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ORIGINAL CONTRIBUTION

Effect of a neonatal low-protein diet on the morphology of myotubes in culture and the expression of key proteins that regulate myogenesis in young and adult rats

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Abstract
Aim To investigate the effects of a neonatal low-protein diet on the morphology of myotubes in culture and the expression of key proteins that regulate myogenesis in young and adult rats.
Methods Male *Wistar* rats ($n = 18$) were suckled by mothers fed diets containing 17% protein (controls, C) or 8% protein (undernourished, UN). All rats were fed a normal protein diet after weaning. Muscles were removed from the legs of 42-, 60- and 90-day-old rats. Muscle cells were cultured to assess cell number, morphology and the expression of major proteins involved in myogenesis (Pax7, cadherins, β1 integrin, IL-4Rα and myogenin) by western blotting. IL-4 levels in culture supernatants were measured by ELISA.
Results Offspring from mothers fed a low-protein diet showed a lower body weight gain. Cell number and myotube expansion were reduced in cultured muscle cells from UN, but the expression of myogenic marker proteins was unaltered.
Conclusions Dietary restriction during lactation had no impact on the synthesis of myogenic marker proteins, and myocyte differentiation occurred normally in the muscles of offspring aged 42, 60 or 90 days. Nevertheless, the number and morphology of the myotubes are altered.

Keywords Critical period of development · Programming · Satellite cells · Neonatal undernutrition · Skeletal muscle · Rats

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Introduction

Maternal and neonatal undernutrition not only causes prolonged growth retardation but also modifies the programming of biochemical mechanisms related to endocrine-metabolic control [1]. Fetal programming is the phenomenon whereby changes in the critical period of development in response to the environment may have permanent effects on the organs and tissues [2]. Perinatal undernutrition-induced metabolic diseases in adult life have been demonstrated in experimental models in rats [3, 4]. In humans, epidemiological studies have also revealed an inverse relationship between birthweight and type 2 diabetes mellitus, hypertension, hyperlipidemia, obesity and insulin resistance in adult life [5–7]. It has been speculated that early influences on the growth and development of skeletal muscle fibers may underlie this relationship [8].

Perinatal undernutrition on muscle-fiber development and composition has been described in a variety of mammalian species [9]. Wilson et al. [10] showed a reduction in secondary fibers in both *soleus* and *lumbrical* muscles and

a deficit in the growth rate of the newborn rat, when the maternal diet was restricted to 30% of the control animals. Maternal low-protein exposure (9% casein) during mid- to late pregnancy reduces the numbers of fibers formed within the *soleus* and *gastrocnemius* muscles of young rats [9]. On the other hand, Bayol et al. [11] found no effect of maternal undernutrition on numbers of fibers in the *semitendinosus* muscle at 21 days postpartum but an increased muscle-cell proliferation and a trend toward increased satellite-cell activity.

Satellite cells are responsible for the growth, repair and maintenance of the skeletal muscle [8]. In response to environmental activating signals derived from injury, satellite cells undergo asymmetric cell divisions for the purpose of self-renewal, giving rise to committed myogenic cells, myoblasts, thereby sustaining postnatal muscle repair and growth [8]. Adhesion molecules, growth factors and cytokines released by the neighboring cells can also activate satellite cells [12]. The myogenic process is regulated by transcription factors, such as Pax3/Pax7, and members of the family of the myogenic regulatory factors (MRFs), including MRF4 (Myf6), Myf5, MyoD and myogenin [12].

Myogenin and MyoD may have important functions in later development, since they bind directly to the enhancers and activate transcription of the creatine kinase and myosin light chain [8]. Several gene products are crucial for the fusion of mammalian myoblasts, including cell adhesion molecules (N and M cadherins) and the $\beta 1$ integrins [13]. It was recently demonstrated that newly formed myotubes also secrete IL-4, which interacts with the IL-4 α receptors (IL-4Rα) present on surrounding myoblasts to promote their fusion with existing myotubes, thereby increasing myotube size [14].

Previous studies have reported the relationship between maternal undernutrition and the expression of genes that control muscle growth in offspring at weaning [11]. Little is known about the long-term effects on the satellite-cell activity in adult rats, and the mechanisms by which maternal nutrition may influence muscle growth. In the present study, we evaluated the effects of neonatal undernutrition on the morphology of myotubes in culture and the expression of proteins that regulate myogenesis in rats aged 42, 60 and 90 days.

