Neuroendocrinology Letters ISSN 0172–780X

ORIGINAL ARTICLE

Assessment of the relationship between dynamic pattern of nighttime levels of melatonin and chosen biochemical markers of bone metabolism in a rat model of postmenopausal osteoporosis

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Submitted: *January 21, 2001*
Accepted: *February 06, 2001*

Key words: **melatonin; bone metabolism; model of postmenopausal osteoporosis; female rats**

*Neuroendocrinology Letters 2001; **22**:129–136 pii: NEL220201A07 Copyright © Neuroendocrinology Letters 2001*

Abstract

BACKGROUND: Lately, there have been suggestions that bone mass changes occurring in postmenopausal women may remain related to melatonin.
OBJECTIVE: To assess the relationship between the dynamic pattern of nighttime levels of melatonin and chosen biochemical markers of bone metabolism in ovariectomized rats – a model of postmenopausal osteoporosis.
METHODS: Mature Wistar female rats were either ovariectomozed or underwent a sham operation. Following this they were killed at 02:00AM at weekly intervals for 8 weeks after surgery. Serum levels of MEL at death related to the chosen biochemical markers of bone formation (alkaline phosphatase - ALP; carboxyterminal propeptide of type I procollagen - PICP, both in serum) and resorption (cross-linked carboxyterminal telopeptide of type I collagen - ICTP in serum; hydroxyproline - HYP and total calcium - Ca, both excreted in urine).
RESULTS: In all ovariectomized rats changes of examined indices of bone tissue metabolism were found to be dynamic and statistically significant relative to the control group; however the changes were more pronounced regarding resorption markers. Following ovariectomy, the increase in ALP and PICP values was found to begin at the 4th and the 1st week, while that in ICTP, HYP and Ca at the 2nd, the1st and the 1st week, respectively. The ALP and PICP values remained at a similar level until the end of observation, whereas ICTP, HYP and Ca gradually decreased. MEL levels were decreased during the 2nd week following surgery and slightly increased 2 weeks later. The serum MEL levels in the ovariectomized group were significantly and negatively correlated with serum ICTP and both urinary HYP and Ca levels.
CONCLUSION: Our findings in rats seem to corroborate the concept of secondary changes in MEL levels co-participating in the development of bone mass changes characteristic for postmenopausal osteoporosis.

Abbreviations

ALP	alkaline phosphatase
C	control group
Ca	total calcium
5 – HT	5 hydroxytryptamine
HYP	hydroxyproline
ICTP	cross-linked carboxyterminal telopeptide of type I collagen
IGF-I	insulin-like growth factor
LD	light:dark cycle
MEL	melatonin
M – Oval	preparation contains 75 mg of MEL and a minor amount of estrogen
O	intact rats
Ovx	ovariectomized rats
PICP	carboxyterminal propeptide of type I procollagen
PTH	parathormone
RIA	radioimmunological methods

Introduction

The influence melatonin exerts on bone tissue has been the subject of numerous reports. They concerned primarily the participation of this hormone in the development of postmenopausal osteoporosis [1], the latter being the commonest metabolic disease of the bone tissue and subject of a great deal of interest.

There have been suggestions in the literature that characteristic changes of bone mass in osteoporosis, resulting from predominance of resorptive processes over those leading to bone tissue formation, may be related to the melatonin level [1]. It has been concluded that secretion of this hormone decreases with age [2, 3, 4, 5, 6] and that menopause is time-related with substantial decrease in melatonin secretion and with increased rate of pineal calcification [3, 4, 5, 6, 7, 8]. Experimental studies have shown that melatonin is an important modulator of calcium and phosphorus metabolism. Most probably, it exerts its effect indirectly, by influencing activity of parathyroids and secretion of calcitonin as well as prostaglandins, important regulators of calcium and phosphorus balance and bone metabolism [9, 10, 11, 12, 13, 14, 15, 16].

Studies by Sandyk et al. [1] indicate that abrupt decrease of melatonin concentration in early menopause phase may be an important co-factor in osteoporosis development. It is thus believed that assessing melatonin concentration at such time might be a useful indicator and perhaps even a marker of postmenopausal osteoporosis susceptibility. On the other hand light therapy, oral administration of melatonin or factors which induce melatonin secretion might be used in prophylaxis and treatment of osteoporosis. Interesting observations concern women who were taking M-Oval preparation elaborated by Cohen et al. [17] in order to alleviate climacterium-related ailments. This preparation contains 75 milligrams of melatonin and a minor amount of estrogen. At the outset of three-year-long therapy an increase of bone density was noted in subjects taking the preparation.

