Zagazig University Medical Journal
www.zumj.journals.ekb.eg
http:// 10.21608/ZUMJ.2024.273508.3214 Volume 30, Issue 4, July 2024
Manuscript ID ZUMJ-2402-3214 (R1)
DOI 10.21608/ZUMJ.2024.273508.3214

ORIGINAL ARTICLE

Musculoskeletal effect of ActRIIB-Fc Fusion protein ligand alone or concomitant with swimming exercise in ovariectomized adult albino rats

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Submit Date 04-05-2024
Revise Date 30-05-2024
Accept Date 2024-05-31

Abstract:

In postmenopausal osteoporosis, hormonal alteration leads to bone turnover and muscle weakness. Activin typeIIB receptors were revealed to increase bone & muscle mass. This experiment was designed to explore ActRIIB-Fc-ligands administration alone or with swimming-exercise might restore musculoskeletal system homeostasis followed negative effects of estrogen deficiency in ovariectomized rat model.

Materials & Methods: Fifty adult female albino rats were divided into 5groups: Group I (control sham rats) were subjected to i.p. PBS. In the other groups rats were exposed to ovariectomy procedures. Group II, III, IV rats were exposed to i.p. PBS, exercise-training-protocol, i.p. ActRIIB-Fc (5mg/kg/week) 7weeks respectively, while groupV rats were exposed to exercise-training-protocol and i.p ActRIIB-Fc. Finally, body weights & muscle strength tests were examined for all animals. After scarification, uterine weights and muscle mass (quadriceps femoris & gastrocnemius) measurements were obtained. Serum biochemical and histopathological parameters were examined; also, RNA was extracted from their right femora to perform genes expression.

Results: Exercise-training and ActRIIB-Fc were capable to reverse changes of muscle strength and bone mass measurements of OVX rats. Significant elevation in osteocalcin & P1NP and significant reduction in CTX, TOS, TAC, IL-1β, IL-6, and CK values, moreover, down-regulation of RANKL and up-regulation of OPG genes expression were detected. These significant values and histomorphometric osteoporotic variations were more pronounced in groupV.

Conclusion: Addition of ActRIIB-Fc ligand to exercise-training-protocol act as a musculoskeletal-enhancing agent in OVX rats. These results were proved by reduction of osteoclast function and elevation of some osteoblast genes expression; therefore, it could be a respectable choice in musculoskeletal disorders management.

List of abbreviations:
(ActRIIB-Fc) Activin Receptor IIB Fc-ligand
(i.p.) Intraperitoneal
(PBS) Phosphate Buffered saline
(OVX) Ovariectomy
(CTX) C-terminal cross-linking telopeptide of type I collagen
(P1NP) Procollagen type 1 N-terminal Propeptide
(CK) Creatine Kinase
(OPG) osteoprotegerin
(RANKL) Receptor Activator of Nuclear factor Kappa beta Ligand

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Introduction

Ovarian function decline in elderly women leads to sex hormones insufficiency and menopause. Estrogen-dependent signaling disturbances have been shown to promote osteoclast activity, disturb osteoblastic functions and increase the hazard of bone fractures [1]**.** Also, skeletal muscle tissue has estrogen receptors. These receptors modify the expression of myosin heavy chain (MHC) isoform and reduce muscle damage [2].

Estrogen deficiency induces decrease phosphorylation of muscle protein kinase Akt, which decline muscle mass, strength and physical activity [3]. Release of cytochrome c to the cytosol from mitochondrial membrane initiates caspase-3, caspase-9 and results in DNA fragmentation and cell death. Estrogen has a critical role in the inhibition of mitochondria mediated apoptosis in skeletal muscles [4].

Exercise training is considered a safe low-cost non-pharmacological routine for skeletal & muscle health preservation after menopause [5]. It is widely accepted that exercise training increases muscle mass and enhances the osteoblast activity [6]. Exact mechanisms which improve muscle & bone health via exercise are not completely explained yet.

Activins are belonging to the TGF-β superfamily show several systemic and physiological roles [7]. Myostatin inhibits muscle growth and decrease muscle mass. Activins/myostatin signal pathway inhibition via activin IIA or IIB receptors has as a possible therapeutic approach for osteoporosis [8]. Activins role in muscle and bone physiology are still unclear.

Interestingly, ActRIIB-Fc can block both myostatin & activin A [9], that could be possibly very useful in conditions, such as duchenne muscular dystrophy (DMD), which include both bone and muscle [10].

According to the preceding data, this experiment aimed to detect the exercise effects on musculoskeletal system in ovariectomized rat model as well as the interaction of ActRIIB-Fc ligand blocking with the physical activity.

Material and methods

Fifty adult female wistar albino rats (140–230 g) were collected from Animal House, Faculty of Medicine Zagazig University then kept under hygienic conditions in steel wire cages (50 x 30 x 20 cm), 5 rats per cage under a strict a 12:12 h dark–light sequence at a 20 to 24 °C with usual diet pellets and freely access to tap water. This experiment was done from November 2022 to April 2023. The protocol of this study was done according to the data guiding animal research and approved by Institutional Animal Care and Use Committee (IACUC), Zagazig University (**ZU-IACUC**/3**3/F/2**5 6**6/20**2**23).**

After 2 weeks of acclimatization, we equally divided all rats randomly into 5 groups; (*n*=10 rats in each group):

1. **Group (I) control sham operated group:** The ovaries of each rat were exposed but left intact and after 6 weeks they will be intraperitoneally (i.p.) injected with the phosphate buffered saline (PBS) vehicle once weekly for 7 weeks.
2. **Group (II) ovariectomized group (OVX):** Rats in this group underwent surgical bilateral ovariectomy after anesthetization and after 6 weeks injected intraperitoneally (i.p.) with (PBS) vehicle once/week for 7 weeks.
3. **Group (III) OVX+exercise trained group:** 6 weeks after ovariectomy rats in this group underwent resistant swimming training (30 min 5 days/week for 7 weeks) and intraperitoneally (i.p.) injected with (PBS) vehicle once weekly.
4. **Group (IV) OVX+ActRIIB-Fc treated group:** 6 weeks after ovariectomy, rats were injected intraperitoneally (i.p.) with ActRIIB-Fc (purchased from Phoenix Pharmaceuticals; Belmont, CA, USA, catalog no. VAC-0340-0.5) dissolved in PBS 5 mg/kg once /week for 7 weeks [11].
5. **Group (V) OVX+exercise trained+ActRIIB-Fc treated group:** 6 weeks after ovariectomy, rats in this group underwent resistant swimming training 30 min 5 days/week and injected intraperitoneally (i.p.) with ActRIIB-Fc dissolved in PBS 5 mg/kg once/week for 7 weeks.

