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Inhibition of the RANK RANK-L OPG/Cathepsin-K pathway on osteoclast activity by a treadmill in the osteoporosis induced by D-gal in the male rats

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Abstract

Osteoporosis is bone loss density and deterioration of bone tissues; osteoclasts are generally regarded as the only cells capable of resorbing to bone physical exercise by Treadmill, which has positive preventive and therapeutic effects on osteoclast activity. The experiment employed 24 male rats, randomly divided equally into three groups: the first group was the control group, and the second group of rats was given daily 200 mg/kg BW D-gal injected intraperitoneally for sixty days. The third group, the rats, were given 200mg/kg BW D galactose daily using a treadmill at 25 m/min for five days weekly for 60 days. Serum blood was drawn for biochemical analysis to evaluate the bone densitometry by measuring bone resorption and formation biomarkers represented by receptor activator of nuclear factor kappa-B (RANK), receptor activator of nuclear factor kappa-B ligand (RANK-L), cathepsin, and osteoprotegerin (OPG). Femur bone tissues were removed and processed for light and electron microscopy inspection for light and electron microscopy inspection. The results investigate a significant increase in serum RANK, RANK-L, and cathepsin-K levels in osteoporotic rats treated with D-gal. At the same time, OPG analysis revealed a substantial decline in these groups. The impact of the treadmill showed a significant decrease in serum levels, RANK, RANKL, and cathepsin K compared with the D-gal group. The femur rats' histological examination of the osteoporotic group showed resorption and marked trabecular thinning with numerous osteoclasts. In contrast, using an electron microscope, the D-gal treadmill group showed a newly added lamellar structure and several healthy osteocytes with fewer cavities in the bony plates and reduced micro-fissures in femur compact bone.

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Introduction

Deterioration of bone microarchitecture decreased bone mineral density, and an increased risk of fractures are the hallmarks of osteoporosis, a severe health issue (1-3). Maintaining a healthy bone mass requires careful balancing between osteoblastic bone production and osteoclastic bone resorption (4,5). Conditions affecting the bones, such as osteoporosis, can develop when this balance is upset (6,7). Three essential proteins, RANK, RANK-L, and OPG, work together to regulate the development and activation of osteoclasts (8). In addition, osteoclast-mediated bone resorption is aided by the cysteine protease cathepsin K, which is involved in the breakdown of proteins that make up the bone matrix (9-11). Cathepsins are endopeptidases found in most cells, which take part in cell autolysis and self-digestion of tissues (12). In humans, there are 11 members of cathepsins which are distinguished by their structures, catalytic mechanisms, and which proteins they cleave (13). Cathepsin K (Cat K) is predominantly secreted by activated osteoclasts to degrade collagen and other matrix proteins during bone resorption (14). Effects of cathepsin K

degradation: The proteolytic activity of cathepsin K on the bone matrix results in weakened bone structure and loss of bone density, contributing to the development of osteoporosis. This increases the risk of fractures and bone deterioration. The serum Cat K levels were significantly elevated in women with postmenopausal osteoporosis (15). Osteoporosis treatment strategies that focus on these mechanisms appear promising (16,17). In experimental models, D-galactose (D-Gal) has been widely used to induce osteoporosis because of its capacity to speed up the aging process and cause oxidative stress (18,19). Several organs can experience aging effects due to increased oxidative stress, apoptosis, and inflammation when an exogenous amount of D-galactose is administered beyond the average concentration (20,21). In long bones, increased porosity close to the periosteal surface produces a more significant loss of strength than increased porosity near the surface (22-24). As a result, the bone cortex becomes more porous with age, increasing surface area but weakening strength (25). Physical activity, especially on a treadmill, has emerged as an effective treatment option as a modulator of bone remodeling (26,27). Treadmill exercise accelerates the dynamic process of bone remodeling (28). Osteoclasts, which degrade old or damaged bone, and osteoblasts, which produce new bone, work together to remodel bone (29,30). In the case of working, the mechanical loading triggers osteoblasts' production of bone matrix (31). The total adaptive response of bone to physical activity includes this process, which is critical for the repair of microinjury within the bone (32,33). Treadmill exercise during D-Gal-induced osteoporosis in male rats has been shown to alter the RANK/RANK-L, OPG, and Cathepsin K pathways. Still, the molecular processes underlying these effects have yet to be addressed (34,35). By studying osteoclast activity in male rats with D-Gal-induced osteoporosis, this study hopes to shed light on the complex relationship between treadmill exercise and the aforementioned biochemical pathways (36). Our goal is to provide helpful information that might lead to the creation of osteoporosis-specific treatment strategies by investigating the regulation of the RANK/RANK-L, OPG, and Cathepsin K pathways (37). Treadmill activity is considered a possible moderator in this work, which uses a robust experimental design incorporating D-Gal induction to mimic accelerated aging and osteoporosis (38). Understanding the molecular mechanisms of treadmill exercise's effects on osteoclast activity will shed light on bone homeostasis and provide new avenues for the research and development of treatments to prevent and treat osteoporosis and improve bone health (39). Exercising on a treadmill raises your metabolic rate because it demands more energy from your muscles, which in turn causes your muscle mass to grow and your energy expenditure to rise (40). As an alternative to storing galactose, the body can try to convert it into energy. Enhanced oxidative stress is the result (41,42).

