Use of recombinant human thyroid-stimulating hormone for thyrotropin-stimulation testing of euthyroid cats

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Objective—To evaluate response of euthyroid cats to administration of recombinant human thyroid-stimulating hormone (rhTSH).

Animals—7 healthy cats.

Procedure—Each cat received each of 5 doses of rhTSH (0, 0.025, 0.050, 0.100, and 0.200 mg), IV, at 1-week intervals. Serum concentration of total thyroxine (TT_4) and free thyroxine (fT_4) was measured immediately before each injection (time 0) and 2, 4, 6, and 8 hours after administration of each dose.

Results—Overall TT_4 response did not differ significantly among cats when administered doses were ≥ 0.025 mg. Serum TT_4 concentrations peaked 6 to 8 hours after administration for all doses ≥ 0.025 mg. For all doses ≥ 0.025 mg, mean ± SEM TT_4 concentration at 0, 6, and 8 hours was 33.9 ± 1.7, 101.8 ± 5.9, and 101.5 ± 5.7 nmol/L, respectively. For all doses ≥ 0.025 mg, mean fT_4 concentration at 0, 6, and 8 hours was 38.7 ± 2.9, 104.5 ± 7.6, and 100.4 ± 8.0 pmol/L, respectively. At 8 hours, the fT_4 response to 0.025 and 0.050 mg was less than the response to 0.100 and 0.200 mg. Adverse reactions after rhTSH administration were not detected.

Conclusions and Clinical Relevance—The TSH stimulation test can be performed in cats by IV administration of 0.025 to 0.200 mg of rhTSH and measurement of serum TT_4 concentrations at time of injection and 6 or 8 hours later. Clinical validation of the TSH stimulation test would facilitate development of additional tests of thyroid gland function, such as a TSH assay. (*Am J Vet Res* 2003;64:149–152)

The **thyroid-stimulating hormone** (**TSH**; thyrotropin) stimulation test is considered the criterion-referenced standard for evaluation of thyroid functional reserve in dogs and cats.[1] The principal use of the TSH stimulation test is for the identification of hypothyroidism. When clinicians evaluate only serum **thyroxine (T_4)** concentrations, it can be difficult to differentiate hypothyroidism from euthyroid sick syndrome in which cats with nonthyroidal illness can have substantial reductions in circulating T_4 concentrations.[1-4] Hypothyroidism in cats can be congenital,[5-9] acquired,[10] or iatrogenic (as a result of treatment of hyperthyroidism).[11-14]

In the past, the TSH stimulation test involved the use of bovine TSH, but this hormone is no longer commercially available as a pharmaceutical preparation.[1] It has been replaced with the more expensive **recombinant human TSH (rhTSH)**.[15] This has necessitated development of new protocols for the TSH stimulation test in companion animals. A new protocol has been described for dogs,[16] but we are not aware of a similar protocol for cats. We hypothesized that rhTSH could be safely and effectively used in place of bovine TSH for TSH stimulation testing of cats.

In humans and dogs, the serum concentration of endogenous TSH is useful in establishing a diagnosis of hypothyroidism.[17-24] This readily available and inexpensive test has reduced the need for TSH stimulation testing in clinical practice, but attempts to develop an assay for endogenous TSH in cats have been hampered by lack of a criterion-referenced standard (ie, TSH stimulation test) to define thyroid function in test subjects.[a] Therefore, the objective of the study reported here was to initiate development of the TSH stimulation test as a criterion-referenced standard for assessment of thyroid gland function in cats, which would assist researchers in the redevelopment of less costly and simpler assays (such as an assay for endogenous feline TSH).

Materials and Methods

Animals—Seven young adult (7 to 8 months old) cats (4 sexually intact females and 3 sexually intact males) were used in the study. These were specific-pathogen-free cats that were acquired from a commercial vendor and vaccinated against feline rhinotracheitis virus, calicivirus, panleukopenia virus, and rabies virus. Cats were housed in 2 groups on the basis of sex and fed a commercial maintenance diet. Complete physical examination, CBC, serum biochemical analysis, urinalysis, and determination of baseline **total T_4 (TT_4)** and **free T_4 (fT_4)** concentrations were performed to ensure that cats were healthy prior to the start of the study. All cats were allowed to acclimate to their surroundings and to handling procedures for 1 week prior to testing.

