Administration of bovine, porcine and equine growth hormone to the horse: effect on insulin-like growth factor-I and selected IGF binding proteins

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Abstract

This study investigated the biochemical effects of administration of three types of recombinant growth hormone (GH; somatotropin) to the Thoroughbred horse. Equine or bovine or porcine GH was administered at a recommended dosage to 3–5-year old Thoroughbred geldings, for up to 21 days. It was shown that, in addition to equine GH, bovine and porcine GH were active in the horse; however, porcine GH caused injection-site reactions that were so serious that administration had to be terminated. The concentrations of a range of GH-related serum protein markers were determined before, during and after the administration period. Because of the short half-life of GH itself, the objective was to identify GH-related markers that showed changes in concentration and which could be used as indicators of the abuse of these hormones.

Among the possible markers identified, serum total insulin-like growth factor (IGF)-I was shown to be the most promising, increasing to 270% of the basal concentration for equine GH administration. After GH administration, IGF-I took longer to attain baseline concentrations than the time required for GH concentrations to recover to normal. The concentration obtained from the administration significantly exceeded natural concentrations for IGF-I, as was determined from a population of more than 2000 Thoroughbred horses in three continents. The concentrations of serum free IGF-I and IGF binding protein 3 (IGFBP-3) were also shown to be significantly affected by equine and bovine GH.

Journal of Endocrinology (2001) **171**, 163–171

Introduction

It is well-known that growth hormone (GH; somatotropin) has anabolic effects, and its consequent abuse is a concern in many sports, including horseracing. The recent commercial availability of recombinant equine GH (eGH) in Australia (BresaGen Press Release 1998) has attracted the attention of horseracing authorities worldwide and prompted the pursuit of methods for the detection of its abuse. It has however also been shown that recombinant bovine somatotropin (bovine GH, bGH), which is available in many countries for the improvement of milk production in lactating cows, and recombinant porcine somatotropin (porcine GH, pGH), which is used for the promotion of protein deposition in the growing pig in Australia, are both active in the horse (Buonomo *et al.* 1996). This aspect has to be considered when the objective is to design a method of detection universally applicable to the abuse of different types of GH in the horse.

In the horse, GH is released naturally in a pulsatile fashion, typically increasing from basal quantities of about 1–2 ng/ml to peak at 8 ng/ml and to return to basal values

within about 3 h (Christensen *et al.* 1997). Such secretions typically occur between two and five times over a 24 h period (Thompson *et al.* 1992). Compared with the naturally occurring hormone, recombinant eGH possesses an additional amino-terminal methionine residue (BresaGen Press Release 1998); there are also significant differences between recombinant bGH (89% sequence homology) and recombinant pGH (98% homology) (Secchi & Borromeo 1997). These differences in structure provide a possible means of detecting recombinant GH presence. However, the limitation of this approach is the fast serum clearance rate of the protein, which has a half-life of less than 15 min (Hindmarch *et al.* 1989), implying a 'window' for the detection of abuse that is less than 1 day. It is clear that, in order to extend the period of detection, other markers of GH abuse needed to be investigated.

The main site of GH action is the liver, where the production of insulin-like growth factor (IGF)-I, a 7.6 kDa protein that acts as a potent mediator of cell proliferation, is known to be stimulated (Daughaday & Rotwein 1989). On release of GH, the increases in IGF-I were shown to

persist for longer periods of time than those for GH. This is mostly due to the binding of IGF-I with up to six IGF binding proteins (IGFBPs) in circulation. The exact role of these IGFBPs is not clear, but it is known that they increase the effective lifetime of IGF and act to deliver and provide a reservoir of IGFs (Baxter *et al.* 1990). Pharmacokinetic studies have indicated, however, that a small fraction of IGF-I is not associated with IGFBPs (Zapf *et al.* 1986). There is not consensus on the significance of so-called free IGF-I but, in theory, this could be the metabolically active form (Guler *et al.* 1987, Nam *et al.* 1997). In a quest to identify suitable markers of the abuse of GH in the horse, it is clear that both total and free IGF-I require investigation.

