Title

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Journal

Journal of Medical Primatology, 32(3)

ISSN

0047-2565

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Publication Date

2003

Peer reviewed

J Med Primatol 2003: 32: 148–160

JOURNAL OF MEDICAL PRIMATOLOGY
ISSN 0047-2565

Cortisol responses to immobilization with Telazol or ketamine in baboons (*Papio cynocephalus/anubis*) and rhesus macaques (*Macaca mulatta*)

Bentson KL, Capitanio JP, Mendoza SP. Cortisol responses to immobilization with Telazol or ketamine in baboons (*Papio cynocephalus/anubis*) and rhesus macaques (*Macaca mulatta*). J Med Primatol 2003; 32:148–160. © Blackwell Munksgaard, 2003

Abstract: Little is known about the influence of Telazol on cortisol or of anesthetic agents on immunological measures, and reports of ketamine's effect on cortisol are inconsistent. We measured effects of Telazol, ketamine and blood sampling on cortisol in male rhesus macaques and male savannah baboons. We also obtained leukocyte counts in the macaques. In macaques, Telazol reduced cortisol in the morning but not in the afternoon; ketamine had no effect on cortisol in these animals. In baboons, cortisol changed little post-Telazol but increased post-ketamine. In macaques, lymphocyte numbers decreased following afternoon injection of Telazol, ketamine or saline. The injection and blood sampling process increased cortisol levels in monkeys not trained to extend an arm but exerted no effect on cortisol in trained macaques. Thus, the animals' physiological responses to blood sampling and immobilization are influenced by such variables as anesthetic agent, species, time of day, and familiarity with the blood sampling process.

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Key words: psychoneuroimmunology – lymphocytes – leukocytes – circadian – biorhythm – benzodiazepines – novelty – training – benzodiazepine receptors – blood sampling – venipuncture

Accepted February 7, 2003.

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Funding: Research was supported by Grants RR00166, HL50351, RR00169, and MH 49033 from the National Institutes of Health.

Introduction

Researchers and health care professionals have many incentives to be able to measure plasma cortisol reliably. Cortisol, the principal glucocorticoid in primates, can be an important indicator of pathophysiological processes, and the cortisol response to an event is often considered a useful indicator of stress. Health may be impaired by repeated transient increases in cortisol and by exceptionally high or low basal levels [19, 43]. Basal levels of cortisol in non-human primates have been related to such psychosocial factors as rank in a dominance hierarchy [42], personality [11], and group composition [14]. Cortisol affects levels of many physiological substances, including those involved in metabolism, reproduction, and immunity. If the process of obtaining blood to measure such substances alters cortisol levels, which in turn affect the substance being measured, results that are obtained may provide misleading clinical or research data.

Because psychological stress influences glucocorticoid concentrations, the blood collection process may alter cortisol levels if the process of obtaining blood is stressful. Cortisol has been observed to increase when blood is obtained via venipuncture from animals unfamiliar with the blood collection process [37]. Removal of animals from their home environment, a procedure that commonly accompanies clinical treatment and data collection, may also increase glucocorticoid levels [36]. Cortisol concentrations might also be influenced by immobilization, a procedure that is commonly used to obtain blood samples in non-human primates. Because glucocorticoid concentrations in blood may be affected by a combination of psychological and pharmacological factors, it is important to

consider the combined influence of both components.

Two dissociative anesthetics are widely used in research and clinical settings: ketamine hydrochloride, which was introduced in 1957 [28], and Telazol (tiletamine hydrochloride and zolazepam), which was developed in the late 1960s [26] and has been administered to more than 50 species of non-human primates [44]. Telazol is a one-to-one combination of tiletamine hydrochloride, the anesthetic agent, and zolazepam, a benzodiazepine that serves as a muscle relaxant and anticonvulsant. Telazol is more potent than ketamine, and its duration of action is longer [26]. The small volume of Telazol required to immobilize animals contributes to its popularity for use in the wild, where volume-limited projectile devices typically are used to deliver the anesthetic agent [44].

No studies have investigated the effect of Telazol on plasma cortisol in non-human primates. We predicted it would inhibit the cortisol response to blood collection and immobilization because of its benzodiazepine component; numerous studies have demonstrated an inhibitory effect of benzodiazepines on the activity of the hypothalamo–pituitary–adrenal axis [e.g., 9, 21, 34, 39]. In studies involving rhesus macaques, ACTH and/or cortisol secretion are reduced by the related benzodiazepine alprazolam [22, 49].

Although the cortisol response to ketamine has not been reported for male savannah baboons (*Papio cynocephalus/anubis*), ketamine does not significantly influence cortisol levels in female savannah baboons [2, 50] or male hamadryas baboons (*Papio hamadryas*) [24]. The effect of ketamine on cortisol in male rhesus macaques (*Macaca mulatta*) is not clear. In a study with a sample size of three monkeys for each condition, already elevated levels of cortisol increased further after multiple injections of ketamine but not of distilled water or after a single ketamine injection [35]. In an experiment involving four male rhesus macaques, cortisol was unchanged for 2 hours, rose in the next 60 minutes, and was lower at all time points than levels in blood collected from conscious monkeys [52]. Cortisol levels rose in four female rhesus macaques following ketamine or saline administration; the increase in response to ketamine was not different than the increase post-saline [17].

To investigate effects of blood sampling and immobilization with Telazol or ketamine on plasma cortisol concentrations in male baboons and rhesus macaques, we conducted the following four experiments.

Experiment 1 (baboons, a.m.)

Rationale/objectives

Because ketamine does not affect cortisol in female savannah baboons or male hamadryas baboons, we predicted that male savannah baboons would show no cortisol response to administration of ketamine. The psychological aspects of immobilization and sample collection would not be expected to differ between the ketamine and Telazol conditions, so any significant difference in cortisol levels after Telazol vs. ketamine administration would be assumed to result from the action of Telazol. Because of the benzodiazepine component of Telazol, we predicted that the change in cortisol would be lower post-Telazol than post-ketamine. We did not measure the cortisol response to saline administration in the male baboons because they were group-housed and not trained to allow blood to be withdrawn while they were conscious.

