Impact of infrasound exposure and glucose intolerance on bone composition in Wistar rats

Luísa Zagalo (analuisazagalo@gmail.com)
Center for Interdisciplinary Research Egas Moniz (CiiEM)
Gonçalo Pereira
Center for Interdisciplinary Research Egas Moniz (CiiEM)
Diogo Casal
Lisbon University Hospital Centre
Luísa L. Gonçalves
Center for Interdisciplinary Research Egas Moniz (CiiEM)
Carlos Zagalo
Center for Interdisciplinary Research Egas Moniz (CiiEM)
Maria João Oliveira
University of Porto
Pedro Oliveira
Center for Interdisciplinary Research Egas Moniz (CiiEM)
José A. A. Brito
Center for Interdisciplinary Research Egas Moniz (CiiEM)

Article

Keywords: X-ray fluorescence spectroscopy, Elemental concentration, Infrasound, Glucose intolerance, Diabetes, Wistar rat

Posted Date: March 16th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1448044/v1

Abstract

Introduction: The concentrations of some chemical elements may differ between healthy and diseased tissues. The knowledge of these differences is pivotal to understand the metabolic processes involved in both physiological and diseased states. The aim of this study was to evaluate the elemental composition of calcium (Ca) and phosphorus (P) in the bone of rats subjected to glucose intolerance and/or infrasound.

Materials and Methods: X-ray fluorescence spectroscopy was applied in the concentration determination of Ca and P in samples of Wistar rat tibiae. Tibiae from glucose intolerant, non-intolerant, exposed and non-exposed to infrasound rats were analysed.

Results: There was a significant decrease in bone P concentration in glucose intolerant rats compared to non-intolerant animals. Similarly, the Ca/P ratio was higher in glucose intolerant animals. There were no significant differences for bone Ca concentration in both studied groups, nor between animals exposed and non-exposed to infrasound in either studied element.

Discussion and Conclusions: Glucose intolerant rats had lower bone P concentration and unaltered bone Ca concentration compared to non-glucose intolerant rats. Infrasound exposure was not associated to changes of bone Ca or P. The reduction of bone P concentration may be associated with the augmented risk of bone fractures in diabetes.

1. Introduction

Although some biological trace elements, such as phosphorus and calcium are involved in important physiological processes, their concentrations may differ significantly between healthy and diseased individuals [1]. The knowledge of changes in calcium (Ca) and phosphorus (P) concentrations is pivotal to understand both normal metabolic processes and pathophysiology in a tissue like the bone [2, 3], and these may be affected by both biochemical and mechanical stimuli.

However, the investigation of the role of each element considered in isolation has been questioned since it ignores potential interactions between them [1]. For all that, even when concentrations of several elements are obtained in the same study, comparisons between healthy and diseased tissues, or correlations between the various elements, both intrinsically multivariate, are often implemented with univariate methods, which may result in observed effects or the inability to detect such effects [4, 5].

Diabetes, a growing global epidemic, is a biochemical stimulus that can be harmful to bone health [6]. Low bone mineral density is associated with type 1 diabetes (T1DM), while in type 2 diabetes (T2DM) bone mineral density is comparable to or greater than in non-diabetic subjects. Nevertheless, latest cohort studies and meta-analyses corroborate that T1DM and T2DM have an increased risk of osteoporotic fracture as a chronic complication [6], with various factors seemingly affecting the likelihood of fractures [7].

Other studies also show that bone plays an essential part in regulating intermediary metabolism, acting as pathophysiological factor in the disease itself [8]. Because of its ability to secrete osteocalcin, bone may be considered an endocrine organ [9]. The connection between osteocalcin and metabolic factors, namely glycemia, β-cell proliferation, insulin secretion, and lipid profile, unravelled the possible effect of the skeleton on energy control and glucose metabolism [9].

Similarly, infrasound, a physical stimulus defined by acoustic vibrations whose frequency is below the low frequency limit of normally audible sound, is ubiquitously present [10]. In fact, many studies suggest that infrasound pollution is on the rise worldwide, particularly in industrial and urban settings [11]. Osteoblasts and osteoclasts functioning are affected by vibrations, suppressing bone resorption, and encouraging bone formation [12].

Furthermore, bone remodelling is apparently promoted by this type of stimulation, which indicates that it has an impact on bone mineralization [13]. Additionally, it was demonstrated that infrasonic exposures may possibly stimulate osteoblast-like cells growth and secretion activity *in vitro* [14]. There also evidence that that infrasound may possibly promote osteogenesis and fracture healing *in vivo* [15]. It was also shown that infrasound and low frequency noise induce alterations in various animal tissues, such as cardiac atrium [16], salivary glands [17], periodontium [18] and liver [19].

So, little is known regarding the effects of both diabetes and infrasound on elemental bone composition, and even less regarding the effects of the potential interaction of both. For major human pathologies, such as diabetes, a full variety of well-described induced models is accessible, namely in rats. [20].

X-ray fluorescence spectroscopy (XRF) has been applied in determining the concentration of major and vestigial elements in biological tissues, namely humans and animals, and presents important methodological advantages for this type of studies compared to other techniques [4, 5, 8, 21]. The main methodological advantages of XRF spectroscopy compared to other techniques are the minimum preparation of the sample, eliminating the risk of contamination or loss of elements of interest, and the simultaneous determination of the concentration of several elements, providing a fast, accurate and sensitive elemental analysis. This technique has been applied in studies of histological alterations in metabolic disorders such as diabetes and osteoporosis, which have demonstrated its validity, when complemented with a correct data analysis, to distinguish between normal and altered tissues [1, 2, 22, 23]. Moreover, animal models have been shown to be an apt model for investigating the relationships between Ca and P concentration in bone [21].