Ovariectomy procedure*:*

After overnight fasting, rats were anesthetized with 2% Na pentobarbital by (0.2 mL/100 g i.p). Central midline slit of 1 cm was made, and then the ovaries were palpated and bilaterally excised. Then the peritoneum and the muscle layer were closed separately. The skin was sutured; then, post-

operative analgesia and gentamicin will be administered. In sham-operated groups the similar procedures were done but the ovaries were not removed. Afterward, recovery was allowed for rats and then re-grouped. Then, hormonal status was inspected by vaginal smears attained at 10 am daily [12].

Determination of estrous cycle

Daily at 10 am vaginal smears were achieved to determine estrous phases. Unstained vaginal fluids were examined microscopically and comparison between the groups done in the mean frequency of diestrus, metestrus, proestrus, and estrus phases 4-5 days cycles were considered regular. Success of the ovariectomy procedure was confirmed by constant leukocytes in the vaginal smears [13].

Training protocol:

It was done once daily 5 days/week according to **Cunha et al.** [14]. Group III&V rats were trained by swimming (30 min/day) in tank *(80 cm depth, 60 cm width and 75 cm length).* It was occurred one week for adaptation. In second week, exercise training was performed as follow; 4 sets of 10 jump with recovery periods about 30s in between sets. For augmentation of the exercise strength rats were allowed to carry additional load, the extra load during the second week (10% body weight tied to the chest), during third and fourth weeks (20% body weight) & during the sixth and seventh weeks (30% of body weight).

Muscle strength evaluation:

Wire hang test

To assess motor strength, it was done according to **Nishitani et al.** [15]. A 60 cm rope was fixed above floor and each rat was exposed to grasp it with forelimbs. 6 point scale scores: {0-fall off}; {1-hanging the rope by forelimbs}; {2-same as 1 but attempting to climb the rope}; {3-same as 1 plus one or both hind limbs}; {4-same as 3 plus tail wrapped around the rope}; and {5-escaping}. Latency to fall was also measured. Maximum score and duration attained from the 3 trials was documented.

Rotaroad test

To assess motor ability, rotarod device was used; thirty min training were done on the rotating rod. Then, rats were examined on the rod at speed (5min). The test was done 3trials. Maximum time among the 3trials was documented [16].

Uterine weight (UW) and muscle mass measurements of the studied groups:

All rats were weighed once/week. After sacrification of all rats, quadriceps femoris and gastrocnemius muscles were immediately weighed and frozen in liquid nitrogen [17]. Its weights reported as the average right and left leg weights. Also, rat uteri were isolated and weighed with analytical scale immediately after dissection to determine estrogenic status.

Serum samples and biochemical analysis:

Blood were collected under anesthesia from the tail vein, serum obtained by centrifugation then preserved at−80°C [18] until they were tested. Commercial ELISA kits were used to measure serum levels of serum estradiol (E2) (Antibodies .com, USA, Catalog No.A80286), vitamin D3 (25OHD3) (Eaglebio.com, USA, Catalog No.VID21-K01), calcium (Ca) (Mybiosource.com, USA, Catalog No.MBS3807893), phosphorus (P) (Mybiosource.com, USA, Catalog No.MBS3809130), osteocalcin (Eaglebio.com, USA, Catalog No.OST31-K01), procollagen type 1 N-terminal propeptide (P1NP) as bone-formation marker (Elabscience.com, USA, Catalog No.E-EL-R1414), C-terminal cross-linking telopeptide of type I collagen (CTX) as a bone resorption marker (Elabscience.com, USA, Catalog No.E-EL-R1456), creatine kinase (CK) as muscle damage marker (Mybiosource.com, USA, Catalog No.MBS1600481), total antioxidant capacity (TAC) as a measure of antioxidant defense system (Afgsci.com, USA, Catalog No.EK720634), total oxidant status (TOS) as oxidant stress parameters (Afgsci.com, USA, Catalog No.EK720417) and interleukin-1β (IL-1β) (Elabscience.com Catalog, USA, No.E-EL-R0012), interleukin-6 (IL-6) (Elabscience.com, USA, Catalog No.E-EL-R0015) as inflammation markers were measured.. Serum osteocalcin concentration was assessed according to **Risteli and Risteli** method [19]. VitD (25OHD3) was tested using hormone autoanalyzer in the Biochemistry Laboratory, Faculty of Medicine Zagazig University [20].

Measurement of Gene Expression

RNAs were extracted from the right femora of all rats by osteotomy to proximal &distal parts of the bone then centrifuging it to take out bone marrow. Total RNA was isolated followed by DNAase treatment and RNA clean-up. The cDNA was then created from 1 µg of RNA with Sensi-FAST probekit. Quantitative PCR was done using iQ SYBR Green Supermix (Bio-Rad laboratories, USA). The osteoprotegerin (OPG) and NF-κB receptor activator ligand (RANKL) mRNA expression levels were measured using the $2^{-\Delta\Delta C}T$ method. Internal control *β-actin* was used. Primer sequences are listed in table1.

