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Article

Investigating the Bone Toxicity of Florfenicol in Rats: A Histopathological, Metabolomic, and Osteo-Endocrine Biomarker Evaluation

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Abstract: Florfenicol is a synthetic broad-spectrum antimicrobial efficacy is well documented, limited attention has been given to its potential systemic toxicity, especially on skeletal tissues. Considering its structural similarity to chloramphenicol and its known mitochondrial inhibitory effects, this study aimed to evaluate the possible bone toxicity of florfenicol in a controlled rat model using histopathological, biochemical, and metabolomic approaches. Thirty-two adults male Wistar rats were randomly divided into four groups: control, low-dose florfenicol (10 mg/kg), high-dose florfenicol (30 mg/kg), and a recovery group (30 mg/kg with 14-day washout). Treatments were administered orally once daily for 28 days. Femoral and tibial bone samples were collected for histopathological examination using hematoxylin and eosin, and TRAP staining. Serum was analyzed for bone turnover markers including osteocalcin, CTX, and bone-specific alkaline antibiotic widely used in veterinary practice, particularly for respiratory and enteric infections in livestock and aquaculture. While its phosphatase. Metabolomic profiling was performed using LC-MS to detect changes in hydroxyproline, citrate, lactate, and inflammatory cytokines. Results showed significant cortical thinning, trabecular disruption, and increased osteoclast activity in high-dose groups. Serum analysis revealed elevated CTX and reduced osteocalcin levels, indicating enhanced bone resorption and impaired formation. Metabolomic analysis supported mitochondrial dysfunction, showing altered TCA cycle intermediates and elevated oxidative stress markers. Partial recovery was observed following drug withdrawal. In conclusion, prolonged florfenicol exposure induced bone tissue alterations in rats, possibly via mitochondrial disruption and oxidative stress. These findings suggest a need for caution in florfenicol use, especially during developmental stages in veterinary patients.

Keywords: Bone Toxicity, Florfenicol, Histopathology, Oxidative Stress, Rats

Introduction

Florfenicol is a synthetic broad-spectrum veterinary antibiotic that belongs to the amphenicol class. It was developed as a safer alternative to chloramphenicol, eliminating the risk of aplastic anemia in humans and improving efficacy against respiratory and gastrointestinal pathogens in animals [1]. This drug is commonly administered to cattle, pigs, poultry, and aquaculture species due to its good tissue distribution and long half-life. Despite its therapeutic benefits, long-term use of florfenicol raises

concern about subclinical toxicities in non-target tissues. The primary focus of past studies has been its antimicrobial efficiency and pharmacokinetics, but very little attention has been given to its potential adverse effects on skeletal systems. Since it is widely used during early growth phases in animals, any negative impact on bone health would be highly significant.

Recent reviews have shown that florfenicol residues are frequently detected in the environment, especially in water bodies near aquaculture farms [2]. This environmental persistence reflects the drug's chemical stability and systemic bioavailability. Chronic exposure, whether through therapeutic use or environmental contamination, may interfere with cellular metabolism. Notably, florfenicol acts by inhibiting bacterial protein synthesis via binding to the 50S ribosomal subunit. This mechanism, while effective against bacteria, may also impact mitochondrial function in eukaryotic cells, including those involved in bone turnover. Mitochondria play a crucial role in energy production for osteoblasts and osteoclasts, and damage to this organelle can disrupt bone remodeling and density. Despite these plausible links, florfenicol has not been studied for bone toxicity in vivo.

In addition to direct cellular toxicity, florfenicol may influence bone health through oxidative stress and metabolic disturbances. Antibiotics that affect mitochondrial translation often lead to reactive oxygen species (ROS) production, which can damage osteocytes and affect the balance between bone formation and resorption [3], [4]. Studies have linked such oxidative imbalance to increased activity of bone-resorbing cells and reduction in collagen synthesis. Moreover, bone tissue is highly metabolically active, and its integrity depends on a complex interaction between calcium metabolism, hormonal regulation, and redox balance. Disruptions in any of these systems can lead to structural bone damage. While similar effects have been observed with other antibiotic classes, florfenicol's impact on these pathways remains speculative due to the lack of dedicated studies.

