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Evidence of Free Leptin in Human Seminal Plasma

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Leptin is an adipose tissue–secreted hormone that actively participates in the regulation of energy homeostasis. Besides this principal role, leptin has been implicated in a large variety of neuroendocrine, paracrine, and autocrine actions involved in the regulation of reproductive function in both experimental animals and humans. Although the participation of leptin in female reproduction is well established, any role in male reproductive function is at best tenuous. The aim of this study was to ascertain whether true leptin is present in human seminal fluid and the tissue of its production. Pooled human seminal plasma obtained from healthy donors showed by direct radioimmunoassay (RIA) the presence of radioimmunoassayable leptin. Serial dilutions of unextracted semen paralleled the RIA standard curve, also devoid of interference in the assay. To prove that this activity was true leptin, seminal plasma was subjected to size-exclusion chromatography, which showed that leptin immunoreactivity eluted with the same partition coefficient as cold leptin, ^{125}I-leptin, and ^{125}I-leptin preincubated with seminal plasma. The results demonstrate that true leptin was present in semen in a free form, i.e., without binding proteins. The presence of leptin charge variants in seminal plasma was assessed by anion-exchange chromatography, which showed two peaks of leptin inmunoreactivity, while ^{125}I-leptin eluted as a single peak. Preincubation of ^{125}I-leptin with seminal fluid converted the single peak into a double peak, indicating that components of the seminal fluid introduce a charge variation in leptin. Leptin levels in seminal plasma of 40 healthy men were 0.95 ± 0.19 ng/mL while in 5 vasectomized men the levels were 0.92 ± 0.25 ng/mL, suggesting that testicular tissues were not the source of seminal leptin. No correlation was observed between leptin concentrations in semen and the physical characteristics of semen samples or physical characteristics of spermatozoids, such as concentration, motility, vitality, or morphology. In conclusion it was unambiguously demonstrated that human leptin is present in seminal fluid, with at least two charge variants and no binding proteins, the most likely source being either seminal vesicles or prostate tissue. The role of seminal fluid leptin in the male reproductive function or sperm capacitation is at present unknown.

Key Words: Leptin; semen; seminal plasma; body mass index.

Introduction

Leptin is a pleiotropic hormone produced by white adipose tissue, acting as the afferent loop to the hypothalamus, and contributing to energy balance by informing the brain of the amounts of adipose reserves in a given individual *(1)*. Although it was originally thought of as the hormone regulating obesity, it soon became clear that leptin is actually the hormone regulating fasting, since its reduction in the situation of low energy intake puts into gear the multiple hormonal adaptations of fasting *(2)*. In addition to its role in the regulation of energy homeostasis, leptin has been implicated in several neuroendocrine activities, such as regulation of growth hormone *(3)* and adrenal *(4)* and thyroid hormone secretion *(5)*. Leptin either produces or acts in several tissues in a paracrine-autocrine way *(6,7)*; however, the physiologic role, if any, of such actions has yet to be resolved.

In addition to regulation of body composition, the physiologic area in which leptin has been most seriously implicated is reproduction. The overwhelming data on this topic can be summarized as follows: First, circulating leptin levels are higher in females than in males of any species, and this is so even at birth *(8–11)*. Second, leptin seems to exert a permissive role for puberty in both female experimental animals *(2,12)* and women *(13–15)*. Third, serum leptin levels change through pregnancy and postpartum period *(16)*. Finally, leptin exerts regulatory actions on pituitary gonadotropins and at the gonadal level *(17–20)*. Interestingly, leptin also seems to be implicated in some local or paracrine actions that are still poorly understood. For example, it is present in milk crossing the neonatal intestinal barrier *(21)*; it is present in relevant concentrations in placenta *(22,23)*;

