საქართველოს მეცნიერებათა ეროვნული აკადემიის მოამბე, ტ. 15, №2, 2021
BULLETIN OF THE GEORGIAN NATIONAL ACADEMY OF SCIENCES, vol. 15, no. 2, 2021

Medical Science

Influence of Calcium and Vitamin D3 on the Mineral Metabolism in Rats. Experimental Study

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(Presented by Academy Member Nodar Mitagvaria)

Calcium is not only a necessary chemical element but also a regulator, accelerating the inevitable processes in a body. It supports homeostasis and metabolism in different tissues. Calcium deficiency triggers a range of bone tissue diseases. Nowadays, we use the medications with the different content of calcium and vitamin D3 to prevent and treat musculoskeletal diseases. There is no common idea on the sufficient amount of daily intake of calcium. The experiment on 50 rats revealed the influence of the extra calcium and vitamin D3 on the mineral metabolism of an organism. It was found that additional 60 mg calcium within 30 days reduces its level in blood serum by 35%, followed by the blood serum decrease in the concentration of active metabolites of the vitamin D3: D_3-1.25$(OH)_2D_3$, and 24,25$(OH)_2D_3$ by 50% against the background of 25OHD_3 level. Extra 10 mg calcium and 20 IU of vitamin D3 promoted achieving an optimal calcium concentration in the blood serum. For correction of structural and functional conditions of bone tissue, pharmaceutical preparations should be preferred with composition ensuring efficient absorption and digestion of calcium. The intake of large doses of calcium causes significant disorders in mineral metabolism and metabolism of vitamin D. The results confirm the absence of a single-focused correlative dependence between increased calcium intake with food and mineral metabolism of a body. *© 2021 Bull. Georg. Natl. Acad. Sci.*

Bone metabolism, calcium, vitamin D3, rats, experiment

Calcium is not only a necessary chemical element but also a regulator, accelerating the inevitable processes in a body. It supports homeostasis and metabolism in different tissues. Calcium deficiency triggers a range of bone tissue diseases. Insufficient admission of calcium with food leads to disorders of growth and formation of peak bone mass in children, the occurrence of osteoporosis in adults [1-4]. Calcium deficiency may be both exogenous and of endogenous origin. The level of calcium nutrient availability decreases with ageing in 40% of the population due to intestinal diseases. Metabolites of vitamin D have significant influence on the availability of calcium. Proliferation and differentiation of bone cells, synthesis of the specific proteins, enzymes, their activity, mineral

metabolism are regulated by the vitamin D3 forming in a living body hydroxylase enzymes under the influence of vitamin D3. Among the regulators of the action of vitamin D3 25-hydroxylase enzymes, the most important is active vitamin D3 metabolite – 1.25-Dihydroxycholecalciferol ($1.25(OH)D_3$) and ions Ca^{2+} [5]. The increase of intracellular concentration of Ca^{2+} inhibits these enzymes' action. Such increase of hormonally induced Ca^{2+} in cytosol originates either from higher cell input or output due to the reduced activity of a specific adenosine triphosphatase or Ca^{2+} mobilization from the intracellular pools [6,7].

Nowadays, to prevent and treat musculoskeletal diseases, we use the medications with different content of calcium and vitamin D3. Still, there is no common idea on the sufficient amount of daily intake of calcium. It made us study the impact of different doses thereof on the mineral metabolism through an experiment.

Materials and Methods

The study was performed on Wistar rats weighing 130-150 g. The animals were taken from the vivarium of the Institute of Biochemistry, National Academy of Sciences of Ukraine. The rats were housed in a controlled environment with a 12 h light/dark cycle, constant temperature (22°C) and humidity (70%), and free individual access to food and water. It revealed biochemical values of mineral metabolism and histomorphological structural and functional conditions of growth plates and bones of the rats. Test animals were divided into four groups (10 per each) plus a control group. The rats in 1-3 groups obtained orally 10, 20, 60 mg Ca in the form of calcium oxide within 30 days. In addition to 10 mg calcium, the rats in the 4th group received an extra 20 IU of vitamin D3. The study of blood serum of the test animals in dynamics covered the levels of calcium, ALP, and vitamin D3 active metabolites.

For study we took femoral bones from five animals in each group. Preparation of the samples included their cutting into pieces, fixation in 10% formalin solution, degreasing and dehydration in spirits and acetones fot increasing strength, placing into celloidin blocks, processing into histologic sections, dying with hematoxylin and eozyne, in picro-fuchsin, and exposure to histomorphologic examination.

