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Research Article

SEASONAL FEATURES OF EXOGENOUS MELATONIN AND DOSED HYPOXIA INFLUENCESON BONE REMODELING OF YOUNG RATS

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ABSTRACT

The study was conducted on 72 young (3 months) male Wistar rats in spring (March-April) and autumn (October-November).Each experiment lasted 28 days. Animals of the experimental groups were breathing the gas mixture (GM, PO_2 = 90 mmHg, the equivalent of 12volume per cent in nitrogen) in the mode 15 min deoxygenation/15 min reoxygenation during two hours.The other group of animals, exceptGM, was orally administered 1 ml of an aqueous suspension of melatonin at pharmacological dose of 5 mg/kg of body massat 10.00 a.m. In spring, combined effect of dosed hypoxia (DH) and pharmacological doses of melatonin reduced the activity of alkaline phosphatase (ALP), but did not affect the activity of acid (ACP) and tartrateresistant acid phosphatase (TRACP).This effect has boosted the concentration of glycosaminoglycans (GAG), which may cause a slowdown in bone remodeling of young rats. In spring, changes in the concentration of amino acids (AA)which are directly involved in the synthesis of bone collagen of young animals after exposure to DH or combined effect of DH and exogenous melatonin at pharmacological doses were unidirectional. In most cases, the concentration of AA significantly reduced, which can be the reason of collagen synthesis inhibition in organic matrix of bone tissue (BT).In autumn osteoblast activity in young rats after exposure to DH and after its joint action with exogenous melatonin in pharmacological doses unchanged. However, activity of lysosomal enzymes after the influence of mentioned factors increased. At the same time, concentration of GAG increased. It may be the outcome of communication between GAG and collagen fibers after exposure to DH, ie destructive changes of the organic matrix of bone. In autumn the increase of total and free cholesterol (TC and FC respectively) concentrations in BT ofratsafterexposure as to DH, so to its jointaction with melatonin, couldbe considered as a sign of possibleseasonal intensification of bone mineralization.

INTRODUCTION

The physiological remodeling ofBT is a complex process that is controlled by the system hormones, numerous growth and local regulatory factors. Melatonin is a hormone which plays a significant role in this process. It is established that melatonin modulates the process of osteoblast and osteoclast differentiation (Reiter, 2001). It promotes mineralization of matrix in cell culture, enhances the synthesis of collagen and non-collagen proteins of bone matrix, it inhibits the development of osteopenia by activating the secretion of growth hormone in rats (Wolden-Hanson, 2000).

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In cultures of human osteoblasts, melatonin in pharmacological doses stimulates ALP proliferation and its activity in these cells; promotes collagen type I, osteopontin, bone osteocalcin and sialoprotein expressions; stimulates the formation of mineralized matrix. These changes have a clear seasonal character (Nakade, 1999 and Satomura, 2007). In our previous studies it was investigated the effect of pharmacological dose (5 mg / kg) of melatonin on parameters of bone remodeling in 3-month-old Wistar rats within 28 days in autumn and spring seasons. In autumn the administration of melatonin increased the activity of ALP by 43,9%, hyaluronidase activity (HA) by 15,4% and reduced the concentration of GAG by 46,7%. The concentration of total lipids and triglycerides (TG) decreased. In spring the administration of melatonin suppressed physiological bone remodeling of rats, reduced activity of ALP and increased

