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Protective Effect of Bisphosphonates on the Pathological Changes in the Blood and Tissues in Case of Experimental Atherosclerosis

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Abstract
The protective effect of the three bisphosphonates, synthesized in Russia, on the pathological changes in the blood vessels and tissues in experimental atherosclerosis were determined. Atherosclerotic pathology was reproduced on white outbreed rats with the help of 1% cholesterol diet, sub-toxic doses of vitamin D_2 and the line implantation into the abdominal aorta. The protective effect of xydifon (K, Na-etidronate), mediphone (olpadronate) and zoledronate, administered per os was studied. The positive effect of all three preparations in comparison with the untreated control was noted. There were observed the reduction of LDL, accumulation, cholesterol of triglycerides, atherogenic index and toxicity index for albumin, uric acid in the blood. The positive biochemical features were correlated with the morphological image: bisphosphonates significantly reduced the accumulation of Fe, Cu in the aortic wall and deposition of calcium granules. The usefulness of bisphosphonates in the atherosclerosis' treatment complex is discussing.

1. Introduction

Atherosclerosis is one of the most common chronic diseases of cardiovascular system [1, 2]. Its main pathologic substrate is the formation on the inner surface of the blood vessels, the so called atheroma and "atherosclerotic plaques". The components of the atherosclerotic plaque are the following: fats, fat-like substances (primarily cholesterol)

and fibrin and some other [3, 4]. Gradually, the lipoid, mainly as a result of the accumulation of calcium, and begin to pose a serious threat to the circulation. There are reports about the value of the excess accumulation in atherosclerotic vessels and micronutrients [5, 6, 7]. The current problems of diagnosis, prevention and treatment of excessive accumulation of minerals and calcium in tissues, blood vessels with human and animal diseases, including atheroma and plaques are remain [8, 9, 10]. It is known that Cu and Fe are powerful catalysts of peroxide processes, particularly in atherosclerosis [5, 6, 11, 12].

In order to prevent an excessive accumulation of microelements, a number of drugs in patients with genetically determined diseases are used: desferrioxamine in the presence of hemochromatosis [13], D-penicillamine during Wilson-Konovalov's disease with deposition of Cu in the tissues. However, these medications cause many adverse effects up to an acute renal failure, although their appliance must be practically constant. At the same time, the deposition of Fe, Cu, Ni, Zn in atherosclerotic vessels always occurs with Fe and Cu have prooxidant characteristics, promote peroxidation with concomitant formation of pro-inflammatory mediators and development of proliferous process in the intimae of vessels.

The deposition of excessive amounts of microelements and calcium in modified atherosclerotic vessels and plaques in adults, as well as in an urine microprotein with cardiomyopathies were detected earlier [7, 11, 13, 14, 15]. Preventing the accumulation of microelements in atherosclerotic blood vessels is a relevant problem of biology and medicine [16].

There is some evidence on the study of the effectiveness method of pharmacological correction by violation of microelement composition in the atheroma with experimental reproduction atherosclerosis in genetically exposed to mouse's atherosclerosis (apo E / LDRL), kept on the atherogenic diet, by using one of antiatherosclerotic preparations - perindoprilat (inhibitor of angiotensin converting enzyme - ACE inhibitor) for 2 months.

It was noted that this preparation caused the "tendency" to approach of concentration Cl, Cu, Fe, Se, Zn in the vascular atheroma to the concentration of animals, which are contained in the cholesterol-free diet [6].

However, long-term use of perindopril (noliprel) such complications quite often occur: hypokalemia, renal dysfunction, fluid and electrolyte balance, can be neutropenia, angioneurotic edema, an occurrence of dry cough, there are contraindications to the use of perindopril in heart failure, renal failure, ischemic heart disease, lack of cerebral circulation, i.e. when the anti-atherosclerotic drugs is needed [6].

Therefore the aim of the presented studies was to determine the possibility of medicamentous correction of biochemical and tissular (including cardiovascular) excessive deposition of microelements Fe, Cu, Cr, Zn, and calcium to prevent the damage of blood vessels and tissues under the chronic metabolic disorders (in atherosclerosis and other excess microelementoses), also the reduced risk of complications by using of bisphosphonates in an antiatherosclerotic treatment complex, particularly the following preparations: xyidifon, medifone, zoledronate.