For statistical processing of the results, we used generally accepted methods involving criteria of difference between two totals, disperse and correlation analysis.

All animal procedures in the laboratory were performed in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes [8], Basel Declaration Society. Basel declaration: A call for more trust, transparency and communication on animal research [9].

Results and Discussion

The research has demonstrated the absence of single-focused correlative dependence between the increase in Ca consumption and its level in the blood serum (Table 1). Thus, the animals from the 4th group demonstrated its increased content in the blood serum after intake of 10 mg calcium. The animals in the 2nd and 3rd groups had reduced level of calcium in their blood serum after insertion of 20 and 60 mg of calcium, respectively.

In the animals of the 1st group, after one month of observation the level of calcium in their blood serum remained almost unchanged, within the limits of standard values. At that, the content of phosphorus increased slightly, and the activity of alkaline phosphatase dropped. The 2nd group of the animals, obtaining 20 mg of calcium, compared to the values at the beginning of the experiment had reduced activity of alkaline phosphatase, increased level of phosphorus, but calcium level in their blood serum dropped reliably by 11%. The

significant influence of extra calcium appeared in the rats of the 3rd group. Upon insertion of 60 mg Ca^{2+}, its level in the blood serum dropped by 35.7 %. At that, the activity of alkaline phosphatase in the blood serum increased by 12.7%. The levels of 1.25- and 24,25-dioxymethabolites of vitamin D_3 rose by 55.5% and 52.1% respectively if compared to their initial levels (Table 2).

Table 1. Influence of the extra calcium on its content in the blood serum of the experimental animals, M±m

Groups of the animals	Number of the animals	The content of calcium in the blood serum of the animals at the begi-nning of the test, mMol/l	The content of calcium after 30 days of the experiment, mMol/l
I (10 mg Ca)	10	2.60±0.04	2.7±0.02 p> 0.05
II (20 mg Ca)	10	2.71±0.06	2.4±0.07 p> 0.05
III (60 mg Ca)	10	2.46±0.03	1.58±0.04 p< 0.01
IV (10 mg Ca + 20 IU vitamin D_3)	10	2.40±0.02	2.64±0.03 p> 0.05
Control group	10	2.56±0.07	2.52±0.04 p> 0.05

Table 2. Influence of extra 60 mg of calcium on some biochemical values of the rats, M±m, n=10

Studied values (blood serum)	Before the experiment	30 days later
Calcium, mmol/l	2.46±0.03	1.58±0.04 p< 0.01
Alkaline phosphatase, u/l	228±7	257±5 p> 0.05
$25OHD_3$, ng/ml	8.1±0.8	7.8±0.9 p> 0.05
$1,25(OH)_2D_3$, ng/ml	0.090±0.003	0.04±0.002 p< 0.01
$24,25(OH)_2D_3$, ng/ml	4.6±0.3	2.2±0.1 p< 0.01

The level of calcium in the second group of rats rose by 10% compared to the initial values. At that, the levels of phosphorus and alkaline phosphatase grew insignificantly.

Histomorphologic study revealed that the rats in the control group, obtaining their standard food, had femoral bones growth zones of standard structural and functional conditions (Fig.1). Specific features of their femoral growth zones histomorphological structure included: an irregular decrease in height, fewer proliferation areas and epiphyseal cartilage columns on bigger spaces, almost no signs of endochondral ossification process, a layer of initial spongiosis. The central and penetrating vessel canals in the middle third of a femoral diaphysis are equally-spaced, but their quantity rose gradually towards the articulated ends of the bone.

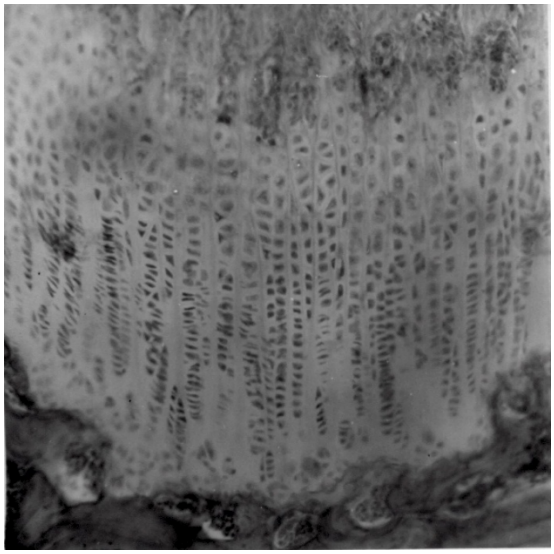

Fig. 1. Physiological structural and functional conditions of a distal femur. Hematoxylin-eosin. Amplification X 80.