Materials and methods

Animals and diet

Pregnant female *Wistar* rats were purchased from Centre d'élevage Dépré (Saint-Doulchard, France). Animals were handled in accordance with the guidelines and regulations of the Ethics and Safety Committee of the Compiègne University of Technology and housed under conditions of controlled temperature (22 ± 1 °C), 12-h light–dark cycle. The standard animal laboratory food and water were given ad libitum. At the time of delivery, the litter size and pups' birth weight were recorded. During the suckling period, the offspring were kept in groups of six pups, randomly distributed into two nutritional groups according to their mothers' diet: a well-nourished group (control, C) fed by mothers receiving a 17% protein (casein) diet and a low-protein group (undernourished, UN), fed by mothers receiving a 8% protein (casein) diet (Table 1). After weaning (at the age of 22 days), only male offspring were used (nine per group) and kept in the collective cage, receiving standard animal laboratory food (Teklad Global 18% protein rodent diet; Harlen Teklad) until the 60-day-old ad libitum. Subsequently, offspring were fed by a 14% protein diet (Teklad Global 14% protein rodent diet; Harlen Teklad) until the 90th day of life. Body weights were recorded every 5 days (Mettler Toledo XS4001S, 0.1 g readability, Inc, Columbus, OH) during lactation and at 42, 60 and 90 days. Body weight gain was calculated as follows: percentage weight gain = [body weight (g) × 100/birthweight (g)] − 100 [11].

Offspring were anesthetized with Ketamin (45.2 mg/kg)/Xylazin (6.8 mg/kg) by intramuscular injection at different ages (42, 60 and 90 days, $n = 6$ per age). The muscles from both legs were removed and immersed in ice-cold 15 mL phosphate-buffered saline (PBS) containing 0.15 g glucose

Table 1 Composition of the diets (control 17% and low protein 8%)

Ingredients	Amount for 1 kg of diet	
	Low protein	Control
Casein	79.3 g	179.3 g
Vitamin mix[a]	10 g	10 g
Mineral mixture[b]	35 g	35 g
Cellulose	50 g	50 g
Choline bitartrate	2.5 g	2,5 g
DL-methionine	3.0 g	3,0 g
Soya oil	70 mL	70 mL
Corn starch	750.2 G	650.2 g

[a] Vitamin mixture contained the following (mg/kg of diet): retinol, 12; cholecalciferol, 0.125; thiamine, 40; riboflavin, 30; pantothenic acid, 140; pyridoxine, 20; inositol, 300; cyanocobalamin, 0.1; menadione, 80; nicotinic acid, 200; choline, 2,720; folic acid, 10; p-aminobenzoic acid, 100; biotin, 0.6

[b] Minerals mixture contained the following (mg/kg of diet): $CaHPO_4$, 17,200; KCI, 4,000; NaCl, 4,000; MgO, 420; $MgSO_4$, 2,000; Fe_2O_2, 120; $FeSO_4{\cdot}7H_2O$, 200; trace elements, 400 ($MnSO_4{\cdot}H_2O$, 98; $CuSO_4{\cdot}5H_2O$, 20; $ZnSO_4{\cdot}7H_2O$, 80; $CoSO_4{\cdot}7H_2O$, 0.16; KI, 0.32; sufficient starch to bring to 40 g [per kg of diet])

and 1% antibiotic solution (Gibco, InVitrogen Cergy Pontoise, France). The animals were killed after the procedures.

Primary culture of rat skeletal muscle cells

All products for cell culture except Matrigel and DNAse were from Gibco (InVitrogen, Cergy-Pontoise, France). Primary culture of rat skeletal muscle cells was performed as previously described [15]. Briefly, the *soleus*, *gastrocnemius* and *quadriceps* muscles were dissected, cut into small pieces and digested with 2% type II collagenase, 0.25% trypsin and 0.1% DNAse (Sigma) for isolation of myoblasts. Cells were placed into 80-cm^2 Pétri dishes (Nunclon®) coated with 2 mL Matrigel® (BD Biosciences, Le Pont de Claix, France) diluted 1:500 in DMEM. Cells (1.6×10^6 cells/80-cm^2 dishes) were cultured in DMEM containing 10% fetal bovine serum, 10% horse serum, 4mMol/L L-glutamine and 1% antibiotic solution. After 48 h of culture, a differentiation medium (DM) with 7% horse serum, 4mMol/L L-glutamine and 1% antibiotic solution was used and changed every 2 days for a total of 10 days.

