Defining normal adrenal function testing in the intensive care unit setting: A canine study*

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Objective: **To determine whether intensive care medicine therapies and testing influence hypothalamic-pituitary-adrenal test results. It is routine in intensive care medicine to measure hypothalamic-pituitary-adrenal function, commonly utilizing the adrenocorticotropic hormone stimulation test to diagnose absolute or relative adrenal insufficiency.**

Design: **Prospective, 96-hr animal study.**

Setting: **Research laboratory.**

Subjects: **Twenty-four healthy canines.**

Interventions: **Animals were randomized into two groups—awake and unrestrained or treated with intensive care medicine therapies, including sedation, intubation, and mechanical ventilation. Animals were further randomized to receive dexamethasone (or placebo) or undergo either a total of four or seven adrenocorticotropic hormone stimulation tests over 96 hrs.**

Measurements and Main Results: **Sedation, intubation, and mechanical ventilation transiently increased both basal and postadrenocorticotropic hormone total and free cortisol concentrations >2-fold as compared with baseline for the first 24 hrs ($p \leq .05$ for both). Performance of seven stimulation tests increased both basal and postadrenocorticotropic hormone total and free cortisol concentrations from baseline by >1.5-fold for the duration of the 96-hr study ($p \leq .05$). Neither sedation, intubation, and mechanical ventilation nor the performance of more stimulation tests affected delta cortisol measurements (total or free cortisol, p = NS). In contrast, dexamethasone suppressed basal total cortisol concentrations by >2-fold ($p \leq .005$) at all time points and transiently increased delta total cortisol by approximately 35% during the first 24 hrs of the study ($p \leq .05$).**

Conclusions: **Total and free cortisol measurements—whether pre- or post- adrenocorticotropic hormone or as a calculated delta—were altered by intensive care therapies or frequent adrenocorticotropic hormone stimulation testing with one exception. Delta free cortisol was the only hypothalamic-pituitary-adrenal measurement unaffected by sedation, intubation, and mechanical ventilation, completion of more adrenocorticotropic hormone stimulation tests, or dexamethasone therapy. These findings support the need to determine normal ranges for hypothalamic-pituitary-adrenal testing in subjects receiving intensive care medicine before establishing laboratory criteria for the diagnosis of relative adrenal insufficiency. (Crit Care Med 2010; 38:553–561)**

KEY WORDS: hypothalamic-pituitary-adrenal stimulation test; canine; critical illness; glucocorticoid and relative adrenal insufficiency

The use of the adrenocorticotropic hormone (ACTH) stimulation test to establish the diagnosis of absolute adrenal insufficiency in the outpatient setting is well established. By comparison, the concept of relative adrenal insufficiency (RAI) was introduced in the 1990s and has only recently gained acceptance as a clinical diagnosis (1). The premise of RAI is that a basal cortisol concentration which is elevated may, nonetheless, be inadequate in the setting of critical illness. Proponents of RAI would further argue that critically ill patients with concomitant RAI, like patients with absolute adrenal insufficiency, benefit from steroid therapy.

The interpretation of cortisol concentrations in critical illness has proven to be complex (2). As such, there is yet to be a diagnostic criterion for RAI that prospectively identifies patients who benefit from glucocorticoid therapy. To date, different total serum cortisol levels, including a baseline <18 μg/dL or 25 μg/dL, an absolute post-ACTH stimulation level <18 μg/dL, or an ACTH-induced increment (i.e., delta) in total cortisol <7 μg/dL or 9 μg/dL, have all been used to define RAI in published studies (3). Uncertainty also exists as to whether free serum cortisol measurement, as opposed to total cortisol, is a superior test, especially in critically ill patients with hypoalbuminemia (2, 4). If ACTH stimulation testing is to be used to diagnose RAI, it is imperative that the reference range of results be defined in terms of both total and free serum cortisol and that treatment factors which influence test results be identified. Only then can the ACTH stimulation test be used to differentiate normal from abnormal adrenal function in critical illness.

The purpose of this study was to evaluate the impact of various intensive care medicine therapies and interventions on tests of pituitary and adrenal function in otherwise healthy animals. First, the effect of sedation, intubation, and mechanical ventilation (SIM) on pituitary and adrenal function testing was assessed. We

*See also p. 721.

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also compared animals receiving a dexamethasone infusion with controls, as this glucocorticoid is used in intensive care units (ICUs) because it is believed to neither affect performance of assays that measure cortisol concentrations nor, in the short-term, alter the absolute post-ACTH cortisol concentration (5). Finally, we randomized animals to receive either four or seven ACTH stimulation tests with low- and high-dose ACTH over the 96-hr time period to evaluate the effect of performing more than one test in a day—what can occur clinically when practitioners either accidentally repeat the test or decide to confirm the results. Besides comparing the low- and high-dose ACTH stimulation tests, this design allowed us to evaluate the precision of the ACTH stimulation test (i.e., the reproducibility of results) and determine whether the administration of ACTH itself affected test results. The impact of all of these interventions on serum cortisol concentrations (both total and free) measured at baseline, peak response, or the calculated increment of change from baseline was assessed, as each of these measurements has been used to diagnose RAI in critically ill patients.

