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ORIGINAL CONTRIBUTION

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Biotin deficiency in mice is associated with decreased serum availability of insulin-like growth factor-I

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Abstract *Background* Biotin deficiency leads to decreased weight and nose-rump length in mice. *Aim of the study* The mechanisms underlying this impairment in body growth are yet unclear. Biotin restriction, however, could affect the availability of growth hormone (GH) and/or insulin like growth factor-I (IGF-I) since both hormones control body growth. We then conducted a correlative study aimed at establishing whether biotin dietary restriction is associated with decreased GH/IGF-I serum concentrations. *Methods* Levels of GH and IGF-I were measured through ELISA in serum samples of male BALB/cAnN mice fed with: 1] standard chow diet (control diet); 2] 30% egg-white biotin-deficient diet; or 3] 30% egg-white diet supplemented with 16.4 μmol biotin per kilogram (biotin sufficient diet). Relative food consumption, as adjusted per gram of body weight, was also determined. GH and IGF-I measurements were taken individually for 20 weeks beginning at the postnatal week 3, when the animals started consuming the corresponding diets. In addition, femur's weight and longitudinal growth and the organization of its growth plate were all analyzed as indicators of GH/IGF-I function. *Results* No differences in relative food consumption were observed among the three groups of mice along the experimental period that was evaluated. IGF-I serum levels, but not GH ones, were decreased in biotin deficient mice. These animals also showed decreased femur's longitudinal growth, speed of lengthening and weight gain, as well as shorter and disorganized growth plates. *Conclusions* This study shows that biotin dietary restriction is indeed associated with decreased availability of IGF-I and diminished long bone growth and elongation. These conditions could explain the impairment of longitudinal body growth previously reported in biotin deficient mice. Although cause-effect studies are still needed, we believe our results support the notion that biotin might modulate the availability of IGF-I.

Key words body growth – body size – bone growth – nutrition – vitamins – growth hormone

Abbreviations BD: Biotin deficient, BS: Biotin sufficient, GH: Somatotropin or growth hormone, IGF-I: Insulin-like growth factor I

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Introduction

Biotin is a vitamin of the B complex that functions as a cofactor of the carboxylases that catalize key steps along various biochemical pathways leading to fatty acid synthesis (Acetyl Co A carboxylases 1 and 2; ACC1, ACC2 respectively), amino acid metabolism (Propionyl and Methylcrotonyl Co A carboxylases; PCC and MCC respectively) and gluconeogenesis (Pyruvate carboxylase; PC) [13, 18]. In addition, biotin modulates cell proliferation [47], gene transcription and protein translation [9, 38, 39]. More recently it has been also implicated in DNA repair [47].

Evidence obtained in murine and other animal models suggests that biotin may also be involved in the regulation of body growth because its dietary restriction leads to decreased adult body size [1–3, 5, 13, 16, 35, 40]. The physiological processes that underlie this effect are unclear. Body growth and size, however, are complex traits controlled by the interaction of genetic and epigenetic factors [10]. Among the latter are the endocrine messengers GH and IGF-I. The coordinated actions of these hormones on diverse target tissues modulate somatic growth [4, 25, 26, 41, 44]. Accordingly, the deletion of either GH receptor gene, IGF-I gene, or both leads to decreased body size [26]. Similar results are observed following GH gene mutations [10].

The main form of GH is a protein of 22 kDa secreted into the blood stream by the somatotrophs located in the adenohypophysis. GH is an anabolic hormone that stimulates amino acid uptake in muscle cells, increases protein synthesis in several tissues and promotes longitudinal bone growth [23, 25, 33, 37]. The synthesis and secretion of GH is stimulated by the somatotropin-releasing hormone and inhibited by somatostatin; both hormones are produced/released by hypothalamic neurons [31]. GH secretion is also down regulated through negative feedback loops mediated by IGF-I [43].