One of the most promising agents for the calcium' accumulation in the soft tissues are bisphosphonates, it is structural analogues of inorganic pyrophosphate (PPI), one of the major metabolites are in the cell, which is involved as a product or substrate is in more than 60 biochemical reactions and it is a major regulator of calcium distribution in cells and tissues. Bisphosphonates is PPI synthetic analogues as well as pyrophosphates form soluble chelate complexes with metals [15, 17, 18].

In previously published studies it is shown that xydifon reduces the deposition of calcium in the elaboration area in the vessels of atherosclerotic plaques [15]. The advantage of bisphosphonates in comparison with natural inorganic pyrophosphates is their resistance to spontaneous and enzymatic hydrolysis. A human does not have ferments that destroy bisphosphonates, which allows to get the effect of calcium metabolism regulation, even when using small doses of the drug.

The polyphosphate's ability to inhibit scaling (salt deposition) was discovered in the late thirties of the twentieth century [19, 20].

The bisphosphonate's distinctive features in comparison with inorganic pyrophosphates are as follows: 1) the presence of the phosphonic bonds P-C-P in the molecule instead of orthophosphoric P-O-P; 2) Phosphonic bonds provide the resistance of bisphosphonates molecules to spontaneous and enzymatic degradation in the organism, therefore the bisphosphonates are not metabolized in the human's body and animals unlike the inorganic pyrophosphate; 3) due to the bisphosphonates' stability in the body, it can be taken orally or transdermally, most importantly if situation so requires for prolonged drug usage; 4) the bisphosphonates effect on the organism basically similar to the inorganic pyrophosphate effect, and on the one hand, it prevents calcium salts deposition in soft tissues, and on the other hand, it normalizes the skeletal ossification process (reduction of bone resorption, improving calcium absorption in the bones) [17, 18]. Bisphosphonates exert their activity in minimal doses - 4-10 mcmol / l [17, 18]. Bisphosphonates have been very effective medication in preventing sedimentation of slightly soluble substances such as calcium carbonate, calcium sulfate, calcium phosphate, strontium sulfate and barium sulfate, hydroxide aluminum, hydroxide cobalt, hydroxide cuprium and many other [18].

2. Materials and Methods

Bisphosphonates were administered per os into experimental animals after the reproduction of an atherosclerotic process with the formation of vascular plaques (atheroma) using the line associated with cholesterol

(1%) diet and subtoxic doses of vitamin D_2. It is used 3 bisphosphonate synthesis: xyidifon (oxyethylidenbis-phosphonate K, Na salt), mediphone ([3-(dimethylamino)-1-hydroxy propylidene] bis-phosphonate: olpadronate) and zoledronate ((1-hydroxy-2-imidasole-1-y1) ethyliden bis-phosphonate,). Control over the content of microelements and calcium in serum and blood vessels (abdominal aorta) was carried out using X-ray fluorescence analysis in the geometry of total external reflection, on a laboratory spectrometer S2 PICOFOX. This method allows to conduct the study of microelement composition in the minimum level of the studied material (mcg) [18, 21, 22, 23, 24]. The indicator of metabolic processes was investigated: serum content of high and low density lipoproteins (HDL and LDL) cholesterol, triglycerides, atherogenic index was calculated. The state of the blood albumin, providing the lowest level of the index of blood toxicity was determined with the help of fluorescent probes [25]. Peroxidation processes were determined by the activity in the serum of prooxidant enzyme of xanthine oxidase, total antioxidant activity of serum and serum content of one of the key end delivery products of lipid peroxidation – malonic dialdehyde [15, 26]. Furthermore, using standard set of HUMAN (Germany) firms on the biochemical analyzer KONELAB in the serum was determined the activity of enzymes such as lactate dehydrogenase (LDH), involved in the metabolism of lactic and pyruvic acid - activity indicators anaerobic (hypoxic) glycolysis, the activity of creatine phosphokinase (CPK), which includes a myocardial isoenzyme CPK, the alkaline phosphatase activity - a regulator of calcium-phosphorus metabolism in the organism. It was determined the cholesterol's level in the blood, triglycerides, uric acid, creatinine.

