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Effects of 4-hexylresorcinol on facial skeletal development in growing rats: Considerations for diabetes

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Objective: To investigate the long-term effects of 4-hexylresorcinol (4HR) on facial skeletal growth in growing male rats, with a focus on diabetic animal models. **Methods:** Forty male rats were used. Of them, type 1 diabetes mellitus was induced in 20 animals by administering 40 mg/kg streptozotocin (STZ), and they were assigned to either the STZ or 4HR-injected group (STZ/4HR group). The remaining 20 healthy rats were divided into control and 4HR groups. We administered 4HR subcutaneously at a weekly dose of 10 mg/kg until the rats were euthanized. At 16 weeks of age, whole blood was collected, and micro-computed tomography of the skull and femur was performed. **Results:** All craniofacial linear measurements were smaller in the STZ group than in the control group. The mandibular molar width was significantly smaller in the 4HR group than in the control group (P = 0.031) but larger in the STZ/4HR group than in the STZ group (P = 0.011). Among the diabetic animals, the STZ/4HR group exhibited significantly greater cortical bone thickness, bone mineral density, and bone volume than the STZ group. Serum testosterone levels were also significantly higher in the STZ/4HR group than in the STZ group. **Conclusions:** 4HR administration may have divergent effects on mandibular growth and bone mass in healthy and diabetic rats. In the context of diabetes, 4HR appears to have beneficial effects, potentially through the modulation of mitochondrial respiration.

Key words: 4-hexylresorcinol, Micro-computed tomography, Diabetes mellitus, Testosterone

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INTRODUCTION

The development of the maxillofacial skeleton is derived from the neural crest and is primarily governed by genetics, resulting in comparable facial characteristics among siblings.[1-3] Nonetheless, external factors, such as nutritional intake, can affect gene expression. Orthopedic interventions during the growth period can also serve as environmental influences on skeletal growth.[4,5] Although environmental elements can alter gene expression, the complete mechanisms of epigenetic regulation remain unclear.

Histones are proteins that play a fundamental role in gene regulation by binding to DNA and organizing it into a compact structure called chromatin, which is composed of nucleosomes.[6] The binding of histones to DNA can limit the access of transcription factors to gene promoter regions, resulting in reduced gene expression. However, the transfer of acetyl groups to specific sites on histones can make chromatin structure more open and accessible to transcription factors. This process, known as histone acetylation, can increase the expression of certain genes by allowing transcription factors to bind to their promoter regions.[7] While only a small proportion of genes (less than 20%) are affected by histone acetylation, many of these genes play important roles in skeletal tissue development. For instance, histone acetylation can activate the genes associated with the transforming growth factor-β family, which is involved in bone formation and remodeling.[8,9] Additionally, the Runx2 transcription factor can bind to the promoters of several genes involved in bone development, and histone acetylation can enhance this binding and increase gene expression.[10,11]

Histone acetylation levels can be increased by 4-hexylresorcinol (4HR), a histone deacetylase inhibitor,[12] increasing the expression of certain genes, such as those associated with bone morphogenic protein-2 and Runx2 in osteoblast-like cells.[13,14] However, 4HR has been shown to suppress testosterone production and mandibular growth in healthy growing male rats,[14] and it is unclear if this effect is permanent or temporary. The potential inhibition of mitochondrial metabolism by 4HR may suppress testosterone production and skeletal growth as they depend on mitochondrial function.[15] Diabetes mellitus (DM) is a well-known mitochondrial dysfunction-related disease.[16,17]

Antioxidants are sometimes administered to potentially prevent or mitigate complications associated with DM.[18] Interestingly, 4HR may protect cells in this scenario through its antioxidant effect.[19] However, to date, there are on only a few studies reporting the effect of 4HR on facial skeletal growth in diabetic conditions. Thus, this study aimed to examine the long-term effects of 4HR on facial skeletal growth in growing male rats and to expand the investigation to include diabetic animal models.

