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Estradiol and/or Ibandronate Therapy Ameliorates Oxidative Status in Livers of Ovariectomized Rats

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Author's contribution

Author SMK is responsible for the design of the study, wrote the protocol, managed the analyses of the study, performed the statistical analysis, managed the literature, wrote the first draft of the manuscript, read and approved the final manuscript.

Original Research Article

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ABSTRACT

Background: Overiectomized rats suffer from osteoporosis that mainly results from oxidative stress (OS). Studies revealed that the levels of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-*S*-transferase, catalase (CAT), and glutathione reductase could be used to determine and follow-up OP. The anti-oxidant activity of anti-osteoporotic drugs needs further investigations to be proved to add more confidence in the ability of these drugs to control the disease.
Objective: To investigate the possible anti-oxidant effect of treatment with ibandronate, a highly potent nitrogen-containing bisphosphonate, on activities of catalase and glutathione peroxidase anti-oxidant enzymes, thiobarbituric reactive substance (TBARS), as a marker of lipid peroxidation and DEPPD free radicals in liver homogenates of ovariectomized rats.
Methods: Fifty adult female albino rats were divided into five equal groups (n=10 rats): Group (1): served as control injected with saline, 2nd group: rats were subjected to ovariectomy, 3rd group: rats were exposed to ovariectomy and treated with estradiol, 4th group: were ovariectomized and treated with ibandronate and 5th group: were ovariectomized and treated with both estradiol and ibandronate. Duration of therapy with either drug was 12 weeks.
Results: Estradiol alone or in combination with ibandronate to ovariectomized rats showed significant increase in activities of anti-oxidant enzymes. Both drugs reduces

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hepatic TBARS and DEPPD free radicals in hepatic homogenates in this model of OP.
Conclusion: The present study can conclude that ovariectomy leading to oxidative changes liver of tested rats. Co-administration of estradiol and ibandronate provides a kind of protection against alterations in anti-oxidative/oxidative balance. The results support the hypothesis stated by experimental studies that anti-osteoporotic treatment could also possess anti-oxidative protective property.

Keywords: Ovariectomy; estradiol; ibandronate; anti-oxidant enzymes; DEPPD free radical; rat's liver.

1. INTRODUCTION

Osteoporosis (OP) is a disease characterized by a reduction in bone mass without alteration in bone mineralization. These changes predispose to an increase in the risk of fracture. To induce a model of osteoporosis (OP) simulating that occurs in menopausal women, ovariectomized rats are used provide a valid and reliable model to determine the effects of OP. Bonee resorption in ovariectomized rats is highly simulate to the postmenopausal bone loss in women [1,2].

Oxidative stress (OS) plays an important role in the development of many diseases e.g. OP, renal disorders, rheumatoid arthritis, diabetes, sepsis, and Alzheimer's disease. The levels of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-*S*-transferase, catalase (CAT), and glutathione reductase could be used to determine and follow-up OP. OP is thought to be the result of accumulation of reactive oxygen species (ROS) and lipid peroxidation by estimating the thiobarbituric acid reactive substance (TBARS) [3,4] .

When estradiol diminishes in menopausal females, the risk of occurance of OP is increased and some antioxidant enzymes are decreased [4]. These observations suggest that antioxidants may be beneficial in the treatment OP cases [5].

Bisphosphonates are commonly prescribed to control cases of OP in postmenopausal females. Their major mechanism of action is to inhibit bone resorption by osteoclasts, and hence keep its normal density. They include include etidronate, risedronate, ibandronate, and alendronate [6].

The evaluation of the role of oxidants and antioxidants in OP shows interesting results [7]. Any imbalance in hormonal balance affects proper functions of living organism including liver with great affection of metabolic process. Disorders in oxidative/anti-oxidative status in a vital organ as liver will result in an increase in peroxidative damage of protein and lipid metabolism. This damage is primarily related to the accumulation of reactive oxygen species (ROS) [8].

Ovariectomy in rats is a useful animal model for studying the influence of oestrogen deficiency and metabolic changes related to organs responsible for metabolism notably the liver [9]. The influence of metabolic disturbances on liver is of interest from clinical point of view because it may play a potential role in aggravating liver diseases via generation of ROS excess.