activity of ACP and TRACP by 78 and 72% respectively. Concentrations of free amino acids (fAA) and GAG increased (Berezovsky, 2015; Litovka, 2016 and Zhernoklov, 2016). In autumn melatonin administration increased the concentration of free thyroxine (fT4) by 30%. Concentration of pyridinoline (PYD) decreased by 48%. In spring period the results were significantly different. The concentration of free triiodothyronine (fT3) decreased by 18%. FT4 and PYD concentrations increased by 31% and 42% respectively. The data obtained indicate that melatonin inhibits the physiological remodeling of BT in spring. Such changes may disrupt the link between GAG and collagen fibrils and weaken the fixation of inorganic component of connective tissue - hydroxyapatite crystals (Zhernoklov, 2016). Itis known that in a dosed short-term reduction of partial pressure of oxygen (PO_2) in the inhaled air, protective and adaptive reactions are activated. There are always ready and fully formed genetic, physiological and biochemical mechanisms for the implementation of them in the body (Franke, 1995 and Arnett , 2003). Our previous studies confirmed that dosed intermittent normobaric hypoxia at sanogenic level (12% O_2 in nitrogen) changes the concentrations of parathyroid hormone, osteocalcin, PYD and C-terminal propeptide collagen I type. These biologically active substances can influence the rate of bone remodeling and organic matrix plays the main role in it (Berezovskaja, 2002; Litovka, 2011 and Litovka, 2014).

Aim

To study the joint influence of exogenous melatonin and hypoxia on biochemical markers of bone tissue of Wistar rats in autumn and spring.

MATERIALS AND METHODS

The study was carried out on young 3 month old rats of Wistar line in spring (March – April) and autumn (October-November). 72 animals were used in experiments: 36 rats in spring and 36 – in autumn. The rats were divided into 3 groups, 12 animals in each one: I – control group, II - group of animals that within 28 days were breathing gas mixture (GM, PO_2 = 90 mmHg, the equivalent of 12 volume per cent in nitrogen) in the mode 15 min deoxygenation/15 minreoxygenation during two hours, III – group of animals which except GM, was orally administered 1 ml of an aqueous suspension of melatonin ("Unipharm Inc.", USA) in pharmacological dose of 5 mg/kg of body mass at 10.00 a.m. At the same time, control group of ratsreceived equivalent quantity of physiological solution.The animals were received from nursery of vivarium ofInstitute of physiology named after O. O. Bogomolets, NAS of Ukraine. They were kept in standardconditions of vivarium at natural cycle light/darkness and received the ordinary food allowance.
Biochemical markers of BTwere studied using spectrophotometric and chromatographic methods.Using the standard sets of reagents in the blood serum of rats (blood sampling was carriedout at 29th day of experiment at once after decapitation of animal) were determined: the alkalinephosphatase (ALP, "Lachema", Czechia) – index of bone tissue formation and also acid phosphatase (ACP, "Bio Systems", Spain), tartrate resistant acid phosphatase (TRACP, "BioSystems", Spain), glycosaminglycans by orcine method by Klyatskin (GAG) [14]– as resorption parameters. In the BT extract uronic acids (UA) were studied by Leontiev's method (Leontiev, 1976). The method of thin layer chromatographydetermined the concentration of free amino acids (fAA) in serum and BT, and lipids - in BT.To determine the major lipid fractions in BT, the femur was purified from muscle and washed from bone marrow. Bone sample (100 mg) was degreasedand dehydrated in alcohol: acetone (1: 2).Then alcohol: acetone was evaporated and the lipids dry residue was dissolved in 100 mcl mixture of chloroform-benzene-acetone (1: 2: 1) and applied by microsyringeon marked chromatogram. Chromatographic separation of TL was performed on factory manufactured plates Silufol (Czech Republic) 15 × 15 cm, after activating them during 1 hour in an incubator at 110^0C. At the same time, filter paper is added and the mixture of solvents (hexane-diethyl ether-acetic acid (7: 23: 1)) is pouredin the chromatographic chamber for better saturation (Veselskiy, 1999). To determine AA composition of BT, degreased and dehydrated femur was subjected to hydrolysis at 100^0C for 20 minutes in a solution of 0, 04 M CH3COOH (1:10). It was centrifuged during 30 minutes at 3000 rev/min. The supernatant was evaporated at 40-60^0C and dissolved in 0,1ml of 50% C2H5OH. It was applied to marked chromatograph20 ml by microsyringe. Solvent systemwas used for the distillation, which included isoamyl alcohol, butyl alcohol, acetic acid, formic acid-water (9: 7: 4: 25) by volume (Kaznacheeva, 1976 and Korobeinikova, 1981). Statistical analysis of the received results was carried out on personal computer using the programANOVA. The arithmetical mean (M) and standard mistake (m) were determined. The reliabilityof difference between control and experimental samples was assessed by Student t-criterion.

