Impact of Vitamin D Treatment versus Sporting Exercises on Irisin Level in Vitamin A-Induced Osteoporosis in Male Rats

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Abstract

Background: Osteoporosis is a skeletal disorder. It leads to weak fragile bones with pathological fractures. It develops over several years and is diagnosed when bone breaking happens. Vitamin A has a demonstrated role in causing osteoporosis. Numerous previous studies have indicated the essential role of exercise and vitamin D in treating osteoporosis.

Objective: To investigate changes of serum irisin in the Vit. A osteoporosis- induced group and the osteoporosis- treated groups with either vitamin D or exercise.

Materials and methods: Forty four equal groups of mature male rats were created. Group I (Control group) was given distilled water orally. Group II (OST group) was given retinoic acid at a dose of 75 mg/kg/day for 2 weeks. Group III (OST+vitamin D group) was given retinoic acid for 2 weeks followed by 4 weeks of SC 4300 IU of vitamin D3/rat/week. Group IV (OST+Swim group) was given retinoic acid for 2 weeks followed by swimming exercises for 8 weeks. Serum irsin, calcium, phosphorus, and alkaline phosphatase levels were measured, and bone histopathological examination was undergone.

Results: The study revealed significant increases in serum irisin and calcium in OST+vit D (4.74±2.492) and OST+Swim (4.573±0.246) groups in comparison to OST group (2.15±1.05; P=0.003). Serum irisin was positively correlated with serum calcium levels (r=0.566, P<0.001) and negatively correlated with phosphorous (r=-0.557, P<0.001). Histopathological examination revealed that the OST+vit D and OST+Swim groups had less bony destructive changes.

Conclusion: Vitamin D and exercise can be used as a type of adjuvant agent of osteoporosis treatment.

Keywords: Osteoporosis; Vitamin D; Irisin; Exercise.
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Introduction

As a metabolic disorder affecting the bones of the skeleton, osteoporosis is defined by the reduction of bone mass, bone-thinning and increased risk of bone fragility and fracture as a result of disruption of bone architecture **(Qaseem et al., 2017; Li et al., 2023).** As osteoporosis's the rate of occurrence keeps going up, it has become a public health problem worldwide, due to its related fragility fractures, the increased risk of mortality, and economic burden **(Harvey et al., 2010; Wang et al., 2023).**

A fat-soluble vitamin, vitamin A is critical for development, immunity, healthy skin and is used in treatment options for cancer and severe nodulocystic acne **(Dattola et al., 2020)**. The word "vitamin A" refers to a class of organic molecules that share certain chemical properties which is found in eggs, milk, fortified cereals, and liver in the form of retinol, retinal, and retinoic acid, and in vegetables in the form of carotenoids, most notably beta-carotene **(Carazo et al., 2021a; Yee et al., 2021a).** At a higher concentration, vitamin A has proven to be toxic on bones showing osteopenia, fractures, and even growth arrest **(Liu et al., 2020).**

Irisin is a myokine derived from its precursor (FNDC5) in skeletal muscle cells in response to exercise **(Boström et al., 2012; Zhao et al., 2023).**Several studies had proven that irisin has numerous physiological activities including changing white fat into brown fat, enhancement of glucose tolerance with the improvement of tissue sensitivity to insulin, and increasing nitric oxide release and vasodilation **(Fu et al., 2016; Kim et al., 2023).**

Several experimental studies proved that serum level of irisin showed a positive correlation with the density of bone mineral, and it promotes osteoblast proliferation and differentiation, which plays a significant role in bone metabolism and health **(Qiao et al., 2016).**

One steroid that is lipophilic is vitamin D. Ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3) are its two main forms. Serum 25(OH)D levels can be raised more effectively by vitamin D3 than by vitamin D2 **(van den Heuvel et al., 2024).** Calcium homeostasis and bone metabolism, as well as a number of other areas such as infections, cancer, cardiovascular diseases, and immune-mediated ailments, depend heavily on vitamin D **(Dominguez et al., 2021)**. Vitamin D supplementation has been generally considered very useful to prevent and treat of osteoporosis **(Voulgaridou et al., 2023).**

Physical activity provides clear benefits for people with osteoporosis, including increasing bone mineral density and decreasing the likelihood of falls **(Daly et al., 2019; Beck, 2022).**

As an exercise therapy, swimming has been proven to improve cardiopulmonary function and delay aging through its antihyperlipidemic effects and increasing the antioxidant capacity. Swimming is a suitable physical activity for all ages to boost the density of bone mass in individuals with osteoporosis **(Su et al., 2020a).**

The aim of the current study was to detect the effects of either exercises or vitamin D treatment in osteoporosis and correlating the level of serum irisin with the bone mineral status in the groups of the study.