To assess relationship between melatonin secretion and the metabolism of bone tissue we chose a very useful postmenopausal osteoporosis animal model, frequently used in experimental studies, i.e. female ovariectomized rats.

The purpose of this study was: a) the assessment of influencing ovariectomy and female sex hormone deficiency, which becomes evident as a result of this operation, on the bone tissue metabolism in female rats; b) to determine if probable secondary changes in melatonin (MEL) concentration can participate in generating expected disturbances of bone metabolism.

Material and Methods

102 sexually mature female Wistar rats weighing 140 ± 8 g were included in the study. During the experiment the animals were housed under conditions of uniform temperature (20 to 22°C), air humidity (80–85%) and light (12 hours of illumination and 12 hours of darkness – LD 12:12, light from 07:00 to 19:00). Rats were fed at the onset of the dark phase using a standard diet suitable for conducting bone metabolism research in experimental animals (Altromin Standard Diäten, Austria) and were given drinking water "at libitum".

After 2 weeks of adjustment, vaginal swabs were taken daily between 08:00 and 08:30. After establishing the estrus cycle, 6 animals were left intact (group 0 - before surgery), 48 underwent ovariectomy in the estrus phase (group Ovx) and the remaining ones underwent sham operation (control group - C). After the surgery the rats were divided into eight equally numerous subgroups (each consisting of 6 animals). At the appropriate time, i.e. prior to surgery and at weekly intervals following it (groups 1–8) the animals were marked and placed separately in metabolic cages for 3 hours (from 00:30 until 03:30) in order to collect urine aliquots for HYP and Ca determinations. Within the next 24 hours the rats were decapitated (at 02:00). The blood was collected into test tubes with granulated mass (Sarstedt) and centrifuged immediately. The obtained serum, as well as urine specimens, were stored frozen at –75°C until determination of concentration of MEL, ALP, PICP and ICTP. The study was conducted with the permission of Ethics Committee at the Silesian Academy of Medicine in Katowice.

MEL and the majority of bone metabolism markers were measured with comercially available RIA

kits: MEL (DRG, USA); PICP, ICTP (Farmos, Finland). Serum ALP activity and urinary excretion of Ca were determined using ALPHA DIAGNOSTICS kits (Poland). Urinary excretion of HYP was determined according to Drózdz et al. [18]. The sensitivity of assays was as follows: MEL 0.2 pg/tube, PICP 1.2 µg/l, ICTP 0.34 µg/l, HYP 6.6 µmol/l. The linearity for ALP and Ca methods were: 16 mg/dl and 1000 U, respectively. The respective intraassay and interassay coefficients of variations were: MEL 11 and 12 %, PICP 3.1 and 5.8 %, ICTP 4.5 and 6 %, HYP 5.5 and 7.2 %, ALP 7.8 and 8.5 %, Ca 4.7 and 6.8 %.

The statistical significance of results was assessed based on variance analysis for parametric tests when distribution of the variable was normal. When the variable distribution departed from normal, the variance analysis was conducted according to the Kruskal-Wallis method for nonparametric tests. The dependency of MEL concentrations and values of bone metabolism exponents was assessed using the Pearson correlation test (when variable distribution was normal) or the Spearman correlation test (when the variable distribution departed from normal).

Results

In all rats with ovaries removed and, resulting from this manipulation, reduced concentrations of female sex hormones, it has been found that there are dynamic, statistically significant, changes in values of examined indices of bone tissue metabolism in relation to the control group. However, the changes were more intensified regarding resorption markers (Figures 1, 2 and 3). Following ovariectomy, the increase in ALP and PICP values was found to begin at the 4th and the 1st week, while that in ICTP, HYP and Ca at the 2nd, the 1st and the 1st week, respectively. The ALP and PICP values remained at a similar level until the end of observation, whereas ICTP, HYP and Ca levels gradually increased so that after a lapse of the 6th, the 7th and the 7th weeks, respectively, after the operation underwent a decrease.

MEL levels were decreased during the 2nd week following surgery and slightly increased 2 weeks later (Figure 3).