MATERIALS AND METHODS

Experimental design

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Gangneung-Wonju National University (GWNU-2021-21 and GWNU-2021-22). In total, 40 male rats were used, all of whom were 4 weeks of age and had the same pair of grandparents. The rats' initial weight was 87–90 g. Half of them were assigned to the diabetic model and allowed to grow until they reached 7 weeks of age without any treatment due to the high mortality rate observed in young animals following experimental induction of DM. At the time of streptozotocin (STZ) injection, the rats' body weight was 270–290 g. The rats were fasted for 6–8 hours before STZ injection and provided regular water supply. High-dose STZ (40 mg/kg) was injected intravenously through the tail vein to induce diabetes. On day 3 after STZ injection, the rats' fasting blood glucose levels were measured. All STZ-injected animals exhibited blood glucose levels of more than 300 mg/dL, confirming their diabetic status. Subsequently, they were randomly assigned to either the STZ or 4HR-injected group (STZ/4HR group). We administered 4HR subcutaneously at a weekly dose of 10 mg/kg until the end of the experiment. Two rats from the diabetic model died: 1 from the STZ group and the other from the STZ/4HR group. The remaining 20 rats were assigned to the healthy group. Half of them received the same dose of 4HR every week as those in the diabetic model, whereas the other half were fed a normal diet and served as controls (Figure 1). Ultimately, 18 diabetic rats and 20 healthy rats were included in this study and humanely sacrificed at 16 weeks of age. The rats were anesthetized with enflurane, and a whole blood sample was collected from the heart. Supernatant serum was obtained by centrifuging the blood sample, and all animals were sacrificed by injecting paraformaldehyde. The head and femur samples were sent for microcomputed tomography (μCT) analysis.

Microcomputed tomography

We performed μCT of the heads of the animals to evaluate their mandibular growth using μCT50 (Scanco Medical, Brüttisellen, Switzerland) with an aluminum filter. The source voltage was set to 90 kVp with an image pixel size of 9.04 μm. The images were saved as digital imaging and communication in medicine files. Three-dimensional images were reconstructed using the OnDemand 3D software (Cybermed, Seoul, Korea). The

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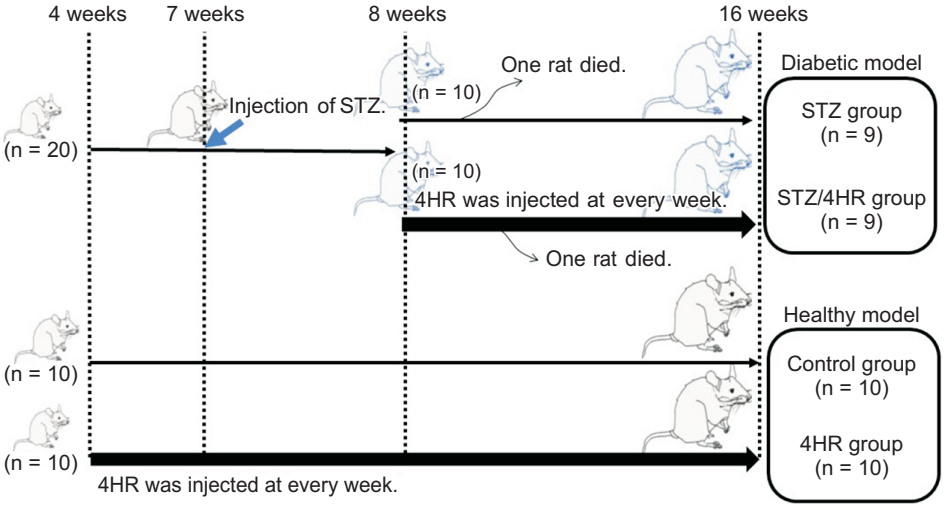

Figure 1. Experimental design.
STZ, streptozotocin; 4HR, 4-hexylresorcinol.

landmarks used in this study are defined in Table 1. Fourteen linear variables were measured by a single examiner (Figure 2). To evaluate the method error, 10 μCT images were randomly selected, and the measurements on these images were repeated at 1-week intervals. The method error ranged between 0.04–0.27 according to Dahlberg's formula.

Further, μCT with Quantum FX (Perkin Elmer, Walthamm, MA, USA) was performed to analyze the femur samples. The samples were carefully positioned in the scanner to ensure their proper orientation and alignment. The scanning parameters were set as follows: voltage, 90 kVp; current, 180 μA; field of view, 10 mm; voxel size, 9.7 μm; and scan time, 3 minutes. Cross-sectional images of the femur samples were acquired. Then, a region of interest (ROI) was identified for cortical thickness analysis. This ROI was located 0.3 mm from the growth plate, allowing for accurate cortical thickness measurement.