The histomorphologic study of structural and functional conditions of the growth areas and bone tissues of the rats revealed the following. The rates from the first series have shown the activation of endochondral ossification as in proximal, as in distal growth area of a femur. However, endochondral osteogenesis was more active in the distal growth area, compared to the proximal. Their epiphyseal cartilage was of correct zonal structure: proliferation zones and the places of chondrocyte columns were distinct, especially in the distal femoral portion.

The histomorphologic structure in the 1st and the 2nd groups was almost identical and did not differ

reasonably from the control one. As for the 4th group of animals, structural and functional conditions of their epiphyseal cartilage and growth areas were very close to those observed in the animals from the 1st and 2nd groups. Noteworthy was the activity of chondrogenesis and endochondral ossification, especially in the growth area (Fig. 2). The structure of the compact bone layer in the middle third of the diaphysis was almost identical to the control and the first two groups of rats.

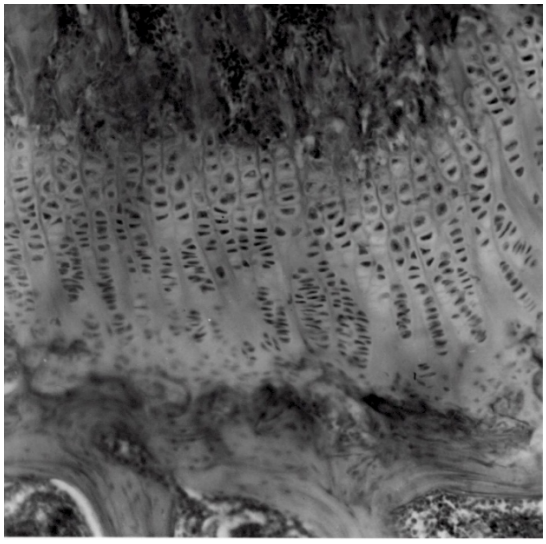

Fig. 2. The activation of the endochondral ossification in the distal growth area of a rat's femur. Hematoxylin-eosin. Amplification X 80. 4th group.

Surprising were the results in the 3rd group, in which the animals obtained an extra 60 mg of calcium within 30 days daily. Structure and function of their epiphyseal cartilage, chondrogenesis and endochondral ossification changed significantly (Fig.3).

The zonal structure of the epiphyseal cartilage was indefinable; the proliferation area was almost absent. The space of the cartilage cell columns disappeared, and the processes of endochondral ossification stopped. These changes were more vivid in a femur proximal growth area. The compact layer of the middle third of femoral diaphysis was specific by the increased number of vessel canals, while the endosteal surface thereof becomes significantly uneven.

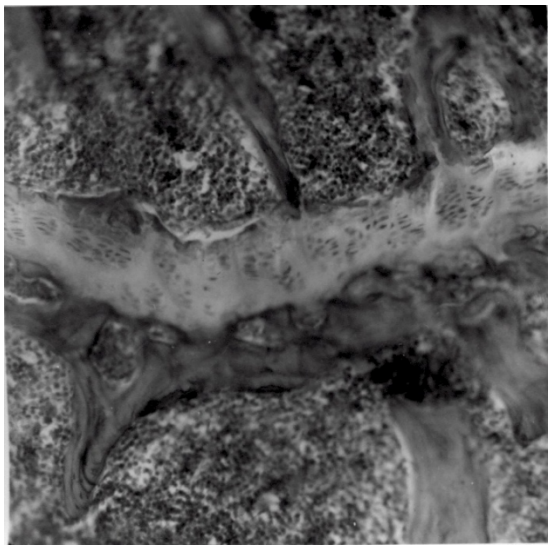

Fig. 3. The structural disturbance of the rat's distal femoral epiphyseal cartilage. No endochondral ossification. Hematoxylin-eosin. Amplification X 40. 3rd group.

The comparison of thickness in the middle third of femoral diaphysis and compact layer measured using radiograms showed the difference between all series of study animals (Table 3).

The thickness of femoral diaphysis and compact layers significantly increased in the animals from the 1st and the 4th series, while in the 3rd group it dropped reasonably compared to the control group.

