Original Article

Impacts of Valproic Acid on Systemic Bone Loss Associated with Experimental Model of Rheumatoid Arthritis in Rats

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Abstract

Background: Valproic acid (VA) is a compound used for many neurological disorders, which is also known as a histone deacetylase inhibitor. The impacts of VA on adjuvant-induced arthritis (AIA) in rats were investigated. **Objectives:** To investigate the possible osteoprotective and anti-inflammatory effects of VA in rheumatoid arthritis (RA). **Materials and Methods:** AIA was achieved through subdermal injection of complete Freund's adjuvant (CFA) into the rat hindpaw. Forty Swiss albino rats were recruited in this study. These rats were divided into four groups: the normal control group received 0.1 mL phosphate buffer saline intraperitoneally each day, the positive control group received 0.75 mg/kg intraperitoneal once weekly dose of methotrexate, the AIA group received CFA without treatment, and the VA group received 300 mg/kg/day intraperitoneal dose of VA. Statistical analysis was done, and a *P* value of less than 0.05 was considered as statistically significant. **Results:** VA (300 mg/kg body weight) administered intraperitoneally could significantly reverse the effect of adjuvant arthritis on bone mineral density and bone mineral content in addition to an improvement in bone formation markers (osteoprotegerin, osteoprotegerin/RANKL ratio). Serum levels of tumor necrosis factor and interleukin 1b were significantly reduced by VA treatment. Reduced serum malondialdehyde with the elevated superoxide dismutase level was also achieved. **Conclusion:** These findings support the evidence that VA has a positive effect on bone anabolism and its anti-inflammatory properties could be considered as a bonus in inflammatory disorders induced bone loss such as RA.

Keywords: Adjuvant-induced arthritis, osteoporosis, valproic acid

Introduction

Rheumatoid arthritis (RA) is considered as the most common chronic inflammatory arthritis throughout the world.[1] The prevalence of RA is approximately 1%; it is about 3–5 times more common in women than in men. The second to fourth decades of life is considered as the peak incidence, but no age is immune.[2]

The clinical course is prolonged with exacerbation and remission. RA is associated with increased rate of mortality with the reduction of lifespan by about 8–12 years.[3,4]

RA is initiated by infiltration of the synovium with plasma cells, lymphocytes, and macrophages. CD4 T cells play the crucial role by interacting with other inflammatory cells in the synovium. T cells stimulate B cells to produce antibodies including rheumatoid factor and at the same time stimulate the production of inflammatory cytokines from macrophages.[5]

The proinflammatory tumor necrosis factor α (TNF-α) plays a significant role in the process of arthritis by regulating productions of other inflammatory cytokines. Inflammatory granulation formed around the articular cartilage is progressively eroded and destroyed. Moreover, activated T-cells in RA lead to express impressive amount of receptor activator nuclear factor kappa B ligand (RANKL). RANKL induces osteoclast differentiation and plays a key role in the bone resorption seen in destructive lesions of RA.[6] The resulting chronic synovitis progresses to destroy articular cartilage and underlying bones.

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The overexpressed RANKL binds to receptor activator of nuclear factor kappa B (RANK) on macrophage. This osteoclastogenic activity of RANK–RANKL pathway is regulated by OPG secreted from stromal cells and osteoblasts called. OPG binds to RANKL and thus blocks its binding to RANK. Binding of OPG to RANKL prevents the formation of osteoclast and thus their bone resorbing function.[7]

A number of studies suggested that valproic acid (VA) could have a positive impact on bone mass and bone formation.

One of the mechanisms suggested for the effect of VA on bone osteogenesis is through its effect on Notch signaling pathway.[8]

Notch signaling is a signaling pathway system that acts intracellularly and governs cell proliferation and differentiation, affects cell fate, and participates in cellular processes in both embryonic and adult tissue, such as skeletal tissue formation and regeneration.[9]

Intermittent activation of Notch signaling pathway has been shown to have a significant role in increasing bone formation in different studies held in mice, rat, and overiectomized rat.[10]

The other suggested mechanism discussed the effect of VA as a histone deacetylase (HDAC) inhibitor on bone formation.

HDAC inhibitors can selectively inhibit osteoclastogenesis mainly by suppressing RANK ligand-induced translocation of nuclear factors and moreover increasing the mRNA level of interferon-β, an inhibitor of osteoclastogenesis.[11]

Treatment of adjuvant-induced arthritis (AIA) in rats revealed that the use of an agent with HDAC-inhibiting activity prevent disease development in a prophylactic model in addition to the inhibition of bone destruction in a therapeutic model. These findings imply that a HDAC inhibitor suppresses osteoclastogenesis and bone degradation.[11-13]

Materials and Methods

Process of induction of arthritis

AIA was achieved through immunization of rats with CFA.[14] Two injections of 0.1 mL (10 mg/mL) CFA subdermally into the footpad were carried out. The first injection was given two days after starting treatment with the assigned regimen for each animal group, and the second immunization or a booster dose was given 2 days after the first injection to augment the effect of the first dose. This was a modified model set based on previous trials to induce a clinically manifested inflammation of the joints as most of studies used a single injection with a lower dose of CFA.

