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Catch-up growth in intrauterine growth retarded rats: its correlation with histomorphometric changes of the pituitary somatotrope cells

E.E. Oyhenart[1,2,3], F.A. Quintero[1,3], A.B. Orden[1,2], M.C. Fucini[1,4], L.M. Guimarey[1,5,6], M. Carino[6,7], C. Ferese[6,7] and G.M. Cónsole[6,7]

1- Centro de Investigaciones en Genética Básica y Aplicada (CIGEBA), Facultad de Ciencias Veterinarias, niversidad Nacional de La Plata (UNLP)
2- Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)
3- Cátedra de Antropología Biológica IV, Facultad de Ciencias Naturales y Museo, UNLP
4- Cátedra de Radiología, Facultad de Odontología, UNLP
5- Unidad de Endocrinología y Crecimiento, H.I.E.P., "SSM Ludovica"
6- Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CICPBA)
7- Cátedra de Citología, Histología y Embriología, Facultad de Ciencias Médicas, UNLP

Summary

The aim of the present study was to examine the long-term effects of prenatal injure on body and craniofacial growth in intrauterine growth retarded (IUGR) animals and its correlation with histomorphometric changes of the pituitary somatotrope population. IUGR model was carried out by means of uterine vessels ligation in pregnant rats at 14th day of pregnancy. Control and sham-operated animals were also included. The animals were X-rayed at 1, 21, and 84 days of age. Body weight, neural and facial variables were measured. Pituitaries were processed for light microscopy and immunolabeled with anti-GH sera. Morphometry was performed by means of an image-analysis system. Data were processed by ANOVA, and Wilcoxon tests. Body weight was significantly lower in newborn IUGR rats compared with that of their control counterparts, even during postnatal growth. Both neurocranium and face were similarly affected at birth and weaning. At 84 days of age, despite facial growth exhibited a partial recovery, cranial volumes remained smaller in IUGR animals. Quantitative immunohistochemistry revealed a significant decrease in the volume and cell densities in IUGR compared to control age peers. Adequate nutritional and environmental conditions were insufficient to reverse the effects of a reduced uteroplacental blood supply on fetal growth. The timing and duration of the growth insult seem to be crucial for the occurrence of catch-up body weight and cranial growth in the rat. The lack of complete catch-up in these IUGR animals may be associated to an alteration in the GH production.

Key words: IUGR – Catch-up growth – Somatotrope cells

Introduction

Intrauterine growth retardation (IUGR), caused by maternal undernutrition or placen-

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Correspondence to:
Evelia Edith Oyhenart. Centro de Investigaciones en Genética Básica y Aplicada (CIGEBA), Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata (UNLP). Calles 60 y 118 S/N, CC 296, 1900 La Plata, Argentina.
Phone: 54 –221-421-1799. E-mail: eoyenart@fcv.unlp.edu.ar

tal insufficiency, is usually associated with disproportionately large reductions in the growth of some fetal organs and tissues and impaired cellular development of other tissues (Greenwood and Bell, 2003). Head growth seems to be spared when IUGR, which is associated to placental dysfunction tends to occur latter in pregnancy (Falkner et al., 1994). However, the skull is not a single developing unit; rather, it has two distinct regions: the face or viscerocranium and the neurocranium (Cheverud, 1982). The former appears more susceptible to epigenetic factors than the neurocranium (Pucciarelli, 1981; Fields, 1991) because of the different functional requirements and growth patterns (Miller and German, 1999). In this sense, previous studies have demonstrated that when IUGR is experimentally induced in early gestation, facial growth resulted more affected than neurocranium in newborn rats (Oyhenart et al., 2003).

Epidemiological findings and experimental studies in animals have shown that individual tissues and whole organ systems can be programmed in utero during critical periods of development with adverse consequences for their function in later life (Fowden and Forhead, 2004). Many children experiencing IUGR show catch-up growth within the first 2 years of life, but a significant fraction (15 to 40%) fail to do so (Karlberg et al., 1997). Our studies in IUGR animals have indicated that a limited supply of substrates during pregnancy results in an irreversible slowing of cranial growth rate, which persists even after the substrate limitation was removed during lactation (Oyhenart et al., 2003). Similar results were found in the postcranial skeleton after nutritional rehabilitation during the post-weaning period (Oyhenart et al., 2002).