Cell number and morphology of the myotubes in culture

Cell number was colorimetrically evaluated by measuring intracellular lactate dehydrogenase (LDH) activity. Cells in culture for 10 days were lysed in serum-free DMEM and transferred to a 96-well plate. The LDH concentration was quantified with the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega Corporation, Madison, WI, USA) in accordance with the manufacturer's instructions. The absorbance (490 nm) of the red formazan produced by LDH was measured in a microplate reader (Model 680, Bio-Rad). The absorbance was proportional to the total number of cells. LDH activity is given as the number of cells, read off from a standard curve of absorbance plotted against an increasing number of lysed cells.

The morphology of myotubes in culture for 10 days was observed using a contrast microscope (Olympus CKX41, Japan) coupled to a digital camera (Canon A620). Qualitative analysis was performed from 5 fields by dish at 100× magnification.

Expression of myogenic marker proteins

Proteins were extracted with lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton× 100, 1 M NaF, 100 mM PMSF, 10 mg/mL aprotinin, 10 mM orthovanadate). Aliquots of supernatants were used for the measurement of total protein content, as described by Bradford [16]. Protein lysates from muscle cells cultured for 0, 4, 7 and 10 days were mixed with Laemmli buffer and boiled for 5 min. Equal amounts of proteins of each sample (40 μg) were subjected to SDS–PAGE (7.5% or 12% gels) and transferred to nitrocellulose membranes (BioRad, Marnes-la-Coquette, France). The nitrocellulose filters were soaked in blocking solution (PBS containing 0.1% Tween 20 and 5% non-fat dried milk) at room temperature for 1 h and subsequently incubated overnight at 4 °C with rabbit anti-Pax7 (Santa Cruz Biotechnology, Tebu-Bio, Velizy, France), anti-pan-cadherin (Sigma, Saint-Quentin Fallavier, France), anti-IL-4Rα (Santa Cruz Biotechnology, Tebu-Bio, Vélizy, France) antibodies or a goat anti-β1 integrin antibody (Santa Cruz Biotechnology, Tebu-Bio, Vélizy, France). The membranes were then incubated with horseradish peroxidase-linked goat anti-rabbit immunoglobulin G or a peroxidase-linked donkey anti-goat immunoglobulin G (Jackson ImmunoResearch, Interchim, Montluçon, France) for 1 h at room temperature. Membranes were incubated for 1 h at room temperature with mouse monoclonal anti-myogenin antibody (BD Biosciences, Le Pont de Claix, France), followed by a goat anti-mouse IgG coupled to horseradish peroxidase (Jackson ImmunoResearch, Interchim, France). Immunoreactivity was detected by chemiluminescence (ECL and ECL hyperfilm, GE Healthcare Europe, Saclay, France). The molecular weights of the immunoreactive bands were established by comparison with stained standards (BioRad, Marnes-la-Coquette, France). Western blots for actin and α-tubulin were used to check gel loading. The ratios of proteins to actin or α-tubulin were determined, and band intensities were quantified using KODAK 1D Image Analysis Software (Scientific Image System, Rochester, NY, USA).

Concentration of IL-4 in the supernatants of muscle cells in culture

Interleukin-4 (IL-4) released was measured by ELISA in the supernatants from cells cultured for 10 days (DuoSet ELISA Kit, R&D Systems, Minneapolis, MN, USA). The IL-4 concentration was normalized to 10^5 cells using the LDH dosage.

Statistical analysis

The data are presented as mean ± SEM. For body weight analysis, two-way repeated measures analysis of variance ANOVA, followed by Bonferroni's post hoc test, was used. For LDH and IL-4 levels in the supernatants of cultured cells and immunoblots quantitation groups were compared using Student's t-test. Results were considered statistically significant for $p < 0.05$ (NS non-significant, $*p < 0.05$). The GraphPad Prism 5 software (Graph Pad Software, Inc., San Diego, CA, USA) was used.

