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Long-Term Intake of a High-Protein Diet with or without Potassium Citrate Modulates Acid-Base Metabolism, but Not Bone Status, in Male Rats 1

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1 **Long-term intake of a high protein diet with or without potassium citrate**
2 **modulates acid-base metabolism in male rats, but not bone status.**

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13 **Running title:** High-protein intake and bone health

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19
20 Abbreviations: BMD, bone mineral density; DPD, deoxypyridinoline; HP, high protein diet;
21 HPKcit, high protein diet supplemented with potassium citrate; IGF-1: Insulin-like growth
22 factor 1; NP, normal protein diet; NPKcit, normal protein diet supplemented with potassium
23 citrate; OC, osteocalcin.

24

25 **Abstract**

26 High dietary protein intake generates a substantial fixed acidity, which may adversely
27 affect bone health. Alkaline potassium citrate (Kcit) may contribute to the neutralization of
28 the protein-induced metabolic acidosis. We investigated the impact of two levels of protein
29 intake and Kcit supplementation on acid-base metabolism and bone status in rats. Two
30 months-old Wistar male rats were randomly assigned to four groups (n=30 per group). Two
31 groups received a normal-protein content (13 %) (NP) or a high-protein content diet (26 %)
32 (HP) for 19 months. The two other groups received identical diets supplemented with Kcit
33 (3.60 %) (NPKcit and HPKcit). Rats were pair-fed on the *ad libitum* intake of the HP group.
34 At 9, 16 and 21 mo of age, 10 rats of each group were killed. The HP diet induced a metabolic
35 acidosis characterized by hypercalciuria, hypermagnesuria and hypocitraturia at all ages. Kcit
36 supplementation neutralized this effect, as evidenced by decreased urinary calcium and
37 magnesium excretion by the HPKcit rats. Femoral bone mineral density, biomechanical
38 properties, bone metabolism biomarkers (osteocalcin and deoxypyridinoline) and plasma IGF-
39 1 levels were not affected by the different diets. Nevertheless, at 21 mo of age, calcium
40 retention was reduced in the HP group. This study suggests that lifelong excess of dietary
41 protein results in low-grade metabolic acidosis without affecting the skeleton, which may be
42 protected by an adequate calcium supply.

43

44

45 **Introduction**

46 Compared to “optimum” nutritional requirements, which are genetically determined,
47 Western diets are characterized by an excessive protein intake, frequently associated with
48 insufficient fruit and vegetable consumption. It has been suggested that this mismatch could
49 lead to a number of pathological conditions (1).

50 In particular, an imbalanced protein-potassium ratio results in low-grade metabolic
51 acidosis (2). Protein-rich diets are considered acidogenic due to the excess of sulphuric anions
52 produced by the catabolism of sulphur amino acids. Moreover, a low fruit and vegetable
53 intake results in decreased mineral and organic anion provision, including potassium citrate
54 and malate. Thus, the availability of alkaline organic salts potentially is insufficient to
55 neutralize the endogenous acid production associated with excessive protein intake.

56 To counteract the excess dietary acid load, the homeostatic systems excrete increased
57 amounts of urinary calcium and phosphorus (3,4). Hypercalciuria can have multiple
58 pathological consequences (5), including nephrolithiasis, and it may contribute to the
59 pathogenesis of osteoporosis. Acidosis promotes continual release of alkaline calcium salts
60 from the skeleton, to maintain the acid-base balance (6,7), and this may result in bone loss in
61 the long-term. Indeed, metabolic acidosis is associated with lower bone mineral density in
62 women (8) and rats (9). In an *in vitro* model for metabolic acidosis, significant bone calcium
63 efflux was observed (10) and cell function was impaired as evidenced by stimulation of
64 osteoclastic bone resorption and inhibition of osteoblastic formation (11,12).

65 Similarly, high protein intake and the associated acidosis are considered potentially
66 detrimental for bone health in humans (13,14) and rats (15). However, in contrast to the well-
67 characterized effects of dietary protein on urinary calcium excretion, the effect on bone status
68 needs further insight. Actually, the impact of dietary protein on bone remains somewhat
69 controversial as several reports have emphasized that high protein intake benefits bone health

70 (16-18). Indeed, protein intake may favourably influence bone metabolism by stimulating the
71 anabolic action of IGF-1 (Insulin-like Growth Factor 1) on bone (19).

72 Fruit and vegetable intake provides potassium salts, especially potassium citrate.
73 Previous investigations have reported beneficial effects of dietary potassium and potassium-
74 rich foods (fruits and vegetables) on bone health (20-23). Hypothesizing that a high-protein
75 diet could induce osteopenia, potassium citrate intake would neutralize the diet-induced
76 metabolic acidosis, and may be associated with a higher bone mass (24).

