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THE EFFECT OF DIETARY SUPPLEMENTATION WITH CALCIUM SALTS ON SKELETAL CALCIUM IN SUCKLING RATS

Veda Marija VARNAI, Marija ŠARIĆ, Gordana MOKROVIĆ, Martina PIASEK, Maja BLANUŠA, Jasminka BULJAN CULEJ, Marijana MATEK SARIĆ, and Krista KOSTIAL

Institute for Medical Research and Occupational Health, Zagreb, Croatia

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This study aimed at identifying a calcium compound which could serve as an effective and safe dietary supplement in suckling rats over the period of intense growth and development. The main objective was to assess the effect of additional calcium intake on skeletal calcium in suckling pups. Suckling Wistar rats were fed using a pipettor with one of the following calcium salts from day 6 to 14 after the birth: gluconate, hydrogenphosphate, carbonate (each suspended in cow's milk), or chloride (in demineralised water). Control rats received only cow's milk. Calcium in the carcass (body without organs and skin) was analysed by atomic absorption spectrometry. The only effective dietary supplement that produced no risk for the suckling pups' growth was calcium hydrogenphosphate in cow's milk in the total amount of 340 mg. That dose increased the daily calcium intake 3 to 4 times compared to non-supplemented controls, increasing carcass calcium content by about 16 per cent. Other calcium compounds were either inefficient (carbonate) or had adverse effects on pups' growth (chloride and gluconate).

KEY WORDS: *calcium carbonate, calcium chloride, calcium gluconate, calcium hydrogenphosphate, calcium in bone, suckling period*

Osteoporosis, a disease characterised by a deficiency of bone tissue relative to bone volume, poses an important public health problem in the western world (1, 2). Osteoporotic bone loss leads to bone fractures, which significantly increase immobility and mortality in the elderly population (2, 3). Since the bone mass is one of the most important determining factors for osteoporotic fractures, it is assumed that the milestone of osteoporosis prevention is to attain the highest possible peak bone mass during bone growth. Peak bone mass is defined here as the highest level of bone mass achieved as a result of normal growth (2). Peak bone mass is determined by heredity, mechanical loading, hormonal and nutritional factors, especially adequate calcium intake (4, 5). Population studies revealed a relationship between dietary calcium intake during growth and bone mass later in life (6-8). It was shown that lower calcium intake during

skeletal formation is associated with lower bone mass in adulthood and greater risk of fractures (6, 7). It is assumed that the critical period for peak bone mass formation is during puberty and early adolescence (2). However, according to calcium balance studies, the highest requirements for calcium are during infancy and adolescence, and then during childhood and young adulthood (9). Considering the whole life cycle, the fastest increase of body calcium content in relation to body size occurs in the first year of life (2). The relationship between the level of calcium intake during infancy and the peak bone mass in adult life is not yet clarified and requires further investigation. Due to ethical reasons, certain studies during this earliest period of life have to be carried out on animal models. In this respect, we tried to find a calcium compound which could serve as an effective dietary supplement for rats in their suckling period, without producing

harmful effects on their growth. The main objective was to assess the effect of additional calcium intake on skeletal calcium in suckling pups. Four calcium compounds were tested in suckling rats: calcium gluconate, calcium hydrogenphosphate, calcium carbonate, and calcium chloride. Similar data in sucklings are not available in the literature.

MATERIAL AND METHODS

Animals

Experiments were performed on suckling Wistar rats bred in the Laboratory Animals Unit of the Institute for Medical Research and Occupational Health, Zagreb. At the beginning of the experiments, the pups were 6 days old with average body weight of 15.6 g (range 11.2-21.1 g). The animals were kept with their mothers, six to eight per litter (the number of pups was reduced on the second day of life) in individual polycarbonate cages (26x20x14 cm, Ehret, Austria) with stainless steel lids and bottoms (Ehret, Austria). The cages were cleaned and pine-shaving bedding was changed daily. Body weights of pups were recorded every morning. The pups from each

litter were randomly distributed into experimental groups, two to three pups per litter in each group. That way, each litter was equally present in each experimental group. Altogether, 19 litters totalling 100 pups were used for the experiments. At the beginning, experimental groups did not differ in body weight of animals.

All research procedures were performed according to the national law on the care and use of laboratory animals and were officially approved by the Croatian Ministry of Agriculture and Forestry.