Methods

Subjects and living arrangements

The subjects were six male savannah baboons (*Papio cynocephalus/anubis*), 5–10 years old and weighing 20–37 kg. They were born in Africa or in outdoor breeding facilities in the USA. They were housed in social groups of two males and one or two females at the Washington Regional Primate Research Center. Food and water were available *ad libitum*; biscuits (Purina monkey chow) were provided at noon. The baboons were trained to enter a holding area (series of cages) that was adjacent to the compound. End cages in the holding area contained squeeze mechanisms that were used when anesthetic agents were administered. Each subject was familiar with the process of immobilization in the holding area.

Experimental design and procedure

Samples were collected between 8:15 an 10:00 a.m. from two baboons per day. After the baboons entered the holding area, the subjects were separated from the other members of the group, moved into the end cages, restrained by use of the squeeze mechanism, and injected with Telazol (4 mg/kg, Fort Dodge, IA, USA) or ketamine hydrochloride (10 mg/kg, Ketaset, Fort Dodge, IA, USA). Since a single dose of ketamine did not provide a sufficient length of immobilization, supplemental doses (3 mg/kg) were provided when baboons began to initiate sustained unpatterned movements; two baboons received one, and two baboons received

two supplements. No Telazol supplements were needed. Each baboon received Telazol and ketamine in random order on separate days that were at least 2 weeks apart. Blood samples were collected after the baboons were removed from the holding area and placed on carts. The initial sample was collected as soon as baboons were sufficiently immobilized (4–9 minutes, mean 6.4 minutes post-injection). Additional samples were collected 15, 30, 45 and 60 minutes post-injection. The 3-ml samples were drawn from a femoral vein, transferred to heparin-containing evacuation tubes, and stored on ice until they were processed. Plasma was stored at −80°C. Cortisol was assayed with the use of ^{125}I radioimmunoassay kits with antibody-coated tubes (Incstar, Stillwater, MN, USA). The inter-assay coefficient of variation (CV) was 8.3%; the intra-assay CV was 2.5%.

Data analysis

A repeated-measures ANOVA was performed with independent variables agent (Telazol and ketamine) and time (15, 30, 45 and 60 minutes after injection) and the dependent variable 'change in cortisol.' Changes were calculated by subtracting, for each blood draw sequence, the initial cortisol value from values obtained at subsequent time points. Since the initial value of cortisol may influence the magnitude of its change and two baboons had initial values more than one standard deviation above the mean, data from those baboons were excluded from statistical analysis. The Kolmogorov–Smirnov test indicated that the assumption of normality was not violated. The level for statistical significance was set at $P \leq 0.05$. Bonferroni corrections were made when *post-hoc* analysis required performance of more than one *t*-test on the same set of data.

Results

Initial cortisol levels of 23.2 ±0.8 μg/dl were similar in the Telazol and ketamine conditions (Table 1). Values did not change under either condition for the first 15 minutes. Cortisol increased between 15 and 60 minutes after ketamine was administered (Fig. 1A). Cortisol was unchanged for 30 minutes post-Telazol; at 60 minutes, it was a highly variable 7.0 ± 7.0 μg/dl higher than the initial level. There were significant main effects of agent [$F_{(1,3)} = 13.32$, $P < 0.05$] and time [$F_{(3,9)} = 4.89$, $P < 0.05$] on changes in cortisol. The interaction between agent and time was not significant.

To determine whether the unexpected rise in cortisol following ketamine administration was reliable, we conducted a separate series of *t*-tests for pair measures. Results indicated that cortisol was significantly higher than the initial level 60 minutes after ketamine administration (see Table 1; $t_3 = 5.56$, $P \leq 0.011$). Cortisol post-Telazol did not differ from the initial level at any time point. See Table 3 for a summary of significant results from all of the experiments.

Discussion

The prediction that changes in cortisol in the male baboons would be lower post-Telazol than post-ketamine was confirmed, supporting the hypothesis that the zolazepam in Telazol interacts with benzodiazepine receptors to inhibit hypothalamo–pituitary–adrenal activity and depress cortisol levels. Contrary to our prediction, however, cortisol rose significantly after administration of ketamine.

A number of factors may account for the unexpected cortisol increase following ketamine administration. First, ketamine may exert a pharmacologic effect on cortisol in male savannah baboons. Ketamine is a phencyclidine derivative

Table 1. Baseline cortisol levels and significant ($P \leq 0.0125$) changes from baseline (μg/dl ± S.E.)

Experiment/group	Agent	Baseline	15 Minutes	30 Minutes	60 Minutes	120 Minutes
Experiment 1: untrained baboons, a.m. (n = 4)	Ketamine	22.5 ± 3.5			39.4 ± 5.8 (↑)	
	Telazol	24.0 ± 2.7				
Experiment 2: trained rhesus macaques, p.m. (n = 17)	Saline	20.5 ± 1.9		17.1 ± 1.4 (↓)	15.2 ± 1.2 (↓)	14.0 ± 1.3 (↓)
	Ketamine	18.8 ± 1.3				
	Telazol	19.7 ± 1.7	17.7 ± 1.4 (↓)			
Experiment 3: untrained rhesus macaques, p.m. (n = 4)	Saline	23.9 ± 4.1				
	Ketamine	20.2 ± 0.8				
	Telazol	22.8 ± 1.2				
Experiment 4: trained rhesus macaques, a.m. (n = 16)	Saline	26.1 ± 1.6				
	Ketamine	26.9 ± 1.5				
	Telazol	27.9 ± 1.7	23.8 ± 1.6 (↓)	22.4 ± 1.7 (↓)	21.2 ± 2.0 (↓)	

