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## Monitoring of bone matrix acidification by TRAP and ERK biomarkers in the chronic hypercholesterolemia male rats

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### ABSTRACT

**Background:** Hypercholesterolemia is frequently linked to an elevated risk of cardiovascular diseases, including heart attacks and strokes. Additionally, it could be connected to a higher susceptibility to osteoporosis. Hypercholesterolemia can stimulate the differentiation and activity of osteoclasts, leading to enhanced bone reabsorption and a subsequent net loss of bone tissue.

**Aim:** The purpose of this study was to examine the influence of a high-cholesterol diet on osteoporosis in male rats with differences in biological and oxidative indicators in the hypercholesterolemia diet in male rats.

**Methods:** The samples in this study were twenty male rats, ranging between 1.5 and 2 months, were separated into two groups. In one group, 10 rats were fed a regular diet, while in another group, 10 rats were fed a high-cholesterol diet (2%) over the course of 8 weeks. Samples of blood were obtained at the last stage of the experiment. To calculate physiological and biological markers including extracellular signal-regulated kinase (ERK), tartrate-resistant acid phosphatase (TRAP), hormones, malondialdehyde (MDA), and glutathione (GSH).

**Results:** The results of this study demonstrated a decrease in GSH levels, an increase in ERKs, no significant change in serum TRAP levels, an increase in MDA levels in the blood, and elevated levels of parathyroid hormone, calcitonin, and vitamin D in the cholesterol group.

**Conclusion:** Increased oxidative stress, altered signaling, and disruptions in calcium/bone metabolism associated with cholesterol-related conditions and monitoring biomarker ERK can provide valuable information about disease progression.

**Keywords:** Hypercholesterolemia, Osteoporosis, ERK, TRAP.

### Introduction

Osteoporosis is a metabolic bone condition that affects many people around the globe. It is represented by a major reduction in the mineral and protein content of the bones. The prevalence of the disease increases with age, creating a substantial strain on healthcare systems and economies worldwide (Sobh *et al.*, 2022). Osteoporosis is a prevalent, long-term metabolic bone disorder characterized by a decrease in bone mass, degradation of bone structure, and heightened vulnerability to fracture (Wawrzyniak and Balawender, 2022). Elevated levels of cholesterol can contribute to a range of Physiological disorders like fat accumulation, Cardiovascular disorders, and Alzheimer's disease. leading to adverse impacts on the body (Jeong *et al.*, 2018; Raheem *et al.*, 2023). Additionally, high cholesterol levels can negatively affect the microstructure of bones, leading to conditions like osteopenia and osteoporosis, as well as reduced bone strength. These, in turn, increase the risk of fracture, particularly in animals that have low

calcium and vitamin D levels or engage in excessive dieting with cholesterol (Perticone *et al.*, 2019). Hypercholesterolemia is a complex disorder influenced by a combination of lifestyle choices and genetic predisposition. Furthermore, it serves as a contributing factor to cardiovascular diseases (CVDs), which result in approximately 172 million fatalities each year (Fang *et al.*, 2021). A correlation exists between a decrease in bone mineral density (BMD) and the consumption of a high-cholesterol diet (Qiao *et al.*, 2021).

Emerging studies have revealed that the consumption of a high-fat diet (HFD), which is rich in fat content. Contributes to both obesity and interferes with metabolic processes. and impacts bone health. Specifically, the effect of this is a decrease in bone density and reduced bone integrity. thereby elevating the potential for both spontaneous and traumatic bone fractures (Duran *et al.*, 2020). During this process, the generation of reactive oxygen species (ROS) can exacerbate inflammation and contribute to the progression of atherosclerosis (Nowak *et al.*, 2017).

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Increased lipid levels, particularly cholesterol, in the bloodstream, along with the production of the ROS, play a vital role in the advancement of coronary artery disease and atherosclerosis, in order to counteract the detrimental effects of various oxidizing agents, the organism bodies have developed an intricate defense mechanism involving antioxidant enzymes. This defensive system consists of three enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) works in tandem to protect the organism from oxidative damage (Demirci *et al.*, 2022).

In rats, hypercholesterolemia was found to be linked to a decrease in bone density, an increase in bone resorption, and a decline in bone formation. Moreover, *in vitro* investigations demonstrated that free cholesterol hindered the proliferation and differentiation of osteoclast, while simultaneously elevating levels of malondialdehyde (MDA) and reducing the activity of glutathione (GSH). Additionally, hypercholesterolemia resulted in increased osteoblast activity (Arai *et al.*, 2007; Jähn and Bonewald, 2012).