The analysis of correlation has shown that the alterations in the values of ICTP, HYP and Ca correlated statistically, significantly and negatively with changes in MEL concentrations both in ovariectomized and control rats (Table 1). This relation was more intensified regarding ovariectomized group. However, the changes in ALP and PICP concentrations correlated only to a little degree with the variations in concentrations of MEL.

Discussion

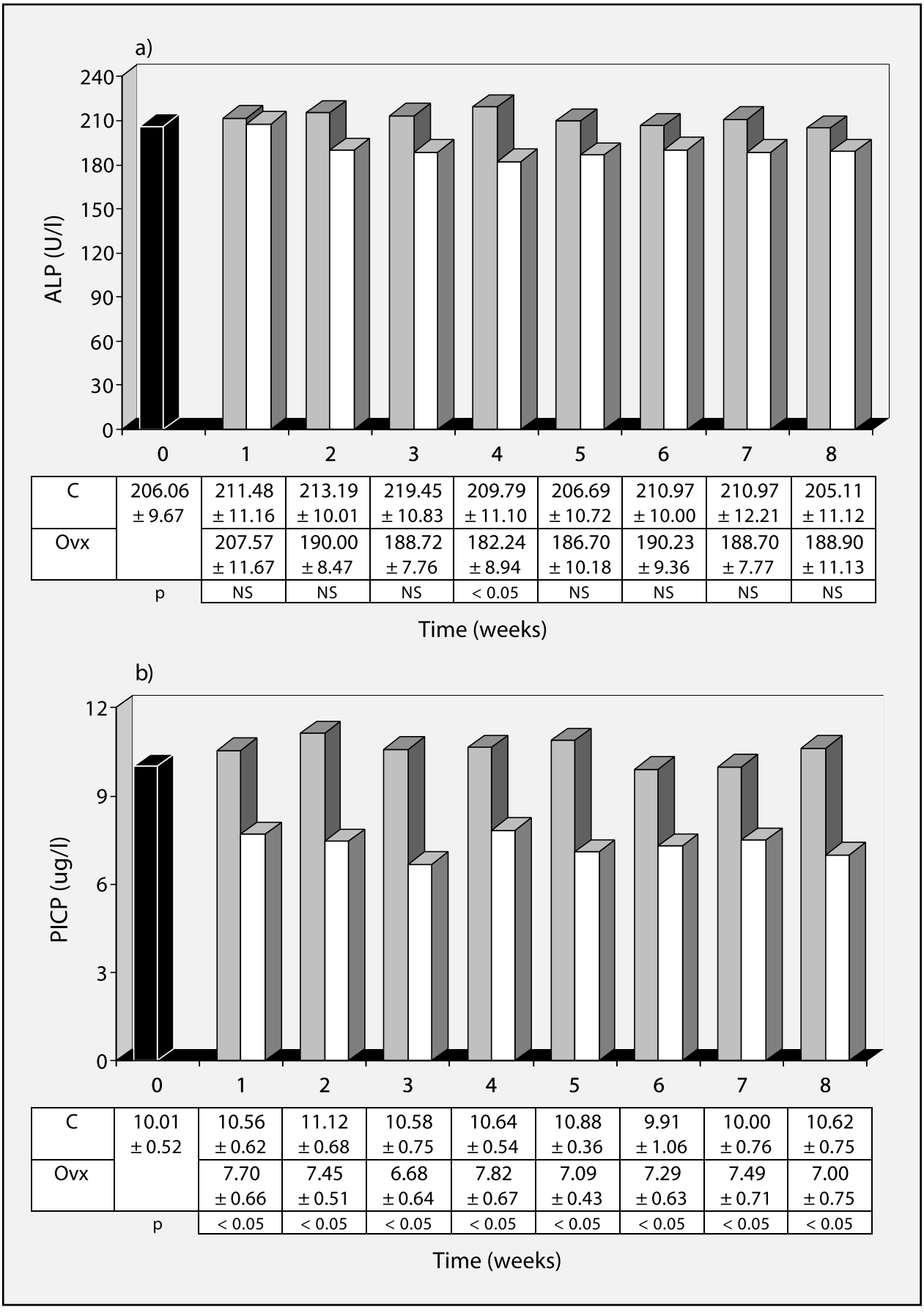
Bone is a dynamic tissue continually in reconstruction. In adults, processes of its formation and resorption remain in equilibrium. Disturbing this equilibrium leads to osteoporosis and can result from both an increased resorption of bone tissue due to increased activity of osteoclasts (as in postmenopausal osteoporosis) or a decrease in bone formation processes due to lesser activity of osteoclasts (as in senile osteoporosis) [14, 15, 16].