Table 1: Primers sequence for OPG, RANKL and β-actin genes

Primer	primer sequence
OPG	F: GTAGGTGCCAGGAGCCATT R: CAATGAACAAGTGGCTGTGC
RANKL	F: TGAAGACACACTACCTGACTCCTG R: CCACAATGTGTTGCAGTTCC
β-actin	F: CGTGGGCCGCCCTAGGCACCA R: TTGGCCTTAGGGTTCAGGGGG

F forward primer, *R* reverse primer

Histopathology sampling

After scarifying of all rats, left femurs were immediately fixed in 10% formal saline solution. Then, the upper portions were immersed in EDTA. Samples fixed in paraffin and sectioned into 6-µm slice and stained by:

- Hematoxylin&Eosin (H&E) [21].
- Immunohistochemical OPN, bone remodeling biomarker (osteopontin) staining of specimens: sections were incubated in a rabbit anti-OPN polyclonal antibody (San Diego, CA, USA). OPN +ve immunoreactivity showed cement lines, bone matrix osteocytes and osteoblasts. 1ry antibodies were exchanged with a buffer solution to get -ve control sections, while osteosarcoma sample was used as a +ve control [22].

Bone histomorphometry

Morphometry was performed in Zagazig University (Anatomy and Embryology Department) Faculty of Medicine, using analyzer (Leica Qwin 500 image analyzer computer system), each H&E stained section was subjected for measuring the cortical thickness (µm) using image analysis system in random microscopic regions at 100 power fields. Ten interpretations were achieved for each specimen; 5 on the lateral and 5 on the medial cortical bone [23]. Subsequently, the area % of +ve OPN immune-staining was obtained.

Statistical Analysis:

All studied parameters, were analyzed by (ANOVA) one-way analysis of variance and Tukey HSD for Post hoc multiple comparisons were used to compare means. Data were offered as mean±standard deviation (SD). The software, IBM Statistical Package for Social Sciences (SPSS) Version 26 Software for Windows (SPSS, Inc., Chicago, IL, USA) was used. P value ≤ 0.05 was set as statistically significant.

Results

Wires hang test results in the studied groups:

OVX rats were not capable to wrap their tails around the rope and fell down rapidly than control sham rats. Average maximum score to fall in OVX group were significantly (P<0.001) reduced than those of control sham rats: score, (2.0±0.8 vs. 3.7±0.4; latency, 28.4±3.0 vs. 55.2±3.7 (Table2). On the contrary, exercise and ActRIIB-Fc treatment led to improve its results in the OVX+exercise trained group, OVX+ActRIIB-Fc treated and OVX+exercise trained+ActRIIB-Fc treated groups when compared with OVX group. The average maximum score to fall in OVX+exercise trained, OVX+ActRIIB-Fc treated and OVX+exercise trained+ActRIIB-Fc treated rats were significantly (P<0.001) better than those of OVX rats: score, (2.7±0.6, 2.8±0.7, 3.2±0.6 respectively vs. 2.0±0.8; latency, (35.6±4.2, 39.3±5.8, 42.9±4.3 respectively vs. 28.4±3.0) (Table2).

Rotarod tests results in the studied groups:

To evaluate motor ability, we made rotarod tests. The maximum latency to fall in OVX rats was significantly shorter (P<0.001) than control sham rats: 58.5±15.4 s vs. 172.3±13.9 s. Exercise and ActRIIB-Fc treatment significantly (P<0.001) improve the maximum latency to fall of OVX+exercise trained, OVX+ActRIIB-Fc treated and OVX+exercise trained+ActRIIB-Fc treated rats when compared with OVX rats: 88.3±22.2, 104.5±18.7, 126.4±25.6 respectively vs.58.5±15.4 s (Table 2).

Table 2: Muscle strength tests evaluation in different studied groups

Muscle tests	Control group	OVX group	OVX+exercise trained group	OVX+ActRIIB-Fc treated group	OVX+exercise trained+ActRIIB-Fc treated group
Wire hanging test *Score*	3.7±0.4	2.0±0.3 [a]	2.7±0.6 [a&b]	2.8±0.7 [a,b&c]	3.2±0.6 [a,b,c&d]
Wire hanging test *duration* (sec)	55.2±3.7	28.4±3.0 [a]	35.6±4.2 [a&b]	39.3±5.8 [a,b&c]	42.9±4.3 [a,b,c&d]
Rotarod test Latency (sec)	172.3±13.9	58.5±15.4 [a]	88.3±22.2 [a&b]	104.5±18.7 [a,b&c]	126.4±25.6 [a,b,c&d]

Data was expressed as mean±SD. [a] P<0.05 when compared with control sham group, [b] P<0.05 when compared with OVX group, [c] P<0.05 when compared with OVX+exercise trained group, [d] P<0.05 when compared with OVX+ActRIIB-Fc treated group.

Body weight changes, uterine weight and muscle mass in all studied groups:

Significant increase in final body weight and significant decrease in uterine weight and quadriceps femoris & gastrocnemius mass values (p<0.001) were observed in all OVX groups in comparison to control sham group. The OVX+exercise trained, OVX+ActRIIB-Fc treated and OVX+exercise trained+ActRIIB-Fc treated groups showed significant decrease in final body weight and a significant increase in uterine weight and quadriceps femoris & gastrocnemius mass values (p<0.001) in comparison to that in OVX group. These significant values were more pronounced in OVX+exercise trained+ActRIIB-Fc treated rats when compared with exercise OVX and ActRIIB-Fc treated OVX groups**,** while the changes in the uterine weight and quadriceps femoris & gastrocnemius mass showed no significance between OVX+exercise trained and OVX+ActRIIB-Fc treated groups (Table 3).