This study aims to investigate the effect of treadmill exercise on the inhibition of the RANK, RANK-L, OPG pathway and its impact on osteoclast activity in male rats with osteoporosis induced by D-galactose.

Materials and methods

Ethical approve

Under the reference number UOK.VET. PH.2023.075, the study was conducted at the Kerbala University, College of Veterinary Medicines' anatomical facility in Iraq.

Experimental design

Twenty-four male rats, each weighing between 190 and 210 g, were employed in this investigation. The rats were kept in clean, comfortable cages with free access to food and water (ad libitum), a 12-hour cycle of light and darkness, 50±5% relative humidity, and a constant temperature (18±2ºC). They were kept for two weeks to acclimate to the usual experimental setting. For sixty days, the three groups of rats were divided at random (8 rats per group). Control group rats received 0.2 ml of normal saline injected intraperitoneally. D-gal group rats in the D-gal group received 200 mg/kg B.W. of D-galactose. D-gal+ treadmill group rats in the D-gal+ treadmill group received the same injections plus the added benefit of running on a treadmill for 25 m/min, five days a week, for an hour, for sixty days. Twenty-four rats were randomly assigned to one of three groups for sixty days, with eight rats per group. For the first group of rats, which served as a control, 0.2 ml of normal saline was injected intraperitoneally. The rats in the D-gal group received 200 mg/kg B.W injected intraperitoneally of D-galactose (36), while the rats in the D-gal+ treadmill group received the same injections plus the added benefit of running on a treadmill for 25 m/min, five days a week, for an hour, for sixty days (43) (Figure 1).

Figure 1: The treadmill machine 25 m/min used in the experiment was designed by the researcher.

In designing a treadmill for rats, measuring and understanding the speed at which it operates accurately is crucial. This ensures that experiments or training sessions are conducted consistently and effectively. Here's a detailed breakdown of how we calculated the conversion from treadmill rotations per minute (RPM) to meters per second (m/s): Firstly, we started by determining the length of the treadmill. Measurement revealed that the size of the treadmill was 70 centimeters. This measurement provides a fundamental parameter for our subsequent calculations. Next, we examined the treadmill's RPM, recorded at 36 rotations per minute. This RPM value indicates how often the treadmill belt rotates within a minute under the specified conditions. To find out the total distance covered by the treadmill belt in one minute, we multiplied the length of the treadmill (70 cm) by the RPM (36 rotations per minute), resulting in a total distance of 2520 centimeters per minute. Since the standard unit for speed is meters per second (m/s), we needed to convert the total distance covered per minute from centimeters to meters. To do this, we divided the total distance (2520 cm/min) by 100 cm, yielding a distance of 25.20 meters per minute. Finally, to express the speed in meters per second, we converted the speed from meters per minute to meters per second. By dividing the speed (25.20 meters per minute) by 60 seconds (the number of seconds in a minute), we obtained a final speed of approximately 0.42 meters per second (43).

