Effect of Naloxone Hydrochloride on Osteogenesis in Chick Embryos

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Naloxone hydrochloride, an opioid receptors blocker, was administered to chick embryos. Morphological analysis of femoral bones of embryos showed an appreciable increase in the thickness of the perichondral bone cuff in the tubular bone diaphyses and increased mitotic activity in the zone of proliferating young cartilage of the epiphyseal plate.

Key Words: *naloxone hydrochloride; osteogenesis*

Bone tissue remodeling is regulated by body systems including calcium-regulating and other systemic hormones (glucocorticoids, estrogens, androgens, progestins, thyroid hormones, retinoid), systemic and local factors (insulin-like growth factors 1 and 2, bone morphogenetic proteins 2, 3, 4, 7), including those produced by osteoblasts and osteoclasts [3]. The regulation of embryonic osteogenesis remains little studied.

It was shown that injection of [Met (5)]-enkephaline to pregnant rats decreases DNA synthesis in fetal cells representing all three primordial; this effect can be abolished by application of naltrexone and is receptor-mediated [7]. Autocrine regulation of the production of this peptide production, denoted as opioid growth factor, and endocrine opioid regulation of organogenesis as a fundamental component of mammalian embryogenesis were hypothesized [6,7]. Presumably, this regulation involves bone tissue formation. This hypothesis is supported by detection of opioid receptor mRNA and its expression in MG 63 human osteoblastic cells and naloxone-inhibited capacity of μ- (but not δ-) agonists to appreciably suppress the production of osteocalcitonin (osteoblastic activity marker) [5]. It is also possible that opioid regulation of osteogenesis is typical of not only mammal, but other organisms as well.

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We studied the role of opioid regulation in embryonic osteogenesis by evaluating its course under conditions of opioid receptor blockade during different periods of embryonic development.

MATERIALS AND METHODS

Chick embryos aged 10 and 14 days were used. Opioid receptors (μ-, σ-, and κ-) were blocked with naloxone hydrochloride (NH; Sigma) [4].

All embryos were divided into 5 groups, 10 per group. In one group NH diluted in $^1/_{15}$ M phosphate buffer (pH 7.36) was injected (0.1 ml) into chorion allantoic vein on days 11, 13, and 15 of development (experiment 1); in another group it was injected on days 14, 16, and 18 (experiment 2). Chick embryos injected with 0.1 ml phosphate buffer (PB) on the same days served as control 1 and control 2. Intact embryos served as the reference group.

The chicks were sacrificed by decapitation 2 h after hatching (on day 21 of incubation) and on day 7 of life (experiment 2 and control 2). Osteogenesis was evaluated by morphological analysis of the femoral bone. Histological preparations of embryonic bone tissue were made [2] and paraffin sections were stained with hematoxylin and eosin. Osteocytes were visualized by staining after Schmorl. Morphometrical analysis of the preparations was carried out by Avtandilov's method [1]. The number of osteoblasts in the perichondral bone cuff, mitotically active cells in

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the zone of proliferating young cartilage of the epiphyseal plate, osteocytes, and thickness of the perichondrial bone cuff (TPBC) were evaluated in each experimental series. All parameters were examined in at least 10 visual fields of Avtandilov's grid and their area per mm^2 was calculated.

The results were statistically processed using Student's *t* test at normal distribution. The differences were considered significant at $p<0.05$.

RESULTS

Phosphate buffer injected during week 2 of embryonic development (control 1) did not modify the studied parameters in comparison with intact chicks (Table 1). Injection of NH during week 2 of embryogenesis (experiment 1) led to a 2-fold increase in TPBC in comparison with intact and control groups (Fig. 1). The density of osteoblasts in the diaphysis and mitotic activity in the epiphysis growth zone did not change, while osteocyte density in the diaphysis somewhat decreased (by 15.4%). Morphological analysis of the bone cuff revealed its common structure: cubic or cylindrical osteoblasts at the periphery of the osteoid and osteocytes identified as mononuclear squamous cells in bone lacunae. All these data indicate that NH treatment during week 2 of embryogenesis stimulated TPBC growth at the expense of synthetic activity of osteoblasts, but not their increased number. Presumably, decreased density of osteocytes under these conditions was due to lower rate of their formation in comparison with the production of bone matrix.

Bone tissue of chicks treated with PB during week 3 of embryogenesis and collected 2 h after hatching also did not differ from that of intact embryos. Application of NH during this period led to an appreciable increase in the number of mitoses in the epiphysis growth zone, 5.68 and 6.6 times surpassing the levels in intact and control groups, respectively. The density of cells in the zone of proliferating cartilage increased ($51.0\pm1.1/mm^2$) in comparison with analogous zones in chicks hatched from intact embryos (40.0±0.8) and