Measurement of TT_4 concentrations—Serum TT_4 concentrations were measured by use of a commercially available ^{125}I solid-phase competitive radioimmunoassay kit.[b] Precision of the kit was assessed within and between assays by 10 replicate analyses of 3 concentrations of pooled feline serum. Intra-assay **coefficients of variation (CVs)** were 4, 4, and 5% for pooled serum that contained 10, 40 and 140 nmol/L, respectively. Interassay CVs were 15, 12, and 10% for pooled serum that contained 13, 63, and 116 nmol/L, respectively.

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Accuracy of the method was documented by recovery of 96 to 102% of known quantities (26, 52, and 78 nmol/L) of T_4 added to pooled feline serum. Limit of detection of the assay was calculated as 3 nmol/L.

Measurement of fT_4—Serum fT4 concentrations were measured by use of a sensitive solid-phase radioimmunoassay for T_4 that followed equilibrium dialysis. Concentrations were determined by use of a commercially available kit[c] that had been validated for use in cats.[2,4]

TSH—The rhTSH was a commercially available, highly purified recombinant form of human TSH[15,d] that was produced in a genetically modified Chinese hamster ovary cell line. Each vial contained 1.1 mg of thyrotropin α that, when reconstituted with 1.2 mL of sterile water, contained 0.9 mg of thyrotropin α/mL (pH, 7.0). Each single-use vial did not contain preservative; thus, a new vial was used each week (ie, a new vial was used for each dosing period).

Various protocols have been used for bovine TSH and TSH stimulation testing of cats.[1] Use of a recommended dose of 0.1 U of TSH/kg would result in a total dose of approximately 0.4 to 0.5 U for many cats. Because 1 mg of rhTSH approximates 4 U of biological activity,[15] the equivalent dose of rhTSH would be 0.100 to 0.125 mg/cat. However, a study[16] conducted in Beagles revealed that a maximal response was achieved with a total dose of only 0.050 mg of rhTSH/dog. Response in that study was most consistent when the compound was administered IV to the Beagles. In the study reported here, we performed a dose response study that included 4 doses of rhTSH (0.025, 0.050, 0.100, and 0.200 mg) as well as a sham injection (0 mg). The sham dose was provided as 0.10 mL of saline (0.9% NaCl) solution and served as a control treatment for effects of repeated handling and venipuncture.

Protocol—Once each week for 5 weeks, cats were administered a dose of rhTSH, IV. For each cat, the 5 doses were randomly allocated to the 5 periods to minimize potential confounding effects of period and dose. Blood samples were collected into serum-separator tubes immediately before injection (time 0) and 2, 4, 6, and 8 hours after injection for use in measurement of TT_4 and fT_4 concentrations. Cats were monitored for adverse effects such as vomiting, diarrhea, or anaphylaxis that could have resulted from IV administration of the foreign antigen.

Statistical analysis—Statistical analysis was conducted by use of a 5-factor ANOVA.[e] The analysis was conducted in accordance with the following model:

$$Y = \mu + \alpha + C(\alpha) + \beta + \pi + error_2 + \tau + \tau \times \beta + error_3$$

where Y was the response variable (concentration of TT_4 or fT_4), μ was the overall mean, α was the fixed effect of sex, C(α) was the random effect of cats within sex and served as the error term for sex, β was the fixed effect of dose, π was the fixed effect of period, $error_2$ served as the error term for dose and period, τ was the fixed effect of time after injection, and $error_3$ served as the error term for time and the time-by-dose interaction. Post-hoc analysis to detect differences between doses at specific time points was performed by use of the Bonferroni *t*-test for multiple comparisons. Values of $P < 0.05$ were considered significant.

