ADVANTAGE OF CARBONATE- VERSUS CITRATE-BASED ALKALINIZATION ON BONE METABOLISM IN MODERATELY EXERCISING AGED MALE RATS FED AN ACIDOGENIC DIET

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This study aims to determine the effects of different alkaline supplementations on high protein diet-induced abnormalities affecting bone metabolism in rats which were also undergoing physical exercise of moderate intensity. Sixty elderly Sprague-Dawley rats were randomly divided into four groups of 10 rats each and treated for 16 weeks as follows: baseline control group fed normal food (C); acidic high-protein diet supplemented group (chronic acidosis, CA group), bicarbonate-based alkaline formula (Basenpulver, Named, Italy) supplemented chronic acidosis (BB-CA) and citrate-based alkaline supplement (CB-CA). Throughout the supplementation period, rats were put on a treadmill training mimicking a moderate level of exercise. In the CA group, 24-hour urinary calcium (Ca) and phosphorus (P) excretion were increased over 30% ($p<0.05$ *vs* normal diet controls). However serum Ca was not significantly changed. Femural and tibial BMD and BMC was significantly decreased in the CA group ($p<0.05$) but both alkaline supplementations prevented such phenomenon ($p<0.05$ *vs* CA), without significant difference between the two formulations although the BB-CA group showed significantly more preserved trabecular bone volume ($p<0.05$ *vs* CB-CA group). An increased level of over 50% of urinary Dpd observed in the CA group ($p<0.001$) was reverted to normal by both supplementations ($p<0.001$ *vs* CA group). The same applied to urinary net acid excretion ($p<001$) with BB-supplementation performing better than CB-supplementation ($p<0.05$). Moreover, while the latter did not modify N-terminal telopeptide value, BB-supplementation significantly normalized this parameter ($p<0.05$ *vs* CA group) which exercise and acidic protein diet had modified ($p<0.01$ *vs* control diet). Overall, the present study shows that a bicarbonate-based alkaline formula, when administered to a dose amenable to clinical use, may significantly protect bone structure in exercising aged animals to a greater extent than a quali/quantitavely similar citrate-based formula.

Bone mass is affected by hormonal modulation changes along our different biological life cycle and by lifestyle factors such as physical loading where exercise plays an important role in maintaining and

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increasing bone morphogenic proteins and bone mass (1). However, intense physical activity may bring about a decrease in blood pH as a result of increased lactate and blood CO_2 and the possible supervening acidosis may have a detrimental effect in the long run (2). It is known that dietary protein promotes peripubertal bone formation (3) and has been positively associated with higher bone mass (4) and lower hip fracture incidence in adults (5). The pH of bone is a very large ion exchange buffer system, and a diet rich in protein and low in fruits and vegetables may result in a low-grade, chronic metabolic acid load. This is because the metabolism of protein releases non-carbonic acids into the bloodstream in amounts that override the alkalinizing effect of potassium in vegetables (6). Thus, unbalanced protein intake has been shown to increase urinary calcium excretion (7) and exert negative effects on calcium absorption (8-9). Indeed, an acidic environment reduces osteoblastic activity (9) and increases osteoclastic activity (10). Studies have found that the addition of alkaline salts lowered urinary calcium excretion (11) and biochemical markers of bone turnover during short-term administration (12), suggesting beneficial effects on bone preservation. Indeed, a decrease in bone resorption due to potassium citrate (13) and potassium bicarbonate (14) supplementation has been observed in postmenopausal women. Potassium citrate has also been shown to prevent increased bone resorption, as measured by urinary N-telopeptide excretion caused by high salt intake in postmenopausal women (15). However, a recent study showed that administration of an alkaline salt of potassium citrate in rats in combination with a high-protein diet improved calcium retention but failed to demonstrate beneficial skeletal effects (16). We have recently shown that a specifically balanced bicarbonate-based alkaline supplementation is able to beneficially counteract the derangement of bone metabolism due to an acidic diet in rats (17) while also potentiating the bone preservation effect of a isoflavone-containing red clover preparation in ovariectomised rats (18). The alkaline supplementation we employed in the present study has been shown by in-house testing to yield a significantly higher buffering capacity than an equal amount of sodium bicarbonate *per sè* (ratio 1.6), besides providing a much lesser content of sodium, and we have recently shown that it beneficially influences muscle metabolism in aged rats undergoing exhaustive exercise (19). On the other hand, citrate is known to be converted into bicarbonate within the liver and in acute clinical settings, such as continuous veno-venous hemofiltration for acute kidney injury, it has been observed that the two different alkali-based substitution fluids are comparable concerning acid-base balance and electrolyte control (20). However, in more physiological conditions, bicarbonate proved to be a better buffer compared to lactate and citrate in exhaustive physical exercise (21). This is possibly because, especially in an acute physical exercise setting, an increase in intracellular citrate concentration after ingestion of citrate could reduce generation of ATP via inhibition of the enzyme phosphofructokinase (22). It has to be considered that, under certain conditions, dietary unbalance among them, moderate-intense exercise may be associated with a decrease in bone mineral density (23). One potential mechanism is increased bone resorption due to an exercise-induced increase in parathyroid hormone (PTH), possibly triggered by dermal calcium loss. Thus, the present study was designed to investigate whether a bicarbonate-based or a citrate-based alkaline supplementation would have a more favourable impact on indices of bone conservation in a dietary protein-induced acidic environment and ongoing moderate physical activity.