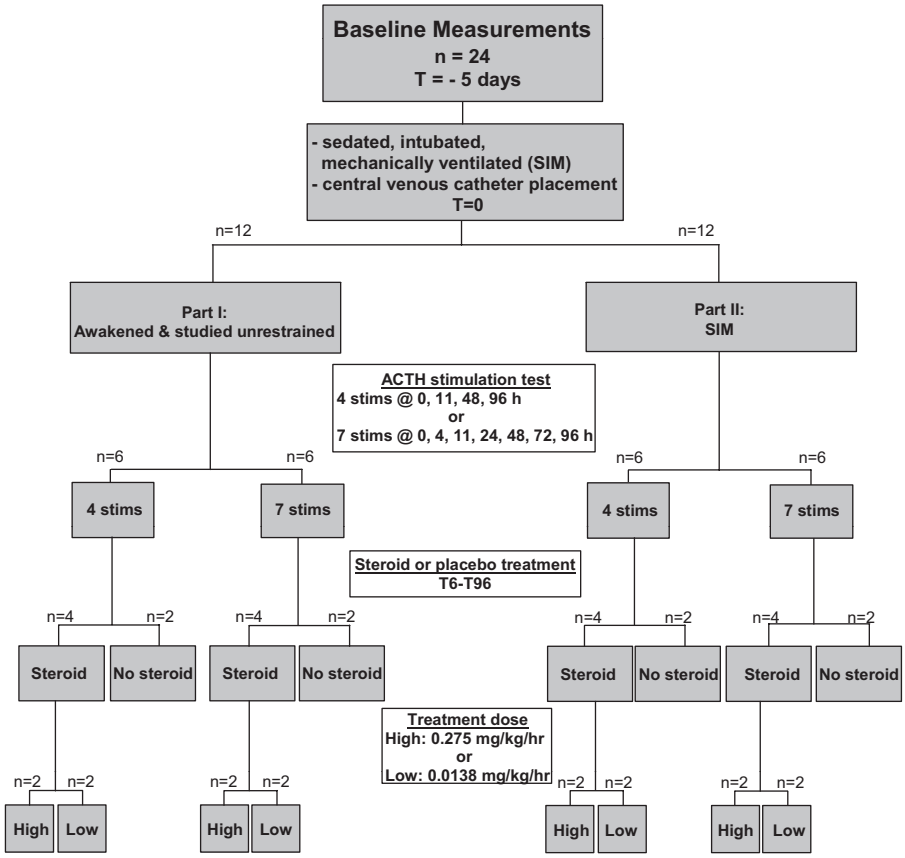


Figure 1. Study design. *ACTH*, adrenocorticotropic hormone.

MATERIALS AND METHODS

Study Design

The experiments described below were performed as part of a protocol approved by the Animal Care and Use Committee of the Clinical Center at the National Institutes of Health. At baseline, all animals (n = 24, 12–18 months old, 10–12 kg, male, purpose-bred beagles) had a peripheral venous catheter placed aseptically (Fig. 1). A blood sample was drawn from each animal for assessment of basal pituitary and adrenal function via measurements of serum total and free cortisol, aldosterone, and antidiuretic hormone (ADH) concentrations and plasma endogenous adrenocorticotropic hormone (eACTH) concentration. Low- and high-dose synthetic ACTH (cosyntropin, Cortrosyn, Amphastar Pharmaceuticals, Inc., Rancho Cucamonga, CA) stimulation tests were then performed sequentially. One hour after administration of low-dose ACTH (1.0 μg IV), a blood sample was drawn, and immediately thereafter, high-dose ACTH (5.0 μg/kg IV) was administered; another blood sample was drawn 1 hr later. Serum free and total cortisol concentration was determined in both post-ACTH samples. The peripheral catheter was then removed, and the animals were returned to standard holding.

Five days after baseline evaluation, all animals were taken to a surgical suite and placed under general anesthesia. A central venous catheter was inserted in all animals; completion of this procedure was designated as $t = 0$. In Part I, animals were then awakened. In Part II, animals had femoral arterial and urinary catheters placed and a tracheostomy was performed. After these procedures were completed (approximately 20 mins), general anesthesia was converted to continuous sedation (fentanyl, midazolam, and medetomidine), and mechanical ventilation was initiated and continued for the duration of the study (96 hrs).

All animals in Parts I and II had blood drawn for measurement of plasma eACTH concentration and serum aldosterone, ADH, and basal free cortisol and total cortisol concentrations at $t = 4$ hrs, 11 hrs, 24 hrs, 48 hrs, 72 hrs, and 96 hrs after catheter placement. Animals in both parts were further randomly assigned to undergo a total of either four (t = baseline, 11 hrs, 48 hrs, or 96 hrs; n = 6) or seven (t = baseline, 4 hrs, 11 hrs, 24 hrs, 48 hrs, 72 hrs, 96 hrs; n = 6) sequential low- and high-dose ACTH stimulation tests. Animals in Parts I and II were also randomly assigned to receive continuous intravenous infusion (1.7 mL/hr) of low-dose dexamethasone (0.0138 mg/kg/hr; n = 4), high-dose dexamethasone (0.275 mg/kg/hr; n = 4), or saline (n = 4). The doses chosen are 10× and 200× as potent as the 24-hr cortisol dose used to treat adrenal insufficiency in canines, and are thus comparable to the higher-dose cortisol therapy tested in early human randomized controlled trials and the more recent, lower-dose cortisol therapy (300 mg/day) used more commonly today (6, 7). Infusions were initiated at $t = 6$ hrs and continued until the end of the study ($t = 96$ hrs). Prophylactic oxacillin (20 mg/kg IV every 8 hrs) was administered, beginning at $t = 0$ hr and continued until the end of the study.

Surgical Procedures

Animals were fasted for 18 hrs before anesthesia induction, and all procedures were performed, using aseptic technique. After intravenous induction with propofol (4–6 mg/kg), tracheal intubation (6F, Rusch, Duluth, GA), and initiation of mechanical ventilation (Fabius Trio, Drager Medical, Telford, PA), anesthesia was maintained with isoflurane (0.5–1.5%). A 20-gauge catheter (Maxxim Medical, Athens, TX) was then inserted in the external jugular vein and covered with a self-adhesive, 1.5 inch × 2 inch elastic bandage (Co-Flex, Andover Coated Products, Andover, MA) to prevent catheter access by the animal. In Part I, animals were then awakened, extubated, and placed in individual cages with free access to food and water. Animals in Part II remained

anesthetized and underwent femoral artery catheter (20-gauge, Maxxim Medical, Athens, TX) and urinary catheter (Foley 8F, 55 cm, Cook, Bloomington, IN) placement. A tracheostomy was also performed on each animal (8).