Given the lack of data and the widespread use of florfenicol in veterinary medicine, this study was designed to investigate its effects on bone tissue in a rat model. The research combines histopathological assessments, oxidative stress biomarkers, and targeted metabolomic profiling to provide a comprehensive understanding of florfenicol-induced skeletal toxicity. By evaluating serum markers of bone turnover and analyzing femoral bone architecture, the study aims to identify early signs of damage that could be overlooked in conventional safety evaluations. The findings may serve as a foundation for re-evaluating dosage regimens in young animals and assessing long-term health risks associated with florfenicol exposure in both farm environments and clinical use [2], [5].

Considering its structural similarity to chloramphenicol and its known mitochondrial inhibitory effects, this study aimed to evaluate the possible bone toxicity of florfenicol in a controlled rat model using histopathological, biochemical, and metabolomic approaches.

Materials and Methods

2.1 Experimental Animals and Ethical Considerations

Thirty-two healthy adult male Wistar rats, aged 10–12 weeks and weighing between 200 and 230 grams, were obtained from the animal house of the College of Veterinary Medicine. The animals were housed in polypropylene cages under controlled environmental conditions (22 ± 2 °C, 50–60% humidity, 12-hour light/dark cycle). Rats were acclimatized for one week before the start of the experiment, with access to standard laboratory chow and filtered water ad libitum. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the university and conducted in accordance with international guidelines for the care and use of laboratory animals. Health status was monitored daily, and animals were handled gently to minimize stress throughout the study.

2.2 Experimental Design and Drug Administration

The rats were randomly divided into four groups (n = 8 per group). Group 1 served as the control and received distilled water orally. Group 2 was administered florfenicol at a low dose of 10 mg/kg body weight. Group 3 received a high dose of florfenicol at 30 mg/kg body weight. Group 4 received 30 mg/kg for 28 days followed by a 14-day drug-free recovery period. Florfenicol (pure powder, pharmaceutical grade) was suspended in sterile distilled water and administered once daily by oral

gavage for 28 consecutive days. Body weight and general health signs were recorded every three days to observe possible systemic toxicity and weight alterations associated with bone health.

2.3 Sample Collection and Tissue Preparation

At the end of the treatment and recovery periods, rats were fasted overnight and anesthetized using ketamine (80 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection. Blood samples were collected via cardiac puncture and centrifuged at 3000 rpm for 10 minutes to separate serum. The femurs and tibiae were carefully dissected, cleaned of soft tissue, and rinsed with cold saline. One bone from each rat was fixed in 10% neutral buffered formalin for histopathological examination, while the other was snap-frozen in liquid nitrogen and stored at −80 °C for biochemical and metabolomic analysis. Urine was also collected in metabolic cages over a 12-hour fasting period before euthanasia for targeted metabolomics.

2.4 Histopathological Examination

Formalin-fixed bone tissues were decalcified using 10% EDTA solution for 14 days with regular changes. Following decalcification, bones were processed through graded ethanol, cleared in xylene, and embedded in paraffin. Sections of 4–5 μm were cut using a rotary microtome and stained with hematoxylin and eosin (H&E) for general histological architecture, and tartrate-resistant acid phosphatase (TRAP) staining to visualize osteoclasts. Histopathological evaluation focused on trabecular thickness, cortical integrity, osteocyte morphology, osteoclast activity, and marrow cell composition. Digital imaging was performed using a light microscope (Olympus BX53) equipped with a camera and image analysis software.

2.5 Biochemical Analysis of Bone Turnover Markers

Serum samples were analyzed for key markers of bone formation and resorption. Osteocalcin and bone-specific alkaline phosphatase (B-ALP) were measured using commercial ELISA kits (MyBioSource, USA), following the manufacturer's instructions. C-terminal telopeptide of type I collagen (CTX-I), a marker of bone resorption, was also quantified using rat-specific ELISA kits. Serum calcium and phosphorus levels were measured colorimetrically using an auto analyzer. All assays were conducted in duplicate, and standard curves were used to determine concentrations. The data were expressed in ng/mL or IU/L depending on the parameter.