MATERIALS AND METHODS

Animal handling and supplementation schedule

Sixty male Sprague-Dawley rats, sixteen-months old and weighing 280–310 g, were used in the experiments. The animals were individually housed in aluminium metabolic cages in a temperature- and dark/light cycle-controlled vivarium and strict sanitary control was observed. The animals were fed controlled amounts of control or modified food and water *ad libitum* until the day prior to the study. Following a 1-week adaptation to the new environment, the rats were randomly divided into four groups: Baseline same age control group (n. 10, C), chronic acidosis group (n. 10, CA), chronic acidosis group supplemented with bicarbonate-based alkaline compound (n. 10, BB-CA) or with same amount of citrate-based alkaline compound (CB-CA) (containing the same amount of calcium and potassium as the bicarbonate-based

supplement). Indeed, both K and Ca supplementation may have beneficial effects on bone through separate mechanisms. K in the form of citrate or bicarbonate affects bone by neutralizing the acid load caused by a high protein intake or a low intake of alkalizing foods, i.e. fruits and vegetables. Ca is known to decrease serum PTH concentration and bone resorption. Indeed, both K and Ca supplementation may have beneficial effects on bone through separate mechanisms; K in the form of citrate or bicarbonate affects bone by neutralizing the acid load caused by a high protein intake or a low intake of alkalizing foods, i.e. fruits and vegetables; Ca is known to decrease PTH concentration and bone resorption. While the control group was given standard chow food, chronic acidosis was induced in the latter four groups by a high protein diet (a 46% protein diet composed of casein, RMH 3000 protein chow, and dextrin -40, 35, 25; wt/wt/wt). All rats had unrestricted access to distilled water *ad libitum*. The modified alkaline supplementation was administered mixed with chow food at a daily dose of 16 mg (BB: calcium bicarbonate, sodium bicarbonate, magnesium and potassium carbonate and disodium phosphate, Basenpulver, Named, Italy or quail-quantitatively similar CB composition prepared in-house). Every day it was checked whether any food was left and if present, this was added and completely ingested the following day. Observation was carried out for 16 weeks. Body weights were measured once a week. During the last three days of the study, three 24-hour urine collections were carried out for each animal. The urine was collected under mineral oil and thymol crystals placed in the urine container to prevent bacterial overgrowth. Before sacrifice, urine was directly drawn for 24-hours and frozen at -30°C until assay. At the end of the experimental period, the animals were fasted overnight and sacrificed by cervical dislocation while the blood was being collected from the carotid artery. The serum obtained by centrifugal separation was stored at -30°C until assay. The femur and tibia were removed and bone status was measured.