Materials and methods

Chemicals and Drugs

- Vitamin A was purchased from Sky Medical Company, Assiut, Egypt.
- Vitamin D (cholecalciferol) was purchased from Memphis for pharmaceutical and chemical industries, Cairo, Egypt.
- Kits for serum calcium, phosphorus, alkaline phosphatase, and irisin were purchased from Sky Medical Company, Assiut, Egypt.

Animals and Experimental Design

The Medical Physiology department of South Valley University's Faculty of Medicine in Qena, Egypt, is where this study was carried out. Forty three-month-old male Sprague-Dawley rats in good clinical health, weighing between 200 and 250 grams, were obtained from the animal facility of South

Valley University's Faculty of Veterinary Medicine in Egypt.

Rats were housed in hygienic 42 x 21 x 20 cm stainless steel cages that were well-ventilated and exposed to a natural light/dark cycle. The rats were given unlimited access to clean drinking water and a standard, sanitary laboratory pellet diet. Before the trial started, the rats were given a week to get used to the lab environment.

Official approval for the experimental technique came from the Qena Faculty of Medicine, South Valley University Medical Ethics Committee. Ethical approval code (SVU/MED/PHY003/1 /22/8/422).

Forty male rats were randomly categorized into 4 groups (10 rats per group):

- **Group I (control group):** rats were given distilled water orally.
- **Group II (osteoporosis-model group, OST):** rats were given retinoic acid at a dose of 75 mg/kg body weight/ day by gastric gavage for 2 weeks **(Cheng et al., 2019).**
- **Group III (OST+vitamin D):**rats were given retinoic acid in the same dose and as group II for 2 weeks, followed by 4 weeks of subcutaneous injection of vitamin D (cholecalciferol) at a dose of 4300 IU/rat/week dissolved in 0.5 ml semsem oil **(Nadimi et al., 2019).**
- **Group IV (OST+Swim):**rats were given retinoic acid in the same dose as group II for 2 weeks, followed by low-intensity swimming exercises for additional eight weeks **(Kang et al., 2019).**

Low-intensity swimming exercises

For eight weeks, the rats in the exercise-treated group participated in a swimming program (as swimming reduce stress on bone and joint, support muscle strength that help in support bone, improve balance which lower the risk of fall as well as it improves cardiovascular and respiratory health). For three days (10–45 minutes per day), the rats were acclimated to a 70 cm x 70 cm circular water tank with water kept at 28 ± 2°C. In the first two weeks, the rats swam for 45 minutes daily, and in the next six weeks, they swam for 60 minutes daily, starting at 10 AM **(Kang et al., 2019).**

After the experiment was over, a top-loading balance (Model D0030, A & D Company Limited, USA) was used to weigh the rats. 50 mg/kg of thiopental sodium was used to put the rats to sleep. Rats were put to death by being beheaded.

Blood sampling and biochemical assays

Samples of blood were collected by a cardiac puncture. Once the animal has reached a surgical plane of anesthesia, the animal was positioned in lateral recumbency. Under aseptic conditions with the elbow used to help indicate location along the rib cage, the chest was palpated for strong heartbeat and the needle was inserted into the thoracic cavity where the heartbeat was the strongest.

Blood samples were centrifuged at 2000 rpm for 15 min within 30 min of collection to the determination of the following as follow:

- Serum calcium and phosphorus were measured by the ISE method using a commercial colorimetric kit with Cobas c311 (Roche Diagnostics, USA). The electrolytes levels were measured using a spectrophotometer (UV/VIS spectrophotometer UV752, PEC Medical USA) at a wavelength setting of 540 nm.
- Alkaline phosphatase was measured by colorimetric assay Beckman Coulter OSR6004 device (Beckman Coulter Diagnostics, Ireland). The electrolytes levels were measured using a spectrophotometer (UV/VIS spectrophotometer UV752, PEC Medical USA) at a wavelength setting of 540 nm.
- Irsin level was measured using commercially available ELISA kits (Catalog No.: EKF58236, Biomatik, Ontario, Canada). The O.D. absorbance at 450nm in Microplate Reader provided with the kit immediately after adding the stop solution (Catalog No.: EKF58236, Biomatik, Ontario, Canada).