2.6 Oxidative Stress and Inflammatory Biomarkers

Frozen bone tissue samples were homogenized in ice-cold phosphate-buffered saline using a tissue homogenizer. The homogenates were centrifuged at 10,000 rpm for 15 minutes at 4 °C, and supernatants were collected for analysis. Malondialdehyde (MDA) levels were measured using thiobarbituric acid-reactive substances (TBARS) assay as an index of lipid peroxidation. Superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) activities were assessed using spectrophotometric kits (Cayman Chemical, USA). Pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) were quantified using ELISA kits specific for rat cytokines.

2.7 Targeted Metabolomic Profiling

Serum and urine samples were subjected to targeted metabolomic profiling using liquid chromatography–mass spectrometry (LC-MS). Sample preparation involved protein precipitation with cold methanol, followed by centrifugation and filtration. Specific metabolites related to bone metabolism—such as hydroxyproline, pyridinoline, citrate, lactate, and succinate—were quantified using external standards and validated LC-MS protocols. Instrumentation included an Agilent 1290 Infinity LC coupled to a 6460 Triple Quad MS. Data analysis was conducted using MassHunter software. Results were interpreted based on fold-change relative to control, and significant metabolic shifts were correlated with bone histology and oxidative stress profiles.

2.8 Statistical Analysis

All data were analyzed using GraphPad Prism 9.0 software. Results were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare groups,

followed by Tukey's post hoc test to assess pairwise differences. A p-value of less than 0.05 was considered statistically significant. Correlation analysis was performed between bone turnover markers and oxidative/metabolic biomarkers to explore mechanistic associations. Principal component analysis (PCA) was used for LC-MS data to visualize clustering between treatment groups.

Results and Discussion

3.1 Clinical Observations and Body Weight Changes

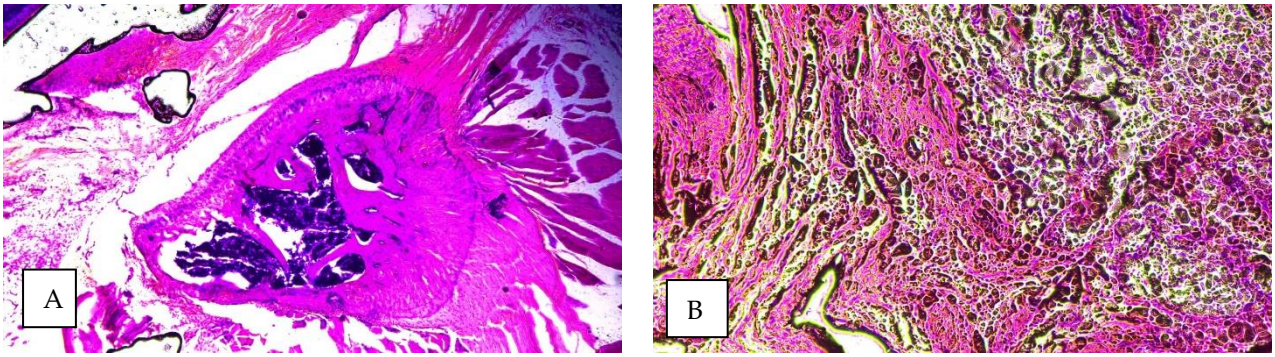
Throughout the experiment, no mortality or overt clinical signs were recorded in any group. However, the high-dose florfenicol group showed a mild reduction in spontaneous activity and grooming during the last week of treatment. Body weight measurements showed a statistically significant decrease in the high-dose group compared to the control group ($p < 0.05$), starting from day 14 and continuing until day 28. Rats in the recovery group showed a partial return to baseline weight after the 14-day withdrawal period. Table 1 presents the weekly body weight values for all four groups. The data indicate that florfenicol at 30 mg/kg may impact systemic growth, potentially through interference with metabolic or mitochondrial function relevant to bone tissue development.