RESULTS OF RESEARCH

In spring the ALP activity in blood serum after a 28-day exposure to DH did not change significantly. There was only a trend towards its increase by 20,4%. Joint action of DH and oral administration of pharmacological doses of melatonin in young animals led to significant decrease of ALP activity by 56% ($P<0,05$) in blood serum compared with control (Table 1). Markers of BT resorption under the same experimental conditions, namely ACP and TRACP activity in blood serum, significantly did not change in these animals. GAGconcentration in blood serum significantly increased by 275% ($P<0,05$) after exposure to DH and its joint actionwith melatonin in pharmacological doses compared with control. We found no significant alteration of UA concentration, but only a tendency to its increase after a joint action of factors.

A common biochemical feature of BT is high content oflipids. They are important components of connective tissue, but their role in bone mineralization is poorly studied. Lipid fractions in the femoral of control group of rats were distributed as follows: 41,54% was accounted for the total phospholipids, 19,74% - total cholesterol (TC), 23,33% - free fatty acids (fFA) and 13,85% - triglycerides (TG). Correlations between these fractions in the total pool of lipids was equal to 0,42: 0,20: 0, 23: 0, 14. After exposure to DH total lipid concentration increased by 11% ($P<0,05$). It was found a significant increase of TG concentration by 62% after the jointaction of DH and melatonin compared with the control group. The concentration of TC did not change significantly in any of the studied groups of animals. However, after the exposure to DH the concentration of cholesterol esters increased by 68% ($P<0,001$) and free cholesterol decreased by 48% ($P<0,01$) compared with

the control group. Aftera jointaction of DH and melatonin the concentration of cholesterol esters increased by 58% (P<0,001) compared with the control group (Table 2). Amino acids (AA) are the basal structures of which initial and all subsequent levels of proteins are created. The concentration of most fAA investigated in BT of young Wistar rats after 28-day exposure to DH in spring decreased. Fourteen groups ofAA were studied.We noted a significant reduce in the concentration in 4 cases: arginine, histidine and taurine (Arg+His+Tau) - by 29% (P<0,001), lysine and asparagine (Lys+Asn) - by 14% (P<0,001), valin and tryptophan (Val+Trp) – by 25% (P<0,01), serine and aspartic acid (Ser+Asp) - by 50% (P<0,05) compared withcontrol (Figure 1).

synthesis of collagen in BT of young animals were unidirectional after as exposure to DH, so to its joint action with exogenous melatonin in pharmacological doses. In most cases, these rateswere significantly decreased. This could indicate the inhibition of collagen synthesis in organic matrix of BT. Inautumn ALP activity did not change in any of the groups of animals. ACP activity increased by 85,5% (P<0,05) after the jointaction of DH and melatonin compared with control group(Table 3).TRACP activity increased as after exposure to DH (by 61,8%, P<0,05), so after its joint action with melatonin (by 85,5%, P<0,05) compared with control (Table 3).

Table 1. Biochemical indicators of bone tissue of young Wistarrats in spring (M±m, mg/g of wet bone, n=12)

Index	Unit of measurement	Group I (Control)	Group II (Dosed hypoxia)	Δ, %	Group III(Dosed hypoxia+melatonin 5 mg/kg of body mass)	Δ, %
ALP	MU/l	32,96±1,95	39,69±2,73	+20,4	18,52±1,77*	-43,8
ACP	MU/l	36,04±6,85	40,52±8,18	+12,4	38,08±5,44	+5,7
TRACP	MU/l	20,40±4,26	24,62±7,42	+20,7	15,42±1,76	-24,4
GAG	g/l	0,03±0,003	0,05±0,01*	+46,9	0,12±0,04*	+ 275
UA	mg/g	43,75±1,95	43,94±1,25	+0,43	45,76±1,19	+4,6