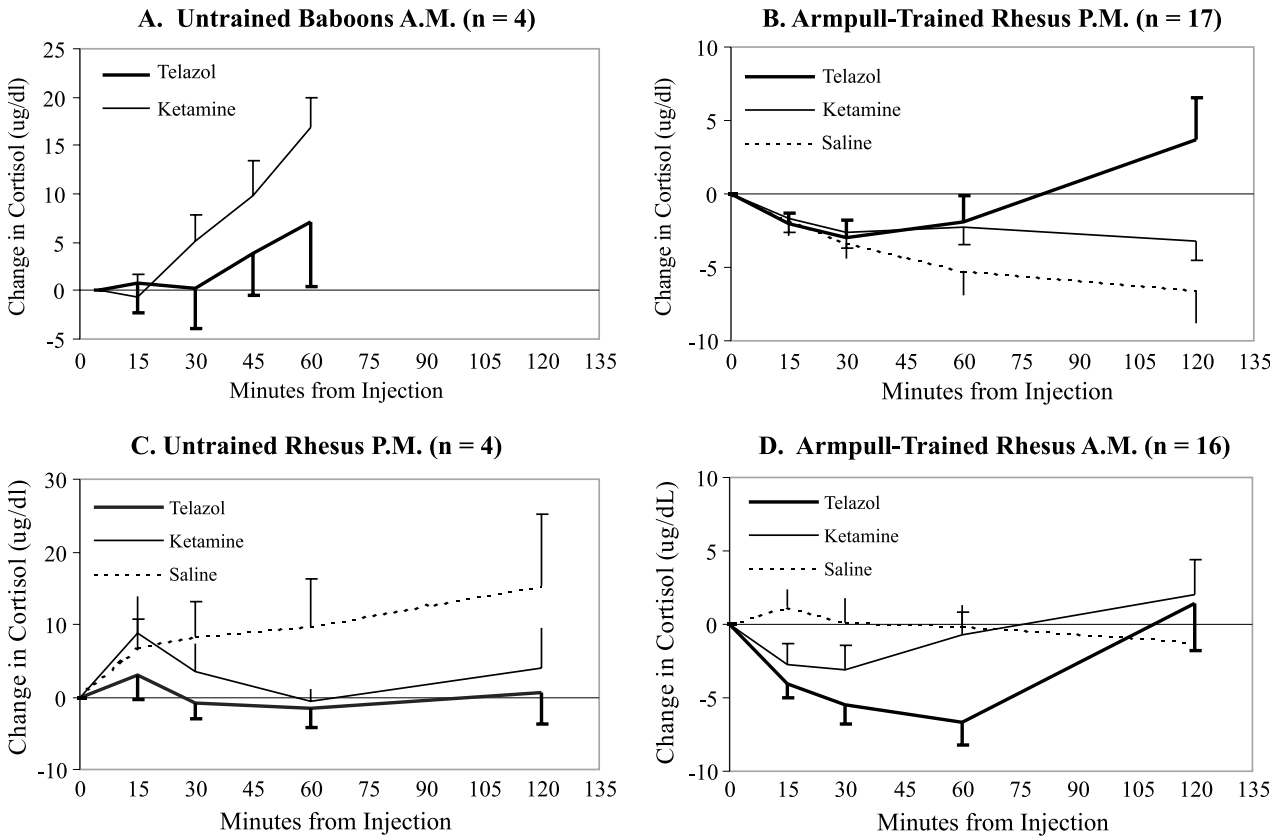

Fig. 1. Changes (mean ± S.E.) in cortisol following administration of Telazol, ketamine, or saline. (A) Effect of agent is significant. (B) Interaction between agent and time is significant. Change in cortisol post-Telazol is significantly higher than post-saline at 120 minutes. (D) Effect of agent and interaction between agent and time are significant. Change in cortisol post-Telazol is significantly lower than post-saline at 15, 30, and 60 minutes.

Table 2. Afternoon changes in leukocyte cell counts (cells per μl of blood)

Cell types	1:00 mean (S.E.)	3:00 mean (S.E.)	Change from 1:00 to 3:00 p.m.[1]	Afternoon circadian pattern in humans[2]	Change from 1:00 to 3:00 in chaired macaques[2]
Total lymphocytes	3389 (203)	2812 (192)	↓ ($P \leq 0.001$) s	↑ or no change	↓
$CD8^+$ lymphocytes	1164 (107)	894 (80)	↓ ($P \leq 0.002$) s	Little or no change	↓
$CD4^+$ lymphocytes	1191 (103)	992 (89)	↓ ($P \leq 0.001$) s	↑	↓
$CD4^+$:$CD8^+$ ratio	1.13 (0.11)	1.22 (0.11)	↑ ($P \leq 0.001$) s	Mixed reports	↑
Neutrophils	4528 (252)	5203 (322)	↑ ($P \leq 0.071$) ns	Little or no change	↑

[1] Repeated-measures ANOVAs used to assess effect of time on cell counts; s = significant, ns = non-significant.
[2] See experiment 2 discussion for references.

[46], and male savannah baboons immobilized with phencyclidine exhibit a sustained rise in cortisol [42]. It unclear, however, why such a pharmacological effect of ketamine is not seen in female savannah baboons [2, 50]. Another possibility is that the dose of ketamine administered in this experiment (10 mg/kg with 3-mg/kg supplements) is too low to maintain a sufficiently deep plane of anesthesia and prevent disorientation accompanying emergence from anesthesia. Such disorientation is associated with increases in cortisol [42, 48]. Studies with cats have suggested that lower dosages may be associated with large and sustained increases in cortisol, whereas higher dosages result in lower and more constant concentrations of cortisol [12]. A third possibility is that multiple injections of ketamine may, through a physiological stress of prolonged immobilization, increase cortisol levels. Cortisol increased in three male rhesus macaques that received multiple intramuscular injections of

Table 3. Summary of significant changes in cortisol following injection of Telazol, ketamine, or saline