Experimental atherosclerosis reproduction and plaque formation in the vessels was performed using white rats, male, weighing 250 grams, in 5 randomized groups. The line's fragments were implemented into the abdominal aorta for reproduction atheroma and plaque formation in all animals. 1% of cholesterol was included into the diet of all the animals starting from the 2nd group. Furthermore, at the first 3 days in the groups 3 and 5 it was inserted ergocalciferol, vitamin D_2, 75 mg / kg for reproduction vascular calcification through the gastro-duodenal tube into the stomach. As a therapeutic factor of animals' selected groups one of the bisphosphonates: xydifon, medifone or zoledronate a dose of 50 - 20 mg / kg in the form of liquid was injected through the gastric gavage starting from the 4th day during the entire period of observation. At the end of the experiment a set of morphological, histochemical and morphometric data by using light microscopy was conducted. Biochemical study of microelement composition and calcium in the tissues of the aorta atheroma was conducted.

The study about the correcting effect of bisphosphonates on the excessive accumulation of minerals and calcium in atherosclerotic vascular changes was conducted with laboratory animals during experimental modeling of atherosclerosis vascular calcification.

The experimental material was divided into five groups:

Group 1 – Experimental atherosclerosis, caused by the intravital injection of the line into laboratory rats' aorta;

Group 2 - Experimental atherosclerosis, caused by the intravital injection of line into the aorta of rats and the injection of cholesterol and vitamin D_2;

Group 3 - Experimental atherosclerosis caused by intravital injections of the line into the rats' aorta and injection of cholesterol and vitamin D_2 + xydifon oral exposure in a dose of 50 mg / kg daily.

Group 4 - Experimental atherosclerosis caused by intravital injection of the line into the rats' aorta and injection of cholesterol and vitamin D_2 + medifone oral exposure in a dose of 20 mg / kg daily.

Group 5 - Experimental atherosclerosis caused by intravital injection of the line into the rats' aorta and injection of cholesterol and vitamin D_2 + zoledronic acid oral exposure in a dose of 20 mg / kg daily.

As a control, the data was received from the intact animals.

3. Results and Discussions

During the material's analysis obtained from the animals of 2-5 research groups it was revealed an atherosclerotic pathomorphosis of the moderate aortic wall. These changes were accompanied by calcification of the aortic wall. The results of the morphometric analysis of the distribution micro-conglomerates of calcium salts in histochemical preparations of the aorta and the results of biochemical blood tests are presented in Table 1.

The results obtained in the analysis of the morphometry histochemical preparations of the aortic experimental animals, generally confirm the underlying hypothesis in the study that the use of bisphosphonates (particularly ksidifon) has a positive effect on tissue processes in the vascular wall in case of atherosclerosis in the reducing form of the micro-conglomerates salts calcium' disposition.

Table 1. *Biochemical indicators of the blood serum and changes in the vascular tissues of animals in case of experimental atherosclerosis (treatment by xydifon, medifone and zoledronic acid).*

Indicators (norm)	**Groups**				
	1 Line	**2 Line + cholesterol + D_2**	**3 Line + cholesterol + D_2 + xyidifon**	**4 Line + cholesterol + D_2 + medifone**	**5 Line + cholesterol + D_2 + zoledronic acid**
Index of toxicity <0,2 units	0,1±0,01	0,33±0,04	0,16±0,01	0,13±0,01[2]	0,16±0,01[2]
LDL >0,8 г/л	0,15±0,01	0,22±0,012	0,17±0,01[1]	0,13±0,01[1, 2]	0,16±0,01[2]
HDL 0,25 g/l	0,62±0,06	0,52±0,05	0,55±0,05	0,57±0,05[1]	0,73±0,08
Index of atherogen. 2,1 units	3,03±0,3	3,55±0,3	3,5±0,3	3,2±0,3	2,4±0,2[2, 3]
Creatinine<60 mmol/l	45,80±4,16	48,98±4,83	47,66±4,0	50,54±5,15	52,00±4,83