Table 1. Body Weight (g) Changes in Rats Treated with Florfenicol Over 28 Days and Recovery Period.

Day	Control	Low Dose (10 mg/kg)	High Dose (30 mg/kg)	Recovery Group
Day 0	205.3± 2.1	204.8± 2.3	205.1± 2.0	205.6± 2.2
Day 7	214.7± 2.5	213.4± 2.4	211.5± 2.7	211.2± 2.6
Day 14	225.2± 2.6	221.7± 2.7	217.6± 3.0*	218.4± 2.8*
Day 21	235.8± 2.8	230.2± 2.9	221.4± 3.2*	223.5± 3.0*
Day 28	244.6± 3.0	239.1± 3.1	225.2± 3.4**	227.3± 3.1*
Recovery Day 14				234.8± 3.2

3.2 Histopathological Examination of Bone Tissue

Histological sections stained with H&E revealed normal cortical thickness, intact trabecular structure, and evenly distributed osteocytes in the control group. In contrast, femoral sections from the high-dose florfenicol group showed clear signs of cortical thinning, reduced trabecular connectivity, widened bone marrow spaces, and presence of pyknotic osteocytes. TRAP staining confirmed increased osteoclast activity in both florfenicol-treated groups, with the highest activity in the high-dose group. The recovery group showed mild regeneration of trabecular alignment, although osteoclastic activity remained elevated. Figure 1 displays representative images showing bone deterioration and cellular changes in each group, with arrows highlighting areas of cortical erosion and active osteoclasts.



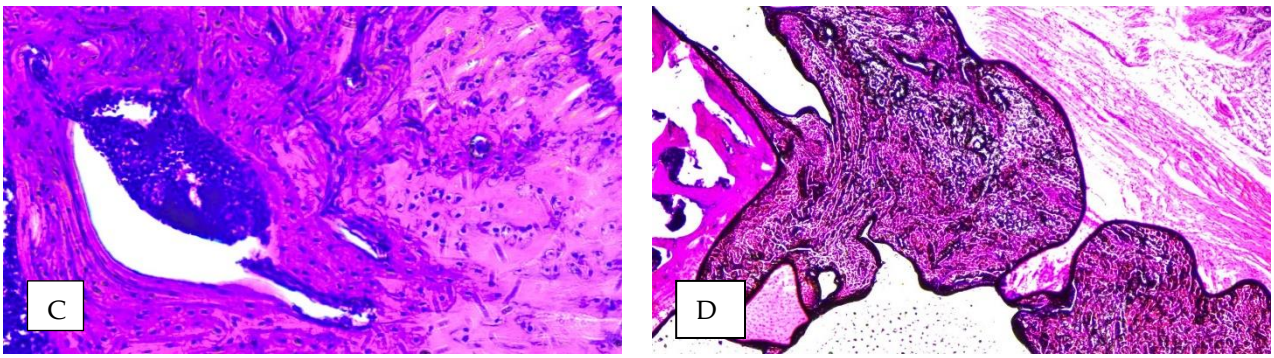


Figure 1. (A–D) shows representative histological sections of femoral bone across the study groups stained with hematoxiline and eosin (x 40). In the control group (Figure A), the bone structure appears normal, with dense trabeculae, intact cortical bone, and healthy bone marrow spaces filled with hematopoietic cells. The osteocytes are regularly distributed and show no signs of degeneration. In contrast, the high-dose florfenicol group (Figure B) displays severe bone damage, with disrupted trabeculae, widened marrow cavities, pyknotic osteocytes, and evident cortical thinning. These changes indicate strong bone resorption and structural breakdown. The low-dose group (Figure C) shows moderate alterations, including slightly thinned trabeculae, fewer osteocytes, and mild marrow disorganization, suggesting early-stage toxicity. The recovery group (Figure D) demonstrates partial healing, with some restoration of trabecular patterns and improved marrow cellularity, though irregularities in bone formation remain. Together, these images confirm that florfenicol induces dose-dependent histological changes in bone, with only partial reversal after drug withdrawal.