Mechanical Ventilation

In Part II, the ventilator (Servovent 300, Siemens Medical, Sweden) was initially set with a fraction of inspired oxygen (F_{IO_2}) = 25%, positive end expiratory pressure (PEEP) = 5 cm H_2O, tidal volume = 20 mL/kg, and ventilatory rate = 15 breaths/min. Tidal volumes of 15 mL/kg to 20 mL/kg are standard in ICUs treating critically ill septic canines (8, 9). An oxygen saturation <92% was treated by alternately increasing F_{IO_2} in increments of 25% or the PEEP, initially by 5 cm H_2O followed by 2 cm H_2O. The maximum settings were F_{IO_2} = 100% and PEEP =12 cm H_2O. Both the F_{IO_2} and PEEP were reduced by the same decrements if the oxygen saturation was >93% for 6 hrs. Blood gas determinations (every 2 hrs until t = 8 hrs and then every 8 hrs thereafter) were used to set ventilatory rates on the mechanical ventilator. Ventilatory rates were increased in increments of 5 breaths/min to maintain P_{CO_2} [<35 torr (4.65 kPa); alternatively, the ventilatory rate was decreased by 5 breaths/min if both the pH was <7.3 and P_{CO_2} was ≤30 torr (4.0 kPa)]. The minimum and maximum settings were 15 breaths/min and 35 breaths/min, respectively.

Other Intensive Care Unit Therapies

In addition to human therapies, veterinary critical care specifically for animals was instituted in Part II of the study based on the standard of care for critically ill large animals requiring sustained mechanical ventilation in the clinical setting (9, 10). Animal mouth and endotracheal tube care along with body positioning and intravenous catheter site dressings were attended to at scheduled intervals (8). Humidity in the ventilator tubing was maintained, using a humidifier (Conchatherm III, Hudson RCI-AB, Temecula, CA) attached to the airway system. Throughout the study, a heated water blanket and other heavy blankets were used to maintain a core body temperature between 36.5°C and 37.5°C. Famotidine (1 mg/kg IV q 12 hrs) was given to prevent stress-induced stomach ulcers and unfractionated heparin (3000 IU IM, q 8 hrs) was administered for venous thrombosis prophylaxis.

Sedation Management

In Part II, adequacy of the level of sedation was evaluated and adjusted continuously by a clinician or trained technician constantly present in the animal laboratory for 96 hrs after initiation of administration of midazolam (0.2 mg/kg loading dose, 50 μg/kg/min IV infusion) and fentanyl (5 μg/kg loading dose, 0.7 μg/kg/min IV infusion). Both the fentanyl and midazolam doses were increased in 25% increments every 5 mins until adequate sedation was obtained. Medetomidine infusion (2–5 μg/kg/min) was used to supplement sedation as needed according to set criteria. Criteria for adequacy of sedation were monitored continuously (8).

Laboratory Data

Blood samples used for measurement of serum aldosterone and total and free cortisol concentrations were allowed to clot and were centrifuged, and the serum was separated and frozen in plastic tubes at −20°C until assayed in duplicate. For measurement of serum aldosterone concentration, an assay was performed, using a previously validated (11) radioimmunoassay kit (Coat-A-Count Aldosterone Assay, DPC, Los Angeles, CA). The sensitivity of the assay was 20 pg/mL; all measurements below the standard curve were recorded as 10 pg/mL. Measurement of ADH as arginine vasopressin was determined, using a radioimmunoassay kit (Nichols Institute, Diagnostics San Juan Capistrano, CA) (12, 13). For measurement of total cortisol concentration, assay was performed, using a previously validated (14) radioimmunoassay kit (Coat-A-Count Cortisol Assay, DPC, Los Angeles, CA). The sensitivity of the assay was 14 nmol/L; all measurements below the standard curve were recorded as 7 nmol/L.

For measurement of serum free cortisol concentration, the samples were assayed by a modified ultrafiltration technique. A sample volume of 500 μL serum was placed in a micropartition device (Centrifree Micropartition Device, Millipore Corporation, Billerica, MA). The device was covered to prevent evaporation, warmed to 37°C, and centrifuged in a swinging bucket rotor (37°C, 1200× g, 30 mins). The sample remaining in the top of the device ("top") and the ultrafiltrate were harvested and stored at −80°C until analysis. All samples were analyzed in duplicate as above. The percent free cortisol in each sample was determined by the formula: (Cortisol Ultrafiltrate/Cortisol Top) × 100. The free cortisol concentration in each sample was determined by multiplying the percent free cortisol by the total cortisol concentration measured in the noncentrifuged sample.

To validate the procedure, free cortisol percent and concentration were determined in 53 samples obtained from healthy animals before and after administration of either 1 μg ACTH per animal or 5 μg/kg body weight. A significant ($p < .0001$) linear relationship between total plasma cortisol and percentage free cortisol was found by linear regression. The regression equation (% free cortisol = 6.5 + [0.04 × total cortisol concentration]) was similar to that found by Kemppainen et al when free cortisol was measured in canine plasma by a centrifugal ultrafiltration-dialysis technique (% free cortisol = 8.7 + [0.05 × total cortisol concentration]) (15). In addition, a single basal sample from an animal was divided into six equal aliquots and processed as described. The cortisol concentration was measured in all six ultrafiltrate portions obtained. The coefficient of variation was 3.2% showing repeatability of the technique.

Samples for measurement of plasma eACTH concentration were collected at the time of the pre-ACTH sample. A blood sample was collected in EDTA tubes containing the proteinase inhibitor aprotinin (final concentration 1000 kallikrein inhibitor U/mL blood) (16). Samples were centrifuged immediately; plasma was separated and placed in plastic tubes and stored at −20°C until assayed in duplicate, using a previously validated (17) immunoradiometric kit (ACTH assay, Nichols Institute, San Clemente, CA). The sensitivity of the assay was 4.4 pg/mL. All measurements below the standard curve were recorded as 2.2 pg/mL.

