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The influence of prolactin-releasing prokinetic drugs on bone microarchitecture and biomechanics in streptozotocin-diabetic rat

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ABSTRACT

To understand the potential impact of antidopaminergics such as Domperidone (DOM), Metoclopramide (MCP), and Trimethobenzamide (TMB) used in diabetic gastroparesis, this study examined their effects—both harmful and beneficial—on bone fragility. A diabetic model was created in male Wistar rats by a single intraperitoneal injection of Streptozotocin (STZ, 60 mg/kg). Our groups consisted of: Control (no diabetes or treatment), STZ (diabetes) group, STZ+DOM (diabetes with DOM) group, STZ+MCP (diabetes with MCP) group, and STZ+TMB (diabetes with TMB) group. Treatments were administered daily during the last 2 weeks of the 8-week study. Bone biomechanical properties were evaluated using a three-point bending test (TPBT), while histopathological analysis and serum receptor activator of nuclear factor kappa-B ligand (RANKL) levels were also assessed. TPBT results indicated increased bone loss in the STZ group, and histological analysis revealed elevated lipid content in the femoral metaphysis. Rats in the STZ group showed significant alterations

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in bone biomechanical and histological parameters. Our study suggests that inducing diabetes with STZ in rats leads to bone loss within eight weeks, which may be valuable for testing new drugs aimed at treating osteoporosis in diabetic patients. Except for the doubling of RANKL levels with TMB among prolactin-releasing anti-dopaminergic agents in diabetic rats, these agents had no significant effect on bone mineral density or microarchitecture. Therefore, their use can be considered safe in sensitive diabetic populations.

Keywords: antidopaminergic drugs, diabetic gastroparesis, rational drug therapy, seconder osteoporosis, streptozocin

INTRODUCTION

Diabetes mellitus can lead to many serious complications, including an increased risk of cardiovascular diseases, retinopathy, nephropathy, neuropathies, gastroparesis, and osteoporosis. Making healthy lifestyle choices, especially regarding diet and exercise, is crucial to preventing these complications[1,2]. However, many patients still require pharmacological treatments. When choosing medications, the safest options to both treat diabetes and prevent complications need to be identified. Unfortunately, some diabetes medications (such as thiazolidinediones and SGLT2 inhibitors) may have side effects on bone health[1].

While metoclopramide (MCP) is the only medication approved by the FDA specifically for gastroparesis[3], other drugs such as domperidone (DOM) and trimethobenzamide (TMB) can be used to relieve gastrointestinal symptoms such as gastroesophageal reflux disease, diabetic diarrhea, dysphagia, dyspepsia, and emesis. These medications are also known as antidopaminergic prokinetics. MCP, DOM[4], and TMB[5] block central and peripheral dopamine receptors; as a result, an inevitable increase in prolactin levels occurs immediately.

Prolactin (PRL) is a hormone with many functions, including helping to regulate blood glucose (BG) levels. Interestingly, high prolactin levels seem to be linked to a lower risk of type 2 diabetes[2], while lower PRL levels have been associated with glucose impairment[6,7]. PRL receptors have been located in the gastrointestinal tract, kidneys, and bones, where they appear to play a role in calcium metabolism[2,8,9]. Non-physiological hyperprolactinemia has been reported to have negative effects on infertility-related issues, as well as on glucose and bone metabolism[4]. However, Chen et al. suggested that slightly increased PRL levels may improve bone mineral density in men with type 2 diabetes, although this effect was not observed in women[2].

The slope of the elastic region of the force-displacement curve represents the extrinsic stiffness or rigidity of the structure. The elastic modulus is a measure of the intrinsic stiffness of the material. The maximum stress the bone can sustain is referred to as the ultimate strength; these strength values are independent of the size and shape of the bone. However, the force required to break the bone differs from intrinsic strength because this breaking load, or fracture force, varies with bone size. It is crucial to keep this distinction in mind because intrinsic strength and breaking load can exhibit different trends in drug studies, especially if the drug affects bone size[10].