Measurement of serum testosterone level

The serum samples were used for analysis of testosterone levels with a commercially available kit (CAT#: ab285350; Abcam, Cambridge, UK). The subsequent procedures were performed as described in our previous publication.[13,14]

Statistical analysis

Descriptive and statistical analyses were conducted. The normality and homogeneity of variances were confirmed for analysis of variance (ANOVA). Each observation was independent. Once the assumptions were met, one-way ANOVA was performed to test the null hypothesis that there would be no significant difference between the group means. If ANOVA revealed significant differences, the least significant difference test was performed post-hoc. The level of significance was set at $P < 0.05$. Post-hoc power analysis (β) was conducted for the variables that exhibited statistically significant differences between groups ($P < 0.05$).

Table 1. Definitions of landmarks

Landmark	Definition
Co	Most postero-superior point on the mandibular condyle
Go	Most posterior point of the bony contour of the gonial angle of the mandible
Gn	Most inferior point of the bony contour of the gonial angle of the mandible
Me	Most inferior point of the mandibular symphysis
Id	Most inferior point of the marginal alveolar bone of the lower central incisor
B1	Most anterior point of the marginal alveolar bone on the lingual surface of the lower central incisor
M1	Most mesial point of the marginal alveolar bone on the mesial surface of the lower first molar
L1	The deepest point on the mesial occlusal fossa of the lower first molar
L1'	Projected point of L1 on the line connecting Gn and Me
L2	Most lateral point on buccal surface of the lower first molar
Cp	Most superior point of the coronoid process
Cp'	Projected point of Cp on the line connecting Gn and Me
Zy	Most lateral point of the zygomatic arch

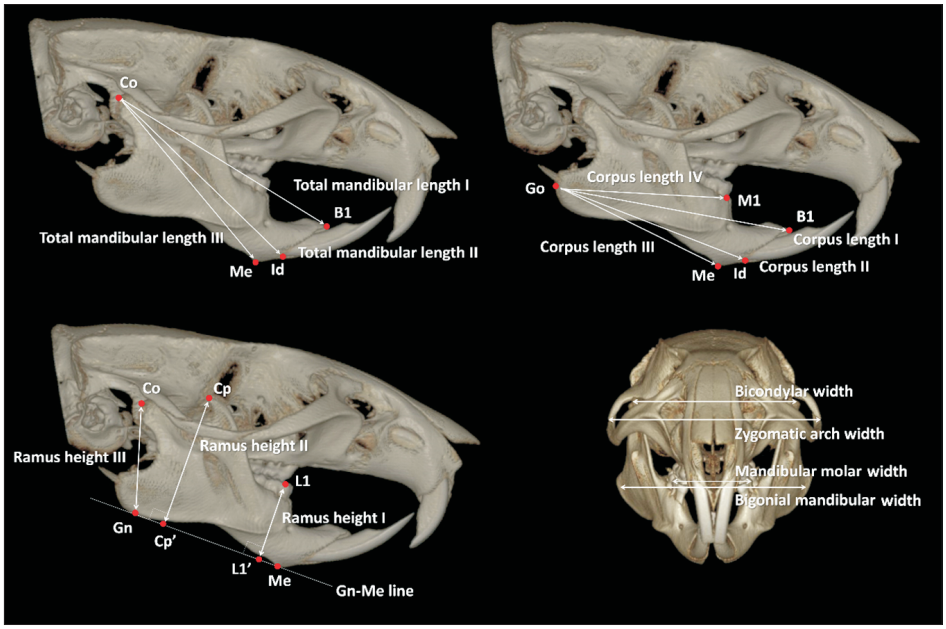

Figure 2. Linear measurements on cone-beam computed tomography images.

RESULTS

All measurement values were significantly lower in the STZ group than in the control group ($P < 0.001$, Table 2). Administration of 4HR produced different effects in healthy and DM animals. The mandibular molar width in the 4HR group (9.82 ± 0.26 mm) was significantly lower than that in the control group (10.03 ± 0.22 mm) ($P = 0.031$, $\beta > 0.9$). Further, the mandibular molar width in the STZ/4HR group (9.66 ± 0.12 mm) was significantly higher than that in the STZ group (9.40 ± 0.19 mm) ($P = 0.011$).

