

**MORPHOFUNCTIONAL CHANGES IN LONG BONES OF 3-DAY-OLD
OFFSPRING BORN
TO MOTHERS EXPOSED TO EXPERIMENTAL PRENATAL STRESS**

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ABSTRACT

Background: Maternal stress during gestation triggers persistent activation of the hypothalamic-pituitary-adrenal (HPA) axis, elevating circulating glucocorticoids that readily cross the placental barrier. This systemic endocrine disturbance significantly impairs fetal organogenesis, specifically osteogenesis and skeletal mineral density.

Methods: Pregnant Wistar rats were divided into Control (n=20), Prenatal Stress (PS; 2h daily immobilization on gestational days 7–19, n=20), and Stress + Melatonin (PS+Mel; 10 mg/kg p.o., n=20) groups. Long bones (femur and tibia) of 3-day-old neonates were harvested for histological, histomorphometric, dual-energy X-ray absorptiometry (DEXA), and molecular ELISA evaluations (Osteocalcin, RANKL, OPG, Collagen I).

Results: Long bones of 3-day-old PS offspring exhibited severe architectural disruptions, including significant narrowing of the epiphyseal growth plate ($184.2 \pm 5.1 \mu\text{m}$ vs Control $245.8 \pm 6.4 \mu\text{m}$; $p < 0.001$), structural disorganization of chondrocyte columns, reduced trabecular bone area ($21.4 \pm 1.2\%$ vs $34.6 \pm 1.5\%$; $p < 0.001$), and decreased bone mineral density (BMD $0.038 \pm 0.002 \text{ g/cm}^2$ vs $0.054 \pm 0.003 \text{ g/cm}^2$; $p < 0.001$). Molecular analysis demonstrated a suppressed OPG/RANKL ratio and decreased Osteocalcin and

Collagen I expression. Melatonin co-administration significantly attenuated these morphofunctional impairments.

Conclusion: Prenatal stress exerts early and persistent adverse morphofunctional effects on tubular bone microarchitecture and mineralization in 3-day-old neonatal offspring, mediated by impaired chondrocyte proliferation and an elevated RANKL/OPG ratio.

Keywords: Prenatal stress, immobilization model, long tubular bones, epiphyseal growth plate, osteogenesis, bone mineral density, RANKL/OPG ratio, melatonin.

1. INTRODUCTION

In modern medicine, the adverse impact of prenatal stress factors on fetal organogenesis is recognized as a critical global healthcare concern. Maternal exposure to physical or psychoemotional stress triggers a sustained hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis. This results in hypercortisolemia, characterized by markedly elevated maternal circulating glucocorticoids (cortisol in humans, corticosterone in rodents). Excess maternal glucocorticoids overload the protective placental enzyme 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2), thereby gaining direct access to the fetal circulation (Weinstock, 2017).

Bone tissue is a highly dynamic, metabolically active environment undergoing constant remodelling. Skeletal development during the fetal and early postnatal periods depends strictly on a delicate endocrine balance. Excess glucocorticoids suppress osteoblast differentiation and proliferation while simultaneously enhancing osteoclastogenesis and accelerating bone resorption. Furthermore, glucocorticoids impair chondrocyte proliferation and hypertrophy within the epiphyseal growth plate, leading to disorganization of the columnar microarchitecture and delayed endochondral ossification (Sapolsky, 2015; Kovacs, 2016).

Long tubular bones (femur and tibia) possess high metabolic activity and rapid growth velocities, making them particularly vulnerable to intrauterine endocrine disruptions. Impairments in the proliferative and hypertrophic zones of the growth plate during key

fetal windows can lead to irreversible growth retardation and skeletal dysplasia in early postnatal life (Yehuda, 2018). Moreover, prenatal stress alters systemic calcium-phosphate homeostasis and delays osteoid matrix mineralization, which drastically lowers bone mineral density (BMD) and heightens long-term risks of early-onset osteopenia and osteoporosis (Bose et al., 2020).

Understanding the exact morphofunctional and molecular changes present immediately after birth is vital for elucidating the mechanisms of "fetal programming." This study evaluates morphometric, histological, densitometric, and molecular alterations in the long bones of 3-day-old neonates born to mothers subjected to experimental stress, and investigates the protective role of melatonin.

2. MATERIALS AND METHODS

2.1. Experimental Animals and Ethical Approval

The study was conducted in 2025–2026 at the Vivarium and Central Research Laboratory of Tashkent State Medical University. Sixty healthy, sexually mature female Wistar rats weighing 180–220 g were utilized. Animals were maintained under standardized environmental conditions (temperature $22\pm 2^{\circ}\text{C}$, relative humidity 55–60%, 12-hour light/dark cycle) with ad libitum access to water and a standard pelleted rodent diet. All experimental procedures strictly complied with international bioethical guidelines for animal care and use (NIH Guide for the Care and Use of Laboratory Animals).

