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Skeletal Growth after Oral Administration of Demineralized Bone Matrix

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Oral administration of bone extracts obtained from bovine demineralized bone matrix to rats has a direct effect on bone metabolism, affecting bone proportions and some markers of bone formation such as bone malate dehydrogenase, serum alkaline phosphatase and serum osteocalcin. Futhermore collagen deposition, bone protein synthesis and nucleic acids content were significantly increased by the treatment.

Key words: Bone metabolism, Bone extracts, Bone growth, Oral administration. Rat.

The extraordinary capacity for growth, remodeling and regeneration of bone has been attributed to the induction of the differentiation of mesenchimal type cells into cartilage and bone, and to the proliferation of predifferentiated osteoprogenitor cells (20). The process of bone formation is regulated by hormones and systemic growth factors (EGF, FGF, etc.) acting on specific bone target cells and also by local growth factors through autocrine and paracrine mechanisms (13). These factors, generally referred as bone derived growth factors (BDGF's), apparently may modulate bone cell replication and differentiated function, which are primarily represented by changes in bone collagen synthesis (4). Bone is unique among tissues in the variety of polypeptide growth factors, which it harbors (32); however, the role of the extracellular bone matrix proteins has not been characterized fully (33). Biological activities of BDGFs have been estimated to date by using implant models (23, 29) or bone cell cultures (5, 8). In this context, this experiment evaluates the effects of oral administration of bone preparations of xenogeneic origin on rat bone metabolism.

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Materials and Methods

Freshly excised maxillary bones of young bovine animals recently killed (2-3 hours) were carefully scraped, cleaned of

soft tissue and thoroughly washed with distilled water and later dried at 38 °C until constant weight. Then, the bones were broken into fragments and pulverized in a hammer mill to particle sizes ranging from 74 to 420 μm. The bone powder was defatted with ethilether (1:2 v/v) during 60 min under continuous stirring. The resulting dried powder was demineralized in 0.6 N HCl at 2-4 °C (25 mmol/l) for 24 hours. The acid insoluble precipitate was repeatedly washed in cold distilled water and later liophilized before oral administration.

The characterization of bone extracts, included determinations of water, ash, fat and protein (23). Also hexosamine, phosphorus, DNA and RNA contents were measured (23).

A pilot study was carried out by gavaging three different doses of bone extracts (0.1, 0.25 and 0.5 g/kg) or casein (0.5 g/kg) to male Wistar rats (n = 6) weighing about 110 g, which were fed on a semipurified diet containing casein as the source of protein. Serum alkaline phosphatase was measured in order to evaluate dose-dependency on bone formation (23, 28). Also, a group of untreated rats to show the possible effects on daily body gain of the gavage, was included. The animals received daily casein as control protein or the bone extract by oral administration in aqueous vehicle by means of gavage, following a previously described dosification pattern for anabolic agents (9:00 h and 20:30 h) (19).

In a second trial, animals were divided in two groups (n = 9) and casein or bone extracts (0.5 g/kg) were administered by means of gavage. In both experiments body weights were recorded daily.

After an experimental period of 36 days, liver, gastrocnemius muscle and tibial and femoral bones of both legs were carefully excised, weighed and frozen prior to analysis. Bone hydroxyproline was determined according to WOESSNER (31) as indicator of collagen content:

$$\% \text{ hydroxyproline} \times 7.14 = \% \text{ collagen}$$

Bone malate dehydrogenase (MDH), bone lactate dehydrogenase (LDH) and MDH/LDH ratio were measured as indices of cellular metabolism (22) using the whole bone. Calcium (2), phosphorus (7) and osteocalcin (15) were determined in serum as markers of bone metabolism. Bone protein synthesis (Ks; %/day) was performed using the phenylalanine flooding dose method (21) as validated for intraperitoneal injection (17). Radiolabelled phenylalanine (50 μCi/ml) was combined with unlabelled phenylalanine (150 mM) and the rats were injected with 1 ml/100 g BW. Finally, DNA bone content was determined through a modification of Burton's method (3, 10) as an indirect index of number of cells in the tissue (6), and bone RNA, by the orcinol method (25) as a second stationary index of protein synthesis capacity (30). The data were statistically analyzed through the one tailed Student's «t» test.

Results and Discussion

Bone extract obtained from bovine maxillaries contains a high proportion of proteins (94.0 %) and low levels of water (2.0 %), ash (3,1 %) and fat (1.2 %). Other components analyzed on bone extracts were hexosamines (0.1 %), phosphorus (0.4 %) and DNA (< 1 %) and RNA (< 1 %).

The pilot study showed that no changes in growth rates could be ascribed to the route of administration. Thus, the values of body daily gain were similar in all experimental groups: untreated (6.2 ± 1.0 g/day), control of casein with gavage (6.2 ± 0.9 g/day), 0.1 g/kg of bone extracts (6.6 ± 0.8 g/day), 0.25 g/kg (6.0 ± 0.7 g/day) and 0.5 g/kg (5.8 ± 0.9 g/day). However, a statistically significant increase in serum alkaline phosphatase ($p < 0.05$) was found for the 0.5 g/kg group as compared

Table I. *Bone (tibia and femur), liver and gastrocnemius weights, expressed as percentage of body weight, in male rats orally treated during 36 days with control or bone protein (means ± SD)*

Variables (% BW)	Control	Bone protein
Liver	4.33±0.54	4.18±0.43 NS
Gastrocnemious	0.62±0.04	0.59±0.04 NS
Tibia	0.14±0.01	0.15±0.01*
Femur	0.25±0.02	0.27±0.02**

Statistics: *p < 0.05; **p < 0.01; NS, not significant.

with casein controls (624 ± 204 U/l vs 410 ± 99 U/l). Therefore, a second trial was performed by using this higher dose and more specific markers of bone metabolism were assessed.