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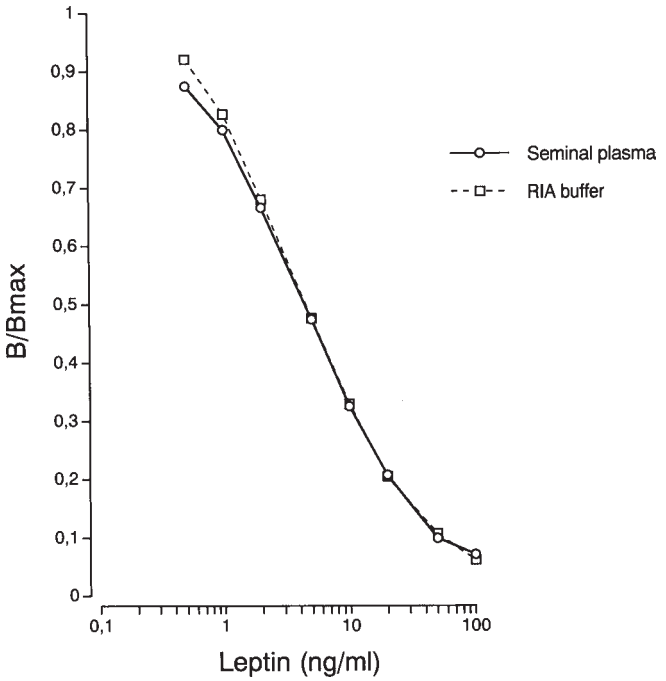

Fig. 1. RIA standard curves of leptin in absence (– – □ – –) or presence (—○—) of 100 µL of human seminal plasma, which substituted the assay buffer.

and it has been detected in follicular fluid *(24)*, where it is a marker of the quality of ovarian follicles and oocytes *(25)*.

Although the role of leptin in male gonadal function is less evident than in females *(1)*, a participation of this hormone in seminal production or sperm capacitation has not been previously assessed. The aim of the present work has twofold: to study the presence of true leptin in human seminal fluid, and to assess the reproductive tissue producing it.

Results

Leptin was identified in seminal plasma by a direct radioimmunoassay (RIA). As additional criteria of identity, RIA standard curves were compared with curves obtained when 100 µL of human seminal plasma substituted the same volume of buffer. The new curve overlapped with the RIA curve (Fig. 1), indicating that human seminal plasma did not interfere with the RIA of leptin.

In addition, size-exclusion chromatography was used as a qualitative approach to identify the presence of true leptin in seminal plasma. As Fig. 2 shows, ^{125}I-leptin (Fig. 2A) and cold leptin (2 mg) (Fig. 2B), both taken as controls, and leptin immunoreactivity from pooled seminal plasma (Fig. 2C) all eluted as single peaks, showing the same elution volume partition coefficient (K_{av}) respective to free and monomeric leptin (16 kDa). The absence of additional peaks after the chromatography of ^{125}I-leptin previously incubated with seminal plasma (Fig. 2D) confirmed that leptin was present in a free form. These results would seem to rule out the possibility of complexes with macromolecules (binding proteins) as happens in serum, in which leptin is known to be of both monomeric (free) and higher molecular mass (bound).

To investigate the presence of charge variants, anion-exchange chromatography on Fast Flow Q was realized (Fig. 3). Both labeled leptin and cold leptin eluted in the same elution volume and conductivity (Fig. 3A,B). However, when seminal fluid was chromatographed, a broad peak was observed by RIA. This peak was characterized by a well-defined peak and a shoulder suggesting the presence of possible charge variants (Fig. 3C). When seminal plasma was incubated with ^{125}I-leptin, the expected broad peak appeared as two well-resolved peaks of radioactivity in the elution profile (Fig. 4A). Peak 1 showed the same elution volume and conductivity as the single peak observed for ^{125}I-leptin, in the absence of seminal plasma (Fig. 3A). To demonstrate that peak 2 could not be the result of the presence of binding proteins, on the basis of size-exclusion chromatography results, excess cold exogenous leptin was added to seminal plasma plus ^{125}I-leptin. Cold leptin should be effective in replacing ^{125}I-leptin in bound form and, therefore, in producing a decrease or disappearence of radioactivity associated with peak 2. However, the addition of 2.5 mg/mL of leptin did not produce a significant reduction in the peak 2 (Fig. 4B). The presence of two radioactive peaks might be explained by a transference and equilibrium of radioactive isotope between charge leptin variants during the incubation period. This result supports the idea that leptin in seminal plasma was in free form. The majority of leptin immunoreactivity in semen was associated with peak 1 (Fig. 3C), corresponding to the peak of true leptin. These results suggest that the leptin exists in seminal plasma as two clearly different charge variants.

The leptin value (mean ± SE) in seminal plasma from 40 healthy men was 0.95 ± 0.19 ng/mL (Fig. 5). In a group of five vasectomized men, the mean value was 0.92 ± 0.25 ng/mL. These results rule out the possibility that testes contribute to leptin secretion, and, therefore, the source of leptin may be the seminal vesicle or the prostate tissues.