Experimental design

Four separate experiments were performed to test four calcium compounds as calcium supplements in suckling rats: calcium carbonate (CaCO_3 , *p. a.*, Kemika, Zagreb), calcium chloride (CaCl_2 , *p. a.*, Kemika, Zagreb), calcium gluconate ($\text{C}_6\text{H}_{11}\text{O}_7 \times \frac{1}{2} \text{Ca}$, Sigma Chemical Co, USA), and calcium hydrogenphosphate ($\text{CaHPO}_4 \times 2\text{H}_2\text{O}$, *p. a.*, Kemika, Zagreb). Calcium gluconate, hydrogenphosphate, and carbonate were suspended in cow's milk (commercial sterilised cow's milk, Vindija, Varaždin, Croatia, with 2.8 % milk fat), and calcium chloride was dissolved in demineralised

Table 1 Total calcium dose, total body weight gain, wet carcass weight, calcium concentration and content in the carcass of 15-day-old suckling rats artificially fed on cow's milk (control) or on cow's milk supplemented with one of the calcium compounds from day of birth 6 through 14

Experimental groups	Total Ca dose (mg)	No. of rats	Total body weight gain (g)	Wet carcass weight (g)	Ca carcass concentration (mg/g wet wt.)	Ca carcass content (mg)
<u>Experiment 1</u>						
Control	7	5	19.2±4.75	15.1±1.79	12.1±0.592	182.6±29.4
CaCO_3	350	5	19.7±5.27	14.9±1.72	13.1±0.841	195.9±30.3
<u>Experiment 2</u>						
Control	7	12	25.9±1.18	16.8±1.20	11.8±0.761	197.3±16.2
CaCl_2	340	12	10.9±0.939*	11.9±1.19*	14.0±1.27*	164.6±10.6*
<u>Experiment 3</u>						
Control	5	12	21.9±3.86 ^a	16.7±1.69 ^a	12.7±2.81	209.3±40.6
Ca gluconate	80 [§]	12	17.1±4.41 ^b	14.8±2.14 ^b	13.2±1.35	195.6±34.8
CaHPO_4	240	12	21.5±4.04 ^a	16.8±1.83 ^a	12.1±1.52	203.7±35.3
<u>Experiment 4</u>						
Control	7	15	21.2±4.10	15.6±1.83	12.5±2.49	205.1±32.1
CaHPO_4	340	14 ^{§§}	20.8±3.92	15.4±1.80	15.4±0.925*	237.4±40.8*

* Statistically significant difference between groups (by t-test, $P < 0.05$).

a,b Statistically significant difference among groups (by one-way ANOVA followed by Duncan's multiple range test, $P < 0.05$).

§ Due to lower body weight gain in pups supplemented with calcium gluconate, both supplemented groups received lower doses of calcium than in other experiments.

§§ One animal died during the experiment.

water, since it could not be suspended in milk. Due to lower solubility, calcium gluconate was prepared as a 4 % calcium suspension, while other compounds were administered as a 6 % calcium suspension or solution. The suspension and the solution were administered using the method of artificial feeding. This method, introduced by *Kostial and co-workers* (10), closely resembles bottle-feeding of infants. Each morning, the pups were separated from their mothers, placed in labelled boxes, and kept warm during treatment. Solutions were given drop-by-drop by an automatic pipettor, allowing pups to swallow the drops. Feeding lasted about 7 hr per day (from 9:00 a.m. to 4:00 p.m.) for nine consecutive days; from day 6 through 14 after birth. After the treatment, the pups were returned to their mothers for the rest of the day and over night. The number of drops administered to pups increased every day, according to pups' weight gain.

In all experiments the control group received cow's milk and other groups received one of the following calcium supplements (total calcium dose in brackets):

Experiment 1: calcium carbonate (350 mg);

Experiment 2: calcium chloride (340 mg);

Experiment 3: calcium gluconate (80 mg) or calcium hydrogenphosphate (240 mg);

Experiment 4: calcium hydrogenphosphate (340 mg)

The number of pups per group for each experiment is given in Table 1.

