Vol 12 No 2, p 111-116

VALIDATION OF A HETEROLOGOUS RADIOIMMUNOASSAY FOR INSULIN-LIKE GROWTH FACTOR-I IN CAMELS

M. Hammadi[1], T. Khorchani[1], D. Portetelle[2] and R. Renaville[2]

[1]Livestock and Wildlife Laboratory, Arid Lands Institute, 4119 Médenine, Tunisia,

[2]Biology and Biochemistry Applied Department, Animal and Microbial Unit, Gembloux Agricultural University, Avenue Maréchal Juin, 13, B-5030 Gembloux, Belgium

ABSTRACT

This study was undertaken to develop a heterologous radioimmunoassay method for measuring IGF-I in camel serum. Precision, accuracy and limit of quantitation were the principal parameters used to judge validity of an acid-ethanol extraction method. Blood was taken from 20 suckling dromedary females and their calves. Dams were divided into 2 body condition score groups (group 1: 5.2 ± 0.9 and group 2:3.8 ± 0.6; P <0.0001).

The precision expected by the intra and inter-assay variation was in the range of 5-10% and 14-16%, respectively. In dilution buffer, recovery ranged from 98.4% in highest added quantity to 126.6% in lowest added quantity of IGF-I. In camel serum, recovery ranged from 92.4% to 107%. Sensitivity or displacement of the concentration of IGF-I in the diluted serum was acceptable until dilution 8 fold after what the precision of the recovery was very low.

Dams and calves in group 1 had elevated (P<0.01) serum IGF-I concentration as compared to those in group 2 (41.6 ± 14.3 ng/ml and 248.3 ± 101.9 ng/ml vs. 26.6 ± 2.9 ng/ml and 90.1 ± 77.2 ng/ml, respectively). IGF-I was found to be less concentrated in dams than in calves in which the IGF-I serum concentration averaged 34.1 ± 12.7 ng/ml and 169.2 ± 119.7 ng/ml, respectively.

Key words: Camel, IGF-I, radioimmunoassay

Camel forms an integral part of the desert environment with an ability to survive on scarce and poor vegetation and water and to produce milk, meat and fibre during stress. It also provides a means of regular transport for nomadic people. However, it is well documented that productive and reproductive parameters in classical farm animals are modulated by endocrine system.

Insulin-like growth factor-I (IGF-I), also termed somatomedin-C, is 70 amino acid single chain polypeptide involved in the growth and differentiation of many cell types. In serum, the IGF-I is present as 7.4 to 7.65 kDa molecular protein. The blood concentration of IGF-I primarily reflects growth hormone activity, nutritional status, gender, age and oestrogen levels (Renaville *et al*, 2002; Hagino *et al*, 2005). It has been reported that in adults of many species, more than 95% of IGF-I circulates as complexed to 6 distinct IGF-binding proteins (IGFBPs), which regulate its bioactivity and bioavailability (Clemmons, 1993).

For many years, studies concerning IGF-I are restricted to human, rodent, fish, and classical farm animals. Despite the great structural similarities (96% - 100%) in IGF-I across several species, it has been documented that effects of IGF-I on cultural cells differ among species (Sara and Hall, 1990; Humbel, 1990). There are very few reports on this metabolic factor in camels (Hammadi *et al*, 2001 a, b; 2002).

Radioimmunoassay (RIA) technique is one of the sensitive and quantitative analytical methods used to determine the concentration of IGF-I in blood. Daughaday *et al* (1980) and Underwood *et al* (1982) proposed an extraction process to determine the concentration of IGF-I in blood. Blum and Breier (1994) used a nonextraction process with samples diluted in acidic buffer. The use of one of these two methods depends on availability of materials and reagents. This paper deals with the validation of the acid-ethanol extraction method for the measurement of IGF-I in camels (*Camelus dromedarius*). The effects of level of nutrition, revealed by the body condition score (BCS), on the concentration of IGF-I in blood is studied.