Leptin levels in the subjects tested did not show any correlation with sperm parameters such as concentration, motility, vitality, morphology, head alterations, and volume, nor was any correlation shown with the body mass index of donors (data not shown).

Discussion

In this work the presence of true leptin in human semen was observed for the first time. This finding opens a new field of observation in order to assess fully whether leptin plays any role in spermatozoid capacitation or whether it merely reflects an endocrine or paracrine activity. Although poorly defined, the role of leptin in energy homeostasis and neuroendocrine modulation is currently well accepted *(1)*.

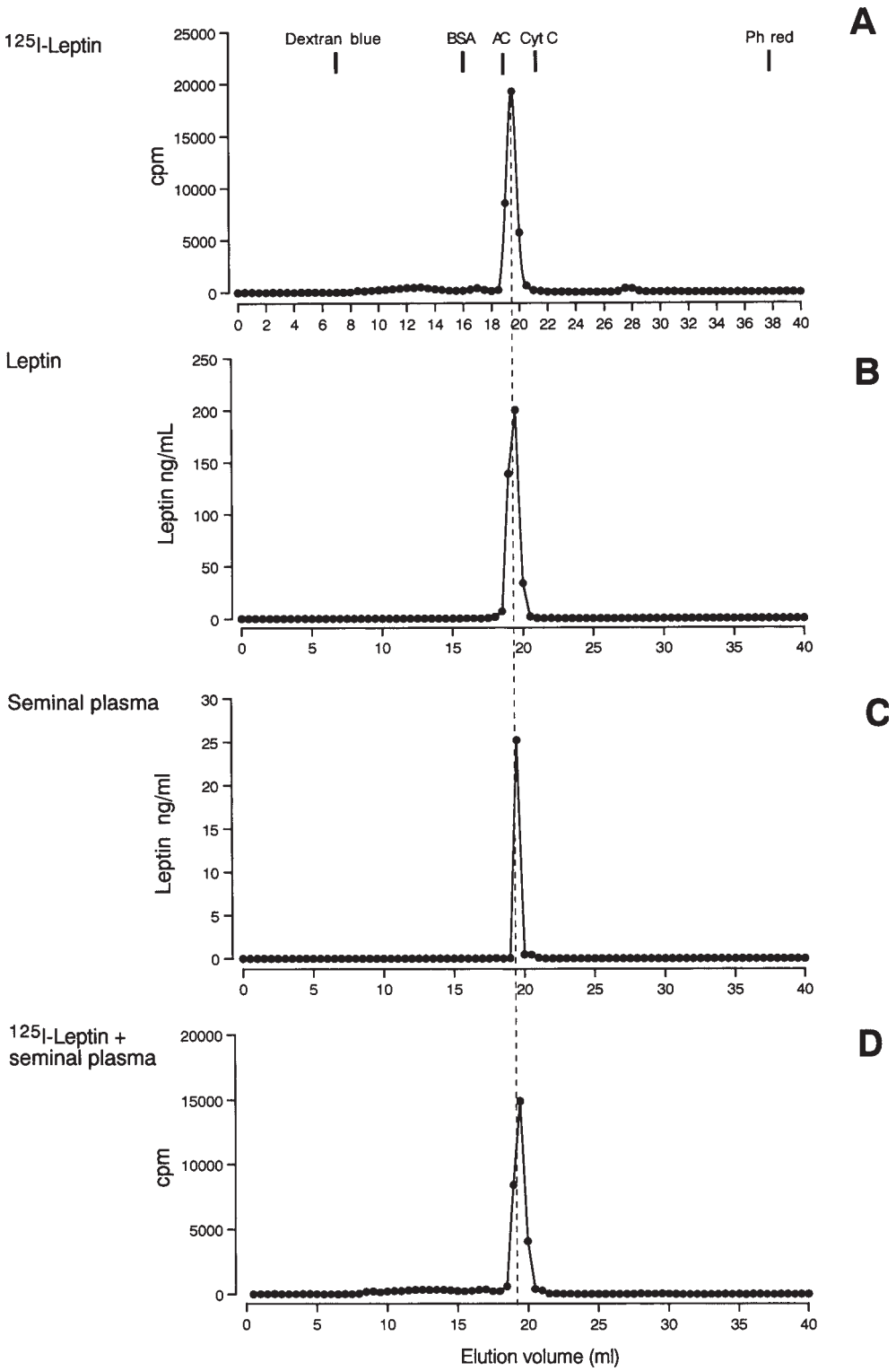

Fig. 2. Identification of leptin in seminal plasma samples. Elution profiles from different Superose-6 Fast Protein Liquid Chromatography (FPLC) size-exclusion chromatographies are shown: (**A**) ^{125}I-leptin; (**B**) cold leptin immunoreactivity; (**C**) seminal plasma sample; (**D**)^{125}I-leptin