The lack of catch-up growth in IUGR animals makes them an attractive model for studying the mechanisms responsible for growth retardation in low birth weight humans who never attain normal adult stature (Huizinga et al., 2000). The persistent postnatal growth failure may be attributable in part to abnormal GH secretion. Whether putative alterations in GH secretion are the result of abnormalities intrinsic to the pituitary or reflect changes in the production of GH-releasing hormone or somatostatin (SS) is unclear (Huizinga et al., 2000). Studies in undernourished squirrel monkeys (Cónsole et al., 2001a, b) have reported a decreased number of somatotrope cells as well as ultrastructural changes. We believe that similar changes in the pituitary cells may also be found in IUGR animals.

This paper addresses to examine the long-term effects of prenatal injure on body and craniofacial growth of IUGR animals, and its correlation with histomorphometric changes of the pituitary somatotrope population.

Materials and Methods

Animals and Experimental Design

The animals employed in this experiment were *Rattus norvegicus albinus*, var. Wistar, brought from the Comisión Nacional de Energía Atómica in 1997. They were grown as an outbreed colony in the animal house of the Centro de Investigaciones en Genética Básica y Aplicada (CIGEBA-UNLP), for about twelve generations up to the experiment. The outbreeding condition was assured by maintaining a minimal stock of two hundred rats free of experimentation. Periodic genetic monitoring was carried out to avoid bottlenecks or similar effects. The animals were kept free of pathogens and treated in compliance with standardized institutional guidelines.

Rats were individually housed in solid stainless steel cages (12? x 12? x 6.8?), which were cleaned three times a week. The room temperature ranged from 21 to 25ºC and the photoperiod was 12h of light, from 6:00 a.m. to 6:00 p.m. They were fed on a pelleted and sterilized commercial stock diet containing proteins (23%), carbohydrates (44%), lipids (11%), water (8%), fiber (5%), mineral mixture (3%) and vitamin mixture (1%).

Adult male and female rats were mated overnight. The beginning of pregnancy was determined by the presence of spermatozoa in vaginal smear. Pregnant rats were housed in individual steel boxes, fed on stock diet and water *ad libitum*, and assigned to one of three experimental groups: control (CTR), IUGR, and sham-operated (SH). Control dams did not receive any treatment. A lower midline laparotomy was done in mothers of the IUGR group at 14th day of pregnancy. Light-ether anesthesia was given during surgery. The uterine vessels near the base of each uterine horn were partially ligated with a 3-0 silk suture (see Oyhenart et al., 1998, for details). Pregnancy was allowed to proceed until delivery. The SH dams were submitted to a laparotomy,

without vessel ligation, in order to isolate the effects of surgery from those of vessel bending.

After delivery, CTR pups (16 males and 15 females) were suckled by their own mother and IUGR (14 males and 14 females) and SH (15 males and 15 females) pups cross-fostered to a well-nourished control dam. Litters were reduced to four males and four females each, to make lactation uniform across the groups. Pups suckled *ad libitum*. A standard diet was available *ad libitum* to mothers and pups during post lactation.

Measurements

Body weight (BW) was recorded three times: at birth, at weaning, and at the end of the experiment (84 days old).

At 1, 21 and 84 days of age, all the animals were x-rayed under light-ether anesthesia on the dorsal and lateral planes. The following measurements were taken on each radiograph (digital Mitutoyo calliper, 0.05 mm precision): neurocranial length (NL) from de mid point of the frontal-nasal suture to the opistocranium, neurocranial width (NW) from eurion to eurion, neurocranial height (NH) from the spheno-occipital synchondrosis to vertex; facial length (FL) from rhinion to the mid-point of the frontal-nasal suture, facial width (FW) maximum premaxillary distance, and facial height (FH) from the upper first molars to the frontal-nasal suture. The size variation of each component was estimated by calculating two volumetric indices: Volumetric Neural Index (VNI)=$\sqrt[3]{(NL*NW*NH)}$ and Volumetric Facial Index (VFI)= $\sqrt[3]{(FL*FW*FH)}$.