Collection of blood samples and histology preparation

After starving for the whole night, the animals were given ketamine and xylazine to help them calm down and self-regulate before blood samples were taken. The animal was placed on its back, and a direct cardiac puncture was used to introduce a sterile medical syringe containing five milliliters of blood into its heart. The serum was placed in a different gel tube without an anticoagulant and centrifuged for five minutes at 3000 r/min. Blood sera stored at -20ºC for biochemical analysis, including Serum RANK(ELK5786) RANKL(ELK2647), Cathepsin K (ELK1912) and osteoprotegerin (ELK2530) determined by special Elisa kit from ELK China. After the experiment's end, Femur bone specimens were taken, and the muscles surrounding the bone were removed. They were kept in buffered formalin at 10% and then washed with distilled water. After washing, the bones were put in formalin and nitric acid (1:3) overnight for the decalcification process, and then they were passed into the histokinate (44). Histological sections were made using light and a Scanning Electron Microscope (ESM).

Statistics analysis

The statistical Graph Pad Prism 8.0 was for one-way analysis of variance (ANOVA), with a significance level of P≤0.05 being selected as mean±SD.

Results

The increase in the RANK in the d-gal group was significant at 0.190±0.015 compared to the control group 0.159±0.0013, as shown in figure 2. However, there was a significant increase in the RANKL at 0.195±0.01 in the d-gal group compared to the control group 0.155±0.01, as shown in figure 3. Figure 4 of the current study demonstrated that the d-gal group's serum OPG concentration was significantly lower than the control group's 7.94±0.01, while figure 5 demonstrated that the d-gal group's Cathepsin K concentration was significantly higher than the control group's 163.07±15.09 (Figures 2-13).

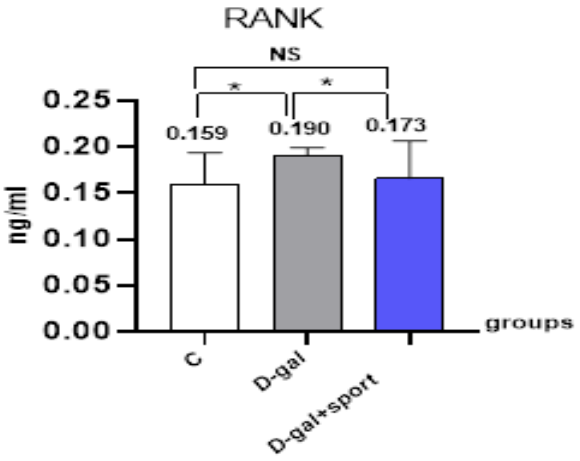

Figure 2: Effect of 200 mg/kg of D-gal and 25m/min for 1hr/5 days treadmill sport for 8 weeks in the serum RANK concentration on male rats. Values are expressed as mean±SE. n=5/ group.

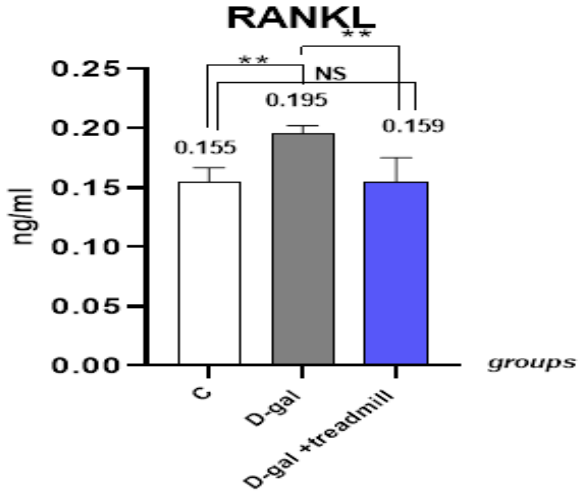

Figure 3: Effect of 200 mg/kg of D-gal and 25m/min for 1hr/5 days treadmill sport in the serum RANKL concentration on male rats. Values are expressed as mean±SE. n=5/ group.