Three primary types of bone cells play crucial roles in maintaining bone homeostasis. The types of cells are osteoblasts, osteocytes, and osteoclasts. They are actively involved in bone formation and remodeling processes (Sims and Vrahnas, 2014). The cell types mentioned are derived from two separate stem cell lineages (Triffitt *et al.*, 2022). Osteoblasts and osteocytes are derived from the mesenchymal lineage, while the hematopoietic lineage is accountable for the generation of osteoclasts (Kim *et al.*, 2020). Osteoclasts are large, multinucleated cells derived from hematopoietic origins, and their primary function is the resorption of bone tissue (Wojda and Donahue, 2018). Maintaining a delicate equilibrium between osteoclastic bone resorption and osteoplastic bone formation is crucial for maintaining overall bone homeostasis. Direct connections exist between parathyroid hormone (PTH) and cholesterol metabolism, where vitamin D (regulated by PTH) affects cholesterol production. Nevertheless, they lack a direct relationship or influence over each other. PTH primarily regulates calcium and phosphate levels, while cholesterol has its own metabolic pathways and regulatory mechanisms (Song, 2017). PTH is a hormone secreted by the parathyroid glands; very small glands situated in the neck. PTH is essential for controlling calcium and phosphate levels in the body. It affects the structure of the skeleton. maintain appropriate levels of calcium and phosphate in the bloodstream. PTH helps increase calcium levels by promoting its release from bones (Wein and Kronenberg, 2018).

Calcitonin is a hormone secreted by the thyroid gland. The primary role is to control the amounts of calcium and phosphate in the body. When blood calcium levels are too high, calcitonin works to decrease them by inhibiting calcium release from the bones and increasing

calcium excretion by the kidneys. Calcitonin also plays a role in inhibiting the activity of osteoclasts, which are cells responsible for breaking down bone tissue (Sheweita and Khoshhal, 2007). Vitamin D plays a vital function in maintaining skeletal health by influencing the process of bone mineralization and the balance of calcium and phosphate levels (Xia, 2022). It also helps regulate the PTH (Warren *et al.*, 2021).

Vitamin D and cholesterol metabolism are closely linked through the same biosynthetic route. Cholesterol is a multifunctional lipid. It is an essential element of cell membranes. (Zidovetzki and Levitan., 2007). The study aimed to determine some physiological parameters and hormones in the hypercholesterolemic diet in male rats.

## Materials and Methods

### Experimental design

This study exploited an experiment of twenty Wister male rats. The rats were acquired from the Faculty of Pharmaceutical at the University of Kerbala in Iraq. Each rat had an average and standard deviation weight of  $100 \pm 7.5$  g, with a standard deviation of 75 g. The rats, aged between 4 and 6 weeks, were housed in sterile, specialized plastic enclosures. To initiate the experimental design, the rodents were placed in a clean compartment within a box. The study employed a 12-hour photoperiod and maintained a moisture content of the air at  $50\% \pm 5\%$ . The participants were kept for two weeks to adapt to the Typical testing conditions. The experiment commenced on September 25th. and ended on November 23rd. The experiment involved regulating the room temperature to a range of  $23^{\circ}\text{C}$ – $26^{\circ}\text{C}$ . Utilizing a room thermostat. The room's air was always replenished through continuous ventilation suction, and the animal was provided with nourishment through pre-prepared fresh feed pellets.

Twenty whitish male rats were allocated at random and administered the following treatments for eight weeks. The control group consisted of half of the rats in this group who were given a normal diet that was consumed orally. The remaining half of the rats were administered a high-cholesterol diet containing 2% cholesterol by weight for eight weeks (Cunha *et al.*, 2021).

### Collecting blood samples

After 8 weeks, the blood samples were taken from animals following the administration of ketamine and xylazine to ensure they were calm and still. The collected serum was subjected to centrifugation for 5 minutes at a speed of 4,000 revolutions per minute using a specialized gel tube, following the heart puncture method. Upon separation, the serum was directly transferred into 1.5 microcentrifuge tubes for preservation and stored in a freezer at a temperature of  $-30^{\circ}\text{C}$ . The concentrations of the extracellular signal-regulated kinase (ERK, tartrate-resistant acid phosphatase (TRAP)), GSH, and MDA were measured using ELISA kits (Fine Test, Wuhan, China) and a

special blood analysis company (SQLab, Seoul, Korea) assessed the levels of PTH, calcitonin and vitamin D in the blood.

#### Statistical analysis

GraphPad Prism 8.0 was used to conduct the statistical analysis. The standard of significance for the analysis was  $p < 0.05$ , and the data points were reported as mean-standard error.

