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# THE EFFECT OF CHRONIC ACIDOSIS ON BONE COMPOSITION IN ADULT MALE AND FEMALE RATS

DARINKA DEKANIĆ

*Institute for Medical Research and Occupational Health, Zagreb*

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Four-month old male and female rats received a diet supplemented with 1. 98 per cent of calcium chloride over a period of eight months. Bone changes were analysed by determining weight, volume, density, ash, organic and marrow content of the femur. In chronic acidosis the bone changes observed were generally identical in male and female rats. A development of bones similar in dimensions to those of the control animals but deficient in mineral component was noted. In females this effect of chronic acidosis was still clearly evident after the determination of parameters commonly used to estimate bone composition while in male rats the effect was less obvious. As regards the bone marrow content and bone density sex differences were more evident in the acid loaded than in the control animals. The advantage of using bone without marrow substance is presented.

Skeletal demineralization in chronic acidosis in human beings has been well documented[1—5]. Direct experimental evidence has been provided to the effect that an excessive amount of acidifying salts caused increased resorption of bone in adult rats[6,7]. However, the question whether the bone loss caused by chronic acidosis was due to a proportional loss of organic and mineral components as found by *Barzel and Jowsey*[7] in adult male rats or whether the loss of mineral component was greater as established by *Delling* and *Donath*[8] in female rats has as yet not been cleared. On the other hand *Newell* and *Beauchene*[9] reported that acid stress did not significantly affect bone composition in rats.

Since the response to chronic acidosis has been suggested as a possible etiologic factor of osteoporosis[10], a parallel study of bone mineralization in male and female rats in chronic acidosis has been made in this investigation.

## MATERIAL AND METHODS

### *Animals and diet*

Four-month old male and female albino rats of our own strain were used in this study. From the weaning to the beginning of the experiment the animals were fed a basic-stock diet (»Pliva«, Pharmaceutical Works, Zagreb) composed as follows (in g/100 g wet diet):

| | |
|---|---|
| crude proteins | 19.81 |
| crude fat | 5.16 |
| crude fibers | 2.73 |
| carbohydrates | 55.92 |
| Ca | 1.00 |
| P | 0.70 |
| NaCl | 0.90 |
| $D_3$-vitamin (i. u.) | 200 |
| Fe | 0.04 |

The control diet with high (2 per cent) calcium content was produced by adding 4.3 per cent of calcium hydrogen orto-phosphate dihydrate ($CaHPO_4 \cdot 2H_2O$) to the basic diet. The experimental acid loaded, high calcium diet was produced by adding 1.98 cent of calcium chloride ($CaCl_2 \cdot 2H_2O$) to the basic diet. These diets were fed to the animals for a period of eight months following which they were killed under aether anaesthesia.

### *Preparation of bone samples*

The right femur was dissected and thorough cleaning removing all traces of superficial soft tissues was accomplished by scraping with a sharp blade. The nutrient foramen was enlarged by means of a hand borer to a diameter of 1—2 mm and the available marrow substance was removed from the femur by centrifugal force (10 000 × g for 30 min.) as described previously[11,12]. The use of a femur freed of marrow permits direct determination of the actual volume of the space which was occupied by the marrow according to the method of *Robinson* and *Elliott*[13] and modified by *Mueller* and co-workers[14]. On the other hand it permits calculations of femur density with and without marrow substance by summing up fractional volumes (g/cm³) occupied by ash, organic matter, absorbed water and marrow.

*Treatment of bone samples*

The femur was placed in cold distilled water (4° C) under vacuum 5-7 mm Hg) for about one hour to allow the air to be replaced by water. Upon return to ambient atmospheric pressure the specimen was allowed to stand for at least one hour before analysis to ensure sufficient time for the water to replace the space left by the removal of gas. All femora were individually weighed under the following conditions:

— after cleaning of the soft tissue - wet weight;

— after removal of the marrow substance - wet weight without marrow;

— submerged in water after degassing - submerged weight;

— in air after rapid removal from the water - fully hydrated weight (this value is the average of ten weightings of the same sample);

— after being wrapped in filter paper and centrifuged at 10 000 x g for 30 min. - hydrated weight;

— after having been dried for 36 hours in a drying oven set at 105° C

— dry weight;

— after ashing in a muffle furnace 600° C for 36 hours - ash weight.