Results

Time, dose, and the time-by-dose interaction significantly affected TT_4 concentrations (**Fig 1**). Mean ± SEM TT_4 concentrations did not differ significantly ($P = 0.25$) between male (70.8 ± 1.7 nmol/L) and female (68.0 ± 1.4 nmol/L) cats. Response for the control dose (0 mg) was stable over time (ie, TT_4 concentration did not differ significantly with time). Overall, TT_4 responses did not differ significantly after administration of doses ≥ 0.025 mg. The TT_4 concentration at time 0 was less than the concentration at 2 hours, which was less than the concentration at 4 hours, which was less than the concentration at 6 or 8 hours; TT_4 concentrations were similar at 6 and 8 hours. For all doses ≥ 0.025 mg, mean TT_4 concentration was 33.9 ± 1.7 at time 0 and 101.8 ± 5.9 and 101.5 ± 5.7 nmol/L at 6 and 8 hours after injection, respectively (**Table 1**).

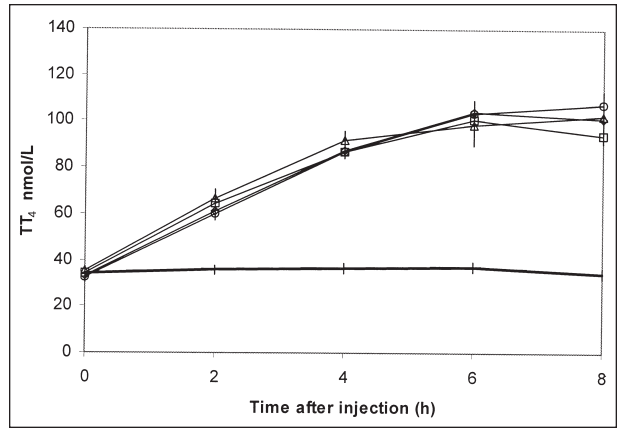

Figure 1—Mean ± SEM total thyroxine (TT_4) concentrations at various time points after IV administration of recombinant human thyroid-stimulating hormone (rhTSH; 0 mg, thick black line without symbols; 0.025 mg, open square; 0.050 mg, open triangle; 0.100 mg, open diamond; 0.200 mg, open circle). The SEM for between-group comparisons was 3.85, and SEM for between-time comparisons was 3.01. Time 0 = Time of injection.

Table 1—Mean ± SEM serum concentration of total thyroxine (nmol/L) after IV administration of recombinant human thyroid-stimulating hormone (rhTSH) to 7 cats

rhTSH (mg)	Time after administration (h)				
	0	2	4	6	8
0	34.3 ± 1.7^{aA}	35.6 ± 1.9^{aA}	36.4 ± 2.0^{aA}	37.0 ± 2.3^{aA}	34.3 ± 2.2^{aA}
0.025	34.1 ± 1.6^{aA}	64.3 ± 2.6^{bB}	86.7 ± 2.4^{cB}	100.7 ± 4.5^{dB}	94.3 ± 3.3^{cdB}
0.050	35.1 ± 1.3^{aA}	66.6 ± 3.9^{bB}	91.6 ± 4.0^{cB}	98.3 ± 8.4^{cB}	102.7 ± 6.1^{cB}
0.100	33.1 ± 1.6^{aA}	61.1 ± 3.0^{bB}	87.1 ± 3.2^{cB}	104.4 ± 4.1^{dB}	101.4 ± 7.0^{dB}
0.200	32.6 ± 2.2^{aA}	59.7 ± 2.6^{bB}	86.9 ± 2.7^{cB}	103.9 ± 5.5^{dB}	107.7 ± 5.6^{dB}

^{a,b,c,d}Within a row, values with different superscript letters differ significantly ($P < 0.05$). ^{A,B}Within a column values with different superscript letters differ significatly ($P < 0.05$).

Time, dose, the time-by-dose interaction, cat, and period all significantly affected fT_4 concentrations (**Fig 2**). Mean ± SEM fT4 concentrations did not differ significantly ($P = 0.29$) between male (69.3 ± 3.7 pmol/L) and female (75.1 ± 3.2 pmol/L) cats. Similar to the TT_4 concentrations, fT_4 concentrations did not differ significantly over time after administration of 0 mg of rhTSH. At 6 and 8 hours after administration, fT_4 concentrations for the lowest doses of rhTSH (0.025 and 0.050 mg) were less than the concentrations for the highest doses of rhTSH (0.100 and 0.200 mg). The fT_4 concentration at time 0 was less than the concentration at 2 hours, which was less than the concentration at 4 hours, which was less than the concentration at 6 or 8 hours; fT_4 concentrations were similar at 6 and 8 hours. For all doses ≥ 0.025 mg, mean fT_4 con-