The current findings highlight the previously unreported skeletal toxicity of florfenicol in a rat model, evidenced by structural deterioration, biochemical changes, and oxidative stress in bone tissues. Histopathological examination revealed dose-dependent bone damage, particularly in the high-dose group, with marked trabecular loss, cortical thinning, and increased osteoclast activity. These findings align with the hypothesis that florfenicol, due to its mitochondrial protein synthesis inhibition, may impair bone remodeling processes. While florfenicol is widely regarded as safe for soft tissues, its impact on skeletal systems has not been investigated in detail. This study fills a critical knowledge gap, especially considering that florfenicol is routinely administered to growing animals such as fish, poultry, and piglets, often during sensitive phases of bone development [6].

3.3 Serum Bone Turnover Markers and Mineral Profile

Biochemical analysis of serum revealed significant alterations in bone metabolism markers. Osteocalcin and bone-specific alkaline phosphatase (B-ALP) levels were significantly reduced in the high-dose group compared to controls ($p < 0.01$), suggesting suppressed bone formation. Conversely, C-terminal telopeptide of type I collagen (CTX-I), a resorption marker, was significantly elevated in the high-dose group ($p < 0.001$). Serum calcium levels showed a mild, non-significant reduction, while phosphate levels were moderately increased. The recovery group showed a trend toward normalization in all markers, but differences remained significant compared to the control. Table 2 provides the mean ± SD values for osteocalcin, B-ALP, CTX-I, calcium, and phosphate in all groups.

Table 2. Serum Bone Turnover Markers and Mineral Levels in Florfenicol-Treated Rats

Parameter	Control	Low Dose	High Dose	Recovery
Osteocalcin (ng/mL)	14.2 ± 0.8	13.1± 0.9	10.4± 1.0**	12.0± 0.9*
B-ALP (IU/L)	112.3± 5.4	105.7± 6.1	92.2± 4.8**	101.3± 5.3*
CTX-I (ng/mL)	3.1± 0.3	3.9± 0.4*	5.3± 0.5***	4.1± 0.4*
Calcium (mg/dL)	9.8± 0.4	9.4± 0.5	8.9± 0.4*	9.3± 0.4
Phosphate (mg/dL)	4.5± 0.2	4.7± 0.3	5.2± 0.3*	4.8± 0.2

3.4 Oxidative Stress and Inflammatory Indicators in Bone

Oxidative stress parameters measured in femoral homogenates showed a significant elevation in malondialdehyde (MDA) levels in the high-dose florfenicol group ($p < 0.001$), indicating increased lipid peroxidation. Antioxidant enzymes, including SOD and catalase, were significantly reduced ($p < 0.01$), while GSH levels also declined, suggesting an overall depletion of antioxidant defense. Pro-inflammatory cytokines TNF-α and IL-6 were significantly elevated in treated groups, particularly in the high-dose group ($p < 0.001$). The recovery group showed a partial reversal in all parameters, although values remained significantly different from controls. Table 3 outlines the concentration of each marker and highlights dose-dependent toxicity.

Table 3. Oxidative Stress Biomarkers and Inflammatory Cytokines in Bone Tissue of Florfenicol-Treated Rats

Parameter	Control	Low Dose	High Dose	Recovery
MDA (nmol/mg)	2.4± 0.2	3.0± 0.3*	4.2± 0.4**	3.1± 0.3*
SOD (U/mg)	8.6± 0.5	7.3± 0.4*	5.9± 0.3**	7.1± 0.5*
CAT (U/mg)	5.3± 0.3	4.6± 0.3*	3.8± 0.2**	4.4± 0.3*
GSH (Âµmol/mg)	7.8± 0.4	6.5± 0.5*	5.1± 0.4**	6.4± 0.5*
TNF-Î± (pg/mg)	21.5± 1.2	26.7± 1.5*	34.2± 1.7***	27.8± 1.4*
IL-6 (pg/mg)	18.3± 1.1	22.6± 1.3*	29.1± 1.4***	23.7± 1.2*