Results

The birth weight of neonates did not differ when groups were compared ($C = 5.9 \pm 0.4$ and UN $= 5.8 \pm 0.3$). During lactation, offspring from mothers fed a low-protein diet showed a lower body weight than controls at 10, 15 and 21 days. The pups from undernourished mothers continued to exhibit a lower body weight than the control group throughout the experiment: 42 days ($C = 208.6 \pm 4.3$; UN $= 183.3 \pm 4.3$), 60 days ($C = 308.7 \pm 6.6$; UN $= 246.1 \pm 5.6$) and 90 days ($C = 378.2 \pm 7.7$; UN $= 313.1 \pm 4.3$; Fig. 1).

Between days 4 and 7 in differentiation medium (DM), both myoblasts from controls and undernourished rats began to form myotubes into the standardized mode. Examination of cultures under the phase contrast microscope suggested that on the 10th day of culture, myotubes from control rats are large and numerous, while the number and the size of myotubes were low in undernourished rats (Fig. 2a). LDH measurement showed a lower cell number in the UN group when compared with cells from the *C* group (*C* group $21.8 \pm 0.19 \times 10^3$, UN group $18.9 \pm 0.2 \times 10^3$; Fig. 2b).

Myotubes from UN 90-day-old offspring were less ramified than those from their controls (Fig. 3).

The expression of Pax7 protein in lysates of cells taken from 42-, 60- and 90-day-old pups and cultured for 0 and 4 days was studied by western blotting. The expression remained roughly constant in cells from offspring aged between 42 and 90 days at all culture times (Fig. 4). There was no difference in the Pax7 content of cells from UN and *C* pups. Similarly, the cadherin (N and M) content of cells cultured for 4 days was assayed using a pan-cadherin antibody. The amounts in 42-, 60- and 90-day-old pups appeared to be similar. There was no difference in the expression of cadherin in cells from UN and *C* pups (Fig. 4).

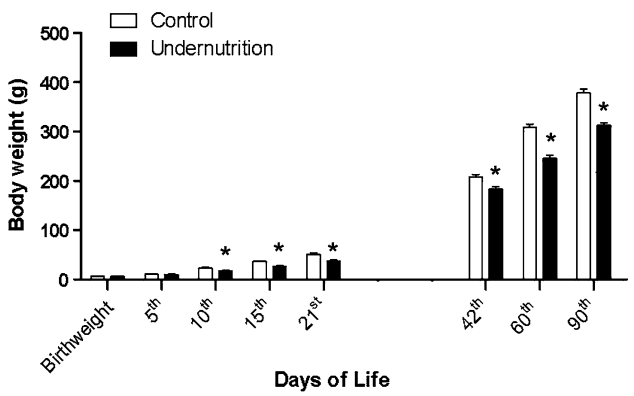

Fig. 1 Body weight of pups from mothers fed a low-protein diet (undernutrition, casein 8%) or a control diet (casein:17%) during growth. Analysis was performed every 5 days during lactation ($n = 9$ in each group) and at 42, 60 and 90 days ($n = 3$ in each group). The values are presented as means + SEM. $p < 0.05$ compared with control group using two-way ANOVA and Bonferroni's post hoc test

The expression of the $\beta 1$ integrin subunit in cells cultured for 4 or 7 days did not vary with the age of the pups providing the muscle samples. The content of the $\beta 1$ integrin subunit in muscle cultures of UN and C offspring was similar. Cells from 42-, 60- and 90-day-old pups cultured for 7 days showed similar contents of IL-4Rα, but IL-4Rα was not detected in cells from 90-day-old offspring. The expression of IL-4Rα protein in cells from UN and *C* offspring cultured for 7 days was the same.

Muscle cells cultured for 10 days all showed the same content of myogenin protein, regardless of the age of the offspring providing the muscle. There was also no difference in the expression of myogenin in cells cultured for 10 days from 60-day-old UN and C offspring.

The concentration of IL-4 in the supernatants of muscle cells from 60-day-old offspring cultured for 10 days, normalized using LDH activity, showed no significant differences between UN and C offspring.

Discussion

Perinatal undernutrition has been one of the most thoroughly studied programming factors acting in the genesis of metabolic disease in adult life [17]. The mechanisms for the induction of programming appear to include the following: certain clones of cells may be altered by environmental adversity during development; nutrient environment may permanently alter gene expression; and early nutrition may permanently reduce cell numbers in the organs and tissues [18]. In our study, casein (a highly phosphorylated protein) was chosen because it is an important component of breast milk. Casein absorption induces an increase in the concentrations of essential (threonine, valine, methionine, isoleucine, leucine, phenylalanine and lysine) and non-essential amino acids (histidine and arginine) in the neonatal plasma [19]. Moreover, it has been shown that dietary casein intake is the key factor in the stimulation of ribosome aggregation in liver that reflects the protein biological value [19]. Thus, casein has been widely used in previous studies as an inducer of perinatal undernutrition.