The methodologies used in this study provide an important contribute to fill existing gaps in current knowledge of the role of specific chemical elements, such as for e.g. Ca and P, in such metabolic pathways. Thus, the aim of this study was to evaluate the elemental composition of calcium (Ca) and phosphorus (P) in the bone of rats subjected to glucose intolerance and/or infrasound.

2. Materials And Methods

2.1 Animal experiments

In agreement with the 3Rs principles [24], this investigation shares resources with a different research on the consequences of infrasound exposure on the function and morphology of the pancreas. Study design and sample size estimation were carried out as previously described, in view of the magnitude of expected effects on pancreatic function [19]. The animal experiments executed in this investigation were all approved by the Ethics Committee of the Instituto Universitário Egas Moniz, by the Portuguese National Authority for Animal Health (project nº 204/2017) and by the Animal Welfare Body (ORBEA) of Abel Salazar Biomedical Sciences Institute, University of Porto (Portugal), under the protocol nº 204/2017. The experimental design was performed in accordance with the PREPARE guidelines [25]. Every animal was handled by accredited researchers (FELASA Category C) and was held in a certified animal facility. All the care was done in agreement with the EU Commission on Animal Protection for Experimental and Scientific Purposes (2010/63/EU) and with the Portuguese legislation (DL 113/2013). The research was done in accordance with the ARRIVE guidelines [26].

2.1.1 Animals

For this study, wild-type Wistar rats (n = 133) were obtained from Charles River Laboratories (Saint-Germain-sur-l'Arbresle, France), aged 11 weeks and weighing 375.95g ± 18.29g. Solely male rats were chosen in order to avoid potential sex-induced differences in results. Every animal passed the Preyer reflex test, a simple method to estimate auditory function [27]. The subjects were accommodated in standard cages, with a 12h light/dark cycle and had unrestricted access to water and food (standard commercial rat chow). Only one to two specimens were kept in each cage. The housing conditions were maintained unchanged throughout the entire experiment. Succeeding one week of acclimatization period, the primary sample of 133 individuals was arbitrarily allocated into two groups adopting open access online software [28]: G1 (no treatment) and G2 (glucose intolerance).

2.1.2 Glucose Intolerance

A high-fat diet (D12492 diet, Research Diets Inc., USA) was fed to the animals for 3 weeks. Nutritionally, the diet comprised 60% fats, 20% carbohydrates and 20% protein (5.21 kcal/g energy density) while the standard rat chow (D10001 diet, Research Diets Inc., USA) comprised 12% fats, 67% carbohydrates and 21% protein (3.86 kcal/g energy density). Following the three-week high-fat diet, a low dosage of streptozotocin (STZ, Sigma-Aldrich, USA) 40 mg/kg was mixed in a sodium citrate buffer 50 mM, pH4.4, and was injected intraperitoneally succeeding a fasting of 6 to 8 h. The rats had unobstructed access to water at all times. This protocol was executed as described by Furman (2015) [29], which is considered to simulate the human disease [30].

Corroboration of glucose intolerance was achieved via an intraperitoneal glucose tolerance test (glycemia ≥ 140mg/dL at 2h), accordingly to Ayala et al. [31]. The specimens were rated glucose intolerant if they had a plasma glucose > 140 mg/dL after 2h of the assessment.

G1 and G2 specimens were provided regular rat ration and posteriorly distributed into two smaller groups: G1s (no treatment, silence), G1n (no treatment, infrasound), G2s (diabetes, silence) and G2n (diabetes, infrasound). All the rats were unsystematically separated into three noise exposure periods and sacrificed after 1, 6 and 12 weeks of exposure.

2.1.3 Infrasound Exposure

Infrasound exposure was implemented as formerly described by Lousinha et al. [32]. The enclosures were sited in a soundproofed room, measuring 217×211×195 cm, facing a noise generator consisting of a subwoofer which repeated an uninterrupted sound signal, earlier recorded, consisting of white noise, amplified and frequency filtered, generating an acoustic environment with high-intensity infrasound. The overall sound pressure level and the spectral characteristics of the consequential acoustic pressure waveform were examined, and the outcomes were at an average sound pressure level of 120 dB in the 2–20 Hz with a tolerance of ± 3 dB in a 30 second timeframe in the entire compartment. Regarding the spectral boundedness of the produced sound field the outcome was 80 dB total out-of-band average sound pressure level (-40 dB lower). The remaining groups, not exposed to infrasound, were held in a comparable room although in quietness.

2.1.4 Tissue and blood samples

After 1, 6 and 12 weeks of noise exposure, an intraperitoneal glucose tolerance test was performed, according to Ayala et al. [31]. Blood glucose measurements during the test were expressed as both a time course of glycemia measurements and as the area under the curve (AUC) for each animal. Blood samples were also collected before and 30 minutes after glucose administration to measure plasma insulin levels. The animals were afterwards euthanized through inhalation of carbon dioxide.

Following blood centrifugation, plasma insulin levels were measured using commercially available ELISA kits (Rat Insulin ELISA kit 10-1250-10, Mercodia), according to the manufacturer's instructions and guidelines. Insulin levels are expressed as micrograms per litre (µg/L).

