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The relative effects of severe burn injury and pre- and post-natal protein deprivation on mandibular condyle morphology

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Summary. The mandible has a mixed embryological origin, and its growth is associated with the secondary cartilage of the condyle process (CP). In this area, growth depends on an array of intrinsic and extrinsic factors that influence protein metabolism. In the present study, we used an adolescent rat model to evaluate the growth and development of the CP under conditions of pre- and postnatal protein deficiency, combined with or without the stress of severe burn injury (BI). We found that protein deficiency severely undermined the growth of the CP, by altering the thickness of its constituent layers. BI is also capable of affecting CP growth, although the effect is less severe than protein deficiency. Interestingly, the summed effect of protein deficiency and BI on the CP is less severe than protein deficiency alone. A possible explanation is that the increased carbohydrates in a hypoproteic diet stimulate the production of endogenous insulin and protein synthesis, which partially compensates for the loss of lean body mass caused by BI.

Key words: Undernutrition, Burn injury, Secondary cartilage, Rat, Craniofacial growth

Introduction

Craniofacial growth and development involves a constant process of bone remodelling and sutural apposition (Enlow, 1968, 1975; Schumacher, 1999). This process is governed by intrinsic genetic and hormonal factors, as well as extrinsic mechanical and nutrition factors (Newman et al., 1982; Moyers, 1991). The mandibular condyle process (CP) exemplifies this growth phenomenon.

Ontogenetically, the mandible derives from the juxtaposed central primary cartilage, and fibrocellular tissue arises from neural crest cells that migrate to the mandibular region (Hall, 1994). During embryogenesis, primary cartilage emerges from the chondroskeleton, known as Meckel's cartilage. The posterior part of Meckel's cartilage forms the malleus of the middle ear (Ten Cate, 1998). The CP is derived from the secondary condylar cartilage, independent of the embryonic chondroskeleton. Remnant fibrocellular tissue material is present in the fully formed structure and forms the articular surface (Delatte et al., 2004).

There are several histological zones that define this area. Subjacent to the articular surface is the prechondroblastic zone, which contains undifferentiated, potentially cartilaginous cells. The next layer is beneath the chondroblastic zone, where extracellular matrix secretion occurs. Below this zone is the inner cell layer, which forms the lowest hypertrophic zone, where the subchondral bone is formed (Carlson et al., 1980; Fuentes et al., 2002).

For this complex process of CP growth to proceed normally, balanced protein intake is essential. Insufficient protein can result from a variety of factors. The most evident is restriction of proteinaceous food intake (Shrader and Zeman, 1973). Another factor that can cause protein deficiency is severe burn injury (BI), which impacts protein catabolism (Hart et al., 2000). A useful comparison is the relative impact of these two types of factors on CP growth.

The relationship between maternal nutrition and foetal alterations in bone morphology has been well documented. In several species models, maternal protein

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food restriction affects multiple foetal tissues, such as skin (Naeye, 1965; Lansdown, 1978), locomotor (Shrader and Zeman, 1969; Adams, 1971) and central neural tissue (Yamano et al., 1980). Indeed, malnutrition stands out as a determinant extrinsic factor capable of affecting craniofacial growth, but there is a lack of detailed knowledge on the subject (Moyers, 1991). During the postpartum lactation period of undernourished rats, there is clear deviation of normal growth in the offspring (Pucciarelli and Oyhenart, 1987). During pregnancy, protein-caloric undernutrition caused specific alterations in endochondral and intramembranous ossification (Nakamoto et al., 1983). After weaning, mandibles are deformed (Alippi et al., 1984).

In patients with BIs, there is hypermetabolism of proteins that ultimately decreases the reserve of protein available for organic physiological processes. Specifically, bone turnover decreases, and bone mineral density is reduced (Klein et al., 1997; Edelman et al., 2003). Also, there is higher muscular catabolism (Hart et al., 2000), and profound calcium loss through urine. The most important organ affected by BI is the liver and its function. Interestingly, these deleterious effects persist even after all lesions are healed (Hart et al., 2000).

In our study, we aim to co-evaluate the possible morphological alterations caused by both protein calorie deficiency and BIs. These two extrinsic factors affect large populations of children in developing countries. We used a well-defined rat CP model (Sardi et al., 2007) to characterize the specific impact of these two factors on craniofacial growth and development.

Materials and methods

Experimental groups

Young male and female 280-320 g Wistar rats were housed together in mating groups for 7 days in a temperature-controlled room (21-24°C), and under a regular light-dark cycle. Mating groups received one of two protein-modified AIN-93G diets: protein-nourished, containing 20% casein (N), and hypoproteic-undernourished containing 5% casein (U). All diets were prepared according to "American Institute of Nutrition" protocol (Reeves et al., 1993) in a specialized laboratory (Rhoster®). After mating, dams were caged individually. After the 21st postnatal day, the pups were weaned and adapted to new cages over the course of 5 days. These young adults were then organized into four groups according to both diet and exposure to burn (n = 5 each): N, U, UI (undernourished and burn injured), and NI (nourished and burn injured).