For graphical comparisons, mean values were standardized by Percentual Differences between Means (PDM%). For treatment comparisons: PDM%= $100*(X_1 - X_2) / X_1$, where: X_1 = the mean values in the SH group, and X_2 = the mean values in the IUGR group). This standardization method has been frequently employed (Oyhenart et al., 1998). In its current form, it reduces any difference to a percent value, which cannot be affected by scaling and sense.

Inmunohistochemistry

Briefly, pituitaries tissues from 6 animals of each group was fixed in Bouin's fluid and embedded in paraffin. Serial sections of 4µm were obtained at different levels of the blocks following a ventral-to-dorsal sequence. The sections were immunostained, and then incubated for 1 h at room temperature with the primary anti-GH antibody (murine, Dako, CA, USA), diluted 1:100. Thoroughly washed sections were then treated for 30 min with a ready-to-use EnVision reaction system (Dako, CA, USA). The peroxide-sensitive chromogen was diaminobenzidine. In all instances, the specificity of the primary antiserum was monitored either by observing its ability to block the immunocytochemical reaction after its preabsorption with an excess of the related antigen or by its replacement with normal rabbit serum or phosphate-buffered saline.

Morphometry

Morphometry was performed as reported in detail previously (Cónsole et al., 2002). Briefly, measurements of immunostained pituitary cells were made by videomicroscopy (Imaging Technology, Optimas 5.2). The cells and reference area (RA) were analyzed in each field for an average of ten micrographs taken from two levels (e.g. a and b). These measurements were recorded and processed automatically and the following parameters subsequently calculated: volume density (VD = Σ cell area/RA) and cell density (CD = number of cells/RA). RA (reference area) represents the total adenohypophyseal (*pars distalis*) area scanned, in which the pituitary cells were scored. Thus, this area divided into the sum (Σ) of the individual cell areas (A) yielded VD, which parameter represents an estimate of cell mass according to generally accepted criteria. The number of cells (CD) was calculated by dividing the immunostained area of the pituitary tissue by the mean individual cell area. For this parameter, 100 cells were recorded in each field.

Data Analysis

The goodness of fit for the frequency distributions was estimated by the Kolmogorov-Smirnov for one sample. Normal distributions were found in all cases. Data were processed by multifactor analysis of variance (ANOVA), post-hoc Least Square Difference (LSD) for multiple comparisons and Wilcoxon-Mann-Whitney tests. Statistical procedure was performed with the SPSS 7.0 package.

RESULTS

Body weight and cranial measurements

The differences between control and sham-operated males and females were non signifi-

cant. Therefore, sham-operated animals were used as controls.

Descriptive statistics at 1, 21 and 84 days of age; PDMs (percent difference of the means) and its statistical significance are described in Table 1.

At birth, IUGR rats were significantly lighter than CTR age peers (25.5% and 33.3% in males and females, respectively) (Table 1). Except NW in males and FH in both sexes, the cranial differences were also statistically significant. Although facial component was relatively more affected than the neurocranium, differences in their growth retardation were non significant (Fig. 1).

At weaning, IUGR animals remained significantly lighter compared to CTR. The growth retardation for BW was about 15.9% in males and 2.2% in females. Except FW in females, IUGR also had smaller neurocranial and facial dimensions than their CTR counterparts (Table 1). Both volumetric indices were similarly retarded in males and females (Fig. 1). At 84 days of age, IUGR animals were still 13.5% (males) and 9.6% (females) lighter than CTR counterparts and had lower neurocranial variables. Facial dimensions were less affected and differences were restricted to FH in males and females and FL in females. At variance,

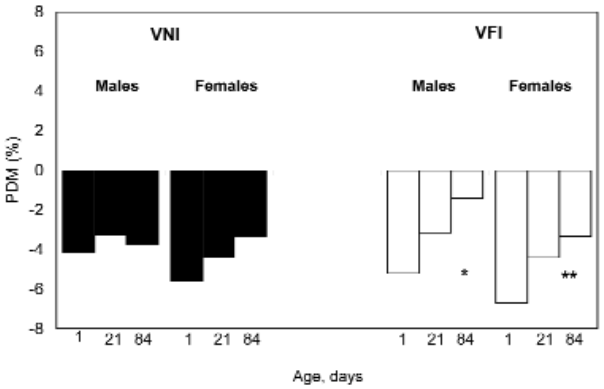

Figure 1. Comparison of growth retardation (%) between VNI (black bar) and VFI (white bar), at 1, 21 and 84 days old in males and females. Bars represent the growth retardation. **p<0.01.