The majority of the somatotrophic actions of GH are mediated by IGF-I. This last hormone is an integral component of signaling systems that control body growth and muscle metabolism [14, 15]. Levels of IGF-I in the blood are the highest in the body and strongly correlate with body size. Although the main source of plasmatic IGF-I (75%) is the liver, it is also produced by a variety of local sources, thus exerting auto/paracrine actions [25]. IGF-I is synthesized and released by hepatocytes through a constitutive secretory mechanism that is stimulated by GH serum concentrations [22, 25]. Once in circulation, the majority of the IGF-I (70–80%) is found bound to IGF-I-binding protein 3 (IGFBP3) and to the acid labile subunit (ALS); both proteins are synthesized and constitutively released by hepatocytes in response to GH [22, 25]. The association of IGF-I with IGFBP3 and ALS prolongs its half life, thus extending the period through which IGF-I exert its growth promoting actions.

Having all this information in mind, this work was aimed essentially at evaluating whether biotin restriction might be associated with altered availability of GH and/or IGF-I. Decreased availability of these hormones could in part explain the body growth impairment and the shortening of adult body size observed in rodents subjected to biotin deficiency.

Materials and methods

Mice

BALBc/AnN, 3-week-old male mice raised in the in-house animal facility at the *Instituto de Investigaciones Biomédicas* were used to carry out the experiments described in the present work. All of the animals were maintained under barrier conditions in light (12 h light/12 h dark cycles) and temperature controlled rooms having free access to food and water. The experimental protocol used in this study has been described previously [1, 2]. Briefly, six lots of mice were divided into three experimental groups. Three of these lots were used to keep records of the food consumed per animal in each experimental group for the entire duration of the experiment. The remaining three lots were use to measure all of the parameters described below at different time points along the experiment. The control groups were fed with the standard diet (F-2018S, Harlan Teklad, 3.4 Kcal/g). Mice of the biotin-deficient (BD) groups were fed with a diet lacking biotin and containing 30% dry egg white as the source of protein (TD-01363, Harlan Teklad, 3.8 Kcal/g); the egg white contains avidin, a glycoprotein that binds biotin with high affinity and impedes its absorption leading to biotin deficiency [13]. Mice of the biotin sufficient (BS) group were fed with a diet having the same composition as the biotin-deficient diet, but supplemented with 0.004 g (16.4 μmol) of biotin/kg (TD-01362, Harlan Teklad). This amount of biotin fully neutralizes the avidin present in the diet, and supplies the nutritional requirements of the vitamin to assure normal mice growth [1, 2]. The detailed composition of the diets has been published elsewhere [1].

Mice in the control, sufficient and deficient groups were weighed individually every week. The mice of the different groups were sacrificed at 0, 2, 4, 8, 12, 16 and 20 weeks, after having started consuming the

corresponding diets. The day of experimentation, 3–5 mice per group fasted during 6–7 h before being anesthetized with diethyl ether for weighing and subsequent exsanguination. Mice were sacrificed by cervical dislocation and the tissue samples were then taken (see below). Animal handling and experimental procedures were all reviewed and approved by the local Ethical Committee for Animal Experimentation at the Instituto de Investigaciones Biomédicas.

Food intake

It has been reported that rats subjected to biotin deficient diets reduce their food intake when compared with control animals [30, 35]. This has led to the assumption that the clinical features associated with biotin deficiency may be linked to the reduction of food consumption, suggesting that the deleterious effects of biotin restriction on body weight and size predominantly reflect the inability of the afflicted organisms to nourish themselves properly [30, 35]. In fact, protein malnutrition itself could lead to altered GH/IGF-I function [12, 19, 28, 29]. Thus, it was imperative to evaluate whether the effects of biotin restriction on body growth and size were due to undernourishment in our experimental series. We then estimated relative daily food intake per body gram (RDFI) in control, BD and BS mice based upon the following equation:

$$\mathrm{RDFI} = X_{\mathrm{f}}[\mathrm{Fi} - \mathrm{Ff}/d \times n]/\mathrm{X\,bw}$$

in which: X_{f}-mean of three weekly measurements per cage; Fi-Initial weight of the food provided; Ff-weight of uneaten food rations; *d*-number of days between measurements; *n*-number of animals per cage; Xbw mean body weight of mice per cage as measured weekly. This procedure for adjusting and comparing relative food consumption among groups seems adequate since food intake tends to be proportional to body weight under *ad libitum* conditions [36, 46].