77 Thus, further investigations with more long-term outcomes would be needed to
78 completely define the role of high protein intake and acid-base balance in age-related bone
79 loss. The goal of this study was to carefully identify the long-term effects of protein content in
80 the diet on bone status and to test if alkaline-forming supplements had favourable effects on
81 bone health. Using rats as a model, the study was designed to address two specific questions:

- 82 i) Does a long-term high-protein diet (HP: 26%) have beneficial or detrimental
83 effects on bone status when compared to a normal-protein diet (NP: 13%).
- 84 ii) Does potassium citrate supplementation (Kcit) modulate the bone response to
85 dietary protein intake.

86

87 **Materials and methods**

88 *Animals and experimental design*

89 The study was conducted in accordance with the regional Ethics Committee for animal
90 experiments, in France. One hundred and twenty male Wistar rats (2 mo old, mean weight
91 316 g) were purchased from INRA (Institut National de la Recherche Agronomique,
92 Clermont-Ferrand/Theix, France). The rats were housed individually in plastic cages at 25°C,
93 in 12/12 h light/dark cycles and had free access to water. Body weight and food intake were

94 measured weekly. Male rats were chosen in order to eliminate the potential confounding
95 effect of hormonal fluctuations.

96 At the beginning of the study, the rats were randomly assigned to four groups (n=30).
97 Two groups received diets containing either a normal protein content (13 % (g/100g)) (NP) or
98 a high protein content (26 %) (HP). The other two groups received identical diets
99 supplemented with potassium citrate (3.60 %) (NPKcit and HPKcit) (**Table 1**). All diets were
100 purchased from INRA (Jouy en Josas, France). All rats were fed a measured amount of diet,
101 corresponding to 90-95 % of the mean *ad libitum* intake of the HP groups, which had the
102 lowest food intake. The pair-feeding allowed us to energy match the food intake, providing
103 healthy, non-obese control animals for the study (25). All diets contained the same amount of
104 lipid, fiber, vitamins and minerals, excepting potassium (0.36 % vs. 1.66 % in supplemented
105 groups). Thus, all rats consumed similar amounts of dietary calcium (0.25 %) and phosphorus
106 (0.20 %) (Ca/P ratio=1.25).

107 At 9, 16 and 21 months of age, 10 rats from each group were killed. One week before
108 the end of the study, the rodents were transferred to individual metabolic cages specially
109 designed for rats. After a three-day adaptation period, food intake was controlled daily, and
110 urine and faeces were collected for three consecutive days. At the end of the experiment, the
111 rats were food-deprived for 12 h then killed by injection of a lethal dose of anaesthetic
112 (Imalgen 1000, Merial, Lyon, France; 0,75mL/kg bw and Vetranquil 1%, Ceva santé animale,
113 Libourne, France; 0,25mL/kg bw). Blood samples were collected from the abdominal aorta
114 and frozen at -20°C until analysis. Femurs were cleaned from adjacent tissues. Left femurs
115 were harvested in saline solution (9 g NaCl/L) and frozen (-20°C) until mechanical testing.
116 Right femurs were kept in 80 % alcohol until bone mineral density was measured.

117

118 *Biochemical analyses*

119 *Minerals.* Urine samples were appropriately diluted in lanthanum oxide solution.
120 Urinary calcium and magnesium concentrations were determined by atomic absorption
121 spectrophotometry (AA800, Perkin Elmer, Norwalk, CT, USA). Sodium and potassium
122 concentrations were measured by atomic emission spectrophotometry. 200 mg of dried
123 samples (food and faeces) were dried-ashed (10 h at 500°C) and extracted until discoloration
124 at 130°C in HNO₃-H₂O₂ (2:1) (Suprapur; Merck, Darmstadt, Germany). For calcium
125 determination by atomic absorption spectroscopy, the final dilution was performed with
126 lanthanum oxide. Calcium retention ($\mu\text{mol} / 24 \text{ h}$), defined as [calcium intake – (urinary
127 calcium excretion + faecal calcium excretion)] was calculated.

128 *Organic anions.* Urine was diluted 400-fold with milli-Q water. Chloride, phosphate,
129 sulphate and citrate concentrations were determined by ionic chromatography (DX320,
130 Dionex, Sunnydale, CA, USA). The anions were separated on a 4 × 250 mm AS 11 column /
131 AG 11 precolumn (flow rate 1 mL/min). An EG40 eluent generator controlled the elution
132 using an OH⁻ gradient (0.5 to 35 mmol/L in 20 min) and the conductimetry detector was
133 preceded by an ASRS self-regenerating suppressor.