Statistical Methods

Treatment effects found at various contiguous time points (4 hrs, 11 hrs, and 24 hrs; 72 hrs and 96 hrs) were qualitatively similar for all interventions studied and therefore combined for ease of presentation and to enhance our ability to find significant effects. Pituitary-adrenal function parameters were analyzed, using an analysis of variance procedure (18). Serum bound cortisol concentration was calculated as the difference between serum total and free cortisol concentrations. A delta cortisol concentration was calculated by subtracting a pre-ACTH serum total or free cortisol concentration from its respective post-ACTH serum total or free cortisol concentration. To analyze the effect of dexamethasone and ACTH stimulation, the analysis of variance had two main effects, time and dose of dexamethasone (low and high). When two-way interaction indicated results were qualitatively similar, data from the animals receiving the two doses of dexamethasone were pooled to increase the power of the study to find time effects. In addition, single *df* tests were used to compare subsequent time points with the baseline data. To analyze the effect of SIM, a three-way analysis of variance was used; the three factors in the analysis of variance model included time, dose of dexamethasone (low and high), and each intervention analyzed separately (e.g., sedation, steroids, and number of ACTH stimulation tests), as well as all interactions. As in the model above, when interactions associated with the dose of dexamethasone showed qualitatively similar results, data from the animals receiving the two doses of dexamethasone were pooled to increase study power. Correla-

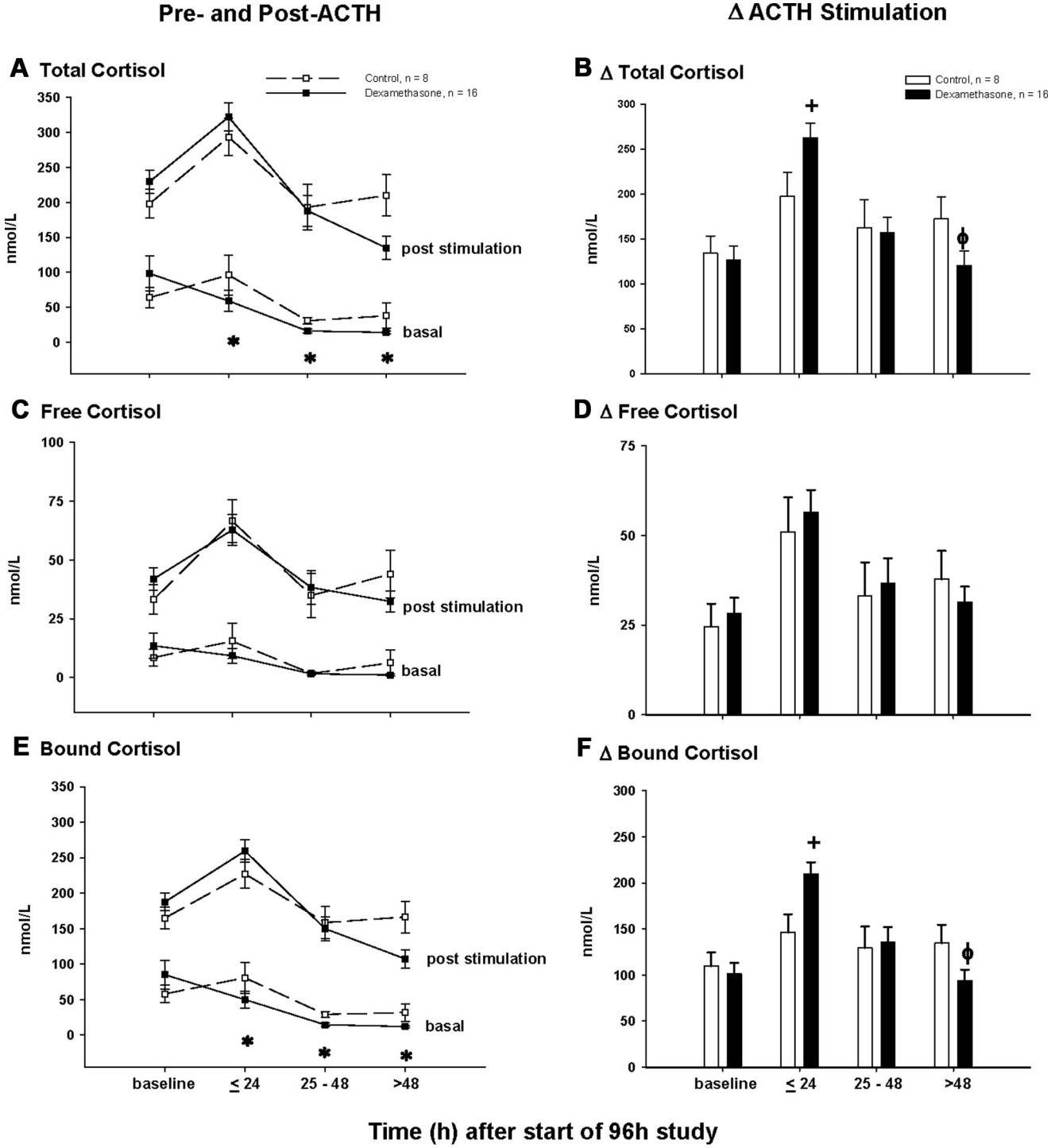


Figure 2. For dexamethasone-treated animals (*closed squares connected by a solid line*) and controls (*open squares connected by a dashed line*), the mean concentrations (nmol/L) ± SEM pre- and postadrenocorticotropic hormone (*ACTH*) stimulation (results of low and high dose ACTH challenge combined) at serial time points are shown for total cortisol (*A*), free cortisol (*C*) and protein-bound cortisol (*E*). For dexamethasone-treated animals (*closed bars*) and controls (*open bars*), the mean ± SEM change from pre- to post-ACTH stimulation (i.e., delta cortisol; results of low- and high-dose cosyntropin challenge combined) is shown for total cortisol (*B*), free cortisol (*D*), and protein bound cortisol (*F*).

tions between pre- and post-ACTH serum cortisol concentration were assessed by using a general linear model, treating the baseline values as a continuous variable, and controlling for animal-to-animal variation within the linear model (19).