The objective of the present study is to investigate whether ibandronate, alone or in combination with estradiol, could possess an anti-oxidative and/or an anti-lipid peroxidative effects on hepatic homogenates of ovariectomized rats.

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2. MATERIALS AND METHODS

2.1 Drugs and Chemicals

Ibandronate sodium (Shreeji Pharma International Co, India). Estradiol Valerate vials (Cadila Healthcare, USA). BIOXYTECH GPx-340TM Assay kit was purchased from OXIS International, Inc., USA for determination of glutathione peroxidase enzyme. All other chemicals and kits were purchased from Sigma chemical company (St Louis, MO, USA). Both drugs were freshly prepared by dissolving their powders in saline before each injection. Both drugs were injected subcutaneously (Sc) daily for 12 weeks.

2.2 Animals

Fifty female albino rats raised in the same environment and weighing 200-250 g were used in this study. They were maintained in a well-ventilated controlled room at 20–22ºC on a 12-h light/12-h dark schedule. The study protocol was approved by the Ethical Committee of Faculty of Medicine, Ain Shams university, Cairo, Egypt. The rats were randomly divided into five groups (n = 10 rats/group):

Group (1): served as control injected with saline, as the solvent of both estradiol and ibandronate, 2nd group: rats were subjected to ovariectomy and injected with saline, 3rd group: rats were exposed to ovariectomy and treated with estradiol dissolved in saline, 4th group: were ovariectomized and treated with ibandronate dissolved in saline and 5th group: were ovariectomized and treated with both estradiol and ibandronate dissolved in saline.
The rats had access to food and water until 2 h prior to the anesthesia.

2.3 Anesthesia

After anesthesia (intraperitoneal ketamine, 50 mg/kg), bilateral ovariectomy was performed with a ventral approach. A period of 100 days after overiectomy was allowed for the development of OP [10]. Surgical procedure was not performed to the control group. After surgical procedure, Ibandronate was injected Sc in 4th and 5th groups at a dose of 25 µg/kg/day. Estradiol was also injected Sc at a dose of 250 µg/kg/day to rats of 3rd and 5th groups. The duration of therapy was 12 weeks.

All doses were determined from a pilot study done before the full original research. These doses were found to induce the best results for the measured parameters.

2.4 Measurements

2.4.1 Preparation of liver specimens from rats of all tested groups

After the treatment, the animals were dissected under ketamine anesthesia. The livers were quickly excised, rinsed in ice-cold 0.175 M KCl/25 mM Tris–HCl (pH 7.4) to remove the blood, weighed, finely minced in the same solution, and homogenized by means of a homogenizer with a Teflon pestle. The liver homogenates were centrifuged at 10,000 ×*g* for

15 min. The supernatants were then used for lipid peroxidation determination and antioxidant enzyme assays.

2.4.2 Liver SOD and CAT determination

The SOD activity was measured by the inhibition of nitroblue tetrazolium (NBT) reduction due to O2 -generated by the xanthine/xanthine oxidase system as described by Sinha [11] . One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the NBT reduction rate. The CAT activity of tissues was determined according to the method of Sinha,1972. The enzymatic decomposition of H2O2 was followed directly by the decrease in absorbance at 240 nm. The difference in absorbance per unit time was used as a measure of CAT activity. The enzyme activity is given in U/mg of protein.

2.4.3 Determination of glutathione peroxidase enzyme activity

Glutathione peroxidase (GPx) activity in the liver homogenates was measured by the method described by Rotruck [12]. Glutathione peroxidase (GPx) was assayed in the liver sample homogenized in 8 volumes of cold buffer (50mM Tris-HCl, pH 7.5, containing 5 mM EDTA and 1 mM 2-mercaptoethanol), next centrifuged 8500xg for 10 minutes at 40C. GPx activity was determined in supernatant using BIOXYTECH GPx-340TM Assay kit produced by OXIS International, Inc., USA. The GPx assay was based on the oxidation of NADPH to NADP+, which is accompanied by a decrease in absorbance at 340nm. The rate of this decrease is directly proportional to the GPx activity in the sample.