Both hind limbs were manually removed from the already deceased animals. Samples of muscle, cartilage, periosteum and tibiae were then removed using a #22 surgical blade. The samples were lyophilised (in the ModulyoD-230 freeze dryer, Thermo Savant) for 48 hours at a temperature of -50°C, to reduce the water content in the tissues, contributing to the reduction of background radiation and optimising the determination of elements of interest. After lyophilisation, the samples were reduced to fine powder whose particles averaged 180 µm in size and kept cold. The mass of the samples ranged between 0.7149 and 1.1910 g.

2.2 X-ray fluorescence spectroscopy

Bone samples were prepared and analysed by Wavelength Dispersive X-ray Fluorescence Spectrometry (WDXRF), after system calibration and method validation. WDXRF is suitable for the direct assessment of a wide range of elements in the periodic table with the required sensitivity and detection limits.

The elemental concentrations of calcium (Ca) and phosphorus (P) of the tibiae of the Wistar rats were carried out with the WDXRF method, using a 4 kW commercial X-ray fluorescence spectrometer (S4 Pioneer, Bruker AXS) equipped with a rhodium (Rh) X-ray tube with a 75 mm beryllium (Be) terminal opening and a 34 mm diameter collimator mask.

Polyethylene cups measuring 35.8 mm in diameter assembled with a 4 µm Prolene® film were used to support the bone powder sample. The polyethylene cups were placed in steel sample cup holders with an opening diameter of 34 mm. The mass of the samples ranged between 0.7149 and 1.1910 g.

Calibration of the WDXRF system was accomplished by preparing a set of synthetic standards of 1 g each, in triplicate and doped with known amounts of Ca and P. Bone matrix effects were simulated by a mixture of calcium carbonate and disodium hydrogen phosphate. The WDXRF measurements were performed in helium mode, with measurement times fixed (30 s) based on measuring scans on multiple standard samples, to account for a maximum counting statistical error of 5% for a 3σ criterion.

The validation of the analytical method was performed according to ICH guidelines (ICH Q2 (2005) [33]), for specificity, linearity, detection and quantification limits, precision and accuracy. The estimated detection limits were 0.6 and 0.2% for Ca and P respectively. The intra-assay precision, which was determined at 3 concentration levels for each element as the coefficient of variation of 6 repeat measurements, was below 5% for both elements. Accuracy of the method was established by measuring certified reference material (Caprine Bone NYS RM 05 – 01/ 05 – 04).

2.3 Statistics

Since elemental concentrations and their ratio were determined in the same animals, the degree of intercorrelations between those variables may warrant a multivariate approach to the data. The need of such approach, however, was excluded in view of the value obtained for the Kaiser-Meyer-Olkin measure (0.257). Therefore, to assess the effects of exposure and metabolic condition on bone composition, univariate general linear models (GLM) were applied, after assessment of suitable covariates. Normality and homoscedasticity assumptions were checked using the Shapiro-Wilk and Levene tests, respectively. Statistical analysis was performed with the statistical software Statistical Package for Social Sciences (SPSS; IBM SPSS Statistics. Version 26.0, Armonk, NY: IBM Corp.). Correlations between variables were assessed using the Pearson correlation coefficient. Statistical significance was set at the 5% level.

3. Results

To account for the effects of concomitant variables in the relationships between exposure to infrasound and metabolic condition and bone composition, represented by the set of dependent variables Ca and P concentrations and their ratio in bone, suitable covariates were considered and their correlations with the dependent variables were assessed. These included the age of animals at sacrifice and duration of protocol, which were considered to model potential bone maturation effects, and body weight and the Area Under the Curve (AUC) derived from the Oral Glucose Tolerance Test (OGTT), at baseline and

sacrifice, to model potential effects of adipose tissue and impaired glucose metabolism on bone physiology.

Regarding insulin, complete records were not available for all animals due to the limited volume of plasma sample collected in some specimens, making it impossible to quantify insulin in these cases. The levels of plasma insulin, glucose AUC, plasma corticosterone and GLUT4 transporter in the skeletal muscle of these animals were previously published [34]. In the set of animals with complete record for all variables (n = 86), significant correlations were found only between AUC at baseline and Ca ($r = -0.320$; $p = 0.003$) and P ($r = -0.261$; $p = 0.015$) concentrations in bone. Therefore, AUC at baseline was considered as covariate in the GLMs used for those elements, after validation of the assumption of homogeneity of regression lines between subgroups, as discussed next.

For each of these three models (where Ca and P concentrations and Ca/P ratio are the dependent variables), the assumptions of normality and homogeneity of variances of the dependent variables were previously validated, using the Shapiro-Wilk and Levene tests, respectively, which prove that these assumptions are valid, except in the case of P concentrations in a subgroup, in what concerns normality. However, this violation does not prevent the use of GLM, since the P concentration data are symmetric and mesokurtic in that subgroup. Moreover, whenever the interaction between the covariate AUC at baseline and the main factors was included in the GLM approach to Ca and P data, such interaction was observed to be non-significant. Therefore, the assumptions for the use of GLM were considered validated.

Table 1 presents the descriptive statistics (mean and standard deviation) of the bone composition variables considered in this study, for the set of animals with complete records for the variables of interest (n = 86).

Table 1
Mean and SD of Ca, P and Ca/P in bone of animals with different status of glucose metabolism and infrasound exposure. Infrasound: 1 – exposed; 0 – non-exposed; GI: 1 – glucose intolerant; 0 – non-glucose intolerant.