Despite the current medical use of prolactin-releasing antidopaminergic drugs for diabetic gastroparesis, there is a lack of clear evidence in the literature regarding their impact on bone health in diabetic osteoporosis. This study aims to investigate these potential effects.

METHODOLOGY

All experiments were conducted after obtaining the relevant permission from the Aydin Adnan Menderes University Animal Experiments Committee (HADYEK 64583101/2024/35). This study primarily aimed to understand the vascular and metabolic effects of prolactin release caused by antidopaminergics. To minimize animal use in medical research while maximizing the information gained, eye, testicular, and aortic tissues were also evaluated following proper ethical approvals. DOM (Motilium®, Sanofi, Turkey), MCP (Metpamid®, Sifar, Turkey), and TMB (Emedur®, Opella, Turkey) were purchased from a local pharmacy. Treatments with the drugs were adjusted weekly according to changes in body weight.

Control Group: The control group received no intervention.

STZ Group: Streptozotocin (60 mg/kg; Sigma-Aldrich) was administered once. Blood glucose (BG) levels were measured on the 3rd day, and the rats were monitored for 8 weeks.

STZ+DOM Group: Streptozotocin (60 mg/kg; Sigma-Aldrich) was administered once. BG levels were measured on the 3rd day, and the rats were monitored for 6 weeks. Then, DOM (10 mg/kg/day) was administered orally twice daily. At the end of the sixth week, DOM (10 mg/kg/day) was administered orally twice daily for 15 days.

STZ+MCP Group: Streptozotocin (60 mg/kg; Sigma-Aldrich) was administered once. BG levels were measured on the 3rd day, and the rats were monitored for 6 weeks. Then, MCP (2 mg/kg/day) was administered twice daily via the intramuscular injection. At the end of the sixth week, MCP (2 mg/kg/day) was administered by intramuscular injection twice daily for 15 days.

STZ+TMB Group: Streptozotocin (60 mg/kg; Sigma-Aldrich) was administered once. BG levels were measured on the 3rd day, and the rats were monitored for 6 weeks. Then, TMB (4 mg/kg/day) was administered twice daily via the intramuscular injection. At the end of the sixth week, TMB (4 mg/kg/day) was then administered by intramuscular injection twice daily for 15 days.

At the end of the study, blood samples were collected via cardiac puncture under anesthesia induced by ketamine (50 mg/kg) and xylazine (5 mg/kg). Serum was isolated by centrifugation (1000 xg for 10 minutes) and stored at -20°C for ELISA analysis. The muscles and soft tissues attached to both femurs were removed, the bones were then weighed and adjusted according to the animal weighting of each animal. The right femurs were wrapped in gauze soaked in 0.9% NaCl solution and stored at -20°C until mechanical tests were performed on the thawed bones. The left femurs were kept in 10% formalin solution for histopathologic evaluation.

ELISA analysis

Receptor activator of nuclear factor kappa-B ligand (RANKL), a member of the tumor necrosis factor receptor superfamily, is known as a type 2 transmembrane protein and is considered the most important factor for osteoclastogenesis. Therefore, in our study, we examined RANKL as an important bone biomarker[11]. Commercially available receptor activator of nuclear factor kappa-B ligand (RANKL) with a sensitivity of 5.7 pg/mL (ELK Biotechnology CO. Ltd., USA) was employed according to the manufacturer's protocol. Plate readings were conducted at the specified wavelength using a MultiscanGo spectrophotometer (Thermo Fisher Scientific Inc., USA).