*Calculations*

The calculation of the femur volume was based on Archimedes's principle as the difference between the weight in the air (fully hydrated weight) and the submerged weight. The weight of the marrow substance was determined from the difference in total femur wet weight and wet weight without marrow, while the volume of the femur marrow space was calculated according to the method of Mueller and co-workers[14] which previously was used only for the determination of marrow space in bone slices. The weight of organic matter was calculated as the difference between dry weight and ash weight. With all these data fractional volumes of the femur occupied by ash, organic matter and marrow were calculated in addition to densities of femur with and without marrow.

The results are presented as the arithmetic mean and standard errors. The significance of the difference between the groups was tested by the Student's t-test. The level of 5 per cent was taken as the limit of significance.

## RESULTS

The acid loaded diet caused in male and female rats a significant decrease in body weight (Table 1) while femur dimensions (volume, weight) were reduced but never significantly. Femur ash in females receving

animals, while in $CaCl_2$-treated males compared to their sex matched controls the reduction of femur mineral was only marginally significant. The amount of organic matter, marrow substance and volume of marrow space were similar in control and acid loaded animals of both sexes. The well-known sexual differences in bone dimensions[15—17] for age matched males and females mirroring the difference in body weight were once again confirmed in this study (Table 1) for the control animals as well as for the $CaCl_2$-treated animals.

Table 1.

*Body weight, weight and volume of femur and its components (mean of 12 animals ± SE) in control and acid loaded male and female rats.*

| | Male | | Female | |
|---|---|---|---|---|
| | Control animals | Acid loaded animals | Control animals | Acid loaded animals |
| Body weight wet (without) | 406.00 ± 11.00$^{c}$* | 379.00 ± 7.00* | 270.00 ± 8.00$^{c}$ | 242.00 ± 4.00 |
| Femur weights (mg) | | | | |
| marrow) | 812.05 ± 26.34$^{n}$* | 785.56 ± 19.19* | 604.39 ± 20.53$^{n}$ | 568.87 ± 17.00 |
| dry | 668.87 ± 22.73$^{n}$* | 638.05 ± 16.88* | 485.49 ± 15.69$^{n}$ | 436.65 ± 13.88 |
| ash | 483.33 ± 16.35$^{m}$* | 456.41 ± 12.15* | 348.58 ± 11.35$^{b}$ | 308.92 ± 9.59 |
| organic matter | 183.20 ± 5.71$^{n}$* | 181.64 ± 4.80* | 136.92 ± 4.43$^{n}$ | 134.15 ± 4.14 |
| marrow | 164.24 ± 3.05$^{n}$* | 162.24 ± 9.07* | 104.00 ± 3.71$^{n}$ | 104.77 ± 8.02 |
| Femur volumes ($mm^3$) | | | | |
| femur | 654.29 ± 13.38$^{n}$* | 643.90 ± 15.69* | 482.21 ± 14.06$^{n}$ | 458.76 ± 10.96 |
| marrow | 227.68 ± 2.28$^{n}$* | 225.34 ± 10.04* | 168.99 ± 5.43$^{n}$ | 168.83 ± 5.72 |

$^{b}$, $^{c}$ Statistically significant differences between control and acid loaded group at 2 and 1 per cent level respectively.

$^{n}$ Insignificant ($P>0.1$), $^{m}$marginally significant ($P>0.05$).

* Statistically significant differences between sexes for control groups and acid loaded groups at 0.1 per cent level.

The effect of acid stress on femur density is presented graphically in Fig. 1. Generally the values for femur density with and without marrow substance were lower in acid loaded animals of both sexes. However, in males the differences in femur density with and without marrow sub-

