Normal function of the hypothalamic-pituitary growth axis in three dwarf Friesian foals

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Serial blood samples were collected from three dwarf Friesian foals to examine their endogenous growth hormone (GH) profiles, and the integrity of the GH-insulin-like growth factor-1 (IGF-1) axis was tested in one of them by examining its responses to the administration of GH-releasing hormone (GHRH) and to 10 days of treatment with recombinant equine GH. The basal serum concentrations of IGF-1 in the three dwarf foals were compared with those in nine age-matched normal foals. All the dwarf foals secreted endogenous GH. Stimulation with 7·0 µg/kg GHRH led to a 1400 per cent increase in plasma GH concentration in the dwarf foal tested, and 10 daily subcutaneous treatments with 20 µg/kg recombinant equine GH led to a 100 per cent increase in its serum IGF-1 concentration. The basal serum concentrations of IGF-1 in the dwarf foals were not significantly different from those of the normal foals.

DWARFISM has been described extensively in human beings (Donaldson and Paterson 1998) and dogs (Sande and Bingel 1983, Kooistra and others 2000), and the phenotypic characteristics of heritable dwarfism in Friesian horses have recently been described by Back and others (2008). The mechanism underlying the growth disturbance in Friesian dwarf horses is not known, but it would help to determine the genetic defects that predispose to, or are responsible for, dwarfism in this breed.

Growth in animals is generally defined by their age and development-related increases in body size or weight, with particular reference to the skeletal elements (Mullis 2005). Despite genetic and environmental diversity, most animals grow and develop secondary sexual characteristics in a fairly predictable manner (Shulman and Bercu 2000), and any deviation from an established growth pattern may be the first sign of an underlying nutritional disorder, disease of a specific organ system, endocrinopathy or genetic dysfunction (Reiter and Rosenfeld 2002).

Normal somatic growth requires the integrated and concerted action of many hormonal and metabolic pathways, and other growth factors, and is driven by the hypothalamic-pituitary axis (Mullis 2005). In this respect, growth hormone (GH) is the major endocrine regulator of linear growth and, as a result, failure to grow in human beings is often associated with disturbances of the hypothalamic GH-insulin-like growth factor-1 (IGF-1) axis (Donaldson and Paterson 1998). Pituitary GH is usually secreted in an episodic manner as a result of a delicate interaction between the two hypothalamic peptides, GH-releasing hormone (GHRH) and somatostatin (SRIF). GH stimulates the liver to produce IGF-1, which mediates most of the effects of GH via the IGF receptor family; GH also exerts direct effects on, for example, physeal growth plates (Donaldson and Paterson 1998).

Defects in the function of the GH-IGF-1-axis could, in theory, be located at any level from the hypothalamus down to the target receptors in skeletal tissues. Reported defects of the GH-IGF-1 axis in species other than the horse include defects of the growth hormone-releasing hormone (GHRH) receptor (*lit/lit*) in the mouse (Sellier 2000), GH insufficiency syndrome (Mullis 2005), GH insensitivity (primary and secondary), GH receptor problems (Laron syndrome) (Hull and Harvey 1999, Laron 2004), and defects in the synthesis and action of IGF-1 (Mullis 2005).

Diagnostic tests for assessing the function of the GH-IGF-1 axis include measurements of the serum concentrations GH and IGF-1, and various endocrine challenge tests, for example, with GHRH (Bauman 2000). However, there is little experience of the use of these tests in horses.

The aim of this study was to evaluate the function of the GH-IGF-1 axis in three dwarf Friesian horses to determine whether gross disturbances in this endocrine pathway might play a role in dwarfism in Friesian horses.

Materials and methods

Endocrine tests were performed in three Friesian foals (one colt and two fillies) with classical phenotypic signs of dwarfism (Fig 1). They were 11, 18 and 68 weeks old when first examined (Table 1). The colt (case 1) was the only foal in which all the tests were performed. For reference purposes, identical tests were performed simultaneously in an age-matched control Friesian colt (case 4). A further eight normal Friesian foals (three fillies and five colts ranging in age from 23 to 32 weeks) were used to establish normal reference ranges for serum IGF-1 concentrations.

The three dwarf foals (cases 1 to 3) first underwent a thorough clinical, haematological and blood biochemical examination, including serum thyroxine (T_4) concentrations. The function of the GH-IGF-1 axis was tested at up to four different levels as outlined below. The three dwarf foals were finally euthanased on welfare grounds and examined postmortem, with special attention to pathology of the pituitary gland.