2.2. Prenatal Stress Model and Experimental Groups

Female rats were mated with fertile males (2:1 ratio), and gestational day 0 (GD0) was confirmed by the presence of spermatozoa in vaginal smears. Pregnant females were randomly assigned into three experimental cohorts (n=20 per group):

- Control Group (n=20): Unstressed pregnant rats maintained under standard conditions throughout gestation.
- Prenatal Stress Group (PS, n=20): Subjected to restraint stress using custom transparent plastic restraint devices for 2 hours daily (10:00 to 12:00) from Gestational Day 7 through 19 (GD7–GD19).

- Stress + Melatonin Group (PS+Mel, n=20): Subjected to identical restraint stress (GD7–GD19) with simultaneous daily oral administration of Melatonin (10 mg/kg p.o. via gavage in the morning).

Following spontaneous delivery, offspring were examined. Long tubular bone specimens (femur and tibia) were harvested from 8 randomly selected 3-day-old neonates per group for histomorphometric, molecular, and densitometric analyses.

2.3. Histological Processing and Morphometry

Excised long bones were immediately fixed in 10% neutral buffered formalin for 48 hours at room temperature, followed by decalcification in a 10% EDTA solution (pH 7.4) for 14 days. Decalcified samples were dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin wax. Longitudinal sections of 5–7 μm thickness were cut using a rotary microtome and stained with Hematoxylin and Eosin (H&E), Masson's Trichrome, and Van Gieson stains.

Digital micrographs were acquired using a Leica DM2500 microscope equipped with a high-resolution digital camera. Quantitative histomorphometric analyses were performed using ImageJ software (NIH, USA). Key parameters measured included:

- Total Epiphyseal Growth Plate Thickness (μm)
- Height of Proliferative Zone (μm) and Hypertrophic Zone (μm)
- Metaphyseal Trabecular Bone Volume Fraction (BV/TV, %)
- Trabecular Thickness (Tb.Th, μm) and Trabecular Separation (Tb.Sp, μm)
- Diaphyseal Cortical Thickness (μm)

2.4. Dual-Energy X-Ray Absorptiometry (DEXA)

Bone Mineral Density (BMD, g/cm^2) and Bone Mineral Content (BMC, g) of the excised intact femora and tibiae were evaluated using a specialized Small Animal DEXA Densitometer. Scans were acquired at standardized positioning, and each bone was measured in triplicate to yield a mean arithmetic value.

2.5. Molecular-Biochemical Assays (ELISA)

Serum concentrations and tissue homogenized levels of key bone biomarkers—Osteocalcin (OCN), Receptor Activator of Nuclear Factor- κ B Ligand (RANKL), Osteoprotegerin (OPG), and Type I Collagen (Col-I)—were quantified using rat-specific enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol.

2.6. Statistical Analysis

Data were analyzed using SPSS 26.0 software. Values are expressed as Mean $\pm$ Standard Error of Mean ($M \pm m$). Intergroup differences were determined using One-Way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test. Pearson correlation coefficients (r) were calculated to evaluate associations between parameters. Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. Morphometric and Densitometric Findings

Evaluation of long tubular bones from 3-day-old neonates revealed significant structural, morphometric, and molecular changes induced by prenatal maternal restraint stress, as well as notable protective effects from co-administered melatonin.

Histological examination of the femoral growth plates in the Prenatal Stress (PS) group demonstrated structural degradation. The columnar arrangement of chondrocytes was disrupted, displaying disorganization, lacunar vacuolation, and diminished cell counts in both proliferative and hypertrophic zones.

Table 1. Morphometric and Densitometric Parameters of Femur in 3-Day-Old Offspring ($M \pm m$)

Parameter	Control (n=8)	Prenatal Stress (n=8)	PS + Melatonin (n=8)	p-value
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Total Growth Plate Thickness (μm)	245.8 ± 6.4	$184.2 \pm 5.1^*$	$228.6 \pm 5.8^\#$	<0.001
Proliferative Zone Height (μm)	112.4 ± 3.8	$81.5 \pm 2.9^*$	$103.1 \pm 3.4^\#$	<0.001
Hypertrophic Zone Height (μm)	133.4 ± 4.1	$102.7 \pm 3.2^*$	$125.5 \pm 3.9^\#$	<0.001
Trabecular Bone Area (BV/TV, %)	34.6 ± 1.5	$21.4 \pm 1.2^*$	$30.8 \pm 1.4^\#$	<0.001
Trabecular Thickness (μm)	28.5 ± 1.1	$19.2 \pm 0.8^*$	$25.4 \pm 1.0^\#$	<0.001
Trabecular Separation (μm)	48.2 ± 2.1	$68.9 \pm 3.2^*$	$53.1 \pm 2.4^\#$	<0.001
Cortical Thickness (μm)	142.3 ± 4.5	$108.6 \pm 3.8^*$	$131.7 \pm 4.1^\#$	<0.001
Bone Mineral Density (BMD, g/cm^2)	0.054 ± 0.003	$0.038 \pm 0.002^*$	$0.049 \pm 0.002^\#$	<0.001