Three-point bending test studies

The right femurs were thawed at room temperature before mechanical testing. Then the lengths of the bones were measured and the center point was marked, which would be the location of force application during the three-point bending test. For the three-point bending test, a Zwick Roell Z0.5 testing machine was used at Aydin Adnan Menderes University's TARBIYOMER labs. The test parameters were set with a support points (L; Span Length) of 15mm, a preload of 2N, and a strain rate of 1mm/min. The load was applied directly to the midpoint of the bone, pushing it in the cranio-caudal direction. Following the test, the diameters of the fractured bone were measure in various directions (cranio-caudal, medio-lateral) on both the inner (endosteal) and outer (periosteal) surfaces were measured. Using the endosteal and periosteal diameters, the cross-sectional moment of inertia (I) of the bones

were calculated. The stiffness was calculated from linear regression of the Force-displacement graph using the software TestXpert (Zwick/Roell, Ulm, Germany). Using the stiffness, moment of inertia, bone diameter and distance between the support points, ultimate strength and elastic modulus were calculated by using the formulas from the previous literature[10,12,13,14].

Histopathological evaluation

The left femurs were fixed in a 10% buffered formalin solution and then decalcified in a 10% nitric acid solution. After confirming adequate decalcification, the tissues were trimmed. The tissues were then processed through a series of alcohol solutions (70°, 80°, 90°, 96°, and 100°) and xylene treatments (Leica TP1020) before being embedded in paraffin blocks. Sections of 4-5 μm thickness were cut from these blocks using a microtome (Leica RM 2135), transferred to slides, and stained with hematoxylin and eosin (H&E). Microscopic examination was performed using a light microscope (Olympus BX51), and digital photographs were captured and archived. The bone trabeculae, bone marrow, and adipocytes were evaluated and recorded to provide detailed insights into bone structure and composition.

Statistical analysis

Animal weighting was evaluated by a percent change amount the groups. The Mann-Whitney *U* test was used to compare body weight, the combined femur weight/body weight ratio and serum RANKL level. The data of normal distribution was checked with the Shapiro-Wilk test. One-Way ANOVA was performed for normally distributed values and Kruskal-Wallis intergroup comparison was performed for non-normally distributed values. In the One-Way ANOVA test, Levene's test results for homogenous values were checked with the post hoc Bonferoni test. For non-homogeneous values, Welch's test results were checked with post-hoc Tamhane test. Data were expressed as mean ± standard deviation of mean, values of $p<0.05$ was accepted as significant.

RESULTS and DISCUSSION

Clinical follow-up

The percent change in animal weighting of STZ group rats was -8.37 ± 10.61% ($p<0.001$ vs control group). Treatment with DOM was -2.97 ± 15.73%, MCP was -2.80 ± 15.18% and TMB was -1.33 ± 12.81% ($p<0.001$, <0.01, <0.001, respectively vs. control group) at the fifteen days after 8-weeks of diabetes, while the animal weighting of control groups was continued to increase by 36 ± 22.94% (Table 1).

The ratio of total femur mass (Right+Left femur weight) to animal weighting (mg/g) was calculated as 5.39 ± 0.42% for the control group, while it was 6.78 ± 0.81 for the STZ group ($p<0.001$ vs control group). Treated with DOM was 6.94 ± 0.9, MCP was 6.64 ± 0.79 and TMB was 6.92 ± 0.78 ($p<0.01$, <0.01, <0.001, respectively vs. control group) (Table 1).

ELISA analysis results

Serum RANKL levels were similar across all groups, except for the STZ+TMB group, which showed approximately a two-fold elevation in RANKL levels ($p<0.05$ vs. Control group, $p<0.05$ vs. STZ group) (Table 1).