134 *IGF-1.* Serum insulin-like growth factor I concentrations were measured using a two-
135 site immunoassay (OCTEIA Rat/Mouse IGF-1 kit, IDS, Paris, France). The
136 sensitivity of the assay was 82 $\mu\text{g/L}$. Intra- and interassay variations were 5.7 % and 10.7 %,
137 respectively.

138 *PTH.* Plasma parathyroid hormone concentrations were determined using an
139 immunoradiometric assay kit (Immunotopics, Inc., San Clemente, CA, USA). The sensitivity
140 was 1.0 ng/L. The intra- and interassay variations were 4.1 % and 4.5 %, respectively.

141 *Bone biomarkers.* Plasma osteocalcin (OC) concentrations were measured by an
142 immunoradiometric assay (Immunotopics, Inc., San Clemente, CA, USA). The sensitivity was
143 0.01 $\mu\text{g/L}$. The intra- and interassay variations were 2.0 % and 4.5 %, respectively. The
144 urinary deoxypyridinoline (DPD) concentration (nmol/L) was determined using a
145 radioimmunoassay (Pyrilinks-D RIA kit, Metra Biosystems, Mountain View, CA, USA). The
146 sensitivity was 2 nmol/L. The intra- and interassay variations were 4 and 6%, respectively.
147 DPD excretion rates (nmol/24 h) were calculated using the prior 24-hour urine volumes.

148

149 *Physical measurements*

150 *Bone mineral density (BMD).* BMD was assessed by dual-energy X-ray
151 absorptiometry (DEXA), using a Hologic QDR-4500 A X-ray bone densitometer (Hologic,
152 Massy, France). Total femoral BMD (T-BMD), metaphyseal BMD (M-BMD) and diaphyseal

153 BMD (D-BMD) were determined. For M-BMD and D-BMD measurements, scans were cut
154 and analyzed as follow: the first cut of the femur was performed at the upper quarter, and the
155 next cut at the lower quarter. D-BMD corresponded to the density of the second and the third
156 quarter of the femur. M-BMD was calculated as the mean of the upper and the lower quarters
157 of the femur (26).

158 *Mechanical testing of femurs.* Femoral length and mean diaphyseal diameter were
159 measured with a precision calliper (Mitutoyo, Shropshire, UK). Because of the irregular shape
160 of the femoral diaphysis, the femoral diameter used in the calculation was the mean of the
161 greatest and smallest femoral diaphyseal diameters. The femoral failure load was determined
162 using a 3-point bending test (27), with a Universal Testing Machine (Instron 4501, Instron,
163 Canton, MA, USA). The two lower supports were separated by a 20 mm distance and an
164 upper crosshead roller (diameter, 6 mm) was applied in front of the middle of the bone at a
165 speed of 0.5 mm/min until failure, to guarantee that 85-90 % of the bone flexure was due to
166 bending. Load (Newtons) at rupture was recorded.

167

168 *Statistical analyses*

169 Results are expressed as mean \pm standard error of mean (SEM) and were analyzed
170 with XLSTAT (Addinsoft, Paris, France). The sample size was based on a previous study of
171 the effect of protein intake on BMD in 8 months-old male rats (28) followed during 7 months,
172 which used 7 rats per group. Given the duration of the present study, we used 10 rats per
173 group. The normality of the data was verified. All variables were subjected to a three-way
174 analysis of variance (ANOVA), testing for any difference among the groups. Thus, the main
175 effects assessed were protein intake (NP / HP), potassium citrate supplementation (Kcit), age
176 (9, 16 and 21 mo) and their interactions. If a result was statistically significant ($P<0.05$), the
177 Student-Newman-Keuls multiple comparison test was used to determine specific differences
178 between means. Linear regressions were performed to study internal correlations among
179 variables and the Pearson test was carried out to assess their significance.

180

181 **Results**

182 *Body weight and dietary intake.* The body weight of all rats increased between 9 and
183 16 mo of age ($P=0.01$) (**Table 2**). At 21 months of age, body weight appeared to decrease
184 (non significant). There was no difference in body weight between the four groups, whatever

185 the level of protein or the supplementation of potassium citrate in the diet. The mean dietary
186 intake did not vary among the four groups, due to the pair-feeding, and it was 22.1 ± 0.9 g,
187 22.4 ± 1.2 g and 21.0 ± 1.3 g at 9, 16 and 21 months of age, respectively.