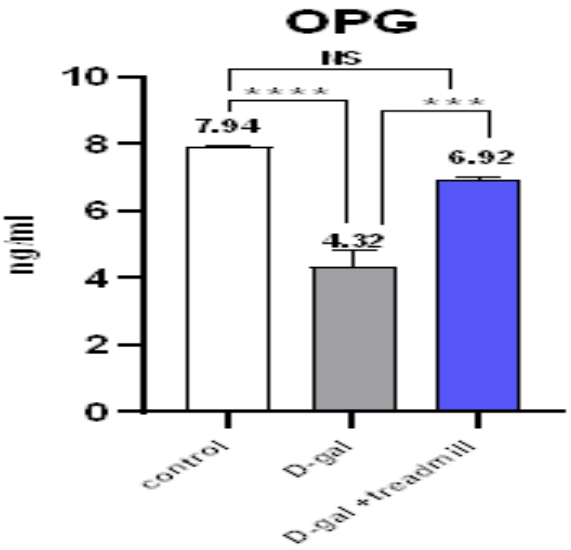

Figure 4: Effect of 200 mg/kg of D-gal daily for 8weeks and 25m/min days treadmill sport in the serum OPG concentration on the male rats. Values are expressed as mean±SE. n=5/ group.

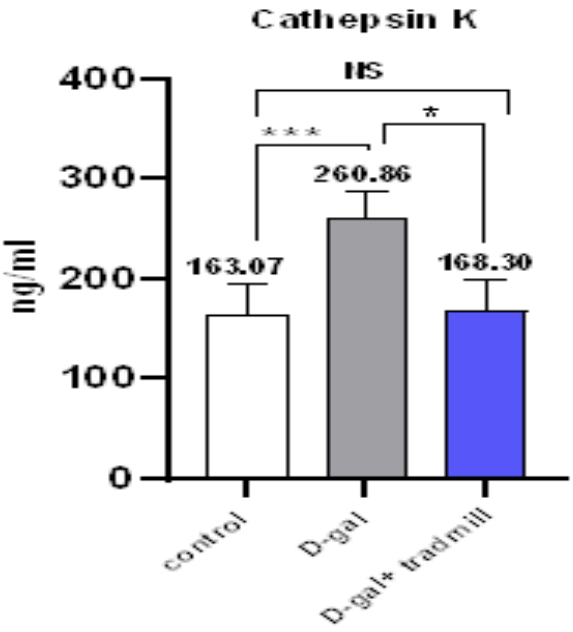

Figure 5: effect of 200 mg/kg of D-gal daily for 8weeks and 25m/min days treadmill sport in the serum Cathapsin concentration on the male rats.

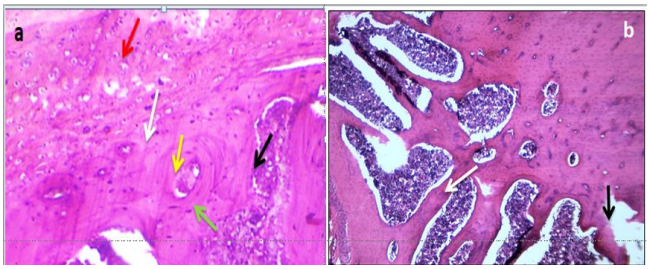

Figure 6: Photomicrograph of bone tissue section for a control animal, showing the normal histology of cortical bone, compact bone plate (white arrow), remarkable canals (yellow arrow) with significant osteocyte lei into their lacunae (red arrow), bone trabeculae (green arrow) surrounding cavities of bone marrow (black arrow). (b) Photomicrograph of bone tissue section for D-gal treated animal, revealing the significant histological alterations manifested by marked thinning of trabeculae (white arrow) with irregular eroded borders (black arrow) (H&E, 4X).

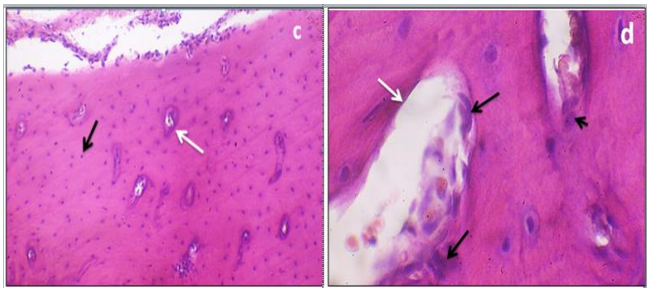

Figure 7: Photomicrograph of bone tissue section for D-gal treated animal, revealing the significant histological alterations manifested by severe resorption and perforation of compact bone (white arrow) with irregular atrophied osteocytes (black arrow), (2d) Photomicrograph of bone tissue section for D-gal treated animal, revealing remarkable histological alterations represented by marked cavities formations (white arrow) with numerous typical large multinucleated osteoclasts released from surface of bony trabeculae (black arrow).(H&E, 40X).