Histological examination

The preparation of the right femur for histological analysis was done with great care.

All animals' femoral heads were taken out, cleaned of any attached muscle, and then cut. Bone samples were stored in 10% formalin, softened with 5% nitric acid, and prepared for paraffin embedding. Using a rotary microtome, specimens immersed in paraffin were cut into slices that were 5 µm thick. The slides were stained by hematoxylin and eosin stain and observed for histopathological changes **(Kumar, 2014).**

Statistical analysis

Data was gathered, encoded, modified, and entered into version 27 of the Statistical Package for Social Sciences (IBM SPSS). One-way analysis of variance (ANOVA) was used to assess the numerical variables with a parametric distribution, which were reported as mean ± standard deviation. For pairwise comparisons, a post hoc Bonferroni adjustment was then applied. To show possible relationships between serum irisin levels and the evaluated biochemical indicators (calcium, phosphorus, and ALP), Pearson's correlation (r) analysis was performed. For all statistical analyses, a P-value of less than 0.05 was deemed statistically significant.

Results

Weight and Biochemical parameters (Table.1,2 & Figs. 1,2,3).

Rat weight significantly decreased in the OST group (Group II: 201.2 ± 6.32 g) compared to the control group (Group I: 285.7 ± 7.68 g). Supplementation with Vitamin D (Group III: 211.1 ± 7.97 g) resulted in a significant increase compared to the OST group ($P < 0.05$) but was lower than the control. The OST + Swim group (Group IV: 226.6 ± 5.9 g) showed a further significant increase in weight compared to both the OST group and the OST + Vit. D group but rats of this group still had lower weight compared to the controls.

Statistical analysis of the mean values of serum irisin in the groups of the study revealed that a notable decline occurred ($p < 0.01$) in the serum irisin in the OST group (2.15 ± 1.05 ng/ml) in comparison to that of the control group (5.35 ± 2.67 ng/ml). There were significant increases ($p < 0.05$) in the serum irisin in the OST+ vit D (4.74 ± 2.492 ng/ml) and OST+ Swim groups (4.573 ± 0.246 ng/ml) in comparison to the OST group, whereas there were non-significant decreases ($p > 0.05$) in the mean values of serum irisin in each of the OST+ vit D and OST+ Swim groups in comparison to the control group. Comparing the serum irisin in the OST+ vit D and OST+ Swim groups, the change was not statistically significant ($p > 0.05$) in the mean values.

Regarding the serum calcium mean value, it was significantly decreased ($p < 0.001$) in the OST group (9.78 ± 0.73 mg /dl) in comparison to that in the control group (17.1 ± 7.11 mg /dl).There were non-significant decreases ($p > 0.05$) in the mean values of serum calcium in each of the OST+ vit D (14.88 ± 2.80 mg /dl) and OST+ Swim (15.18 ± 1.98 mg /dl) groups in comparison to the control group. On comparing the serum calcium in each of the OST+ vit D and OST+ Swim groups to that of the OST group, they showed significant increase ($p < 0.05$) in the mean values. There was a non-significant change ($p > 0.05$) in the mean values when comparing the OST+ vit D group with the OST+ Swim group.

Regarding phosphorus levels in the blood, there was a marked rise ($p < 0.001$) in each of the OST (7.65 ± 1.01 mg /dl), OST+ vit D (5.64 ± 0.88 mg /dl) and OST+ Swim groups (4.76 ± 0.14 mg /dl) when compared to the control group (3.38 ± 0.7 mg /dl). On the other hand, there were a significant decrease ($p < 0.001$) in the mean values of serum phosphorus in each of OST+ vit D and OST+ Swim groups in comparison to that of the OST group. The mean value of serum phosphorus in the OST+ vit D group showed a non-significant change ($p > 0.05$) when comparing with that in OST+ Swim group.