Determination of biotin status

A parameter that provides information on biotin availability is the activity of carboxylases [1]. In fact, the specific activity of PC and PCC is a better indicator of biotin status than the concentration of biotin in serum [1, and Bonjour JP, 1985 and Mock DM, 1999 cited in 1]. Then, the specific activity of PC and PCC was measured in the liver of control, BD and BS mice at different time points during the experiment. Livers obtained from the three experimental groups were frozen at −70°C until they were use to measure the enzymatic activity of PC and PCC with the procedure previously described [1, 2]. Briefly, the day of the assays, livers from control, BS and BD mice were randomly selected. Such livers were thawed and homogenized with a polytetrafluoroethylene piston in ice-cooled PBS. After erythrocytes hemolysis, the isolated hepatocytes were lysed with an ultrasonic homogenizer (4710 Series; Cole Parmer Instrument Co, Chicago). The activity of both enzymes was determined in 10 µl each of the sonicated samples by a radioenzymatic method using $NaH^{14}CO_3$ and it is reported as nmol of fixed CO_2/min × mg protein. The total protein concentration of the sonicated samples was determined by the Bradford method [6]. Two independent replicas of the assay were carried out using liver homogenates obtained from the mice included in the study. The values obtained were finally average per mice and per mice group.

Serum GH and IGF-I quantification through ELISA

Longitudinal body growth is especially sensitive to GH/IGF-I actions, since this endocrine axis regulates the enlargement of the cartilage of growth plates in long bones during infancy and puberty in mammals [8, 11, 20, 21, 26, 34]. After exsanguinations, blood serum was obtained by centrifugation and stored in aliquots at −70°C until GH and IGF-I quantification. The GH and IGF-I serum concentrations were determined using ELISA kits following the manufacturer's directions (Diagnostic Systems Laboratories, Inc). The Active Rat IGF-I EIA kit (DSL-10-2900) estimates total IGF-I (free and bound) with a sensitivity of 30 ng/ml (5.3–7.8 and 4.9–11.7% intra- and inter-assay precision, respectively). The Active Mouse/Rat Growth Hormone ELISA kit (DSL-10-72100) detects GH at a sensitivity of 0.2 ng/ml (2.2–8.6 and 3.4–9.0% intra- and inter-assay precision, respectively). Absorbance readings were made on 96-well plates, using either an ELISA Multiskan Ascent (Thermo Labsystems) or an UltraMicroplate reader El_X808 (Bio-Tek Instruments, Inc.). The GH and IGF-I serum concentrations are reported as average values of three experiments per animal group at different times of the study.

Femur morphology

To corroborate that biotin deficiency associates with an impairment of longitudinal body growth through altering long bones lengthening, we analyzed the femur weight and length in control, BD and BS mice (n = 3–5/group) during the 20 weeks of experimentation. The left femurs were obtained after the mice were exsanguinated and killed by cervical dislocation.

The isolated femurs were weighed and their length determined with a Vernier caliper, after dissecting off the muscles. To microscopically asses the overall structure of the growth plates, the right femurs were dissected from control, BD and BS mice (n = 3–5/group) at 6, 8 and 16 weeks of having started the consumption of the corresponding diets. The samples were transferred to a fixing/decalcifying solution made of 4% paraformaldehyde with 0.2 M EDTA in PBS (pH 7.4) for 15 days at 6°C, under constant shaking. This solution was renewed every 2 days to assure proper decalcification. By the end of this period, femurs were included in paraffin and processed for histological observations according to the procedure described previously [17]. Longitudinal sections (5 μm thickness) were cut at the level of the knee joint, mounted on poly-lysine embedded slides, rehydrated and stained successively with Weigert's hematoxylin, fast green and safranin O. The sections were then dehydrated, mounted with Crystal Mount and observed under bright field microscopy (Nikon, Eclipse E600). Digital images of the growth plates were capture with a Nikon Coolpix 995 digital camera (Nikon, Inc.) and the figures made with Adobe Photoshop. The histological observations were aimed at comparing qualitatively the laminar organization of the growth plates in femurs of control, BD and BS mice.

Statistical analysis

Statistical comparisons among the experimental groups were carried out by using a two-way ANOVA test followed by a Tukey HSD post-hoc tests for multiple comparisons (STATISTICA 5.0; StatSoft). The level of statistical significance was set at a value of $P < 0.05$.