In the present study, we first examined the effects of a low-protein diet during lactation on the body weight of the offspring. We then studied the morphology of myotubes and the expression of some key proteins known to regulate myogenesis, from muscle-cell cultures of young and adult offspring. The results indicate that offspring from mothers fed a low-protein diet showed a lower body weight at adult age (60 and 90 days) than their controls. Our results are in

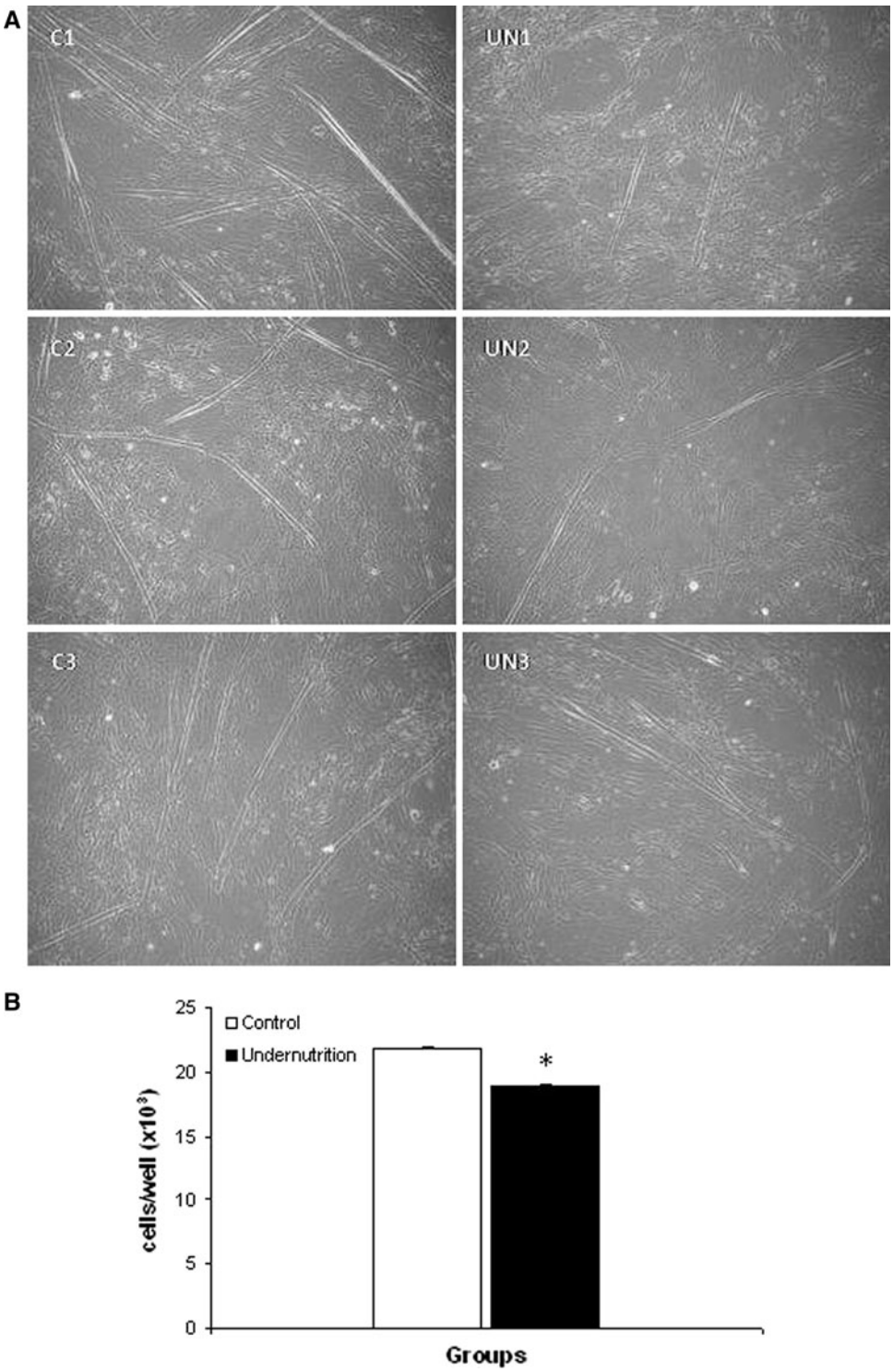

Fig. 2 Morphological observation of muscle cells in culture (**a**) and numbers of muscle cells/well (0.33 cm^2) (**b**) after 10 days in culture (60-day-old offspring). Mothers were submitted to a control diet (C1, C2, C3) or a low-protein diet (UN1, UN2, UN3) during lactation. **a** Cells were cultured in a differentiation medium (DM) and observed at 10 days. Phase contrast objective 10×. *Bar* 100 μm. **b** Data were obtained from LDH activity of adherent cells. Data are expressed as mean + SEM $n = 18$ in each group. *$p < 0.05$ unpaired Student's *t*-test. *C* control group, *UN* undernourished group

agreement with earlier studies using the same experimental model [11, 20, 21]. Nevertheless, 90-day-old rats were not evaluated in those studies. Neonatal maternal protein restriction (8% casein-restricted diet) causes changes in milk composition and volume of the lactating rats, and it may be related to less transfer of nutrients to the offspring and to a long-lasting low body weight [22]. In addition, it has been demonstrated that reduced body weight induced by maternal undernutrition is related to the low number of muscle fibers in offspring [8, 18]. Thus, it can be suggested that changes in the numbers and/or types of fibers present in skeletal muscle may contribute to the risk of developing type 2 diabetes, and/or obesity in later life (developmental origin of adult disease) [17]. However, little is known