BI exposure

The rats were housed in individual wire-bottomed metabolic cages for 5 days before the onset of the study. The wire bottom in these cages deters infection, as it minimizes contact with feces and urine. The tray beneath the wire bottom allows for cage cleaning with minimal disruption to the animal. In addition, both ingestion and body weight can be assessed daily.

Rats were anesthetized with 40mg/kg sodium pentobarbital IP (Hypnol 3%, Fontoveter, Cristália, Produtos Químicos Farmacêuticos Ltda, Brazil), then tracheotomized. A nonlethal scald injury was applied to dorsal and ventral surfaces with an asbestos device, recommended by Walker and Mason (1968). The burn area was calculated from body weight, and represented approximately 45% of the total body surface area, delimited by the asbestos device. Burn areas were then transformed to severe BI by immersion in 87°C water for 10 seconds on the dorsal body surface, then 3 seconds on the ventral body surface. The dorsal surface burn represented 35% of the total body area, and the ventral surface burn represented 15% (Newman et al., 1982; Ibebunjo and Martyn, 2001). All rats were injected subcutaneously with the analgesic buprenorphine (0.2 mg/Kg) immediately after sham or burn injury, and again 24h later.

After BI, rats were returned to individual metabolic cages, and continued the modified diets for 14 more days. At postnatal day 40, all animals in each group (U, N, UI, NI) were weighed and euthanized by a lethal injection of 100mg/kg sodium pentobarbital IP. Then, the temporomandibular joint containing the CP was carefully dissected and removed.

Ethical observations

All procedures were approved by the Committee of Ethics in Animal Experimentation (CEEA - ICB/USP), protocol number 014/03 with declaration 018/2006.

Light microscopy

Join specimens were fixed in 10% formalin for 24 hours and decalcified (Morse, 1945). They were then dehydrated through a series of graded ethanol (70-100%), diaphanized in xylol, and embedded conventionally in paraffin. Semi-serial histological sections were then acquired in the sagittal plane at 6 mm.

Examination of cartilage

Sections were stained with Azan (Romeis, 1968), to identify cell layers of the articular cartilage. The collagen features that compose the extracellular matrix were observed under polarized light in sections that were stained with Picrosirius (Junqueira et al., 1979). For qualitative analysis of protein content in the CP, cartilaginous matrix sections were stained by the Safranin-O method (Luna, 1968).

Qualitative analysis

All sections from each experimental group were

observed and documented by conventional photomicroscopy (Axioscop 40, Karl Zeiss) coupled to a high definition in-line digital camera (Axiocam HRc 12MP, Karl Zeiss). Images were acquired and the articular, prechondroblastic, chondroblastic and hypertrophic zones in CP cartilage were examined. The morphology of each layer was compared, with particular attention to proteoglycans and the collagen components of the extracellular matrix.

Quantitative analysis

Five sections from the most central part of the CP cartilage were selected from each rat joint specimen. Sections were then stained by Azo-Carmim method. Successive mitoses in the chondroblastic zone are the basis of bone growth, so cell number was counted in this zone. Cells were identified and counted by a series of fields that were sequentially organized across the extent of the section.

The cartilaginous zone was also analyzed with image software (Axiovision 4.5, Karl Zeiss), to obtain the density of chondroblastic zone cells. The thickness of each CP layer (articular, prechondroblastic, chondroblastic and hypertrophic zone) was measured. The prechondroblastic and chondroblastic zones (proliferative zones) were measured together, because the borders of these layers were ambiguous in U and UI groups.

For each rat specimen, the parameters evaluated were: body weight, total cell number, cell density per section in the proliferative zone, and layer thickness in the prechondroblastic, chondroblastic and hypertrophic zones.

Statistical Treatments

Data obtained from U, N, UI and NI groups were submitted to an analysis of variance (ANOVA) with 2 factors (group and treatment), and followed with a Tukey's test for multiple comparisons, when necessary (Zar, 1984).

Results

Qualitative analysis showed that, in all groups, the CP fibrous cartilage had a normal organization and distinct layers. In the N group, there was a regular ossification pattern, with zones of balanced proportions, evident limits, and a thin articular layer (Figs. 1, 2Aa,B). This pattern was not observed in the other groups (Figs. 2Ab-d,B). In the NI and U groups, there was a slight loss of definition between the CP cartilaginous layers (Fig. 2Ab,c), and this feature was even more pronounced in the UI group (Fig.2Ad,h). In the NI group, the prechondroblastic and chondroblastic layers showed reduced thickness compared to the hypertrophic layer, which was relatively expanded (Fig. 2Ab,B). In the U group there was drastic loss in thickness throughout all layers (Fig. 2Ac,g,B). In the UI group, the prechondroblastic zone was thicker (Fig. 2Ad,B) than those observed in the NI and U groups. Relative size and proportions of tissue layers in the UI group resembled those of the N group, yet all layers were thinner.