Table 3: Biochemical changes in different studied groups

Parameter	Control group	OVX group	OVX+exercise trained group	OVX+ActRIIB-Fc treated group	OVX+exercise trained+ActRIIB-Fc treated group
Final BW (g)	268±8.92	357±5.01[a]	325±7.23 [a&b]	314±7.02 [a,b&c]	306±10.17 [a,b,c&d]
UW (g)	2.52±0.35	0.69±0.08 [a]	1.25±0.25 [a&b]	1.30±0.33 [a&b]	1.90±0.45 [a,b,c&d]
Muscle mass (mg)	380±7.61	335±6.9 [a]	349±7.2 [a&b]	350±5.8 [a&b]	360±5.7 [a,b,c&d]
E2 (ng/L)	28.4 ±3.5	16.7 ±4.1 [a]	19.2 ± 3.9 [a&b]	22.8 ±5.0 [a,b&c]	23.3 ±4.1 [a,b&c]
VitD3 (25OHD3) (ng/mL)	0.39±0.04	0.20±0.02 [a]	0.27±0.03 [a&b]	0.26±0.04 [a&b]	0.28 ±0.06 [a&b]
Serum ca (mg/dl)	11.37 ±0.45	8.76 ±0.23 [a]	9.35 ±0.29 [a&b]	9.43 ±0.38 [a&b]	10.05 ±0.31 [a,b,c&d]
Serum p (mg/dl)	4.74±0.32	6.03±0.41[a]	5.36±0.43 [a&b]	5.29±0.37 [a,b&c]	5.16±0.29 [a,b,c&d]
Serum osteocalcin (mg/dl)	97.33±2.23	83.27±1.53 [a]	86.54±2.09 [a&b]	88.86±3.42 [a,b&c]	88.94±1.20 [a,b&c]

Parameter	Control group	OVX group	OVX+exercise trained group	OVX+ActRIIB-Fc treated group	OVX+exercise trained+ActRIIB-Fc treated group
Serum PINP (ng/ml)	2.6±0.6	1.18±0.2 [a]	1.20±0.3 [a]	1.93±0.5 [a,b&c]	1.94±0.6 [a,b,c]
Serum CTX (ng/ml)	5.43±0.7	8.91 ±1.4 [a]	7.65±0.9 [a&b]	7.43 ±1.3 [a,b&c]	6.89 ±0.8 [a,b,c&d]
Serum CK (U/L)	274.8±33.5	584.2±45.9 [a]	579.6±36.4 [a]	402.7±27.6 [a,b&c]	493.6±39.2 [a,b,c]
Serum TOS (µmol H2O2 Equiv/l)	1.8±0.5	4.7±.0.7 [a]	3.3±0.5 [a&b]	2.9±0.3 [a,b&c]	2.8±0.4 [a,b&c]
Serum TAC (mmol Trolox Equiv/l)	1.9±0.4	0.6±0.1 [a]	1.0±0.2 [a&b]	1.2±0.3 [a&b]	1.5±0.3 [a,b,c&d]
Serum IL-1β (pg/mL)	148.8±22.5	368.3±35.7 [a]	327.3±41.2 [a&b]	319.5±43.8 [a&b]	290.8±39.3 [a,b,c&d]
Serum IL-6 (pg/mL)	156±37.9	481.6±29.5 [a]	440.4±23.6 [a&b]	437.0±32.3 [a,b]	399.5±25.3 [a,b,c&d]

Data was expressed as mean±SD. a P<0.05 when compared with control sham group, b P<0.05 when compared with OVX group, c P<0.05 when compared with OVX+exercise trained group, d P<0.05 when compared with OVX+ActRIIB-Fc treated group. Final BW, final body weight; UW, uterine weight; E2, estradiol; PINP, procollagen type 1 N-terminal propeptide; CTX, C-terminal cross-linking telopeptide of type I collagen; TOS, total oxidant status; TAC, total antioxidant capacity, IL-1β, interleukin-1β; IL-6, interleukin-6; CK, creatine kinase.

Change of serum estradiol (E2), Vitamin D3 (25OHD3), calcium and phosphorus levels in the studied groups:

The current data shown in table3 revealed significant decline in serum E2, 25OHD3 and calcium levels, also, a significant rise in phosphorus values (p<0.001) in all OVX rats when compared to control sham group**.** On the other hand, compared with OVX group, significant increase was noticed in serum E2, 25OHD3 and calcium levels in addition to significant decrease (p<0.001) in serum phosphorus values in OVX+exercise trained, OVX+ActRIIB-Fc treated and OVX+exercise trained+ActRIIB-Fc treated groups, while the changes in the 25OHD3 and calcium levels showed no significant between OVX+exercise trained and OVX+ActRIIB-Fc treated groups.

Change of bone biomarkers in the studied groups:

Significant decrease was observed in these bone biomarkers of all OVX groups compared to the control sham group. Regarding osteocalcin a significant (P<0.001) increase was noticed in the OVX+exercise trained, OVX+ActRIIB-Fc treated and OVX+exercise trained+ActRIIB-Fc treated groups, while the changes in serum osteocalcin levels were not significant between the OVX+ActRIIB-Fc treated and OVX+exercise trained+ActRIIB-Fc treated groups. However, significant increase was found in P1NP with ActIIb-Fc administrated groups (IV &V) but not with exercise trained group (Table 3).

Moreover, a significant increase was observed in CTX, a bone resorption markers, levels (P<0.001) of all OVX groups compared with the control sham group. Comparison with the OVX group, a significant (P<0.001) decrease was detected in this biomarker in the OVX+exercise trained, OVX+ActRIIB-Fc treated and OVX+exercise trained+ActRIIB-Fc treated groups (Table 3).

Change of total oxidant status (TOS) and total antioxidant capacity (TAC) in the serum of studied groups:

The current data in table 3 revealed significant increases in serum oxidant stress parameters (TOS values) and significant decrease (TAC values) (P<0.001) in all OVX rats when compared to control sham group. On the other hand, comparison with the OVX group, a significant decrease was noticed in serum TOS levels in addition to a

significant increase (p<0.001) in serum TAC values in OVX+exercise trained, OVX+ActRIIB-Fc treated and OVX+exercise trained+ActRIIB-Fc treated groups.

while the changes in serum TAC values showed no significant between the OVX+exercise trained and OVX+ActRIIB-Fc treated groups, But TOS values revealed significant decrease (P<0.001) between both of them and a non-significant changes between OVX+ActRIIB-Fc treated and OVX+exercise trained+ActRIIB-Fc treated groups.

Changes in serum (IL-1β and IL−6) and creatine kinase (CK) among the studied groups:

Values of IL-1β and IL-6 as (pro-inflammatory cytokines), also, creatine kinase (CK) as (muscle damage marker) revealed significant increases in all OVX rats when compared to control sham group.