Assessing the GH-IGF-1 axis

The function of the GH-IGF-1 axis was tested at four levels. The secretion of GH was monitored by measuring its concentrations in serial plasma samples; a GHRH challenge was performed to assess pituitary responsiveness;

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FIG 1: Typical Friesian dwarf foal (case 1) and its age-matched control foal (case 4)

basal serum IGF-1 levels were compared between the dwarf and the normal Friesian foals; hepatic responsiveness to GH was tested by monitoring the IGF-1 response to repeated doses of recombinant equine GH (reGH).

Concentration of GH in serial plasma samples GH is secreted in a pulsatile fashion and measurements of its concentration in serial blood samples are therefore required to determine the extent of endogenous GH secretion. Plasma samples for GH analysis were collected from cases 1 to 4 at 10-minute intervals for 90 minutes via an indwelling jugular catheter. The sampling always began at 09.30, because GH secretion is subject to diurnal variation. In case 1, a further series of plasma samples was collected at 10-minute intervals for five hours between 08.00 and 13.00 because the 90-minute sampling period was too short to obtain useful information on pulsatility.

The concentrations of GH were analysed using a pulse detection program (Cluster 8; UVA Pulse Analysis Software) for the following parameters: mean GH concentration, total AUC for GH and the number of GH concentration peaks (Veldhuis and Johnson 1986).

GHRH stimulation test Synthetic GHRH (Somatorelin; Ferring) was administered as a 1·0 µg/kg bodyweight intravenous bolus to cases 1 and 4 to examine the ability of the pituitary to secrete GH. Plasma samples for GH assay were collected 15 minutes before and 0, 15, 30 and 45 minutes after the GHRH injection via the same indwelling intravenous catheter, which was flushed with 20 ml 0·9 per cent sodium chloride between injections or blood collections.

Basal IGF-1 concentrations Single serum samples were collected from cases 1 to 3 to measure basal IGF-1 concentrations. Similar samples were collected from nine healthy control Friesian foals (three fillies and six colts, including case 4), to establish reference values for serum IGF-1 concentrations for this breed and age of horse.

Effect of exogenous GH on serum IGF-1 concentrations To examine hepatic responsiveness to GH, as an index of peripheral sensitivity, serum IGF-1 concentrations were monitored in case 1 for 10 days (starting when it was 82 weeks of age), during which it received daily intramuscular injections of 20 µg/kg methionyl somatotropin (Equigen; Bresagen) reconstituted immediately before use with distilled water

TABLE 1: Description of four foals used to study the functionality of the growth hormone-insulin-like growth factor-1 axis in dwarf Friesian horses

Case	Phenotype	Sex	Age when examined (weeks)	Outcome
1	Dwarf	Colt	11-158	Euthanased
2	Dwarf	Filly	18-70	Euthanased
3	Dwarf	Filly	68-129	Euthanased
4	Normal	Colt	11-51	Discharged

to a concentration of 2·5 mg/ml. Serum samples for IGF-1 assay were collected before the first administration and thereafter daily before 10.00 on days 1 to 11 and on day 15 after the treatment with reGH began.

Postmortem examination

The three dwarf foals were euthanased as a result of progressive limb and hoof deformities secondary to severe tendon laxity (Back and others 2008) by intravenous administration of an overdose of pentobarbital sodium (Euthasol 400 mg/ml; Produlab pharma). Postmortem examinations paid particularly attention to the size (weight), gross anatomy and histology of the pituitary gland. Sections were stained with haematoxylin and eosin, and immunohistochemically for GH-producing cells with a 1:5000 dilution of a polyclonal anti-human growth hormone antibody (Biogenex).

Blood samples

Blood samples for GH were collected into lithium heparinised tubes (Venoject; Terumo), which were kept on ice until they were centrifuged at 4°C for 10 minutes at 4000 *g*. The resulting plasma was stored at –20°C until assayed for GH.

For IGF-1, blood samples were collected into serum tubes (Venoject; Terumo), allowed to clot and then centrifuged for 10 minutes at 4000 *g*. The serum was stored at –20°C until assayed for IGF-1.

Hormone assays

Plasma GH concentrations were measured with an ELISA validated for equine GH (Diagnostic Systems Laboratories); the assay had a minimum detection limit of 0·11 µg/l and mean intra- and interassay coefficients of variation of 4·5 and 6·5 per cent, respectively. All the GH samples were analysed with a single assay.