At higher magnifications (Fig. 2Ae-h), there were signs of cartilage diminution in the UI and U groups (Fig. 2Ag,h). Tissue degradation was even more pronounced in the UI group (Fig. 2Ah), wherein the layers showed no organizational pattern. Furthermore, the UI prechondroblastic, chondroblastic and hypertrophic layers were larger than those observed in U group. In the N, NI and U groups, high magnification showed hypertrophic zone cells (Fig. 2Ai-l) with an ovoid contour in the nucleus (Fig. 2Ai,j).

Cell dysplasia in the hypertrophic zone was characterized for all experimental groups. In the extracellular matrix the Picrosirius stain under polarized light revealed collagen maturity (Fig. 3a-h, 4, 5) and the Safranin-O stain (Fig. 3i-l) revealed proteoglycans. The CP articular layer and articular disc showed a typical pattern in the N group, with a predominance of mature collagen fibres (type I collagen) and few immature fibres (type III) (Figs. 3a,e, 4a, 5a). However, in the NI group (Figs. 3b,f, 4b, 5c) the predominant collagen was

Table 1. Quantified changes in CP fibrous cartilage after protein deficiency and burn injury.

	Weight (g)	Cell number, chondroblastic zone	Cell density, chondroblastic zone (cells / μm^2)	A thickness (μm)	Proliferative Zone (P+C) thickness (μm)	Articular + Proliferative zone (A + P + C) thickness (μm)	H thickness (μm)
N	*129,85±15,37	38,896±7,73	0,003529±0,00071	22,78±4,65	71,69±11,69	94,47±11,39	*129,27±16,58
NI	*111,456±2,28	36,96±6,29	0,003317±0,00043	18,904±6,77	73,93±16,23	92,84±10,24	*162,05±4,74
U	*26,054±6,14	35,704±5,56	0,003512±0,0006	15,12±5,06	~53,95±19,22	*69,07±22,67	*89,51±13,50
UI	*21,34±4,30	32,98±9,22	0,003181±0,00077	17,01±2,68	60,71±11,76	77,72±12,03	*99,50±7,21

N: nourished; NI: nourished + BI; U: undernourished; UI: undernourished + BI. Parameter labels are as follows: A: articular layer; (P+C): prechondroblastic and chondroblastic zones; (A+P+C): articular, prechondroblastic and chondroblastic zones, H: hypertrophic zone. Values are presented as means ± standard deviation, for all groups (n=5, each). ANOVA and Tukey's test were used to detect significant differences between means (*: $p \leq 0.05$). A large difference that was not significant is also noted (~: p=0.054).

immature (type III). In the U group (Figs. 3c,g, 4c, 5b), the pattern of collagen fibres was similar to that of the N group, predominantly type I collagen. In the UI group (Figs. 3d,h, 4d, 5d), the pattern was similar to the NI group, predominantly type III collagen.

The tissue of the CP is a fibrous cartilage. The amount of extracellular matrix containing type II collagen fibres is relatively smaller than that found in hyaline cartilage. In addition, there is a mixture of type II collagen with types I and III, which are more fibrous components (Fig. 4c).

Intense Safranin-O staining that revealed proteoglycans throughout the extracellular matrix was observed in the N group (Fig. 3i). Comparatively, proteoglycan staining in the NI group was light (Fig. 3j). The U group showed a drastic reduction in proteoglycan staining (Fig. 3k). The UI group showed a moderate reduction in proteoglycan staining (Fig. 3l).

Under polarized light, additional differences in the CP (Fig. 4) and articular disc (Fig. 5) were observed. The U and UI groups showed very similar collagen distribution in the subchondral bone and fibrous cartilage. Though the predominant collagen in the U group was type I, there were some type III fibres. In the UI group, most collagen fibres were type III. The top and bottom surfaces of the articular disc showed a layer of type I in all groups. The central thickness of articular disc did vary; the N and NI groups were thicker, and U