SEND REPRINT REQUEST TO M. HAMMADI email: mohamed.hammadi@ira.rnrt.tn

Materials and Methods

Antiserum production

Antiserum against rhIGF-I (GroPep Pty Ltd., Adelaide, Australia) was raised in one New Zealand White rabbit (Rab 3) using a standard immunisation procedure. The molecule was mixed with complete Freud's adjuvant, in the first booster, and with incomplete Freud's adjuvant in subsequent boosters and injected in 4 to 6 intradermal sites in the back of the animal. Blood samples were withdrawn 14 days after each booster in order to assess the production of antibodies. Last booster was after the 3rd month of immunisation after which the animal was bled by heart puncture and sera 30 ml was aliquoted and stored at –20°C. The rabbit was treated according to international regulations dealing with the treatment of animal and approved the institutional ethical committee applied experimental procedures.

Preparation of standard curve

The rhIGF-I standard preparation was purchased from Gropep (lot EJF102; GroPep Pty Ltd., Adelaide, Australia). One aliquot of the stock solution of IGF-I (40 ng/40 ml), which was stored at –70°C, was used to prepare the standard curve. This aliquot was kept on ice before dilution 1:50 to 20 ng/ml with phosphate buffer (containing 30 mM $NaH_2PO_4H_2O$, 10 mM EDTA, 0.02% Protamine sulfate, and NaN_3 , 0.05% Tween 20, respectively) at pH 7.5 (SmC). Starting from this, 8 working standard samples (20 ng/ml - 0.156 ng/ml) were obtained by additional sequential dilutions. However, using an unknown samples of dams and calves diluted 21 fold and 35 fold, respectively, the corresponding concentrations used in the MULTICALC Software for the standard curve were ranged from 420 ng/ml to 3.28 ng/ml and from 700 ng/ml to 5.47 ng/ml, respectively. After using, the standard samples were kept at –20°C.

Radioimmunoassay procedure

The serum concentration was measured by a double-antibody RIA procedure. Radioiodinated IGF-I and the goat anti-rabbit IgG (GAR) as a second antibody were prepared in the laboratory. Briefly, the IGF-I preparation (1 mg) was iodinated to specific activity 65 µCi/g by using the chloramine-T method (Parker, 1990). The ^{125}I-IGF-I was purified on a Centrifugal filter (Centricon-3, molecular weight cut-off of 3 kDa; Amicon, Millipore Corporation).

Samples were first extracted with an acid-ethanol solution according to the method of Daughaday *et al* (1980) with small modifications. To 100 ml of samples, 400 ml of acid ethanol mixture (12.5% 2N HCl and 87.5% ethanol) was added. This mixture was vortexed and incubated at room temperature for 30 min. After centrifugation at 3000 g at 4°C, 187.5 ml of supernatant were mixed to 75 ml of Tris 0.855M, vortexed and cryoprecipitated (-20°C) for, at least, 1 h. After spinning at 3000 g, at 4°C, 100 ml of supernatant were diluted with 200 ml of SmC buffer.

To 50 ml of this mixture or to standard samples were added 100 ml of antiserum (initial dilution 1/700) and 150 ml of SmC buffer and incubated at room temperature for 1 h. After that, 100 ml of tracer (about 10000 cpm) were added and the mixture was vortexed and incubated for at least 16 h at 4°C. Both GAR and polyethylene (PEG 6000) precipitation were used to separate free from bound ligand. Firstly, 100 µl of normal rabbit serum (1:100 initial dilution) and 100 µl of GAR (1:10 initial dilution) were added to each tube, the tubes vortexed and incubated for 4 h at 4°C. Secondly, 1 ml 6% (w/v) PEG 6000 in 150 mM NaCl was added to each tube, followed by vortexing, incubation on ice for 15 min and centrifugation at 2500 g for 30 min at 4°C. The supernatant was then aspirated and the radioactivity in the pellets was determined by an automatic gamma counter (1470 Wizard).