Six human IGFBPs have been fully characterized and these differ in their binding affinities for IGF-I (Cohick & Clemmons 1993). GH administration studies have shown that, in addition to IGF-I concentrations, those of some of the IGFBPs are also affected by GH (Buonomo *et al.* 1996, Kicman *et al.* 1997). Concentrations of IGFBPs-2 and -3 have been shown to be affected by exogenous GH treatment in the human (Kicman *et al.* 1997). A study in the horse has indicated that concentrations of IGFBPs-1 and -3, but not of IGFBP-4, could be affected by GH administration (Popot *et al.* 2000).

To act as a suitable marker, a significant response must reliably be shown when treatment with a range of GHs, which are active in the horse, is undertaken. Furthermore, the response must be significant in relation to both the concentrations present in non-treated horses and the variation within a population of such horses. There must be a suitable method of analysis available for this entity that gives reliable and reproducible results. In the present study we investigated the suitability of the proteins IGF-I (free and total), IGFBPs-1, -2 and -3, and acid-labile subunit (ALS) as suitable markers for the detection of GH abuse.

Thoroughbred racehorses of ages representative of the racing population were administered eGH, bGH, pGH or placebo in recommended doses. These horses were fed a typical diet and exercised regularly, to simulate a typical racehorse routine. Serum samples were taken at regular intervals before, during and after the period of administration of GH/placebo. Radioimmunoassay (RIA), immunoradiometric assay (IRMA) and enzyme-linked immunosorbent assay (ELISA) immunodiagnostic methods were selected and investigated for suitability in analysing equine serum for the protein markers in question. The significance of any changes in the quantity of the protein markers in the horses in the administration trial was investigated.

Materials and Methods

Hormones

Recombinant methionyl eGH (EquiGen) for the study was obtained from BresaGen, Thebarton, South Australia.

Recombinant pGH (Reporcin) was supplied by Southern Cross Biotech, Toorak, Australia. Injectable recombinant bGH (Hilac controlled-release formula; LG Chem) was obtained from MDB Animal Health, Johannesburg, South Africa.

Immunodiagnostic assays

The immunodiagnostic kits for unbound IGF-I (Free IGF-I IRMA DSL-9400), total IGF-I (IGF-I IRMA Non-Extraction DSL-2800), IGFBP-1 (Total IGFBP-1 IRMA DSL-7800), IGFBP-2 (IGFBP-2 RIA DSL-7100), IGFBP-3 (total IGFBP-3 IRMA DSL-6600) and ALS (ALS Total ELISA DSL-10-8200) were obtained from Diagnostic Systems Laboratories, Webster, TX, USA. For all the ELISA, RIA and IRMA methods used the procedures for the assays were followed as specified by the supplier. For all assays, plain serum collection tubes were used for blood collection (Vacutainer, The Separations Group, Johannesburg, South Africa). The resulting serum samples were stored at -20°C and thawed just before analysis. Radioactivity was read on a Gamby CR gamma counter (DPC, Los Angeles, CA, USA) and ELISA readings were made on an SLT Lab 400 SFC ELISA reader (Labinstruments, Salzburg, Austria). The resulting data was processed on Microsoft Excel (Microsoft Windows 98).

Validation of IRMA assays

Total IGF-I assay The DSL-2800 IRMA assay for total IGF-I was investigated for recovery. Colt and mare serum were spiked with recombinant IGF-I at six concentrations and were analysed after overnight incubation at room temperature. Regression curves of measured value against expected value were constructed and the percentage recovery at each concentration was determined. Investigation of the linearity of the assay over the concentration range of interest was carried out at six concentrations for colt, gelding and mare serum. Sera were diluted stepwise (1/4, 1/8, 1/16 and 1/32) and plots were constructed from the measured IGF-I values. Comparison of the gradient of these lines with that of the linearity curve of the standard serves as an indication of the parallelism of the assay. Intra-assay variation was determined from the analysis of three colt and three mare sera on different days.

Free IGF-I assay Intra- and inter-assay variation of colt, gelding and mare sera ($n=6$) were determined using the DSL-9400 free IGF-I IRMA.

IGFBP-3 assay Parallelism was determined for the DSL-6600 IGFBP-3 IRMA. Serial dilutions (1/4, 1/8, 1/16 and 1/32) of colt, gelding and mare serum were investigated and plots of the IGF-I values were compared

with that of the linearity curve of the human standard. Intra-assay variation was determined for mare ($n=3$) and colt serum ($n=3$).