RESULTS

Effect of Dexamethasone on Pituitary-Adrenal Function

The effects of low- and high-dose dexamethasone infusion on pituitary-adrenal function were qualitatively similar therefore, data were combined to increase our ability to find significant effects (see Supplemental Table 1, Supplemental Digital Content 1, http://links.lww.com/CCM/A103). Animals assigned to the treatment and control groups had similar basal cortisol (total, free, and bound) (Fig. 2*A*, *C*, and *E*), eACTH (Fig. 3), aldosterone, and ADH concentrations (data not shown). Throughout the dexamethasone infusion, basal cortisol (total and bound) (Fig. 2*A* and *E*) and eACTH concentrations (Fig. 3) significantly declined compared with controls. In contrast, dexamethasone infusion had no significant effect on basal free cortisol (Fig. 2*C*), aldosterone, or ADH concentrations throughout the study (data not shown).

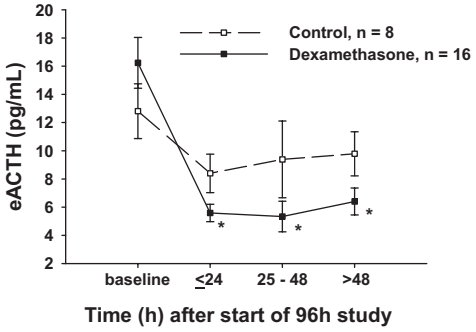
The effect of low- and high-dose ACTH stimulation testing (Fig. 2*A*, *C*, and *E*) on cortisol concentrations (total, free, and bound) were qualitatively similar; therefore, data were combined (Table 1, Supplement). Dexamethasone significantly altered the ACTH-stimulated total and bound delta cortisol concentrations in a time-dependent manner. Delta total and bound cortisol concentrations were augmented by dexamethasone treatment through the first 24 hrs compared with controls (Fig. 2*B* and *F*). After 24 hrs, the effect of dexamethasone on ACTH stimulation testing was reversed, and by the end of the study (>48 hrs), dexamethasone significantly blunted the ACTH response. In contrast, dexamethasone had no significant effect on the delta free cortisol concentration throughout the study (Fig. 2*D*).

Effect of SIM or Increasing the Number of ACTH Stimulation Tests (Seven Versus Four) on Pituitary-Adrenal Function

Both the addition of SIM and the performance of more ACTH stimulation tests (i.e., seven vs. four) raised the basal and ACTH-stimulated serum cortisol concentrations (total and free). Basal and ACTH-stimulated cortisol (total and free) concentrations were significantly increased transiently by SIM in the early stages, i.e., ≤24 hrs, but were similar to controls thereafter (Fig. 4*A*). In comparison, the animals receiving seven vs. four stimulation tests had significantly increased basal and ACTH-stimulated serum cortisol concentrations (total and free) throughout the study after $t = 0$ (Fig. 4*B*). Because both SIM and the performance of seven stimulation tests each increased both basal and ACTH-stimulated cortisol concentrations concomitantly and to similar degrees, the delta cortisol concentrations (total and free) were unchanged by these interventions at all time points studied (data not shown). Thus, more than one stimulation test per 24 hrs (seven vs. four over 96 hrs), on average, elevated basal total and free cortisol >2-fold and likewise elevated post-ACTH concentrations by a similar amount over the course of the 96-hr study.

The addition of SIM and the performance of seven vs. four stimulation tests also induced significant increases in serum aldosterone concentration from baseline (Fig. 5) ($p < .001$ and $p < .05$, respectively). The effect of SIM was again transient, occurring up to 48 hrs after initiation of SIM. However, the significant increase from baseline in serum aldosterone induced by the performance of seven ACTH stimulation tests persisted throughout the study.

The decline in eACTH concentrations over the course of the study seen in both control and dexamethasone-treated animals (Fig. 3) was blunted with SIM ($p = .06$) and the performance of seven vs. four ACTH stimulation tests ($p = .04$) (data not shown). Finally, these two therapies had no effect on serum ADH concentrations throughout the study (data not shown).



*** Decline in dexamethasone group after baseline is greater than control, P ≤ 0.005**

Figure 3. The effect of a 90 h dexamethasone infusion (from $t = 6$ hrs to 96 hrs; results of low and high dose dexamethasone combined) on endogenous adrenocorticotropic hormone (*eACTH*). The mean ± SEM concentrations (pg/mL) at serial time points are shown for dexamethasone-treated animals (*closed squares connected by a solid line*) and control animals (*open squares connected by a dashed line*).

DISCUSSION

We examined the combined effect of SIM on low-dose (1 μg) and high-dose (5 μg/kg) ACTH stimulation tests in 24 healthy canines. In addition, using a factorial design, we investigated if testing frequency or administration of a continuous infusion of low-dose (0.0138 mg/kg/hr) or high-dose (0.275 mg/kg/hr) dexamethasone for 90 hrs altered test results. At baseline, the mean (±SEM) basal total cortisol concentrations were 56 ± 14 nmol/mL (n = 24 animals). Administration of low- and high-dose ACTH resulted in doubling (111 ± 17) and quadrupling (242 ± 27), respectively, of the total cortisol concentrations after 60 mins. We ultimately combined the data obtained from the low- and high-dose ACTH stimulation tests because the results were qualitatively similar, regardless of the variable tested (i.e., SIM, number of stimulations or dexamethasone therapy).