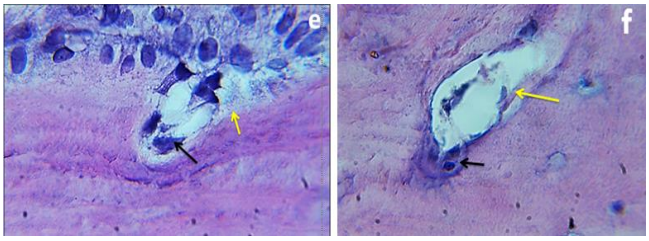

Figure 8: Photomicrograph of bone tissue section for D-gal treated animal, revealing remarkable histological alterations manifested by typical giant multinucleated osteoclasts in bone plate (black arrow) with irregular plates edges(yellow arrow) (3f) Photomicrograph of bone tissue section for D-gal

treated animal, revealing remarkable histological alterations manifested by marked lack of typical large multinucleated osteoclasts in bone plate (black arrow) with irregular plates edges(yellow arrow). (H&E, 100X).

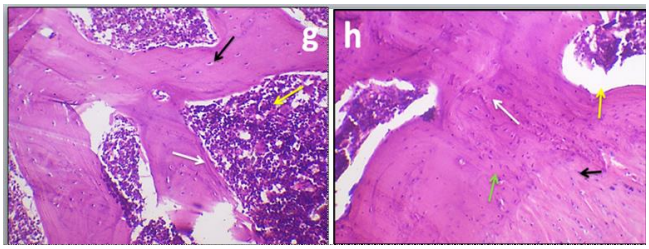

Figure 9: Photomicrograph of bone tissue section for D-gal treated animal with exercise, showing reversible histological changes characterized by marked thickness in bone trabeculae with newly added lamellar structure (white arrow), numerous healthy osteocytes in trabeculae (black arrow) with abundant cellularity red marrow (yellow arrow),(4 h) Photomicrograph of bone tissue section for D-gal treated animal with exercise, showing reversible histological changes characterized by marked thickness in bone trabecular with regular lamination (white arrow), moderate to severe increase on osteocytes (black arrow) with less cavities in bony plate (yellow arrow)and reduction in micro-fissures (green arrow). (H&E, 4X).

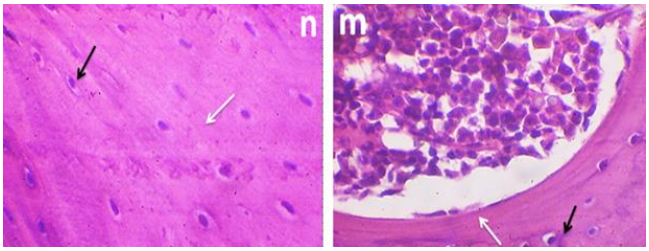

Figure 10: Photomicrograph of bone tissue section for D-gal treated animal with exercise, showing marked reversible histological changes, compact bone matrix (white arrow), and numerous well-differentiated osteocytes settled in their lacunae (black arrow). (5m) Photomicrograph of bone tissue section for D-gal treated animal with exercise, showing reversible histological changes, elongated osteoblasts at the border of the trabeculae (white arrow) with revered osteocytes normal histology (black arrow). (H&E, 40X).

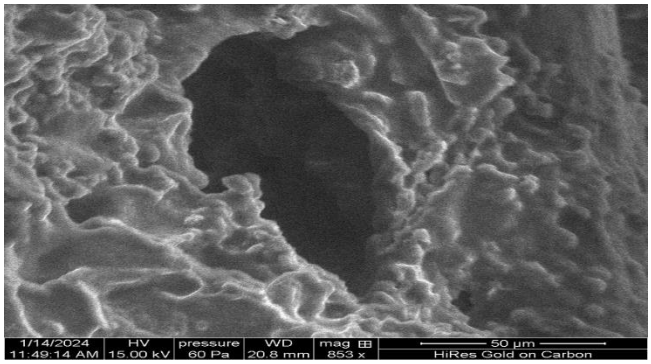

Figure 11: An Electron microscope photograph of the control group showed a reversal of the histological alterations manifested by the marked thickness of trabeculae and lamellar with emulate in the space of the Haversian canal (cavities in the bony plate) and improvement in the bone matrix.