It has been known that the metabolism of bone tissue is modulated by various hormonal and local factors, such as growth and immunological factors [14, 15, 19, 20, 21, 22, 23, 24]. The process of bone formation is controlled by vitamin D, calcitonin, estrogens, androgens and the growth hormone; it also depends on correct functioning of the thyroid [14, 15, 19, 20, 22, 24]. Lately, there have been reports pointing to possible participation of melatonin in modulating the process of bone forming [25]. Resorption, on the other hand, follows from a deficiency of vitamin D, low calcium concentration, surplus of glycocorticosteroids, PTH, thyroid hormones and cytokines [14,

Table 1. Correlation coefficients between the values of melatonin (MEL) and chosen biochemical markers of bone formation (ALP, PICP) and resorption (ICTP, HYP and Ca) in ovariectimized (Ovx) and sham operated (C) rats.

Dependent variable	Independent variables	Groups	
		Ovx (n = 45)	C (n = 45)
MEL (pg/ml)	ALP (U/l)	- 0.253	- 0,160
	PICP (µg/l)	- 0.255	- 0,261
	ICTP (µg/l)	- 0.841*	- 0,529*
	HYP (µmol/l)	- 0.590*	- 0,445*
	Ca (mmol/l)	- 0.537*	- 0,315*

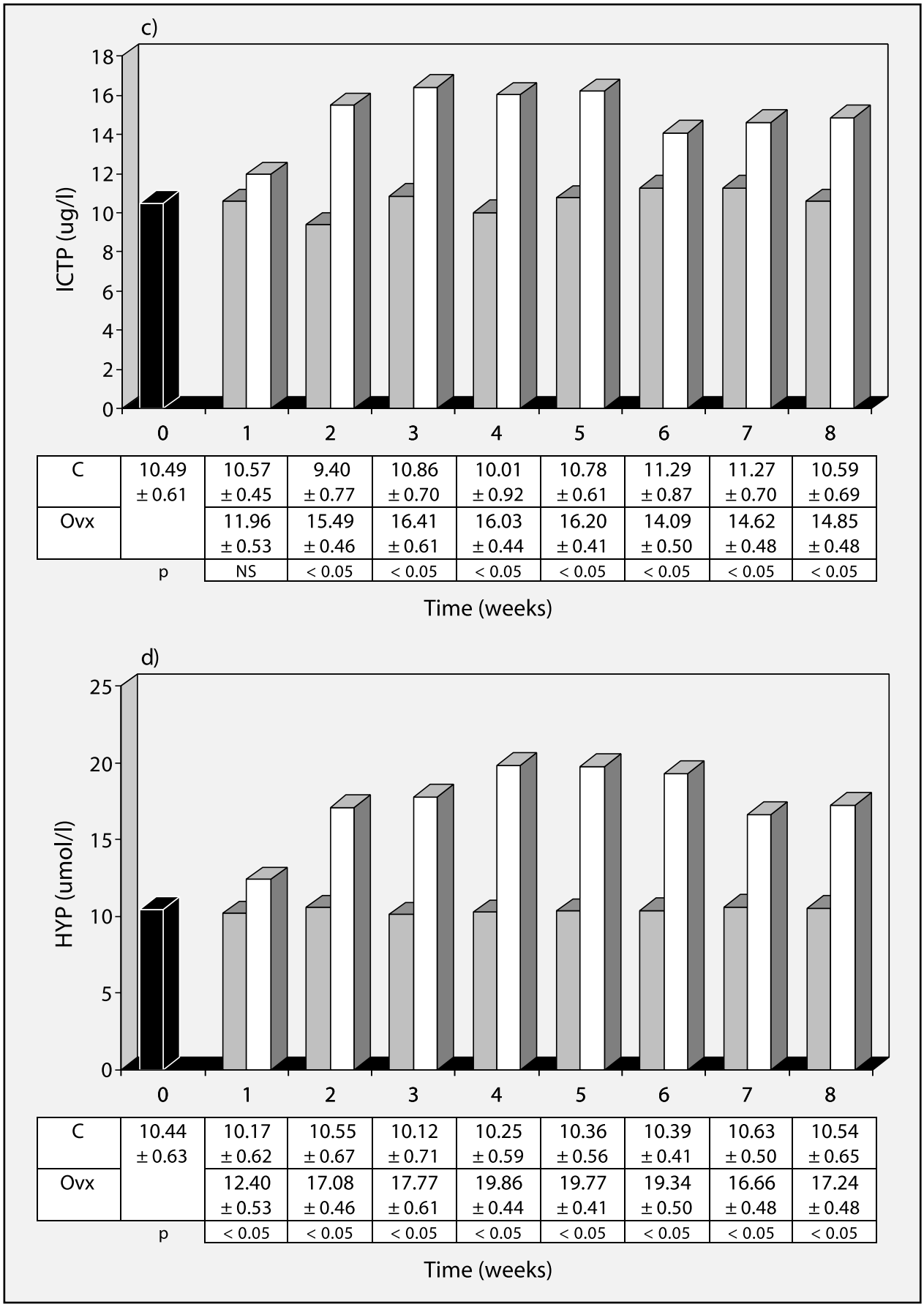
* $p \leq 0.05$



C	206.06 ± 9.67	211.48 ± 11.16	213.19 ± 10.01	219.45 ± 10.83	209.79 ± 11.10	206.69 ± 10.72	210.97 ± 10.00	210.97 ± 12.21	205.11 ± 11.12
Ovx		207.57 ± 11.67	190.00 ± 8.47	188.72 ± 7.76	182.24 ± 8.94	186.70 ± 10.18	190.23 ± 9.36	188.70 ± 7.77	188.90 ± 11.13
	p	NS	NS	NS	< 0.05	NS	NS	NS	NS

C	10.01 ± 0.52	10.56 ± 0.62	11.12 ± 0.68	10.58 ± 0.75	10.64 ± 0.54	10.88 ± 0.36	9.91 ± 1.06	10.00 ± 0.76	10.62 ± 0.75
Ovx		7.70 ± 0.66	7.45 ± 0.51	6.68 ± 0.64	7.82 ± 0.67	7.09 ± 0.43	7.29 ± 0.63	7.49 ± 0.71	7.00 ± 0.75
	p	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

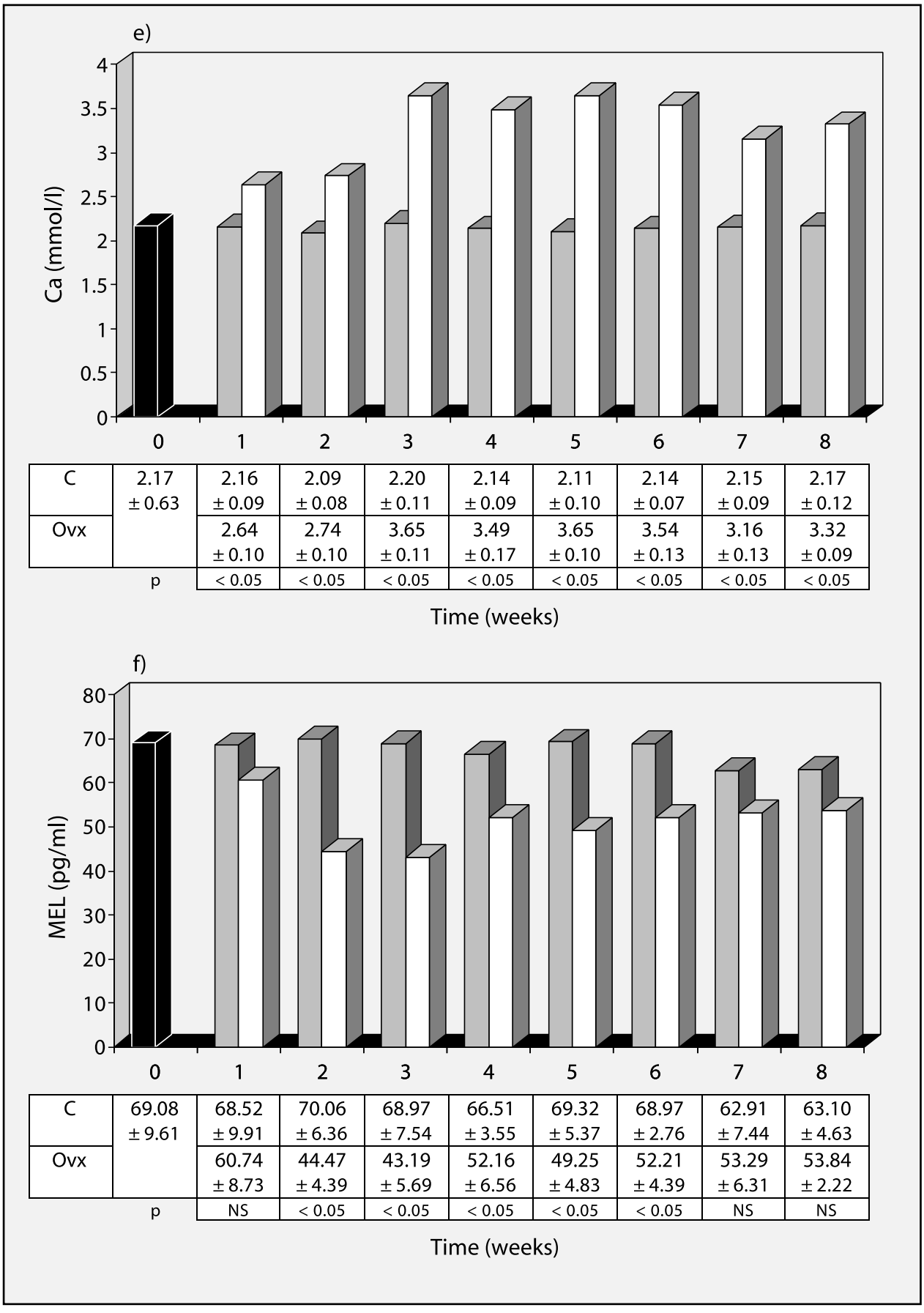
Figure 1. Dynamic pattern of serum alkaline phosphatase (ALP) activity and carboxyterminal propeptide of type I procollagen (PICP) levels in ovariectomized (Ovx), sham operated (C) and intact rats (0)