GH administration trial procedure

Ethics approval and location of study The administration study was authorized by the Animal Use and Care Committee, Faculty of Veterinary Science, University of Pretoria, and was overseen by the Equine Research Centre, University of Pretoria. The importation of GH and its administration was undertaken in accordance with the study procedure and with authorization by the Medicine Control Council of South Africa.

Feeding and exercising Equal amounts of a regular diet were fed at 0730 h and 1600 h only. This diet consisted of grass hay, lucern hay (alfalfa), commercial pelleted concentrate ration and molasses. Water was available *ad libitum*. The horses were treadmill exercised three times a week. This involved a 1 min walk, 5 min trot, 5 min canter and 5 min fast canter. On days of no treadmill exercise the horses were left on pasture for 1 h to exercise freely. Horses that could not be treadmill exercised were lunged for the same time period to an equivalent level of exercise and exertion.

Acclimatization and selection Fourteen Thoroughbred geldings between 3 and 5 years of age were used for the administration trial. They were currently involved in racing or recently retired from racing. Before the trial, the 14 horses were evaluated for suitability. These horses were first acclimatized in the set exercise and feeding routine for at least 7 days. Their total serum IGF-I concentrations were determined for three consecutive days to ensure that the values were typical for horses of these ages as compared with those previously observed in a population of Thoroughbreds (De Kock *et al.* 2000). From this group, the 12 horses with the most typical IGF-I concentrations and best exercise soundness were selected for the trial.

GH administration The horses were randomly assigned to four groups of three horses. The trial consisted of three phases: a pre-administration phase during which drug administration did not take place, an administration phase when GH or placebo was administered (the time period was dependent on the GH administered) as specified, and a post-administration phase when there was no drug administration; this last time period was set in order to determine the effect of termination of drug administration (washout of drug). Administration was undertaken by i.m. injection at 1500 h daily. Horses in group 1 received eGH, group 2 received pGH, those in group 3 were administered a controlled-release bGH formulation, and group 4 was the control group, which was administered sterile water as placebo. The eGH and pGH products were

reconstituted in water for injection to a concentration of 2.5 mg/ml as specified for the eGH product (EquiGen product insert, BresaGen). For eGH the dose was 10 µg/kg for 5 days (as a recommended GH acclimatization period) followed by 25 µg/kg for a period of 16 days. For bGH a controlled release dose of 250 mg was administered, and for pGH the dose was 10 µg/kg each for 6 days.

Serum collection and storage During the three phases of the GH administration study, blood was collected daily at 0800 h only. At least 20 ml of blood was collected by jugular venepuncture into sterile, plain serum tubes. These were left to clot at room temperature for 1 h and left overnight at 4–8 °C. After centrifugation at 1000 *g* for 15 min at 4–8 °C, the serum supernatants were removed and frozen at –20 °C until required for analysis.

Results

Immunodiagnostic assays

It is known that the amino acid sequence for horse IGF-I is identical to that of human IGF-I (Nixon 1999). In theory, therefore, the anti-human antibodies from the commercial immunodiagnostic assay kits used should show a 100% cross-reactivity with horse IGF-I. For the horse protein markers other than IGF-I, the structural homology between these and the human protein counterparts is mostly unknown, or otherwise it is known that the amino acid sequence is not the same as that of humans. Cross-reactivity of the equine serum proteins with the IGFBPs-2 and -3 assay methods were observed. For these proteins the assays were found useful in indicating the effect that GH treatment has on the relative amount of these proteins during and after the trial, compared with before the trial. The human immunodiagnostic methods for the proteins ALS and IGFBP-1 were observed not to show an above-background response with horse serum samples. This is probably due to extremely low or no cross-reactivity with the antibodies of the method.