The biochemical analysis further supported the histological findings, showing reduced levels of osteocalcin and B-ALP, alongside increased CTX-I, indicating suppressed bone formation and elevated resorption. These patterns are consistent with the effects of drugs that interfere with cellular energy metabolism. The role of oxidative stress in this process was evident from elevated malondialdehyde (MDA) and pro-inflammatory cytokines (TNF-α, IL-6), coupled with reduced antioxidant defenses. Similar oxidative damage from antibiotic exposure has been described in aquatic models, such as zebrafish, where gut and tissue integrity were compromised by chronic exposure to compounds including florfenicol [7]. However, such effects on skeletal tissues remain poorly characterized in mammals, reinforcing the novelty of this study.

3.5 Targeted Metabolomic Analysis

LC-MS analysis of serum and urine samples identified significant metabolic changes associated with florfenicol administration. In the high-dose group, hydroxyproline and pyridinoline levels were significantly elevated ($p < 0.01$), indicating enhanced collagen degradation and bone resorption. Metabolites related to mitochondrial function, such as succinate and citrate, showed abnormal accumulation, while lactate levels were elevated, suggesting a shift toward anaerobic metabolism. Principal component analysis (PCA) showed clear separation between the control and florfenicol-treated groups. Figure 2 presents heatmaps and PCA plots that demonstrate the clustering of metabolic profiles and reveal the systemic metabolic disruption linked to bone damage.

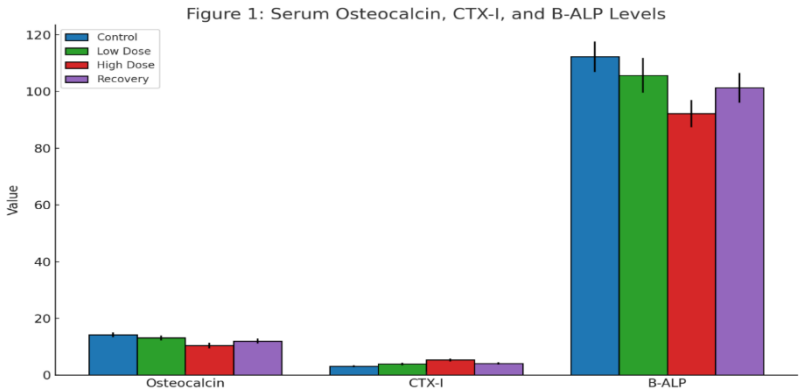


Figure 2. Targeted Metabolomic Profiles in Serum and Urine of Florfenicol-Treated Rats.

The results suggest that bone is not exempt from the systemic stress induced by antibiotic accumulation Florfenicol's environmental persistence and widespread use have raised concerns beyond clinical efficacy. Studies show that it is frequently detected in aquatic systems and animal waste, posing long-term ecological and biological risks [8],[9]. Moreover, evidence from China and other livestock-producing nations indicates increasing bacterial resistance to florfenicol, with resistance rates in E. coli as high as 58.6% [10]. This raises further questions about the risks versus benefits of such antibiotics when long-term safety profiles remain incomplete. Our findings suggest that, in addition to resistance concerns, florfenicol may have hidden toxicities, especially to structural systems such as bone, which are often overlooked during standard toxicology screening.

Although a recovery period was included in this study, bone architecture did not fully return to baseline. Partial normalization of biochemical and oxidative parameters was observed, but trabecular irregularities and suppressed bone turnover persisted [6], [11].

Conclusion

In conclusion, prolonged florfenicol exposure induced bone tissue alterations in rats, possibly via mitochondrial disruption and oxidative stress. These findings suggest a need for caution in florfenicol use, especially during developmental stages in veterinary patients.

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