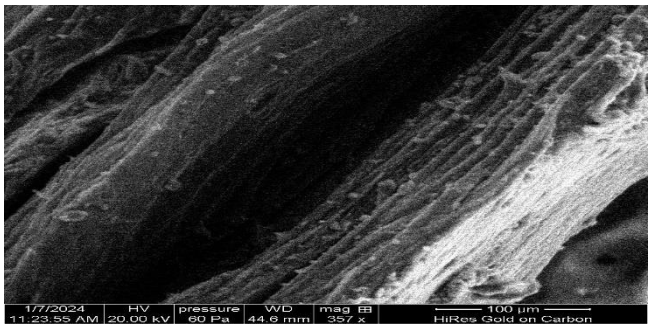

Figure 12: Electron microscope photograph of the D-gal group showed a significant histological alteration manifested by marked thinning and irregular trabeculae and lamellar with enlarged in the space of the Haversian canal (cavities in the bony plate) resorption in the bone matrix.

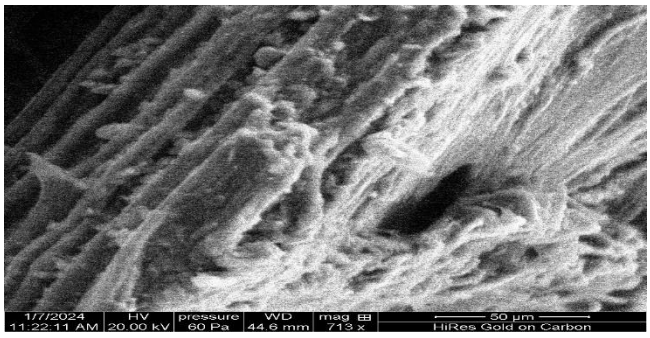

Figure 13: An Electron microscope photograph of the D-gal + treadmill group showed reversible histological alterations manifested by the marked thickness of trabeculae and bone lamellar in the space of the Haversian canal (cavities in the bony plate) in the bone matrix.

Discussion

Excessive D-galactose can be converted to hydrogen peroxide and aldose using galactose oxidase, culminating in free radicals' generation (FRs), eventually impairing many cellular functions (45). Decreased antioxidant enzyme activity, increased oxidative stress, cognitive dysfunction, mitochondrial dysfunction, accumulation of metabolites, and diminished immune response (46,47). The level of cathepsin K rises with high D-galactose consumption, which is thought to be a sign of worsened bone loss (48). Cellular stress-inducing intrinsic damage, such as DNA damage or increased reactive oxygen species (ROS), eventually converges at the mitochondria, where the cell's fate is decided. Under pathological conditions, not only is the expression and activity of Cat B increased, but lysosomal membrane permeabilization also leads to the release of Cat B into the cytoplasm, initiating various types of PCD (49). Cystatins, a superfamily of tight-binding inhibitors of papain-like cysteine peptidases, are widely applied to inhibit Cat B (50).

Activity Studies have found that consuming D-galactose exacerbates age- and obesity-related bone loss, leading to an elevation in the serum level of cathepsin K (51). Cathepsin K is responsible for the degradation of type I collagen in osteoclast-mediated bone resorption (52). Cathepsin K is a cysteine protease of the papain family, now considered the primary enzyme responsible for the degradation of the organic bone matrix. It is highly and selectively expressed in osteoclasts and, under acidic conditions, has the unique ability to degrade type I collagen helical regions (53).

The abundant expression of Cat K instead of the other cathepsins was previously identified in osteoclasts (54). Furthermore, a study has shown elevation in the serum RANK, RANK-L OPG in the D-gal group compared with other groups and an increase in the serum Cat K expression, which is regulated by the receptor activator of nuclear factor κB ligand (RANKL)-RANK signaling (55), the critical signaling pathway of osteoclast genesis. The activation of the RANKL-RANK signaling pathway in osteoclast precursors stimulates the pro-osteoclastogenic transcriptional factor NFATc1 (nuclear factor of activated T cells) to initiate the transcription of Cat K (56). In bone resorption, Cat K is secreted from mature osteoclasts into the sealing zone, a dynamic actin-rich cell-matrix adhesion structure that defines the resorption area of bone (57); it is known that Cat K can efficiently degrade type I collagen (52). D-galactose is a type of sugar. Studies have shown that excessive consumption of sugars, including D-galactose, may be detrimental to bone health. Dietary sugar levels may impact the ratio of proteins connected to bone, such as OPG (58). The specific mechanisms by which D-galactose affects OPG levels may involve complex interactions within the biological context and environmental conditions (59).