Validation of IRMA assays

Total IGF-I assay Study of the DSL-2800 IRMA method indicated a recombinant IGF-I recovery of 93.07% ($n=6$ concentrations, relative standard deviation (R.S.D.) 5.36%) for colt serum and 96.06% ($n=6$ concentrations, R.S.D. 2.76%) for mare serum. In order to allow for the association of exogenously added IGF-I with IGFBP (Guler *et al.* 1987, Lewitt *et al.* 1993) in these recovery studies, spiked serum samples were analysed only after overnight incubation. Good linearity and parallelism were observed for colt, gelding and mare serum with linearity gradients varying between 1.137 and 1.143

($R^2=1.000$) compared with the curve gradient for the human standard of 1.143 ($R^2=0.9985$). These highly parallel linearity gradients indicate very limited assay interference when analysing equine serum samples, and a small amount of concentration-dependant interference, if any. Intra-assay variation was determined at an R.S.D. of 5.35% ($n=6$) for mare serum and 6.01% ($n=6$) for colt serum. Inter-assay variation and investigation of IGFBP interference forms part of another study (Noble & Sillence 2000). In this study the absence of IGFBP interference was confirmed using an adaptation of existing acid-chromatographic methods of IGFBP removal (Daughaday *et al.* 1987, Holly & Hughes 1994). The method involved loading of serum samples onto a BSA-coated Sephadex G-100 gel filtration column, followed by elution with a 0.5 M acetic acid solution, which separates IGF-I from IGFBPs. When the IGF-I fraction was analysed, the same value was obtained compared with the sample preparation method of the DSL-2800 IGF-I IRMA, indicating quantitative IGFBP removal by this method (Noble & Sillence 2000). The above results indicate that the DSL-2800 IRMA delivers quantitative IGF-I results from the analysis of equine serum samples within the IGF-I concentration range investigated.

Free IGF-I assay Sample preparation and storage was found to be important when free IGF-I was analysed. Using the DSL-9400 free IGF-I IRMA, repeatable results were obtained when collected serum was frozen at -20°C and thawed only just before analysis. Extended storage at $4-8^\circ\text{C}$ and repeated thawing of samples increased values significantly. This can in part be ascribable to breakdown of IGFBP through proteolysis or denaturation and subsequent release of bound IGF-I. For colt, gelding and mare serum, an intra-assay variation R.S.D. of 5.11% was obtained; the inter-assay value was 7.89%. Recovery could not be determined, as addition of IGF-I to serum would effect binding to IGFBPs, resulting in low concentrations. Linearity and parallelism studies were not undertaken because dilution affects the IGF/IGFBP equilibrium and distorts results. Such studies were, however, successfully undertaken by the supplier of the assay, using human serum and a synthetic serum substitute for additions (DSL-9400 procedure insert). It was not possible to determine whether the assay method measures the same free component that it was set up to measure in humans. The ratio of total IGF-I to free IGF-I measured in untreated horses (392.2) was, however, similar to the value of 367.7 measured in a human study (Nam *et al.* 1997), which does tend to indicate that a similar free component is measured.

IGFBP-3 assay Parallelism investigation of the DSL-6600 IGFBP-3 assay showed that, when colt, gelding and mare sera were diluted and analysed, curves were obtained that were highly parallel with that of the linearity curve of

the human IGFBP-3 standard. An intra-assay variation R.S.D. of 9.79% ($n=6$) was determined for mare and colt serum. In the absence of horse IGFBP-3 standard, a recovery study was previously undertaken (Noble & Sillence 2000) using human IGFBP-3 standard, yielding a mean recovery of 80.0%.

Clinical effects

No side effects were observed with any of the horses administered eGH. All three horses treated with pGH, however, developed stiffness of the neck during the period of administration. The effect was considered to be a local tissue reaction in the areas adjacent to the site of injection and was so severe that the daily administration of the hormone was terminated after 6 days at a dose of $10\text{ }\mu\text{g/kg}$ per day. However, the effects disappeared within a few days after the last day of administration. Only one of the three horses administered bGH developed similar stiffness of the neck, but this was considered to be a mild effect. No horses developed significant lethargy as is possible with exogenous GH treatment, and in none of the horses given eGH was significant soreness at the site of injection noted.