C	10.49 ± 0.61	10.57 ± 0.45	9.40 ± 0.77	10.86 ± 0.70	10.01 ± 0.92	10.78 ± 0.61	11.29 ± 0.87	11.27 ± 0.70	10.59 ± 0.69
Ovx		11.96 ± 0.53	15.49 ± 0.46	16.41 ± 0.61	16.03 ± 0.44	16.20 ± 0.41	14.09 ± 0.50	14.62 ± 0.48	14.85 ± 0.48
	p	NS	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

C	10.44 ± 0.63	10.17 ± 0.62	10.55 ± 0.67	10.12 ± 0.71	10.25 ± 0.59	10.36 ± 0.56	10.39 ± 0.41	10.63 ± 0.50	10.54 ± 0.65
Ovx		12.40 ± 0.53	17.08 ± 0.46	17.77 ± 0.61	19.86 ± 0.44	19.77 ± 0.41	19.34 ± 0.50	16.66 ± 0.48	17.24 ± 0.48
	p	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

Figure 2. Dynamic pattern of serum cross-linked carboxyterminal telopeptide of type I collagen (ICTP) levels and urinary excretion of hydroxyproline (HYP) in ovariectomized (Ovx), sham operated (C) and intact rats (0)



C	2.17 ± 0.63	2.16 ± 0.09	2.09 ± 0.08	2.20 ± 0.11	2.14 ± 0.09	2.11 ± 0.10	2.14 ± 0.07	2.15 ± 0.09	2.17 ± 0.12
Ovx		2.64 ± 0.10	2.74 ± 0.10	3.65 ± 0.11	3.49 ± 0.17	3.65 ± 0.10	3.54 ± 0.13	3.16 ± 0.13	3.32 ± 0.09
	p	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

C	69.08 ± 9.61	68.52 ± 9.91	70.06 ± 6.36	68.97 ± 7.54	66.51 ± 3.55	69.32 ± 5.37	68.97 ± 2.76	62.91 ± 7.44	63.10 ± 4.63
Ovx		60.74 ± 8.73	44.47 ± 4.39	43.19 ± 5.69	52.16 ± 6.56	49.25 ± 4.83	52.21 ± 4.39	53.29 ± 6.31	53.84 ± 2.22
	p	NS	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	NS	NS

Figure 3. Dynamic pattern of urinary excretion of total calcium (Ca) and serum levels of melatonin (MEL) in ovariectomized (Ovx), sham operated (C) and intact rats (0)

15, 20, 21, 22, 24]. It is also likely that melatonin deficiency stimulates bone resorption [25].