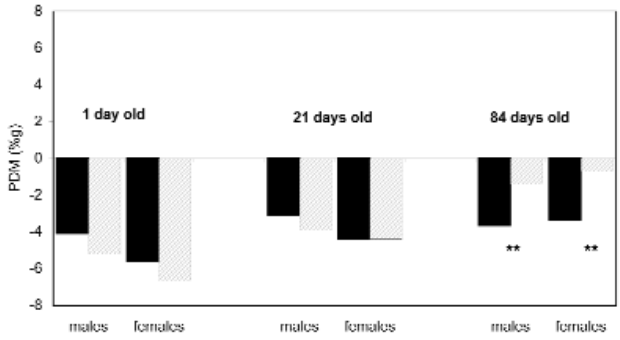

Figure 2. Comparison of growth retardation (%) between 1-21 days of age and 21-84 days of age for VNI (black bar) and VFI (white bar) in males and females. Bars represent the growth retardation. * p<0.05, **p<0.01.

Table 1.-

Mean (M), standard deviation (SD) and percentual differences between means (PDM%) at 1, 21 and 84 days of age.

Variables	1 day old						21 days old						84 days old					
	C		IUGR				C		IUGR				Sham		IUGR			
	M	SD	M	SD	PDM%	p	M	SD	M	SD	PDM%	p	M	SD	M	SD	PDM%	p
Males																		
Body weight	6,9	± 0,1	5,5	± 0,6	-25,5	**	32,5	± 3,4	27,4	± 3,8	-15,9	**	319,9	± 21,4	276,8	± 20,9	-13,5	**
Neurocranium																		
Length	13,7	± 0,5	13,2	± 0,5	-3,6	*	23,3	± 0,7	22,2	± 0,9	-4,7	**	31,9	± 0,7	30,6	± 1,0	-4,0	**
Width	9,9	± 0,3	9,7	± 0,4	-2,0	n.s	14,7	± 0,3	14,3	± 0,2	-2,2	*	16,0	± 0,3	15,6	± 0,3	-2,6	**
Height	7,4	± 0,2	6,9	± 0,4	-6,8	**	10,5	± 0,3	10,3	± 0,3	-2,4	*	12,4	± 0,3	11,9	± 0,4	-4,4	**
Face																		
Length	4,9	± 0,6	4,7	± 0,6	-4,1	**	9,7	± 0,5	9,3	± 0,8	-3,7	**	16,5	± 0,7	16,7	± 0,7	0,9	n.s
Width	7,2	± 0,5	6,7	± 0,7	-6,9	**	10,3	± 0,4	10,0	± 0,4	-3,5	*	14,4	± 0,5	14,3	± 0,5	-0,6	n.s
Height	4,1	± 0,3	4,0	± 0,5	2,4	n.s	7,3	± 0,4	6,9	± 0,4	4,6	*	11,6	± 0,4	11,1	± 0,5	4,5	**
Females																		
Body weight	6,8	± 0,1	5,1	± 0,9	-33,3	**	31,2	± 3,8	30,5	± 4,9	-2,2	**	214,6	± 25,3	194,0	± 12,4	-9,6	**
Neurocranium																		
Length	13,9	± 0,3	12,8	± 0,6	7,9	**	10,2	± 0,6	9,5	± 0,6	6,7	**	29,9	± 0,9	29,0	± 0,7	2,8	**
Width	9,9	± 0,4	9,6	± 0,5	-3,0	*	14,5	± 0,3	14,3	± 0,4	-1,1	*	15,7	± 0,4	15,2	± 0,3	-3,5	**
Height	7,3	± 0,3	6,9	± 0,4	-5,5	**	10,6	± 0,2	10,3	± 0,4	-2,9	**	11,8	± 0,2	11,4	± 0,3	-3,6	**
Face																		
Length	4,8	± 0,6	4,6	± 0,7	4,2	**	9,6	± 0,5	9,4	± 1,0	2,0	**	15,9	± 1,0	15,2	± 0,5	4,1	**
Width	7,4	± 0,5	6,3	± 0,6	-14,9	**	10,2	± 0,6	9,9	± 0,4	-3,4	n.s	13,7	± 0,4	14,4	± 0,6	5,1	**
Height	4,0	± 0,3	4,1	± 0,6	2,5	n.s	7,3	± 0,4	6,7	± 0,4	-7,5	**	11,0	± 0,3	10,6	± 0,3	-3,0	*