Serum IGF-1 concentrations were measured with a radioimmunoassay kit validated for horses, after extraction with acid ethanol. The efficiency of extraction was 85 to 90 per cent. Curves obtained with serial dilutions of equine plasma spiked with IGF-1 were parallel to the standard curve, and the intra- and interassay coefficients of variation were 3·2 and 15·6 per cent, respectively (Nap and others 1993, Sloet van Oldruitenborgh-Oosterbaan and others 1999). All the IGF-1 samples were analysed in a single assay.

Statistics

A Mann-Whitney U test was used to determine whether the basal serum IGF-1 concentrations in the dwarf and control foals were different. A difference was regarded as significant if $P<0·05$.

Results

Clinical, haematological and blood biochemical examinations

The foals had normal vital parameters. There were no audible abnormalities of the respiratory system but heart auscultation revealed murmurs in two of the three dwarf foals, a systolic murmur in case 1 and a diastolic murmur in case 3, both localised over the aortic valve.

The haematological and blood biochemical parameters of the foals were within normal limits, with the exception of a high concentration of β-globulin in cases 2 and 3. The mean (sd) plasma concentration of T_4 in the three dwarf foals was 22·3 (8·3) nmol/l, within the normal reference range of 20 to 42 nmol/l.

Function of the GH-IGF-1 axis

Fig 2 shows the concentrations of GH in cases 1 to 4 over 90 minutes. Pulse detection analysis revealed a mean (sd) AUC for the dwarf foals

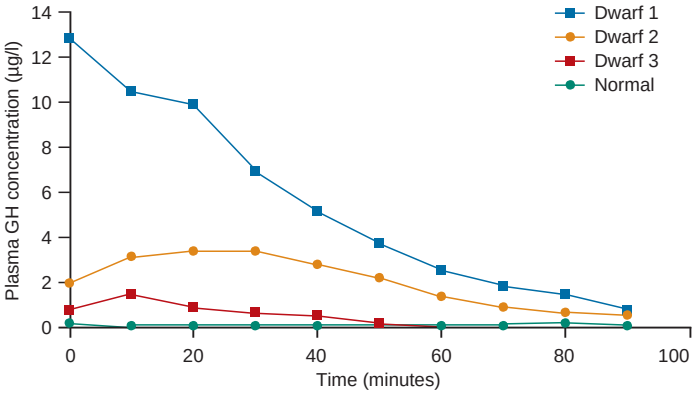

FIG 2: Plasma concentrations of growth hormone (GH) measured every 10 minutes in three dwarf Friesian foals and one normal Friesian foal

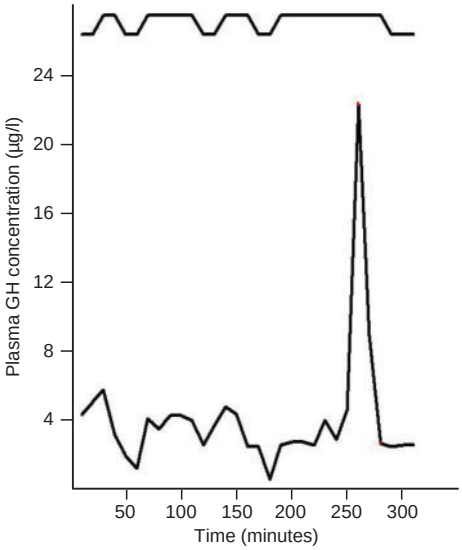

FIG 3: Plasma concentrations of growth hormone (GH) measured every 10 minutes for five hours in a Friesian dwarf foal (case 1)

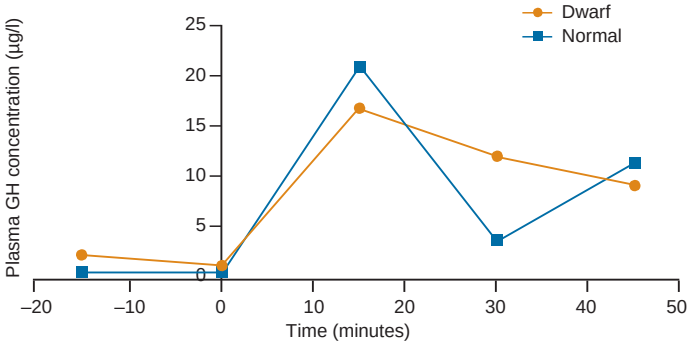

FIG 4: Plasma concentrations of growth hormone (GH) measured at intervals before and after the administration of 1·0 µg/kg synthetic GH-releasing hormone to a dwarf Friesian colt (case 1) and an age-matched normal colt (case 4)