Our results prove the inadequacy of an unidirectional correlation of the increased intake of calcium with food and the conditions of its mineral metabolism. Insertion of low doses of calcium keeps the values within the limits of the physiological norm. High doses of calcium cause significant disorders not only in mineral metabolism but also in the metabolism of D3. The probable mechanism of this process could be the suppression of the synthesis of calcium-linking proteins by the inserted high doses of Ca without vitamin D3.

Table 3. The thickness of diaphysis of femoral bone and its compact layer in the middle third, M ±m, n=10 (for each group)

Groups of the animals	Thickness, mm		
	Diaphysis	The lateral portion of a compact layer	Medial layer of compact tissue
10 mg Ca	3.058 ±0.095	0.568±0.036	0.513±0.019
60 mg Ca	2.659±0.088*	0.442±0.029*	0.376±0.018*
10 mg Ca + vitamin D3	3.378±0.076*	0.683±0.200*	0.586±0.012*
Control	2.895±0.065	0.525±0.029	0.495±0.067

*Probable difference of the values being studied compared to the standard (control group) – $p < 0,05$.

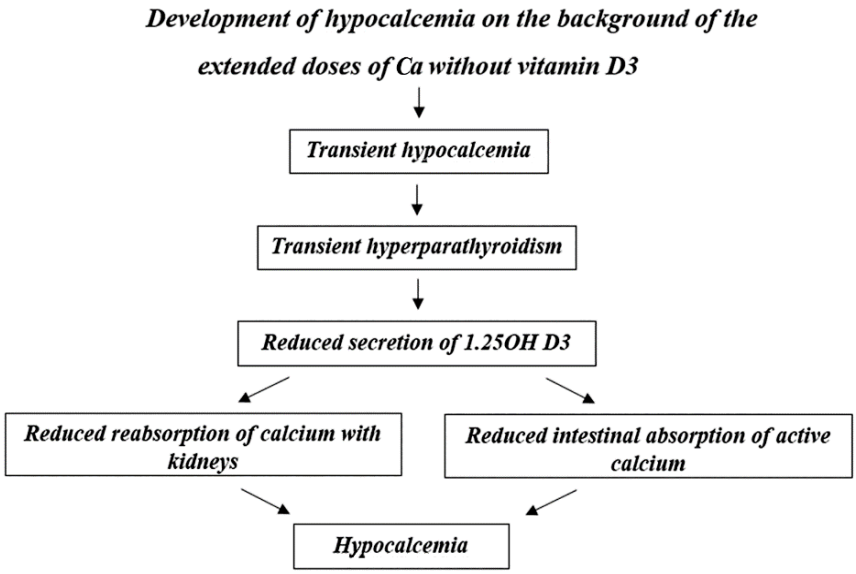

Fig. 4. Schematic diagram of the occurrence of hypocalcemia due to extended doses of Ca without vitamin D.

At that, a probable mechanism of the development of hypocalcemia could be as follows in Fig. 4.

In normal conditions, absorption of calcium rises, if the content thereof in food drops. In such a case, the increased absorption of calcium is indirect, through the increased synthesis of hormonally active forms of vitamin D3 and the activity of 1α-hydroxylase. A significant drop in circulating metabolites of the vitamin D_3 in case of hypocalcemia could lead to metabolic disorders, especially in bone tissue. At the same time, small doses of calcium accompanied by vitamin D_3 have a positive effect on the level of calcium in bone serum.

As a result, the histomorphologic data prove that extra intake of small doses of calcium (10 mg), especially combined with vitamin D3 (20 IU), has a positive effect on structural and functional conditions of the bone tissue. Bone diaphysis and compact layer of a bone become thicker; chondrogenesis and endochondral ossification arise.

After the intake of large doses of calcium (60 mg), diaphysis and compact layer of a test animal's femur became thinner. Besides, it inhibited chondrogenesis and endochondral ossification.

Conclusions

1. The experiment proved that daily adding 10 mg of calcium, combined with vitamin D3, improves structural and functional conditions of bone tissue.
2. After the intake of large doses of calcium (60 mg daily during a month) femoral diaphysis and compact layer of the test animals become thinner. Besides, it inhibits the processes of chondrogenesis and endochondral ossification.
3. The intake of large doses of calcium causes significant disorders to mineral metabolism and metabolism of vitamin D.
4. The results confirm the absence of a single-focused correlative dependence between the increased calcium intake with food and mineral metabolism of a body.
5. To correct structural and functional conditions of bone tissue, the pharmaceutical preparations should be preferred, with composition ensuring efficient absorption and digestion of calcium.