The mean values of the ALP were significantly increased in each of the OST group (306.47 ± 93.36 IU/L, $p < 0.001$) and the OST+ Swim group (270.51 ± 77.01IU/L, $p < 0.01$) in comparison to the control group (133.48 ± 84.8 IU/L), whereas there was a non-significant increase ($p > 0.05$) in ALP in the OST+ vit D group(205.41 ± 62.1 IU/L) in

comparison to the control group. ALP in the OST+ vit D group was significantly decreased ($p < 0.001$) in comparison to that of the OST group, whereas there was a non-significant decrease ($p > 0.05$) in ALP in the OST+ Swim group in comparison to the OST group. The mean value of serum phosphorus in the OST+ vit D group showed a non-significant decrease ($p > 0.05$) when comparing with that in OST+ Swim group.

Table 2 demonstrated the correlations of serum irisin versus all parameters in the groups of the experimental study. Serum irisin showed a significant moderate positive correlation with the serum calcium ($r = 0.566$, p-value < 0.001), whereas a significant moderate negative correlation ($r = -0.557$, p-value < 0.001) was proved between the serum irisin and the serum phosphorous. There was a non-significant correlation ($r = -0.227$, p-value $= 0.160$) between serum irisin, and alkaline phosphatase.

Table 1. Mean ± SD of weight, biochemical and histological parameters of the groups of the experimental study

Variables	Group I (Control group, n=10)	Group II (OST group n=10)	Group III (OST + Vit. D group, n=10)	Group IV (OST + Swim group, n=10)	P-value*
Rat weight (gm)	285.7 ± 7.68	201.2 ± 6.32 [a]	211.1 ± 7.97 [a, b]	226.6 ± 5.9 [a, b, c]	**<0.001**
Irisin (ng/ml)	5.35 ± 2.67	2.15 ± 1.05[a]	4.74 ± 2.492[b]	4.573 ± 0.246[b, #]	**0.003**
Calcium (mg /dl)	17.1 ±7.11	9.78± 0.73[a]	14.88 ± 2.80[b]	15.18± 1.98[b, #]	**0.001**
Phosphorous (mg /dl)	3.38±0.7	7.65 ± 1.01[a]	5.64 ± 0.88 [a, b]	4.76 ± 0.14[a, b, #]	**<0.001**
ALP (IU/L)	133.48± 84.8	306.47± 93.36[a]	205.41 ±62.1 [b]	270.51± 77.01[a,#]	**<0.001**
Bone trabecular thickness (mm.)	225.15± 3.03	149.60±8.91[a]	256.39±11.82[a, b]	234.50± 14.62[b, c]	**P<0.0001**

All values are expressed as mean ± SD. One-way ANOVA test followed by Bonferroni post hoc for pairwise comparison. The mean difference is significant at $P < 0.05$. P-value*, comparing the different groups of the study using one-way ANOVA; a, Significant difference vs. control group; b, Significant difference vs. OST group, c, significant difference between OST+ vit Dand OST+ Swim groups.# , non-significant difference vs. OST+ vit D group.

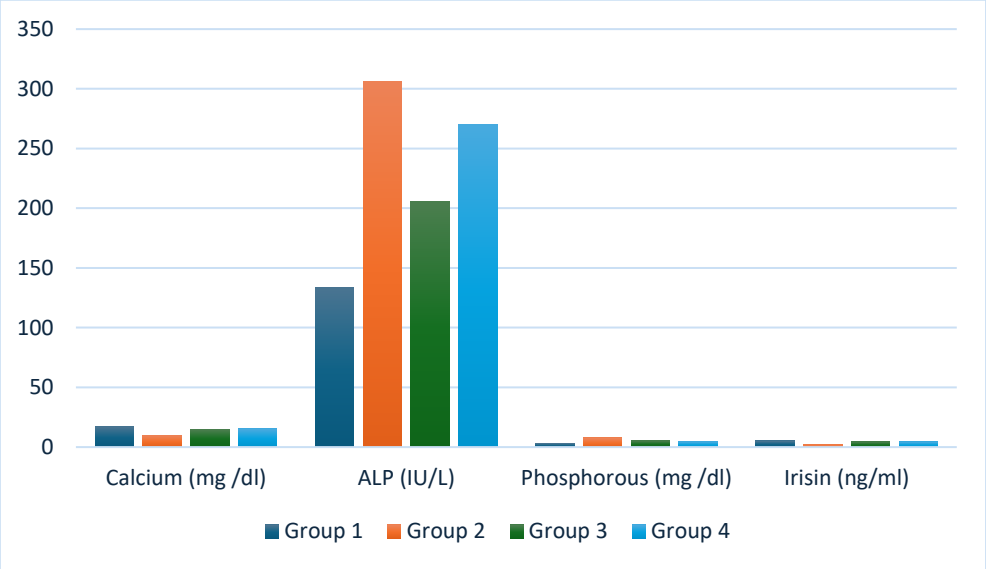

Fig.1. Laboratory data among different tested groups

Table 2. Correlation between Irisin level with serum phosphorous, calcium, and ALP