Experiment	Subjects	Time of day	Sample size	Dependent variable	Agents	Test	Significant effects ($P \leq 0.05$ for repeated measures ANOVAs; *t*-tests Bonforroni-corrected)
1	Baboons	a.m.	4	Change in cortisol	Telazol Ketamine	Repeated measures ANOVA	Main effects of agent and time
				Cortisol	Ketamine	*t*-Test	Cortisol was higher than the initial level at 60 min post-ketamine
2	Armpull-trained macaques	p.m.	17	Change in cortisol	Telazol Ketamine Saline	Repeated measures ANOVA	Interaction between agent and time
				Change in cortisol	Telazol vs. Saline	*t*-Test	Cortisol had decreased more post-saline than post-Telazol at 120 minutes after injection
				Cortisol	Saline	*t*-Test	Cortisol was lower than the initial level at 30, 60, and 120 minutes post-saline
				Cortisol	Telazol	*t*-Test	Cortisol was lower than the initial level at 15 minutes post-Telazol
4	Armpull-trained macaques	a.m.	16	Change in cortisol	Telazol Ketamine Saline	Repeated measures ANOVA	Main effect of agent, interaction between agent and time
				Change in cortisol	Telazol vs. Saline	*t*-Test	Cortisol had decreased more post-Telazol than post-saline at 15, 30, and 60 minutes after injection
				Cortisol	Telazol	*t*-Test	Cortisol was lower than the initial level at 15, 30, and 60 minutes post-Telazol

ketamine but not in three monkeys that received a single injection [35]. However, female baboons that did not exhibit a post-ketamine increase in cortisol received continuous intravenous infusions of ketamine for 90–120 minutes after an initial intramuscular injection [2, 50]; the prolonged immobilization and the greater amount of ketamine *per se* did not increase cortisol levels in the those animals.

Walker et al., on the basis of their work with baboons, stated that '…ketamine prevents the stress-induced rise in [cortisol] typically associated with handling and restraint' [50]. In their experiments [2, 50], the baboons were singly housed and immobilized in their home cages; here, the males were separated from their living quarters and group prior to ketamine injection. Ketamine may not prevent cortisol increases resulting from the nature or intensity of the pre-immobilization separation process.

Experiment 2 (armpull-trained rhesus macaques, p.m.)

Rationale/objectives

Experiment 2 had three objectives. The first was to continue to explore the hypothesis that Telazol inhibits the cortisol response to immobilization/blood sampling. We extended our investigation to a second species of non-human primate, used a larger sample, and added a saline condition so that we could compare the effects of anesthetic agents with effects of a substance that does not pharmacologically affect cortisol. We sought to minimize the psychological component in the saline condition by using singly housed rhesus macaques that could be sampled in their home environment, were trained to extend an arm for sample collection (armpull-trained), and had extensive familiarity with the sampling process. The second objective was to measure the effect of ketamine on cortisol. As no consensus has emerged regarding effects of ketamine on cortisol in rhesus macaques (see Introduction), we made no prediction about the cortisol response to ketamine. Third, we examined the effects of anesthetic agents and blood sampling on a variety of leukocyte cell counts, which are often measured in blood obtained from immobilized animals for diagnostic purposes.

Methods

Subjects and living arrangements

Subjects were 17 male rhesus macaques ranging in age from 10.2 to 13.3 years at the start of the study. All were born in half-acre corrals at the California Regional Primate Research Center and had lived in indoor individual cages for 5 years before this

experiment began. Each subject had been trained to extend an arm and allow blood to be withdrawn, and each had experienced sample collection via armpull approximately monthly for the previous 5 years. Subjects lived in squeezeback-equipped standard laboratory cages located in two rows on one side of a room. Water was available *ad libitum* and monkey chow (Purina) was provided in the morning and afternoon. Samples were collected before the afternoon feeding.

Experimental design and procedure

Data were collected according to a within-subjects repeated-measures design; monkeys received each agent in a counterbalanced order with an inter-test interval of at least 1 week. The agents were ketamine hydrochloride (10 mg/kg; Vetamine, Shering-Plough, NJ, USA), Telazol (5 mg/kg; Fort Dodge, IA, USA), and 0.9% bacteriostatic saline (0.8 ml; Abbott Laboratories, Chicago, IL, USA). Since we could collect blood samples from conscious armpull-trained rhesus macaques in their home cages and we wished to examine the cortisol response to emergence from anesthesia in the absence of novelty, no supplements were administered. One subject was still awake after receiving 10 mg/kg of ketamine and was retested later with a dose of 13 mg/kg. All samples were collected between July and September. Caretaking activities were completed by 12:30 p.m., and data collection began at 1:00 p.m.

Typically, six subjects were tested per day (two monkeys per agent). All blood samples were timed from the point when the door to the animal quarters was opened. Additional times were obtained when the technician stepped in front of an animal's cage and when the needle was withdrawn from the antecubital vein. An initial 3.5-ml blood sample (2 ml for the cortisol assay and 1.5 ml for immune system measures) was collected from each monkey. The order of sample collection was from the front (i.e. nearest the entry door) to the rear of the room; if one subject was housed above another, the subject in the lower cage was sampled first. All initial blood samples were collected within 3 minutes after a technician arrived at a subject's cage, and 46 of 51 samples were obtained within 6 minutes after the technician entered the animals' room; the other five were obtained within 7.5 minutes. After the last baseline sample was collected, the monkey that was sampled first received its injection, with other subjects injected at approximately 2.5-min intervals. At 15, 30, and 60 minutes post-injection, 2-ml blood samples were collected for measurement of cortisol; at 120 minutes post-injection, a 3.5 ml sample was collected for measurement of cortisol and cell counts. All samples were drawn via non-heparanized syringes and transferred to evacuation tubes containing EDTA. Samples for immune system assays were kept at room temperature. The samples for cortisol analysis were kept on ice until they were centrifuged, and plasma was subsequently stored at −80°C. Cortisol was measured with the use of radioimmunoassay kits from Diagnostic Products Corporation (Los Angeles, CA, USA). The inter-assay CV was 5.8%; the intra-assay CV was 7.9%.