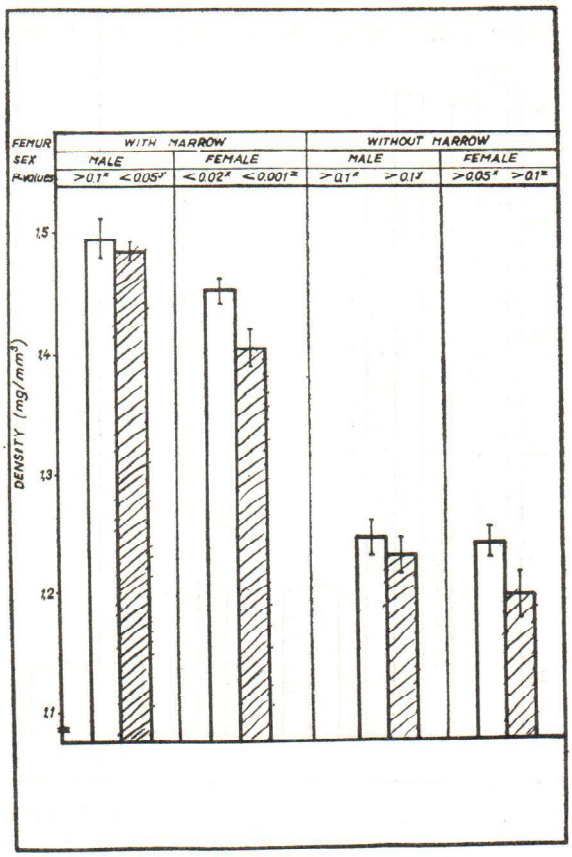


Fig. 1. *The density of femur with and without marrow substance in control (squares) and acid loaded (hatched squares) male and female rats. Values are means of 12 animals ± SE. *Differences in arithmetic means tested by Student's t-test: xbetween control and acid loaded group; ybetween sexes for control groups; zbetween sexes for acid loaded groups.*

stance between control animals and acid loaded animals were insignificant, while in females fed $CaCl_2$ the density of femur with the marrow substance was significantly lower, and marginally lower in femur without marrow substance in comparison with control female rats.

Sex-related differences in femur density, as previously reported from this laboratory[12] were found again in control animals and in acid loaded rats in bone containing the marrow and were highly significant while density of femur freed of marrow substance was also lower in females than in males but never significantly.

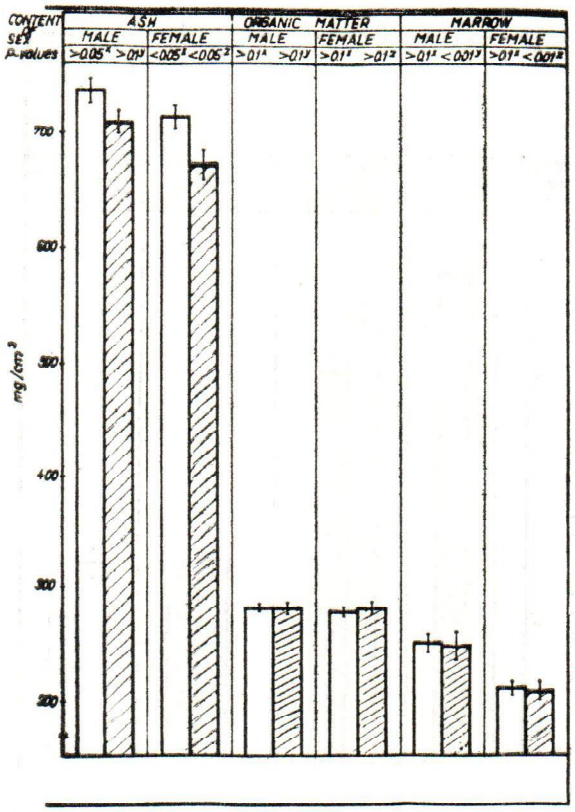


Fig. 2. *The fractional volumes of femur (mg/cm³) made up by ash organic matter and marrow substance in control (squares) and acid loated (hatched squares) male and female rats. Values are means of animals ± SE. *Differences in arithmetic means tested by Student's t-test: xbetween control and acid loaded group; ybetween sexes for control groups; zbetween sexes for acid loaded groups.*

The fractional volumes of femur (mg/cm³) occupied by its main compoments are shown in diagramatic form in Fig. 2. The acid loated diet caused a significant reduction of femur ash content in females while compared to their sex matched controls this reduction was marginally significant in males. Control males and females did not differ significantly in femur ash content, while acid loaded males showed significantly more ash per unit bone volume than identically treated females. The organic content of the femur was essentially identical in all groups. The marrow content was not significantly affected in males or females by the diet loaded with $CaCl_2$.