Serum total IGF-I

Serum total IGF-I was affected by the administration of eGH and bGH. For eGH, the average pre-treatment serum IGF-I concentration was 281.4 ng/ml (S.D. 70.8). At about 19 days after the first administration, the average of the maximum concentration attained in each of the treated horses was 714.3 ng/ml (S.D. 14.7). This is a 270% increase compared with the pre-treatment average. A concentration exceeding 530 ng/ml was attained from day 8 to day 21 of treatment (Fig. 1A). This time period corresponds roughly with the period during which a $25\text{ }\mu\text{g/kg}$ dose of hormone was administered. In two of the three horses, maximum concentrations exceeded 700 ng/ml (Fig. 1B). There was a close correlation between the IGF-I profiles of the three treated horses, especially during the period before treatment. On average, treatment with controlled-release bGH increased serum IGF-I concentrations to 540.1 ng/ml (S.D. 27.4) from a pre-treatment average of 262.3 ng/ml (S.D. 41.3), an increase in concentration of 106% (Fig. 2). This value was achieved 2 days after administration and was sustained for another 6 days. Thereafter, concentrations decreased to 420 ng/ml within the next 6 days. At 15 days after administration, the concentration had decreased to pre-treatment values. When a higher dose was used for bGH, the extent of increase in IGF-I (206%) was less than that for eGH (270%). As mentioned before, pGH administration was terminated after 6 days of administration at a dose of 10 mg/kg per day. As expected, concentrations of IGF-I

Figure 1 (A) Serum total IGF-I concentration and percentage increase from the pre-treatment average after eGH administration. (B) Serum total IGF-I concentration in individual horses after eGH administration. PreAd, before administration; Admin, period of administration; PostAd, after administration.

Figure 2 (A) Serum total IGF-I concentration and percentage increase from the pre-treatment average after bGH administration. (B) Serum total IGF-I concentration for individual horses after bGH administration. PreAd, before administration; Admin, period of administration; PostAd, after administration.

increased only for 6 days and not to high concentrations, the daily increases being very similar to those observed for the first 6 days of administration of eGH.

Serum free IGF-I

As for total IGF-I, the serum concentrations of free IGF-I also showed an increase in horses treated with eGH and bGH. For eGH, concentrations of free IGF-I increased from a measured average of 0.72 ng/ml (s.d. 0.37) to a maximum of 2.8 ng/ml (s.d. 0.5) at about day 13 of administration (a 388% increase; Fig. 3A). Compared with total IGF-I, serum free IGF-I concentrations took longer to increase. These increased concentrations of free IGF-I returned to baseline values during the period when GH administration was taking place. With bGH treatment, increases in free IGF-I concentrations were also observed, from a pre-treatment concentration of 0.70 ng/ml (s.d. 0.48) to a maximum observed concentration of 4.09 ng/ml (s.d. 1.25), corresponding to more than 700% of the pre-treatment value (Fig. 4A). The onset of increase was at the same time as that of total IGF-I, but the decrease took place during a period in which release of bGH was still in progress, as indicated by total IGF-I concentrations. These results should be interpreted taking into account that free IGF-I is buffered by IGFBPs and the concentrations of some binding proteins are themselves stimulated by GH treatment. The decrease in free IGF-I levels before termination of administration, while total IGF-I concentrations were still high, may be attributed to the fact that the concentrations of binding proteins, such as IGFBP-3, are increased at this stage and could account for a greater binding occupancy of IGF-I. This would lead to a lower free IGF-I concentration.

Serum IGFBP-3

With bGH and eGH administration, increases in concentration were noted for total IGFBP-3. Measured natural IGFBP-3 concentrations of horses in this study (404.9 ng/ml with s.d. 88.8) were noted to be significantly lower than those measured for humans (2000–4000 ng/ml). This implies that the cross-reactivity of the antibody is less than 100% with the horse IGFBP-3 serum protein. For the purpose of the study, this was not limiting to the usefulness of the method or the results generated in monitoring changes in IGFBP-3 concentration. For eGH, amounts for all three treated horses increased from a measured pre-treatment average of 380.5 ng/ml (s.d. 69.7) to a maximum average concentration of 630.2 ng/ml (s.d. 96.0), implying an increase to 165% of the pre-treatment value (Fig. 3B). The greatest individual value was measured as 794.1 ng/ml at 19 days after the first daily administration of eGH. For bGH, there was an increase in concentration within a few days of administration (Fig. 4B). However, the concentration decreased

Figure 3 (A) Serum free IGF-I concentration and percentage increase from pre-treatment average after eGH administration. (B) Serum IGFBP-3 concentrations and percentage increase from pre-treatment average after eGH administration. PreAd, before administration; Admin, period of administration; PostAd, after administration.

