications are expensive, have a one-year shelf life, and have considerable vascular adverse effects. As a result, the application of these antibodies in clinical practice is thought to be restricted[33]. There is just one research in the literature that looks at the relationship between IVIG and -catenin. Kranam and colleagues demonstrated that therapeutic normal IgG IVIG treatment resulted in the activation of the -catenin pathway[34]. Our findings validated the previous work, and we also established for the first time that IVIG therapy had a beneficial impact on -catenin in experimentally generated osteoporosis.

In conclusion, this is the first study to demonstrate the protective benefits of IVIG on experimentally induced osteoporosis. Mechanism activities are linked to RANKL, anti-inflammation, and the -catenin pathway. Following more research, anti-inflammatory medicines impacting these pathways, such as IVIG, may be regarded a potential agent in the treatment of osteoporosis without major adverse effects.

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Conflicts of interest

The author declares no conflicts of interest.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article. Furthermore, they have acknowledged and followed the recommendations as per the SAGER guidelines depending on the type and nature of the study.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Use of artificial intelligence for generating text. The authors declare that they have not used any type of generative artificial intelligence for the writing of this manuscript nor for the creation of images, graphics, tables, or their corresponding captions.

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