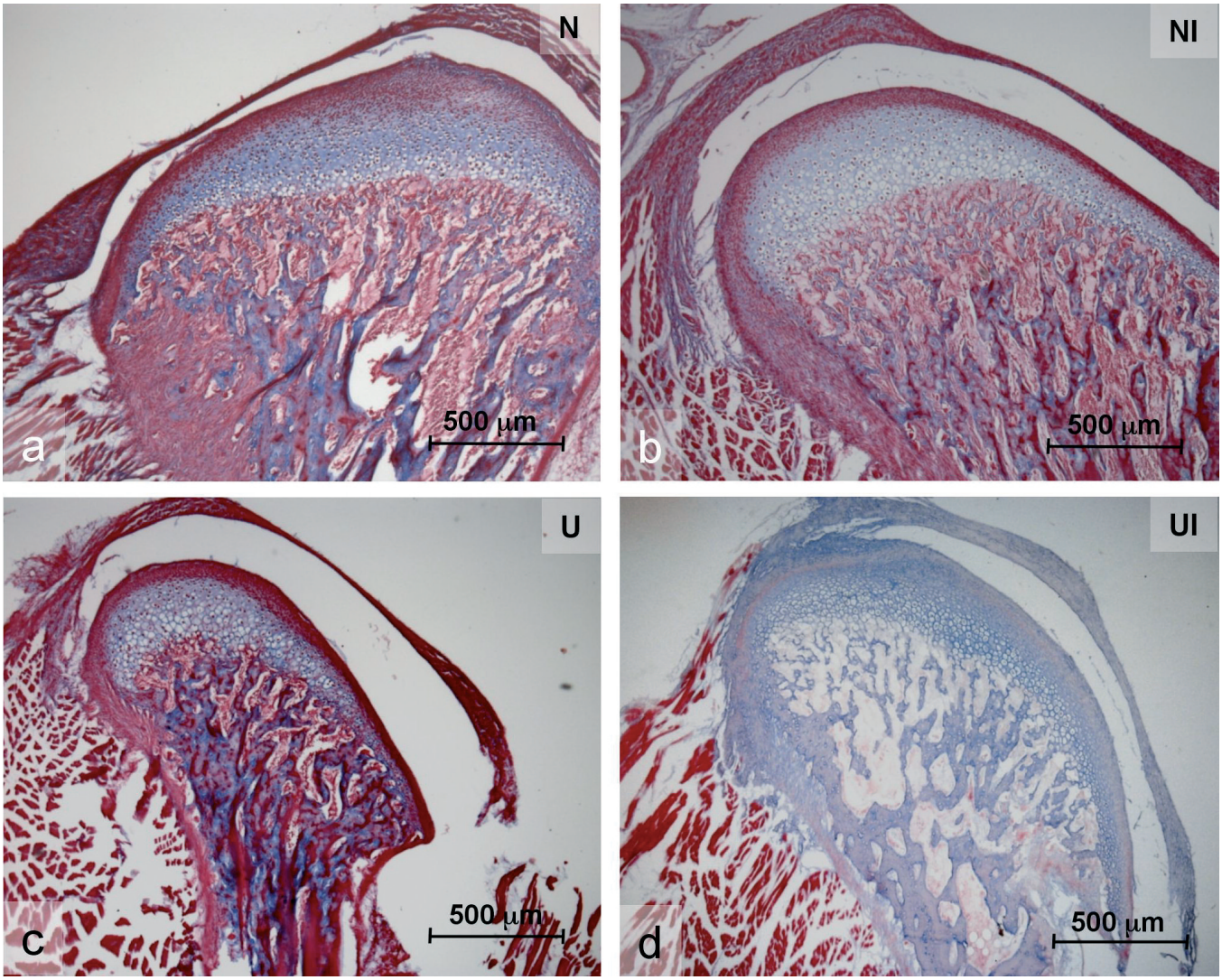

Fig. 1. CP sagittal sections stained by Azan method. For this figures, N = nourished, NI = nourished + BI, U = undernourished, UI = undernourished + BI. **(a-d)**. Representative images from each group.