Assay performance

Assay performance was characterised by assessing precision (intra- and inter-assay variation), accuracy (recovery of added quantities in buffer and in dromedary serum) and sensitivity (limit of quantitation). For this purpose, one preliminary assay was performed to pool samples into three pools of high, medium and low concentration of IGF-I. Some of the high concentration aliquots were diluted, before extraction to 2, 4, 8, 16, 32 and 64 fold and another from the low concentration aliquots were enriched, with known concentrations of IGF-I ranging from 15 ng/ml to 250 ng/ml (covering the working range of the calibration curve). Each sample was assayed in triplicate on the same assay.

The limit of quantitation of the assay was defined as the lowest concentration that could be determined with a precision of ≤ 20%, and an accuracy within ± 20% of the given concentration value.

Animal management and sampling

Samples of serum used in this study were taken from 20 suckling dromedary females of Maghrebi breed and their calves. Females were at 3 to 5 months postpartum belonging to the experimental herd of the

Arid land Institute (Tunisia). Dams were divided into two BCS groups (BCS was 5.2 ± 0.9 and 3.8 ± 0.6 in group 1 and group 2, respectively; P <0.0001) using a dromedary scale of numerical range of 1 to 8 (Tibary and Anouassi, 1997).

The herd was kept in the pens during the night and moved to the range during the day for 7 to 8 hours of grazing. The pasture was dominated by salty native grass species (*Arthrocnemum indicum, Limoniastrum gyunianum, Nitraria retusa, Salsola tetrandra*). At sunset, each female of group 1 received 4 kg/d of concentrated supplement and that of group 2 received only 1kg/d. The concentrate was formulated and quantified for group 1 to supply 60% of total daily requirement energy for a 420 kg female producing 4 litre of milk/day during the 3 months of lactation (Hammadi *et al*, 2002). The animals had access to water twice a day. In the morning, before going to the pasture, blood was taken from the jugular vein of each dam and her calf. Serum samples were centrifuged after an incubation of 3 h at room temperature and were conserved at – 20°C until assayed.

Statistical analysis

Statistical analysis of data was performed with Minitab 13.20 Software (Minitab Inc. PA, U.S.A.). Classical descriptive analysis was determined for the concentration of IGF-I in serum. The paired Student's *t* -test was used to study the effect of BCS on this parameter in dams and calves. Data are expressed as mean ± SD.

Results

Antiserum

The primary production of the polyclonal antibodies against rbIGF-I was observed after 4 weeks of immunisation. However, the plateau was attained after 8 weeks and 2 sera pools were made during the third month of immunisation. Considering that suitable dilution of antiserum in competitive binding radioassays is that which yields a binding between 20% and 50% of the total labeled tracer (TC) in the absence of unlabeled IGF-I (B0), an initial dilution 1/700 of the antiserum, and a 10000 cpm concentration per tube of ^{125}I-IGF-I was added for a value of 25-30% and a non-specific binding (NSB) less than 2%. A typical standard covering the concentration range from 420 ng/ml to 3.28 ng/ml is shown in fig 1. The antiserum showed no cross-reactivity against recombinant bovine IGF-II (rbIGF-II; batch GTS-2, Monsanto, St Louis, OM) or bovine insulin (Sigma, Chemical Co., St Louis, MO) (Renaville *et al*, 1994).

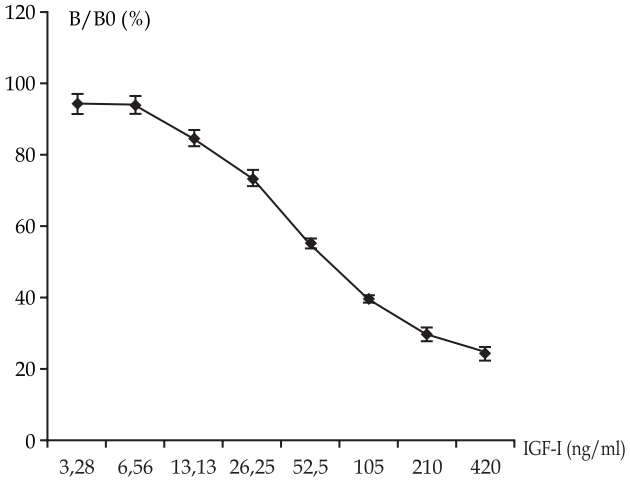

Fig 1. Standard curve for IGF-I assay in dromedary samples. Displacement of ^{125}I-IGF-I by increasing amounts of unlabelled IGF-I. B/B0 values represent the ratio of radiotracer bound to the antibodies in the presence (B) and absence (B0) of unlabelled IGF-I. Each value is the means (± SD) of 3 determinations.