Figure 4 (A) Serum free IGF-I concentration and percentage increase from pre-treatment average after bGH administration. (B) Serum IGFBP-3 concentration and percentage increase from pre-treatment average after bGH administration. PreAd, before administration; Admin, period of administration; PostAd, after administration.

to pre-treatment values within 7 days. This is in contrast to total IGF-I concentrations, which were still high at this stage. Compared with the changes in total IGF-I, the increase in IGFBP-3 values in response to eGH was much more gradual over time. After termination of the daily treatment, the concentrations were also much slower to return to pre-treatment values. At 13 days after the treatment period, amounts were still about 16% in excess of that of the pre-treatment value.

Serum IGFBP-2

With eGH treatment, total IGFBP-2 concentrations remained very constant, at a value of about 1111.7 ng/ml (s.d. 331.6) for all three horses. This was very close to the average concentration of 1022.7 ng/ml (s.d. 368.1) determined for the population of untreated horses and indicated that, in the present study, the concentration of IGFBP-2 was not significantly affected by eGH treatment.

Discussion

For the immunodetection of total IGF-I, the complete removal of IGFBPs is a prerequisite. As IGFBPs in the horse have been shown to differ from those of humans (Malinowski *et al.* 1996), the effectiveness of the human total IGF-I immunodiagnostic method in removing IGFBPs when applied to horse serum was assessed. This was also of importance, as previous studies had shown that the removal of equine IGFBPs might be incomplete in some assay methods, resulting in under-reading of total IGF-I value (De Kock *et al.* 2000). Only serum collected in plain serum tubes was analysed in this study, as a previous investigation indicated that samples collected in serum tubes with clot activator or in plasma EDTA tubes resulted in lower readings for IGF-I (De Kock *et al.* 2000). Using horse serum samples, it could be shown by validation that, within the concentration range investigated, the immunodiagnostic method for total IGF-I delivers quantitative results from the analysis of equine serum samples. Although the method for human total IGFBP-3 showed a less than 100% cross-reactivity with horse IGFBP-3, the method was useful in showing the effect that GH treatment has on the relative amount of total serum IGFBP-3 in horses at different times during the trial. IGFBP-2 did not show a significant response with GH administration, indicating that it may be independent of the GH and IGF-I response in the horse. No results were obtained for ALS and IGFBP-1 as these had extremely low or no cross-reactivity with the antibodies of the diagnostic methods evaluated.

This study confirmed a previous observation that eGH, bGH and pGH are active in the horse and that the concentration of IGF-I is significantly increased by GH administration (Buonomo *et al.* 1996). The potency of

bGH in the horse could not be ascertained in this study, as it was not possible to determine the exact dose of bGH which is released on a daily basis. It can, however, be suggested that the release is relatively constant and controlled, and seems to last for about 14 days. The limited soreness at the site of injection of GH noted in the trial confirms one concern regarding the abuse of this type of GH in the horse. The stiffness of the neck observed in all the horses that had been administered pGH was severe. It was thought that this could possibly be explained as an antigenic response to the amino acid sequence differences between eGH and pGH. Compared with eGH, pGH shows much more structural homology (98%) than does bGH (89%) (Secchi & Borromeo 1997). When this is considered, the clinical result is surprising, as no such effects were observed in the horses administered bGH. In a previous study, pGH was administered for 5 days in a significantly higher dose (Buonomo *et al.* 1996). Mild injection soreness was observed in some horses, and local tissue reactions at the site of injection were observed in others. In the current study, administration of pGH over 6 days resulted in increases in IGF-I concentration very similar to that of eGH over the same period of administration. This indicated that pGH is possibly close in potency to eGH. This is not unexpected, considering the high structural homology to eGH. However, this finding is not significant, as the observed severe soreness in the region of the injection should exclude the use of pGH in the horse.