Due to lower body weight gain in pups receiving cow's milk supplemented with calcium gluconate in Experiment 3 (Figure 3), the number of daily administered drops was reduced in all 3 groups (the control and two groups receiving supplements, see Table 1) to maintain the same experimental conditions (equal stress level) in all experimental groups, since we noticed earlier that the number of drops (i.e. the period of separation from mothers) affected the pups' body weight gain and carcass and organ weights. Consequently, pups from both supplemented groups in this experiment received lower doses of calcium than in other experiments. The increase in daily calcium intake in supplemented pups was calculated as in our previous studies (11) on the basis of calcium concentration of rat's milk measured by *Luckey and co-workers* (12) and *Nicholas and Hartmann* (13), and quantity of milk secreted by lactating rats according to *Kametaka and co-workers* (14), considering litter size and strain of rats used in our experiments.

Analysis of skeletal calcium

One day after the last day of supplementation pups were killed by exsanguination from the abdominal aorta in ether anaesthesia. Carcasses (whole body after removal of the organs, total gastrointestinal tract and skin; equivalent to skeleton) were dissected and used for further analysis. Fresh weights were recorded, and samples were dry ashed at 600 °C in a muffle furnace and dissolved in 5 % nitric acid (15). Calcium was determined by flame atomic absorption spectrometry (Varian AA-375, Australia).

Statistical analysis

Results are presented as arithmetic means and standard deviations of the mean. The concentration of skeletal calcium is expressed in milligrams per gram wet carcass weight, and total skeletal amount of calcium (carcass calcium content) in milligrams. The statistical differences between groups were analysed by the Student's *t*-test or one-way analysis of variance (ANOVA) followed by *post hoc* Duncan's multiple range test (at the level of significance of $P < 0.05$). The homogeneity of variance for both ANOVA and *t*-test was analysed using Leven's test. Statistica for Windows program (StatSoft 1995 package, release 5.0) was used for all statistical analyses.

RESULTS

Table 1 shows the effects of calcium supplementation with different calcium compounds on pups' body weight gain, wet carcass weights, carcass calcium concentrations and contents. Average daily body weights in control and supplemented groups in all experiments are shown in Figures 1 to 4.

Supplementation with calcium carbonate, calcium chloride and calcium hydrogenphosphate (at a total calcium dose of 340-350 mg) increased the average daily calcium intake about 3-4 times above control values. Supplementation with calcium hydrogenphosphate at a lower calcium dose (240 mg) increased calcium intake about 2-2.5 times, and supplementation with calcium gluconate about 1.5 times above control values.

When compared to unsupplemented control, calcium carbonate did not influence body weight gain and wet carcass weights, and had no effect on carcass calcium concentration and content.

Pups supplemented with calcium chloride (dissolved in demineralised water) had a lower body

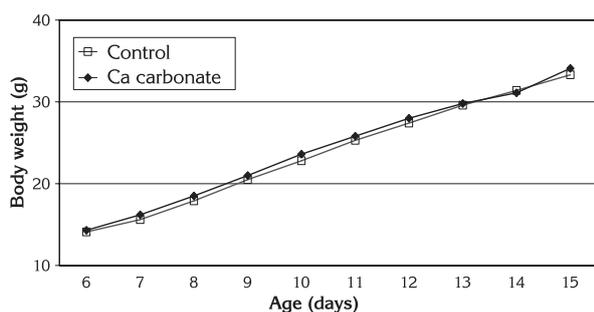


Figure 1 Average daily body weights in suckling rats artificially fed on cow's milk (control) or on cow's milk supplemented with calcium carbonate (total calcium dose 350 mg)

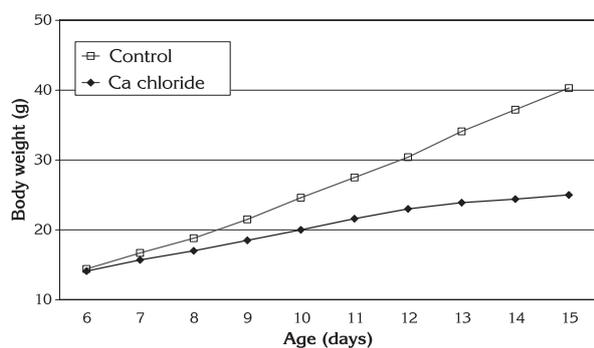


Figure 2 Average daily body weights in suckling rats artificially fed on cow's milk (control) or on cow's milk supplemented with calcium chloride (total calcium dose 340 mg)