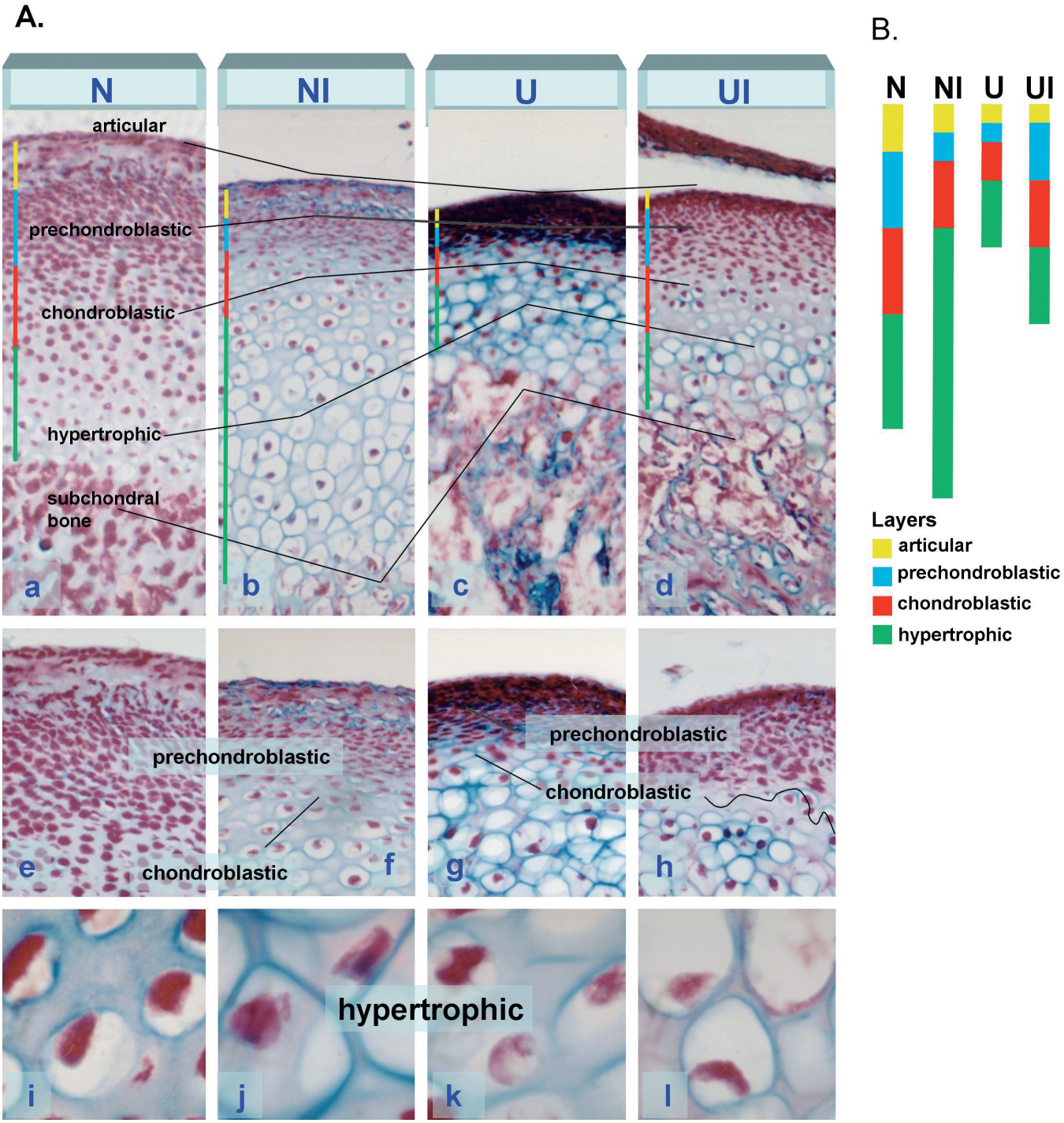

Fig. 2. For subsequent figures, N = nourished, NI = nourished + BI, U = undernourished, UI = undernourished + BI. **A. a-l.** CP sagittal sections stained by Azan method. Representative images from each group are organized into columns. The relative thickness of each cartilage layer was designated with coloured vertical lines in each group panel (top row), to facilitate comparison across treatment groups. Horizontal lines show the relative position of each layer across groups **a-d.** In the UI group, there is disorganization in the chondroblastic zone **h.** compared to those in the N group, UI layers are reduced in size but proportional to each other. Layer thickness is most distorted in group U **c. B.** A closer comparison of coloured vertical lines representing layer proportions in each group. In the NI group, the hypertrophic zone is the largest. Aa-d, x 20; Ae-h, x 40; Ai-l, x 100