Administering D- D-galactose to rats may impact the hormone system, particularly those linked to bone. One protein that helps control bone activity is RANKL, and its balance can be affected by hormonal fluctuations (60,61), which may impact the equilibrium of RANKL and OPG by influencing the equilibrium of proteins linked to bone. Increased bone activity may result from bone resorption or other reactions if there is an increase in RANKL without a matching balance with OPG (62). It may also contribute to inflammatory responses, with RANKL potentially increasing levels in rats (63). Osteocytes could also express and secrete Cat K, which was required for the lactation-induced peri-lacunar resorption to guarantee adequate amounts of calcium in milk for the skeletal development in offspring (64). Mechanical loading could stimulate osteoblasts and osteocytes to express CatK, whereby it could modulate modeling-based cortical bone formation by degrading periostin (65).

Treadmill (66) found that the CatK-deficient osteoclasts would secrete more sphingosine-1-phosphate (S1P) to enhance osteoblastic bone formation. The excess mechanical stress loading could stimulate the CatK expression in human chondrocytes (67), inhibition of CatK by mechanical stress for eight weeks and 25m/min days treadmill sport could suppress the cartilage degradation as well as the systemic and local bone loss (68-71). Since suppressing CatK activity could prevent bone resorption without perturbing bone formation (14), it has become an attractive target for anti-resorptive drug development.

In most studies, exercise and physical activities promote bone health by increasing OPG and decreasing RANKL levels. However, several investigations have reported no change in OPG and RANKL levels after exercise. The current study showed a significant decrease in the serum OPG, which acts as a negative regulator of osteoclast genesis. It helps maintain bone homeostasis by preventing excessive bone resorption. The balance between OPG and RANKL is crucial for maintaining bone density and preventing loss. When RANKL binds to its receptor on the surface of osteoclast precursors, RANK promotes osteoclast formation and activation, leading to increased bone resorption. However, OPG competitively binds to RANKL, preventing its interaction with RANK by binding to RANKL, and OPG inhibits the formation and activation of osteoclasts.

Through the histological results of the bone examined under the microscope, there are significant differences between the control group and the D-galactose group, as the galactose group has activated osteoclast cells, which cause bone necrosis and failure to perform their functions properly, which leads to weakness in the bone tissue. In addition, due to the loss of calcium from the bones, this may have happened because D-galactose leads to bone cell (osteocyte) apoptosis and death in bone tissue, which leads to activity in

the osteoclast, While the control group did not change and was similar to normal bone tissue (72,73).

The combination group showed significant differences compared to the D-gal group, as the treadmill group showed an improvement in bone formation and a decrease in bone osteoclast compared to the D-gal group. Also, the efforts of osteoblasts were in higher quantity in the D-gal group compared to the treadmill group. This result occurs due to the consumption of D-gal before it leads to damage due to the oxidative stress effect it can cause, which leads to preserving the body from the oxidative effect. In addition, exercise has been found to help regulate growth hormones, which work to build bones, produce bone osteocyte cells, and add calcium to the bone (71). The image demonstrates histological changes in the bone due to D-gal treatment, including reduced trabecular density and bone structure erosion. These changes may indicate an increased risk of osteoporosis and bone structural weakness in D-gal-treated animals. The histological alterations observed in the bone tissue sections of D-gal-treated animals suggest significant changes in bone remodeling dynamics. The presence or absence of large multinucleated osteoclasts and irregularities in the bone plate edges indicate aberrant bone turnover and remodeling processes, which could contribute to bone pathology and structural changes associated with D-gal treatment (74).

On the other hand, the examination by electron microscopy showed that the D-gal group showed Significant histological alterations manifested by marked thinning and irregularity of the trabeculae and lamellar with enlarged space of the Haversian canal. This result may be a result of the increased effectiveness of the osteoclast in the bone and thus causes a delay in the area, which leads to its appearance in an irregular shape with the presence of holes, While the group that underwent treadmill did not show the same results as the D-gal group, as its results were closer to the control group, and thus reflects the importance of exercise in preserving the bone and preserving the structural composition of the bone and its safety from pathological injuries (75,76).