Animals of the study

Forty 3-month old male Swiss albino rats recruited in this study. The animals were housed at room temperature (25°C) and a 12-hour light/dark cycle in the laboratory animal house at the college of pharmacy—University of Basrah. The rats had free access to both food and water.

After 2 weeks of adaptation, these rats were randomly assigned into four groups (n = 10 per group) all group were treated with two doses of complete Freund's adjuvant (CFA) for induction of arthritis except for the normal control group.

Group (1) normal control group received 0.1 mL phosphate buffer saline intraperitoneally once daily for 4 weeks. **Group (2)** positive control group received 0.75 mg/kg intraperitoneal once weekly dose of methotrexate for 4 weeks.[15] **Group (3)** AIA group received CFA without treatment and **Group (4)** VA group received 300 mg/kg/day intraperitoneal dose of VA for 4 weeks.[16]

Monitoring of the disease course

For monitoring of the disease course, animals' body weight and paw thickness were measured throughout the study. Each animal was weighed before beginning of the experiment, 14 days after and lastly at the end of the experiment, and mean change in body weight was calculated in order to monitor any effect of the disease on animals' weight. Rats paw thickness was also measured using a digital vernier caliper. Measurement was performed before starting the treatment and then every 4 days till the end of experiment.

Evaluation of bone parameters

After animal sacrifices, lumbar vertebral samples were excised and stored in 10% formalin. Bone mineral density (BMD) and bone mineral content (BMC) of the cancellous lumbar regions were measured using microcomputed tomography. This test was performed at Bruker laboratories, Turkey.

24 hours prior to scanning, samples were removed from formalin and placed in 0.9% physiological saline.

Evaluation of bone formation/resorption markers

For evaluation of bone formation/resorption markers, serum OPG and serum RANKL were measured using enzyme-linked immunosorbent assay kit from Cusabio, USA. These assays utilized the quantitative sandwich enzyme immunoassay technique.

Evaluation of rheumatoid arthritis inflammatory markers

For evaluation of the disease inflammatory markers, serum levels of TNF-α and interleukin 1b (IL-1b) were measured using enzyme-linked immunosorbent assay kit from Cusabio, USA. These assays utilized the quantitative sandwich enzyme immunoassay technique.

Statistical analysis

Results were expressed as means ± standard deviation (SD). Comparisons between different experimental groups were achieved using IBM SPSS Statistics 23, 64-bit edition, 2015 software using analysis of variance followed by LSD tests. A *P* value of less than 0.05 was considered as statistically significant.

Ethical approval

This study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. The study protocol was reviewed and approved by a local ethics committee according to the document number 20200973 in 8/12/2020 to get this approval.

Results

Microcomputed tomography results

Local BMD (g/cm^3) and BMC of the lumbar vertebrae were measured and analyzed by Bruker-Micro CT (SkyScan 1275, Bruker, Co., Ltd., Germany).

Micro-CT analysis revealed a highly significant decline in BMD and BMC in the AIA group when compared to normal nontreated rats ($P < 0.001$). While methotrexate and VA treatment showed a highly significant increase in BMD and BMC as compared to the AIA group ($P < 0.001$) [Figures 1 and 2].

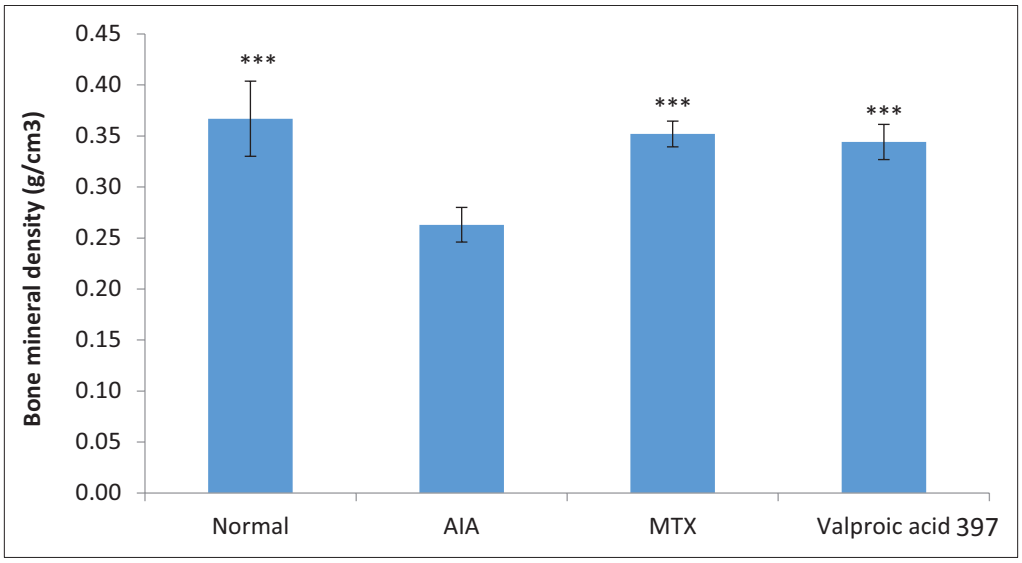

Figure 1: Effect of valproic acid on bone mineral density (g/cm^3) of lumbar vertebrae in experimental groups. Results are expressed as mean ± SD. AIA: adjuvant-induced arthritis, MTX: methotrexate; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared to AIA group