Treadmill training

The treadmill provided an aversive electric stimulus (150 V of alternating current and 3 mA) in the back region of each lane to force the rats to run. The rats were allowed to adapt to treadmill running for 1 week (running for 10 min at 5-10 m/min). Training sessions were always held in the morning (6-12 am). Animals trained 5 times a week for further 15 weeks (altogether 16 weeks training). In the first 3 weeks, exercise intensity was maintained at 16 m/min and duration was increased from 30 to 60 min (15 min increase every week). Exercise sessions were held for 60 min thereafter and the treadmill speed was increased at 1 m/min for each 2 sessions until it reached 20 m/min. Treadmill inclination was maintained at 5° throughout the training period. This long-duration training protocol maintains exercise intensity from 55 to 60% of VO_2 max. This intensity has been regarded as defining a moderate level of exercise (24). All animal completed the experiment.

Bone morphometric assessment histomorphometric analysis of the tibial proximal metaphysis

At the end of experimental period, the left femur and right tibia were collected from every animal. Bones were carefully isolated while the muscles and adherent tissues were cleaned off. The femurs were stored in a freezer (-70°) and processed later for the measurements of the femoral length and bone mineral density (BMD) as described below. The bone mineral density (BMD) and content (BMC) was determined by dual energy X-ray absorptiometry (DEXA) using a Hologic QDR-4500 Plus (Hologic Inc., Bedford, MA, USA). The instrument was adapted for an ultra-resolution mode, with line spacing of 0.0254 cm, resolution of 0.0127 cm, and collimation of 0.9 cm diameter. The tibiae were sliced in half through the mid-diaphyseal shaft and fixed for 24 h in 0.1 mol/L phosphate buffered formalin. The bone tissues were then dehydrated in ethanol and embedded in methyl methacrylate (Fisher, Los Angeles, CA). Histomorphometric measurements of the cancellous bone of the proximal tibia were performed semi-automatically with an Olympus BX 40 microscope and an Olympus DP-70 digital camera (Olympus, Tokyo, Japan). The daily coefficient of variation for the manufacturer-supplied phantom was 0.6%. The precision for the DEXA measurements was estimated by duplicate measurements at the same time point, the CV for repeat scans and standards was <1%. Data were further analyzed using commercially available software (Multi Gauge v2.1, Fuji film Co., Tokyo, Japan) and a morphometry program (OsteoMetrics, Atlanta, GA, USA). The parameters measured for trabecular bone included total tissue volume (TV) and bone volume (BV) and these data were used to calculate the trabecular bone volume (BV/TV) percent in accordance with the standard nomenclature proposed by Parfitt et al. (25). In the present study, the region of trabecular bone measured was 1-4 mm distal to the lower margin of the growth plate in the proximal tibial metaphysis.

Biochemical measurements

Serum calcium, inorganic phosphate and 24-h urinary creatinine were measured by an automated clinical chemistry analyzer (Olympus AU400; Olympus America Inc., Melville, NY). The CVs for these assays ranged from 3.4 to 5.6%. The 24-h urinary calcium (UCa) was measured by direct-current plasma emission spectroscopy

(Beckman SpectraSpan VI Direct Current Plasma Emission Spectrophotometer; Beckman Instruments, Fullerton, CA) with a CV of 3.5%–5.5%. Twenty-four hour urinary deoxypyridinoline crosslink (Dpd) excretion, reflecting bone resorption, was analyzed by an ELISA kit. Twenty-four-hour NAE was measured by a modified titration method. Briefly, titratable acid $HCO3^-$ was assessed after addition of HCl, boiling the sample, and then titrating the sample to neutral pH. To measure the NH4+, formol was added to the sample to release the H+ from NH4+, and the sample was again titrated to neutral pH. All titrations were carried out with a TIM 900 Titration Manager (Radiometer Analytical, Loveland, USA). The precision of NAE measurements in our laboratory was determined by analyzing aliquots of a single 24-h urine collection on 15 different days. The aliquots were stored frozen at -20°C and thawed only once. The CV of these measurements was 10.1%. Serum intact PTH concentration was measured by an immunoenzymometric assay using PTH Kits (Immunodiagnostic Systems Limited, Boldon Colliery, Tyne and Wear, UK). The intra- and inter-assay CV were 2·9 and 5·1%, respectively. Serum intact PTH was measured by chemiluminescent immunoradiometric assays on an automated immunoassay system, (Immulite 1000, Diagnostic Product Corporation, Los Angeles, CA, USA). The CV for this assay ranged from 3–9%. The excretion of urinary N-terminal telopeptide of type I collagen (NTx) was analysed by an ELISA using Osteomark NTx Test Kits (Ostex International, Inc., Seattle, WA, USA). Values were expressed as nmol bone collagen/mmol·creatinine (nMBCE/mM·Cr). The intra- and inter-assay CV were 4.3 and 6.2%, respectively.