plus seminal plasma sample. Marker proteins used for the calibration of the Superose-6 FPLC column are indicated as Dextran blue (2000 kDa), BSA (bovine serum albumin, 67 kDa), CA (carbonic anhydrase, 29 kDa), Cyt C (cytochrome C, 12.4 kDa), and Ph red (phenol red, 1.4 kDa).

However, in the last few years, several reports have shown that leptin is present in unexpected organs, such as the stomach, muscle, and placenta, or in fluids, such as milk *(6,21, 23,26)*, exerting actions of a nonmetabolic nature, the relevance of which is poorly known *(27–30)*. In the field of reproduction *(31)*, leptin acts at the hypothalamic level to facilitate

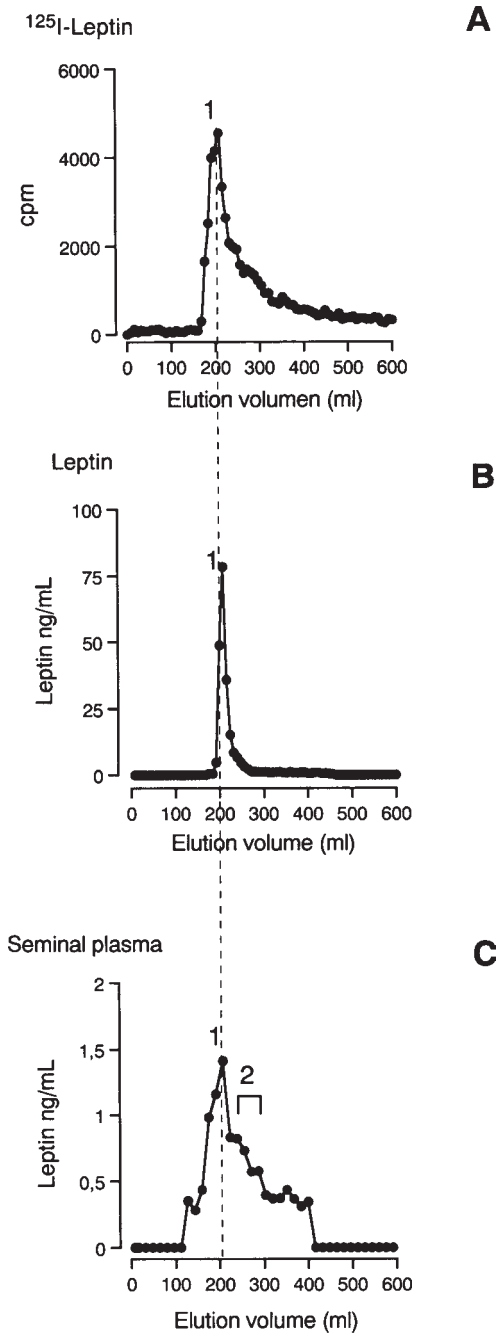

Fig. 3. Leptin charge variants in seminal plasma samples using anion-exchange chromatography (FFQ-Sepharose): (**A**) ^{125}I-leptin eluted as a single peak; (**B**) elution profiles of leptin immunoreactivity detected after adding recombinant h-leptin; (**C**) leptin immunoreactivity of seminal plasma sample.