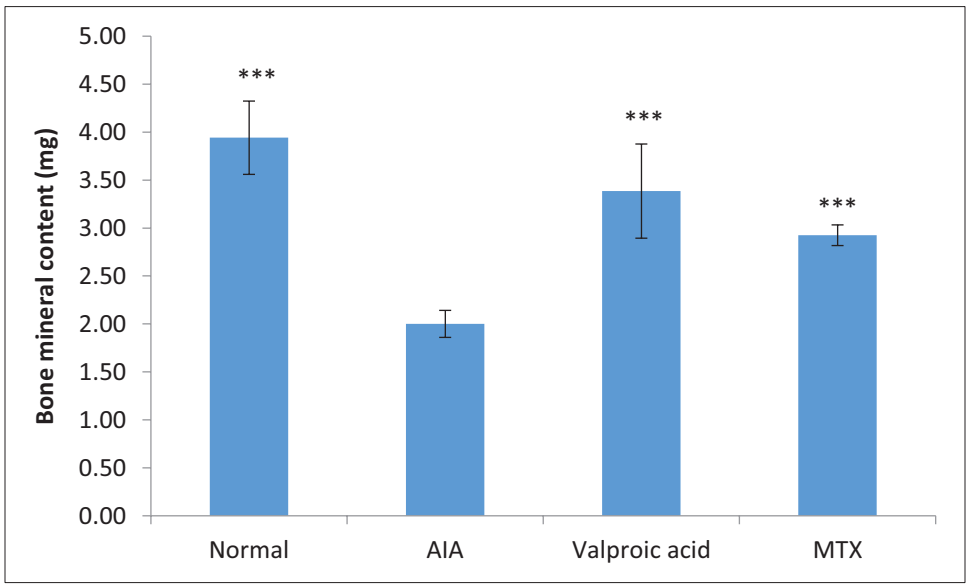

Figure 2: Effect of valproic acid on bone mineral content (mg) of lumbar vertebrae in experimental groups. Results are expressed as mean ± SD. AIA: adjuvant-induced arthritis, MTX: methotrexate; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared to AIA group

Influence on inflammatory cytokines

Induction of arthritis in rats had resulted in a profound increment in serum levels of inflammatory cytokines TNF-α and IL-1b, which was statistically significant compared to the normal group ($P < 0.001$).

In contrast, methotrexate and VA significantly reduced the TNF-α and IL-1b level when the levels were compared to the AIA group ($P < 0.001$) [Figures 3 and 4].

Influence on oxidative stress markers

Induction of adjuvant arthritis had a negative impact on the oxidative status in immunized rats where it resulted in a lower level of the protective antioxidant superoxide dismutase (SOD) enzymes while it increases the level of the harmful oxidative malondialdehyde (MDA). These results were found to be statistically significant against the control group ($P < 0.001$).

While treatment with methotrexate and VA revealed a marked improvement in the oxidative status demonstrated by a highly significant increase in the serum level of SOD with a lower level of MDA when the results compared with the AIA group ($P < 0.001$) [Figures 5 and 6].

Influence on bone resorption/formation markers

Although the AIA group did not reveal a marked difference in the level of OPG; treatment with VA showed a pronounced increment in its level. The result was statistically significant.

However, VA resulted in a highly significant increase in the serum level of OPG compared to the AIA [Figure 7].

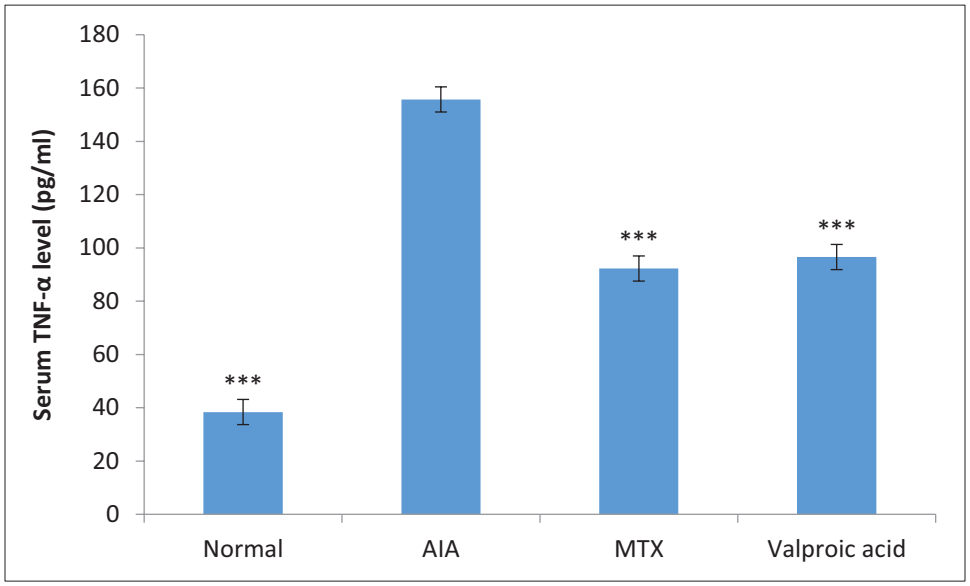

Figure 3: Effect of valproic acid on serum level of TNF-α (pg/mL) in experimental groups. Results are expressed as mean ± SD. AIA: adjuvant-induced arthritis, MTX: methotrexate; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared to AIA