of 274 (264) µg/l (range 52·8 to 566·0), compared with 6·6 µg/l for the normal foal, and a mean (sd) plasma GH concentration for the dwarf foals of 2·7 (2·7) µg/l (range 0·53 to 5·7 µg/l), compared with 0·06 µg/l for the normal foal. No peaks of plasma GH concentration were detected, but the program can identify only complete pulses, that is, with a beginning and an end, within a profile.

Fig 3 shows the concentration of GH in case 1 over 300 minutes. There were four concentration peaks with a mean pulse height of 0·1 (0·1) µg/l, mean pulse mass of 1·3 (1·7) µg/l and a mean pulse interval of 65 (14·7 minutes). The AUC was 1268 µg/l, and the mean plasma GH concentration was 4·1 µg/l.

GHRH stimulation test The injection of GHRH induced an increase in plasma GH concentrations in both case 1 and case 4 (Fig 4). Peak plasma GH concentrations were reached 15 minutes after the injection of both animals, and the concentration increased from 1·2 to 17 µg/l in the dwarf foals and from 0·6 to 21 µg/l in the normal foal.

Basal serum IGF-1 concentrations The mean (sd) basal serum IGF-1 concentration in cases 1 to 3 (at 32, 28 and 76 weeks of age, respectively) was 163 (56) ng/ml (range 120 to 227 ng/ml), and was not significantly different from the value for the nine control foals, 180 (62) ng/ml (range 114 to 327 ng/ml).

Effects of exogenous reGH on serum IGF-1 levels The day after the first administration of reGH to case 1, its serum IGF-1 concentration had increased 2·1-fold to 289 ng/ml; the concentration continued to rise with each successive treatment and reached a peak of 604 ng/ml on day 10. Five days after the end of the daily reGH administration, the serum IGF-1 concentration had decreased to 371 ng/ml, but was still considerably higher than before the treatment (138 ng/ml).

Postmortem examination

The mean (sd) weight of the fresh pituitary glands from cases 1 to 3 was 2·3 (1·0) g. Histological examination revealed no abnormalities, and immunohistochemical examination showed that the distribution and proportion of GH-positive cells in the pars distalis were normal.

Discussion

The hypothesis tested in this study was that dwarf foals might suffer an abnormality of the GH-IGF-1 axis, such as GH deficiency, GH insensitivity, IGF-1 deficiency or IGF-1 insufficiency. GH insensitivity is a term used to describe patients with a GH-deficient phenotype and reduced IGF-1 production, despite normal or high serum GH concentrations. This contrasts with GH deficiency, in which the low serum IGF-1 levels are associated with, and presumably a result of, low serum GH concentrations (Martinelli and others 2007). In theory, this should be a straightforward differential diagnosis. However, it is difficult to assess pituitary GH production because GH secretion is pulsatile and, in human beings, the most consistent secretion occurs during sleep (Reiter and Rosenfeld 2002). Moreover, the regulation of GH secretion involves several factors, including sex, age, pubertal stage and nutritional status (Reiter and Rosenfeld 2002). In addition, between the pulses of GH secretion, serum GH levels are generally low (often less than 0·1 µg/l in human beings) (Reiter and Rosenfeld 2002). As a result, a single random measurement of serum GH concentration is useless for diagnosing GH deficiency, although it can be helpful in the diagnosis of GH insensitivity and GH excess. Multiple samples, collected over a period of 12 to 24 hours, are recommended for an accurate assessment of spontaneous GH secretion, but the expense, discomfort and variability in interpretation have prevented this procedure from being used routinely for establishing a diagnosis of GH deficiency in human beings (Reiter and Rosenfeld 2002).