*Note: * - P<0,05 – comparing with control group of animals, " – " - index decrease in% compared with control, "+" - index increase in% compared with control*

Table 2. The content of lipid fractions in bone tissue of young Wistar rats in spring (M±m, mg/g of wet bone, n=12)

Lipidfractions		Experimentalgroups				
		Group I (Control)	Group II(Dosed hypoxia)	Δ,%	Group III(Dosed hypoxia+melatonin 5 mg/kg of body mass)	Δ,%
Phospholipids		29,52±1,5	32,56±2,4	+10	33,76±3,1	+14
	Esters	6,68±0,5	11,21±0,7***	+68	10,54±0,7***	+58
Cholesterol	Free	6,96±0,6	3,64±0,96**	-48	5,5±1,5	-21
	Total	14,03±0,7	15,19±1,1	+8	15,2±1,9	+8
Freefattyacids		16,58±1,9	19,2±2,7	+16	16,6±2,2	0
Triglycerides		9,84±1,01	12,11±0,96	+23	15,9±2,4*	+62
Totallipids		71,06±4,26	79,1±3,6*	+11	74,26±6,8	+5

*Note: * - P<0,05, ** - P<0,01, *** - P<0,001 – comparing with control group of animals, " – " - index decrease in% compared with control, "+" - index increase in % compared with control*

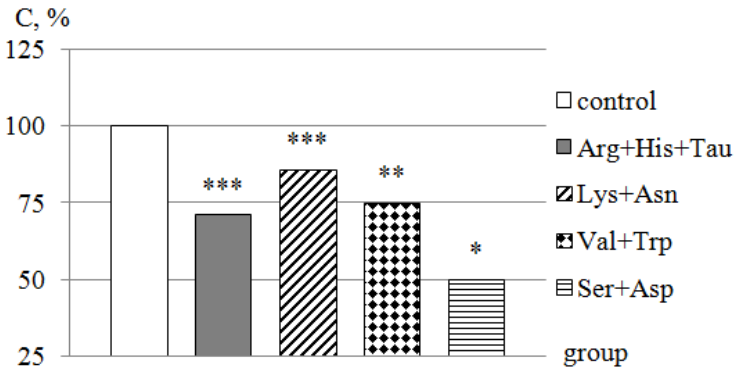

Fig.1. Amino acid composition in bone tissue of young Wistar rats after exposure to dosed normo baric hypoxia in spring: * -P <0,05,

Afterthe joint action of DH and melatonin in Wistarrats the concentrations of cysteine and cystine (Cys-SH+Cys-S-) significantly increased by 96% (P<0,001) and of ornithine and glucosamine (Orn+) - by 60% (P<0,01). In other cases under the same conditions we observed a significant decrease in the concentration of the following: tyrosine and glutamic acid (Tyr+Glu) by 27% (P<0,05), threo nine and izovalin (Thr+)by 40% (P<0,01), proline and hydroxyproline (Pro+) by 49% (P<0,001), leucine (Leu) by 21% (P<0,05) and isoleucine (Ile) by 18% (P<0,05) compared with control group (Figure 2). Changes in AA concentrations that are directly involved in the

GAG concentration increased by 24% (P<0,05) after the exposure to DH and tended to decline by 6% after its joint action with melatonin. The concentration of total lipids in the BT of II and III groups showed a tendency to increase by 6% and 14,4% respectively. The concentrations of TC significantly increased on average by 23% (P<0,05) after the exposure to DH and after its joint action with melatonin (Table 4). Under the same experimental conditions it was revealed a significant increase of FCin rats of II and III groups by 230% and 307% (P<0,05) respectively, compared with control (Fig.3).All other lipid fractions in BT significantly did not change in autumn.