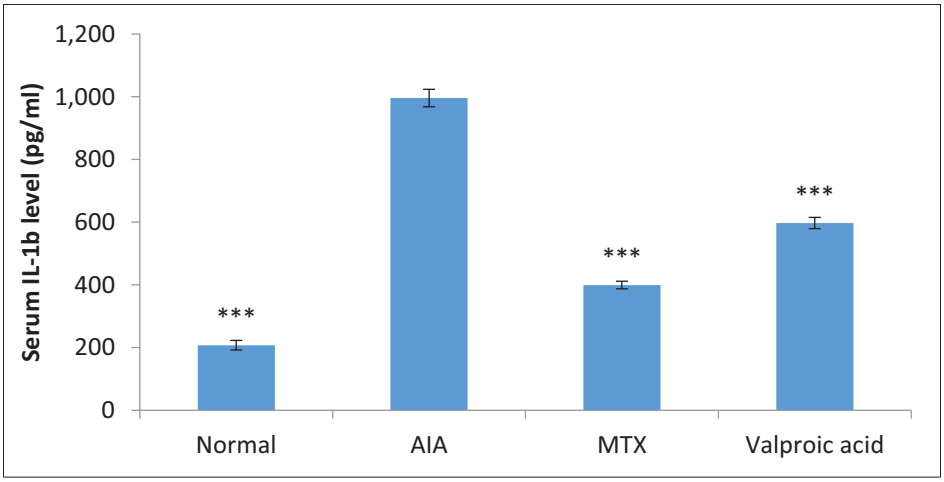

Figure 4: Effect of valproic acid on serum level of IL-1b (pg/mL). Results are expressed as mean ± SD. AIA: adjuvant-induced arthritis; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared to AIA

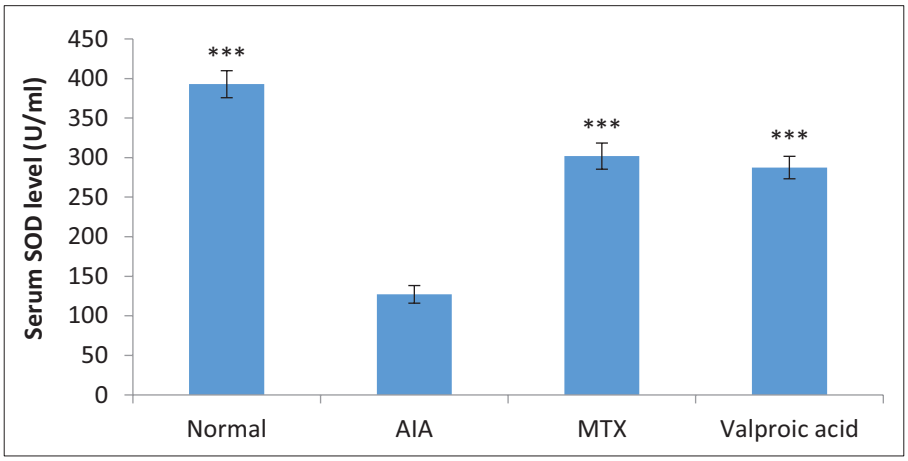

Figure 5: Effect of valproic acid on serum level of SOD (U/mL) in experimental groups. Results are expressed as mean ± SD. AIA: adjuvant-induced arthritis, MTX: methotrexate; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared to AIA group

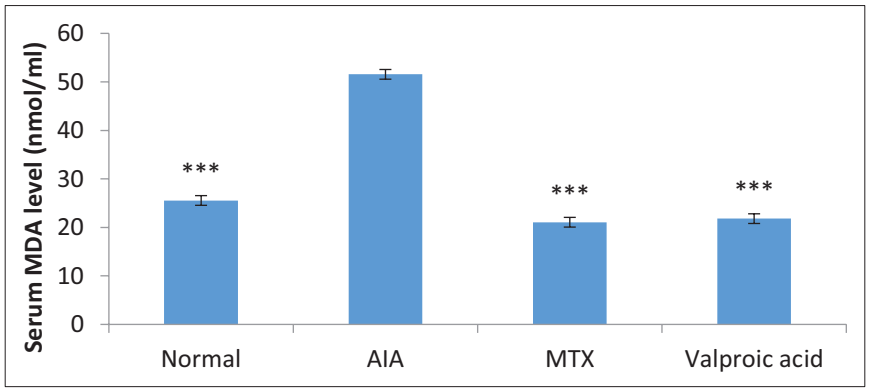

Figure 6: Effect of valproic acid on serum level of MDA (nmol/mL) in experimental groups. Results are expressed as mean ± SD. AIA: adjuvant-induced arthritis, MTX: methotrexate; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared to AIA group