We counted the numbers of white blood cells (WBC), lymphocytes, $CD4^+$ and $CD8^+$ lymphocytes, and segmented neutrophils before and 120 minutes after injection of the agents. A Serono Baker Diagnostic System (Allentown, PA, USA) was used to count WBCs, segmented neutrophils and total lymphocytes. A manual differential was used to verify electronic counts. 50-μl aliquots of whole blood were directly labeled with fluorescein-conjugated anti-human $CD8^+$ (Leu-2a; Becton Dickinson, Mountain View, CA, USA) to detect $CD8^+$ lymphocytes. Phycoerythrin-conjugated anti-human $CD4^+$ (OKT4; Ortho Diagnostic Systems, Raritan, NJ, USA) was used as a label to detect $CD4^+$ lymphocytes [see Reimann et al., 1994]. A Coulter Q-prep (Coulter Corp., Miami, FL, USA) was used to fix lysed red blood cells in paraformaldehyde. Lymphocytes were gated by side and forward light scatter. A FACSCAN flow cytometer (Becton Dickinson) was used to count the lymphocyte subsets.

Data analysis

The Kolmogorov–Smirnov test indicated that the assumption of normality was not violated for any dependent variables. A repeated-measures ANOVA with independent variables agent (Telazol, ketamine and saline) and time (15, 30, 60, and 120 minutes after injection) was performed using the dependent variable 'change in cortisol' (calculated as described for experiment 1). Degrees of freedom were adjusted with a Huyen–Feldt Probability when there were covariance inhomogeneities. Paired *t*-tests comparing anesthetic agents with saline were performed when a repeated-measures ANOVA yielded a significant interaction between agent and time. *t*-tests for effects of Telazol vs. saline on cortisol were one-tailed because we hypothesized that Telazol would inhibit cortisol. Bonferroni corrections were made for *post-hoc* tests. Repeated-measures ANOVAs also were used to assess effects of anesthetic agents and blood sampling on immune system parameters. In

each case, the independent variables were agent (Telazol, ketamine and saline) and time (0 and 120 minutes). The level for statistical significance was set at $P \leq 0.05$.

We used paired *t*-tests to evaluate within-condition (Telazol, ketamine, or saline) cortisol effects. Initial levels were compared with levels at the other time points. The Bonferroni-adjusted criterion for significance was 0.0125.

Results

Cortisol

Initial cortisol levels of 19.7 ± 0.5 μg/dl were similar in the Telazol, ketamine and saline conditions (Table 1). Cortisol decreased for the entire 2 h in the saline condition (Fig. 1B). After ketamine injections, cortisol concentrations declined as in the saline condition for the first 30 minutes, then remained stable for the remaining 90 minutes. After Telazol administration, the cortisol response paralleled the pattern seen post-ketamine for the first hour; it rose during the second hour to 3.6 ± 2.9 μg/dl above the starting level, which was 10.1 μg/dl higher than the change in cortisol from the initial level for the saline condition at that time point. Main effects of time ($P > 0.10$) and agent [$F_{(1.5,24.4)} = 2.7$; $P \leq 0.098$] on changes in cortisol were not significant. The interaction between time and agent was significant [$F_{(3.5,56.4)} = 5.91$; $P \leq 0.001$]. At 120 minutes, cortisol decreased more (not less, as predicted) post-saline than post-Telazol [1-tailed, $t_{(16)} = 2.92$, $P \leq 0.005$].

Within the saline condition, cortisol was lower than the initial level at 30 minutes ($t_{16} = 3.18$, $P \leq 0.006$), 60 minutes ($t_{16} = 3.35$, $P \leq 0.004$) and 120 minutes ($t_{16} = 2.87$, $P \leq 0.011$) post-injection (see Table 1). Under the ketamine condition, cortisol did not differ from its initial level at any time point. Cortisol post-Telazol was lower than the initial level 15 minutes post-injection ($t_{16} = 3.04$, $P \leq 0.008$; see Table 1).

Immune system measures

There was no effect of agent and no interaction between agent and time for any measured immune system parameter. There were, however, significant effects of time for nearly all measures. Because cell counts did not differ by agent, counts for the saline, Telazol and ketamine conditions were averaged. Table 2 lists mean cell counts. By the 120 minutes sample, total lymphocyte, $CD4^+$, and $CD8^+$ cell numbers were lower and the CD4/CD8 ratio was higher compared to the initial numbers (total lymphocytes: [$F_{(1,16)} = 20.47$, $P \leq 0.001$]; $CD8^+$ lymphocytes: [$F_{(1,16)} = 14.16$, $P \leq 0.005$]; $CD4^+$ lymphocytes: [$F_{(1,16)} = 23.03$, $P \leq 0.001$]; CD4/CD8 ratio: [$F_{(1,16)} = 17.34$, $P \leq 0.001$]. Neutrophil numbers increased over the 2 hours, although the effect was not significant [$F_{(1,16)} = 3.75$, $P \leq 0.07$]. Total WBC numbers did not change over time.

Discussion

The decrease in cortisol observed over the course of the afternoon in the saline condition is consistent with the diurnal rhythm for cortisol in rhesus macaques [30, 32, 41]. It appears that blood sampling and saline injection did not affect cortisol in these armpull-trained monkeys sampled in their home cages. A deviation from the cortisol pattern for the saline condition after administration of an anesthetic agent, then, would suggest that the agent exerted a pharmacologic effect. The data from Experiment 2 differ from results of the baboon experiment and do not support the hypothesis that Telazol inhibits cortisol release. Compared with saline, Telazol exerted no effect on cortisol in the first hour in the macaques, and at 120 minutes, the change in cortisol was actually more positive post-Telazol than post-saline. Telazol may exert a pharmacologic effect more than 60 minutes after it is administered, but if so, the effect is opposite to the predicted outcome, namely that the benzodiazepine in Telazol would inhibit cortisol release. Changes in cortisol post-ketamine differed strikingly between the baboons (Fig. 1A) and the rhesus macaques (Fig. 1B), with the sustained rise observed in the baboons following ketamine administration entirely absent in the rhesus macaques. It appears that ketamine exerted no pharmacologic effect on cortisol at any time point in the rhesus macaques.