Table 1. Precision of the assay in camel serum: three different pooled samples were assayed 5 times in triplicates on the same assay.

Pool concentration	Means (SD)	Intra CV	Inter CV
High	105.9 (15.6)	5.0	14.3
Medium	59.2 (9.7)	10.3	16.4
Low	30.3 (4.5)	7.1	14.8

Table 2. Recovery of added quantity of IGF-I in dilution (SmC) buffer.

Given	N	Found ± SD ng/ml	CV(%)	Recovery (%)
250	3	246.1 (16.7)	6.8	98.4
125	3	146.3 (7.8)	5.3	116.8
100	3	115.1 (8.2)	7.1	115.1
75	3	91.1 (8.6)	9.4	121.3
60	3	69.2 (5.5)	7.9	115.0
15	3	19.1 (6.8)	35.4	126.6

Precision, accuracy and sensitivity of the method

The concentration of IGF-I in the 3 pools were 105.9 ± 15.6, 59.2 ± 9.7 and 30.3 ± 4.5 ng/ml, respectively. Inter and intra-assay variations were in the range of 14-16 and 5-10%, respectively (Table 1).

The accuracy was determined by the addition of known quantities in SmC buffer (Table 2) and in camel serum (Table 3). In SmC buffer, recovery ranged from 98.4% in highest added quantity to

126.6% in lowest added quantity. In camel serum, recovery ranged from 92.4 to 107%.

Accepting both variability lower than 20% and recoveries comprised between 80 and 120%, the limit of quantitation of the assay appeared to be between 14.3 ng/ml and 24.3 ng/ml (Table 4). In fact, the ED- 80 in the standard curve for the five different assays varied between 14.2 ng/ml and 23.3 ng/ml with an average of 18.6 ± 3.9 ng/ml. Until dilution 8, recoveries were in the range of 91.6 – 107.1% and CVs were in the range of 14.7 - 25.1%.

Insulin-like growth factor (IGF-I) in camel

The concentration of IGF-I in serum of camel is reported in table 5. Dams and calves in group 1 had elevated (P<0.01) serum IGF-I concentrations as compared to those in group 2 (41.6 ± 14.3 ng/ml and 248.3 ± 101.9 ng/ml vs. 26.6 ± 2.9 ng/ml and 90.1 ± 77.2 ng/ml, respectively). IGF-I was found to be less concentrated in dams than in calves in which the IGF-I serum concentration averaged 34.1 ± 12.7 ng/ml and 169.2 ± 119.7 ng/ml, respectively.

Discussion

The goal of this paper was to test an heterologous radioimmunoassay method for measuring IGF-I in camel serum. Precision, accuracy and sensitivity were the principal parameters used to judge validity of the method. The data presented herein demonstrate the validity of the acid-ethanol extraction method to measure IGF-I in serum of camel. The precision expected by the intra and inter-assay variation was in the range of radioimmunoassay data obtained in bovine blood (8.5% and 7.8% for intra andinter-assay variation, respectively as reported by Roeder *et al* (1994) but was lower than that reported by Yu *et al* (1999) for human. Recovery of added quantities in buffer as well in dromedary serum was acceptable, but it appears that recovery of IGF- I in serum seems to be lesser than in buffer. This could be attributed to the effect of their specific binding proteins in serum. In fact, it is well known that in blood, IGF-I circulates binding to IGFBPs which modulate their bioavailability (Gibson *et al*, 2001) and increase their half-life (Clemmons, 1997 and Lackey *et al*, 1999). Sensitivity or displacement of the concentration of IGF-I in the diluted serum was acceptable until dilution 8 fold after what the precision of the recovery was very low.