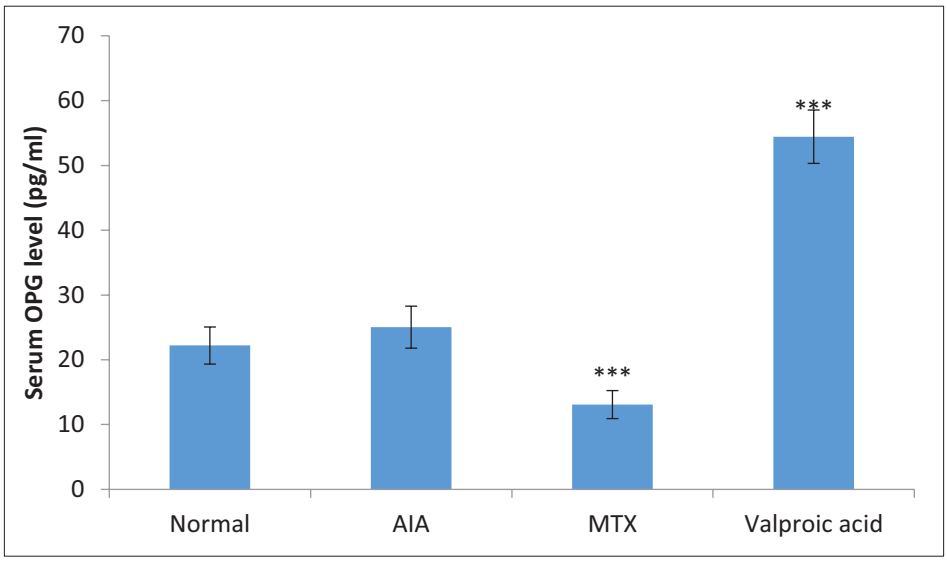

Figure 7: Effect of valproic acid on serum level of osteoprotegerin (OPG) in experimental groups. Results are expressed as mean ± SD. AIA: adjuvant-induced arthritis, MTX: methotrexate; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared to AIA group

In contrast, induction of arthritis resulted in a significant increase in the serum level of RANKL as compared to the normal group ($P < 0.001$).

Treatment with methotrexate and VA lowers serum level of RANKL significantly as compared to the AIA group ($P < 0.001$) [Figure 8].

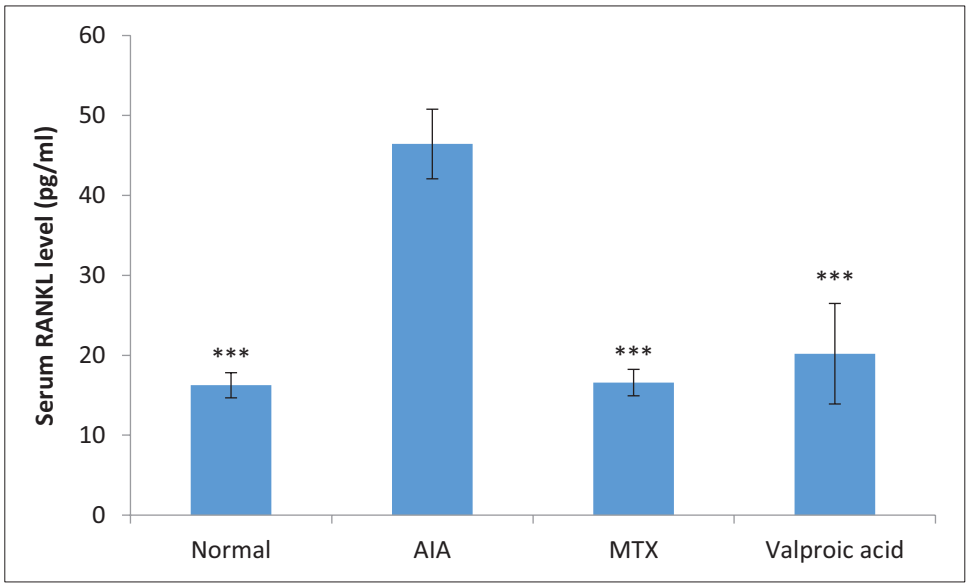

Figure 8: Effect of valproic acid on serum level of receptor activator of nuclear factor kappa-B ligand (RANKL) in experimental groups. Results are expressed as mean ± SD. AIA: adjuvant-induced arthritis, MTX: methotrexate; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared to AIA group