There are three principal differences between experiments 1 and 2 that may contribute to the dissimilarities in results. The first difference is species. There are mixed reports about effects of ketamine on cortisol in rhesus macaques and no reports on ketamine's effect on cortisol in male savannah baboons. Consequently, we cannot rule out the possibility that the different outcomes reflect a true species difference.

The second difference is that the macaques in experiment 2 were more familiar with the blood sampling procedure than were the baboons in experiment 1 and were trained to participate in the procedure. The cortisol response to blood sampling in the macaques may have been influenced by the

animals' longstanding familiarity with the blood sampling protocol, as repeated or chronic exposure to some stimuli blunt cortisol responses [5, 20, 27, 29, 40, 45]. This may be particularly relevant to testing the effect of the benzodiazepine component of Telazol on cortisol. Benzodiazepine receptor levels appear to be altered in humans [7] and rats [8] that exhibit long-term changes in glucocorticoid responsiveness. Comparison of these results with comparable data obtained from untrained monkeys could confirm whether experience does influence cortisol responses to these procedures. We explore this possibility in experiment 3.

We are reluctant to conclude, however, that armpull training and regular blood drawing completely eliminates a stress response to blood sampling. Although cortisol concentrations did not increase in the ketamine and saline conditions, we did find changes in immune cell numbers. The results of our experiment, in which neutrophil numbers increased and lymphocyte numbers decreased, run counter to the diurnal pattern reported in rhesus macaques [30] and humans [1, 4, 23, 25, 38, 47]. In fact, the pattern of cell count changes in this experiment is identical to the pattern reported in a previous study involving these monkeys and others, in which blood was collected at the same time of day while the monkeys were in restraint chairs, a situation that is inherently stressful and that elevates cortisol [10]. It is possible that other factors (perhaps sympathetic nervous system activity [33]) may have influenced lymphocyte trafficking in one or both of the experiments.

The third difference between experiments 1 and 2 is that samples were collected from the baboons in the morning and the macaques in the afternoon. Diurnal variations in basal cortisol and hypothalamo–pituitary–adrenocortical responsiveness are well documented [53]. In addition, there is a circadian rhythm for benzodiazepine receptor activity, and altering the time of administration of benzodiazepines may alter their effect [31]. We explore the possible effect of diurnal rhythms in experiment 4.

Experiment 3 (untrained rhesus macaques, p.m.)

Rationale/objectives

In this experiment, we explored the possibility that a difference in the subjects' training and familiarity with the injection and sampling process contributed to the difference in results obtained in experiments 1 and 2. Briefly, the armpull-trained rhesus macaques had no cortisol response to injection/sampling, even in the saline condition, but the immune system data suggest that the procedure was stressful. Thus, the cortisol responsiveness of the monkeys to this particular stimulus may have been blunted. We predicted that cortisol levels would be higher in response to injection/blood sampling in monkeys that were not armpull-trained or familiar with the procedure, compared with animals that were trained. As we believed this effect was likely to be large and there were concerns about the stressfulness of the experimental procedure in monkeys not familiar with it, we limited the sample size to four.

Methods

Subjects and living arrangements

Subjects were four male rhesus macaques ranging from 6.1 to 8.3 years of age at the start of the experiment. They were born in half-acre corrals at the California Regional Primate Research Center, where they lived until they were relocated to single cages 8 months before the start of experiment 3. These monkeys had not experienced blood sampling via armpull before this experiment; they had been immobilized periodically for routine health care.

Experimental design and procedure

The design and procedure are as described for experiment 2 with the following exceptions: two monkeys were sampled per day, samples were collected between February and April, and no blood was collected for immunological measures. As before, repeated measures ANOVAs were used to examine differences in the cortisol response to ketamine, Telazol, and saline injection over the 2-h time period.

Results

Initial cortisol levels of 22.3 ± 1.1 μg/dl were similar in the Telazol, ketamine and saline conditions (Table 1). Cortisol increased in the untrained macaques for the entire 2 h after injection of saline (Fig. 1C). In contrast, cortisol was essentially unchanged after injection of ketamine or Telazol. The repeated-measures ANOVA revealed no significant effect of agent on changes in cortisol, and there was no agent by time interaction. The effect of time approached significance [$F_{(3,9)} = 3.625$, $P \leq 0.058$]. No within-agent effects were significant.

Discussion

The difference in cortisol patterns following saline injection to the armpull-trained macaques (Fig. 1B) vs. the untrained monkeys (Fig. 1C) is pronounced. Cortisol decreased steadily and significantly in the trained monkeys and increased throughout the experimental period in the untrained animals. At 120 minutes post-saline, cortisol in the untrained monkeys was 15.2 ± 9.8 μg/dl higher than the initial level; in the trained animals, the level was 6.5 ± 2.3 μg/dl lower. In fact, a *post-hoc* statistical comparison across the two experiments revealed a highly statistically significant result ($P < 0.001$), suggesting that training and familiarity with the process do exert an effect on the cortisol response to blood sampling.

The cortisol response to ketamine administration was different in the baboons (Fig. 1A) than in the untrained macaques (Fig. 1C). In the baboons, cortisol rose >15 μg/dl between 15 and 60 minutes after ketamine injection and was significantly higher than the initial level at 60 minutes. In the untrained macaques, cortisol levels were unchanged following administration of ketamine. The outcome in the untrained macaques mirrors the effect observed in female savannah baboons [2]: for both sets of animals, the contrast between a prolonged increase in cortisol post-saline and the lack of sustained increase in cortisol post-ketamine suggests that ketamine may inhibit a cortisol response to stress. Results of this experiment suggest that the effect may last longer than ketamine's ability to keep monkeys immobilized: the macaques were awake and sitting when samples were drawn 60 and 120 minutes post-ketamine, but cortisol did not increase between 30 and 120 minutes after ketamine administration. The lack of increase in cortisol post-ketamine in the untrained male rhesus macaques is different than the post-ketamine increase in cortisol in four female rhesus monkeys [17]. The sample size for the female and untrained male macaque experiments is small, but these results, combined with the contradictory statistically significant results from the male and female baboon experiments, suggest a need for further study of the ability of ketamine to block cortisol responses to stress in non-human primates.