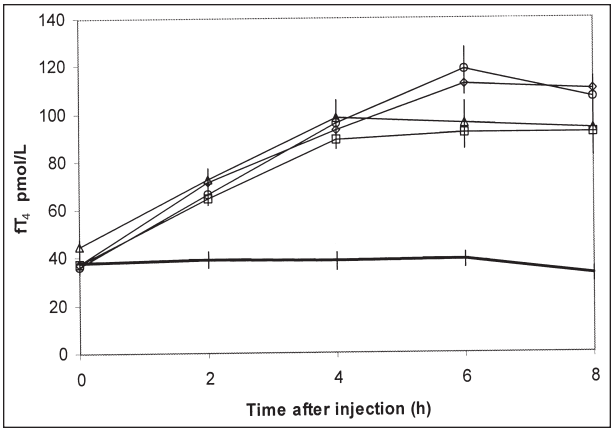

Figure 2—Mean ± SEM free thyroxine (fT_4) concentrations at various time points after IV administration of rhTSH. The SEM for between-group comparisons was 4.71, and SEM for between-time comparisons was 3.71. Time 0 = Time of injection. *See* Figure 1 for key.

Table 2— Mean ± SEM serum concentration of free thyroxine (pmol/L) after IV administration of rhTSH to 7 cats

rhTSH (mg)	Time after administration (h)				
	0	2	4	6	8
0	38.0 ± 3.2aA	39.1 ± 3.4^{aA}	38.3 ± 3.8^{aA}	38.9 ± 2.9^{aA}	32.7 ± 3.0^{aA}
0.025	37.1 ± 2.4^{aA}	64.9 ± 3.3^{bB}	89.0 ± 4.2^{cB}	91.7 ± 6.7^{cB}	91.9 ± 9.9^{cB}
0.050	44.7 ± 2.7^{aA}	72.3 ± 5.0^{bB}	97.9 ± 7.4^{cB}	95.6 ± 9.3^{cBC}	93.3 ± 9.0^{cBC}
0.100	37.6 ± 3.2^{aA}	71.1 ± 3.8^{bB}	93.0 ± 4.7^{cB}	112.3 ± 4.3^{dCD}	110.1 ± 4.8^{dC}
0.200	36.1 ± 3.0^{aA}	66.3 ± 3.4^{bB}	95.9 ± 7.7^{cB}	118.6 ± 8.8^{dD}	106.3 ± 7.4^{cdBC}

^{a,b,c,d}Within a row, values with different superscript letters differ significantly ($P < 0.05$). ^{A,B,C,D}Within a column, values with different superscript letters differ significantly ($P < 0.05$).

centration was 38.7 ± 2.9 at time 0 and 104.5 ± 7.6 and 100.4 ± 8.0 pmol/L at 6 and 8 hours after injection, respectively (**Table 2**).

Discussion

Thyrotropin is a glycoprotein secreted from the pars distalis of the pituitary gland. It is a key regulator in synthesis and secretion of thyroid hormones. Binding of TSH to its receptor on the surface of a cell in the thyroid gland activates adenylyl cyclase through a G protein, which results in an increase in cAMP and subsequent phosphorylation of protein kinases.[16] This stimulates trapping of iodide in cells of the thyroid gland as well as synthesis and secretion of thyroglobulin, triiodothyronine, and T_4.[15,25,26]

The TSH molecule is composed of α and β subunits. The α subunit is the same as that of other glycoprotein hormones produced by the pituitary gland, such as luteinizing hormone and follicle-stimulating hormone, and it is not species specific. The β subunit confers functional specificity of the hormone. Although the exact amino acid sequence of this subunit varies among species, there is biological cross-reactivity such that TSH from 1 species will stimulate the thyroid gland of another species.[1,16] The TSH stimulation test performed in animals takes advantage of this cross-species activity. However, differences in amino acid sequence make the β subunit immunogenic in a nonhomologous species; therefore, clinicians risk hypersensitivity reactions with repeated administration of the foreign hormone. Problems related to immunogenicity when using bovine-source TSH necessitated the development of rhTSH for use in human medicine.[16] Anaphylaxis was not observed after administration of rhTSH in the cats of the study reported. Similarly, anaphylaxis was not a problem in a study[16] in which dogs were administered rhTSH.