Over the first 24 hrs, SIM equally increased both the basal and post-ACTH total and free cortisol concentrations >2-fold such that there was no alteration in the calculated delta total cortisol concentration. After 24 hrs and until the end of the experiment at 96 hrs, SIM had no effect on ACTH stimulation testing.

Increasing the frequency of ACTH stimulation testing to >1 per 24 hrs (i.e., 2 per day) also increased both the basal and post-ACTH total and free cortisol concentrations >1.5-fold and had no effect on the incremental cortisol response to ACTH. As op-

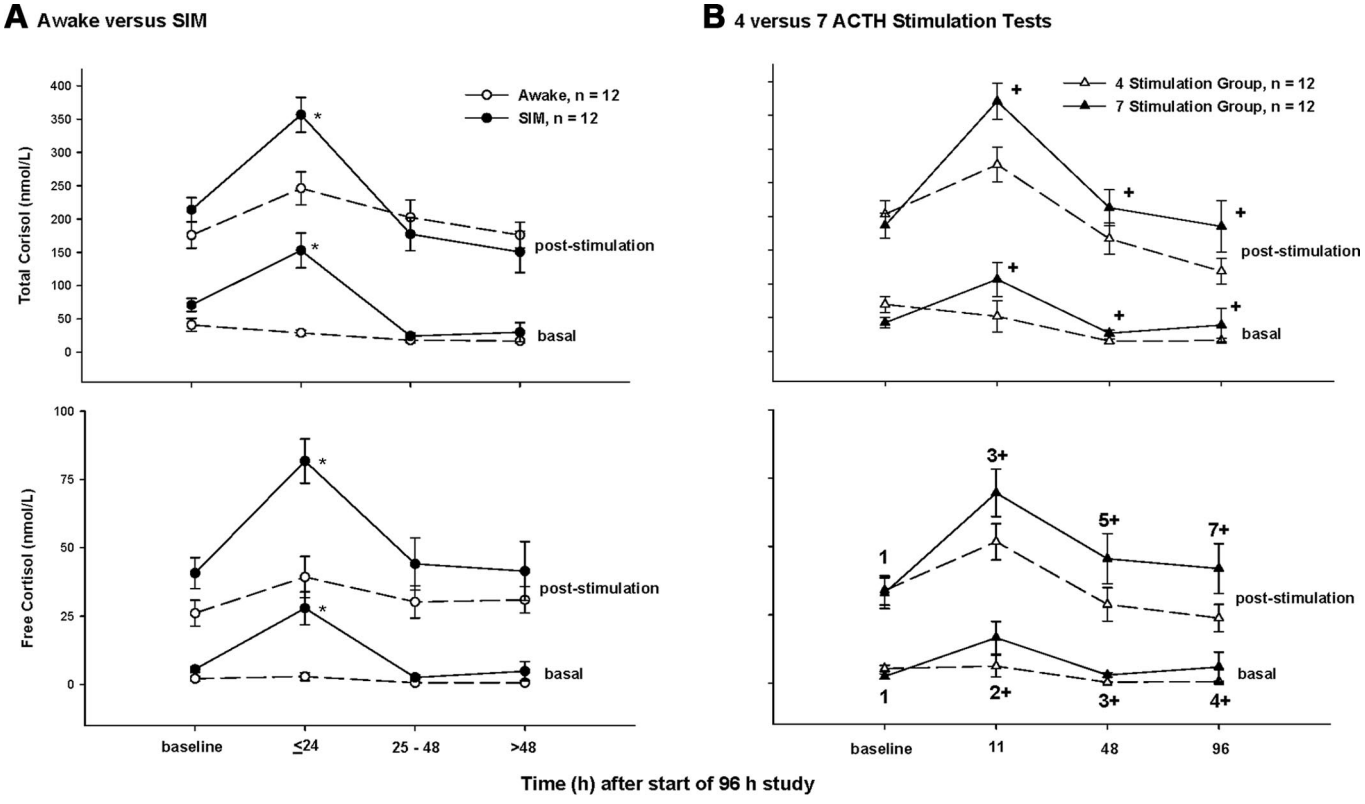
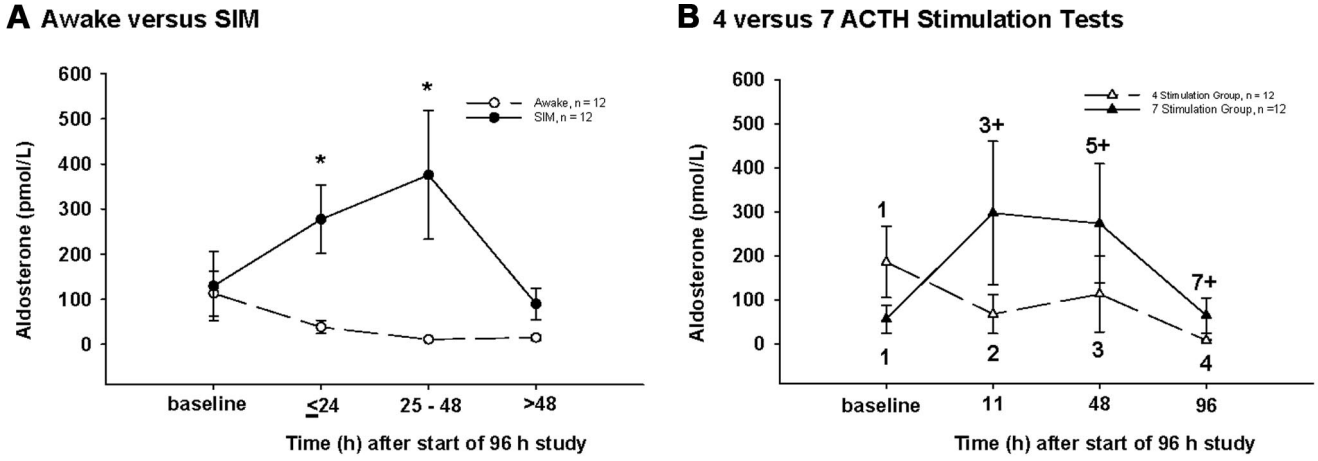