Results

The developmental pattern of body weight and length gain of the three experimental groups is shown in Table 1 and fully accords with data reported previously [2]. No significant differences were found between the control and BS mice for any one of the parameters determined [1, 2]. This confirms that the amount of biotin added to and the composition of the sufficient diet assures proper postnatal growth and development.

Table 1 Body weight of mice at different time points of experimentation

Week of study	Mean ± SEM (n = 19)		
	Control	Sufficient	Deficient
0	11.8 ± 0.3	12.0 ± 1.7	11.7 ± 0.4
8	23.0 ± 2.0	23.4 ± 2.3	18.5 ± 0.4[a]
20	27.5 ± 0.7	26.9 ± 3.1	14.6 ± 1.5[a]

[a]Mean values were significantly different from the control and sufficient groups, $P < 0.05$

Food Intake

As it was mentioned before, the shortage of body size observed in mice subjected to the biotin deficient diet might be taken to result from a decrement in food intake (i.e., under nutrition) and not from the insufficient allotment of biotin. To evaluate this possibility, individual daily food consumption was estimated during the entire duration of the experiment. As it is shown in Fig. 1A, individual control and BS mice

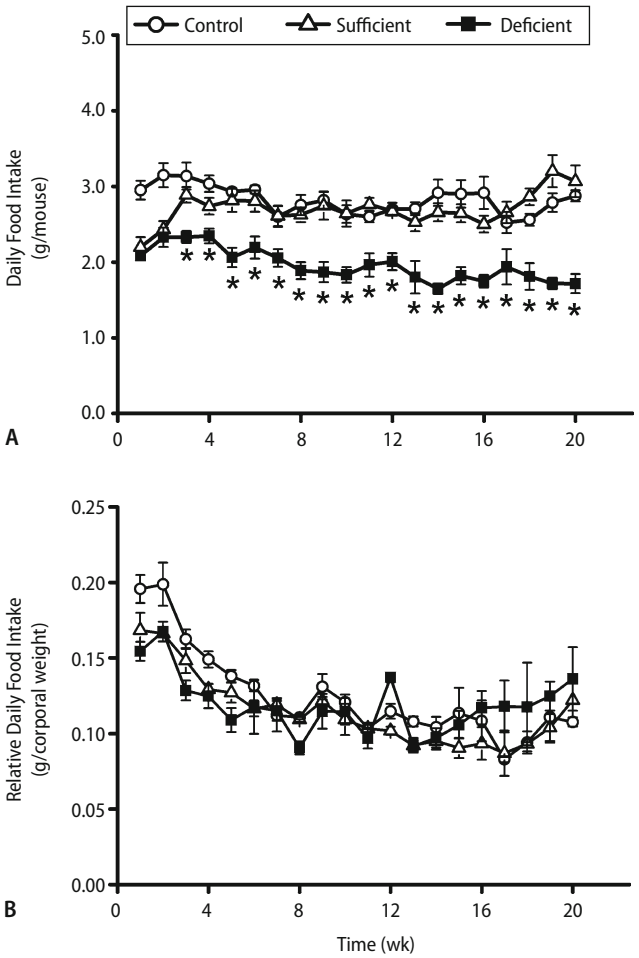

Fig. 1 Food intake. Amount of food ingested by mice fed a commercial standard (control group), biotin-sufficient or biotin-deficient diet. **A** The values represent the daily average consumption of three animal lots per experimental group with their corresponding standard deviations. *Mean values were significantly different from control and sufficient groups, $P < 0.05$. **B** Graph that depicts the average amount of food consumed relative to the average body weight. Data represent the mean ± SD of three animal lots per experimental group. No significant differences were observed among groups

consumed daily more food than BD mice. In spite of this fact, the amount of food consumed daily by control, BD and BS mice was alike when normalized per body weight (Fig. 1B). Hence, it appears reasonable to conclude that biotin deficiency does not diminish food intake as it was previously suggested [35].