Parameters	Irisin	
	r*	P value
Phosphorous	-0.557	**<0.001**
Calcium	0.566	**<0.001**
Alkaline phosphatase	-0.227	**0.160**

r*: Pearson correlation coefficient

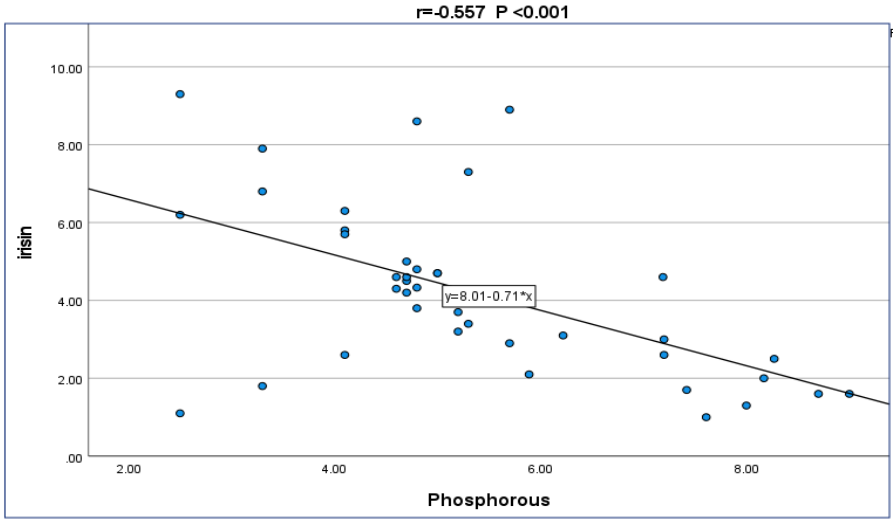

Fig.2. Pearson correlation between irisin and phosphorous

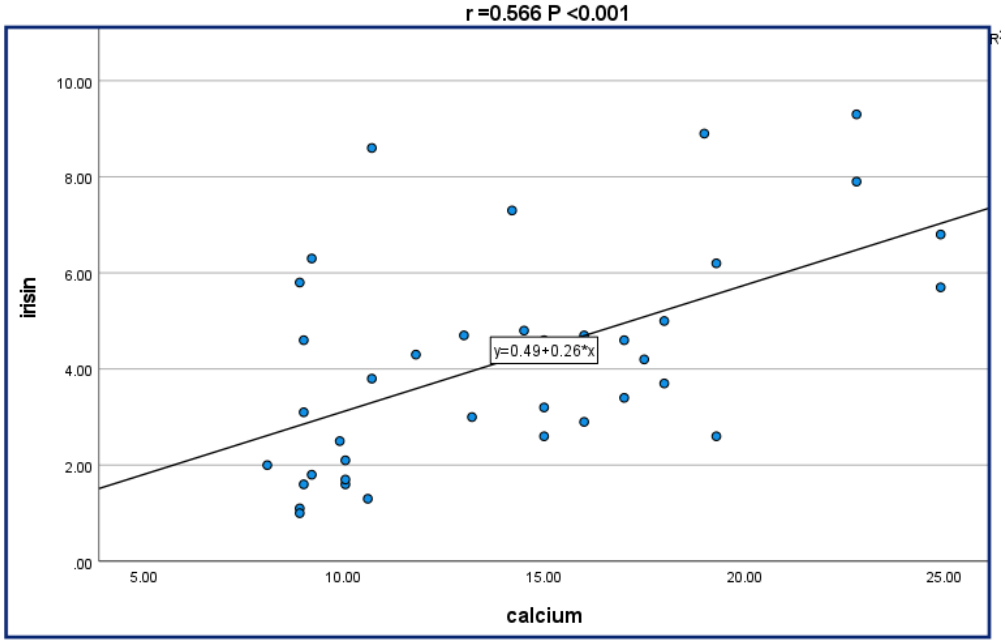

Fig.3. Pearson correlation between irisin and calcium

Histopathological Bone Study: (Figs.4,5)

Slices from the proximal end (head) of the femur in the control group showed a normal anastomosing dense network of bone trabeculae when examined under a microscope. The bone marrow makes up a sizable portion of the intertrabecular cavities and is rich in hematopoietic cells.