#### Ethical approval

The study was conducted at the anatomical facility of the College of Veterinary Medicine at Kerbala University in Iraq, under the reference number (UOK.VET.PH.2023.076).

### Results

The study showed the concentration of antioxidants in rats dosed with cholesterol, it was found a significant increase ( $p < 0.05$ ) in the rate of MDA ( $0.5014 \pm 0.083$ ) concurrent with a significant decrease ( $p < 0.05$ ) in the rate of GSH ( $19.67 \pm 1.46$ ) compare with control groups ( $0.3500 \pm 0.046$ ) and ( $23.03 \pm 2.74$ ), respectively (Fig. 1).

On the other hand, the study found a significant increase in membrane proteins responsible for building bones in mice, as we found a significant increase ( $p < 0.05$ ) in (ERK) in the hypercholesteremic diet group ( $2.161 \pm 0.057$ ) ng/ml compared with control group ( $1.566 \pm 0.034$ ) ng/ml and this study show a no-significant ( $p \leq 0.05$ ) in (tartrate-resistant acid phosphatase (TRAP)) in the hypercholesteremic diet group ( $2.120 \pm 0.036$ ) ng/ml compared with control group ( $1.899 \pm 0.045$ ) ng/ml, respectively (Fig. 2). The results from this study of the present examination revealed significant variations in the concentration of

hormones, including PTH and Calcitonin and vitamin D levels in the rats' blood.

### Discussion

Hypercholesterolemia can cause the buildup of lipids, including cholesterol, in bone tissue, this lipid accumulation within the bone can disrupt the normal bone structure, leading to compromised bone strength. Consequently, the bones become more susceptible to fractures (AL-tememy, 2023). Both hypercholesterolemia and a diet high in cholesterol have detrimental impacts on bone health tissues (Anagnostis *et al.*, 2022). High-cholesterol diets also decrease bone marrow stromal cell proliferation and differentiation, which results in decreased osteoblastogenesis. Under these circumstances, the functionality and equilibrium of osteoblasts are disrupted, leading to an increase in osteoclast activity and quantity, ultimately resulting in a decrease in bone mass (Akhmetshina *et al.*, 2023).

Hypercholesterolemia can impair the function of endothelial cells lining the blood vessels. Healthy endothelial cells produce antioxidants and regulate vascular tone, but dysfunction of these cells can disrupt antioxidant production and promote oxidative stress (Queiroz *et al.*, 2024).

The current study shows a significant reduction in serum GSH in the treated group as compared with the control group as shown in Figure 1, also there is a significant increase in serum MDA in the HCD group as compared with the control group. ROS generation may rise because of elevated blood cholesterol levels, oxidation can occur with elevated cholesterol levels, especially with low-density lipoprotein cholesterol (LDL-C) (Murphy and Johnson, 2008). ROS can be generated as a result of oxidative stress caused by