სამედიცინო მეცნიერება

კალციუმისა და ვიტამინ D3-ის გავლენა მინერალურ მეტაბოლიზმზე ვირთხებში. ექსპერიმენტული გამოკვლევა

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*უკრაინის მედიცინის მეცნიერებათა ეროვნული აკადემიის ტრავმატოლოგიისა და ორთოპედიის ინსტიტუტი, ტრავმული დაზიანებებისა და ოსტეოსინთეზის პრობლემების დეპარტამენტი, კიევი, უკრაინა

(წარმოდგენილია აკადემიის წევრის ნ. მითაგვარიას მიერ)

50 ვირთაგვაზე ჩატარებული ცდის შედეგად აღმოჩენილ იქნა კალციუმისა და ვიტამინ D3-ის გავლენა ორგანიზმის მინერალურ მეტაბოლიზმზე. გამოვლინდა, რომ დამატებითი 60 მგ კალციუმი 30 დღის განმავლობაში ამცირებს მის დონეს სისხლის შრატში 35%-ით, რის შემდეგაც სისხლის შრატში მცირდება D3 – D3 - 1,25(OH)2D3 და 24,25(OH)2D3 ვიტამინის აქტიური მეტაბოლიტების კონცენტრაცია 50%-ით, 25OHD3-ის იმავე დონეზე დარჩენის ფონზე. დამატებითმა 10 მგ კალციუმმა და 20 IU ვიტამინმა D3 ხელი შეუწყო სისხლის შრატში კალციუმის ოპტიმალური კონცენტრაციის მიღწევას. შედეგები გვიჩვენებს, რომ ჰორმონალურად აქტიურ ფორმებში ვიტამინ D3-ის დაბალი შემცველობა ამცირებს ცილებთან დამაკავშირებელი კალციუმის გამომუშავებას. აქედან გამომდინარე, მისმა ნაკლებობამ შესაძლოა გამოიწვიოს ჰიპოკალციემია.

REFERENCES

1. Bolland M.J., Grey A., Avenell A. (2018) Effects of vitamin D supplementation on musculoskeletal health: a systematic review, meta-analysis, and sequential trial analysis. *Lancet Diabetes and Endocrinology,* 6:847–858, doi: 10.1016/S2213-8587(18)30265-1.
2. Bonewald L.F. (2017) The role of the osteocyte in bone and nonbone disease. *Endocrinol Metab Clin North Am,* 46(1):1–18. doi: 10.1016/j.ecl.2016.09.003.
3. Chen C., Ge S., Li S., Wu L., Liu T., Li C. (2017) The effects of dietary calcium supplements alone or with vitamin D on cholesterol metabolism: a meta-analysis of randomized controlled trials. *J. Cardiovasc. Nurs,* 32:496–506. doi: 10.1097/jnc.0000000000000379.
4. Gayko G.V., Brusko A.T., Kalashnikov A.V. et al. (2008) Vitamin D i kostnaia systema, 176 p. [Vitamin D and a Bone System] *Knyha-Plus* (in Russian).
5. Martinaityte I., Kamycheva E., Didriksen A., Jakobsen J., Jorde R. (2017) Vitamin D stored in fat tissue during a 5-year intervention affects serum 25-hydroxyvitamin D levels the following year. *Journal of Clinical Endocrinology and Metabolism,* 102:3731–3738. doi:10.1210/jc.2017-01187.
6. Swanson C.M., Srikanth P., Lee C.G. et al. (2015) Associations of 25-hydroxyvitamin D and 1.25-dihydroxyvitamin D with bone mineral density, bone mineral density change, and incident nonvertebral fracture. *J Bone Miner Res.,* 30(8):1403-1413. doi:10.1002/jbmr.2487.
7. Larsen A.U., Grimnes G., Jorde R. (2018) The effect of high-dose vitamin D3 supplementation on bone mineral density in subjects with prediabetes. *Osteoporos Int.,* 29(1):171-180. doi:10.1007/s00198-017-4222-x.
8. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes; European Commission: Brussels, Belgium, 2010.
9. Basel Declaration Society. Basel declaration: A call for more trust, transparency and communication on animal research. Adopted on 29 November 2010. Available online: http://www.basel-declaration.org/basel-declaration/ (accessed on 13 March 2013).

Received February, 2021