At all time points (Fig. 1C), cortisol levels were lower in the Telazol condition than post-ketamine. This result is consistent with a hypothesis that the benzodiazepine in Telazol inhibits hypothalamo–pituitary–adrenal activity. Benzodiazepine receptor numbers are altered in animals that exhibit a blunted cortisol response [7, 8]. Since the immunological data and comparison of cortisol responses to sampling/injection in the trained vs. untrained macaques suggest the cortisol response in the trained monkeys was blunted, the results of experiments 2 and 3 are also consistent with a possibility that HPA hyporesponsiveness contributed to cortisol not being lower post-Telazol than post-saline or post-ketamine in experiment 2. A larger sample size for untrained monkeys would be required to confirm this statistically.

Experiment 4 (armpull-trained macaques, a.m.)

Rationale/objectives

The existence of diurnal effects on hypothalamo–pituitary–adrenal responsiveness may have played a role in the lack of a cortisol response to afternoon injections of saline in the armpull-trained macaques. If the cortisol response to sampling in the afternoon is different from that in the morning, time of day may also influence the cortisol response to the anesthetic agents. In particular, there is a circadian influence on benzodiazepine effectiveness and receptor levels [31], so time of day may influence responsiveness to the benzodiazepine in Telazol.

Methods

Subjects were 16 of the 17 monkeys from experiment 2 (the remaining monkey was a subject on a different project and was unavailable). The experimental design was similar to that used in experiment 2 except for the following differences: (1) the start time for data collection was 9:00 a.m.; (2) four monkeys were sampled per day (one monkey per agent plus a second monkey for one agent); (3) subjects were fasted overnight and (4) no blood was collected for immunological measures. Experiment 4 was conducted the year after experiment 2. Samples were collected in February, March, June and July. Paired *t*-tests were used to determine whether initial cortisol levels (averaged across the saline, Telazol, and ketamine conditions) in the morning were different from those in the afternoon.

Results

Initial cortisol levels were similar in the Telazol, ketamine and saline conditions (Table 1). Initial levels (averaged across agents for each animal) were

higher in the morning than in the afternoon (27.0 ± 1.2 μg/dl vs. 19.5 ± 1.6 μg/dl, $t_{(15)} = 6.28$, $P < 0.001$). Cortisol was unchanged for the entire 2 h post-saline (Fig. 1D). It was slightly lower than the initial level at 15 and 30 minutes post-ketamine and was similar to the initial level at 60 and 120 minutes. After injection with Telazol, cortisol levels decreased in the first hour, then returned to initial levels. There was a significant main effect of agent on changes in cortisol [$F_{(2,30)} = 3.984$, $P \leq 0.05$], and of the agent by time interaction [$F_{(2.78,41.72)} = 3.436$, $P \leq 0.05$]. The effect of time on changes in cortisol approached significance [$F_{(1.58,23.64)} = 2.91$, $P \leq 0.085$]. One-tailed paired *t*-tests showed a significant effect of Telazol vs. saline at three time points: the change in cortisol was lower post-Telazol at 15 minutes ($t_{15} = 3.07$, $P \leq 0.004$), 30 minutes ($t_{15} = 2.66$, $P \leq 0.009$) and 60 minutes ($t_{15} = 3.44$, $P \leq 0.002$) post-injection. There were no within-agent effects of saline or ketamine on cortisol, but levels post-Telazol were lower than the initial value at 15 minutes ($t_{15} = 4.31$, $P \leq 0.001$), 30 minutes ($t_{15} = 4.18$, $P \leq 0.001$) and 60 minutes ($t_{15} = 4.26$, $P \leq 0.001$; Table 1).

Discussion

Time of day influenced initial cortisol levels and the pattern of cortisol changes post-saline: cortisol decreased in the afternoon (Fig. 1B) but did not change in the morning (Fig. 1D). At both times of day, the cortisol results are consistent with the diurnal pattern for cortisol in rhesus macaques [30, 32, 41]. Psychological aspects of injection/sampling do not appear to have influenced cortisol in the morning or afternoon in the armpull-trained rhesus macaques.

The results of experiments 2 and 4, conducted with the same subjects, indicate that time of day exerted a substantial effect on the cortisol response to Telazol. In the afternoon, cortisol levels showed no deviation from the saline pattern for the first 60 minutes post-Telazol, but at 120 minutes, they were higher than levels post-saline. In contrast, after morning injections of Telazol, cortisol was lower than the initial level at each time point in the first hour and was similar to the initial level 120 minutes post-injection. The morning results provide strong support for the hypothesis that the benzodiazepine in Telazol reduces cortisol. The results are consistent with data indicating that the benzodiazepine alprazolam reduces basal plasma cortisol levels (as well as stress-induced increases in glucocorticoids) [39] in male rhesus macaques. The afternoon results, though, suggest that the Telazol effect, at least in armpull-trained monkeys, is manifested only during a portion of the diurnal cycle. It has been observed that benzodiazepine effects may differ by time of administration [31], and benzodiazepine receptors exhibit a 24-hours rhythm [31]. Melatonin levels also exhibit a circadian rhythmicity and influence benzodiazepine receptor binding in rat brain [18].

General discussion

These experiments and results of previous studies [2, 50, 24, 35, 52, 17, 13, 15, 16, 51] indicate that the effect of immobilization and blood sampling on cortisol in non-human primates is complex and may be influenced by a number of factors including species, gender, familiarity of subjects with immobilization and blood collection procedures, training of subjects to participate in the sample collection process, dose, and time of day. We discuss in turn the influences of Telazol and ketamine on the cortisol response to immobilization, and the effects of the monkeys' training to participate in the sampling process on cortisol levels.