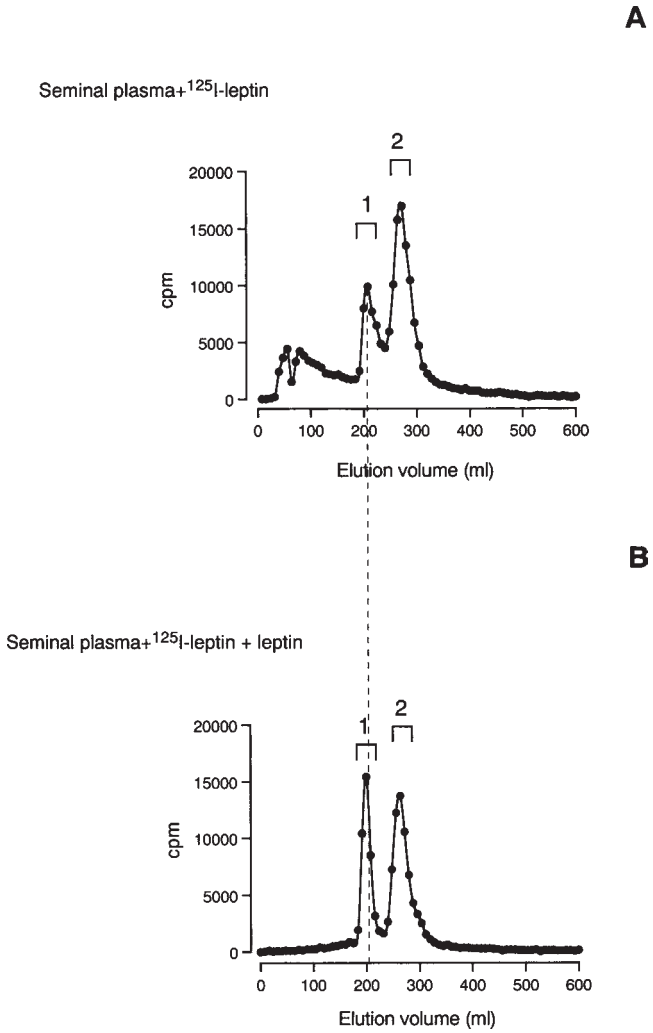

Fig. 4. (**A**) Elution profile obtained from mixture of ^{125}I-leptin + seminal plasma sample. ^{125}I-leptin eluted as two peaks. (**B**) Profile obtained of combination of ^{125}I-leptin + leptin in excess (2.5 mg/mL) + seminal plasma. Peaks did not show any significant modification after incubation with unlabeled leptin.

ovarian cycle and the entrance to puberty, as well as to modulate gonadotropin secretion by the pituitary *(13,32)*. Interestingly, leptin is present and actively participates in the ovarian follicle *(33,34)*, being a marker of the quality of the follicle and the viability of the embryo *(24,25,35,36)*. Leptin acts locally in the steroidogenesis of ovarian cells *(33, 37)*. Several of these actions have been merely descriptive but strongly suggest new and unexpected roles of leptin acting as a paracrine mediator.

The role of leptin in male reproductive function is less well known, mainly because fat mass and energy reserves play no role in male reproductive function, either in experimental animals or in men. In fact, no role for leptin has been observed in the entrance to puberty in boys *(1,13)*, nor has it been reported in the daily gonadal function in men. In the present study, it has unambiguously been demonstrated that leptin-like activity observed in semen by RIA is true leptin, according to the criteria of immunoreactivity, size-exclusion chromatography, and ion-exchange chromatography. Leptin in seminal plasma was presented in free form without binding proteins. This is in contrast with plasma, in which leptin circulates partially complexed with binding proteins that most probably represent the extracellular domain of the leptin receptor *(1)*. On the other hand, the charge variants

