Implications of Creatinine and ALP in the Induction of Osteoporosis by Ovariectomy in Female Wistar Rats

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Histological and biochemical assessment of maxillary bone necrosis induced by therapy with two bisphosphonate types used to treat osteoporosis induced in female Wistar rats, evaluation of effects on creatinine and ALP values at different time intervals. Female Wistar rats were ovariectomized to induce osteoporosis. Blood tests for ALP and creatinine were conducted. They received two types of bisphosphonates. Dental extraction was performed in each rat separately; finally, the rats were euthanized. Microscopic evaluation of the tissue fragments collected from the groups of rats was performed for the following parameters: degree of osteonecrosis, bone resorption, inflammatory infiltrate with neutrophils and mononuclear cells, number of osteoclasts, degree of proliferation of fibrous connective tissue, and local neovascularization. The biochemical and subsequently histological investigations for osteonecrosis of the jaw associated with bisphosphonate therapy in rats with induced osteoporosis provide accurate information about the extent of osteonecrosis, bone resorption, inflammatory infiltrate with mononuclear cells and neutrophils, number of osteoclasts, degree of proliferation of fibrous connective tissue, and local neovascularization. This is necessary for positive and differential diagnosis and for the treatment plan.

Keywords: alkaline phosphatase, creatinine, osteoporosis, rats, bone necrosis

Osteoporosis is defined as a bone cell deregulation, being characterized by a reduction of bone mass and bone tissue integrity, leading to bone fragility and decreased bone hardness. This loss of performance in bone cells leads to a higher risk of fractures, predominantly in the hip and spine. In terms of percentage, 40% of osteoporosis cases occur in postmenopausal women and 15% occur in elderly men [1].

According to recent studies, estrogen deficiency is the main factor causing osteoporosis both in women and men [2]. Estrogen deficiency present at an advanced age is associated with an increase of bone resorption to the detriment of bone formation, which leads to excessive bone loss. From a molecular point of view, it has been recently found that the receptor activator of nuclear factor kβ (RANK)/RANK ligand (RANKL)/OPG triggers an important signal regulating osteoclast formation. Thus, the RANK/RANKL binding inhibits osteoclast formation and differentiation and implicitly slows bone resorption [3]. Recent studies have shown that regulation of RANKL activity at bone cell level and estrogen deficiency in postmenopausal women are responsible for bone resorption [4,5]. Furthermore, experiments conducted in animal models (ovariectomized mice) for induction of osteoporosis have shown that OPG, in addition to preventing bone resorption, increases bone mineral density and in excess, can even lead to osteopetrosis. It was demonstrated that a single injectable OPG dose administered to postmenopausal women can lead to a rapid and marked reduction of bone turnover within 12 hours; this could be proved by biochemical markers and collagen products (NTX urinary N-telopeptide, DPD deoxypyridinoline) [6,7].

According to studies performed so far, bisphosphonates can induce apoptosis in osteoclasts. Injectable bisphosphonate formulas have a direct action on osteocytes, inducing their lysis. Bisphosphonates alter the production of RANKL and OPG, causing a decrease in bone resorption [8,9].

Our study aims to present an experimental comparison made in an animal model – female Wistar rats, under conditions of osteoporosis induced by ovariectomy. The main goal in the performance of this experiment was to observe the fluctuating values of ALP (alkaline phosphatase) and C4H7N3O (creatinine) in the development of osteoporosis. The results obtained will assist veterinarians and other specialists who frequently encounter osteoporosis cases, in their therapeutic approach in these situations.

Creatinine

C4H7N3O (creatinine) (fig. 1) is a metabolic product of creatine phosphate in muscle and is produced at a constant rate in the body, being dependent on muscle mass. In fact, it is the anhydride of creatine (methyl guanidine-acetic acid), being formed in muscle. Creatinine is synthesized in the liver and after being released, it is taken up in a proportion of over 90% by muscle, where phosphorylation occurs. In this form, it plays an important role in storing muscle energy. In cases when this muscle energy is required for metabolic needs, phosphocreatine is broken down to creatine. The amount of creatine converted to

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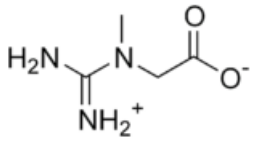