188 *Urinary cation excretion.* After 9 months, the urine pH of rats adapted to the NP diet
189 was slightly acidic, whereas it was markedly lower in rats adapted to the HP diet ($P < 0.0001$)
190 (Table 2). The urine pH of rats fed the diets supplemented with Kcit was around 8.8
191 regardless of dietary protein intake. With age, all groups showed urine acidification: from 9 to
192 21 months of age, the decline in pH was -0.73 in the NP group and -0.57 in the HP group. The
193 age-related decline in urine pH was more pronounced when the rats were fed the Kcit-
194 supplemented diets (-0.87 and -1.94 with NPKcit and HPKcit, respectively). As a result, the
195 urine was still alkaline in the NPKcit group, but marginally acidic in the HPKcit group. There
196 was no difference in urine sodium excretion between the groups, and age had little effect on
197 the efficiency of renal sodium excretion. The rate of potassium excretion was dependent on
198 the dietary potassium level ($P < 0.0001$) and was not affected by the dietary protein level
199 (Table 2). A decline in renal potassium excretion was observed in the 21 months old rats
200 adapted to the NP and HP diets ($P = 0.039$), and it was much less pronounced in the rats
201 adapted to the Kcit-supplemented diets. Urinary calcium (**Table 3**) and magnesium (Table 2)
202 excretion displayed similar features: (i) an increase in rats fed the HP diet when compared to
203 those fed the NP diet ($P = 0.009$ and $P = 0.089$ respectively), (ii) a blunting of this protein effect
204 when Kcit was included and (iii) increased calcium ($P < 0.082$) and magnesium ($P = 0.001$)
205 concentrations in the urine of older rats.

206 *Calcium retention.* Calcium intake was lower in all rats at 21 mo compared to 9 and 16
207 mo ($P < 0.0001$). In parallel, fecal calcium excretion increased significantly from 16 mo of age
208 ($P < 0.0001$). Fecal calcium excretion was also higher in the HP group compared to the other
209 groups at 9, 16 and 21 mo of age ($P = 0.027$). The urinary calcium excretion represented less

210 than 1% of total calcium excretion. Calcium retention decreased during the experimental
211 period (Table 3). This decrease was significant between 9 and 21 mo of age ($P<0.0001$). At
212 21 mo of age, the HP group showed a lower calcium retention rate than the NP, NPKcit and
213 HPKcit groups ($P=0.044$), whereas no differences were detected at the earlier stages.

214 *Urinary anion excretion.* As expected, rats fed potassium citrate excreted larger
215 amounts of citrate in the urine (around 12- to 16-fold more) than those who were not
216 supplemented ($P<0.0001$) (**Fig 1A**). At 9, 16 and 21 mo of age, citrate excretion was
217 modulated by the protein level in the diet ($P<0.0001$): it was lower in the HP group than in
218 the NP group and, at 9 mo, it was lower in the HPKcit group than in the NPKcit group.
219 Similar trends were observed at 16 and 21 mo of age ($P=0.021$). Urinary phosphate (**Fig 1B**)
220 and sulphate (**Fig 1C**) excretion was increased in the groups fed the high-protein diets (HP
221 and HPKcit) ($P<0.0001$), compared with those on the normal-protein diets (NP and NPKcit).
222 At 21 mo, both phosphate and sulphate excretion was significantly lower in the HP group than
223 in the HPKcit group ($P=0.001$). Chloride excretion did not vary between the groups (data not
224 shown).

225 *Bone mineral density.* Total BMD (T-BMD) did not vary between 9 and 16 mo (**Fig**
226 **2A**). M-BMD and D-BMD data are not represented since similar patterns were observed
227 between T-BMD and both M-BMD ($R=0.930$, $P<0.0001$) and D-BMD ($R=0.929$, $P<0.0001$)
228 values respectively. T-BMD ($P=0.004$), M-BMD ($P=0.004$) and D-BMD ($P=0.026$) were
229 reduced at 21 mo compared to 9 and 16 mo. The level of protein intake and potassium citrate
230 supplementation had no effect.

231 *Biomechanical properties.* Femurs from 21 mo old rats had lower resistance to
232 fracture (**Fig 2B**) than those from 9 and 16 mo old rats ($P<0.0001$). The biomechanical data
233 were positively correlated with the BMD data ($R=0.709$, $P<0.0001$). No significant
234 differences were recorded between the four groups at any age.

235 *Bone biomarkers.* Plasma OC was reduced in all groups at 16 and 21 mo ($P<0.0001$)
236 compared to 9 mo (**Fig 2C**). The urinary DPD excretion rate was similar at 9 and 16 mo of
237 age then decreased at 21 mo ($P<0.0001$) (**Fig 2D**). Both bone biomarkers were unaffected by
238 diet protein content and potassium citrate supplementation. Osteocalcin was positively
239 correlated with calcium retention ($R=0.660$, $P=0.014$).

240 *Plasma IGF-1.* Plasma IGF-1 concentrations were lower after 21 mo ($P<0.0001$),
241 compared to 9 and 16 mo old rats (data not shown). No differences were observed between
242 the groups at any age.