Regarding IL-1β and IL-6 a significant decrease were detected in these biomarkers in the OVX+exercise trained and OVX+exercise trained+ActRIIB-Fc treated groups when compared to OVX and OVX+ActRIIB-Fc treated groups respectively, however non-significant changes where found with ActRIIB-Fc administration. However, a significant decrease was found in CK with ActIIb-Fc administrated groups (group IV &V) but not with exercise trained group (group III) (Table 3).

Changes in gene expression of bone formation and resorption among the studied groups:

Compared to sham group, OPG expressions were significantly reduced while RANKL was markedly increased in all OVX rats. On the contrary, exercise and ActRIIB-Fc treatment led to up-regulation of OPG gene, also, down-regulation of RANKL gene in the OVX+exercise trained, OVX+ActRIIB-Fc treated and OVX+exercise trained+ActRIIB-Fc treated groups when compared with OVX group. These significant values were more pronounced in exercise and ActRIIB-Fc treated OVX rats when compared with exercise OVX and ActRIIB-Fc treated OVX groups (Fig.1).

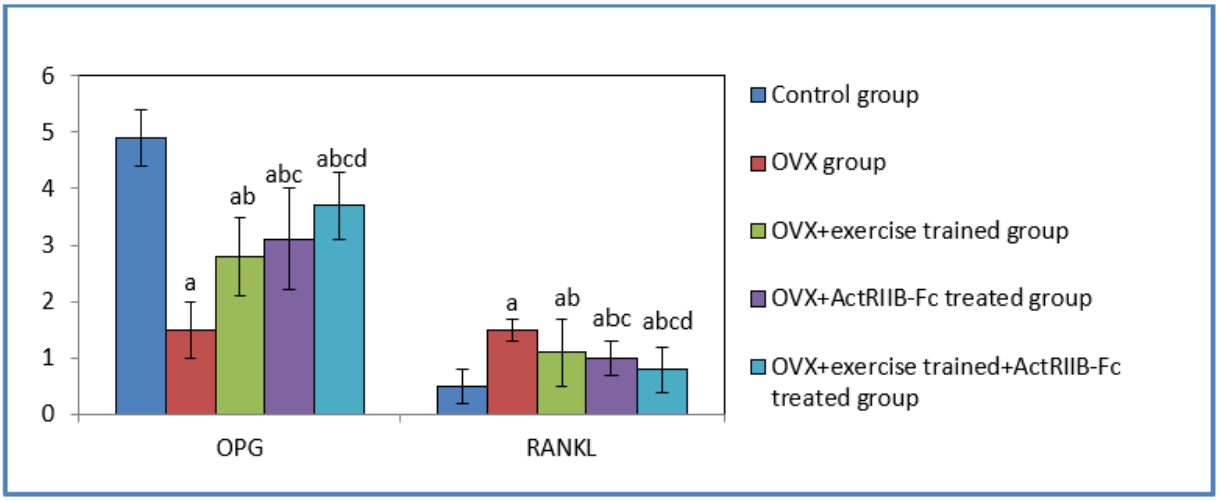

Fig. 1: Expression m RNA levels of OPG and RANKL in each study group: Data was expressed as mean±SD. a P<0.05 when compared with control sham group, b P<0.05 when compared with OVX group, c P<0.05 when compared with OVX+exercise trained group, d P<0.05 when compared with OVX+ActRIIB-Fc treated group. osteoprotegerin gene (OPG**)**; receptor activator of nuclear factor κB (RANKL).

Histopathological results

H&E

Sham group femurs sections showed typical cortical structure, as it appeared with external layer of periosteum, and subperiosteal formation appearance. Additionally, compact bone appeared with homogenous matrix, circumferential lamella, including Haversian canals with concentric lamella arranged around them in addition to osteocytes inside their lacunae. The endosteum was lined with

osteoblasts (Fig. 2a). The cortical bone in the femur of the OVX group also showed thick irregular periosteum, irregular osteoporotic cavities with granulation tissues inside and osteoclasts near them. Osteoclasts appeared with acidophilic cytoplasm and multiple nucleus (Fig.2b). Femur shaft of the OVX+Exercise group seemed to have tinny fibrous periosteum and exhibited many large and empty lacunae and small osteoporotic cavities (Fig.2c). Most of those changes were slighter in the OVX+ActRIIB-Fc treated group , as the longitudinal section of rat femurs revealed outer periosteal layer thinning, the compact bone had circumferential lamellae and osteocytes that were located in their lacunae, with little osteoprotic cavities (Fig.2d). Likewise, the OVX+exercise trained+ActRIIB-Fc treated group femur showed nearly normal cortical structure with few empty lacunae (Fig.2e).