Statistical analysis

All data were obtained from three measurements and expressed as means ± standard deviations. Statistical analysis was carried out using Stat View J-5.0 program on a Macintosh computer (Abacus Concepts). Significance of the results was determined by one-way analysis of variance (ANOVA) with Bonferroni correction as a post hoc analysis. A significance level of $p < 0.05$ was used for all the comparisons.

RESULTS

Adherence to the dietary supplements during the experimental period was over 96% in the placebo group and > 94% in all supplemented groups.

Biochemical markers

Serum calcium and 24-h urinary creatinine did not differ between normally fed animals and the ones given an acidogenic diet, irrespective of the supplementation (data not shown). Moreover, 24-h urinary Ca of the CA group gradually increased during the experimental period, reaching approximately 2.2-fold increase as compared to C group ($p<0.01$) at the end of the experiment (Table I). Both supplementations significantly decreased urinary phosphate and calcium relative to the acidogenic control session ($p<0.01$). Differences were observed in serum PTH concentrations between the study sessions ($p<0.005$; ANOVA). Serum PTH, which was higher in protein-fed rats compared to controls ($p<0.001$), normalized in the citrate- and bicarbonate-treated groups ($p<0.05$ *vs* CA group) and both alkaline supplements reduced serum PTH concentration to a comparable extent. Twenty-four-hour urinary Dpd excretion, reflecting bone resorption, was increased in the CA group ($p<0.01$ *vs* C) during the experimental period. On the other hand, when supplemented with any of the modified alkaline formula, such abnormality was prevented ($p<0.05$ *vs* CA).

Urinary NAE/creatinine ratio was significantly increased in the exercising rats fed a high protein diet ($p<0.001$). Both alkaline supplementations brought about a significant improvement ($p<0.01$). However, only bicarbonate-based supplementation enabled a complete normalization of values ($p<0.05$ *vs* CB-CA group). Urinary NTx decreased in the bicarbonate-based supplementation ($p<0.05$ sessions compared with the control session) while during the citrate-based supplementation session the decrease in urinary NTx relative to the control session did not reach statistical significance (Fig. 1).

Changes of BMD and BMC in the femur and tibia and BV/TV of proximal tibial metaphysis

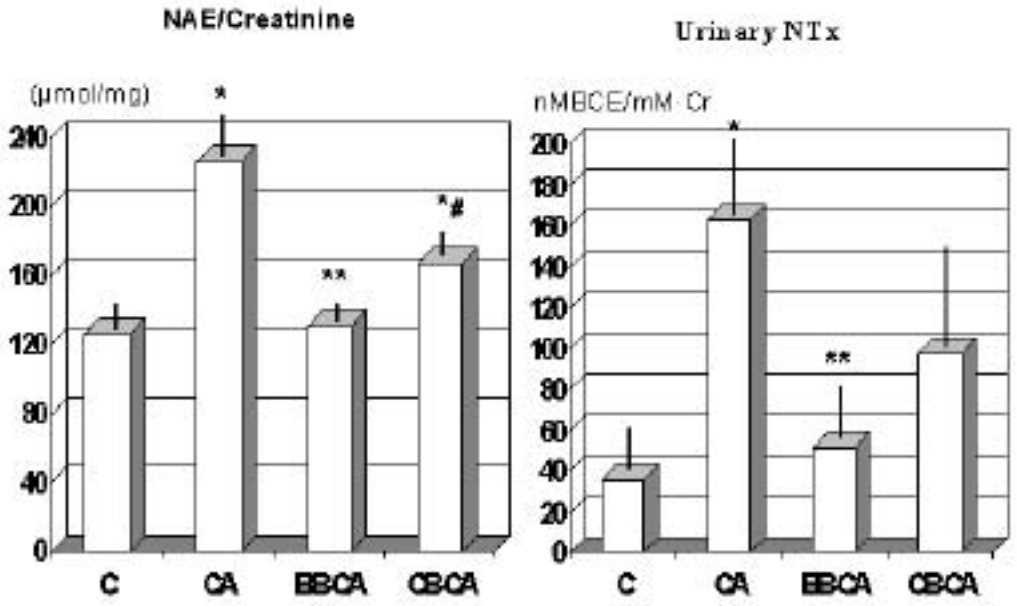
At the end of the study period the femoral BMD (g/cm2, mean±SD) in theCA group was significantly lower than in the C group ($p<0.05$), and all the groups showed a time-course increase ($p<0.01$) during the experimental period (Table II). The tibial BMD of all the groups also showed a similar increase ($p<0.01$) during the experimental period (Fig. 3). The femoral and tibial BMC, weight and length of bone in all the groups showed a similar tendency with BMD (Table II). Supplemented CA group rats showed BMD and BMC values similar to controls ($p<0.05$ *vs* CA group).