Table 1. Clinical follow-up and serum level of the Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) in all groups. Values are mean ± SEM. STZ group: Streptozotocin, STZ+DOM group: Streptozotocin+Domperidone, STZ+MCP group: Streptozotocin+Metoclopramide, STZ+TMB group: Streptozotocin+Trimethobenzamide

	Control group (n=8)	STZ group (n=9)	STZ+DOM group (n=8)	STZ+MCP group (n=9)	STZ+TMB group (n=8)
Animal weighting change %	36 ± 22.94	-8.37 ± 10.61***	-2.97 ± 15.73***	-2.80 ± 15.18**	-1.33 ± 12.81***
Right+Left femur weight/ Body Weight (mg/g)	5.39 ± 0.42	6.78 ± 0.81***	6.94 ± 0.9**	6.64 ± 0.79**	6.92 ± 0.78***
RANKL (pg/mL)	6.77 ± 0.82	5.56 ± 1.00	5.89 ± 0.85	5.87 ± 0.86	11.54 ± 1.82*#

* $p<0.05$ ** $p< 0.01$ ***$p< 0.001$ vs Control, # $p<0.05$ vs STZ

Three-point bending test results

The results of the bone biomechanical tests were given in Table 2 and Table 3. The eight weeks of STZ model has a great impact on bone tissue. Femur length was decreased in the STZ group, STZ+DOM group, STZ+MCP group, and STZ+TMB group compared to the control group ($p<0.001$). The mediolateral external diameter was reduced in the STZ group and STZ+MCP group compared to the control group ($p<0.05$), and there was no statistically significant difference between the other groups. The craniocaudal internal diameter was greater in the STZ+TMB group than in the control group rats ($p<0.05$) (Table 2).

Table 2. The geometrical properties of the femur in all groups. Data presented as Mean±Standart Deviation (Minimum-Maximum). STZ group: Streptozotocin, STZ+DOM group: Streptozotocin+Domperidone, STZ+MCP group: Streptozotocin+Metoclopramide, STZ+TMB group: Streptozotocin+Trimethobenzamide

Morphometric parameters (mm)		Control group (n=8)	STZ group (n=9)	STZ+DOM group (n=8)	STZ+MCP group (n=9)	STZ+TMB group (n=8)	p-value
	L	39.07 ± 1.81[a] (36.22-40.94)	35.25 ± 1.03[b] (33.55-36.48)	34.71 ± 1.37[b] (31.82-36.77)	34.33 ± 1.49[b] (31.54-36.15)	34.76 ± 0.74[b] (33.98-35.98)	<0.001
	$DExt_{ML}$	4.70 ± 0.39[a] (3.96-5.26)	4.13 ± 0.34[b] (3.74-4.70)	4.27 ± 0.26[ab] (3.80-4.58)	4.10 ± 0.20[b] (3.84-4.48)	4.38 ± 0.34[ab] (3.97-5.03)	0.004
	$DExt_{CrCa}$	3.40 ± 0.34 (2.84-3.81)	3.13 ± 0.13 (2.96-3.34)	3.20 ± 0.20 (2.84-3.42)	3.12 ± 0.11 (2.96-3.30)	3.11 ± 0.15 (2.89-3.28)	0.284
	$DInt_{ML}$	2.88 ± 0.29 (2.39-3.29)	2.96 ± 0.32 (2.66-3.69)	3.02 ± 0.35 (2.40-3.49)	3.03 ± 0.35 (2.32-3.38)	3.19 ± 0.24 (2.75-3.61)	0.353
	$DInt_{CrCa}$	1.84 ± 0.31[a] (1.48-2.34)	1.89 ± 0.27[ab] (1.59-2.48)	2.00 ± 0.13[ab] (1.78-2.18)	2.11 ± 0.30[ab] (1.64-2.51)	2.20 ± 0.19[b] (1.96-2.41)	0.027

$DExt_{ML}$: Medio-Lateral external diameter. **$DExt_{CrCa}$**: Cranio-Caudal external diameter. **$DInt_{ML}$**: Medio-Lateral internal diameter. **$DInt_{CrCa}$**: Cranio-Caudal internal diameter. L: Length. a.b: comparison of group

Compared to the control group, a significant decrease in femoral stiffness and strength was observed in the diabetic rats (STZ group, STZ+DOM group, STZ+MCP group, and STZ+TMB group) ($p<0.001$). The cross-sectional moment of inertia was also decreased in the STZ group, STZ+MCP group, and STZ+TMB group compared to the control group rats ($p<0.001$) (Table 3).