		Ca (%)		**P (%)**		**Ca/P**		
Infrasound	GI	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	N
0	0	23.44	1.80	7.61	0.76	3.09	0.25	24
	1	22.87	1.51	7.02	0.83	3.29	0.29	24
1	0	23.90	1.51	7.63	0.76	3.15	0.28	22
	1	22.62	1.54	7.15	0.79	3.19	0.29	16

In the case of Ca concentrations in bone, the GLM approach has shown that no interaction exists between the main factors ($p = 0.272$), in addition to non-significant effects of exposure to infrasound ($p = 0.708$) and glucose intolerance ($p = 0.331$) on Ca concentrations. For P concentrations in bone, the same

approach has shown no interaction between main factors ($p = 0.765$), and no effect of exposure to infrasound on P concentrations ($p = 0.671$). However, glucose intolerant animals show significantly lower P concentrations in bone ($p = 0.040$) than non-glucose intolerant animals, regardless of exposure (Fig. 1.). Finally, no significant effects due to interaction between main factors or to main factors on Ca/P ratios in bone, although higher ratios in the glucose intolerant group were strongly suggested but not statistically supported ($p = 0.062$).

Since insulin is implicated in the metabolic pathways that control energy metabolism and bone mass [6], it seemed important to control for the potential effects of circulating insulin assessed at sacrifice, on the relationships between glucose intolerance and infrasound exposure and bone composition. This was considered for a smaller subgroup of 52 animals with complete records including insulin at sacrifice, which was the only biochemical parameter significantly associated with the bone variables, showing significant ($p = 0.003$) inverse correlation ($r = -0.402$) with P concentration and a significant ($p < 0.001$) positive correlation (0.468) with Ca/P. However, controlling for the effects of insulin does not change the significance of the effects of glucose intolerance and exposure on elemental bone concentrations and ratio observed in the larger subgroup and reported above.

4. Discussion

Our study assessed the elemental composition of calcium (Ca) and phosphorus (P) in the bone of rats subjected to glucose intolerance and/or infrasound. Results show that glucose intolerance decreases the P bone concentration and increases the Ca/P ratio. No differences were found in bone Ca, P or Ca/P ratio between rats exposed to infrasound and rats not exposed.

X-ray fluorescence analysis revealed to be crucial for our investigation. The main methodological advantages of XRF spectroscopy compared to other techniques are the minimum preparation of the sample, eliminating the risk of contamination or loss of elements of interest, and the simultaneous determination of the concentration of several elements, providing a fast, accurate and sensitive elemental analysis [21].

Phosphorus is an essential element, alongside with calcium, to the hydroxyapatite constitution which is deposited throughout the mineralisation of the vertebrate skeleton. Consequently, P is crucial for the mineralisation process [35], to preserve bone strength [36], and during fracture healing and restoration [37]. Phosphorus can promote the proliferation and differentiation of osteoblasts, as it induces the expression of genes essential for mineralisation in osteoblast-like cells, energy metabolism and cell proliferation [38].

Serum levels of phosphorus have been described to be greatly diminished in type 2 diabetic patients, possibly unveiling a phosphorus metabolism diabetic-induced disorder [39]. As such, the low bone phosphorus found in our study may indicate that the bone supplies phosphorus to the serum to try to compensate for the low phosphorus in diabetes mellitus patient’s serum. This corroborates the possible change in the phosphorus metabolism seen in type 2 diabetes.

Also, vitamin D is crucial in calcium and phosphorus homeostasis and bone mineralisation. There is an increase in evidence which associates it with insulin resistance by immune modulation. Moreover, its deficit will promote an increase in inflammatory cytokines [40]. It also may help in insulin secretion and its shortage is associated with an altered insulin secretion in the presence of glucose [41]. Furthermore, hyperglycaemia significantly reduces PTH levels [42]. Both appear to independently contribute to less bone remodeling, conveying less quality to the bone [43].

Calcium is the most abundant element in the body, playing a key part in cell membrane function, intracellular signaling and bone physiology [44]. Our investigation did not reveal significant differences in bone calcium concentrations amongst the studied groups.

Via several pathways at molecular and structural levels, longstanding exposure to a diabetic environment brings on changes in bone metabolism and weakened bone micro-architecture [45]. These modifications predispose the bone to an increased risk of fracture and compromised osseous healing [46]. In type 1 diabetes, low bone mineral density is commonly observed, and other studies indicate that higher bone fracture risk is associated with type 1 diabetes. Even without age-linked comorbidities, fracture peril remains considerably high in young and middle-aged adults with type 1 diabetes [47]. Due to the convoluted connection between bone disease and diabetes, the risk of bone frailty and fractures is affected by several factors. The low bone phosphorus, and consequential alteration in the Ca/P ratio, observed in our study may be a predisposing factor for bone fragility in diabetes. Despite comprehensive evidence of several cellular pathways in experimental models, the importance of each variable to the clinical outcome situation is still uncertain [7].