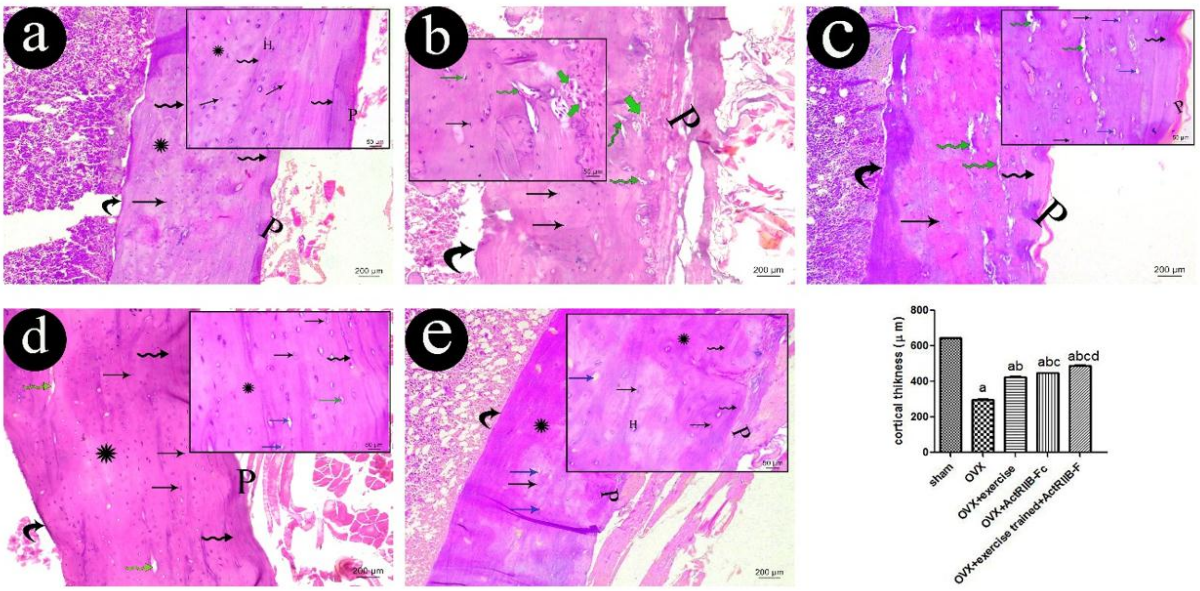

Fig.2. Photomicrograph of rat femur in different experimental groups. (a) the sham group , (b) OVX group, (c) OVX+exercise trained group, (d) OVX+ActRIIB-Fc treated group, (e) OVX+exercise trained+ActRIIB-Fc treated group , femur cortex is formed of periosteum (P), cement basophilic line (zigzag arrow), circumferential lamella around Harversian canal (H), osteocytes within lacunae (arrow), homogenous acidophilic matrix (asterisk), endosteum is lined with osteoblast (curved arrow), multiple cavities (green zigzag arrow), dark stained osteocytes within wide lacunae(green arrow), empty lacunae (blue arrow), osteoclasts (thick green arrow) (H&E X100&400). Bar chart: showing statistical assessment of the cortical thickness (um) in different studied groups. The values are presented as mean ± SD. P value<0.05 was considered significant. (1-a P value<0.05 versus sham group, 2- b P value<0.05 versus OVX group, 3- c P< 0.05 versus OVX+exercise trained group, 4- d P< 0.05 versus OVX+ActRIIB-Fc treated group).

Immunohistochemical results

Osteopontin immune-stained sections of the sham group showed obvious expression in the bone matrix (Fig.2a), whereas OVX group showed obvious reduced expression (Fig.2b). Moreover, the OVX + Exercise group revealed mild OPN expression (Fig.2c). However, OVX+ActRIIB-Fc treated group showed moderate elevation in OPN expression (Fig.2d), OPN expression showed marked increase in cortical bone of OVX+exercise trained+ActRIIB-Fc treated group (Fig.2e).

Morphometric results

Significant decrease in cortical bones thickness of OVX group (295.6±12.32) when compared with sham group (643.1±6.677). Furthermore, compared with OVX groups, including OVX+Exercise (422.8± 13.28), OVX+ActRIIB-Fc treated (447.4±5.863), OVX+exercise trained+ActRIIB-Fc

treated group (487.2±19.41), results only showed a significant increase in cortical bone thickness (Fig.2.Bar chart). The area percentage of OPN expression of bone matrix of femur cortical bone also showed a significant decrease in the OVX group (5.607± 0.8705) compared with that of the sham group (20.74±1.831). On the other hand, compared with the OVX group, including OVX + Exercise (7.353± 0.6893), OVX+ActRIIB-Fc treated (11.23± 1.055), OVX+exercise trained+ActRIIB-Fc treated group (14.27±1.061), results showed a significant increase in the area percentage of OPN expression within the bone matrix of femur cortical bone (Fig.3.Bar chart).

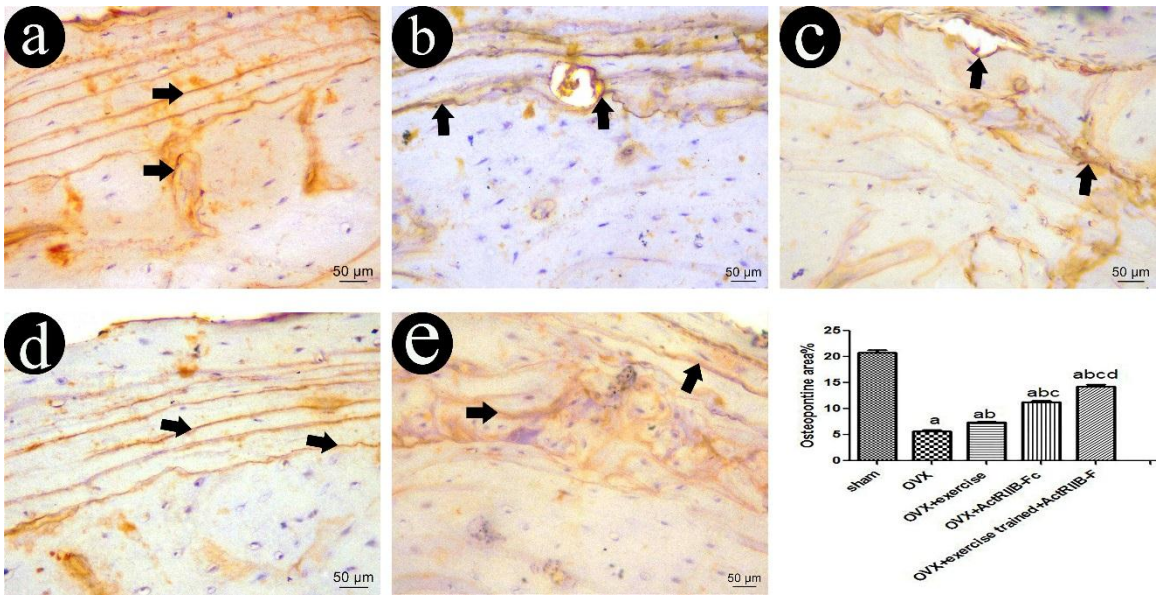

Fig.3. Photomicrograph of rat femurs stained by Osteopontin (OPN) immunohistochemical. (a) Sham group, (b) OVX group, (c) OVX+exercise trained group, (d) OVX+ActRIIB-Fc treated group, (e) OVX+exercise trained+ActRIIB-Fc treated group ,positive expression of osteopontin (thick arrow) (OPN X400). Bar chart: showing statistical assessment of the area % of OPN immune-stained positive expression in different studied groups. The values are presented as mean ± SD. P value<0.05 was considered significant. (1-a P value<0.05 versus sham group, 2- b P value<0.05 versus OVX group, 3- c P< 0.05 versus OVX+exercise trained group, 4- d P< 0.05 versus OVX+ActRIIB-Fc treated group).