Table I. *Biochemical changes during acidic high-protein diet and alkaline supplementation under moderate exercise (mean±sd).*

	CONTROL	CA group	BB-CA group	CB-CA group
24h Ca^{+2}/Creatinine (μmol/mg)	3.2±0.4	10.5±1.6*	4.1±1.1#	3.8±1.4#
Urinary Phos/Creatinine (μmol/mg)	48±9	81±4*	53±6#	51±3#
Serum PTH (μg/ml)	138±22	216±44*	142±26#	153±32#
Dpd (nmol/mmmol/cretatinine)	79±6	133±9*	82±7#	84±11#

**p<0.01 vs control; # p<0.05 vs CA group.*

Fig. 1. *Net acid excretion and urinary NTx during different alkaline supplementation in moderately exercising rats fed and high-protein diet.*



*For abbreviations, see the text. *p<0.01 vs control; ** p<0.05 vs CB-CA group; # p<0.05 vs CA group.*

DISCUSSION

An average western diet is characterised by suboptimal Ca intake (26), excessive protein intake, and low ingestion of potassium-rich, bicarbonate-rich foods (i.e. fruits and vegetables). Indeed, in cross-sectional studies, an association between better bone health and fruit and vegetable consumption – an index of higher intake of K and bicarbonate – has been observed (27-28). Potassium and organic anions are metabolised to alkaline compounds and this works by neutralising the acid load caused by a high protein intake (8). In particular, we made sure to normalize both alkaline compositions in terms of potassium content, given that the skeletal benefit of dietary K has been speculated to be dependent on the accompanying bicarbonate-generating anion (29). Indeed, studies related to metabolic acidosis have

Table II. *Change of femural and tibial bmc during acidic high-protein diet and alkaline supplementation under moderate exercise (mean±sd).*

	CONTROL	CA group	BB-CA group	CB-CA group
FEMUR	**16 week**	**16 week**	**16week**	**16 week**
BMC (mg)	137±14	110±19 *	138±24 #	135±19 #
BMD (g/cm^2)	0.074±0.009	0.056±0.007*	0.069±0.007#	0.071±0.008#
Weight (g)	0.7±0.05	0.5±0.08	0.7±0.03	0.7±0.04
TIBIA				
BMC (mg)	78±21	61±13 *	80±19 #	81±14 #
BMD (g/cm^2)	0.070±0.01	0.051±0.007*	0.067±0.008#	0.069±0.006#
Weight (g)	0.5±0.02	0.5±0.04	0.5±0.02	0.5±0.04
B V / T V (%)	27±3	21±2*	26 ±2**,#	23 ±3#

*BV: bone volume; TV: total tissue volume.*p<0.01 vs control; ** p<0.05 vs CB-CA group; # p<0.05 vs CA group*