Nutritional status plays a major role in regulating levels of IGF system, which includes ligands (IGF-I and IGF-II), receptors (IGF-IR and IGF-IIR), binding proteins (IGFBP-1 to 6) and IGFBP proteases (Renaville *et al*, 2002). Dromedaries in high BCS had more systemic IGF-I than those in low BCS. This finding agrees with previous reports in camel (Hammadi *et al*, 2002) and in cows (Yung *et al*, 1996; Gerrits *et al*, 1998; Bossis *et al*, 1999) indicating that high BCS or nutrient supplementation are associated with high concentrations of systemic IGF-I. Dietary protein in the second trimester of pregnancy affects IGF-I in cows (Perry *et al*, 2002) and an elevated serum concentration of IGF-I in high BCS group of ewes is observed (Snyder *et al*, 1999). Circulating IGF-I concentrations are reduced at the beginning of lactation and undernourished animals, even though the GH contents may be high (Formigoni *et al*, 1996; Ritacco *et al*, 1997). Low level of protein and/or energy intake in growing steers was associated with a reduction in GH binding to hepatic membranes and no IGF-I response to GH administration (Breier, 1999). Also, both moderate and severe food restrictions (Renaville *et al*, 2000) or

Table 3. Recovery of added quantity of IGF-I in camel serum.

Given ng/ml	N	Found (SD) ng/ml	CV (%)	Recovery (%)
250	3	231 (6.0)	2.6	92.4
125	3	116.7 (4.4)	3.8	93.4
62.5	3	66.9 (0.5)	0.8	107.0
37.5	3	37.4 (2.5)	6.8	99.7

Table 4. Sensitivity of the assay in diluted camel serum: Recovery =100 x N x (mean in corresponding dilution/105.9)

Given ng/ml	N	Found (SD) ng/ml	CV (%)	Recovery (%)
1	5	105.9 (15.6)	14.7	-
2	5	51.2 (9.8)	19.2	96.5
4	5	24.2 (3.6)	14.8	91.6
8	5	14.3 (3.6)	25.1	107.1
16	5	9.1 (2.3)	24.8	135.7
32	5	6.2 (2.4)	39.1	186.3
64	5	5.4 (4.3)	79.6	303.2

Table 5. Concentration (ng/ml) of IGF-I in dams and calves.

		n	Mean (SD)	Median	Range
Dams:	Group 1	10	41.6 (14.3)	37.7	17.8 – 68.7
	Group 2	10	26.6 (2.9)	27.42	0.6 – 30.7
	Total	20	34.1 (12.7)	29.71	7.8 – 68.7
Calves:	Group 1	10	248.3 (101.9)	231.81	15.8 – 449.7
	Group 2	10	90.1 (77.2)	57.1	20.0 – 246.0
	Total	20	169.2 (119.7)	165.3	20.0 – 449.7

Paired *t* test : in dams Group 1 vs. Group 2, P < 0.01
in calves Group 1 vs. Group 2, P < 0.001

weaning (Carroll *et al*, 1998) result in a decrease of plasma IGF-I concentrations.

Food deprivation caused a rapid decline in levels of IGF-I in humans and pigs (Clemmons and Underwood, 1991; Thissen *et al*, 1994). Breier *et al* (1986) found that feed restriction in steers decreased IGF-I levels. Hagino *et al* (2005) reported a significant post-eating (8-20 h) decline of plasma IGF-I in sheep when animals were fed an important concentrate diet.

It is well documented that IGF-I in beef calves (Kerr *et al*, 1991; Lents *et al*, 1998; Suda *et al*, 2003) increased with age until puberty where it reached in plateau. However, in camel, serum IGF-I level was elevated in calves than in dams. This finding could be attributed to the nutritional status of animals. Parallel to the serum IGF-I concentration, the daily body gain (DBG) was more important (data not shown) in calves than in dams. Suda *et al* (2003) reported a positive correlation (0.52) between plasma IGF-I and body weight of beef calves.

Conclusion

A quantitative, repeatable, heterologous radioimmunoassay for IGF-I was developed for camel blood. The IGF-I was more concentration in camel in good BCS and determination of this parameter could serve as good indicator for veterinary or physiologist in laboratory to appreciate BCS of animal.

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