Discussion

Bone turnover and muscle weakness occurred in post menopause due to hormonal alterations [24]. In this study, osteoporosis was induced by ovariectomy, and then the musculoskeletal effects of ActRIIB-Fc Fusion protein ligand and physical exercise were examined.

Muscle strength was examined in this study, where it decreased significantly after ovariectomy. However, reverse results in trained exercise group either alone or with ActRIIB-Fc treatment were noticed when compared to the OVX group. These consequences came in accordance with ***Bayarsaikhan et al.*** [25] studies who described that ActRIIB-Fc-treatment in ovarectomized mice enhanced muscle growth and muscle strength. This is because ActRIIB-Fc strongly binds and inhibits TGF family members in muscle such as myostatin, which is a -ve regulator of skeletal muscle cell differentiation and development [26].

This study noticed that the final body weight was increased in OVX groups. This is due to drop in estradiol and increase in FSH [27]. Estradiol adjusts adipose tissue and lipid storage, also, controls food consumption and energy expenditure [28].

On the other hand, quadriceps & gastrocnemius mass and uterine weights were decreased in OVX groups. Reduced quadriceps & gastrocnemius mass may be due to reduced muscle response to anabolic stimuli or muscle catabolism [29]. This came in accordance with ***Greendale et al.*** who reported that rate of fat gain was doubled at the start of the menopause [30].

In this study, these changes were reversed in trained exercise group either alone or with ActRIIB-Fc treatment. This came in in accordance with ***Puolakkainen et al.*** who reported that ActRIIB-Fc treatment in ovarectomized mice reduced systemic adiposity and improved lean mass [31]. Also, ActRIIB-Fc binds myostatin with high affinity thus inhibiting it leading to increase muscle mass in mice [26].

Regarding to vitD3, it was significantly decreased in all OVX groups. However, it increased after exercise training alone or with ActRIIB-Fc treatment. Our results came in accordance with ***Malandish et al.*** who noticed a significant increase in serum vitD3 after chronic endurance exercise [32]. Also, suggested that moderate exercise increased vitD3 in postmenopausal women. However, ***Pilch et al.*** [33] found significant decrease in vitD3 after training exercise and ***Sliwicka et al.*** did not showed any significant changes in it [34].

This conflict may be explained as endurance training can improve vitD3 levels in vitD3 deficiency people. But, in sufficient vitD3 levels, endurance training didn't elevate its levels [32].

Concerning levels of serum ca^{2+} and p, serum ca^{2+} was decreased while serum p was increased significantly in all OVX groups. These findings were reversed in exercise training group alone or with ActRIIB-Fc treatment. Our results came in accordance with ***Mohamed et al.*** [35] who noticed that moderate exercise increased serum calcium while decreasing parathormone in the elderly thus lowering risk of osteoporosis. It was explained by decrease pH after exercise induces more ionized calcium [36]. Also, on line with this study, a preceding study reported an increase in serum calcium levels in ActRIIB-Fc treated mice [10]. But ***Malandish et al.*** found no significant difference in serum ca^{2+} or p levels after exercise [32].

As regards the influence of exercise on bone formation markers in this study, serum osteocalcin and P1NP were significantly reduced in all OVX groups. However, osteocalcin was significantly increased in exercise training alone or with ActRIIB-Fc treatment. Also a significant increase was found in P1NP with ActIIb-Fc administration but not with exercise.

This was reinforced by ***Hiam et al.*** [37] who noticed that acute exercise increased osteocalcin. Conversely, some previous studies showed no significant differences in P1NP levels after a sporadic exercise [38] or a continuous session of running [39]. However ***Civil et al***. [40] showed that P1NP values increased during running and returned to standard levels within one hour. The transient increase in circulating P1NP could be due to outflow of P1NP outside the connective tissue [40]. Consequently, this study reported no significant increase in serum P1NP because samples were collected 24 hours after the last exercise.

Regarding ActRIIB.Fc treatment, these results were in accordance with preceding studies which revealed that bone mineral content & density and serum osteocalcin were significantly greater in ActRIIB.Fc-treated group [41]. Moreover, the same study reported that ActRIIB-Fc administration resulted in persistent bone growth without affecting testosterone serum levels. It may be due to effect of ActIIB-Fc on myostatin which is a -ve regulator of skeletal muscle and bone mass [25]**.** Previous studies showed that myostatin null mice show increased bone bone mineral density and strength [41]. In contrary to these results, ***Puolakkainen et al.*** [31] noticed non-significant changes in osteocalcin or P1NP following ActIIB-Fc treatment.

On the opposite side, serum CTX (bone resorption marker) was increased in all OVX groups. However exercise-trained groups showed a significant decrease, which wasn't observed with ActRIIB-Fc administration. This came in agreement with previous studies of ***Puolakkainen et al.*** [31] who reported that serum concentrations of CTX did not differ after treatment with ActIIB-Fc. Therefore, our results suggest that ActRIIB-Fc may have osteogenic-stimulating action more than osteoclast-inhibiting action. However the significant effect of exercise on CTX came in agreement with previous studies who reported that exercise exerted inhibitory effects on CTX [42]. On the contrary**,** ***Wherry et al.*** reported increase in CTX in response to endurance exercise [43].