Monitoring the functional state of biotin

An effective way to evaluate the magnitude of the deficiency accomplished with the use of diets lacking biotin is to measure the activity of PC and PCC in the liver [1]. The activity of both carboxylases was measured in liver samples obtained from control, BS and BD mice. Our results showed that the average activity of both hepatic enzymes did not differ in control and BS mice (Table 2; see also [2] for a similar result). In sharp contrast, and in accordance with previous results [1, 2, 40], the activity of PCC and PC was lower in BD mice when compared to control and BS mice (Table 2). Our data therefore confirmed that the biotin status in BD mice decays and suggest that the activity of PC and PCC does not depend on absolute food intake but on biotin availability.

Serum GH and IGF-I levels

Body growth is modulated by GH and IGF-I. We measured the serum concentrations of both hormones at various times along the 20 weeks span of the experiment. In agreement with previous observations made in rodents [28, 29], individual measurements of GH serum levels taken at single time points varied greatly among control, BS and BD mice (Fig. 2A). In contrast, IGF-I serum levels were very similar among individuals within the same experimental group along the study. IGF-I serum levels did not differ between control and BS mice (Fig. 2B). These groups had, however, higher IGF-I serum levels than those observed in BD mice. These results suggest that biotin restriction is indeed associated with a decrement of IGF-I serum levels.

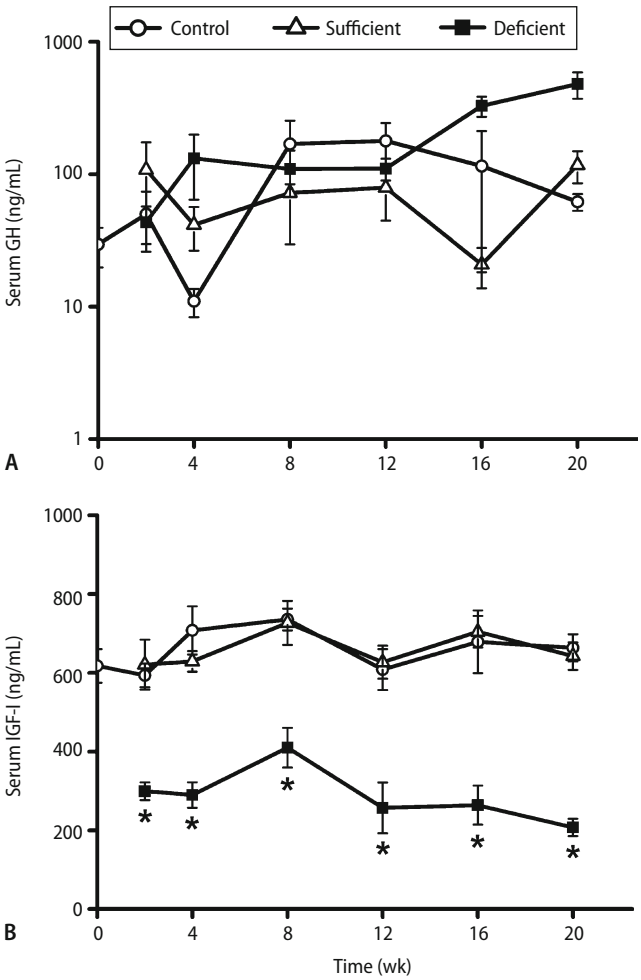

Fig. 2 ELISA measurements in the serum of mice fed a commercial standard (control group), biotin-sufficient or biotin-deficient diets. **A** GH concentration. **B** IGF-I concentration. Values are means ± SEM, $n = 4–12$. The symbol "*" indicates differences between the control and biotin-sufficient groups, $P < 0.0001$

Table 2 Specific activity of hepatic propionyl CoA (PCC) and pyruvate CoA (PC) carboxylases of mice along 20 weeks of experimentation

Enzyme activity	Mean ± SEM ($n = 19$)		
	Control	Sufficient	Deficient
PCC	38.8 ± 15.5	42.3 ± 13.9	9.3 ± 4.9[a]
PC	52.1 ± 1603	47.9 ± 14.5	11.7 ± 3.3[a]

Enzyme activities are expressed in nmol CO_2 fixed × min^{-1} × mg $protein^{-1}$
[a]Mean values were significantly different from the control and sufficient groups, $P < 0.05$