There has been a lot of debate and tumultuous discussion over the past 60 years about whether or not acoustic phenomena can induce extra-auditory effects on living organisms [48]. Long-lasting exposure to industrial noise is acknowledged to disturb biological systems. In industrial settings, the acoustic spectrum is especially rich in high-intensity infrasound (< 20 Hz), whose effects on the bone are unclear [49]. Infrasound has been linked to several health problems, namely cardiovascular diseases, sleep disorders, behavioural and cognitive problems [11]. Vibrations alter the function of osteoblasts and osteoclasts, reducing bone resorption and stimulating bone growth [12]. Additionally, this form of stimulation appears to stimulate bone remodelling, implying it has an effect on bone mineralization [13]. Furthermore, infrasonic exposures have been shown to increase osteoblast-like cell proliferation and secretory activity in vitro [14]. Interestingly, a 2013 [15] paper indicated that osteogenesis and fracture healing *in vivo* may be facilitated by infrasound, due to mechanical stimulation and increased activity of the bone neuro-osteogenic network. The evidence of potential benefits of infrasonic exposure to bone health provided by those studies seems to support the observation in the present work that glucose intolerance did not result in a decrease of the concentration of a major determinant of bone mass, such as Ca. In view of the spectrum of deleterious effects of diabetes on bone micro-architecture, the fact that bone Ca concentrations remained can speculatively be attributed to infrasound exposure, which somehow attenuated the bone effects of glucose intolerance. Therefore, the authors believe the infrasound exposure may have had a benefic effect in the bone maintenance.

In 2019, it was estimated that 9.3% of the global adult population was living with diabetes. This number is anticipated to increase every year [50]. Diabetes and its complications disturb the quality of life of patients negatively and impel a substantial economic burden on society. Changes in ion homeostasis are usual in diabetic patients, as seen in our study, and might be linked with augmented morbidity and mortality [51]. As such, there is need for further studies to clarify the effects of glucose intolerance on phosphorus and bone metabolism.

The authors believe that these findings warrant further animal and clinical studies, in order to better elucidate the role of diabetes and infrasound in bone metabolism.

5. Conclusion

Regarding bone Ca, there were no differences between animals exposed and unexposed to infrasound, as well as between diabetic and non-diabetic animals. As for the bone concentration of P, there were differences between diabetic and non-diabetic animals, with higher concentrations in non-diabetic animals, with no differences between animals exposed and unexposed to infrasound. For the Ca / P ratio, there are differences between diabetic and non-diabetic animals (this ratio being higher in diabetic animals) with no differences between exposed and unexposed animals.

As far as we could ascertain, it is the first time that the elemental concentration of calcium and phosphorus in bone has been described in diabetic animals and concomitantly subjected to infrasound.

The low bone phosphorus observed in our study may be a predisposing factor to the installation of the bone fragility described in diabetes. However, further investigation is needed to clarify the mechanism of action of phosphorus in diabetic bone metabolism.

Declarations

Acknowledgements

The authors wish to thank Egas Moniz – Cooperativa de Ensino Superior, CRL, for the financial support, and Instituto de Ciências Biomédicas Abel Salazar, for their assistance in the animal experiments.

Funding statement

No grants from funding agencies in the public, commercial or non-profit sectors were obtained for this study.

Competing interest statement

No conflict of interest is declared by the authors.

Author contributions statements

Luísa Zagalo, DDs, MSc – Elaboration of the project, execution of the laboratory experimentation and writing of the article

Gonçalo Pereira, DDs, MD, MSc – Execution of the laboratory experimentation and animal handling

Diogo Casal, MD, PhD – Text writing and proofreading

Luísa L. Gonçalves, PhD – Technical guidance for WDRXF calibration and text proofreading

Carlos Zagalo, MD, PhD – Sample preparation and handling and text review

Maria João Oliveira, PhD – Project guidance, supervision and text review

Pedro Oliveira, DDs, MD, PhD - Project guidance, supervision and text review

José A. A. Brito, PhD – Project guidance, supervision and statistical analysis

References

1. Darvish-Molla, S., Al-Ebraheem, A., & Farquharson, M. J. (2014). The Identification and Differentiation of Secondary Colorectal Cancer in Human Liver Tissue Using X-ray Fluorescence, Coherent Scatter Spectroscopy, and Multivariate Analysis. *AppliedSpectroscopy, 68*(1), 79–87. doi:10.1366/13-07047
2. Vidal, B., Cascão, R., Vale, A. C., Cavaleiro, I., Vaz, M. F., Brito, J. A. A., …Fonseca, J. E. (2015). Arthritis Induces Early Bone High Turnover, Structural Degradation and Mechanical Weakness.*PLOS One, 10*(1), e0117100. doi:10.1371/journal.pone.0117100
3. Brito, J. A. A., Costa, I. M., e Silva, A. M., Marques, J. M. S., Zagalo, C. M., Cavaleiro, I. I. B., … Gonçalves, L. L. (2014). Changes in bone Pb accumulation: Cause and effect of altered bone turnover.*Bone, 64*, 228–234. doi:10.1016/j.bone.2014.04.021
4. Silva, M. P., Tomal, A., Pérez, C. A., Ribeiro-Silva, A., &Poletti, M. E. (2009).Determination of Ca, Fe, Cu and Zn and their correlations in breast cancer and normal adjacent tissues. *X-Ray Spectrometry, 38*(2), 103–111. doi:10.1002/xrs.1126
5. Sohrabi, M., Gholami, A., Azar, M. H., Yaghoobi, M., Shahi, M. M., Shirmardi, S., … Ajdarkosh, H. (2017). Trace Element and Heavy Metal Levels in Colorectal Cancer: Comparison Between Cancerous and Non-cancerous Tissues. *Biological Trace Element Research, 183*(1), 1–8. doi:10.1007/s12011-017-1099-7
6. Fernandes, T. A. P., Gonçalves, L. M. L., & Brito, J. A. A. (2017).Relationships between Bone Turnover and Energy Metabolism. *Journal of Diabetes Research, 2017,*1–11.doi:10.1155/2017/9021314