Our own studies conducted previously on starved rats have shown a significant suppressive effect of melatonin on processes of bone forming and resorption; the concentration of PICP in blood serum and hydroxyproline as well as total calcium in urine were evaluated [26]. Also, it has been found that in obesity a significant correlation exists between melatonin and type I collagen metabolism [27]. It has been demonstrated (unpublished data) that lighting conditions, pineal gland removal and administration of exogenous melatonin influence the circadian metabolism of the osseous tissue in rat. A significant role in this relationship is played by changes in endogenous melatonin concentration.

In an experimental model of postmenopausal osteoporosis (generated by ovariectomizing female rats) we have shown a notable decrease in nighttime level of melatonin appearing two weeks after the ovariectomy. However, from the 4th week after surgery the difference in melatonin levels decreased, relative to the control group.

Several studies *in vivo* and *in vitro* have demonstrated sex hormone effects on pineal function. Both 5-hydroxytryptamine (5-HT) and melatonin levels were reduced during proestrus. Ovarian hormones blocked the isoproterenol-induced elevation of pineal melatonin production in the female rat and reduced 5-HT levels in ovariectomized rats [28, 29, 30]. Moreover, ovariectomy increased the density of β-adrenoreceptors whereas estradiol blocked these effects. These data suggest that estradiol directly modulates the responses to dopaminergic neurosecretory system in the hypothalamus to melatonin [29, 31]. It may be primarily associated with the estradiol-induced changes in the density and function of melatonin receptors in the hypothalamus [31]. Judging from our own results it may be supposed that changes in MEL concentration observed in ovariectomized rats within 8 weeks from the surgery, may be related not only to the deficiency of female sex hormones but also to changes in the concentration of other hormonal and, perhaps, local factors [14, 15, 19, 20, 21, 22, 24, 24].

The nocturnal changes in melatonin concentration observed in ovariectomized rats within 8 weeks from the surgery correlate, in an inversely proportional manner, with values of investigated biochemical indicators of bone formation such as alkaline phosphatase and PICP in blood serum as well as with those reflecting bone resorption, i.e. blood serum ICTP and hydroxyproline and total calcium in urine. However, the correlation was significant only with respect to resorption markers which may underlie the domination of resorption processes over those of bone formation. These results seem to corroborate the concept of secondary changes in melatonin concentration (due to the deficiency of sex hormones) co-participating in the development of bone mass changes characteristic for postmenopausal osteoporosis.

The mechanism of melatonin action upon the bone tissue metabolism is difficult to explain on the basis of results obtained during *in vivo* studies since it may reflect not only independent effects of a given hormone action but also its interactions with other endogenous factors, not hormonal in nature. It may only be speculated that such effects could be achieved directly or/and indirectly, *via* endogenous factors (hormonal, growth and immunological), whose synthesis and release are regulated by melatonin.

It has been known that this hormone may impede the development and proliferation of many types of cells including those of connective tissue. Drobnik and Dabrowski [32, 33, 34, 35] postulate the possibility of direct melatonin effect upon collagen and glycosaminoglycans' content in the connective tissue in rats, although their studies were conducted only on healthy skin and wound granulation tissue. According to these authors, melatonin may influence fibroblasts' proliferation and collagen synthesis and the effect is both dose- and time-of-administration-dependent. This may be, in all likelihood, the result of varying sensitivity of melatonin receptors within the 24-hour cycle. No reports were found, although, concerning the presence of melatonin receptors on either osteoblasts or osteoclasts. It has been known that lack of membrane receptors does not necessarily reflect the lack of melatonin influence upon a given type of cells [36]. This hormone easily penetrates intercellular components and may probably act after adhering to intracellular binding sites for example via nuclear uptake receptors RZR/ROR [37]. Through such a mechanism melatonin may influence such basic cell functions as mitotic divisions or scavenging free radicals. The effect of melatonin upon cytokines' secretion is probably achieved also *via* a nuclear receptor [38, 39].

Our own studies (unpublished data) show that melatonin may influence the circadian rhythm of bone formation processes and its resorption also indirectly, by inducing changes of concentration of hormonal factors that significantly influence these processes such as parathormone, calcitonin, thyroid hormones, corticosterone and IGF-I.

Conclusion

1. Our findings suggest that secondary changes in melatonin concentration, due to the deficiency of sex hormones, co-participate in the development of bone mass changes characteristic for postmenopausal osteoporosis.

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