Femur growth and morphology

The growth plates of long bones are especially sensitive to shifts in GH and IGF-I serum concentrations [7, 8]. Accordingly, although the overall anatomical features of femurs dissected from hind limbs of control, BS and BD mice were similar, femur length and weight were found diminished in BD mice, but not in BS mice, as compared with control mice (Fig. 3A, B). This was true for femur's length from week 4 ahead and for its weight from week 8 forward. Interestingly, the final values of the femur's weight were reached approximately 8 weeks earlier in BD mice than in

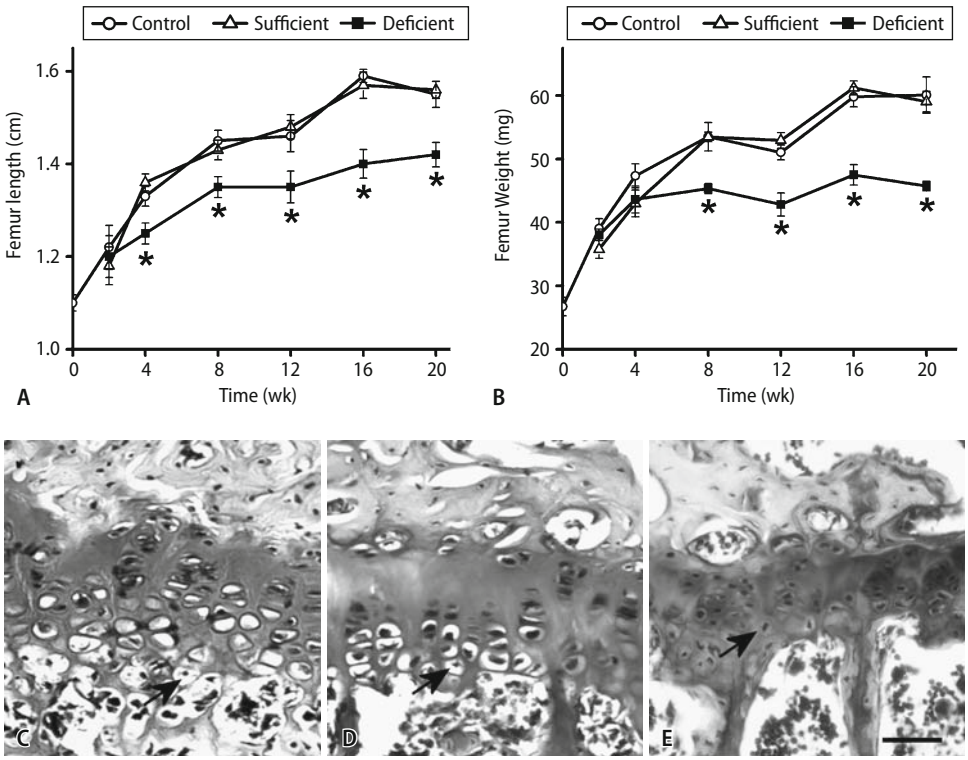

Fig. 3 Femur growth and morphology in mice fed a commercial standard, biotin-sufficient or biotin-deficient diets. (**A**) Femur length. (**B**) Femur weight. Values are means ± SEM, $n = 6–8$. The symbol "*" indicates differences between the control and biotin-sufficient groups, $P < 0.0001$. Histological analysis of femur sections from distal epiphysis. Sections from control (**C**) and BS (**D**) mice show normal histology, whereas in BD mice (**E**) the zones of proliferating and hypertrophic chondrocytes are reduced, and also, a disruption of its normal architecture is observed. *Arrow* shows hypertrophic chondrocytes which are almost absent in the femur of BD mice. Bar scale 50 μm

control or BS mice. In contrast, while in control and BS mice femur's length approximates its final measure by week 16, the same parameter in BD mice is still increasing by the week 20 of experimentation. Hence, biotin deficiency and reductions of IGF-I serum levels are associated with femur's growth impairment.