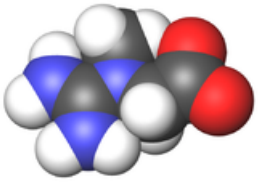

Fig. 1. Chemical and molecular formula of creatinine (https://it.wikipedia.org/wiki/Creatina

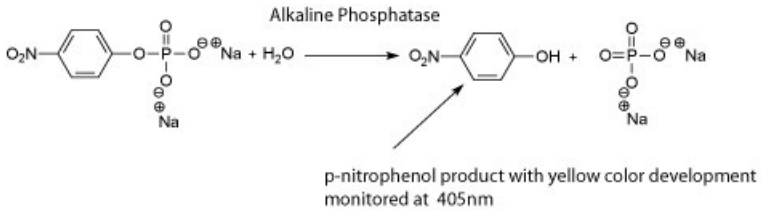

Fig. 2. Chemical formula of alkaline phosphatase (http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/alkaline-phosphatase.html

creatinine is maintained at a constant level, which is directly related to the muscle tissue mass of the body. Creatine derived from food (meat) increases the stores of creatine and creatinine. The reduction of protein intake diminishes the level of creatinine through the absence of the amino acids arginine and glycine, which are creatine precursors. Thus, creatinine is the most stable nitrogen component of blood, being uninfluenced by the majority of foods, by effort, circadian rhythm or other biological constants [10,11].

Under conditions of decreased renal perfusion, serum levels increase more slowly than urea. Since a 50% loss of renal function is necessary for an increase of creatinine values from 1.0 to 2.0 mg/dl, serum creatinine cannot be considered a sensitive indicator in case of mild to moderate renal lesions [11]. Serum levels can be used to estimate the glomerular filtration rate only under balance conditions, when the rate of creatinine synthesis is equal to that of creatinine excretion. To verify this state, two measurements 24 h apart are required; differences of more than 10% can indicate the absence of such a balance state. In renal function alterations, the glomerular filtration rate is overestimated based on serum creatinine levels, because creatinine excretion is not due to glomerular filtration, but to tubular secretion and thus, creatinine is also eliminated through the intestinal mucosa, being apparently metabolized by intestinal bacterial creatininases. A diet with an excessive meat content may cause serum creatinine increases (15-30% of daily excreted creatinine is derived from food) [10]. The normal creatinine values in rats are: 0.0-0.9 mL/dL [12].

From a biochemical point of view, in our experiment we monitored the changes in alkaline phosphatase and creatinine, under conditions of induced osteoporosis associated with bisphosphonate treatment, as compared to normal values.

ALP

ALP (alkaline phosphatase) (fig. 2) was discovered a long time ago, being a metalloenzyme linked to the cell membrane. It is distributed in the liver, placenta and bone. Bone-specific alkaline phosphatase is in fact an isoenzyme involved in bone formation processes and the main enzymatic mediator in the removal of inorganic pyrophosphate, an inhibitor of bone mineralization [13]. Cholestasis-inducing drugs (aminosalicylic acid, amitriptyline, anabolizing steroids, androgens, azathioprine, benzodiazepine, carbamazepine, carbazone, chlorothiazide, chlorpropamide, clavulanic acid, dapsone), drugs that induce hepatocellular damage and many other drugs may cause increases of alkaline phosphatase, which in general are transient, but indicate hepatotoxicity in certain cases. The normal values of alkaline phosphatase are 0-130 I.U./L [12].

Experimental part

The experiment described in this article was carried out at the Biobase of UMPh Cluj-Napoca and in the experimental laboratories of USAMV Cluj-Napoca, according to the standards and directives of the Ethics Commission, in compliance with Law 43/2014 (published in Monitorul Oficial, Part I, no. 326, of 6 May 2014) regarding the protection of animals used for scientific purposes. Our experiment was performed in a rat model, and attention was focused on changes in values compared to normal values, under conditions of induced osteoporosis. The measurement of alkaline phosphatase is commonly used for the differential diagnosis of liver diseases. Another area of clinical use includes bone disorders, alkaline phosphatase being currently the only enzyme of practical importance for bone tissue pathology.

The present study is an extension of a study undertaken by us previously [13] and has as its main purpose, to observe in female rats, the development of osteoporosis induced by ovariectomy and the clinical implications of creatinine and alkaline phosphatase in this process.