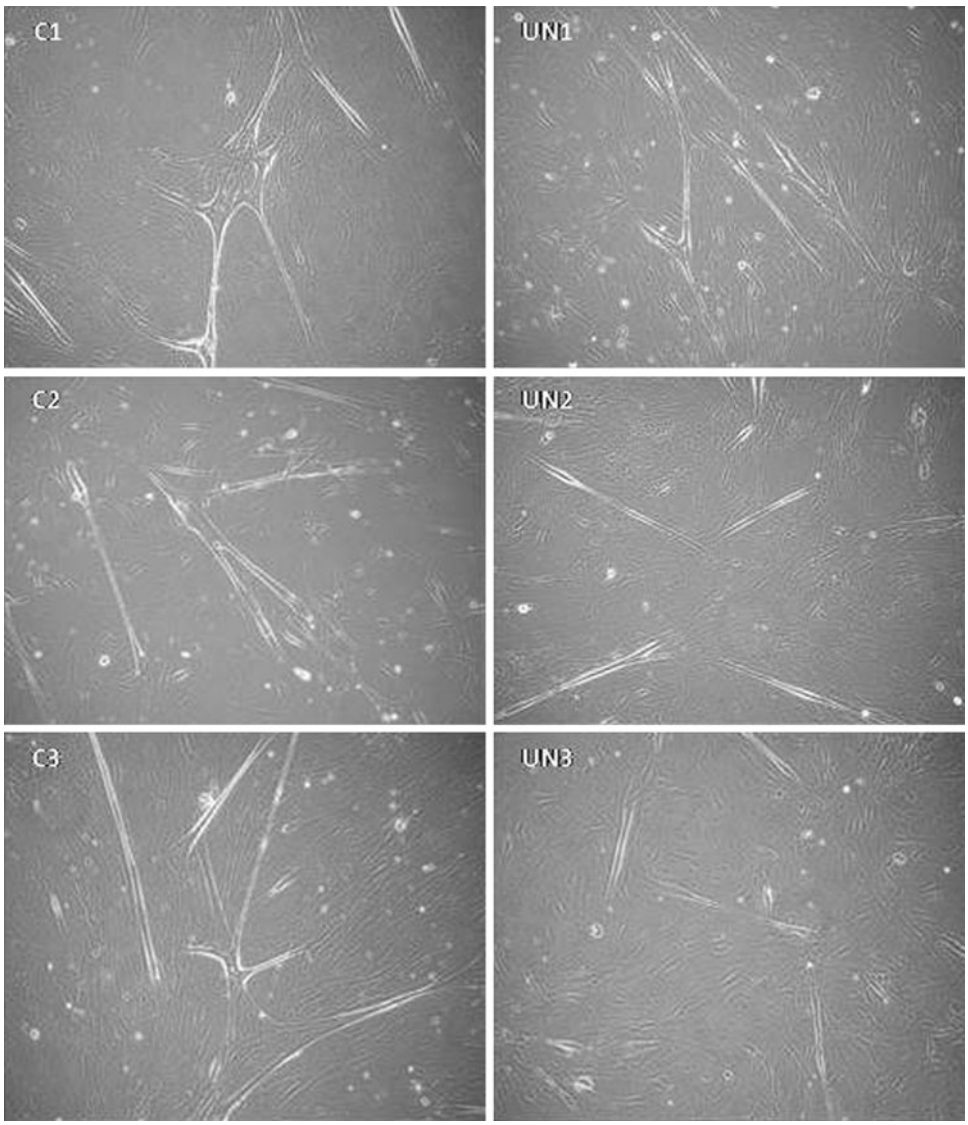

Fig. 3 Morphological analysis of muscle cells in culture (90-day-old offspring). Mothers' offspring were submitted to a control diet (C1, C2, C3) or a low-protein diet (UN1, UN2, UN3) during lactation. Cells were cultured in a differentiation medium (*DM*) and analyzed on the 10th day. Phase contrast objective 10×. *Bar* 100 μm

about the molecular mechanisms by which maternal nutrition influences muscle growth.

In order to test the hypothesis that neonatal undernutrition can impair myogenesis, the capacity of muscles from offspring of different ages (42, 60 and 90 days) to produce myogenic markers of satellite cells, myoblasts and myotubes in culture was assessed. Our data demonstrate that muscle cells from 60-day-old UN offspring cultured for 10 days grew more slowly than did muscle cells from C offspring. There were fewer myotubes in samples from 60-day-old UN offspring, and the myotubes from 90-day-old offspring expanded more slowly than those from their C counterparts. It has been demonstrated that muscle-fiber formation and postnatal growth are regulated by insulin-like growth factor (IGF)-1 [23]. Systemic IGF-1 levels are low in both mothers and fetuses following maternal undernutrition, suggesting that impaired myogenesis could be the result of diminished levels of IGF-1 [24]. After birth, IGF-1 has been shown to promote muscle-fiber hypertrophy by a combination of protein synthesis and activation of satellite cells [25]. We therefore performed key protein-expression analyses of satellite cells, myoblasts and myotubes in the offspring.

Pax7 transcription factor and cadherins (especially N and M cadherins) are involved in initial cell to cell recognition during myogenesis and play an important role as regulators of muscle-cell specification and tissue formation during development [12, 13]. Pax7 is expressed in quiescent satellite cells, serving as a marker for their localization beneath the basal lamina [12]. M-cadherin accumulates with β-catenin at the areas of contact between fusing myoblasts and β-catenin colocalizes with actin in pre-fusing myoblasts [13]. Satellite cells contain β-catenin that remains present in them during activation and proliferation.