Figure 4. *A*, The mean concentrations (nmol/L) ± SEM of pre- and postadrenocorticotropic hormone (*ACTH*) total and free cortisol for sedated, intubated, and mechanically ventilated (*SIM*) animals (*closed circles connected by a solid line*) and awake animals (*open circles connected by a dashed line*). *B*, Mean concentrations ± SEM of pre- and post-ACTH total and free cortisol for animals receiving a total of seven (*closed triangles connected by a solid line*) and four (*open triangles connected by dashed lines*) stimulation tests.



*** Increase from baseline is greater in sedated, intubated, and mechanically ventilated animals, P ≤ 0.001**
+ Increase from baseline is greater with more stimulations, P ≤ 0.05
 # Number of stimulation tests completed at time point for each group. The 7 stimulation group had additional tests performed at 4,24, and 72 h.

Figure 5. *A*, Mean basal aldosterone concentration (pmol/L ± SEM) in sedated, intubated, and mechanically ventilated (*SIM*) animals (*closed circles connected by a solid line*) and awake animals (*open circles connected by a dashed line*). *B*, Mean basal aldosterone concentration (pmol/L ± SEM) in animals undergoing seven (*closed triangles connected by a solid line*) vs. four (*open triangles connected by a dashed line*) adrenocorticotropic hormone (*ACTH*) stimulation tests.

posed to SIM, this effect was sustained throughout the 96-hr experiment.

Low- and high-dose dexamethasone therapy altered pituitary-adrenal function similarly, so we combined the data to increase our ability to find a significant effect. Beginning before 24 hrs and continuing until the end of the experiment at 96 hrs, dexamethasone therapy persistently lowered basal total cortisol concentrations approximately seven-fold compared with controls. In contrast to SIM and performance

of more ACTH stimulation tests, dexamethasone therapy affected the delta total cortisol concentration; from 4 hrs to 24 hrs, the delta total cortisol concentration increased approximately 33%. Over the remainder of the study, dexamethasone therapy caused a greater decline in the delta total cortisol as compared with control.

We also measured free cortisol levels under the same experimental conditions. The effect of SIM and increasing frequency of ACTH stimulation tests on free cortisol concentrations (basal, post-ACTH and delta) were identical to that for total cortisol. However, in contrast to total cortisol, free cortisol was unaffected by dexamethasone treatment throughout the 96 hrs (basal, post stimulation, and delta free cortisol) (Figs. 2 and 4).

There were aspects of this study that could conceivably limit the clinical applicability of our results. This was a relatively small study (n = 24) performed in healthy, not sick, animals. Nonetheless, we used a rigorous design that tested multiple factors in each animal to increase power and control for animal-to-animal variability. Our objective was to determine a normal range for hypothalamic-pituitary-adrenal (HPA) testing in healthy animals so that we could identify the effect of intensive care therapies on testing without the confounder of critical illness. In addition, some of the interventions may have been different from standard clinical practice. For example, dexamethasone was administered as an infusion, whereas it is commonly given clinically as repeated boluses. Similarly, performance of seven ACTH stimulation tests within 96 hrs would be unusual; however, one could imagine performing multiple stimulation tests during a patient's ICU course, including two within 24 hrs, for which the results presented are applicable.

A recent consensus statement from The American College of Critical Care Medicine contains recommendations regarding how to best diagnosis RAI (i.e., delta cortisol <9 μg/dL or a random total cortisol concentration <10 μg/dL), yet concludes that ACTH stimulation testing should not be used to decide which patients with septic shock or acute respiratory distress syndrome should be treated with glucocorticoids (20). There are two possible explanations as to why no HPA test has prospectively been proven to identify critically ill patients with RAI who would benefit from glucocorticoid therapy: either RAI is not a true entity or the current method of diagnosing RAI is imperfect. In this study, we show that, in otherwise healthy animals, intensive care substantially impacts many of the current HPA testing modalities. Our results suggest that delta free cortisol measurement—by virtue of the fact that it was not affected by common critical care therapies, dexamethasone treatment, or multiple ACTH stimulation tests—may represent the single best test for the evaluation of RAI in subjects in an ICU.

Dexamethasone, whether administered as a high- or low-dose continuous infusion, immediately and persistently lowered basal total serum cortisol and eACTH concentrations. This was consistent with basic science supporting the use of the dexamethasone suppression test used to diagnosis Cushing's disease, whereby dexamethasone suppresses the activity of an ACTH-secreting pituitary tumor and subsequently lowers the serum cortisol level (21, 22). The transient, early increase in delta total cortisol observed in animals treated with dexamethasone has been reported in prior canine studies (23, 24). The mechanism for this effect is not known, although in a study of guinea pigs dexamethasone treatment resulted in increased binding of ACTH by the adrenal glands (25). In contrast, toward the end of the study, dexamethasone infusion caused a blunted total cortisol response to ACTH stimulation, likely a result of glucocorticoid feedback (26) on eACTH secretion and resultant adrenocortical atrophy. The exact timing of the change from hyper- to hyporesponsiveness probably depends on the glucocorticoid dose or possibly route of administration. Experiments using single, small injections of dexamethasone (0.01 or 0.1 mg/kg) have shown no effect on ACTH-stimulated total cortisol concentrations when testing was performed 4 hrs to 48 hrs post dexamethasone treatment (27, 28). However, when an ACTH stimulation test was conducted after administration of a higher dexamethasone dose (1.0 mg/kg or 5.0 mg/kg), the post-ACTH total cortisol concentration was decreased beginning at 72 hrs and 24 hrs, respectively (28).