In the OST (vit. A- Induced osteoporosis) group, histological analysis demonstrated alterations characterized by the thinning and discontinuity of bone trabeculae, accompanied by regions of reduced bone density, fissures, and microfractures. The gaps in the bone marrow are enlarged

between the trabeculae, exhibiting a higher fat content compared to the control group.
Stained sections from femur heads from either the OST+ vit D and OST+ Swim groups showed apparently similar histological features as in the control group with fewer harmful alterations compared to the OST group. There were relatively residual thinning, pores and splitting in some bone trabeculae which was variable from one animal to other. Bone marrow shows relatively fewer fatty changes than observed in OST group.

As shown in (**Fig.4**), measuring the thickness of bone trabeculae in the groups of the study revealed that there was a significant decrease ($p < 0.0001$) in the mean bone thickness in the OST group in comparison to the control group. There were significant increases in the trabecular bone thickness in the OST+ vit D group in comparison with each of the control group ($p < 0.0001$), OST group ($p < 0.01$)and OST+ Swim group ($p < 0.0001$). Comparing the OST+ Swim group to the control group, there was a non-significant increase ($p > 0.05$) in the bone trabecular thickness mean value.

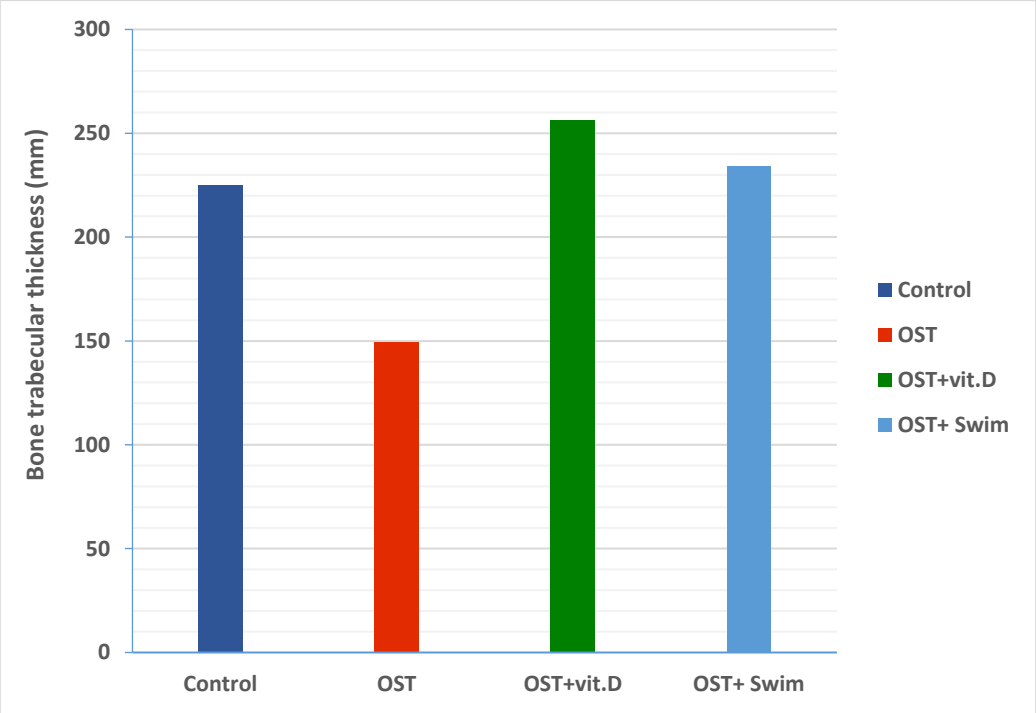

Fig.4. Showing the column graphic presentation for the mean values of the trabecular bone thickness in the experimental groups of the study.