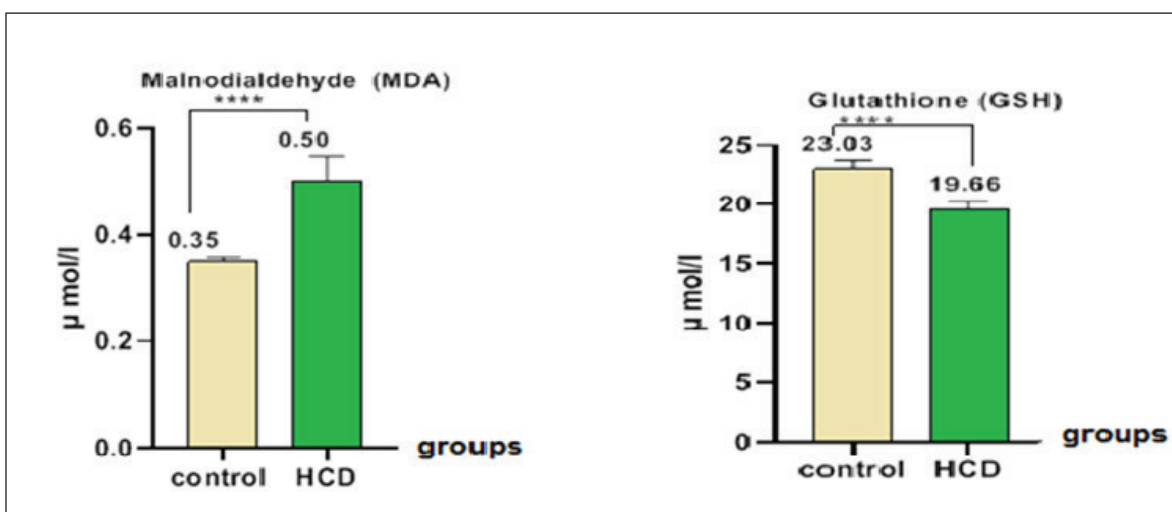
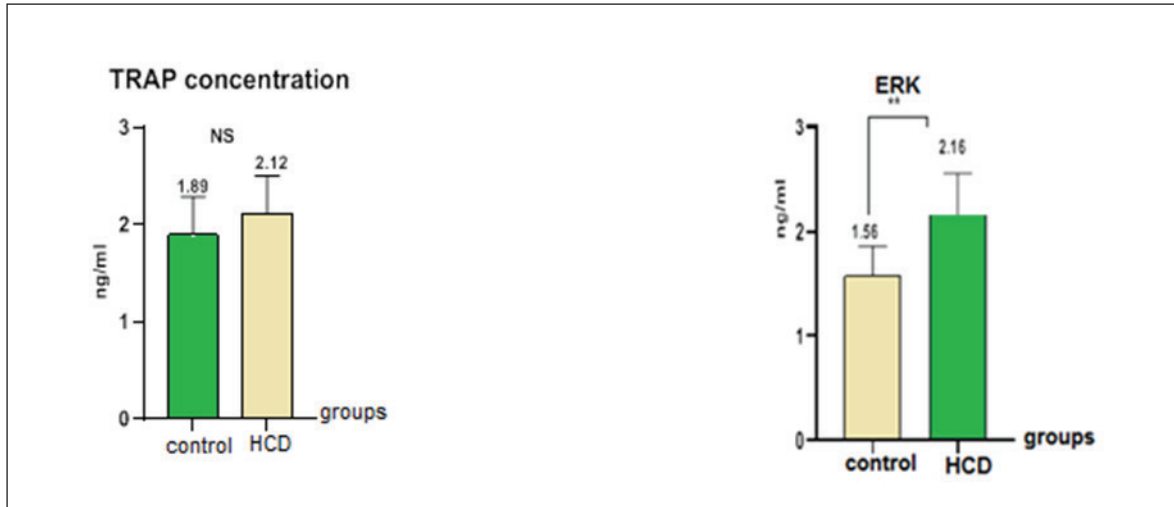
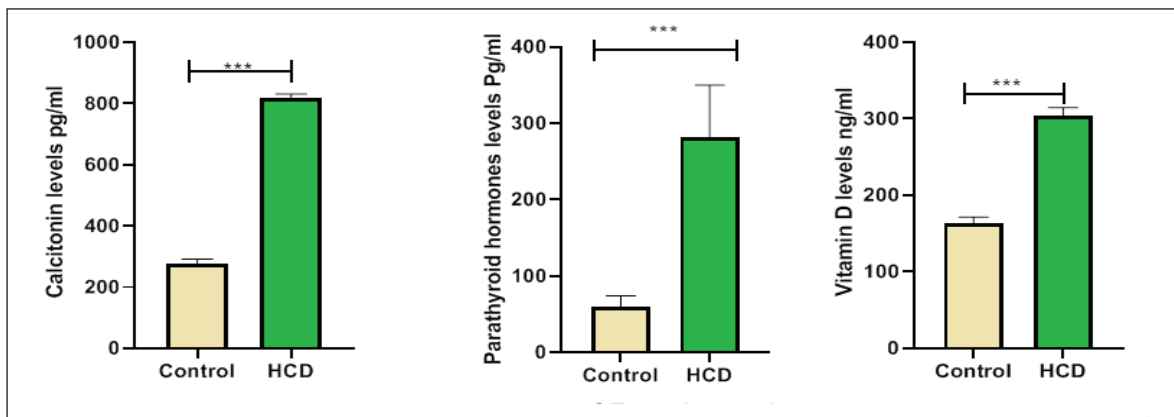


Fig. 1. Malnodialdehyde and GPx concentrations in male rats after hypercholesteremic diet, rats in control group were fed normal diets daily for eight weeks, while rats in hypercholesteremic-diet (0.2 mg/kg BW cholesterol) were fed normal diets daily for eight weeks.



**Fig. 2.** TRAP and ERK concentrations in male rats after hypercholesteremic diet; TRAP concentration in ng/ml; ERK concentration in ng/ml; rats in control group were fed normal diets daily for eight weeks, while rats in hypercholesteremic-diet (0.2 mg/kg BW cholesterol) were fed normal diets daily for eight weeks.



**Fig. 3.** PTH and calcitonine and vitamin D in male rats after hypercholesteremic diet, rats in control group were fed normal diets daily for eight weeks, while rats in hypercholesteremic-diet (0.2 mg/kg BW cholesterol) were fed normal diets daily for eight week.

oxidises low-density lipoproteins, known as ox-LDL (Safo and AlDulaimi, 2022).