Indicators (norm)	Groups				
	1 Line	**2 Line + cholesterol + D_2**	**3 Line + cholesterol + D_2 + xyidifon**	**4 Line + cholesterol + D_2 + medifone**	**5 Line + cholesterol + D_2 + zoledronic acid**
LDG <400 units/l	322,00±30,0	494,30±32,50[1]	345,70±35,00[1]	280,60±26,0[2]	283,20±28,0[2]
Alkaline phosph-for <300 units/l.	201,3±20,60	281,40±16,50[1]	259,00±25,0[1]	249,0±24,0[1]	164,0±16,0[2]
Cholesterol <2,5 mmol/l	2,23±0,15	2,0±0,13	1,39±0,1[1, 2]	1,3±0,1[1, 2]	1,6±0,1[1, 2]
Triglycerides <1,0 mmol/l	0,62±0,16	0,62±0,11	0,55±0,05[1]	0,57±0,06 [1,2]	0,73±0,11
UA<0,28 mmol/l	0,42±0,04	0,57±0,06	0,43±0,03[2]	0,38±0,03[2]	0,33±0,03[2, 3]
Quantity of granules*	3600±350	4738±560[1]	1016±736[1, 2]	1980±424[1, 2]	1898±315[1, 2]
∑ granules square*	4431±400	5827±400[1]	1646±1100[1, 2]	2336±380[1, 2]	2277±395[1, 2, 3]
Ca 8 ±0,5 mg/g tissue	11,1±1	15,8±2,0	8,0±0,8[1, 2]	8,5±0,8[1, 2]	8,5±0,7[1, 2]
Fe 1,8±0,2 mcg/g tissue	3,4±0,1	5,0±0,6	3,5±0,6[2]	3,0±0,3[2]	3,1±0,3[2]
Cu 0,05 mcg/g tissue	0,08±0,001	0,09±0,001	0,04±0,001[2]	0,045±0,001[2]	0,045±0,001[2]
Zn 2,2±0,2 mcg/g tissue	2,7±0,2	2,0±0,2	1,6±0,15[1, 2]	1,5±0,15[1, 2]	1,7±0,12[1, 2]

LDH - lactate dehydrogenase; alkaline. phos-for - alkaline phosphatase, UA- uric acid, LDL - low-density lipoprotein, HDL - high-density lipoprotein
* The amount of calcium granules and Σ granules' square per unit of a cross section of the aorta. The reliability of differences: p ≤0,05 compared to [1] - the first group, [2] - the second group, [3]- the third group.

The data in the table shows typical symptoms of atherosclerosis in animals under the influence of the diet enriched with cholesterol, sub-toxic doses of vitamin D_2 and lines, implanted into the abdominal aorta for the atheroma reproduction and atherosclerotic plaque, as well as the vascular wall inflammation. The most characteristic features was the increase of the content in the blood serum of the low density lipoprotein (LDL), high density lipoproteins decrease (HDL), transporting cholesterol from tissues, the increase of uric acid level, triglycerides, atherogenic index, albumin toxicity index. All the mentioned changes of the blood serum composition were significantly decreased under the action of all three bisphosphonates, though in varying degrees: albumin toxicity index was the lowest in the group treated by xydifon, as well as triglycerides and cholesterol. Under the influence of a mediphone toxicity index for albumin, cholesterol and triglycerides were minimal among the indicators of animals groups treated by bisphosphonates. However, the most numerous and prominent (6 out of 10) were positive signs in the group 5, treated by zoledronic acid: a significant reduction in atherogenic index, LDL, alkaline phosphatase activity and lactate dehydrogenase, the amount of uric acid in a significant increase of HDL - antiatherogenic proteins. In contrast to the groups treated by xydifon and medifone, the treatment by zoledronic acid is still remained a moderate increase of triglycerides and cholesterol in serum blood. These data shows the differences of pharmacological properties of bisphosphonates and the advisability of periodic alternation of the different bisphosphonates' application. The positive effect of the three bisphosphonates, which had been used in this experiment, the study confirmed the content of microelements (Fe, Cu) and calcium in the damaged locus tissues of the aorta, as well as in morphological studies. It is noted that the atherogenic factors reduce the amount of Zn in the tissues. This effect also remains in case of positive effect of bisphosphonates on an atherosclerosis, obviously it is the basis for an additional injection into the organism of zinc supplements during the treatment of atherosclerosis, and the other authors pointed on that as well [27].

The results from the analysis of the morphometry of histochemical preparations of the aortic experimental animals, generally confirms the underlying hypothesis of the study that the use of bisphosphonates has a positive effect on the tissue processes in the vascular wall in case of the atherosclerosis, reducing the degree of calcium salts mikrokonglomerates' deposition (Table).

It is remarkable that the used bisphosphonates have different effects on the evaluated by the histochemical features deposition of calcium in the aortic wall.