243 *Plasma PTH.* Plasma PTH concentrations increased at 16 and 21 mo ($P=0.001$)
244 compared to 9 mo of age. There were no significant variations between the four groups (data
245 not shown) at any age.

246

247 **Discussion**

248 A relatively high dietary protein intake can generate a substantial fixed acidity, which
249 inconsistently has been reported to adversely affect bone health (13-15). Potassium citrate
250 (Kcit) could possibly be used to neutralize this acid load. We investigated the effect of two
251 levels of dietary protein (13% or 26%), in the presence or absence of Kcit supplementation,
252 on acid-base metabolism and bone status. Male Wistar rats were used as an experimental
253 model for age-related bone loss (29) and were monitored until 21 months of age.

254

255 *Characterization of high-protein diet-induced low-grade metabolic acidosis*

256 The high-protein diet (HP) elicited an acid load, which was characterized by a
257 decreased urine pH when compared to the NP diet group at all ages (Table 2). The HP diet
258 was associated with significant calciuria (Table 3). Hypercalciuria is correlated with net acid
259 excretion (30) and is a well-known consequence of high-protein diet ingestion (31,32). In the

260 kidney, it has been attributed to an increased glomerular filtration rate and a decreased tubular
261 calcium reabsorption, which neutralizes SO_4^{2-} generated by catabolism of sulphur amino acids
262 (4). The changes in magnesium excretion were similar (30% increase between the NP and HP
263 groups). This trend is consistent with previous short-term studies and suggests that
264 magnesium, in addition to calcium, compensates for metabolic acidosis in the long-term (33).
265 Increased urinary sulphate and phosphate excretion was observed with the HP diet (Fig 1).
266 The increase in sulphate elimination resulted from a greater sulphur amino acid intake (34).
267 The higher phosphate excretion rate could be due to the presence of more organic phosphorus
268 in the HP diet (35), but impaired tubular reabsorption of phosphate (15) is another possibility.
269 Hypocitraturia was another major factor associated with dietary protein excess (Fig 1A)(36).
270 This is consistent with reports of renal acidosis resulting in decreased proximal tubular
271 reabsorption of citrate and impaired citrate transport (37,38).

272

273 *Potassium citrate supplementation affects acid-base metabolism*

274 In contrast to western diets, the NPKcit and HPKcit diets provided a relative excess of
275 organic anions (Kcit: 35.95 g/kg) compared to sulphur amino acids (around 6.9 g/kg in the NP
276 diets and 13.8 g/kg in the HP diets). The protein-potassium ratio is a reliable predictor of diet-
277 net-acid-load and these two diets were previously shown to prevent metabolic acidosis (2).

278 Indeed, potassium citrate supplementation significantly increased urine pH, consistent
279 with previous work (22,39). The administration of Kcit resulted in increased urinary
280 potassium (Table 2) and citrate excretion (Fig 1) in both the NPKcit and HPKcit group.
281 However, the 26% protein diet limited the effect of the Kcit supplementation on citraturia.
282 The Kcit neutralizing effect was further confirmed by lower urinary calcium and magnesium
283 excretion (Table 2, Table 3). The potassium citrate probably exerted its effects by neutralizing
284 endogenous acidity by KHCO_3^- generation, glutamine sparing (40,41) and modulation of

285 hepatic urea synthesis for removal of bicarbonate (42). Furthermore, potassium influences the
286 expression of the proximal tubular Na/sulphate-cotransporter. Although Kcit supplementation
287 may have restored SO_4^{2-} reabsorption through a reversal of transporter down-regulation by
288 acidosis, it increased sulphate excretion (Fig 1C). This was consistent with data from Sabboh
289 *et al.* (35) who suggested that sulphate reabsorption could not match increased generation of
290 SO_4^{2-} . Moreover, it seems the capacity of the kidney to excrete sulphate and phosphate was
291 maintained during aging with Kcit supplementation, whereas it was altered after 21 mo in the
292 NP and HP rats.

293

294 *High-protein diet and potassium citrate supplementation had no effect on bone quality*

295 The sole change reported on bone status was a marked age-related bone loss at the end
296 of the experimental period (21 mo of age) (Fig 2). Bone density changes were consistent with
297 the observed biomarker variations and resulted in decreased bone strength (Fig. 2B). These
298 alterations may be related to the decreased IGF-1 secretion, IGF-1 being a bone anabolic
299 hormone. The lower plasma IGF-1 levels probably result age-induced perturbation of the
300 hypothalamo-adenohypophysial-somatotrophic axis (43,44). Secondary hyperparathyroidism
301 has been reported in old rats and was suggested to contribute to bone alteration. In parallel,
302 these changes were associated with impaired ability of the kidney to excrete the dietary acid
303 load with aging, and characterized by acidification of excreted urine and increased loss of
304 urinary magnesium and calcium.