7. Luisa Isidro, M., &Ruano, B. (2010).Bone Disease in Diabetes. *Current Diabetes Reviews, 6*(3), 144–155. doi:10.2174/157339910791162970
8. Geraki, K., Farquharson, M. J., & Bradley, D. A. (2003). X-ray fluorescence and energy dispersive x-ray diffraction for the quantification of elemental concentrations in breast tissue.*Physics in Medicine and Biology, 49*(1), 99–110. doi:10.1088/0031-9155/49/1/007
9. Zoch, M. L., Clemens, T. L., & Riddle, R. C. (2016). New insights into the biology of osteocalcin.*Bone, 82, 42–49.* doi:10.1016/j.bone.2015.05.046
10. Leventhall, G. (2007). What is infrasound?*Progress in Biophysics and Molecular Biology, 93*(1-3), 130–137. doi:10.1016/j.pbiomolbio.2006.07.006
11. Baliatsas, C., van Kamp, I., van Poll, R., &Yzermans, J. (2016).Health effects from low-frequency noise and infrasound in the general population: Is it time to listen? A systematic review ofobservational studies. *Science of The Total Environment, 557-558*, 163–169.doi:10.1016/j.scitotenv.2016.03.065
12. Yao, R., Nishii, K., Kito, T., Teranishi, T., Sugiyama, T., Sakai, K., …Yamada, K. (2019). A novel device to prevent osteoporosis by promoting bone metabolism using a newly developed double-loading stimulation with vibration and shaking. *Okajimas Folia Anatomica Japonica, 96*(1), 13–21. doi:10.2535/ofaj.96.13
13. Li, Y., Liu, G., Yu, J., Li, C., Tan, L., Hao, B., …Zhang, X. (2018). Effects of continuous or intermittent low-magnitude high-frequency vibration on fracture healing in sheep. *International Orthopaedics, 42*(4), 939–946. doi:10.1007/s00264-018-3759-4
14. Bing, W., Jing-Zao, C., & Zhen-Guo, G. (2006).Pressures on the Biological Features of Osteroblast-Like Cells in Vitro.*Journal of Low Frequency Noise, Vibration and Active Control, 25*(3), 215–219. doi:10.1260/026309206779800452
15. Long, H., Zheng, L., Gomes, F. C., Zhang, J., Mou, X., & Yuan, H. (2013). Study on osteogenesis promoted by low sound pressure level infrasound in vivo and some underlying mechanisms.*EnvironmentalToxicologyandPharmacology, 36*(2), 437–442.doi:10.1016/j.etap.2013.04.015
16. Lousinha, A., Pereira, G., Borrecho, G., Brito, J., de Carvalho, A. O., Freitas, D., …Antunes, E. (2020). Atrial fibrosis and decreased connexin 43 in rat hearts after exposure to high-intensity infrasound.*Experimental and Molecular Pathology, 104409.* doi:10.1016/j.yexmp.2020.104409
17. Oliveira, P., Pereira da Mata, A., Martins dos Santos, J., da Silva Marques, D., Branco, N., Silveira, J., & Correia da Fonseca, J. (2007).Low-frequency noise effects on the parotid gland of the Wistar rat.*Oral Diseases, 13*(5), 468–473.doi:10.1111/j.1601-0825.2006.01322.x
18. Cavacas, M. A., Tavares, V., Borrecho, G., Oliveira, M. J., Oliveira, P., Brito, J., … dos Santos, J. M. (2015).Industrial Noise and Tooth Wear - Experimental Study. *International Journal of Medical Sciences, 12*(3), 264–269.doi:10.7150/ijms.11309
19. Martins Pereira, G., Pereira, S. S., Santos, M., Brito, J., Freitas, D., Oliveira de Carvalho, A., …Oliveira, P. (2020). Effects of high-intensity infrasound on liver lipid content of rats. *Heliyon, 6*(7), e04383.