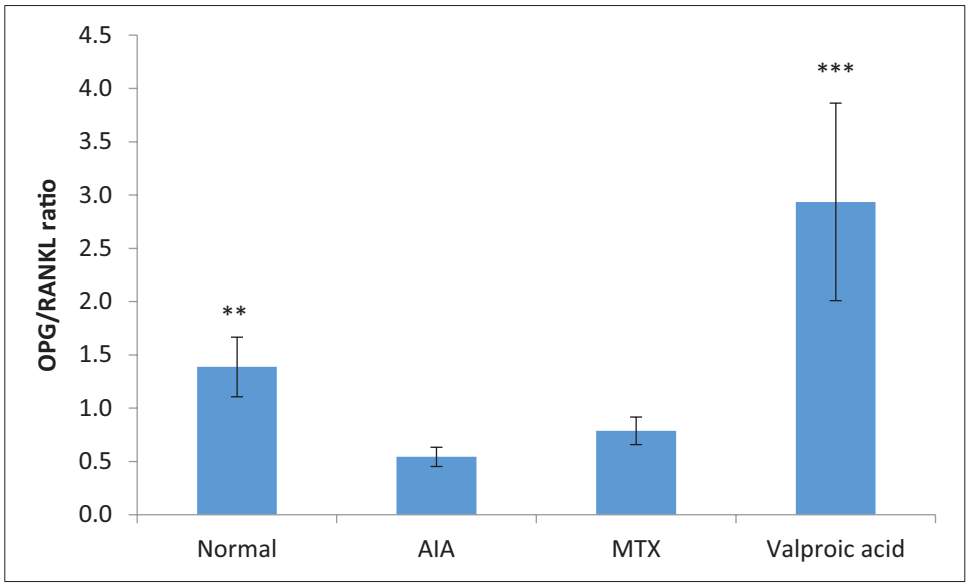

Figure 9: Effect of valproic acid on osteoprotogerin/receptor activator of nuclear factor kappa-B ligand ratio OPG/RANKL in experimental groups. Results are expressed as mean ± SD. AIA: adjuvant-induced arthritis, MTX: methotrexate; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared to AIA group

OPG/RANKL ratio was also calculated and estimated in the different experimental groups where the AIA group showed a significant decrease in the OPG/RANKL when compared with the normal group ($P < 0.01$).

In contrast, treatment with VA had a positive impact on OPG/RANKL ratio. This ratio was significantly higher compared to AIA group ($P < 0.001$), while there was no significant statistical difference in the methotrexate-treated rats ($P > 0.05$) [Figure 9].

Influence on body weight

CFA-treated rats showed a significant decline in the percentage of change in body weight as compared to normal group ($P < 0.001$). Treatment with methotrexate and VA did not result in significant change in the mean body weight when compared with the AIA group ($P > 0.05$) [Figure 10].

Influence on paw swelling

Injection of CFA into rats' hind paw had resulted in a very obvious swelling and inflammation of the rats' joints

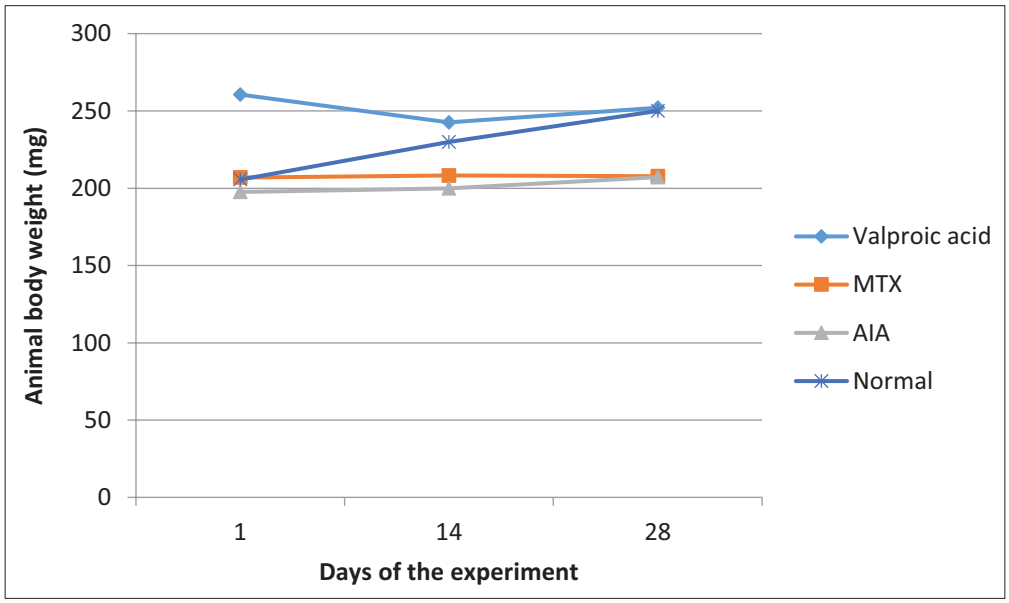

Figure 10: Effect of valproic acid on change in animal body weight (mg) in different experimental groups. Results are expressed as means. AIA: adjuvant-induced arthritis, MTX: methotrexate