*p<0.05

**p<0.01;

n.s. non significant

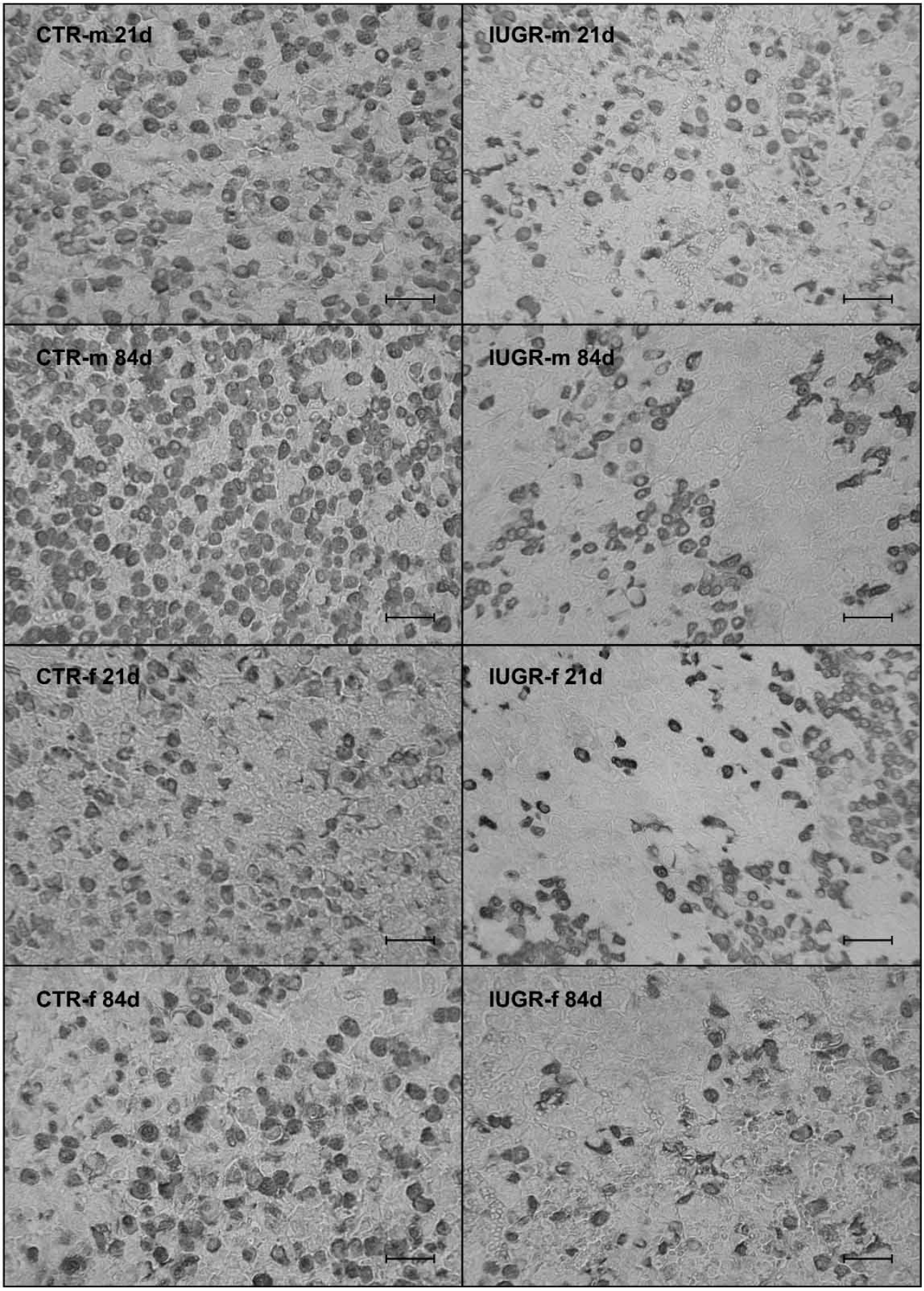

Figure 3. Representative fields of specifically immunostained somatotrope cells of the pars distalis. IUGR and CTR males (m)-females (f) of 21 and 84 days. EnVision peroxidase system. Scale bar: 30 μm.

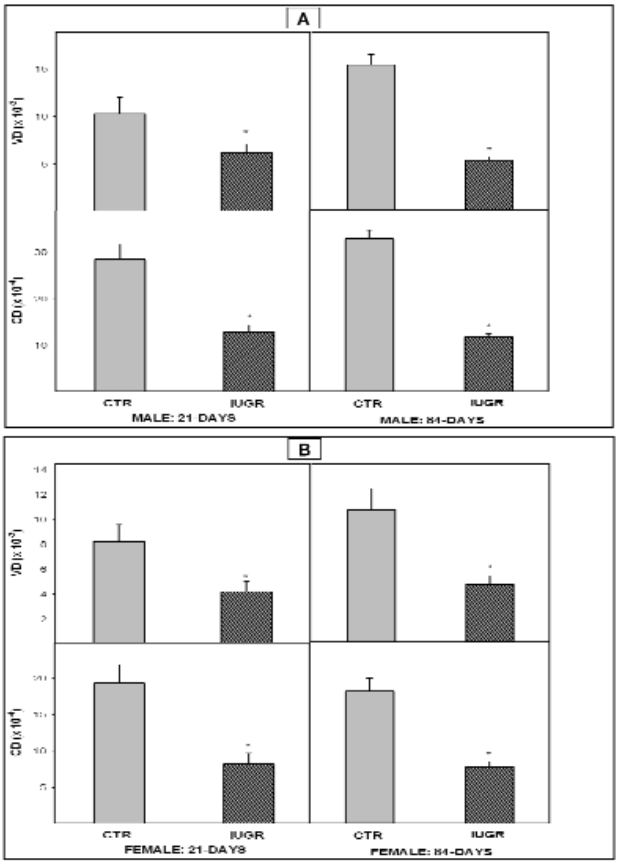

Figure 4. A: Volume density (VD) and cell density (CD) in IUGR males (hatched bar) of 21 and 84 days of age compared to CTR age peers (grey bar) ($p<0.01$).
B: Volume density (VD) and cell density (CD) in IUGR females (hatched bar) of 21 and 84 days of age compared to CTR age peers (grey bar) ($p<0.01$).

FW in IUGR females was significantly larger than CTR peers (Table 1). The differences between volumetric indices were highly significant, since the face grew relatively more than the neurocranium (Fig. 1).

Figure 2 shows differences between ages. It can be observed that both in males and females VNI did not vary significantly from birth to weaning and from weaning to adulthood. On the other hand, the facial index varied significantly between 21 and 84 days of age, indicating the persistence of growth retardation in this component (4-6%).

Morphometric immunohistochemical studies on somatotrope population

We detected changes in the histometrical analysis on somatotrope population of *pars distalis* (Fig. 3). IUGR, males and females, of 21 and 84 days of age showed a significant decrease ($p<0.01$) in the cell density (CD) and the volume density (VD) compared to CTR counterparts. Males had lower values than females in the histometric analysis (Figs. 4A, 4B).

Discussion

Unilateral and bilateral uterine artery ligation in the pregnant rat during late gestation is an animal model which appears to mimic third trimester IUGR in the human (Bussey et al., 1985; Ogata et al., 1990; Huizinga et al., 2004). In previous studies, we found that IUGR induced at the first day of pregnancy produced a significant reduction of body weight at birth –13.2% in males and 4.9% in females– (Oyhenart et al., 2003). Nevertheless, in this study the growth retardation was greater –25.5% in males and 33.3% in females– indicating that uterine vessels ligation in the last week of pregnancy is much more severe possibly because of the short time for maternal-fetal adaptation.