The effect of exercise on increasing bone mass is known to be caused by accelerated osteogenesis3 and inhibition of bone resorption, but its specific mechanism is still unclear. Recent studies have shown a lack of evidence supporting the inhibition of bone resorption upon exercising (77). The binding of RANKL and its receptor, RANK, triggers the activation of signaling related to the formation and differentiation of osteoclasts and regulates bone resorption. Additionally, osteoprotegerin (OPG) acts as a decoy receptor that binds to RANKL, thereby blocking osteoclast differentiation and activation (77). One study showed the serum concentrations of RANKL and OPG and the mRNA expression of RANK, RANKL, and OPG were not significantly changed upon acute treadmill exercise training, reaching 60 or 80% VO- 2max in healthy college female subjects18 (78). The binding of RANKL, which is secreted in osteoblasts, to its receptor RANK, which is secreted in osteoclasts, promotes osteoclast differentiation into cells with multiple nuclei, and the expression of specific cytokines and hormones increases in osteocytes. The effect of sufficient exercise to improve cardiorespiratory fitness, the impact on RANKL/RANK/OPG signaling, and bone-resorptive cytokines will be limited (79).

Conclusion

The present study demonstrates that treadmill exercise stimulates bone formation and suppresses bone resorption; exercise increases cancellous bone mass through increased bone formation, decreased bone sorption, and increased cortical bone mass due to increased periosteal bone formation for facilitating this study.

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Conflict of interests

The authors have not received any funding or benefits from industry, financing agencies, or elsewhere to conduct this study.

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تأثير جهاز المشي على تثبيط مسار رانكل والرانكل لام والكثابسين ك ونشاط الخلايا العظمية في تصلب العظام الناجم عن الكالكتوز-د في الجرذان الذكور

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الخلاصة

هشاشة العظام هي فقدان كثافة العظام وتدهور أنسجة العظام، وتعتبر الخلايا العظمية الهادمة بشكل عام هي الخلايا الوحيدة القادرة على إعادة امتصاص العظام .ممارسة الرياضة البدنية بواسطة جهاز المشي له تأثيرات وقائية وعلاجية إيجابية على نشاط ناقضات العظم. استخدمت التجربة ٢٤ فأرا ذكرا، تم تقسيمهم عشوائيا بالتساوي على ثلاث مجموعات، المجموعة الأولى كمجموعة سيطرة وتم إعطاء الجرذان المصابة بهشاشة العظام ٢٠٠ ملجم/كجم من وزن الجسم يوميا لمدة ٨ أسابيع. المجموعة الثالثة هي الجرذان أعطيت ٢٠٠ ملجم/كجم من وزن الجسم يوميا باستخدام جهاز المشي ٢٥ متر/الساعة يوميا لمدة ٥ أيام أسبوعيا جهاز المشي) لمدة ٨ أسابيع .تم سحب دم المصل للتحليل الكيميائي الحيوي لكل من رانكل والرانكل لام والكثابسين ك وتمت إزالة أنسجة عظم الفخذ ومعالجتها للفحص المجهري الضوئي والإلكتروني. أظهرت النتائج وجود زيادة معنوية في تركيز كل من رانكل والرانكل لام والكثابسين ك في الجرذان المصابة بهشاشة العظام مقارنة مع المجموعات الأخرى، وأظهر تحليل هشاشة العظام انخفاضا معنويا في مجموعة الجرذان المصابة بهشاشة العظام مقارنة مع المجموعات الأخرى .بينما أظهر تأثير جهاز المشي انخفاض معنوي في مستوى مصل رانكل والرانكل لام والكثابسين ك مقارنة مع مجموعة السيطرة . أظهر الفحص النسيجي لفئران الفخذ للمجموعة المصابة بهشاشة العظام ارتشافا شديدا وترققا ملحوظا في التربيق مع العديد من الخلايا العظمية بينما أظهرت المجموعة الرابعة بنية صفائحية مضافة حديثا وعدد الخلايا العظمية السليمة مع تجاويف أقل في الصفائح العظمية وانخفاض في الخلايا العظمية الدقيقة.