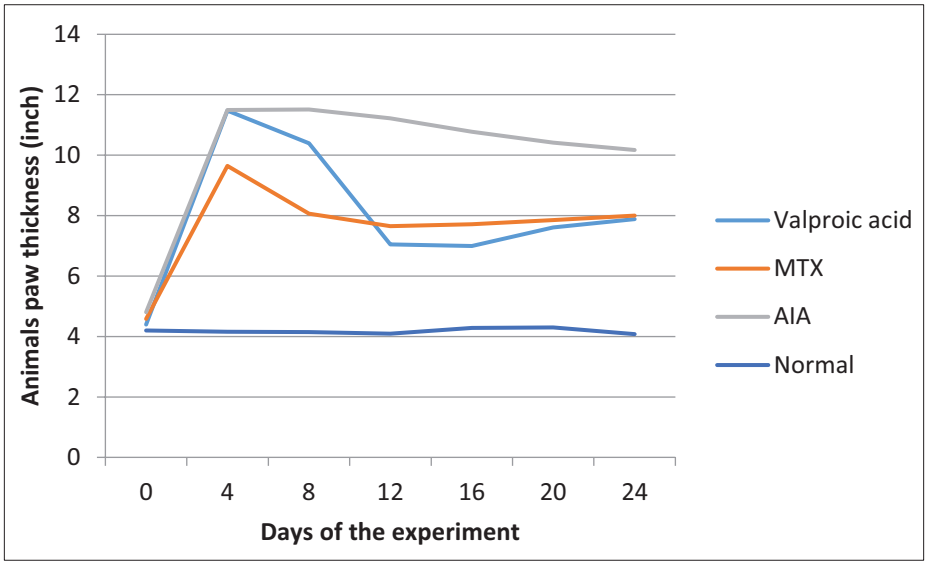

Figure 11: Effect of valproic acid on paw volume thickness (inch) in experimental groups. Results are expressed as means. AIA: adjuvant-induced arthritis, MTX: methotrexate

manifested as a significant increment in paw thickness compared to normal rats ($P < 0.001$).

Treatment with methotrexate and VA remarkably reduce the paw swelling associated with arthritis compared to AIA group ($P < 0.01$, $P < 0.05$, respectively) [Figures 11 and 12].

Discussion

The current study focused on one of the most manifested systemic complications of rheumatoid disease which is generalized bone loss or osteoporosis.

An animal model of RA was used in this study and the impact of VA on this particular complication as well as the general course of the disease was examined.

The model used for induction of arthritis in rats was AIA.

Injection of CFA (which is basically a suspension of *Mycobacterium* in mineral oil) into rats resulted in an immune reaction that involves severe inflammation with destruction of cartilage and bone of the distal joints at the same time; swelling of surrounding tissues also observed. AIA in rats is commonly employed to investigate compounds that might be of potential benefit for the

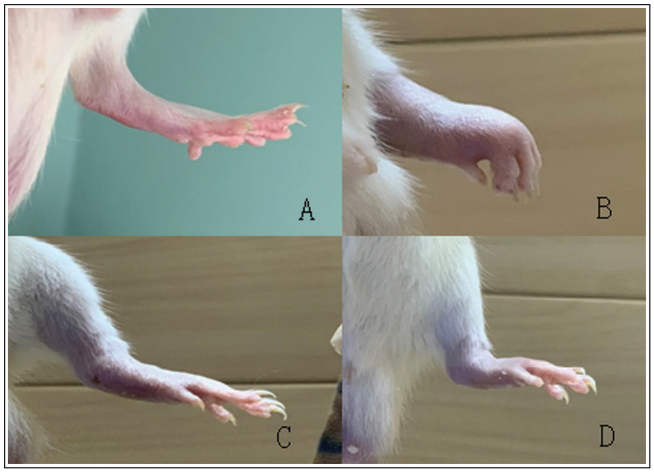

Figure 12: Images of rat paw thickness in different experimental groups. (a) Normal control group; (b) AIA group; (c) methotrexate group; (d) valproic acid group

management of RA and other chronic inflammatory conditions.[17]

Influence on bone mineral density

The current study showed that induction of arthritis resulted in a significant decrease in the BMD among the adjuvant arthritis-induced group.

This result agrees with many previous studies that demonstrated a decrease in the BMD especially in cancellous bone during the induction of arthritis in animals.

Fukata *et al*. founded that while investigating the effect of intermittent administration of thyroid hormone on arthritis and BMD as measured by u-CT in collagen-induced arthritis in rats, that BMD of the proximal metaphysis and diaphysis of the left tibia was decreased at the time of induction of arthritis and was significantly lower 4 weeks after the induction as compared to the control group.[18]

Takagi *et al*. also observed a decrease in local and systemic BMD occurred with induction of adjuvant arthritis in rats. The reduction in BMD was significant at regions of femur and tibia on day 7 following CFA injection ($P < 0.05$).[19]

The current study also showed a significant increase in BMD of rat lumbar vertebra in VA- treated group 4 weeks after induction of adjuvant arthritis.