Analysis of results of the study reported here indicated sufficient cross-species reactivity for rhTSH such that it could be used in the assessment of thyroid gland function in cats. Of interest was the difference between the fT_4 response to the lowest 2 doses of rhTSH, compared with the response for the 2 highest doses. The TT_4 responses did not differ among doses of rhTSH. One possible explanation for this phenomenon may have been saturation of the thyroxine-binding capacity of plasma proteins. Typically, > 99% of T_4 in the circulation is bound to carrier proteins such as albumin and transthyretin.[25,26] When the TT_4 concentration increases acutely to extremely high amounts, binding capacity of the carrier proteins can be exceeded, thus increasing the amount of T_4 that remains in an unbound (free) state without substantially increasing the measured amount of TT_4 (ie, protein-bound T_4).

The results reported here revealed that TT_4 concentrations peaked 6 to 8 hours after injection of rhTSH. For several reasons, we suggest collection of samples before and 6 hours after injection. First, TT_4 concentrations for all doses ≥ 0.025 mg appeared to have reached maximum values by 6 hours. Second, because the concentrations at 8 hours after injection did not yield results that differed significantly from concentrations at 6 hours after injection, collection of a sample at 6 hours after injection would make the test more expeditious and practical. Finally, protocols for the TSH stimulation test that used bovine TSH often involved collection of a blood sample at 6 hours after injection, so the protocol for use of rhTSH would not deviate from protocols for the use of bovine TSH.

Doses ≥ 0.025 mg of rhTSH resulted in the same response in TT_4 at 6 hours after injection. Therefore, any dose between 0.025 and 0.200 mg could be used. Use of the lowest dose will not result in cost savings unless multiple cats are being tested at the same time, because the vial should be discarded 24 hours after it is reconstituted, regardless of how much has been used. Although adverse effects were not seen for any doses in these cats, it would appear logical that use of the lowest dose (0.025 mg) would minimize the risk of adverse effects. The dose of 0.100 mg was the lowest dose that caused a sustained maximal response in TT_4 and fT_4 concentrations. Therefore, choice of dose should be left to the discretion of the investigator when selecting among doses of 0.025 to 0.200 mg.

The effect attributable to cats was expected and reflects differences that exist among clinically normal cats. The effect of period was minor and probably reflected contributions from interassay variation and variations in total dose administered during each treatment. There were 5 treatments and 7 cats. Therefore, the distribution for the doses varied among study days. Analyses of T_4 concentrations were performed weekly following the completion of each period rather than as a single batch at

the end of the study, which allowed for the possibility of interassay variation to have some effect.

Although a relatively small number of cats were used in this study, we believe the results were quite consistent, and the data gathered answered the question of whether rhTSH could be used safely in cats. These results also provide a framework for additional studies in which reference ranges could be determined by the use of a larger number of cats, with the doses and time of collection of blood samples selected on the basis of results of the study reported here.

We concluded that rhTSH can be used for TSH stimulation testing of cats by use of IV administration of a dose of 0.025 to 0.200 mg, and serum samples should be obtained 6 or 8 hours after administration for the measurement of TT_4 concentrations. Given the additional expense and limited availability of fT_4 analysis and our finding that it did not appear to add useful information to the test protocol, we recommend measurement of only the TT_4 response for the rhTSH stimulation test. Additional studies are warranted to evaluate the response to rhTSH stimulation in a larger number of healthy cats, in cats with naturally occurring and iatrogenic hypothyroidism, and in cats with nonthyroidal illness.

[a]Graham P, Refsal K, Nachreiner R, et al. The measurement of feline thyrotropin using a commercial canine-specific immunoradiometric assay (abstr). *J Vet Intern Med* 2000;14:342.

[b]Gamma Coat M (^{125}I) total T4 radioimmunoassay kit, DiaSorin Inc, Stillwater, Minn.

[c]Free T4 100T kit, Nichols Institute Diagnostics, San Clemente, Calif.

[d]Thyrogen, Genzyme Corp, Cambridge, Mass.

[e]NCSS 6.0.22, Statistical System for Windows, NCSS, Kaysville, Utah.

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