doi:10.1016/j.heliyon.2020.e04383

20. Hau J, Hoosier GLV, editors. Handbook of Laboratory Animal Science, 2nd ed., Vol. II.Boca Raton: CRC Press; 2003 [Ch.10].
21. Carvalho, M. L., Brito, J., & Barreiros, M. A. (1998).Study of trace element concentrations in human tissues by EDXRF spectrometry.*X-Ray Spectrometry, 27*(3), 198–204.doi:10.1002/(sici)1097-4539(199805/06)27:3<198::aid-xrs265>3.0.co;2-b
22. Santos, C.A., Fonseca J., Brito J., Fernandes T., Gonçalves L., Guerreiro A.S. (2014), "Serum Zn levels in dysphagic patients who underwent endoscopic gastrostomy for long term enteral nutrition", *Nutricion Hospitalaria, 29*: 359. doi:10.3305/nh.2014.29.2.7035
23. Brito J, Sena V, Zagalo C, Gonçalves L, Silva AM.(2018).Investigation of type 1 Diabetes Mellitus as risk factor for secondary Osteoporosis. *Translational research and Innovation in Human and Health Science, Annals of Medicine, 50*:sup1, S10-S170, doi: 10.1080/07853890.2018.1427445
24. Tannenbaum J, Bennett BT. (2015). Russel and Burch's 3Rs then and now: the need for clarity in definition and purpose. *J Am Assoc Lab Anim Sci; 54*:120-132. PMCID: PMC4382615
25. Smith, A. J., Clutton, R. E., Lilley, E., Hansen, K. E. A., & Brattelid, T. (2017). PREPARE: guidelines for planning animal research and testing. *Laboratory Animals, 52*(2), 135–141. doi:10.1177/0023677217724823
26. Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., … Würbel, H. (2020).*The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. British Journal of Pharmacology.* doi:10.1111/bph.15193
27. Jero, J., Coling, D. E. & Lalwani, A. K. Te use of Preyer's refex in evaluation of hearing in mice. Acta Otolaryngol. 121, 585–589 (2001).
28. Suresh, K. (2011). An overview of randomization techniques: An unbiased assessment of outcome in clinical research. *Journal of Human Reproductive Sciences, 4*(1), 8. doi:10.4103/0974-1208.82352
29. Furman, B. L. (2015). Streptozotocin-Induced Diabetic Models in Mice and Rats. *Current Protocols in Pharmacology, 5*.47.1–5.47.20. doi:10.1002/0471141755.ph0547s70
30. Kleinert, M., Clemmensen, C., Hofmann, S. M., Moore, M. C., Renner, S., Woods, S. C., … Tschöp, M. H. (2018). Animal models of obesity and diabetes mellitus. *Nature Reviews Endocrinology, 14*(3), 140–162. doi:10.1038/nrendo.2017.161
31. Ayala, J. E., Samuel, V. T., Morton, G. J., Obici, S., Croniger, C. M., … Shulman, G. I. (2010). Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice.*DiseaseModels&Mechanisms, 3*(9-10), 525–534. doi:10.1242/dmm.006239
32. Lousinha, A., R. Oliveira, M. J., Borrecho, G., Brito, J., Oliveira, P., Oliveira de Carvalho, A., …Antunes, E. (2018). Infrasound induces coronary perivascular fibrosis in rats. *Cardiovascular Pathology, 37*,39–44. doi:10.1016/j.carpath.2018.10.004
33. International conference on harmonization of technical requirements for registration of pharmaceuticals for human use (ICH) (2005) Validation of analytical procedures: Text and Methodology Q2 (R1,1-13)

34. Pereira GM, Santos M, Pereira SS, Borrecho G, Tortosa F, Brito J, Freitas D, de Carvalho AO, Águas A, Oliveira MJ, Oliveira P. (2021) High-intensity infrasound effects on glucose metabolism in rats.*Sci Rep.* 2021 Aug 26;11(1):17273. doi: 10.1038/s41598-021-96796-5.

35. Serna, J., & Bergwitz, C. (2020). Importance of Dietary Phosphorus for Bone Metabolism and Healthy Aging.*Nutrients, 12*(10), 3001.doi:10.3390/nu12103001

36. Follet, H., Boivin, G., Rumelhart, C., & Meunier, P. (2004).The degree of mineralization is a determinant of bone strength: a study on human calcanei.*Bone, 34*(5), 783–789.doi:10.1016/j.bone.2003.12.012

37. Oryan, A.; Monazzah, S.; Bigham-Sadegh, A. (2015). Bone injury and fracture healing biology. Biomed. *Environ. Sci, 28*, 57–7. doi:10.3967/bes2015.006

38. Camalier, C. E., Yi, M., Yu, L.-R., Hood, B. L., Conrads, K. A., Lee, Y. J., … Beck, G. R. (2013). An integrated understanding of the physiological response to elevated extracellular phosphate.*Journal of Cellular Physiology, 228*(7), 1536–1550.doi:10.1002/jcp.24312

39. Fang, L., & Li, X. (2016). Level of serum phosphorus and adult type 2 diabetes mellitus.*Journal of Central South University. Medical Sciences*,*41*(5). doi: doi:10.11817/j.issn.1672-7347.2016.05.009

40. Mayne, D., Stout, N. R., &Aspray, T. J. (2010). *Diabetes, falls and fractures.Age and Ageing, 39(5), 522–525.* doi:10.1093/ageing/afq081

41. Pittas, A. G., Lau, J., Hu, F. B., & Dawson-Hughes, B. (2007).*The Role of Vitamin D and Calcium in Type 2 Diabetes. A Systematic Review and Meta-Analysis. The Journal of Clinical Endocrinology & Metabolism, 92(6), 2017–2029.* doi:10.1210/jc.2007-0298

42. Polymeris, A. D., Doumouchtsis, K. K., Giagourta, I., & Karga, H. (2011). *Effect of an Oral Glucose Load on PTH, 250HD3, Calcium, and Phosphorus Homeostasis in Postmenopausal Women.Endocrine Research, 36(2), 45–52.* doi:10.3109/07435800.2010.496761

43. Dobnig, H., Piswanger-Sölkner, J. C., Roth, M., Obermayer-Pietsch, B., Tiran, A., Strele, A., … Fahrleitner-Pammer, A. (2006). *Type 2 Diabetes Mellitus in Nursing Home Patients: Effects on Bone Turnover, Bone Mass, and Fracture Risk. The Journal of Clinical Endocrinology & Metabolism, 91(9), 3355–3363.* doi:10.1210/jc.2006-0460

44. Sun M, Wu X, Yu Y, et al. Disorders of Calcium and Phosphorus Metabolism and the Proteomics/Metabolomics-Based Research. Front Cell Dev Biol. 2020;8:576110. Published 2020 Sep 10. doi:10.3389/fcell.2020.576110