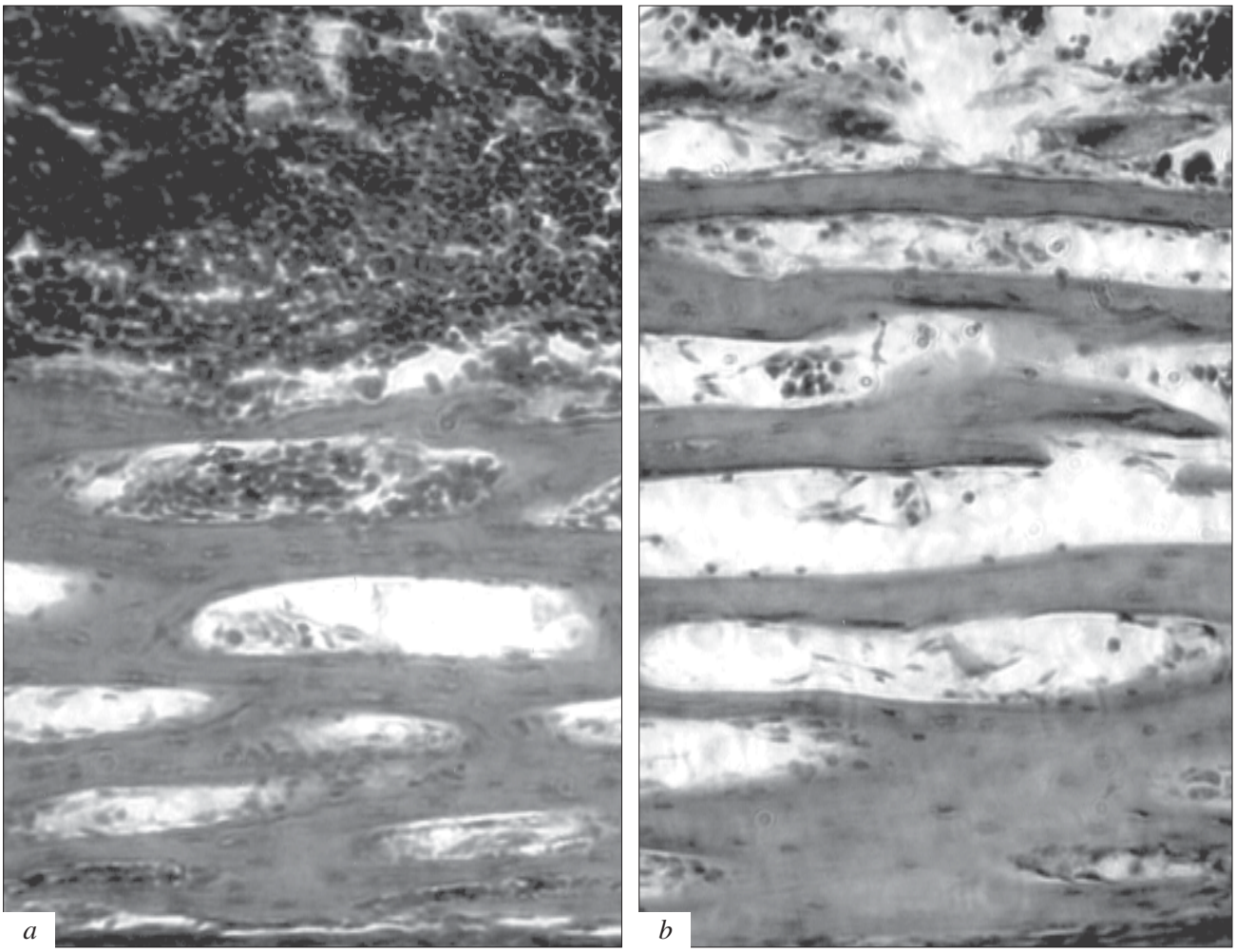

Fig. 1. Chick embryo tubular bone diaphyses. *a*) intact embryo; *b*) embryo after injection of naloxone hydrochloride during week 2 of development; ×200.

TABLE 1. Effect of NH on Bone Tissue Cell Populations and TPBC, Depending on the Time of Injection (*M*±*m*)

Group of embryos	Period after hatching	Number of osteoblasts/mm^2	Number of mitoses per 100 cells	Number of osteocytes/mm^2	TPBC thickness, μ
Intact (*n*=10)	2 h	34.8±1.7	2.8±0.3	30.8±1.1	91.7±1.4
Control 1 (*n*=10)	2 h	34.4±1.5	2.7±0.3	30.1±0.9	92.0±1.4
Experiment 1 (*n*=10)	2 ч	33.3±1.8	3.4±0.3	25.4±1.0*+	188.0±1.7*+
Control 2 (*n*=20)	2 h	34.7±1.6	2.4±0.3	29.3±1.2	93.4±1.3
	days 7	32.8±1.1	2.5±0.3	30.3±1.1	93.3±0.9
Experiment 2 (*n*=20)	2 h	34.8±1.7	15.9±1.8*+	19.4±1.3*+	128.3±1.8*+
	days 7	33.0±1.3	3.0±0.4	25.8±1.0*+	184.0±1.8*+

Note. $p<0.05$ compared to: *intact embyos, +respective controls.

controls (41.0±1.1). Increase of TPBC (by 27.4% *vs.* control) indicated that along with intense formation of osteoblasts, the synthetic activity of these cells in the diaphysis zone was realized. The decrease in osteocyte density (by 33.8% *vs.* control) can reflect inhibition of the final stage of osteoblast differentiation during their intense formation. It is obvious that embryonic osteogenesis is stimulated by NH used according to this protocol as well; in contrast to NH application during week 2 of embryogenesis, this treatment led not only to an increase in synthetic activity of osteoblasts, but to their more intense formation as well.

This difference could be due to either greater dependence of the bone tissue mitotic mechanisms on the embryonic opioid regulation during week 3 of development, or to longer realization of NH effect in embryos injected with the drug during week 2 of development. In the latter case it was impossible to record early changes in bone tissue using our protocol of experiment. We therefore compared bone tissue the status in chicks on day 7 after hatching from embryos treated by PB or NH during week 3 of embryogenesis. In this case the morphology of bone tissue virtually completely corresponded to that in the experimental group 1: 2-fold increase of TPBC with retained osteoblast density and mitotic index, paralleled by decreased (by 17.4%) osteocyte density.

Hence, our findings indicate the participation of opioid regulation in osteogenesis processes in chick embryos.

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