shown that a small reduction in extracellular pH leads to increased osteoclastic activity, indicating an increase in bone resorption (30). Dietary alkali is a potentially effective mechanism to buffer metabolically-generated acid and in *in vitro*, simulated metabolic alkalosis decreases bone calcium efflux by suppressing osteoclasts while stimulating osteoblastic type I collagen production. Indeed, previous studies have shown that alkali substances affect Ca and bone metabolism through four separate mechanisms: by enhancing reabsorption of Ca in the kidneys (11), by enhancing Ca absorption in the intestine, by inhibiting osteoclastic bone resorption and by stimulating osteoblastic bone formation (12). In our study we intentionally put animals on a protein-based acidogenic diet and in this situation it is known that the kidneys counteract such dietary acid challenge with net acid excretion, as well as ammonium and titratable acid excretion, as we also observed. Directly connected to this phenomenon a significant calciuria occurs as a sign of concurrent tentative buffering through active resorption of bone. In clinical practice the addition of exogenous buffers, such as fruits and vegetables, to modulate such balance is not a simple nutritional issue given that different food proteins differ greatly

in their potential acid load and therefore in their acidogenic effect, and potassium-rich dietary fruits and vegetables are not readily measurable in their buffering capacity. Moreover, resistance exercise is known to increase calciuria, independently of immediate osteoclastic activity (31), and the applied moderate exercise we used in our experiment is likely to have brought about a further detrimental factor added to the acidogenic diet (data compared to our prior study referenced as 18). The resulting chronic metabolic acidosis may cause osteomalacic and osteopenic bone diseases as it appeared when BMD and BMC was measured at the end of 16-week study period. Moreover, chronic metabolic acidosis increases corticosteroid production and acts as a signal causing accelerated muscle proteolysis with increased urinary nitrogen excretion (32).

In this setting, both alkaline supplementations proved to equally normalize PTH, calciuria and urinary and serum phosphate abnormalities. These data are of interest when considering the very recent clinical data from Barry et al. (23) which showed that calcium supplementation before exercise attenuates the increase of PTH and possibly reduces exercise-induced demineralization in the long run. Moreover, the effects of dietary protein are likely to be even more pronounced with aging kidneys which excrete less hydrogen ions as compared to young kidneys, with consequent higher blood hydrogen ions and lower blood bicarbonate (33). Accordingly, PTH levels are higher in older adults and since PTH influences plasma CO_2 as well as plasma phosphate levels, the overall buffering capacity tends to decrease. Therefore, aging per se renders more sensitive to the effect of acidic diets and this requires more buffer than younger people for the same dietary acid load. It has to be pointed out that resistance training is a mode of exercise that can be used to build peak bone mass during youth but with advancing age and without proper pH adjustments the issue might be less predictable. As a matter of fact, Iwamoto et al. demonstrated that there was no significant effect on lumbar bone mass after moderate exercise in ovariectomized rats (34). In addition, long treadmill experimental studies (13 weeks and 28 weeks) conducted by Gala et al. (35) showed significant loss of lumbar BMD after ovariectomy (19.5% and 30.3%) compared to the control group. In view of a clinical extrapolation, these latter data suggest that menopausal women should be considered at higher risk if regular exercise and unbalanced diet occur.

Interestingly, in our study bicarbonate-based supplementation proved to yield significantly better results than citrate-based formula as far as NAE, urinary NTx and BV/TV are concerned ($p<0.05$). It has to be considered that sodium citrate does not buffer directly like sodium bicarbonate: the dissociation constant for citrate/citric acid lies well outside the body's pH range although the consumption of protons during its oxidation effectively generates bicarbonate. McNaughton (36) found that ingestion of sodium citrate had a positive effect on work output, but failed to have a significant effect on performance in other studies (37), unlike bicarbonate formulations (38) which indeed have shown to be more beneficial to sprint performance than lactate and probably citrate (21); this may also be related to its recently demonstrated mitochondrial adaptation with greater improvements in muscle oxidative capacity and time to exhaustion (39). In a comparative clinical study testing the effects of calcium carbonate, calcium citrate and potassium citrate on markers of calcium and bone metabolism in young women, all the supplements showed some beneficial effect but only calcium carbonate decreased bone resorption relative to the control session (40). It is likely that calcium carbonate and calcium citrate may have different effects on calcium and bone metabolism and a recent 2-year trial showed that potassium citrate supplementation did not affect bone turnover (41). Overall, the present study shows that a bicarbonate-based alkaline formula, when administered to a dose amenable to clinical use, may significantly protect bone structure in exercising aged animals to a greater extent than a quali/quantitavely similar citrate-based formula. Whether the positive effect of an alkali administration persists with moderate protein consumption is unknown (42) and a matter of further research.

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