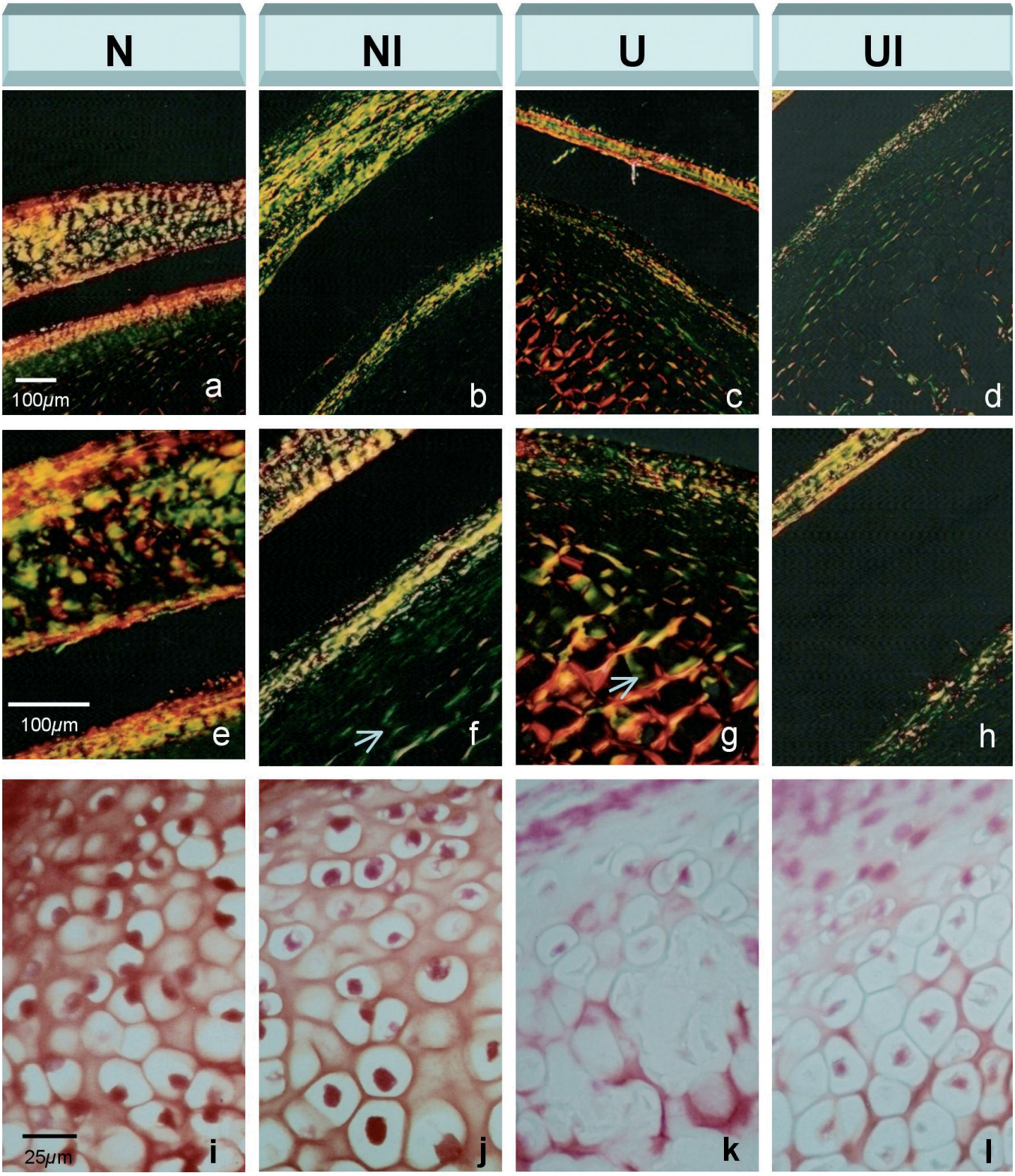

Fig. 3. CP sagittal sections stained with Picrosirius and observed under polarized light **(a-h)** (N: nourished, NI: nourished + BI, U: undernourished, UI: undernourished + BI). The Safranin-O method was also used to observe proteoglycan components **(i-l)**. The same pattern of collagen is observed in N **(a, e)** and U groups **(c, g)** wherein type I collagen is predominant (red, yellow and orange colour), and there is only scant type III collagen (green colour). In groups NI **(b, f)** and UI **(d, h)** type III collagen was predominant (green). In the bottom row, the stain indicates the presence of proteoglycans. The stain is saturated in both the N and NI groups **(i, j)**, compared with U and UI groups **(k, l)**.

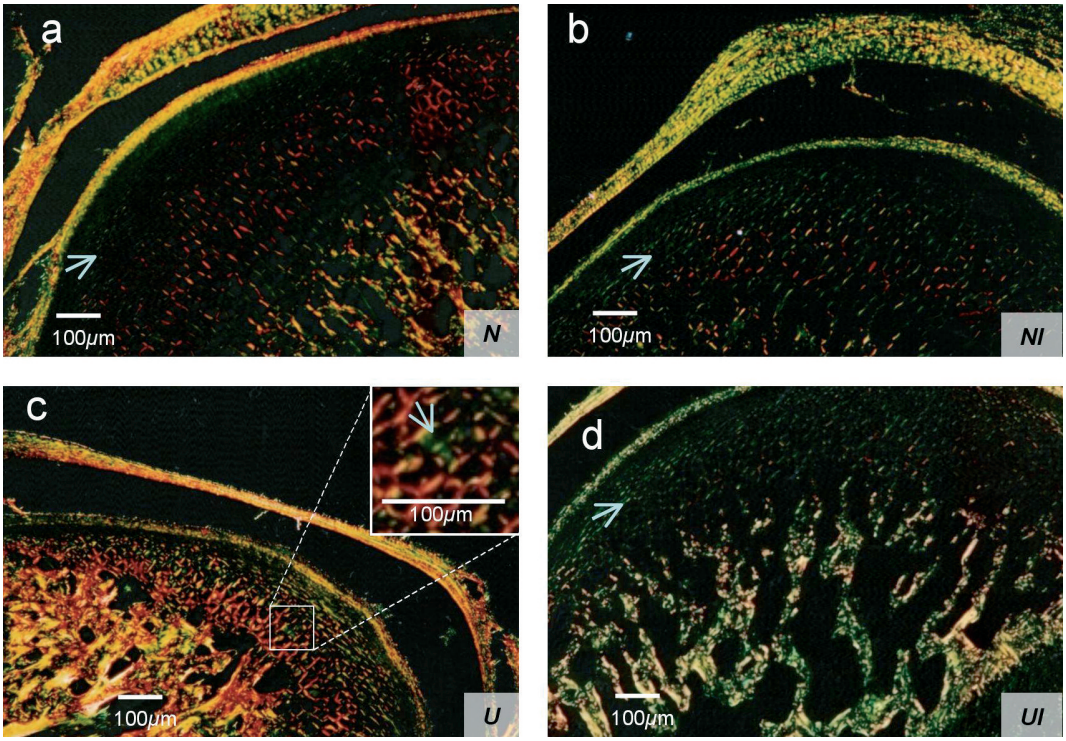

Fig. 4. CP sagittal sections stained with Picrosirius method and observed under polarized light (N: nourished, NI: nourished + BI, U: undernourished, UI: undernourished + BI). Overview includes subchondral bone. In the N and U groups **(a, c)**, type I collagen (yellow, red and orange) is prevalent. In the NI and UI groups **(b, d),** type III collagen (green) is prevalent. In the upper right portion of panel **c**, a closer view of type III collagen (in an aquamarine colour) shows its context in the extracellular matrix of the cartilage. Here, type III collagen lies in the centre of a fibrous component of the CP, alongside type I and III fibres.