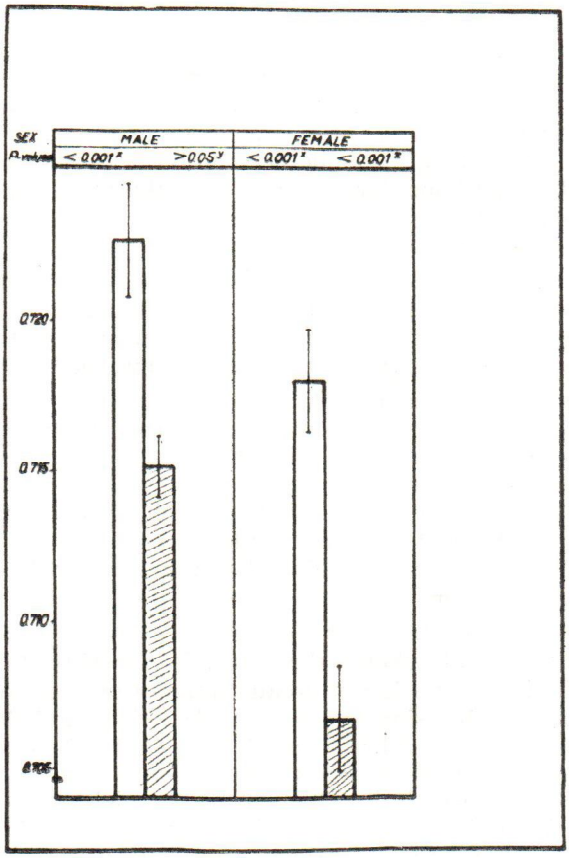


Fig. 3. *The ratio ash to dry weight in control (squares) and acid loaded (hatched squares) male and female rats. Values are means of 12 animals ± SE. *Differences in arithmetic means tested by Student's t-test: ˣbetween control and acid loaded group; ʸbetween sexes for control groups; ᶻbetween sexes for acid loaded groups.*

However, sex differences of high significance for this parameter were found again both in controls and animals receiving $CaCl_2$, as recently reported[12] for animals on basic-stock diet.

The degree of mineralization estimated by ratio of ash to dry weight (Fig. 3) has shown that chronic acidosis caused significantly higher removal of bone mineral than the organic bone component in both sexes. When this ratio was compared in control males and females the marginal level of significance was established while in acid loaded rats the ratio was significantly lower in female than in male animals.

## DISCUSSION

In chronic acidosis the observed bone changes are generally identical in male and female rats. It was noted that the development of bones was similar in dimensions to those of the control animals but deficient in mineral component. In females this effect of acidosis was clearly evident and statistically significant after determination of parameters ordinarily used to establish the bone composition (i. e. the weight of ash and organic component and the fractional volumes of these components) while in males the effect (if estimated by the parameters mentioned above) was less obvious. When degree of mineralization was compared on the basis of a more sensitive parameter i. e. ratio of ash to dry weight, the same high level of significance was found after $CaCl_2$ treatment in male and in female rats as compared to their sex matched controls.

A diet with a high level of calcium was given to the animals because preliminary results in this laboratory indicated that with such a high level of dietary calcium a higher degree of mineralization of bone matrix might be achieved in comparison to animals fed the basic-stock diet. However, even with this high level of calcium in the diet the loss of mineral due to chronic acidosis could not be prevented as previously noted by *Barzel*[6] and *Barzel* and *Jowsey*[7] with several lower levels of dietary calcium.

A characteristic bone lesion in spontaneous human renal acidosis is osteomalacia. This study shows that chronic experimental acidosis in rats also results in a condition of greater amount of organic matter than mineral. In their study using female animals *Delling* and *Donath*[8] stated on the basis of an electron-microscopical analysis that bone loss in chronic acidosis was due to increased resorption on bone surfaces and to increased osteocytic osteolysis. This statement might be postulated for both sexes on the basis of this study. However, the possible direct effect of metabolic acid-base disorders on bone cell function will have to be fully examined.

It is interesting to note that sex differences with regard to bone marrow content and bone density with and without marrow substance, which were recently reported[12] for the first time, were more evident in acid loaded animals.

Subsequently the results of this study showed that even with simple physico-chemical methods the differences in bone mineralization could be successfully tested. Adequate parameters should be used and the bone sample should be prepared to ensure that the organic substance taken into account is only that of bone tissue origin, as has been done in this study by using the femora freed of marrow substance.

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*Sažetak*

## UTJECAJ KRONIČNE ACIDOZE NA SASTAV KOSTI ODRASLIH MUŽJAKA I ŽENKI ŠTAKORA

U pokus su uzeti četveromjesečni mužjaci i ženke bijelog štakora. Životinje su osam mjeseci primale hranu s dodatkom 1,98% kalcijeva klorida. Promjene u kostima uzrokovane kroničnom acidozom određene su na osnovi mjerenja težine, volumena, gustoće te sadržaja pepela organske tvari i srži u femuru i bile su općenito uzevši podjednake u mužjaka i u ženki. U oba spola životinje u kroničnoj acidozi imale su kosti podjednake veličine kao i kontrolne, ali s nižim sadržajem minerala. U ženki taj je efekt kronične acidoze bio očit već poslije određivanja osnovnih parametara koji karakteriziraju sastav kosti, dok je u mužjaka on ustanovljen na osnovi specifičnijih parametara. Spolne razlike u sadržaju srži u kosti i gustoći kosti bile su jače izražene u životinja u kroničnoj acidozi nego u kontrolnih.

*Institut za medicinska istraživanja i medicinu rada, Zagreb*

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