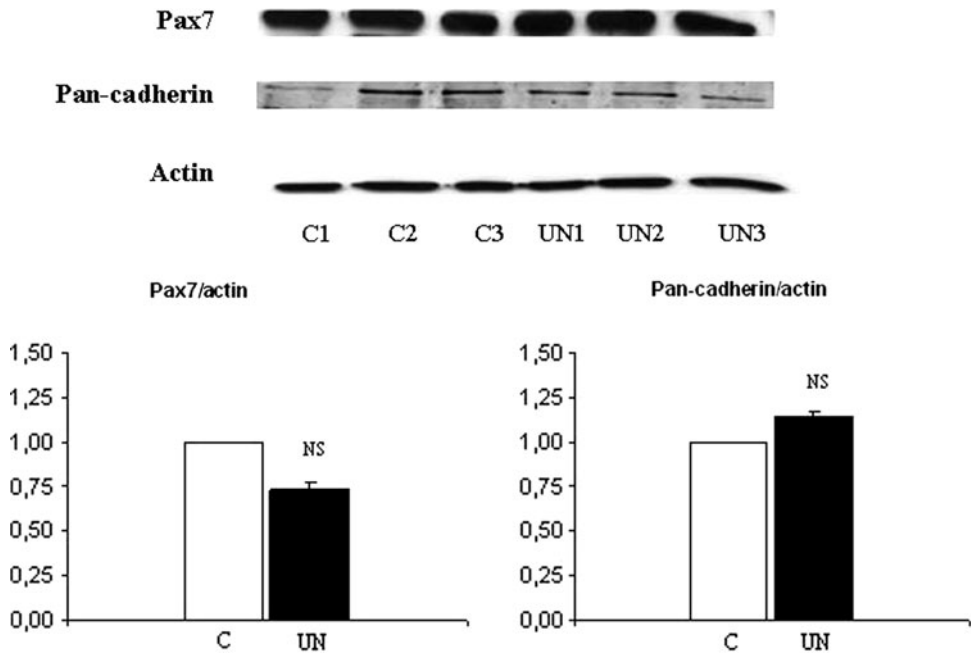

Fig. 4 Immunoblots of Pax7 and M–N (pan) cadherins in muscle cells—from 60-day-old rats. Muscle cells from control (C1, C2, C3) and undernourished offspring (UN1, UN2, UN3) were cultured for 4 days. The lysates were separated by 7.5% SDS PAGE. Actin was used as control for gel loading. The amount of each factor relative to that of actin is shown below. *NS* non-significant

In the present study, we found no difference in the expression of Pax7 and cadherin proteins in cells from UN and *C* offspring, regardless of the age of the donor offspring or the time in culture. The expression of those proteins seems to remain roughly the same in offspring aged between 42 and 90 days. Most satellite cells expressing Pax7 up-regulate MyoD and enter into a proliferative state [12, 26]. N and M cadherins play a role in the initiation of myoblast fusion to form multinuclear myotubes [27]. Our results show that the initial processes of proliferation and differentiation in myogenesis were not affected by neonatal undernutrition.

Skeletal muscle cells are known to produce a wide variety of integrins. The β1 subunit is produced throughout myogenesis, and different variants are present in myoblasts and myotubes [28]. Pure populations of β1-null myoblasts and satellite cells isolated from β1-null chimeric embryos and chimeric newborn mice differentiate in vitro and fuse to form multinucleated myotubes [29]. Other studies have demonstrated that β1-deficient myoblasts adhere to each other, but that their plasma membrane breakdown is defective [30]. The amounts of the β1 integrin protein did not seem to change with the age of the donor pups, and we found no differences in its expression between cells from UN and *C* pups. Many cell adhesion proteins, including the integrins and cadherins, have been shown to be important for myoblast fusion, but how exactly they regulate cell fusion remains largely unknown. However, the focal adhesion kinase (FAK) involved in integrin signaling appears to be a mediator by which integrins regulate myoblast fusion [31]. IL-4 is not required for fusion between mononucleated myoblasts, but it is required for myotube maturation. It may favor myogenic cell migration and the integrin-modulated interaction of these myoblasts with newly formed myotubes [14].