The fact that dexamethasone significantly altered total cortisol measurements but did not affect free cortisol is a clinically relevant finding and may simply be a function of the power of the study—i.e., if we had tested more animals, then a significant difference may have been detected. Alternatively, variation in serum cortisol-binding globulin (CBG) concentration or the affinity of CBG for cortisol could account for the lack of concominant changes in free cortisol. Prior *in vitro* investigations have shown that dexamethasone has varying effects on CBG synthesis and secretion depending on species of the cell studied (29–31). We did not measure CBG concentrations or affinity for cortisol to determine whether such changes played a role in the current study. Similarly, we did not analyze plasma albumin levels, which can also impact the amount of measured free cortisol and are, *in vitro*, increased in the setting of dexamethasone (2, 32). Regardless, this experiment highlights the difficulty in interpreting total serum cortisol levels when dexamethasone is administered and brings into question the clinical utility of this test in patients receiving dexamethasone, despite earlier guidelines suggesting that dexamethasone could be administered in septic patients before performing an ACTH stimulation test (33). In contrast, measurements of free cortisol (basal, post-ACTH, and delta) were unaffected by dexamethasone therapy.

The combination of intubation, sedation, and mechanical ventilation caused a transient but significant increase in both basal and post-ACTH total and free cortisol concentrations. A prior study in human surgical patients also documented an elevation of the basal cortisol concentration, beginning 30 mins after intubation (34). The normalization of basal and post-ACTH cortisol concentrations over the remainder of the study, despite continued mechanical ventilation, is potentially due to more pronounced sedation over time, although the relative contribution of the three sedatives used is difficult to determine. For example, it is possible that intensive veterinary care induced stress that activated the HPA axis, but the stress was effectively managed once midazolam and fentanyl levels were optimized. On the other hand, the administration of sedatives (35, 36) have been shown to suppress the HPA axis, especially medetomidine (37), and its infusion beginning at 24 hrs may have lowered cortisol concentrations in this current study. Neither sedation nor any other aspect of intensive care therapies, including intubation and mechanical ventilation, had an effect on delta total or free cortisol in these otherwise healthy animals. It is also interesting to note that a concomitant increase in endogenous ACTH was not observed at the time that SIM caused a transient increase in cortisol. This would suggest that, instead of

ACTH, either cytokines or more likely sympathetic innervation of the adrenal gland is the mediator responsible for the transient increase in cortisol associated with SIM (38–40).

The performance of seven ACTH stimulation tests vs. four over 96 hrs significantly up-regulated the HPA axis as demonstrated by the elevation in basal and ACTH-stimulated total and free cortisol concentrations throughout the study. When one compares the animals at different time points when both groups of animals have received the same number of tests, the animals in the four stimulation group have lower cortisol levels. Thus, the difference in basal and ACTH-stimulated total and free cortisol between the two groups is most likely a function of the fact that the seven stimulation animals received more frequent testing rather than a greater total of tests. The increase seen with more frequent testing may be due to the trophic effects of ACTH on the cells of the adrenocortical zona fasiculata (41). Alternatively, more frequent ACTH stimulation testing may simulate chronic or repeated stress, and the increased pituitary-adrenal tone may be a function of corticotrophic facilitation (42, 43). Thus, our study suggests that either repeated administration of exogenous ACTH within 24 hrs or repeated stresses may augment HPA axis activity. As was the case with combined intensive care therapies, the delta total and free cortisol measurement were not statistically different between dogs receiving seven vs. four stimulation tests.

The effects of SIM and the performance of seven stimulation tests on aldosterone concentrations mirrored those seen with cortisol. SIM caused early and transient increases in aldosterone concentration, whereas completion of seven stimulation tests resulted in a persistent elevation in aldosterone concentration. PEEP has been shown in human studies (44, 45) to increase aldosterone concentrations, likely secondary to hemodynamic changes occurring both systemically and at the level of the kidneys. Aldosterone concentration may have normalized after 48 hrs in animals receiving mechanical ventilation due to continued fluid resuscitation. The increase in aldosterone with additional stimulation tests was also expected as ACTH stimulates aldosterone release (46). However, ACTH plays a minor role in control of aldosterone secretion overall, and the lack of an effect of dexamethasone therapy on aldosterone concentrations observed in this study may be due to lack of change in the major stimulants of aldosterone secretion. The feedback effects of glucocorticoids on aldosterone secretion have not been widely studied in dogs, but our results are contrary to a prior study in canines albeit under different conditions (47, 48).

Although the reference range of results for HPA testing in healthy adults in the outpatient setting has been previously defined (49, 50), what constitutes a normal HPA test result in a subject receiving intensive medical or veterinary care is less well established. The three variables that we prospectively evaluated—dexamethasone treatment, SIM, and frequency of ACTH stimulation testing—all impacted the results of various HPA tests in otherwise healthy animals. Thus, interpretation of traditional ACTH stimulation testing of HPA function becomes extremely complicated when these tests are performed in patients receiving critical care therapies. For example, our data would suggest that performing an ACTH stimulation test in an ICU patient who was intubated in the past 24 hrs would yield a poststimulation total cortisol concentration that would be significantly higher than if the test had been performed before intubation or 48 hrs after intubation. It has been argued that the measurement of serum free cortisol concentration provides better assessment of adrenal gland function in critically ill patients, which may be especially true for patients with septic shock or multiple trauma where CBG levels are extremely low (2, 4, 51). The results of this study would further suggest that measurement of the delta free cortisol—the only HPA measurement unaffected by the variables tested—may represent the single best test for the evaluation of the HPA axis in subjects receiving intensive care therapies. Regardless, it is essential that a normal HPA test range be determined before establishing laboratory criteria defining relative adrenal insufficiency in subjects receiving critical care treatment.

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