Under the action of xydifon the number of granules decreased by half compared to the same in the animals who received only calcific agents. The size of the granules was also reduced, and in this case xydifon was the most effective among the used bisphosphonates. The size of the granules in most parameters were lower not only those parameters in the animals of the second group (calcification), but also in the animals of the first group (line). It should be noted that the feature of this group was the maximum value of the minimum coefficient of Feret (minimum size of granules' projection) (Table). This indicates on the fact that in case of xydifon's effect, additionally, the relative number of the smallest granules reduces. However, not only the size and number of microconglomerates granules calcium salts decreases in the action of xydifon. Significantly their optical density also decreases.

The mediphone effect on the amount of calcium-containing granules was even more effective than the xydifon's effect. However, the granules' dimensions are the maximum in this group of animals in comparison to the other. Also, the average and the optical density of the granules were the maximum.

The most reduction (five-fold compared to the animals of the second group) of the calcium salt granules was observed in animals who treated by zoledronic acid. The granules' size slightly decreased in comparison to the second group, but they were significantly bigger than in animals who received xydifon. Thus the average optical consistence of the granules was the maximum.

Among the three studied preparations xydifon can be considered as the most effective as it affects all certain

morphometric parameters of calcium inclusions. Despite the fact that the amount of the granules under the influence of mediphone, especially zoledronate becomes less than under the effect of xydifon, the increase of their size and consistence does not allow interpreting clear the clinical efficacy of these preparations.

It should be noted that when comparing the received biochemical indicators with morphometric parameters with deposition of calcium in the aortic wall, it was revealed strong positive correlation between the size of the granules of calcium salts and the LDL level (r= 0,90-0,91), alkaline phosphatase level (r = 0,90-0,91), triglycerides level (r = 0,60-0,62), uric acid levels (r = 0,64-0,66). In contrast to the size, the consistence of calcifications is conversely correlated with an atherogenic index (r = -0,61), triglycerides level (r = -0,8) and uric acid (r = -0,79). In other words, the granules were characterized by more consistency of calcium deposition while reducing the level of triglycerides, uric acid and the level of an atherogenic index, it can be regarded as a factor of an ordering the calcium distribution sin the cells, it was marked by us earlier under the influence of the calcium regulator – xydifon [15].

4. Conclusions

1. Biochemical attributes of an atherosclerosis risk were HDL reduction, LDL and an atherogenic index rise, toxicity index for albumin increase, high triglycerides and uric acid in blood serum, as well as increasing the activity of an alkaline phosphatase.
2. Subtoxic doses of vitamin D_2 injected into the animals at the beginning of the experiment per os were caused an increase of the index of toxicity after one month, the content of MDA and CPK in the blood serum. However, it is noted a moderate decrease in the activity of xanthine oxidase, the concentration of triglycerides and uric acid.
3. Bisphosphonates that used for medicinal purposes raise the level of HDL, reduce an atherogenic index, the level of triglycerides and uric acid.
4. The usage of two calcium metabolism regulators: therapeutic doses of bisphosphonates for a long time and a short first course of sub-toxic doses of vitamin D_2 – by the end of the month it reduces LDL and atherogenic index, significantly reduces the toxicity index for the albumin, the concentration of uric acid and the content triglycerides in the blood's serum.
5. The studied biochemical blood's parameters are significantly correlated with the morphological image of the calcium depositions level in the wall of the aorta, besides the size of calcifications and their consistence depends on the different experimental conditions and treatment with bisphosphonates.
6. A positive effect of xydifon, medifone and zoledronic acid on the content of microelements and calcium in vascular tissues was determined, which correlates with their anti-atherosclerotic effect on biochemical indicators of atherosclerosis risk factors in the blood.
7. Despite the absence of antioxidants in a medical complex, xydifon in combination with the short course sub-toxic doses of vitamin D_2 had the greatest impact on the various aspects of metabolic processes. They are typically combined with a high risk of atherosclerosis development.
8. Based on the data, a method of medicamental correction of minerals' excessive accumulation and calcium in the blood vessels in atherosclerosis using anti-atherogenic medications, for the prevention and correction of changes in the microelement's composition, calcium deposition in the blood vessels and reduction of the risk of side effects. The following bisphosphonates such as xydifon, medifone or zoledronate are included in the complex of therapeutic measures. Besides the normalization of microelement composition of the vascular wall, they reduce the degree of change in the biochemical markers of atherosclerosis: atherogenic index, the content of calcium in the aorta, the amount of LDL the level of uric acid in the blood serum.

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