Management of the animals

30 female Wistar rats, with a weight of 180-200 g and a mean age of 8 weeks, were acquired by UMPh Cluj-Napoca. They were kept in special boxes – 5 rats per box (fig. 1), which were cleaned every day. The light-dark cycle (12:12 h) started at 06:00 a.m., and the room temperature was maintained constant at 20-23°C. All the rats received adequate food (pellet rat chow), according to nutritional requirements (12% fat, 63.2% carbohydrates, 24.3% protein) for rats, and had free access to fresh water in special containers [14].

Osteoporosis induced by ovariectomy

Ovariectomy was initiated by sedating the rats using a combination of xylazine 5.00 mg/kg body weight and ketamine hydrochloride 50.00 mg/kg body weight, intramuscularly. The midventral area caudal to the third pair of mammary papillae was shaved and cleaned with a chloramine and alcohol disinfecting solution. A 2 cm incision was performed [15], the skin was separated from the muscle, and the ovaries were exposed. These were removed, using a special ophthalmological catheter. After the ovaries were removed, the peritoneum and the white line were closed with a running absorbable 3.0 suture and the skin was sutured with an interrupted absorbable 4.0 thread [16,17]. Postoperatively, the rats were covered with a gauze dressing to avoid hypothermia [15], and adequate antibiotic and antiinflammatory treatment was administered.

The operative protocol was performed on 3.02.2015, and during 3.5 months (28.05.2015), the development of osteoporosis was monitored by serial radiographs. During this period, 6 rats died from internal hemorrhage [18] or possibly sepsis [19].

Results and discussions

At the end of the period of development of osteoporosis, we divided the rats into three groups and we analyzed the blood collected from the infra-orbital fossa in order to monitor the fluctuating values of alkaline phosphatase and creatinine. The results obtained in this experiment clearly show that alkaline phosphatase and creatinine values play an exceptional role in the induction of osteoporosis by ovariectomy in female Wistar rats. The data obtained based on the blood tests for alkaline phosphatase (U/L) and creatinine (mg/dL) at 7, 14, 30 days in the three groups were centralized in tables. The ANOVA test was performed to compare values between the groups and see whether significant differences were present or not, and REGRESSION correlations were established, where R^2 showed correlations between the values obtained in each diagram.

ALP

It can be seen that alkaline phosphatase values (U/L) decreased at 7, 14, 30 days under conditions of induced osteoporosis, which means that values changed in group 1. In group 2, fluctuating alkaline phosphatase values were observed. Thus, at 14 days, higher values were found compared to those at 7 and 30 days, respectively. The control group (group 3) had a gradual increase of alkaline phosphatase values at the three time points. By centralizing all values, fluctuating levels can be observed at the three time points in the three groups (table 1).

$C_4H_7N_3O$

Creatinine values (mg/dl) decreased at 7 and 14 days, and at 30 days they slightly increased under conditions of induced osteoporosis, which means that this blood test parameter also underwent changes. In group 2, fluctuating creatinine values (mg/dL) were observed. Thus, at 7 days from administration, higher values were found than those at 14 and 30 days, respectively. The control group (group 3) had fluctuating creatinine (mg/dL) values. At 7 days the highest values were seen, while at 14 days the values decreased and at 30 days they slightly increased (table 2).

Based on all these results, we made a comparison of data regarding blood creatinine and alkaline phosphatase values at the three time points in the three groups and we calculated a mean of values, as shown in the tables below.

Osteoporosis is defined as a bone cell deregulation, being characterized by a reduction of bone mass and bone tissue integrity, leading to bone fragility and decreased bone hardness. The induction of osteoporosis in female Wistar rats allowed in our experiment a perspective on changes that this can generate in bone and blood parameters. The most important bone markers for observing bone disequilibrium are creatinine and alkaline phosphatase, which in fact we aimed to assess for changes in their values.

Creatinine is the anhydrous form of urinary excretion of creatine, a component mostly present in muscle. For this reason, creatinine is the most stable nitrogen parameter of blood, which is correlated with muscle mass volume and is not influenced by periodic food intake, diuresis level, or muscular effort. Creatinine is completely eliminated by glomerular filtration, and through its blood level it can provide indications of the number of functional nephrons [20]. Other studies have also evaluated hepatic and renal toxicity in rats receiving creatine supplements. However, the methods used in these studies are different from those of our study. For example, Taes et al. investigated the effect of creatine on preexisting renal disease. They used rats with renal failure induced by the surgical removal of 2/3 of renal tissue. The animals were divided into four groups: falsely operated controls, falsely operated rats supplemented with creatine, 2/3 nephrectomized controls, and 2/3 nephrectomized rats supplemented with creatine. After four weeks of supplementation, data regarding the clearance of inulin, urea, creatinine and albumin as well as urinary protein excretion showed no effect of creatine supplementation in rats without nephrectomy or rats of the placebo group. The authors concluded that supplementation does not impair renal function, even in the case of preexisting renal disease, which was also observed in the present study, when animals with renal failure showed no changes after supplementation [21].