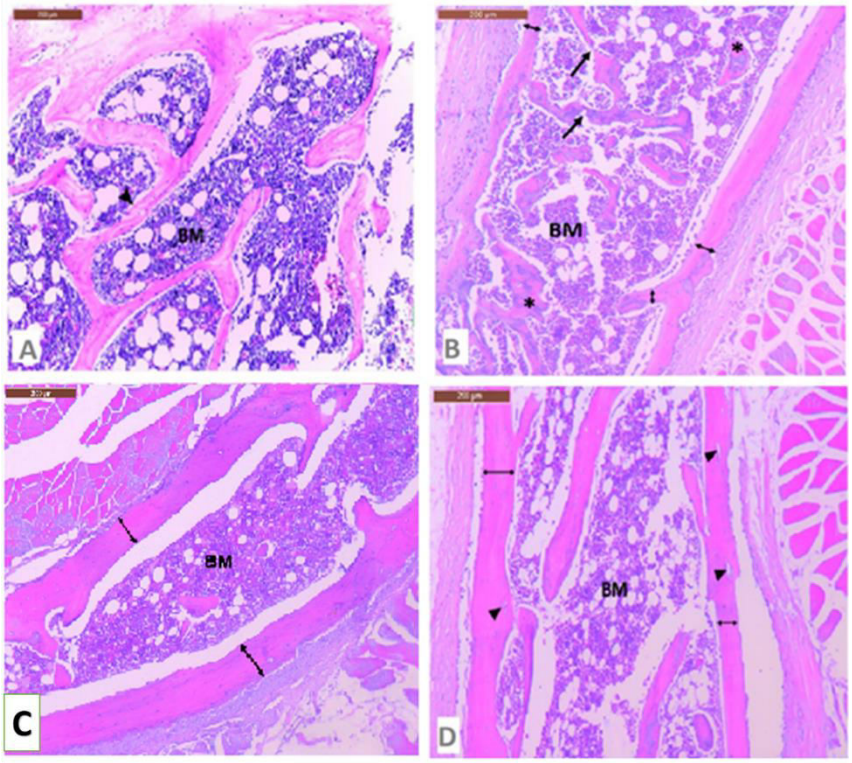

Fig.5. Photomicrographs of sections from the proximal end of the rat femur; **A:** In the control group showing normal trabecular bone structure (black arrow) and bone marrow (BM) fills a large proportion of intertrabecular cavities, (H&E stain, x100). **B:** In OST group (vit. A-induced osteoporosis) showing thinning (double-headed arrow) and discontinuation (arrows) of bone trabeculae with areas of low bone density (asterisks) exhibited by faint blue coloration of bone matrix. Bone marrow spaces (BM) are wide between the trabeculae with increased fat (adipocytes) content (vaculations in bone marrow). (H&E stain, x100).**C**: In the OST+ vit. D group showing increased thickness (Double-headed arrow) of bone trabeculae. There is a residual splitting in some bone trabeculae (curved arrows). Bone marrow (BM) show relatively fewer fatty changes than the untreated diseased group, (H&E stain, x100). **D:**in the OST+ Swim group showing increased thickness (Double-headed arrow) of bone trabeculae, but with reduced bone density at certain parts (asterisks) exhibited by faint blue coloration of bone matrix. There is a residual thinning and pores in some bone trabeculae (triangles). Bone marrow (BM) shows relatively few fatty changes, (H&E stain, x100).

Discussion

Bone loss from osteoporosis often goes unnoticed, making it a silent disease, this disease is regarded one of the most health problems for women at menopause, it results in rapid loss of bone density **(Kulkarni and Kour, 2024).** Among Egyptian women, the prevalence of osteoporosis was 28.4% while among men it was 21.9% **(El Miedany et al., 2024).** Additionally, southern regions had a greater prevalence of hip fractures caused by osteoporosis than northern regions **(El Miedany et al., 2023).**

An essential fat-soluble vitamin, vitamin A improves prenatal growth, reproductive health, immune system performance, and vision. Although getting enough is important, taking too much can be harmful **(Carazo et al., 2021b).** Although it is possible to consume too much preformed vitamin A from animal sources, such as liver, toxicity is mostly linked to the overuse of supplements and the prescription of particular medications **(Carazo et al., 2021b).** These medications are used to treat psoriasis and severe acne **(Al-Abadie et al., 2020).**

In our study, supplementation with vitamin D ± exercise resulted in significant increase in the body weight compared to the osteoporosis group. These findings are supported by **Choi et al., 2013**, who found that adding vitamin D supplementation was associated more increased in the body weight of 9 osteoporotic rats.