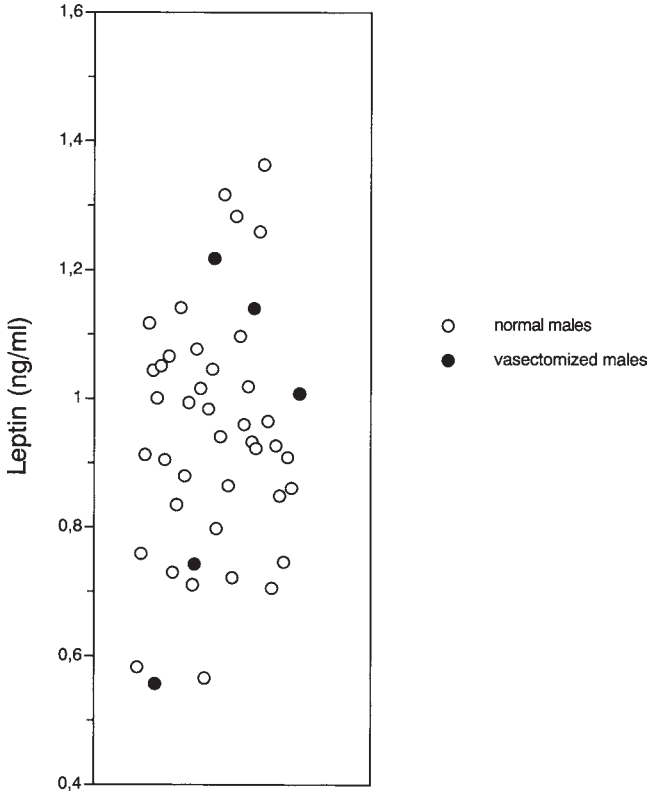

Fig. 5. Individual seminal plasma leptin levels from normal (○) and vasectomized (●) male subjects.

observed herein could be exerted by seminal fluid in a time-related manner, probably owing to the strongly basic properties of this fluid. Since the leptin content in semen from vasectomized subjects is similar to that of normal subjects, the possibility that leptin was passively filtrated from plasma or synthesized at testicular levels is unlikely, with prostatic tissue and seminal vesicles being possible candidates for local synthesis and production. The physiologic role, if any, of seminal leptin is unknown at present but fits in well with the expanding autocrine-paracrine roles attributed to this hormone. No correlation between leptin concentrations and semen or spermatozoid characteristics such as viability or motility was observed; however, the number of subjects studied was too limited to allow any definitive conclusions to be drawn.

In conclusion, true leptin is present in human seminal fluid, in an unbound state and showing at least two charge variants. Prostate or seminal vesicles are likely production sites for this hormone, the role of which at the local level is still unknown.

Materials and Methods

Patients

Forty healthy men ages 33.7 ± 6.7 yr (range: 20–46 yr) participated in the study after providing informed consent. The semen samples were obtained by masturbation after 4 d of sexual abstinence and were collected in sterile collection tubes. They were allowed to liquefy for approx 30 min at 37ºC, after which different aliquots were taken to perform an analysis for measuring the following parameters: volume, spermatozoid concentration, motility, vitality, morphology, and head alterations. Seminal plasma samples were obtained after centrifugation (8000*g* for 15 min) and stored at –80ºC.

Chromatography Procedures

Sample Processing

Chromatography steps were developed on the basis of pooled seminal plasma samples. Samples were dialyzed twice at 4ºC against 10 L of 10 m*M* KCl/25 m*M* Tris-HCl (pH 8.0) using a 3500-Dalton cutoff membrane (Spectra-Por 3; Spectrum Medical, Houston, TX). Dialysates were cleared by centrifugation.

Size-Exclusion Chromatography

Aliquots 0.5 mL of seminal plasma samples were injected into a Superose-6 FPLC size-exclusion column (Pharmacia) preequilibrated with 100 m*M* KCl/25 m*M* Tris-HCl (pH 7.4) and eluted at 0.2 mL/min with the same buffer. Some samples were incubated with ^{125}I-leptin (approx 35,000 cpm) for 24 h at 4ºC and then chromatographed under the same conditions *(21)*. Fractions (0.5 mL) were collected, and the chromatography process was monitored for radioactivity (hot sample) or analyzed by human-leptin RIA (cold sample).

Anion-Exchange Chromatography

Dialyzed seminal plasma (20 mL), incubated or not with ^{125}I h-leptin (15×10^5 cpm) for 24 h at 4ºC (hot), was loaded into a Fast Flow Q Sepharose anion-exchange column (10 × 1 cm) (Pharmacia) preequilibrated with 10 m*M* KCl/25 m*M* Tris-HCl (pH 8.0) at 2.0 mL/min. The column was washed with 10 vol of 10 m*M* KCl/25 m*M* Tris-HCl (pH 8.0), and the bound material was eluted with a linear gradient (45 vol) of 10–1000 m*M* KCl in 25 m*M* Tris-HCl (pH 8.0) at 2.0 mL/min *(21)*. Fractions (8 mL) were collected, and the chromatography process was monitored for either radioactivity (hot) or human-leptin RIA (cold).

Radioimmunoassay

Leptin levels were measured in duplicate by a specific RIA for human leptin using commercial kits (Linco, St. Charles, MO) according to the manufacturer's instructions. The detection limit was 0.5 ng/mL using 100-µL samples. The inter- and intraassay coefficients of variation were 8.3 and 6.2%, respectively.

Acknowledgments

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