Table 3. The mechanical properties of the femur in all groups. Data presented as Mean ± Standart Deviation (Minimum-Maximum). STZ group: Streptozotocin, STZ+DOM group: Streptozotocin+Domperidone, STZ+MCP group: Streptozotocin+Metoclopramide, STZ+TMB group: Streptozotocin+Trimethobenzamide

		Control group (n=8)	STZ group (n=9)	STZ+DOM group (n=8)	STZ+MCP group (n=9)	STZ+TMB group (n=8)	p-value
Mechanical Parameters	I (mm^4)	8.48 ± 2.83[a] (3.97-12.18)	5.17 ± 1.07[b] (3.60-7.14)	5.77 ± 1.48[ab] (2.96-7.49)	4.69 ± 0.71[b] (3.79-5.85)	4.81 ± 1.10[b] (3.44-6.74)	<0.001
	F (N)	155.63 ± 17.34[a] (130.00-176.00)	93.11 ± 18.32[b] (54.90-116.00)	103.63 ± 18.27[b] (65.80-124.00)	89.88 ± 16.49[b] (68.80-112.00)	102.57 ± 14.96[b] (82.10-130.00)	<0.001
	Deformation (mm)	0.65 ± 0.10 (0.50-0.81)	0.56 ± 0.18 (0.34-0.83)	0.80 ± 0.21 (0.61-1.30)	0.72 ± 0.19 (0.45-1.05)	0.80 ± 0.20 (0.50-1.06)	0.054
	Stiffness (N/mm)	372.59 ± 56.87[a] (271.51-442.39)	241.64 ± 54.28[b] (141.47-310.06)	237.50 ± 71.42[b] (97.71-310.90)	182.17 ± 74.95[b] (111.78-297.43)	230.16 ± 35.78[b] (183.14-279.57)	<0.001
	Strength (MPa)	125.05 ± 9.40 (93.20-174.40)	107.88 ± 8.41 (68.90-148.50)	110.54 ± 4.83 (92.50-135.80)	113.86 ± 8.69 (82.30-161.40)	128.63 ± 9.57 (84.80-181.30)	0.314
	Elastic Modulus (GPa)	3.40 ± 1.26 (2.36-6.13)	3.34 ± 0.72 (2.25-3.98)	2.94 ± 0.79 (1.67-4.17)	2.77 ± 1.22 (1.67-5.39)	3.51 ± 0.90 (2.07-5.04)	0.499

I: Cross-sectional moment of inertia. F: Force.a.b: comparison of group

Histopathological evaluation

Histological examination of the femoral metaphysis in the diabetic group showed significant morphological changes. Compared to the control group, the STZ group exhibited thinner and more fragmented trabeculae, increased trabecular spaces, decreased bone marrow density, and an increased number of lipid droplets (Figure 1).

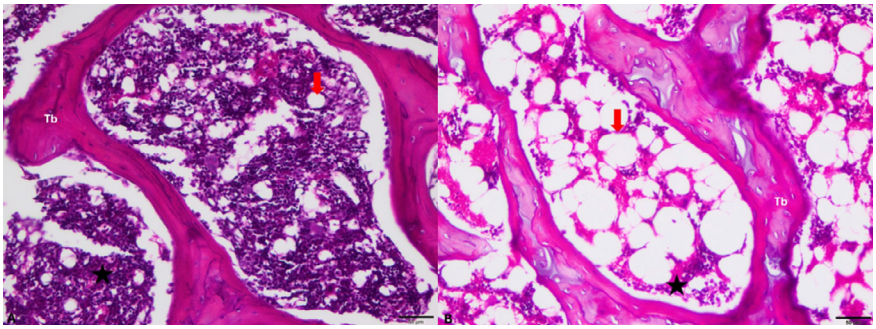

Figure 1. The femur metaphysis in a rat of A: control group; B: STZ (diabetic) group. Hematoxylin and eosin staining, 20 X magnification. Tb: Trabecular bone, Star: Bone marrow, Arrow: Adipocyte.