2.4.4 Measurement of hepatic thiobarbituric acid-reactive substances (TBARS) as a marker of lipid peroxidation [13]

Liver homogenates were rinsed with cold 0.14 M sodium chloride and homogenized in 25% ice-cold 50 mM Tris-HCl buffer (pH 7.4).8 150 µL of the tissue supernatant of samples were diluted to 500 µL with deionized water. Two-hundred and fifty (250) µL of 1.34% thiobarbituric acid was added to each tube, followed by the addition of an equal volume of 40% trichloroacetic acid. The mixtures were then shaken and incubated for 30 minutes in a boiling water bath. Tubes were allowed to cool to room temperature, and the absorbance was then read at 532 nm, using zero concentration

2.2.5 The determination of DEPPD radicals in hepatic supernatants

The method was based on the estimation of radical cation formed in the reaction of alkoxy and peroxy radicals derived from the hydroperoxides by use of N,N,diethyl-p-phenylene diamine (DEPPD; Sigma) as described by Alberti [14]. Incubation mixture contained 1 ml acetate buffer (pH 4.8), 10 µL aqueous solution of DEPPD (0.37 mol/l) and 20 µL supernatant. After 1.5-h incubation at 37ºC absorbance was read at 505 nm against distilled water. Control sample contained distilled water instead of supernatant. Calculations were based on standard curve prepared with different dilutions of H_2O_2. The results were expressed as µmol/g protein (mean ± SD).

3. PROTEIN DETERMINATION

The protein content of liver homogenates was determined by spectrophotometer according to the method of Bradford [15]. The aim is to relate the oxidative marker concentrations to the total tissue protein.

4. DATA ANALYSIS

The results are presented as mean ± standard deviation (SD) and evaluated using one-way analysis of variance (ANOVA), followed by Tukey's post hoc determination, using Graph Pad Prism (version 3.00; Graph Pad Software, La Jolla, CA, USA).

5. RESULTS

Table (1) shows changes in the activities of anti-oxidant enzymes CAT, GPx enzymes in liver of tested rats. There is a significant ($p<0.05$) reduction in their activities in model un-treated group (2) compared to control group (1). However, estradiol, either alone or in combination with ibandronate, shows significant ($p<0.05$) increase in their activities.

Table 1. Changes in the activity of liver catalase (CAT) and glutathione peroxidase (GPx) anti-oxidant enzymes

Group	Catalase Unita/mg tissue protein[a]	Glutathione peroxidase Unit[b]/mg tissue protein
Control (Group 1)	66.30 ± 1.22	10.5 ± 0.50
Ovariectomy alone (Group 2)	4.55 ± 0.74*	0.45 ± 0.06*
Ovariectomy and treated with estradiol (Group 3)	40.90 ± 2.0**	6.93 ± 0.5**
Ovariectomy and treated with ibandronate (Group 4)	77.33 ± 3.73**	12.67 ± 3.45**
Ovariectomy and treated withboth estradiol and ibandronate (Group 5)	90.43± 4.55***	20.34± 5.60***

Notes: [a] = Moles of hydrogen peroxide consumed per minute; [b] = µg of glutathione consumed per minute

*. *P < 0.05, significant reduction in activity of both enzymes versus Group 1.*

***P < 0.05, significant increase in activity of both enzymes versus Group 2;*

****P < 0.05, significant increase in activity of both enzymes versus Group 3, 4.*

Fig.1. Effect of 12-weeks administration of tested drug on levels of superoxide dismutase [SOD] enzyme in IU/mL in erythrocyte lysates of the tested rats. Results are expressed as mean ± SD (n = 10 rats/group).

Fig. (2): Effect of 12-weeks administration of tested drugs on thiobarbituric acid-reactive substance (TBARS) in nmol/mg tissue protein of the liver tissue homogenates of the tested rats.

Table (2) shows changes in concentration of DEPPD free radical in liver homogenates of tested rats. There is a significant ($p<0.05$) increase in its concentration in model un-treated group (2) compared to control group (1). However, estradiol, either alone or in combination with ibandronate, shows significant ($p<0.05$) decrease in its concentration.