45. Cipriani C, Colangelo L, Santori R, et al.The Interplay Between Bone and Glucose Metabolism. Front Endocrinol (Lausanne). 2020;11:122. Published 2020 Mar 24. doi:10.3389/fendo.2020.00122

46. Murray, C. E., & Coleman, C. M. (2019). Impact of Diabetes Mellitus on Bone Health.*International Journal of Molecular Sciences*,*20*(19), 4873. doi: 10.3390/ijms20194873

47. Thong, E. P., Herath, M., Weber, D. R., Ranasinha, S., Ebeling, P. R., Milat, F., &Teede, H. (2018). Fracture risk in young and middle-aged adults with type 1 diabetes mellitus: A systematic review and meta-analysis.*Clinical Endocrinology, 89*(3), 314–323.doi:10.1111/cen.13761

48. Alves-Pereira, M., & Castelo Branco, N. (2007).Vibroacoustic disease: Biological effects of infrasound and low-frequency noise explained by mechanotransduction cellular signalling.*Progress in*

Biophysicsand Molecular Biology, 93(1-3), 256–279. doi:10.1016/j.pbiomolbio.2006.07.011

49. Lousinha, A., R. Oliveira, M. J., Borrecho, G., Brito, J., Oliveira, P., Oliveira de Carvalho, A., …Antunes, E. (2018). Infrasound induces coronary perivascular fibrosis in rats. Cardiovascular Pathology, 37, 39–44. doi:10.1016/j.carpath.2018.10.004
50. Saeedi, P., Petersohn, I., Salpea, P., Malanda, B., Karuranga, S., Unwin, N., … Williams, R. (2019). Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: results from the International Diabetes Federation Diabetes Atlas, 9th edition. *Diabetes Research and Clinical Practice, 157*, 107843. doi:10.1016/j.diabres.2019.107843
51. Ahn, C., Kang, J.-H., &Jeung, E.-B. (2017). Calcium homeostasis in diabetes mellitus. *Journal of Veterinary Science, 18*(3), 261-266. doi: 10.4142/jvs.2017.18.3.261

Figures

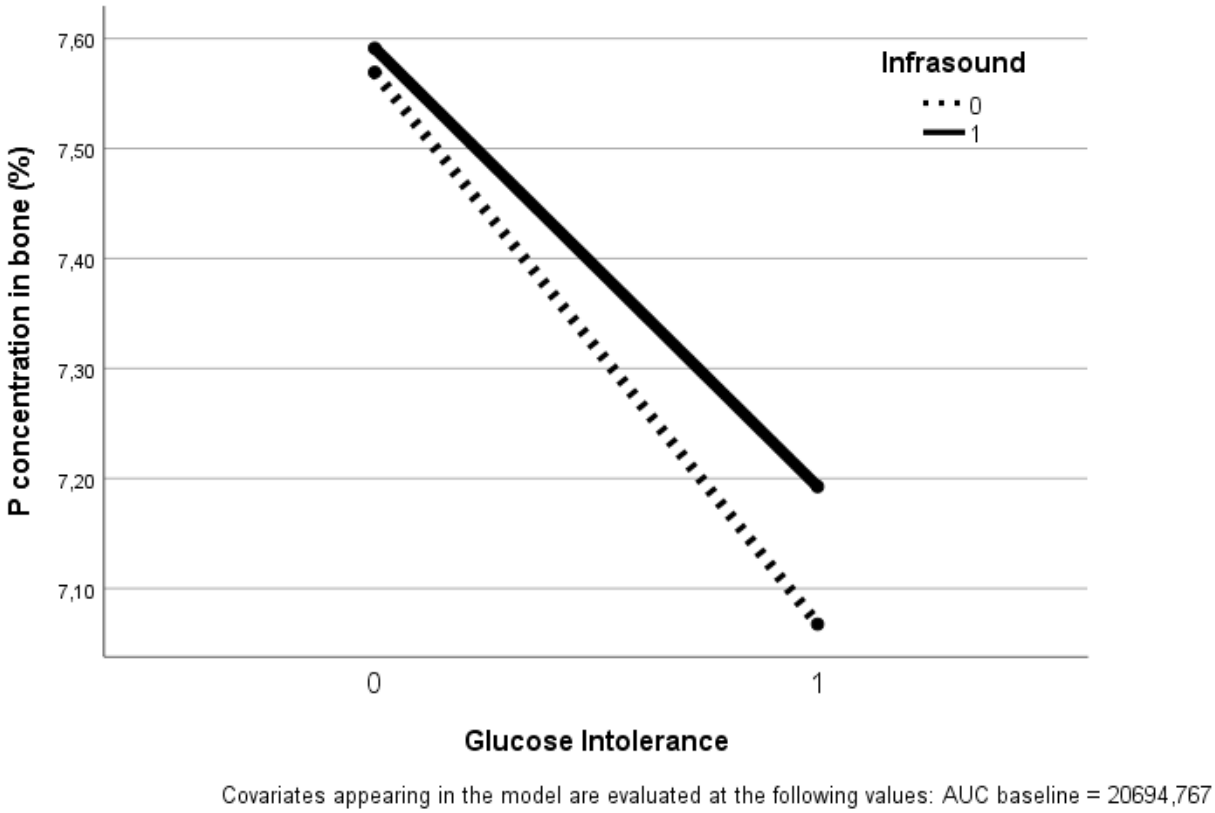

Figure 1

Profile plot of P concentration on bone of animals with different status of glucose metabolism and infrasound exposure, considered in GLM.