Compared to the treatment groups, the STZ-DOM group exhibited an increase in adipocytes, toward decreased bone marrow density compared to the STZ-MCP and STZ-TMB groups (Figure 2). However, overall, all treatment groups displayed slightly different morphological features of osteoporosis, when compared to the STZ group. In other words, the drugs did not significantly improve or worsen the diabetic bone structure.

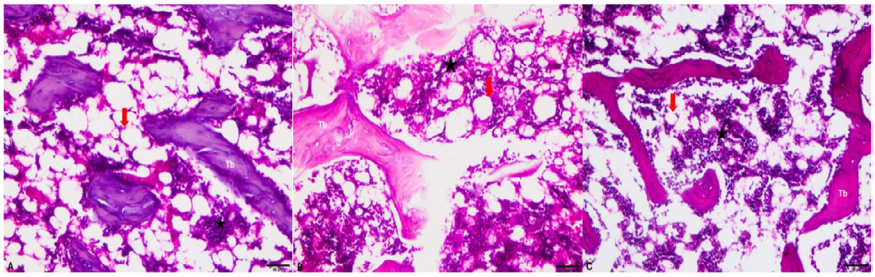

Figure 2. The femur metaphysis in a rat of A: Streptozotocin+Domperidone group; B: Streptozotocin+Metoclopramide group; C: Streptozotocin+Trimethobenzamie group. Hematoxylin and eosin staining, 20 X magnification. Tb: Trabecular bone. Star: Bone marrow. Arrow: Adipocyte.

Anti-dopaminergic agents that increase PRL secretion, which are used in the treatment of diabetic gastroparesis, did not significantly improve or deteriorate femoral stiffness, strength, elastic modulus, or deformation when use in short term. It is known that hyperprolactinemia decreases sex steroid production, which directly affects bone metabolism in both genders[8]. In one study, a daily dose of 200 micrograms of MCP in 0.9% saline solution was administered to oophorectomized mice for 50 days. This dose of MCP was shown to increase serum PRL levels. This study demonstrated that degenerative processes of articular cartilage in the epiphyseal growth plate and bone formation in hyperprolactinemia can be prevented by estrogen, progesterone, and testosterone[8].

In the present study, we considered the impact of antidopaminergics on bone metabolism in diabetes. Diabetes induction in rats led to a significant amount of weight loss over eight weeks; however, the ratio of combined femur weight to animal weight increased. This suggests that muscle mass may have been lost at a faster rate than bone mass. None of the gut motility drugs helped to prevent diabetic cachexia. Remarkable weight loss was observed in all STZ groups, with or without treatment.

Previous studies have demonstrated that STZ induces significant destructive changes in bone mineralization and mechanical properties within 6 weeks[1]. A more recent study by Tomaszewska et al. provided a detailed analysis of the ongoing effects of diabetes, reporting that the diabetic model reaches a chronic

stage by 8 weeks[15]. They observed that rats in the STZ group exhibited shorter bones compared to the control group. The anteroposterior outer diameter was increased at week 8. Consistent with the findings of Tomaszewska et al.[15], we observed a decrease in femur bone length at 8 weeks of diabetes in our study. The mediolateral external diameter was also decreased in all STZ groups. Additionally, bone width, cross-sectional moment of inertia, force, and stiffness values were reduced. The craniocaudal internal diameter was wider in the STZ group compared to the control group.