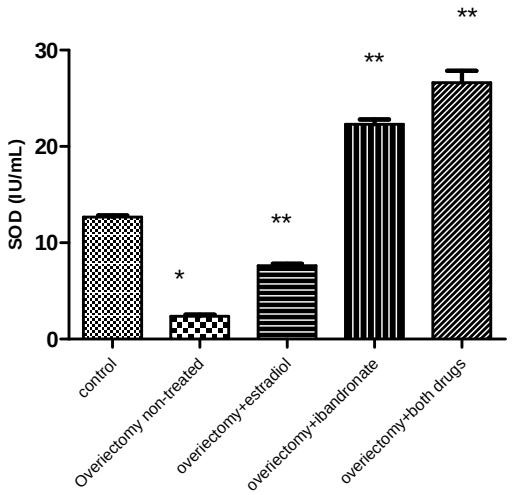

Fig. 1. Measurement of SOD enzyme levels (IU/mL) in erythrocyte lysates of all tested rats.

** p<0.05= significant decrease in SOD enzyme levels in group (2) compared to the control non-treated albino rats group (1) ** p<0.05 = significant increase in SOD enzyme levels in all-treated groups (3,4,5) compared to the control –non treated albino rats group (2)*

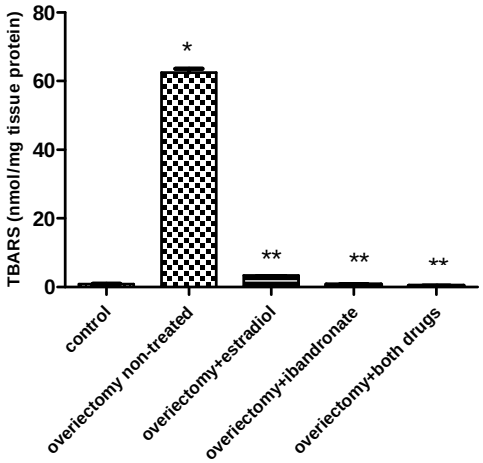

Fig. 2. Effect of 12-weeks administration of tested drugs on thiobarbituric acid-reactive substance (TBARS) in nmol/mg tissue protein of the liver tissue homogenates of the Tested rats. Results are expressed as mean ± SD (n = 10 rats/group). A significant (*P < 0.05) decrease in TBARS levels in comparison to the model untreated Group 2.

** p<0.05= significant increase in TBARS levels in group (2) compared to the control non-treated rats group (1) ** p<0.05 = significant decrease in TBARS levels in either estradiol, ibandronate or both drugs -treated group (4 &5) compared to the control –non treated albino rats group (2)*

Table 2. *N*,*N*,diethyl-*p*-phenylene diamine (DEPPD) free radical concentration in liver homogenates of tested rats of all groups.

Group	DEPPD free radical concentrations in µmol/g protein
Control (Group 1)	0.2±0.01
Ovariectomy alone (Group 2)	2.1±0.3*
Ovariectomy and treated with estradiol (Group 3)	0.9±0.01**
Ovariectomy and treated with ibandronate (Group 4)	0.83±0.02**
Ovariectomy and treated withboth estradiol and ibandronate (Group 5)	0.03±0.001**

**p < 0.05 = Significant increase in DEPPD in group (2) compared to group (1)*
***p < 0.05 = Significant decrease in DEPPD in all treated groups (3,4,5) compared to group (2)*

6. DISCUSSION

There is increasing evidence suggesting the increase in production of free radicals in ovariectomized rats as a result of the process of oxidative stress with oxidative damage to cells [16]. The anti-oxidant defense mechanism adapted by liver in ovariectomized animals remains obscure. The present study investigated the impact of ovariectomy (OVX) on some liver antioxidant enzymes, TBARS, as a marker of lipid peroxidation and DEPPD radicals in hepatic supernatants in ovariectomized rats

The results of the present study revealed that activities of CAT, GPx anti-oxidant enzymes was significantly reduced in ovariectomized rats and that treatment with estradiol or ibandronate or both drugs significantly increased their activities. This increase was more apparent with therapy by both drugs. SOD enzyme level was significantly increased in all treated groups compared to model non-treated group. Additionally, TBARS level, as a marker of lipid peroxidation, was markedly reduced in treated groups. DEPPD free radical was obviously reduced by anti-osteoporotic drugs in all treated groups.