Oral administration of bovine bone extracts to rats induced an increase in the relative weights of tibial and femoral bones without changes in liver or gastrocnemious muscle weights (table I), suggesting a possible specific effect on skeletal growth. Also, collagen deposition, as measured by the rise in bone concentration of hydroxyproline, was increased. Bone MDH and MDH/LDH measurements have been validated as indices of chondrogenesis induction (2), which were significantly increased by the treatment with bone extracts. On the other hand, an increase in serum calcium levels in the acid-insoluble bone powder treated animals was found, while a slight reduction in serum phosphorus was detected (table II). Further, the process of bone formation, assessed by serum osteocalcin, was markedly increased in those animals orally administered with bone extracts. The values of bone RNA, DNA and protein synthesis rate were higher in the demineralized matrix treated animals, as compared with controls (table II).

Xenogeneic bone formation in rats at extraskeletal sites has been repeatedly reported after subcutaneous implantation of bone preparations (23, 29, 16). On the other hand, some of these bone preparations containing local bone growth factors, induce bone formation in cell cultures (5, 8), while some systemic actions on metabolism have been observed with other growth factors, such as epidermal growth factor (EGF) (29, 11) or insulin-like growth factor (IGF) (12, 27), and also

Table II. *Bone collagen, bone dehydrogenase activities (MDH and MDH/LDH), serum calcium, phosphorus and osteocalcin, bone nucleic acids content and daily protein synthesis rates of orally administered male rats during a period of 36 days with control or bone protein (Means ± SD).*

Variables	Control	Bone protein	Statistical analysis
Bone collagen (%)	11.50 ± 2.10	13.09 ± 1.63	*
Bone MDH (U/g bone)	449.30 ± 64.60	686.70 ± 75.60	***
Bone MDH/LDH (U/U)	0.51 ± 0.06	0.64 ± 0.06	**
Serum calcium (mg/dl)	8.97 ± 1.10	10.78 ± 3.63	NS
Serum phosphorus (U/dl)	8.14 ± 1.57	7.49 ± 1.98	NS
Serum osteocalcin (U/ml)	82.50 ± 3.00	377.20 ± 153.20	**
Bone RNA (mg/g bone)	4.00 ± 0.12	4.24 ± 0.20	**
Bone DNA (mg/g bone)	5.26 ± 0.44	5.86 ± 0.49	*
Bone protein synthesis (Ks; % day)	60.20 ± 20.40	95.90 ± 35.70	*

Statistics: *p < 0.05; **p < 0.01; ***p < 0.001; NS, not significant.

by growth hormone exogenously administered to intact rats (18). Recent observations suggest that most of the active compounds inducing bone formation are polypeptides or glycoproteins (33, 14) and some homologies between inductive proteins from different species exist (24).

Our experimental results show a direct effect of some unidentified proteins (putative bone growth factors) of likely osteoblastic origin (13), when administered orally, on bone metabolism, which is in good agreement with the only report found in the literature where new bone growth was observed on aluminium oxide implant contact surfaces after oral administration of an ossein-hydroxyapatite compound to rats (26). In the light of these findings, it can be concluded that bone proteins of bovine demineralized bone matrix act increasing the protein content and protein synthesis rate in bone as assessed by the values of bone hydroxyproline and bone collagen, bone protein synthesis and bone RNA.

In this context, it is of special interest to note that several peptide hormones are known to be biologically active when they are administered orally, such as thyrotropin releasing factor (TRH), luteinizing hormone releasing factor, vasopresin or even large doses of insulin (9), in order to understand a potential effect of oral administration of extracellular matrix derived proteins. Furthermore, osteoblastic low molecular-weight peptides of 770 daltons obtained from bone extracts have been recently shown to stimulate osteoblast mitogenesis (1), which could cross the intestinal wall without change.

Further experiments are needed in order to evaluate the nature of the bone growth factors involved and the possible influence of the length of treatment, the age and sex of animals, etc. which may have important biological implications and clinical applications in the treatment of bone metabolic diseases (osteoporosis, etc.) or in the healing of fractures.

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Resumen

Se muestra que la administración por vía oral de extractos óseos obtenidos a partir de maxilares de ganado vacuno tiene un efecto específico sobre el metabolismo del hueso, afectando tanto a la proporción relativa del fémur y la tibia como a algunos índices de formación ósea como la malato deshidrogenasa, la fosfatasa alcalina y la osteocalcina sérica. Además, el tratamiento con el extracto óseo aumenta el contenido en colágeno y ácidos nucleicos y la síntesis de proteína en el hueso.

Palabras clave: Metabolismo óseo, Extractos óseos, Osteogénesis, Administración oral, Rata.

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