305 The femoral BMD was not affected by protein intake or Kcit supplementation (Fig
306 2A). Indeed, no significant differences were recorded among the groups at any of the time
307 points. Corresponding femoral biomechanical properties remained unchanged as well (Fig
308 2B). These results are consistent with previous observations in rats by Whiting & Draper (45),
309 Calvo *et al.* (46), Goulding & Campbell (47), showing no change in femoral bone mass or

310 macro-mineral composition in response to dietary protein excess (30%, 35% and 60% excess,
311 respectively), despite hypercalciuria. In contrast, using histomorphometric analysis,
312 Amanzadeh *et al.* (15) observed bone loss in rats fed a 48% casein diet during 59 days and
313 displaying hypercalciuria and hypocitraturia.

314 The physical bone measurements were consistent with the bone biomarker levels,
315 which did not vary between the four groups (Fig 2C and Fig 2D). Yet, metabolic acidosis has
316 been demonstrated to stimulate osteoclastic resorption and inhibit osteoblastic collagen
317 synthesis *in vitro* (12,48). As far as hydroxyproline can be considered as a relevant indicator
318 of bone resorption, high protein intake was associated with increased resorption levels
319 (32,47,49). However, Creedon & Cashman (50) and Shapses *et al.* (51) reported no effect of
320 high-protein diets on urinary excretion of collagen pyridinium crosslinks in rats and human
321 subjects. In this study, the absence of variation in bone metabolism can be attributed to stable
322 circulating PTH levels, a potent stimulator of bone resorption.

323 Thus, all examined bone parameters concur to conclude that long-term low-grade
324 metabolic acidosis, induced by a slightly hyperproteic diet (26% casein), does not affect the
325 skeleton. However, it would be interesting to repeat the analysis with female rats that are
326 subject to greater hormonal variations. Actually, it appears that the effects of dietary protein
327 on bone status depend on multiple, interacting parameters (52), including additional dietary
328 factors. Protein and calcium, in particular, seem to act synergistically on human bone (53,54).
329 An adequate calcium supply, as in the present study, may reduce or offset the negative effects
330 of protein on bone mass. Here, calcium retention was not significantly affected by the high-
331 protein (26%) diet or citrate salt intake at 9 and 16 mo of age (Table 3), although calcium
332 fecal excretion was increased. Nevertheless, calcium retention was altered in the HP rats
333 compared to the NP rats after 21 mo. This was a result of both higher fecal and urinary
334 calcium excretion. Calcium retention did not vary between the NPKcit and the HPKcit

335 groups. In summary, in the older rats the HP diet had deleterious effects on calcium retention
336 and Kcit supplementation could prevent this impairment. The impact of high-protein intake
337 on calcium retention is controversial since Creedon and Kashman (50) found no effect of this
338 parameter in growing rats. Femoral BMD was unchanged in this study at 21 mo, but it is
339 conceivable that bone density would be altered at later time points. These results remain to be
340 confirmed in humans, given the differences suspected in intestinal calcium absorption.

341 The role of dietary protein in bone health is currently debated. Some authors argue that
342 high-protein diets diminish bone health (13,55), whereas others speculate that it is favourable
343 for the skeleton (17,56). Considering that dietary protein supplies the amino acid building
344 blocks for the bone matrix and stimulates the IGF-1 system, it may have a magnitude-
345 dependent anabolic effect on bone. We did not observe any differences in plasma IGF-1 levels
346 between the four groups. However, IGF-1 is an osteotropic factor and it was previously
347 shown to be regulated by nutritional status, especially dietary protein intake (44).
348 Inappropriate protein intake (2.5 % casein diet) impairs the IGF-1 system and has been
349 suggested to lead to decreased bone mineral mass and fragility in rats (57). The absence of
350 variation in IGF-1 levels in this study is consistent with the BMD values, and probably
351 reflects that our diets were less restrictive, particularly with respect to protein content.

352

353 In conclusion, the high-protein diet induced a low-grade metabolic acidosis, which
354 persisted over the entire duration of the experiment. Potassium citrate supplementation had
355 alkalinizing effects and neutralized the increased acid load. Neither beneficial nor detrimental
356 effects associated with high-protein intake or potassium citrate supplementation was reported
357 on bone status or metabolism under our experimental conditions. This could be explained by
358 an adequate calcium supply because calcium intake may offset the effects of a protein-
359 generated acid load on bone mass.