The impact of DM on femur findings was analogous to that on facioskeletal measurements (Table 3). Most measurements in the control group were significantly greater than those in the STZ group ($P < 0.001$). The cortical bone mineral density (BMD) was significantly higher in the 4HR group (1,645.65 ± 21.75 mg/cc) than in the control group (1,623.13 ± 12.60 mg/cc) ($P = 0.015$). However, the bone volume (BV), BV to total volume (TV) ratio, and trabecular number were significantly lower in the 4HR group than in the control group ($P < 0.05$). Among the diabetic animals, the STZ/4HR group displayed a significantly higher cortical bone thickness than the STZ group ($P = 0.001$). Additionally, the cortical BMD and BV were significantly higher in the STZ/4HR group than in the STZ group ($P < 0.05$).

As shown in Figure 3, the serum testosterone level was 6.66 ± 4.00 ng/mL in the control group and 3.93 ± 2.38 ng/mL in the 4HR group, with no significant differences between both groups ($P > 0.05$). However, the serum testosterone level of the STZ group (2.67 ± 0.29 ng/mL) was significantly lower than that of the STZ/4HR group (3.17 ± 0.24 ng/mL).

DISCUSSION

The present findings confirm that STZ-induced diabetes significantly affects facial bone growth and bone structure. The STZ group displayed significantly lower facial measurement values ($P < 0.001$), BV, and BMD ($P < 0.001$) than the control group. On the other hand, the 4HR-injected diabetic model (STZ/4HR group) exhibited a significantly greater mandibular molar width, cortical BV, cortical BMD, and cortical bone thickness than the STZ group ($P < 0.05$). These findings indicate that DM severely impairs facial bone growth and reduces bone mass, whereas 4HR administration mitigates these DM-induced alterations.

Diabetes can disrupt the balance between bone formation and resorption, leading to a decrease in bone mass and quality.[20,21] STZ-induced diabetes has been associated with decreased BMD in both cortical and trabecular bones.[22] A lower BMD can increase the risk of fractures and osteoporosis. Diabetes can negatively affect bone microarchitecture, leading to changes, such as reduced trabecular number and BV/TV ratio.[23] In this study, STZ-induced diabetes resulted in impaired facial bone growth and significantly lower bone mass compared those of the healthy controls (Tables 2 and 3). Our results concur with those of an earlier study on bone metabolism in diabetic pigs,[24] which revealed decreased sizes of osteocytes and lacunae in the mandibular cancellous bone of diabetic animals.

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Table 2. Linear facioskeletal measurements

	Healthy model					Diabetic model						
	Control group (n = 10)		4HR group (n = 10)		*P* value	STZ group (n = 9)		STZ/4HR group (n = 9)		*P* value	Control group vs. STZ group	4HR group vs. STZ/4HR group
	mean	SD	mean	SD		mean	SD	mean	SD			
Antero-posterior measurement												
Total mandibular length I (Co-B1)	28.10	0.42	28.09	0.59	0.954	25.81	0.51	26.07	0.65	0.157	< 0.001*	< 0.001*
Total mandibular length II (Co-Id)	27.06	0.44	27.30	0.58	0.169	25.09	0.45	25.31	0.63	0.210	< 0.001*	< 0.001*
Total mandibular length III (Co-Me)	24.84	0.37	24.95	0.59	0.485	22.74	0.50	22.89	0.54	0.395	< 0.001*	< 0.001*
Corpus length I (Go-B1)	28.89	0.33	28.74	0.52	0.329	26.34	0.74	26.50	0.36	0.349	< 0.001*	< 0.001*
Corpus length II (Go-Id)	26.03	0.40	26.15	0.51	0.448	23.90	0.69	24.10	0.40	0.235	< 0.001*	< 0.001*
Corpus length III (Go-Me)	22.93	0.24	22.89	0.52	0.829	20.67	0.82	20.83	0.44	0.377	< 0.001*	< 0.001*
Corpus length IV (Go-M1)	21.22	0.46	21.21	0.41	0.974	19.33	0.64	19.50	0.40	0.294	< 0.001*	< 0.001*
Vertical measurement												
Ramus height I (L1-L1')	9.16	0.28	9.18	0.22	0.776	8.26	0.19	8.37	0.22	0.150	< 0.001*	< 0.001*
Ramus height II (Cp-Cp')	15.10	0.45	8.37	0.22	0.660	8.37	0.22	13.81	0.35	0.389	< 0.001*	< 0.001*
Ramus height III (Co-Gn)	12.16	0.47	13.81	0.35	0.701	11.12	0.45	11.04	0.48	0.623	< 0.001*	< 0.001*
Transverse width												
Bicondylar width	18.82	0.34	18.48	0.41	0.075	17.69	0.52	17.68	0.32	0.968	< 0.001*	< 0.001*
Bigonial mandibular width	22.01	0.94	21.82	0.44	0.565	19.02	0.91	19.67	0.47	0.065	< 0.001*	< 0.001*
Mandibular molar width	10.03	0.22	9.82	0.26	0.031*	9.40	0.19	9.66	0.12	0.011*	< 0.001*	0.106
Zygomatic width	25.29	0.54	25.06	0.54	0.316	23.16	0.55	23.07	0.38	0.735	< 0.001*	< 0.001*

STZ, streptozotocin; 4HR, 4-hexylresorcinol.
*$P < 0.05$.