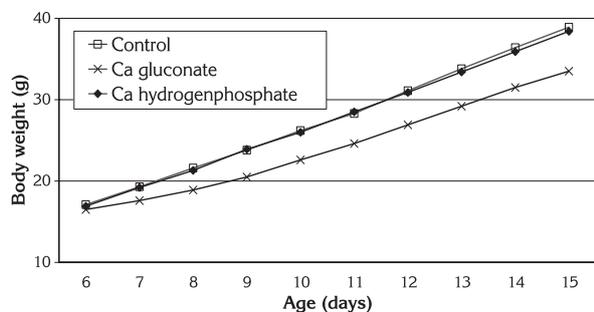


Figure 3 Average daily body weights in suckling rats artificially fed on cow's milk (control) or on cow's milk supplemented with calcium gluconate (total calcium dose 80 mg) or hydrogenphosphate (total calcium dose 240 mg)

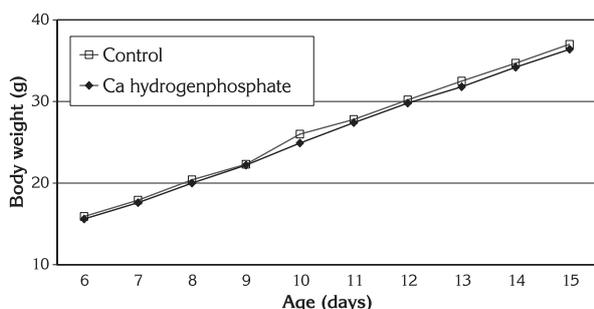


Figure 4 Average daily body weights in suckling rats artificially fed on cow's milk (control) or on cow's milk supplemented with calcium hydrogenphosphate (total calcium dose 340 mg)

weight gain and carcass wet weights compared to control. Although calcium concentration in the carcass increased by approximately 18 % in supplemented animals, the total amount of calcium in the carcass was lower than in controls. This can be explained by the lower carcass wet weights in supplemented group.

Calcium gluconate supplementation in cow's milk caused a decrease in body weight gain and carcass wet weights. Calcium concentrations and content in the carcasses were not affected by supplementation.

Supplementation with calcium hydrogenphosphate suspended in cow's milk, either at lower (240 mg) or higher (340 mg) total calcium dose, had no adverse effects on pups' growth and wet carcass weights. In pups supplemented with the higher dose of calcium hydrogenphosphate, carcass calcium concentration increased about 18 %, and carcass calcium content about 16 % when compared to unsupplemented controls. The lower dose, however, had no such effect.

DISCUSSION

Our results show that only a 3 to 4-fold increase in calcium intake by supplementation with calcium hydrogenphosphate successfully increases skeletal calcium without disturbing pups' growth. Other calcium compounds are either not as efficient (calcium carbonate), or even produce adverse effect on the growth of suckling animals (calcium chloride and gluconate).

Calcium absorption is determined by the chemical form of the calcium compound, other components of the diet, and by the absorption capacity of the intestines, which is physiologically controlled by different calcium requirements in the body (16, 17). These depend on growth (18, 19), special physiological conditions such as pregnancy and lactation (20), and on previous calcium supply (21). Gastrointestinal and endocrine pathology, which are common in the elderly (5), as well as pH values in the gastrointestinal tract (22), also affect calcium absorption. Calcium absorption in the intestines is, of course, only the first step in calcium bioavailability, and has to be followed by incorporation of absorbed calcium into the bone (21). Therefore, calcium bioavailability from different salts is determined by the effect of their anions on calcium

solubility in the intestines, calcium incorporation in the bone and urinary calcium excretion (23). It is observed, for example, that certain anions, such as sulphate and chloride, increase the loss of calcium in the urine, thus impairing calcium bone incorporation (21).