360

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503

504

505 Table 1. Diet compositions.

Component (g/100 g)	NP	HP	NPKcit	HPKcit
Casein	13.0	26.0	13.0	26.0
Corn starch	57.7	47.1	54.8	44.2
Sucrose	14.4	11.8	13.7	11.1
Groundnut and rape oils	6.0	6.0	6.0	6.0
Cellulose	4.0	4.0	4.0	4.0
Potassium citrate	0	0	3.60	3.60
L-cystin	0.19	0.39	0.19	0.39
Choline bitartrate	0.25	0.25	0.25	0.25
Mineral mix ¹	3.50	3.50	3.50	3.50
Vitamin mix ²	1.00	1.00	1.00	1.00

506

¹ Expressed in g/kg of mixture: CaCO₃, 178.5; KH₂PO₄, 250; K₃C₆H₅O₇, 28.00; NaCl,

507

74; K₂SO₄, 46.60; MgO, 24.00; FeC₆H₆O₇: 6.06; ZnCO₃: 1.65; Na₂SiO₃•9H₂O, 1.45;

508

MnCO₃, 0.63; CuCO₃, 0.30; CrK₈S₂•12H₂O, 0.275; H₃BO₃, 0.0815; FNa, 0.0635;

509

NiCO₃, 0.0318; LiCl, 0.0174; Na₂SeO₄, 0.01025; KIO₃, 0.010; (NH₄)₆Mo₇O₂₄•4H₂O,

510

0.00795; NH₄VO₃, 0.0066.

511

² Expressed in g/kg of mixture : nicotinic acid, 3.000; calcium pantothenate, 1.600;

512

pyridoxine-HCL, 0.700; thiamine-HCL, 0.600; riboflavin, 0.6000; folic acid, 0.200;

513

biotin, 0.020; cyanocobalamin, 2.500; all-rac- α -tocopheryl acetate (500 IU/g), 5.000;

514

all-trans-retinyl palmitate (500000 IU/g), 0.800; cholecalciferol (400000 IU/g), 0.083;

515

phyloquinone, 0.075.

516

517 Table 2. Body weight, urine pH, urinary potassium, sodium and magnesium excretion in rats fed a
 518 normal protein diet, a high protein diet, a normal protein potassium citrate supplemented diet and a
 519 high protein potassium citrate supplemented diet for 9, 16 and 21 months.

520

		NP	HP	NPKcit	HPKcit	Statistical significance, <i>P</i> values :
Body weight (g)	9 mo	640.8 ± 20.0	651.2 ± 14.9	642.8 ± 16.2	653.9 ± 15.8	Age: <i>P</i> =0.01
	16 mo	719.1 [†] ± 29.3	732.0 [†] ± 33.4	724.2 [†] ± 32.3	734.1 [†] ± 27.4	
	21 mo	692.4 [†] ± 17.3	696.2 [†] ± 23.8	688.1 [†] ± 15.3	704.3 [†] ± 18.8	
Urinary parameters						
pH	9 mo	6.68 ^b ± 0.19	6.12 ^c ± 0.21	8.76 ^a ± 0.07	8.82 ^a ± 0.12	Age: <i>P</i> <0.0001 Kcit: <i>P</i> <0.0001
	16 mo	6.18 ^c ± 0.14	5.72 ^{d†} ± 0.05	8.37 ^{a†} ± 0.06	7.26 ^{b†} ± 0.47	Protein: <i>P</i> <0.0001 Age x Protein: <i>P</i> =0.03
	21 mo	5.95 ^{c†} ± 0.03	5.55 ^{d*} ± 0.04	7.89 ^{a*} ± 0.23	6.88 ^{b*} ± 0.16	Age x Kcit: <i>P</i> =0.0001 Protein x Kcit: <i>P</i> =0.0001
potassium (mmol/24 h)	9 mo	2.109 ^b ± 0.126	2.316 ^b ± 0.072	8.568 ^a ± 0.385	8.160 ^a ± 0.534	Age: <i>P</i> <0.0001
	16 mo	1.995 ^b ± 0.149	2.007 ^b ± 0.056	7.890 ^a ± 0.474	7.337 ^a ± 0.320	Kcit: <i>P</i> <0.0001
	21 mo	1.357 ^{b*} ± 0.085	1.331 ^{b*} ± 0.162	7.749 ^a ± 0.445	7.192 ^a ± 0.900	Kcit x Age: <i>P</i> =0.039
sodium (mmol/24 h)	9 mo	1.127 ± 0.072	1.221 ± 0.050	1.184 ± 0.066	1.272 ± 0.060	
	16 mo	1.124 ± 0.086	1.255 ± 0.048	1.130 ± 0.071	1.163 ± 0.083	
	21 mo	1.122 ± 0.080	1.180 ± 0.097	1.151 ± 0.086	1.093 ± 0.104	
magnesium (μmol/24 h)	9 mo	44.03 ± 7.00	57.37 ± 6.58	47.74 ± 8.64	49.38 ± 6.58	Age: <i>P</i> =0.001
	16 mo	68.72 [†] ± 6.58	78.89 [†] ± 2.06	62.55 [†] ± 5.35	70.37 [†] ± 7.00	
	21 mo	66.25 [†] ± 7.82	78.24 [†] ± 8.64	60.49 [†] ± 4.53	71.19 [†] ± 4.53	

521

522 Values are expressed as means±SEM, n=10 per group.

523 *: significantly different from 9 and 16 mo of age.