The measurements of plasma GH over a 90-minute period clearly showed that endogenous GH secretion occurred in the dwarf foals. However, the period of sampling was too short to obtain useful information on its pulsatility. It appeared that case 1 was in the middle of a pulse of GH secretion, and pulsatile release was confirmed in this foal when four distinct pulses were recorded in the samples taken over 300 minutes. Compared with the normal Friesian foal (case 4), the dwarf foals secreted more GH during the sampling period, as indicated by their larger AUCs. A mean (sd) plasma GH concentration of 7·4 (0·4) µg/l has been reported for foals (Davicco and others 1993), considerably higher than the mean value recorded for the three dwarf foals. However, it is difficult to compare GH concentrations between studies because of the differences between GH assays, and the diurnal variation in its secretion. The mean plasma GH concentrations reported by Davicco and others (1993) were based on samples collected over 24 hours, which included peaks of GH secretion that would have increased the mean plasma GH concentration.

Because monitoring the pattern of spontaneous GH secretion is labour-sensitive and difficult, challenge tests to measure GH 'secretory

reserves' are often used instead. The peak response to GHRH stimulation tests is generally a good indicator of the amount of GH that can be secreted during 24 hours (Donaldson and Paterson 1998, Reiter and Rosenfeld 2002). In children, the diagnostic cut-off at which GH deficiency can be excluded is an increase in plasma GH concentrations to above 10 µg/l after GHRH stimulation (Wilson and Frane 2005). In the case of an attenuated response, another test is used to confirm the negative result. Increased responses to GHRH stimulation tests have been reported in patients with GH insensitivity, and an upper cut-off limit of 20 µg/l has been proposed (Martinelli and others 2007). In this study, both case 1 and the normal colt responded similarly to the intravenous administration of GHRH. That the dwarf foal responded with an increase in its plasma GH concentration to above 10 µg/l, but below 20 µg/l, suggests that its pituitary GHRH receptor was intact and that its pituitary gland was able to respond normally to stimulation with GHRH.

GH deficiency and GH insensitivity also influence the serum concentrations of IGF-1 and IGF-2 and their binding proteins, which makes the latter good indices of GH status. Furthermore, the serum concentrations of IGF-1 and IGF-2 are relatively constant during the day, so that stimulation tests or multiple sampling is not necessary (Reiter and Rosenfeld 2002). Serum IGF-1 was chosen to test the baseline GH status of the dwarf foals because it is more GH-dependent than IGF-2. However, serum IGF-1 levels are age-dependent, and a group of age-matched normal foals was therefore tested for comparative purposes. The basal serum IGF-1 concentrations of the two youngest dwarf foals (cases 1 and 2) were within the reference range.

When evaluating short children for IGF-1 deficiency, an increase in serum IGF-1 levels of less than twice the intra-assay variation in response to the subcutaneous administration of GH for four days is considered an important diagnostic criterion (Savage and others 1993, Reiter and Rosenfeld 2002). When case 1 was treated with reGH, its serum IGF-1 concentrations increased by more than 200 per cent, considerably more than twice the intra-assay variation, and comparable to the response of two- to three-year-old normal horses in which the administration of reGH induced an increase in serum IGF-1 concentrations to 2·3 times basal levels within 24 to 36 hours (Popot and others 2001). Similarly, Capshaw and others (2001) reported that when four-month-old foals were treated daily with reGH, their serum IGF-1 concentrations approximately doubled in the first five weeks. It therefore appears that case 1 responded normally to treatment with reGH in terms of its secretion of IGF-1, although the decline in its serum IGF-1 concentration after the administration of reGH was discontinued was slower than in two- to three-year-old horses, in which it returned to basal values within three days (Capshaw and others 2001); in case 1 the IGF-1 concentration was still more than double the pretreatment concentration three days after the last dose of reGH. Nevertheless, it can be concluded that the production of IGF-1 in response to GH was not disturbed in this dwarf Friesian foal.

The concentrations of β-globulin were high in cases 2 and 3, and although this is generally a sign of intestinal parasitism, the foals had been dewormed regularly with ivermectin. The high β-globulin levels were due to a severe infestation with *Fasciola hepatica*, an organism that is not sensitive to ivermectin, recorded at postmortem examination.

The study found no evidence of hypothalamic-pituitary dysfunction or failure of IGF-1 production in three dwarf Friesian horses. It appears that the cause of their congenital growth abnormality was located distal or peripherally to the level of the GH receptor in the liver, and could have been a defect in a peripheral IGF-1 or GH receptor, or not involve the GH-IGF-1 axis at all. A detailed comparison of the musculoskeletal development of dwarf foals with that in age-matched normal foals is therefore required to determine the cause of the phenotypic abnormalities and to identify the genes involved.

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