An increase of total and free cholesterol in BT of Wistar rats after exposure to DH and its joint action with melatonin should be considered as a sign of a possible intensification of BT mineralization in autumn.Changes in the content of fAA in 3 months old Wistar rats after exposure to DH and its jointactionwith melatonin were insignificant. However, the concentration of ornithine and glucosamine significantly reduced by 29% (P<0,0001) after exposure to DHcompared with control. The joint action of DH and melatonin resulted in a significant alteration only in concentrations of alanine and globulin (+92,5%, P<0,001), proline and oxyproline (-39,2%, P<0,0001), leucine (+60%, P<0,0001), phenylalanine (+20,8%, P<0,001) and isoleucine (+43,8%, P<0,0001) compared with control group. The rest of AAunder the studydid not change.

DISCUSSION OF THE RESULTS

The activity of ALP in the blood serum of Wistar rats significantly decreased after the jointaction of DH and pharmacological doses of melatonin in spring. ACP and TRACP activitiesdid not change. Under the same conditions GAG concentration significantly increased in blood serum. The obtained results we consider as a significant imbalance of formation and destruction processes of bone and violation of connection between GAG and collagen fibrils.It is known that lysosomal enzymes can be in two different physiological states, active and inactive. In inactive state, the intensity of lysosomal enzymes secretion is reduced, similar to that is recorded in literature for muscle and secretory cells (Heersche, 1978).

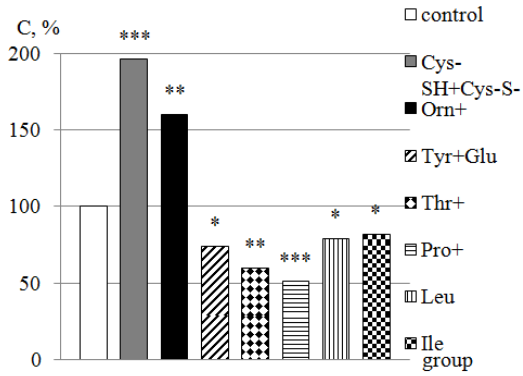

Fig. 2.Amino acid composition of bone tissue of young Wistar rats after joint action of dosed normobarichypoxia and melatonin (5 mg/kg of bone mass) in spring: * -P <0,05, ** - P <0,01, * - P <0,001 - compared with control group – the fact data of control group were considered as 100%**

Table 3. Biochemical indicators of bone tissue of young Wistar rats in autumn (M±m, mg/g of wet bone, n=12)

Index	Unit of measurement	Group I (Control)	Group II (Dosed hypoxia)	Δ, %	Group III (Dosed hypoxia+melatonin 5 mg/kg of body mass)	Δ, %
ALP	MU/l	49,1±4,4	58,79±5,1	+19,7	49,74±6,2	+ 1,3
ACP	MU/l	42,2±5,5	59,1±6,3	+40	76,0±7,7	+80
TRACP	MU/l	29,6±4,7	47,9±5,4*	+61,8	54,9±6,6*	+85,5
GAG	g/l	0,17±0,02	0,21±0,021*	+24	0,16±0,03	-6

*Note: * - P<0,05 – comparing with control group of animals, " – " - index decrease in% compared with control, "+" - index increase in% compared with control*

Table 4.ThecontentoflipidfractionsinthebonetissueofyoungWistarratsin autumn (M±m, mg/g of wetbone, n=12)

Lipidfractions		Experimentalgroups				
		Group I (Control)	Group II (Dosedhypoxia)	Δ,%	Group III(Dosed hypoxia+melatonin 5 mg/kg of body mass)	Δ,%
Phospholipids		31,17±2,9	33,2±1,9	+6,5	33,4±0,9	+7
	Esters	10,2±1,1	10,02±0,6	-1,8	10,27±0,8	+0,7
Cholesterol	Free	2,28±0,9	7,52±0,6***	+230	9,28±1,9**	+307
	Total	14,62±1,2	17,6±0,9*	+23,4	17,4±0,8*	+22
Freefattyacids		20,56±1,97	18,9±1,4	-8	24,95±1,5	+21
Triglycerides		17,3±1,0	20,4±1,1	+17,9	21,5±1,5	+24
Totallipids		85,0±6,2	90,16±4,9	+6	97,26±2,7	+14,