The results pointed to the powerful anti-oxidative properties of estradiol and ibandronate They also reduced the availability of DEPPD free radicals in livers of overiectomized rats. These findings are in agree with that recorded by [17], who proved that oestrogens can protect the liver and intestines against oxidative injury possibly by free radicals scavenging activity.

The present results are also comparable to those reported by [16] who demonstrated that the levels of free radicals in bone of overiectomized rats were increased and enzymatic antioxidants like SOD, GPx were decreased when compared to sham-operated control rats. They proved that overiectomy induces an oxidative stress in bone homogenates of overiectomized rats.

Sies [18] observed that glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT) as well as water and lipid soluble vitamins, glutathione, urates protect against any disturbance in anti-oxidative/oxidative balance and prevent the production of markers of peroxidative processes.

Similarly, free radicals and lipid peroxidation were increased and enzymatic antioxidants like SOD, GPx, GST were decreased in ovariectomized animals when compared to control rats as indicated by Muthusami [19].

Sontakke and Tare [20] proved that there is an increase in superoxide free radicals formation by the osteoclasts that leads to active bone resorption and loss of bone density .In vitro or animal studies showed that reactive oxygen species (ROS) deteriorates osteoblastic functions with an increase in risk of occurance of osteoporosis [21].

Previous studies, in the 20th century, showed that ROS would lead to tissue damage by increase in lipid peroxidation that is a well-known tissue damage mechanism in humans and is used in tissue and cells as an oxidative stress (OS) indicator. Lipid peroxides are made of polyunsaturated fatty acids that decompose to complex compounds in rats with overectomy suffering from deficiency of estradiol. These rats show a great activation in ROS that results in bone loss by increase bone resorption [22,23]

Recently, an experimetal study that was conducted on adult rats showed that melatonin supplementation prevents ovariectomy-induced increases in oxidative stress and serum levels of OS markers in cases of hepatic and renal dysfunction. Overall, melatonin supplementation therapy was found to be more potent and safer than estrogen replacement therapy in controlling the augmentation in postmenopausal OS and hepatic and renal dysfunction [24].

It is worth mentioning that the SOD is a powerful anti-oxidant enzyme that supports the body by a great antioxidant defense mechanism. Several studies found that levels of SOD and GPx levels were low in osteoporotic patients and they explained these findings to be as a result of the loss of their antioxidant defense mechanisms that were reported to be reduced in OP by [20 & 25]

Additionally, Ozgocmen et al. [26] reported that erythrocyte CAT enzyme activity is reduced in osteoporotic patients and also revealed that there is a correlation between erythrocyte CAT enzyme activity and proximal femur bone mineral density (BMD). When BMD reduced by OP, serum and erythrocyte CAT enzyme activity is decreased.

7. CONCLUSION

Ovariectomy in albino rats results in oxidative changes liver of tested rats. However, administration of estradiol and/or ibandronate provides a kind of protection against alterations in anti-oxidative/oxidative balance. The results support the hypothesis stated by experimental studies that anti-osteoporotic treatment could also possess anti-oxidative protective property.

ETHICAL APPROVAL

All procedures were in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals, as well as the guidelines of the Animal Welfare Act (www.nih.gov).

JUSTIFICATION OF SAMPLE SIZE

Number of rats in each group = 10 was determined by sample size determination using program of Microlab, version 2005 so as the α value is < 0.05 and β value is > 80%. The 10 rats/group is the minimal number sufficient to produce these statistical values determined by the pilot study done before the full experimental research. Values will be statistically

analysed using Prism version 3.0 to calculate ANOVA between different studied groups and to determine which group is responsible for the significant changes.

COMPETING INTERESTS

The author reports no conflicts of interest in this work.

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