In our study, we succeeded in inducing a model of osteoporosis with a high dose of vitamin. The vitamin A-induced osteoporotic group exhibits a substantial reduction in serum calcium because administering high doses of vitamin A may hinder the effects of vitamin D by competition between retinoic acid receptor and vitamin D receptor **(Rehó et al., 2023)** that lead to hypocalcemia **(Yee et al., 2021b).**Vitamin D improves calcium absorption in addition to directly promoting bone synthesis and mineralization, decreasing osteoclast recruitment, and increasing osteoclast activity on osteoblasts **(Ebeling, 2024).** These findings are confirmed in our study, there is a significant increase in serum calcium in Vitamin D treatable group when compared with the osteoporotic group.

In addition, there is a significant decrease in irisin in the osteoporotic group due to the decrease in the effect of vitamin. Vitamin D may facilitate the activation of some proteins and regulators in skeletal muscle cells, such as AMP-activated protein kinase and the silent information regulator sirtuin 1 which enhances irisin and helps other transcription factors to regulate a complicated network of genes **(Safari et al., 2023).** Sanesi et al study showed that vitamin D treatment on myoblasts enhanced Fndc5 mRNA **(Sanesi et al., 2023).** Additionally, in people with vitamin D insufficiency, vitamin D supplementation may raise serum levels of irisin **(Safari et al., 2023).** Additionally, compared to the osteoporotic group, the vitamin D-treated group's serum irisin levels are much higher.

In the exercise group, there is significant increased serum calcium and irisin because swimming as endurance exercise **(Guest et al., 2021)** increases vitamin D and fat metabolism, increases stores of adipose tissue of vitamin D, and promotes the release of hormones that regulate lipolysis, leading to the release of vitamin D metabolites that enhanced calcium absorption and inhibited the release of calcium from the bone to the blood stream, thereby reducing bone loss, as well as swimming may be raising blood irisin **(Su et al., 2020b; Zhang and Cao, 2022).** Some studies show that physical activity can initiate irisin release within skeletal muscle, thereby prompting the expression of (FNDC5); the precursor of irisin, and subsequently augmenting irisin production **(Dehkordi and Jafari, 2022).**

Also, in the osteoporotic group, there is a significant increase in serum phosphorus due to hypocalcemia **(Lombardi et al., 2020)** and increased alkaline phosphatase because low bone mineral density causes the reactivation of quiescent osteoblasts. These osteoblasts produce a considerable amount of bone alkaline phosphatase **(Cheng and Zhao, 2023)** so total alkaline phosphatase measurement is used as a biomarker for early diagnosis of osteoporosis diagnosis and follow-up treatment **(Shu et al., 2022).** When bone deposition of calcium salt is inadequate, bone tissue production of bone alkaline phosphatase increases **(Cheng and Zhao, 2023).** It was confirmed by histopathology. In our study, there is a significant decrease in serum alkaline phosphatase and phosphorus in Vitamin D treatable group when compared with the osteoporotic group, because hydroxyapatite crystals are created when calcium and phosphorus combine to mineralize and strengthen bone **(Ahmed et al., 2023).** Furthermore, we confirmed by improved findings in histopathological examination in the treated group with vitamin D.

Additionally, when comparing the exercise group to the osteoporotic group, there are non-significant decrease in serum alkaline phosphatase and a significant decrease in phosphorus because swimming improves the effect of vitamin D in the body **(Ahmed et al., 2023).**

Conclusion

This study examines the impact of irisin, vitamin D, and exercise on vitamin A-induced osteoporosis in male rats. The researchers compared the effects of vitamin D supplementation and swimming exercise on improving osteoporosis in the rat model. The study found that vitamin D supplementation over 4 weeks had better effects on improving osteoporosis than exercise over 8 weeks. However, the difference between the two treatments was not statistically significant. The study also measured the levels of calcium, phosphorus, alkaline phosphatase, and irisin in the rats and found significant differences between the osteoporotic group and the control group, as well as differences between the treated groups and the control group.

In comparison to other research in the field, this study adds to the existing knowledge by specifically examining the effects of irisin, vitamin D, and exercise on vitamin A-induced osteoporosis. However, further research is needed to validate these findings and compare them to other studies that have explored similar interventions for osteoporosis treatment.

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