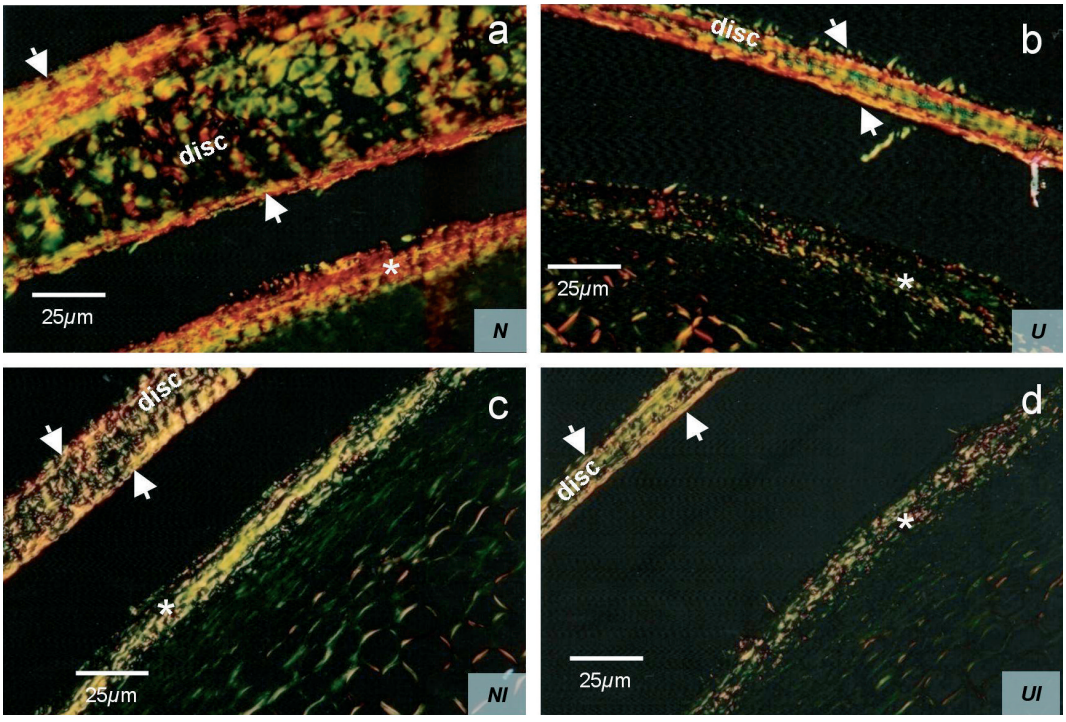

Fig. 5. Higher magnification views of the articular disc (N: nourished, NI: nourished + BI, U: undernourished, UI: undernourished + BI). Type I collagen (red, yellow and orange) at the top and bottom of the articular disc (arrows). Although there is a fibrous component of the articular cartilage, only the N group shows prevalent type I collagen **(a)**.

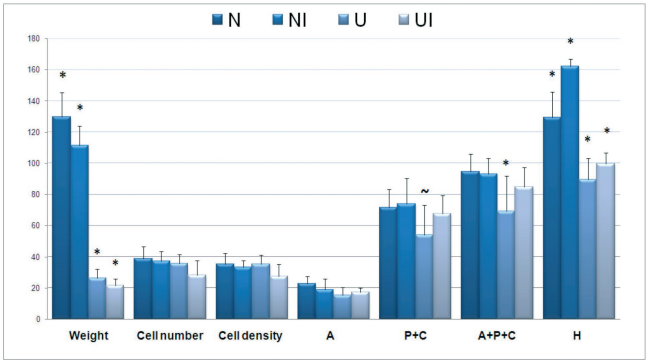

Fig. 6. Mean values of measured tissue parameters compared by group (N: nourished, NI: nourished + BI, U: undernourished, UI: undernourished + BI). Cell number refers to cells in the CP fibrous cartilage. Units are as follows: weight (g), cell density (cells / μm^2 x 10^{-4}) and layer thickness (μm). Parameter labels are as follows: A = articular layer, (P+C) = prechondroblastic and chondroblastic zones, (A+P+C) = articular, prechondroblastic and chondroblastic zones, H = hypertrophic zone. Significant differences are marked with * ($p \leq 0.05$) and values close to significance were marked with ~ ($p = 0.054$).

and UI groups were more slender.