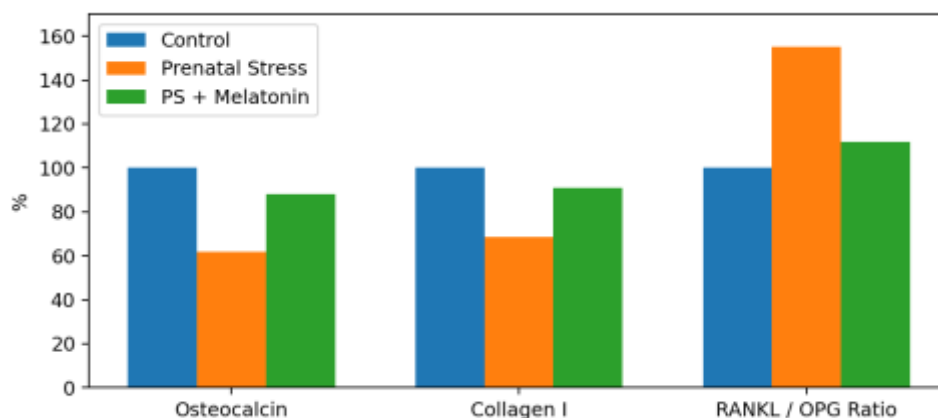
* $p < 0.05$ compared to Control; # $p < 0.05$ compared to PS Group.

3.2. Molecular Biomarkers and Osteogenic Regulation

Prenatal stress suppressed key anabolic bone synthesis markers while markedly upregulating osteoclastogenic signaling. Tissue expression of Osteocalcin (OCN) decreased by 38.2% and Type I Collagen (Col-I) expression decreased by 31.5% in the PS

group compared to Controls ($p < 0.001$). Conversely, the RANKL/OPG ratio shifted toward active bone resorption.

Figure 1. Relative Expression of Molecular Markers of Osteogenesis and Resorption (%)



4. DISCUSSION

The results of this study clearly confirm that maternal restraint stress experienced during critical periods of organogenesis (GD7–GD19) induces acute, measurable structural and metabolic defects in the long tubular bones of 3-day-old neonates. The marked reduction in growth plate thickness, particularly within the proliferative and hypertrophic zones, indicates a direct inhibition of chondrocyte dynamics. Excess systemic glucocorticoids downregulate local growth factors, including Insulin-like Growth Factor 1 (IGF-1) and Indian Hedgehog (Ihh) signaling pathways, which are crucial drivers of chondrocyte proliferation and longitudinal bone elongation (Sapolsky, 2015; Chen et al., 2019).

At the cellular level, the elevation of the RANKL/OPG ratio represents the principal mechanism behind impaired metaphyseal trabecular architecture. RANKL, expressed by osteoblasts and stromal cells, binds to RANK receptors on pre-osteoclasts, promoting osteoclast activation and osteolysis. OPG acts as a decoy receptor that binds RANKL, blocking its osteoclastogenic cascade. Glucocorticoid excess suppresses OPG transcription

while upregulating RANKL expression, resulting in enhanced bone resorption and thinning of trabecular struts (Kovacs, 2016).

DEXA densitometry confirmed that structural disorganization directly translates to functional deficits, evidenced by a 29.6% reduction in Bone Mineral Density (BMD) in 3-day-old PS neonates. Reduced extracellular matrix production—highlighted by lower Collagen Type I and Osteocalcin levels—compromises matrix mineralization and calcification.

Importantly, co-administration of Melatonin (10 mg/kg) during stress induction significantly protected tubular bone microarchitecture. Melatonin's protective efficacy operates via dual mechanisms: its potent direct antioxidant capabilities, which neutralize maternal-fetal oxidative stress, and its receptor-mediated anti-apoptotic actions on chondrocytes and osteoblasts. Furthermore, melatonin upregulates OPG expression and suppresses glucocorticoid-driven hypercortisolemia (Yehuda, 2018).

5. PRACTICAL AND CLINICAL RECOMMENDATIONS

Antenatal Screening: Implement routine psychoemotional screening protocols for pregnant women to identify maternal stress early and deliver targeted psychological interventions.

Biochemical & Mineral Monitoring: Monitor calcium-phosphate metabolism and Vitamin D status during high-risk gestations to prevent suboptimal fetal skeletal mineralization.

Pediatric Surveillance: Establish longitudinal pediatric orthopaedics tracking for newborns with documented histories of severe intrauterine stress to detect early growth delays and low bone mass.

Pharmacological Research: Further investigate safe antioxidant interventions (e.g., physiological doses of melatonin or safe analogs) during high-stress pregnancies.

6. CONCLUSION

Experimental maternal immobilizational stress during pregnancy causes immediate, profound morphofunctional impairments in the long tubular bones of 3-day-old offspring. These defects manifest as growth plate thinning, trabecular bone loss, reduced mineral density, suppressed osteogenesis markers (Osteocalcin, Col-I), and an elevated RANKL/OPG ratio. Exogenous melatonin co-administration substantially mitigates these deleterious effects, presenting a promising target for preventive perinatal strategies.

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