The postnatal period is an opportunity to recover any growth deficit. However, in this experiment, intrauterine restriction resulted in body growth failure, which persisted into adulthood without signs of catch-up growth. Body weight at weaning was clearly reduced in males (15.9%), but at a lesser degree in females (2.2%) suggesting some initial catch-up growth. However, during the extended follow-up period we did not observe a complete catch up in body weight gain. The growth retardation at 84 days of age decreased only 2.4% in males (13.5%) and increased 7.4% (9.6%) in females. These observations are consistent with the hypothesis that the more severe the degree of growth retardation, the longer time it will take to catch up growth (Houdijk et al., 2000).

Only a few studies have looked at the growth of the two major skull components. Oyhenart et al. (2003) observed that these regions are differentially modified by IUGR because of the differences in their critical period of growth, leading to changes of the cranial shape. In the present study, the whole size of neurocranium and face in newborn IUGR rats was smaller than that of their control peers. However, cranial shape was unaltered because of the similar growth retardation in these components. The size differences between groups decreased at weaning, but IUGR rats remained significantly smaller than controls. Again, cranial shape was kept constant. Unlike, at adulthood, the differences in growth of the major components resulted in changes of cranial shape. Despite the trend toward a decrease in cranial differences between IUGR and CTR adult animals (Fig. 2), neurocranial and facial volumes in IUGR remained smaller without a

complete catch up growth: the neurocranium was still significantly smaller and the face only exhibited a partial recovery. It is well stated the greater susceptibility of the facial component to epigenetic factors (Fields, 1991; Miller and German, 1999). The present findings also indicate for a long-term susceptibility of neurocranium, leading to postnatal growth failure. The correlation between impaired cranial growth and cerebral structures is uncertain. In several outcome studies, absence of catch up head growth has been strongly linked to neurodevelopmental impairment in childhood (Hack et al., 1991; Frisk et al., 2002). Furthermore, there is a strong experimental evidence of a deleterious effect of placental insufficiency on fetal brain development, cell number and cell size with overall lighter brain weight (Mallard et al., 1998; Mallard et al., 2000; Dressino and Pucciarelli, 2002). In a recent study in preterm infants with IUGR, Tolsa et al. (2004) found a significant reduction of brain growth, due primarily to the cerebral cortical development. Both brain tissue volume and cortical grey matter volume change over time showed no catch-up growth in the IUGR group.

Intrauterine growth retardation leads to reprogramming of the hypothalamic regulation of GH secretion in the rat (Huizinga et al., 2001) and alterations in the endocrine function of the adult offspring in several species (Fowden et al., 2005). The long-term negative effect of severe IUGR after bilateral uterine artery ligation on postnatal growth in the rat is accompanied by a physiological alteration GH/IGF-I axis (Houdijk et al., 2000). The results of this study indicates a clear decrease in the cell density (CD) and the volume density (VD) of the IUGR somatotrope population in weaned and young adult rats, and suggests a persistent alteration of the *pars distalis*, which cannot be modified. Although the underlying mechanisms are unclear, they are probably involves the growth hormone regulation. This hormone -the central endocrine regulator of somatic growth- is stimulated by the hypothalamic Growth hormone releasing hormone (GHRH) and inhibited by somatostatin (SS). Neuropeptide Y (NPY) stimulates the SS secretion and hence inhibits indirectly the GH secretion (Tannenbaum, 1991; Giustina and Veldhuis, 1998). It has been speculated that during postnatal phase, when growth is under the control of the GH axis, growth retardation in IUGR rats is caused by increased activity of the hypothalamic SS neurons that inhibits GH secretion (Huizinga et al., 2000).

Conclusions

The timing and the duration of the growth-retarding injure seem to be crucial for the occurrence of catch-up in body weight and cranial growth in the rat. Despite the trend toward a decrease in cranial differences of adult animals, the neurocranium remained smaller and the face exhibited a partial recovery. The quantitative differences detected between control and IUGR in the pituitary somatotrope population could be responsible for the lack of catch-up growth, suggesting that intrauterine growth retardation have a long-lasting effect on the GH axis.

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