Quantitative analysis

All quantified data presented in Figure 6 were derived from Azan stained sections, as shown in Figure 1. Final body weight differences were significantly different between groups. Quantitative results (Fig. 6) reflected the qualitative trend seen with the Azan stain (Figs. 1, 2). There were no significant differences in cell number, density, and articular layer thickness across all experimental groups ($p>0.05$) (Fig. 6).

However, there were interesting differences in layer thickness. The combined articular and proliferative zone thickness was significantly smaller in the U group compared to all others. Measures of thickness in the hypertrophic zone showed the most significance. Compared to the N group, thickness was significantly larger in the NI group, and significantly smaller in the U and UI groups. Though UI hypertrophic zone thickness was smaller than that in the N group, it was also significantly larger than that in the U group. For all layer thickness measures, the smallest values were found in the U group.

Discussion

Protein deficiency and BI are different types of stress that can affect the morphology of the mandibular chondyle. With both exposures we observed changes in the CP fibrous cartilaginous tissue. However, protein deficiency appears to have a more negative effect than BI alone. Body weight measures reflected this major difference. Similar cell density values in N and U groups suggest that protein deficiency does not influence this parameter. When BI and protein deficiency occur together (UI rats), tissue layers are difficult to distinguish and there is an increase in the amount of proteoglycans in the extracellular matrix. There is also a similar increase in the proportion of certain cartilage layers in UI rats, a possible sign of improvement. Surprisingly, UI rat cartilage resembles that of age-matched controls.

Despite the smaller size relative to the N group, UI hypertrophic zone thickness is significantly greater than that in the U group. This may indicate that BI causes cells in the hypertrophic zone to become bigger (Fig. 2Ab,d,j,l). Measures in the UI group suggest that the extracellular matrix is more compromised by thermal injury than protein deficiency. In fact, tissue thickness values in the UI group compared to the U group indicate that BI somehow rescues the damage done by protein deficiency.

One of the most interesting results is that secondary cartilage compromised by the lack of protein imposed during the prenatal period showed a slight improvement after BI, despite characteristics that are inherent to a hypermetabolic state (UI group). Both the observed abundance of proteoglycans and the increased thickness of the CP layers in the UI group suggest that the secondary cartilage of the condylar process was able to restore the proper protein balance after BI. There was even some reversal of the poor growth pathology associated with the protein deficiency. Our result may be explained by an increase in protein catabolism that has been reported for post-burn hyperglycaemia (Gore et al., 2002). Thus, control of protein catabolism may have been facilitated by the deficient diet. The hypoproteic diet was also hypercaloric (rich in carbohydrates), so it could stimulate protein synthesis via stimulation of endogenous insulin. Consequently, there would be an increase in lean body mass, possibly reflected here in the secondary cartilage of the CP.

The role of insulin in protein balance and in the maintenance of lean mass after burn injury has been clarified recently (Pidcoke et al., 2007). In an attempt to control the post-burn hypermetabolism and protein catabolism in burn patients, high doses of insulin have been used. The effect is a decreased protein catabolism (Pereira et al., 2005). These findings confirm observations made in humans (Berger and Chiole'ro, 2007). In our study, the observed improvement in layer thickness was not statistically significant for all groups, but our results indicate a trend in this direction (Fig. 2).

The lack of Safranin-O staining in the U group reflected a drastic reduction in its proteoglycan content, which is expected to be high in rats of this age. At the pubertal age, collagen composition is more sensitive to burn injury, regardless of nutritional status (Viau et al., 2005). Normally, the collagen of secondary cartilage extracellular matrix is predominant in type I, yet our results show that in post-burn tissue there is a predominance of type III. Despite the expectation that protein deficiency would most likely retard collagen

maturity, this pathology was clearly seen post-burn, regardless of diet. This observation may be explained by the large size of the BI wound, which prompts a hypermetabolic systemic condition. There is a subsequent release of potentially deleterious factors, pro-inflammatory mediators and abundant cytosine (Piccolo et al., 1999).

The significant post-burn increase in hypertrophic layer thickness suggests that there was a shift to the lower subchondral bone. This shift may indicate a delay of osteogenesis, since the hypertrophic layer cells remain in a degenerative stage longer, and are not replaced by osteoblasts responsible for ossification. The metabolic processes involved in hypertrophic expansion may also be related to the mechanisms of insulin and hypermetabolism described above. Indeed, insulin-like growth factor (IGF) is expressed in the CP layers of Wistar rats at this pubertal stage (Fuentes et al, 2002; Bishara et al., 2008). Unlike in the hypertrophic layer, there was a post-burn decrease in prechondroblastic layer thickness, though it was not significant. This decrease would compromise condylar cartilage growth.

Our results suggest that growth of the condylar process in the pre-pubertal period is affected by either BI or protein deficiency alone. Overall effects in the extracellular matrix vary by exposure; protein deficiency tends to change the amount and content of deposition material, while BI primarily interferes with collagen framework maturity. The reflexive response of the tissue was the initiation of complex metabolic processes. Our results highlight the importance of carbohydrate intake after severe BI, for the growth and preservation of secondary cartilage.

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