Histopathological evaluation has demonstrated that the bone-fat equilibrium is crucial for bone remodeling. An imbalance between these two components can disrupt the differentiation processes of osteogenesis and adipogenesis in bone marrow stem cells[16]. Similar to studies using STZ[16], our histological analysis revealed that diabetes resulted in findings characteristic of bone osteoporosis. Increased bone marrow adipocytes, a hallmark of osteoporosis[17], and decreased trabecular thickness due to osteoporosis-related bone resorption[15,18] were the major osteoporotic findings observed in the STZ group. Our findings strongly suggest that the STZ-diabetes model can be used as an animal model of secondary osteoporosis. This study provides a valuable tool for diabetes research to better understand medication use in this vulnerable disease. Tomaszewska et al. reported a gradual increase in serum RANKL levels from the second week after STZ administration[15]. Contrary to these findings, we did not observe a significant increase in serum RANKL levels in our 8-week diabetic rats.

Chen et al. reported that slightly elevated prolactin (PRL) levels may have beneficial effects on bone mineral density in men with type 2 diabetes, but not in women[2]. However, in our study, treatments with PRL-releasing drugs did not fully restore the bone's geometric properties. Bone length and mediolateral external diameter remained reduced, and the craniocaudal internal diameter remained wider than that of control group rats, with the most noticeable effect observed in the TMB treatment group, which significantly doubled serum RANKL levels. RANKL is a crucial mediator in bone tissue turnover[15]. The presence of prolactin receptors within bone tissue and on osteoblasts has been well documented[2,8,9]. Mice lacking prolactin receptors ($Prlr^{-/-}$) exhibit osteopenia and decreased bone formation in both sexes. It has been suggested that this may be partly due to decreased estrogen levels in female mice. Conversely, increasing pituitary activity through anterior pituitary transplantation in mice led to enhanced bone turnover, characterized by an elevated ratio of RANKL to osteoprotegerin[9]. Therefore, the effect of TMB on bone turnover requires further investigation.

When examining the impact of these drugs on the femur's mechanical properties, we found that DOM slightly increased the cross-sectional moment of inertia, while MCP and TMB further decreased it. However, none of the drugs significantly improved the femur's stiffness, force, strength, elastic modulus, or deformation, which remained significantly lower than those of the control group. Histological analysis revealed that treatments with all PRL-releasing drugs exhibited similar osteoporotic features to the STZ group, suggesting that these drugs had no substantial beneficial or detrimental effects on diabetic bone structure, as observed through hematoxylin and eosin staining.

We initiated prokinetic therapy 6 weeks after STZ administration, when diabetic complications had developed. We limited the study to 15 days to reflect real-world use, as these medications have intermittent usage patterns, corresponding to periodic exacerbations of diabetic gastroparesis. Previous studies have shown that prolonged and high prolactin exposure[6], such as that resulting from pregnancy or prolactinoma, leads to significant changes in the hormonal axes. Therefore, in our study, we avoided prolonged PRL exposure.

This study showed that inducing diabetes with STZ in rats causes bone loss within eight weeks. This suggests that this model could be valuable for testing new drugs aimed at treating osteoporosis in people with diabetes. Since diabetes raises the risk of bone fractures, any medication used to manage diabetes or its complications must be carefully balanced to avoid harming the bones. Prolactin-releasing anti-dopaminergic agents did not have a considerable impact on bone mineral density or microarchitecture in diabetic rats, except for the doubling of RANKL levels with TMB. Therefore, they can be considered safe for use in vulnerable diabetic populations.

STATEMENT OF ETHICS

This study was approved by the Aydın Adnan Menderes University Animal Experiments Local Ethics Committee on March, 21 2024 (Approval No: HADYEK 64583101/2024/35).

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interest.

AUTHOR CONTRIBUTIONS

BOD performed the research, performed the statistical analysis, wrote the manuscript, and had primary responsibility for the final content of the manuscript. ANA performed the bone histopathology. FSK performed the bone biomechanics, performed the statistical analysis, and wrote the draft. SNG

helped the bone histopathology, biomechanics, and the statistical analysis. FB performed the biochemical analysis. TD contributed to conceptualization, methodology, supervision, validation, visualization, review, and editing of the draft. BD contributed to conceptualization, designed the research, formal analysis, methodology, supervision, validation, visualization, writing the original draft, and review and editing of the draft.

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