The newly formed myotubes recruit myoblasts for fusion by secreting IL-4, leading to muscle growth. The transcription factor NFATc2 controls myoblast fusion after the formation of a myotube and is necessary for further cell growth [14]. IL-4 has been identified as a molecular signal that binds to the IL-4Rα subunit on myoblasts to promote fusion and growth [14]. The IL-4Rα subunit of the IL-4R is present on both myoblasts and myotubes and is required for muscle growth. IL-4 controls cell fusion by binding to the IL-4R of myoblasts rather than myotubes, and signaling via the IL-4R in myoblasts is required for cell fusion with nascent myotubes [14]. We believe that the IL-4Rα protein is absent from the muscles of pups aged over 60 days, and this was true for both UN and C rats. Our results suggest that this regulation no longer exists in adult rats (60-day-old). Cells from 60-day-old UN and C rats in culture for 10 days secrete the same amount of IL-4 into the medium. This suggests that myotube function is not altered in UN rats, in spite of the difference observed in their expansion.

Lastly, we analyzed the production of the myogenic regulatory factor myogenin, which is abundant in the muscles of newborn pups. It resides in the nucleus and may function as a sequence-specific DNA-binding factor that interacts directly with muscle-specific genes during myogenesis [12]. The content of myogenin protein in 10-day muscle-cell cultures was not influenced either by the age of the donor rats or whether they were UN or C rats.

Conclusion

In conclusion, casein restriction during lactation followed by the restoration of a normal protein diet at weaning has no impact on the synthesis of myogenic marker proteins, and myocyte differentiation occurred normally in the muscles of offspring aged 42, 60 and 90 days. Nevertheless, the number and morphology of myotubes are affected.

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Conflict of interest The authors declare that they have no conflict of interest.

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