The longitudinal growth of the femur depends upon the constant accretion of cartilage to the growth plate. This process is influenced greatly by the GH/IGF-I axis [4, 7, 20, 21, 34]. We qualitatively analyzed the organization of the femur's growth plate in control, BS and BD mice (Fig. 3C–E). Under normal and biotin-sufficient dietary conditions, the growth plate displayed a layered organization with the germinal zone sitting near the epiphysis and the hypertrophic zone facing the diaphysis close to the calcification/ossification zone. Between these zones, there exists a third layer termed the proliferation zone. In control and BS mice, the proliferation and hypertrophic zones occupied approximately three quarters of the full extent of the growth plate at all time points studied (Fig. 3C, D). In contrast, BD mice showed dysplastic growth plates especially at weeks 8 and 16 (Fig. 3E). There was also a relative thinning of the proliferation and hypertrophic zones that began to be noticeable by week 8; many chondrocytes became picnotic by week 16. Our results then support that BD mice have relatively thinner and disarranged femur's growth plates.

Discussion

Body growth and size are affected by the quality of the diet. In particular, it has been reported that biotin dietary restriction impairs both parameters significantly [1, 2, 5, 13, 16, 35]. Hence, the present results confirm previous findings. Additionally, they reveal that biotin dietary deficiency is associated with decreased IGF-I serum levels and with impaired femur growth. These alterations cannot be simply ascribed to decreased food intake or reduced appetite as previously implied [30, 35] because the relative food intake in mice fed with control, BS and BD diets is fully comparable and relatively constant during the period of experimentation. In addition, the fact that GH serum concentration appeared not to be affected by biotin restriction further supports that the effects of biotin deficiency are not due to overall malnutrition; GH serum levels are known to be reduced following dietary protein restriction [19]. Thus, although causal links are still missing, we think that our results support the notion that decreased body size in BD mice might result from a decrement of IGF-I serum levels. This possibility is strengthened by the fact that the anatomical and histological features described in the femur of BD mice are fully reminiscent of those previously reported in mice displaying a marked reduction of IGF-I [44, 45].

It has long been known that amino acid and carbohydrate dietary deficiency leads to reduced body growth and size in animal species of different taxa [32]. As mentioned before, our results together with other observation suggest that biotin restriction also impairs body growth and size. Interestingly, the effects that nutritional deficiencies of amino acids, carbohydrates [28, 29, 42] or biotin [this study] have on body growth and size are all associated with a reduction of the systemic and local concentrations of GH and/or insulin-like growth factor peptides. Although this association might only be circumstantial, it might also mean that common or distinct endocrine mechanisms, which converge upon insulin-like peptides [10, 26, 32], translate the information provided by nutrients to the body. Surely, studies aimed at restoring body size with IGF-I or assuring higher than normal nutrient intake while keeping the animals under specific nutritional restrictions might help in addressing this possibility.

In the same vein of the preceding paragraph, another issue that would warrant future studies relates with the mechanisms by which amino acids, carbohydrates and biotin might control the availability of insulin-like growth factor peptides. Although little is yet known on such mechanisms, recent evidence showing that, at least in *Drosophila*, the fat tissue posses an amino acid sensor (e.g., the cationic amino acid transporter also called *slimfast*) that allows adipocytes to control the growth of peripheral tissues by directly modulating the availability of insulin-like peptides [32] supports that such nutrient specific mechanisms may indeed exist. In the case of biotin, one might envision levels of insulin-like growth factors being modulated through the binding of this vitamin to chromatin [24] which in turn could control gene expression/transcription [39, 47] and/or through modulating their secretory pathway as it has been shown for insulin [39].

Finally, a somewhat puzzling observation is the relative lack of effect of biotin restriction on GH serum levels. This is especially true if one considers that GH is a major regulator of postnatal body growth and mass, as well as of bone size by stimulating chondrocyte proliferation in the growth plate [7, 20, 21, 33, 34]. Because BD mice displayed impaired body and bone growth, one might have expected to observe a reduction of GH plasma level. This however was not the case. We believe that the data just described support that the association of biotin deficiency with decrements of IGF-I serum levels and with bone growth impairment is to some degree specific. We also think that our results strengthen the notion that some of the IGF-I actions on bone growth occur to some degree independent of GH [44]. Alternatively, it might be that the sampling technique used in the present study lacked the temporal resolution to detect variations of GH serum levels since this hormone is secreted in pulses that vary greatly among individuals along the day [8, 25, 27]. Unfortunately, it is impractical and ethically dubious to withdrawn blood samples from each mouse several times a day in the course of 20 weeks. Clearly more studies are needed to address this caveat of our work.

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