In a study conducted to investigate whether VA has a positive effect accelerating bone defect repair in ovariectomized rat, Tao *et al*. found that treatment with VA resulted in a better bone microscopic parameters including BMD.[10]

Other study performed by Zhou *et al*. also showed a significant increase in BMD of the subchondral area of rat femoral head subjected to a femoral osteonecrosis from glucocorticoids chronic use.[16]

That effect of VA could be attributed to its action on Notch signaling pathway where VA is thought to be a strong activator of this osteogenic promoting pathway. Activation of Notch signaling pathway resulted in proliferation and differentiation of osteoblasts thus increasing osteogenesis and bone mineralization.[8]

The other proposed mechanism that can also explain that effect of VA in increasing BMD is that VA is considered as a HDAC inhibitor[12], and many studies had confirmed that compounds with such activities can increase BMD by suppressing osteoclastogenesis.[11,13]

Influence on inflammatory cytokines

Induction of adjuvant arthritis resulted in a significant increase in the serum level of inflammatory cytokines IL-1b and TNF-α as measured by ELISA.

The increased level of these inflammatory markers is attributed to T-cells response to mycobacterial heat shock protein in CFA and the immune response that driven after.

Many studies have tracked the level of these mediators in AIA in rodents as IL-1b and TNF-α play a pivotal role in the pathogenesis of RA and found a significant increase in serum levels in arthritis-induced rats compared to normal control rat.[20-22]

In our study; VA treatment showed a significant decrease in the level of these markers.

Accumulated experimental and clinical studies showed that the short chain-branched fatty VA had an anti-inflammatory property.[23] Many of these studies examined the effect of VA in several inflammatory disorders such as autoimmune neuritis where it resulted in decreased levels of TNF-α and IL-1b in addition to other proinflammatory mediators. Furthermore, suppressive and therapeutic treatment with VA significantly decreases the invasion of B cells, T cells, and macrophages.[24]

Influence on oxidative stress marker

Induction of arthritis in rats resulted in a significant decrease in the level of SOD as compared with the normal control group while resulted in a significant increase in the level of MDA when compared with the normal control group.

This decline in the level of the antioxidant SOD and the increase in the oxidative stress marker MDA can be explained by the oxidative stress status that is believed to play a pivotal role in many inflammatory diseases such as arthritis where the level of reactive oxygen species increased with the significant reduction in the activity of defense system which manifested by enhanced lipid peroxidation. The same oxidative changes achieved with the injection of CFA in rats.

Studies that had evaluated the oxidative stress status of RA disease showed a significant increase level of

MDA ($P < 0.05$) among RA patients in comparison to control.[25,26]

Different clinical and experimental studies also revealed a significant reduction in the enzymatic antioxidants including SOD.[27,28]

The antioxidative effects of VA could be explained by its inhibitory effects on HDAC. HDACs played a crucial role in the regulation of numerous proteins involved in both inflammation and cell apoptosis.[29]

Many studies suggested that HDAC inhibitors possess immune-modulatory, anti-inflammatory, and antioxidant properties.[30,31]

Influence on bone formation/resorption marker

Injection of CFA in rats did not alter the serum level of OPG significantly. While it resulted in a significant increase in the serum level of RANKL as compared to normal group. OPG/RANKL ratio when calculated also showed a significant reduction as compared to normal control group.

The same findings for OPG and RANKL were found by Ho *et al*. when investigating the effect of relaxin and estrogen on bone remodeling in AIA in rat.[32]

VA treatment resulted in significantly higher level of OPG and a highly significant decrease in serum level of RANKL. OPG/RANKL ratio in VA group also shows a significant increase compared to normal group.

These effects of VA can be linked directly to its action on Notch pathway where activation of Notch signaling resulted in increasing in the ratio of OPG to RANKL.[9]

Chang *et al.* stated that VA can stimulate OPG secretion.[33]

Influence on animals' body weight

Induction of arthritis resulted in a significant reduction in animals' body weight compared to normal control group.

This finding agrees with previous study done by Patil *et al.* where they investigated the effect of *Xanthium strumarium* extracts on CFA-induced arthritis in rats.[34]

VA did not affect body weight significantly compared to the AIA group.

This effect of VA could be explained by the stress expressed by animals due to the process of induction of arthritis and the daily intraperitoneal injections of treatment.

Influence on paw volume thickness

Injection of CFA into rats' footpad showed a profound very severe inflammation that could be confirmed by the increase in paw thickness as measured by digital caliper.

Many studies showed the same finding of our study. One study by Monica and colleagues while evaluated the effect of treatments with *Harpagophytum procumbens* on AIA in rats showed a pronounced local edema after subplantar injection of CFA with a significant increase in paw thickness.[35]

Other study conducted by Gautam *et al.* while studying the effect of rosmarinic acid as an anti-inflammatory in experimentally induced arthritis in rats where rats paw volume was significantly increase after injection of CFA.[36]

Treatment with VA resulted in a significant reduction in animals' paw volume compared to AIA group. That effect occurs as a consequence of the reduction in inflammation achieved by the anti-inflammatory effect of VA.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Author contribution

All authors contributed to the conception and design of the study, analysis of data, statistical analysis, preparation, editing, and reviewing of the manuscript.

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