Serum total IGF-I was found to be a suitable and universal marker for the detection of GH abuse. Concentrations increased significantly for all horses treated with eGH and bGH, and the average increase in concentration was 2.7-fold the untreated value. The concentration of IGF-I remained high for a period of time after the termination of GH administration, providing a longer window for the detection of abuse than is possible when the concentration of GH in itself is under consideration. The assay method used in this study was shown to give good accuracy, linearity and repeatability. These are essential requirements for any programme of detection of hormone abuse. The concentration of free IGF-I was shown to increase with GH administration, more significantly than that of total IGF-I. The extent of the increase was about four to seven times the untreated concentration, depending on the GH used. One should bear in mind, however, that non-ideal methods of sample collection and storage (as is possible during in-the-field collection at race meetings) could lead to binding protein breakdown through proteolysis and denaturation, releasing more free IGF-I and resulting in an increased and therefore unrepresentative value. In contrast, this release of free IGF-I that was IGFBP bound in the serum sample does not affect the concentration of total IGF-I. The above considerations, together with the more rapid decrease in free IGF-I concentration (even at a stage when GH

administration is still in progress), make free IGF-I a less ideal marker than total IGF-I for the screening of GH abuse. With both eGH and bGH, increases in IGFBP-3 concentrations were observed, making it, like IGF-I, a suitable indicator of the abuse of both these types of GH. For eGH an increase to 165% of the pre-treatment concentration was noted. Human studies have indicated that IGFBP-3 concentrations are increased to a level of about 140% (Kicman *et al.* 1997), although this was in response to a significantly greater dose of GH (57 µg/kg per day) than was given in this study of administration in the horse. The observed increase was, however, much less significant compared with that in total IGF-I. As neither free IGF-I nor IGFBP-3 was indicated as a promising marker of GH abuse, in comparison with total IGF-I, no population studies were undertaken to determine the significance of the response. One should, however, not ignore the potential usefulness of IGFBP-3 and free IGF-I in the identification of a serum sample that raises suspicion of GH abuse.

With administration of eGH at a recommended dose, an increase in total IGF-I of up to 728.9 ng/ml (259% that of untreated concentrations) was observed, whereas with bGH an increase in total IGF-I to a concentration of 740.3 ng/ml (282%) was observed. The significance of these responses was investigated in comparison with typical IGF-I concentrations in untreated Thoroughbred racehorses. A similar study has previously been conducted on a population in excess of 2000 horses of a range of ages and sexes and in three continents (De Kock *et al.* 2000). The results indicated that IGF-I concentrations are increased to values greatly exceeding those typically observed in the database. With an average value of 310 ng/ml (s.d. 94) for untreated horses, an increase of up to 700 ng/ml in response to GH administration implies an increase to more than $4 \times$ s.d. The finding of such a high natural concentration implies a probability of 1 in a population of 31 000 Thoroughbred horses. From this result it is confirmed that it will be possible to set a threshold concentration for IGF-I. This study also confirmed that IGF-I concentrations show a steady decrease on termination of administration, which could enable discrimination between exogenous GH administration and a naturally high IGF-I level in any particular horse.

References

- Baxter RC 1990 Circulating levels and molecular distribution of the acid-labile (α) subunit of the high molecular weight insulin-like growth factor-binding protein complex. *Journal of Clinical Endocrinology and Metabolism* **70** 1347–1353.
- BresaGen Press Release. *Horse growth hormone first for South Australian company*, 18 August 1998. Adelaide, Australia: BresaGen.
- Buonomo FC, Ruffin DS, Brendenmeuhl JP, Veenhuizen JJ & Sartin JL 1996 The effects of bovine somatotropin and porcine