Table 3. Measurements obtained from the femur

	Healthy model					Diabetic model					Control group vs. STZ group	4HR group vs. STZ/4HR group
	Control group (n = 10)		4HR group (n = 10)		*P* value	STZ group (n = 9)		STZ/4HR group (n = 9)		*P* value		
	mean	SD	mean	SD		mean	SD	mean	SD			
BMD (mg/cc)	404.51	39.16	368.93	42.36	0.023*	290.38	24.09	269.65	21.22	0.198	< 0.001*	< 0.001*
Cortical BV (mm^3)	21.54	1.51	21.99	1.06	0.481	10.97	1.71	12.47	1.32	0.032*	< 0.001*	< 0.001*
Cortical BMD (mg/cc)	1,623.13	12.60	1,645.65	21.75	0.015*	1,558.14	22.39	1,579.07	20.83	0.031*	< 0.001*	< 0.001*
TV (mm^3)	28.55	3.77	27.56	3.34	0.456	23.91	2.45	21.37	1.27	0.074	0.001*	< 0.001*
BV (mm^3)	2.57	0.84	1.80	0.75	0.007*	0.41	0.28	0.21	0.16	0.486	< 0.001*	< 0.001*
BV/TV ratio (%)	8.98	2.76	6.50	2.57	0.010*	1.70	1.07	0.99	0.74	0.461	< 0.001*	< 0.001*
Trabecular surface area/BV (1/mm)	32.42	2.28	33.91	3.47	0.509	48.58	3.55	48.89	8.59	0.895	< 0.001*	< 0.001*
Trabecular thickness (mm)	0.11	0.02	0.12	0.02	0.310	0.09	0.01	0.09	0.02	0.686	0.029*	0.001*
Trabecular separation/spacing (mm)	0.25	0.05	0.32	0.09	0.994	5.67	3.23	24.51	42.58	0.062	0.573	0.016*
Trabecular number (1/mm)	0.67	0.18	0.50	0.17	0.011*	0.22	0.10	0.12	0.08	0.156	< 0.001*	< 0.001*
Cortical bone thickness (mm)	0.57	0.03	0.59	0.04	0.265	0.33	0.04	0.39	0.04	0.001*	< 0.001*	< 0.001*

BMD, bone mineral density; BV, bone volume; TV, total volume.
*$P < 0.05$.

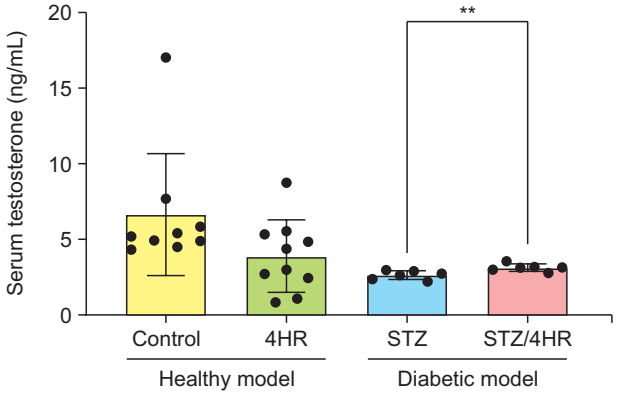

Figure 3. Serum testosterone levels. Hemolysis prevented the separation of serum in one sample from the control group and three samples each from the STZ and STZ/4HR groups. These samples weren't included in the testosterone analysis. The control group displayed higher testosterone levels than the 4HR group, but this difference lacked statistical significance ($P > 0.05$). On the other hand, a marked difference was evident between the STZ group and the STZ/4HR group (**$P < 0.01$).
STZ, streptozotocin; 4HR, 4-hexylresorcinol.