The endothelial cells that line blood arteries may suffer harm from high cholesterol levels and this results in an increase of oxidative stress by inflammation (Fernández-Sánchez *et al.*, 2011). On the other hand, high cholesterol can affect how well mitochondria operate, and when they malfunction, the total amount of the ROS produced might go up which leads to a reduction in GSH levels and elevation in MDA levels (Xiao *et al.*, 2022). The enzyme NADPH oxidase is linked to elevated cholesterol due to its generation of ROS (Masoud *et al.*, 2014).

The current investigation revealed no significant disparity in serum TRAP levels between the control

group and the treated group, as depicted in Figure 2. This outcome may be attributed to rats having individual variability in their TRAP response to osteoporosis induction. Some rats may see a notable rise in serum TRAP levels, while others may not.

This variance may be attributed to hereditary causes, hormone changes, or other underlying variables that impact bone metabolism (Garnero, 2008). This study was in disagreement with some of the studies that have shown that TRAP levels and activity are elevated in individuals with osteoporosis compared to healthy controls. This indicates increased osteoclast activity and bone resorption, which are characteristic features of osteoporosis. TRAP levels have been correlated with BMD and can be used as an indicator of bone turnover



and disease severity (Chung *et al.*, 2005). Also, results demonstrated that the hypercholesterolemic diet groups exhibited elevated ERK activity compared to the control groups (Fig. 3). The ERK signaling pathway has been extensively investigated in the context of osteoporosis, and its activation or inhibition can potentially impact bone health, activation of the ERK signaling pathway has been linked to enhanced osteoblast (cells responsible for bone formation) activity and increased bone formation and conversely, inhibiting the ERK signaling pathway may lead to reduced osteoblast activity and a consequent loss of bone mass (Matsushita *et al.*, 2009). Hypercholesterolemia can lead to alterations in lipid raft composition affecting the localization and activation of signaling molecules including those involved in the ERK pathway. The arrangement of lipid rafts may enhance the clustering and activation of RTKs and downstream effectors, leading to sustained ERK activation (Degirmenci *et al.*, 2020). Also, High cholesterol levels can induce oxidative stress which can activate ERK signaling pathways. Oxidative stress is known to activate various intracellular signaling cascades including ERK in a variety of cell types. Therefore, oxidative stress induced by a hypercholesterolemic diet may contribute to increased ERK activation in bone cells (Rezatabar *et al.*, 2019).

In the current study, there was a significant increase in the (PTH, vitamin D, and calcitonin) in the cholesterol group as compared with the control group as shown in Figure 3. Calcium-sensing receptors (CaSRs) play a crucial role in regulating PTH secretion by sensing changes in extracellular calcium levels and the intracellular. Oxidative stress has been associated with changes in the function of the CaSR (Mroczko *et al.*, 2023). The CaSR is a G protein-coupled receptor that plays a crucial role in maintaining calcium homeostasis in the body by sensing changes in extracellular calcium levels. It is primarily expressed in parathyroid glands (Chavez-Abiega *et al.*, 2020). Oxidative stress can impact the expression levels of the CaSR. Complex intracellular pathways regulate CaSR signaling, and oxidative stress syndrome may impair the receptor's signal-transmitting capacity in response to changes in calcium levels (Kosiba *et al.*, 2020).

Prolonged exposure to oxidative stress may contribute to parathyroid hyperplasia, a condition characterized by the hyperactivity of the parathyroid glands, this activate could be a compensatory response to oxidative damage, aiming to maintain adequate PTH production. However, the hypertrophic changes may lead to dysregulation of PTH secretion, leading to the connection between oxidative stress and the endocrine function of the parathyroid glands. This can interrupt these signaling cascades, which can lead to an imbalance in the level of the (PTH, vitamin D, and calcitonin) in the cholesterol grouping as compared with the control group (Hong *et al.*, 2021).

## Conclusion

In conclusion, these results provide insights into the biochemical and hormonal alterations associated with cholesterol-related conditions; and concluded that increased oxidative stress, altered signaling, and disruptions in calcium/bone metabolism are associated with cholesterol-related conditions.

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## Conflict of interest

The authors declare that there is no conflict of interest.

## Authors' contributions

Duaa Raad Abd Al Ameer was data collection and curation of study; Wefak Albazi was formal analysis and interpretation of results and Hayder ALi muhammed writing the original draft and paragraph and designing the statistical figures.

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## Data availability

All data supporting the findings of this study are available within the manuscript and no additional data sources are required.

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