Alkaline phosphatase is an enzyme belonging to the class of hydrolases (orthophosphoric-monoester phosphohydrolase); it has three main isoenzymatic forms (hepatobiliary, bone, intestinal), and a transient form during pregnancy (placental). Although the hepatic or non-hepatic

7 days		
Group 1		
Mean	189.9875	0.7725
Standard deviation	42.39327566	0.091612538
Group 2		
Mean	168.4375	0.85875
Standard deviation	61.24316726	0.115317884
Group 3		
Mean	208.7685714	0.927142857
Standard deviation	75.68864896	0.087123339
Normal values	130	0.9

Table1
CENTRALIZATION OF DATA REGARDING ALP AND $C_4H_7N_3O$ VALUES; GENERAL NORMAL VALUES AT 7 DAYS

14 days		
Group 1		
Mean	175.375	0.65125
Standard deviation	64.5925637	0.051944338
Group 2		
Mean	189.9875	0.66125
Standard deviation	70.16711557	0.054625347
Group 3		
Mean	219.2428571	0.562428571
Standard deviation	69.45019456	0.234274666
Normal values	130	0.9

Table2
CENTRALIZATION OF DATA REGARDING ALKALINE PHOSPHATASE AND CREATININE VALUES; GENERAL NORMAL VALUES AT 14 DAYS

origin of increased alkaline phosphatase can be reasonably assessed only based on clinical data, there are also biochemical methods that can differentiate between isoenzymes. The measurement of alkaline phosphatase is commonly used for the differential diagnosis of liver diseases. Another area of clinical use includes bone disorders, alkaline phosphatase being currently the only enzyme of practical importance for bone tissue pathology and in hyperparathyroidism. In tumors of various etiologies, alkaline phosphatase has a tumor marker value (detection of liver or bone metastases) [22]. Serum alkaline phosphatase is a member of a family of zinc metalloprotein ezymes, which function to detect a terminal phosphate group from an organic phosphate ester. There are many causes that can induce an increase of alkaline phosphatase activity in the serum, the most frequent being obstructive hepatic diseases and metabolic bone diseases. An enlarged liver or especially the bone isoform (bone-specific alkaline phosphatase) in the serum can provide valuable diagnostic information. The values specific to the alkaline phosphatase isoenzyme are increased as a result of enhanced osteoblast activity. The highest total alkaline phosphatase values have been attributed to an increased bone isoenzyme level due to Paget disease or rickets/ osteomalacia. Enzymatic activity, which is located in the plasma membrane of osteoblasts before extracellular release, is correlated with disease extension in skeletal studies and with bone resorption parameters. This is

30 days		
Group 1		
Mean	168.825	0.66
Standard deviation	53.6036446	0.046904158
Group 2		
Mean	157.525	0.68625
Standard deviation	80.87729242	0.071900228
Group 3		
Mean	308.6714286	0.652857143
Standard deviation	132.4988517	0.054989176
Normal values	130	0.9

Table 3
CENTRALIZATION OF DATA REGARDING ALKALINE PHOSPHATASE AND CREATININE VALUES; GENERAL NORMAL VALUES AT 30 DAYS

normally attributed to an increase of isoenzymes in growing children and adults aged over fifty years. The causes of bone alkaline phosphatase include fracture healing, acromegaly, osteogenic sarcoma, bone metastases, leukemia, myelofibrosis, and rarely myeloma. Thus, alkaline phosphatase is used as a tumor marker. Hyperthyroidism, through its effects on bone, can also increase alkaline phosphatase values [23].

Conclusions

From those presented above and based on the results of our experiment, we came to the conclusion that both creatinine and alkaline phosphatase values are extremely important from a physiological point of view. The induction of osteoporosis by ovariectomy generates important changes in muscle and bone metabolism, with repercussions on bone architecture and bone healing.

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