Previous research has demonstrated that administering 4HR to growing male animals reduces their serum testosterone levels, leading to smaller mandibles than those of control animals.[14] Consistent with these findings, in the present study, the mandibular molar width was significantly smaller in the 4HR group than in the control group ($P = 0.031$; Table 2). Interestingly, the mandibular transverse parameters tended to be narrower in the 4HR group than in the control group and wider in the 4HR/STZ group than in the STZ group, although the difference was not significant, except for the mandibular molar width.

Lower testosterone levels are associated with reduced BMD in both cortical and trabecular bones.[25,26] The changes in bone microarchitecture, including a decrease in trabecular number and reduced BV/TV ratio, could be attributed to testosterone insufficiency. In this study, the average serum testosterone level was lower in the 4HR group than in the control group, although the differences were not statistically significant. Therefore, the lower BV, BV/TV ratio, and trabecular number observed in the 4HR group may be linked to the serum testosterone levels.

In contrast to the control group, 4HR administration appeared to mitigate the DM-induced functional impairment of the testes in diabetic animals. The serum testosterone levels in the STZ/4HR group were significantly higher than those in the STZ group ($P = 0.008$). Administration of 4HR attenuated STZ-induced impaired sper-

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matogenesis. The diabetic rats injected with 4HR exhibited significantly greater cortical bone parameters in the femur than the other diabetic rats, which is in contrast to the findings of the healthy rats who received 4HR. This discrepant response to 4HR between the diabetic and healthy groups may be attributable to variances in cellular response at the mitochondrial level. Mitochondria are the central intracellular organelles responsible for producing testosterone.[27] As 4HR increases mitochondrial stress,[28] testosterone synthesis in mitochondria may be reduced under healthy conditions. The effect of 4HR may be prominent in special conditions that necessitate high metabolic demands, such as growing bones or cancer.[29] However, the reduction in mitochondrial metabolism may be beneficial in other conditions, such as diabetes. In diabetes, the mitochondria receive excessive glucose, leading to the production of a higher amount of reactive oxygen species compared to healthy cells.[30] Accordingly, reduced mitochondrial respiration by 4HR may be beneficial in DM conditions.

Diabetes can increase the risk of periodontal disease, complicating orthodontic treatment and compromising treatment outcomes. Diabetes can impede the healing process and complicate the recovery of patients requiring orthognathic surgery. The potential protective effects of 4HR on mandibular growth and bone health in diabetic conditions may help improve the outcomes of orthodontic or orthopedic treatments in patients with diabetes.

In the present study, the femur was used to assess bone parameters under diabetic conditions. The mandible, which is primarily composed of cortical bone with less trabecular bone, exhibits a slow remodeling rate, which may potentially delay the manifestation of diabetes-induced bone changes. In contrast, the femur, a prominent long bone, can reliably demonstrate the changes in bone structure in response to various conditions, such as diabetes. However, direct extrapolation of our results to facial skeletal development requires caution owing to the anatomical and metabolic differences between the femoral and facial bones.

The limitations of this study are as follows. First, the study had a relatively small sample size, which could affect the statistical power and generalizability of the findings. Second, the study was conducted on growing animals, who may not fully represent the complexity of human bone biology or the various stages of diabetes progression in humans. Third, we did not investigate the potential dose-dependent effects of 4HR administration. Additional research is necessary to elucidate the effects and mechanisms of 4HR and identify potential therapeutic targets.

CONCLUSIONS

This study demonstrated the adverse effects of type 1 diabetes on bone biology, specifically, mandibular growth and femoral bone mass. Our findings highlight the negative effects of diabetes on bone microarchitecture, which increases the risk of fractures and osteoporosis. Furthermore, this study revealed the potential protective effects of 4HR administration in mitigating diabetes-induced alterations in bone growth and mass. While 4HR administration may reduce testosterone levels and bone parameters under healthy conditions, it appears to have beneficial effects in diabetes, possibly through the modulation of mitochondrial respiration.

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AUTHOR CONTRIBUTIONS

Conceptualization: SGK, IJ. Data curation: XC, JYC, JYK. Formal analysis: XC, JYC, IJ, SGK. Investigation: HJ, JYK. Methodology: SGK, XC, IJ. Project administration: IJ, SGK. Software: HJ, JYK. Supervision: SGK, IJ. Validation: HJ, JYK. Visualization: HJ, JYK. Writing–original draft: HJ, JYK, SGK, IJ. Writing–review & editing: SGK, IJ.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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None to declare.

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