524 †: significantly different from 9 mo of age.

525 ^{a-d}: significance of difference between NP, HP, NPKcit and HPKcit groups at a given age (Means without a common letter differ,

526 *P*≤0.05).

527

528 Table 3. Calcium intake, fecal excretion, urinary excretion and retention in rats fed a normal protein
 529 diet, a high protein diet, a normal protein potassium citrate supplemented diet and a high protein
 530 potassium citrate supplemented diet for 9, 16 and 21 months.

531

		NP	HP	NPKcit	HPKcit	Statistical significance, <i>P</i> values :
Ca intake ($\mu\text{mol}/24\text{ h}$)	9 mo	1402 \pm 24	1407 \pm 21	1409 \pm 17	1396 \pm 16	Age: <i>P</i> <0.0001
	16 mo	1412 \pm 27	1417 \pm 16	1401 \pm 24	1404 \pm 25	
	21 mo	1293* \pm 9	1306* \pm 16	1291* \pm 10	1299* \pm 18	
Fecal calcium excretion ($\mu\text{mol}/24\text{ h}$)	9 mo	1158 ^b \pm 38	1267 ^a \pm 41	1206 ^b \pm 37	1173 ^b \pm 26	Age: <i>P</i> <0.0001 Protein x Kcit: <i>P</i> =0.027
	16 mo	1339 ^{b†} \pm 24	1444 ^{a†} \pm 51	1326 ^{b†} \pm 36	1329 ^{b†} \pm 53	
	21 mo	1370 ^{b†} \pm 31	1503 ^{a†} \pm 56	1350 ^{b†} \pm 48	1365 ^{b†} \pm 51	
Urinary Ca excretion ($\mu\text{mol}/24\text{ h}$)	9 mo	8.00 ^b \pm 1.50	15.25 ^a \pm 2.25	8.50 ^b \pm 1.25	9.75 ^b \pm 1.75	Protein: <i>P</i> =0.003 Kcit: <i>P</i> =0.041 Protein x Kcit: <i>P</i> =0.0009
	16 mo	9.25 ^b \pm 2.50	15.50 ^a \pm 3.25	11.25 ^b \pm 2.25	11.00 ^b \pm 1.25	
	21 mo	12.25 ^b \pm 1.25	17.00 ^a \pm 2.75	10.75 ^b \pm 2.50	12.50 ^b \pm 2.50	
Ca retention ($\mu\text{mol}/24\text{ h}$)	9 mo	236 \pm 52	105 \pm 49	199 \pm 52	214 \pm 31	Age: <i>P</i> <0.0001 Age x Protein x Kcit: <i>P</i> =0.044
	16 mo	63 \pm 41	-32.5 \pm 56	64 \pm 53	64 \pm 80	
	21 mo	-89 ^{a†} \pm 51	-214 ^{b†} \pm 42	-70 ^{a†} \pm 55	-78 ^{a†} \pm 79	

532 Values are expressed as means \pm SEM, n=10 per group.

533 *: significantly different from 9 and 16 mo of age.

534 †: significantly different from 9 mo of age.

535 ^{a-d}: significance of difference between NP, HP, NPKcit and HPKcit groups at a given age (Means without a common letter differ,

536 *P*≤0.05).

537

538 Figure 1. Effect of protein intake and potassium citrate supplementation on urinary citrate (A),
539 phosphate (B) and sulphate (C) excretion (mmol/24 h) at 9, 16 and 21 mo of age. Values are
540 expressed as means±SEM, n=10 per group.

541 *: significantly different from 9 and 16 mo of age

542 a-d: significance of difference between NP, HP, NPKcit and HPKcit groups at a given age (Means
543 without a common letter differ, P≤0.05).

544

545

546 Figure 2. Effect of protein intake and potassium citrate supplementation on total femoral BMD (A),
547 femoral biomechanical resistance (B), plasma osteocalcin (C) and the urinary DPD excretion rate (D)
548 at 9, 16 and 21 mo of age. Values are expressed as means±SEM, n=10 per group.

549 *: significantly different from 9 and 16 mo of age

550 †: significantly different from 9 mo of age

551

552