Both human (16, 17, 24-26) and animal studies (27-30) show that there is a difference in calcium bioavailability among various calcium salts, such as citrate, carbonate, gluconate, citrate malate, acetate, lactate, sulphate, and phosphate. Other studies (23, 31-33), however, claim the opposite: that different calcium salts have similar calcium bioavailability. These observations indicate that the significance of chemical form of the calcium compound for calcium bioavailability is not yet clarified. Some of the differences found in these studies could be explained by different methods of evaluation of calcium absorption (21). The presence of phosphorus in the diet may also play a role, since it is necessary for the production of hydroxyapatite. Calcium incorporation into the bone is stimulated by phosphorus. The separation of calcium intake from that of phosphorus may, therefore, restrict bone mineralisation, especially in growing organisms (21). This could explain the efficiency of calcium hydrogenphosphate in increasing skeletal calcium of suckling rats in our study. Similar results were obtained by *Patwardhan and co-workers* (34), who found that supplementation with calcium phosphate was better than supplementation with calcium carbonate and calcium lactate in growing rats during the post-weaning period. Milk is another factor that could be important for calcium bioavailability during supplementation. Studies in growing rats (35) and pigs (36) showed that diet containing milk components provided better bone mineralisation and bone strength than calcium salts in a non-milk diet. It is proposed that certain components of milk, such as lactose, phosphopeptides, proteins and aminoacids (e.g. *L*-arginine, *L*-lysine) increase calcium solubility in the ileum and thus stimulate its passive diffusion (19, 21, 37). This is why all calcium compounds were suspended in cow's milk in our study, except for calcium chloride that was dissolved in demineralised water, since it could not be suspended in milk due to a relatively high dose. Interestingly, supplementation with calcium chloride increased skeletal calcium concentration in our study, indicating good calcium bioavailability from this salt. However, its adverse effects on the pups' growth, rendered it inappropriate for this age group.

CONCLUSION

Our results show that calcium hydrogenphosphate is an effective calcium supplement in suckling rats, and that it increases skeletal calcium without disturbing the pups' growth. The effect of supplementation on skeletal calcium depends on the amount of calcium added. This effect was achieved when daily calcium intake increased about three to four times above the intake in unsupplemented controls. Other tested calcium compounds were either not efficient or had adverse effects on the pups' growth. The question remains whether the increase in skeletal calcium persists in adulthood, that is, what are the long-term effects of calcium supplementation in this age group. Studies to clarify this question are in progress. Our results provide useful information to other researchers studying skeletal calcium accretion in rats, as to what amount of calcium and which supplemental form to use during suckling period for the best effectiveness.

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Sažetak

UTJECAJ DODAVANJA RAZLIČITIH SPOJEVA KALCIJA NA SADRŽAJ KALCIJA U KOSTURU SISAJUĆIH ŠTAKORA

Utjecaj količine kalcija u prehrani dojenčadi na postizanje vršne koštane mase u odrasloj dobi nije još razjašnjen. Zbog bioetičkih razloga dio istraživanja u dojenačkoj dobi potrebno je provesti na životinjskome modelu. Svrha ovog istraživanja bila je pronaći kalcijev spoj odnosno spojeve koji mogu poslužiti kao učinkovit suplement u štakora tijekom razdoblja sisanja, bez štetnog djelovanja na njihov rast. Glavni cilj bio je procijeniti učinak dodavanja kalcija na sadržaj kalcija u kosturu sisajućih štakora. Pokusi su provedeni na štakorima soja Wistar. Mladuncima je tijekom devet uzastopnih dana (od 6. do 14. dana života) ukapavana jedna od kalcijevih soli: glukonat, hidrogenfosfat, karbonat (suspendirani u kravljem mlijeku) ili klorid (otopljen u demineraliziranoj vodi). Kontrolnim skupinama ukapavano je samo kravlje mlijeko. Sljedećeg jutra nakon posljednjeg dana ukapavanja životinje su usmrćene iskrvarenjem iz trbušne aorte u eterskoj narkozi. Nakon disekcije preostali su karkasi (tijelo bez kože i organa, što odgovara kosturu) u kojima je metodom atomske apsorpcijske spektrometrije određen kalcij. Nađeno je da jedino suplementacija mliječnom suspenzijom kalcijeva hidrogenfosfata tijekom razdoblja sisanja u ukupnoj dozi od oko 340 mg povećava količinu kalcija u kosturu za oko 16 %, a da se pritom ne remeti napredovanje mladunaca. Ovom se dozom dnevni unos kalcija povisio 3-4 puta u odnosu prema kontroli. Ostali kalcijevi spojevi pokazali su se ili nedovoljno učinkovitima (kalcijev karbonat), ili su čak štetno djelovali na rast mladunaca (kalcijev klorid i kalcijev glukonat).

KLJUČNE RIJEČI: *kalcij u kosturu, kalcijev karbonat, kalcijev klorid, kalcijev glukonat, kalcijev hidrogenfosfat, sisajući štakori*

REQUESTS FOR REPRINTS:

Veda M. Varnai, M. D., Ph. D.
Institute for Medical Research and Occupational Health
P.O. Box 291, HR-10001 Zagreb, Croatia
E-mail: vvarnai@imi.hr