- somatotropin on growth factors and metabolic variables in horses. *Journal of Animal Science* **74** 886–894.
- Christensen RA, Malinowski K, Scanes CG & Hafs HD 1997 Pulsatile release of somatotropin related to meal feeding and somatotropin response to secretagogues in horses. *Journal of Animal Science* **75** 2770–2777.
- Cohick WS & Clemmons DR 1993 The insulin-like growth factors. *Annual Reviews of Physiology* **55** 131–153.
- Daughaday E & Rotwein P 1989 Insulin like growth factors I and II. Peptide, messenger ribonucleic acid and gene structure, serum and tissue concentrations. *Endocrine Reviews* **10** 68–91.
- Daughaday WH, Kapadia M & Mariz IK 1987 Serum somatomedin binding proteins: physiologic significance and interference in radioligand assay. *Journal of Laboratory and Clinical Medicine* **109** 355–363.
- De Kock SS, Rodgers JP, Swanepoel BS, Faustino-Kemp J, Jackson JS, Houghton E, Noble GK & Sillence MN 2000 Growth hormone abuse in the horse: Establishment of an insulin-like growth factor-1 database. In *Proceedings of the 13th International Conference of Racing Analysts and Veterinarians*, Cambridge, UK, pp 311–313 Eds RB Williams, E Houghton & JF Wade. Newmarket: R&W Publications.
- Guler HP, Zapf J & Froesch ER 1987 Short-term metabolic effects of recombinant human insulin-like growth factor I in healthy adults. *New England Journal of Medicine* **317** 137–140.
- Hindmarch PC, Matthews DR, Brain CE, Pringle PJ, Di Silvio L, Kurtz AB & Brook CGD 1989 The half-life of exogenous growth hormone after suppression of endogenous growth hormone secretion with somatostatin. *Clinical Endocrinology* **30** 443–450.
- Holly JMP & Hughes SCC 1994 Measuring insulin-like growth factors: why, where and how? *Journal of Endocrinology* **140** 165–169.
- Kicman AT, Miell JP, Teale JD, Powrie J, Wood PJ, Laidler P, Milligan PJ & Cowan DA 1997 Serum IGF-1 and IGF-1 binding proteins 2 and 3 as potential markers of doping with human GH. *Clinical Endocrinology* **47** 43–50.
- Lewitt MS, Saunders H & Baxter RC 1993 Bioavailability of insulin-like growth factors (IGFs) in rats determined by the molecular distribution of human IGF-binding protein-3. *Endocrinology* **133** 1797–1802.
- Malinowski K, Christensen RA, Hafs HD & Scanes CG 1996 Age and breed differences in thyroid hormones, insulin-like growth factor and IGF binding proteins in female horses. *Journal of Animal Science* **74** 1936–1942.
- Nam SY, Lee EJ, Kin KR, Cha BS, Song YD, Lim SK, Lee HC & Hub KB 1997 Effect of obesity on total and free insulin-like growth factor (IGF)-I and their relationship to IGF-binding protein (BP)-1, IGFBP-2, IGFBP-3, insulin, and growth hormone. *International Journal of Obesity* **21** 355–359.
- Nixon AJ 1999 Primary nucleotide structure of predominant and alternate splice forms of equine insulin-like growth factor 1 and their gene expression patterns in tissues. *American Journal of Veterinary Research* **60** 1234–1241.
- Noble GK & Sillence MN 2000 Technical considerations when measuring IGF-1 and IGF binding proteins in the horse. In *Proceedings of the 13th International Conference of Racing Analysts and Veterinarians*, Cambridge, UK, pp 314–320. Eds RB Williams, E Houghton & JF Wade. Newmarket: R&W Publications.
- Popot MA, Bobin S, Bonnaire Y, Monget P, Toquets MP, Fortier G, Delahaut P & Closset J 2000 Study related to the detection of equine recombinant growth hormone administration in the horse. In *Proceedings of the 13th International Conference of Racing Analysts and Veterinarians*, Cambridge, UK, pp. 98–104. Eds RB Williams, E Houghton & JF Wade. Newmarket: R&W Publications.
- Secchi C & Borromeo V 1997 Structures and function of bovine growth hormone: bovine growth hormone as an experimental model for studies of protein-protein interactions. *Journal of Chromatography B* **668** 161–177.
- Thompson DL, Rahmanian MS, DePew CL, Burleigh DW, DeSouza CJ & Colborn DR 1992 Growth hormone in mares and stallions: pulsatile secretion, response to growth hormone releasing hormone and effects of exercise, sexual stimulation and pharmacological agents. *Journal of Animal Science* **70** 1201–1207.
- Zapf J, Hauri C, Waldvogel M & Froesch ER 1986 Acute metabolic effects and half-lives of intravenously administered insulin-like growth factor I and II in normal and hypophysectomised rats. *Journal of Clinical Investigation* **77** 1768–1775.

Received 22 March 2001

Accepted 26 June 2001