*Note: * - P<0,05, ** - P<0,01, *** - P<0,001 – comparing with control group of animals, " – " - index decrease in% compared with control, "+" - index increase in% compared with control*

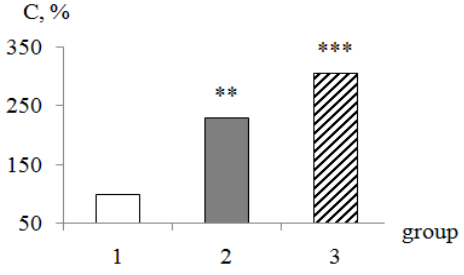

Fig. 3.The concentration of free cholesterol in the blood serum of young Wistar rats fromthe control group (1), after exposure to dosed hypoxia (2) and its joint action with melatonin (3) in autumn: – P<0,01, *** - P<0,001 – comparing with control group of animals – the fact data of control groups were considered as 100 %**

It is possible that with such short-term (28 days) experiment, part of the lysosomal enzymes was inactive. Even so, there are two parallel processes - recovery and destruction of bone, but the balance between them may be broken. The presence of imbalance is confirmed in literature data. The cases of decreased activity of osteoblasts without a significant increase in activity of osteoclasts are described (Grigoriev, 1994). In addition, high concentrations of melatonin may reduce the activity of osteoblasts, which consequently leads to the inhibition of bone resorption (Reiter, 2001; Reiter, 2009; Schroeder, 1981 and Conconi, 2000). It is found that in pharmacological doses, it reduces bone resorption by inhibiting the regulation of RANK-L (Steflik, 1994 and Penarrocha Diago, 2005).The changes we revealed in our experiment can be considered as a consequence of an increase in the number and functional activity of osteoclasts, which prove a dominance of osteoclastic type of bone resorption (Grigoriev, 1994). The results of our experiment give us the reason to suggest that osteoblast activity does not change after exposure to both factors in young Wistar rats in autumn. However, in contrast to the results obtained in spring, in autumn the activity of lysosomal enzymes increases, which are produced by osteoclasts after the exposure to DH and its joint action with exogenous melatonin. However, the increase in the concentration of GAG may indicate abuse link between GAG and collagen fibrils after the exposure to DH, ie qualitative, destructive changes of the organic matrix of bone.

Conclusions

- In spring the jointaction of dosedhypoxia (PO_2=90 mmHg) and pharmacological doses of melatonin (5 mg/kg of body mass) reduced alkaline phosphatase activity, but did not affect the activity of acid and tartrate resistant acid phosphatases. This effect has boosted the concentration of glycosaminoglycans, which may cause a slowdown in bone remodeling of young rats.
- In spring changes in the concentration of amino acids, which are directly involved in the synthesis of collagen in bone of young animals, after exposure to dosed hypoxia and its joint action with exogenous melatonin in pharmacological doses, were unidirectional. In most cases, the concentration of amino acids that can cause inhibition of collagen synthesis of organic matrix in bonesignificantly reduced.
- In autumn osteoblast activity in young Wistarrats as after exposure to dosed hypoxia, so after its joint action with exogenous melatonin in pharmacological doses did not change. However, activity of lysosomal enzymes after exposure to dosed hypoxia andits joint action with exogenous melatonin in pharmacological doses increased. At the same time, anincrease of glycosaminoglycans concentration may be the outcome of communication between them and the collagen fibrils. After exposure to dosed hypoxia, destructive changes of the organic matrix of bonewere occurred.
- In autumn an increase of total and free cholesterol in bone tissue of rats as after exposure to dosed hypoxia, so after its jointactionwith melatonin in pharmacological doses can be considered as a sign of possible seasonal intensification of bone mineralization.

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