Concerning CK, it is a marker of muscle damage. Our study revealed significant elevation in serum level of CK in all OVX groups. However it decreased in groups treated with ActIIb-Fc alone but not with exercise. Previous studies reported a decrease in serum CK after ActRIIB-Fc treatment, indicating preservation of sarcolemmal integrity in mice models of Duchenne muscular dystrophy [44]**.** ActRIIB-Fc to inhibit activinA, myostatin, and growth differentiation factor 11 and consequently muscle hypertrophy [45]. Lack of significant difference in serum CK levels with exercise was in

accordance with ***Laufs et al.*** [46] who reported that regular exercise is not often associated with increased CK. This was in contrary with previous studies which showed raised CK level in athlete's blood [47].

Muscle exercise stress does not frequently increase CK levels. Its levels react to obvious alterations in the intensity and amount of exercise, thus increases after unusual and difficult types of exercise significantly [46].

Regarding oxidative stress markers, our study revealed that TOS was increased while TAC was decreased in all OVX groups. This came in accordance with ***Cao et al.*** [48] who reported that estrogen up-regulates antioxidants expression such as GSH peroxidase and SOD.

Furthermore, in this study, TOS was decreased, while TAC was increased in trained exercise group either alone or with ActRIIB-Fc treatment compared to OVX group. This came in accordance with ***Arazi et al.*** who described that exercise helps to increase antioxidant cells defense ability [49]. However, ***Bloomer et al.*** [50] study showed that carbonyl proteins which are indicators of oxidative stress are acutely elevated after single anaerobic exercise set. Also, ***Hentilä et al.,*** study showed an increase in reduced glutathione 2weeks after ActRIIB-Fc administration [51].

Exercise has positive and negative effects on oxidative stress [52]. High-intensity exercise can lead to a short-term imbalance between the active oxygen/nitrogen species production and elimination, which may cause oxidative stress [53]. However, steady long-term exercise may improve antioxidant defense factors. It can be explained by activation of MAP kinases, which activate (NF-κB) pathway and expression of vital enzymes associated with resistance against ROS as SOD and adaptation to exercise [54]. This explains the increased TAC and decreased TOS in our study.

Antioxidant gene (Aqp8) expression is a mitochondrial function marker and has been shown to be protective by transporting H2O2 out of pancreatic cells in the context of DM [55]. Oxidative stress and mitochondrial dysfunction may occur by downregulation of Aqp8. ActRIIB treatment increased Aqp8 expression, reducing mitochondrial dysfunction and oxidative stress [56].

Regarding inflammatory markers, we reported significant increase in IL-1β & Il-6 in OVX groups. This result was in accordance with ***Fu et al.*** [57] who reported that IL-6, IL-1β and TNF-α levels were elevated after ovarectomy. In current study, these inflammatory markers were significantly decreased with exercise but not with ActRIIB-Fc administration. This was in accordance with ***Lee et al.*** [58] study who found significant decrease in (TNF-α&IL-6) after aerobic exercise in obese children and adolescents & overweight. Exercise down-regulated central and peripheral inflammatory markers associated with chronic unpredictable mild stress [59]. Also, ***Guo et al.*** [60] showed no change in the level of circulating cytokines in immunodeficiency virus-infected rhesus macaques received AcRIIB-Fc.

Our study revealed significant decrease in OPG gene expression in OVX groups. On the other hand it was significantly increased in trained exercise group either alone or with ActRIIB-Fc treatment. OPG is a member of TNF receptor superfamily, which inhibits activity & differentiation of osteoclasts by binding to RANKL. It can inhibit osteoclasts activity and protect bone tissue [61]**.** These results were reinforced by ***Śliwicka et al.*** [34] that noticed significant increase in serum OPG level 24 & 72 hours after in amateur runners. ***De Voogd et al.*** [62] reported that various cytokines as TNFα which stimulated by intense endurance exercise induce synthesis of OPG by immune cells.

Concerning RANKL expression, this study showed significant upregulation in OVX groups. This can be explained by the enhancement in ROS production after ovariectomy that increased RANKL expression leading to osteoclastogenesis [63]. In contrary, its expression was downregulated increased in trained exercise group either alone or with ActRIIB-Fc treatment. These finding were in agreement with ***Puolakkainen et al.*** [31] study that revealed ActRIIB-Fc might have a double effect on bone, acting as an anabolic and anti-resorptive agent. Significant decrease in RANKL expression after ActRIIB-Fc treatment can be explained by inhibition of Activin A (maximum activin in bone), where Activin A has been found to enhance RANKL-induced osteoclastogenesis [64]. Also, ***Peng et al.*** [41] showed that exercise exerted inhibitory effects on the osteoclast-related mRNA expression (RANKL). On the contrary, ***Kim et al.*** noticed that RANKL and OPG expression levels were not affected by exercise [65].

The raised bone mass was also established in our histopathological & morphometric studies. Anti-resorptive effect of exercise and ActRIIB-Fc presented by the inecreased cortical thickness in histopathological as well as increased expression of

osteopontin (OPN, bone remodeling biomarker) genes in our gene expression analyses.

Some limitations of the present study deserve attention. The study lacked the results on human series that differ from those seen in rats. Also, small numbers of rats were used. Moreover, the anti-osteoporotic mechanism of both ActRIIB-Fc ligand & exercise training protocol requires further assessment by using DXA or micro-CT scan. In addition, the beneficial effects of these combined protocol therapy were not assessed on other body systems.

Conclusion: Addition of ActRIIB-Fc ligand to exercise training protocol act as a musculoskeletal-enhancing agent in OVX rats. These results were proved by reduction of osteoclast function and elevation of some osteoblast marker genes expression. Thus ActRIIB-Fc ligand may be a beneficial choice in musculoskeletal disorders management.

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To Cite:

Alsayed, R., Seada, S., Abdul Rahman, M., EL-sayed, Y., Ebrahim, E. Musculoskeletal effect of ActRIIB-Fc Fusion protein ligand alone or concomitant with swimming exercise in ovariectomized adult albino rats. *Zagazig University Medical Journal*, 2024; (1270-1284): -. doi: 10.21608/zumj.2024.273508.3214