Effect of Telazol on cortisol

Benzodiazepines have been shown to reduce activity of the hypothalamo–pituitary–adrenal axis [e.g., 9, 21, 34, 39] by inhibiting release of corticotropin releasing hormone [22]. Administration of the benzodiazepine alprazolam to rhesus macaques reduces adrenocorticotropic hormone levels [22, 49] and may reduce cortisol levels as well [22] (but see 49 for alprazolam's effect on cortisol in response to the stressor hypoglycemia). We examined the possibility that the benzodiazepine in Telazol (zolazepam) inhibits HPA activity by testing the prediction that changes in cortisol concentrations post-Telazol would be lower than changes post-saline or, in the case of the baboons, post-ketamine. The prediction was confirmed for male baboons, and for armpull-trained male rhesus macaques given Telazol in the morning. This result does not appear to be a general response to immobilization since cortisol concentrations post-ketamine did not show a comparable response. Thus, while we cannot be certain that the reduction resulted from the binding of zolazepam to benzodiazepine receptors, the decrease appears to be a pharmacologic effect attributable to an ingredient in Telazol (tiletamine or zolazepam). We did not

directly test zolazepam by blocking benzodiazepine receptors or administering only tiletamine because of the disadvantages of immobilizing animals without the anticonvulsant and muscle relaxant properties of zolazepam.

A comparison of the results of experiments 2 and 4 strongly suggests a diurnal influence on the relation between Telazol and cortisol. The same 16 monkeys whose changes in cortisol were lower post-Telazol than post-saline for the first hour in the morning had no cortisol response to Telazol compared with saline in the first hour of the afternoon. This outcome is consistent with data obtained in other species indicating that time of administration may influence the effectiveness of benzodiazepines [31].

We found that Telazol had no effect on leukocyte cell counts in the rhesus macaques, but we obtained immunological measures only in the afternoon. Given our cortisol results and the influence of circadian rhythms on the effects of benzodiazepines, it is possible that Telazol could affect immune system parameters in the morning. Trafficking of immune cells is influenced both by cortisol and catecholamines, and a related benzodiazepine, alprazolam, inhibits the release of epinephrine [39, 3, 6].

Effect of ketamine on cortisol

In the present study, ketamine did not increase cortisol in any of the experiments involving rhesus macaques, and for armpull-trained animals, cortisol concentrations after administration of ketamine were not substantially different from those after a control injection. This result is at variance with data suggesting a rise in cortisol following ketamine administration [52, 17] in rhesus macaques. Animals in the latter studies, however, received multiple intramuscular injections. Puri [35] found, in three rhesus males, that cortisol rose after multiple injections of ketamine but not after a single injection. The outcome of our experiments with the armpull-trained macaques mirrors Puri's finding that a single injection of ketamine does not increase cortisol levels in male rhesus macaques; the large number of monkeys in the current experiments (16–17 armpull-trained monkeys) makes it likely that an effect would be detectable if it was there.

Cortisol was significantly elevated 1 hour after ketamine injection to male savannah baboons at a dose of 10 mg/kg with 3 mg/kg supplements. The increase may be attributable to a pharmacologic effect of ketamine or its metabolites on cortisol, which rises in male savannah baboons following administration of closely related phencyclidine [42]. It may also result from a stress of prolonged immobilization or a dose of ketamine too low to induce a sufficiently deep plane of anesthesia [12]. We have traditionally limited the delivery of individual ketamine supplements to 3 mg/kg in an effort to minimize physiological stress that may accompany prolonged immobilization or buildup of ketamine metabolites; that dose eliminates visibly observable signs of emergence from anesthesia for a time. Further study is required to determine whether larger supplements will reduce or increase cortisol levels in male savannah baboons.

Effect of the injection/blood sampling process on cortisol and leukocyte cell counts

The sustained post-saline increase in cortisol in our untrained animals is strikingly different than the decline in cortisol levels in our untrained monkeys following afternoon injection of saline. This provides strong support for Reinhardt's [37] contention that training reduces stress for the monkeys. It cannot be assumed, though, that training removes all physiological components of the stress response associated with use of venipuncture to obtain blood from conscious or immobilized animals. Lymphocytes decreased and neutrophils increased in the armpull-trained macaques following administration of ketamine, Telazol, or saline, a response more typical of a stress response than of diurnal influences. It is possible that the hypothalamo–pituitary–adrenal response, but not the sympathetic nervous system response, is attenuated after the training process has occurred.

Training or familiarity with blood sampling does not necessarily eliminate a cortisol response to all aspects of a blood collection process. Male rhesus macaques bled approximately 75 times exhibit an increase in cortisol when removed from their social group and placed in a single cage for sample collection [41]. Female rhesus macaques trained to extend a leg for venipuncture exhibit higher cortisol increases when sampling is carried out (without restraint) in a restraint box than when blood is collected in the animals' home cage [36]. The cortisol increase in these situations may be associated with relocation to a novel environment, a stimulus that has the capacity to increase cortisol levels [36]. One criterion for collecting samples without causing cortisol levels to increase may be to obtain blood without removing animals from their home cage.

Summary

We have shown in male savannah baboons that cortisol increases following injection of 10 mg/kg of ketamine and supplements of 3 mg/kg. Cortisol is lower post-Telazol than post-ketamine in the male baboons. In armpull-trained adult male rhesus macaques, Telazol reduces cortisol in the morning but not in the afternoon. A single injection of ketamine does not affect cortisol in trained male macaques in the morning or afternoon, but it may block an increase in cortisol resulting from psychological aspects of injection/sampling in animals unfamiliar with the blood sampling procedures. In rhesus macaques, leukocyte cell counts after injections of Telazol or ketamine are no different from those after injections of saline. Injection/sampling of any of the agents, although, alters the counts in a pattern that suggests the procedure is not entirely benign, even in armpull-trained macaques that exhibit no post-sampling increase in cortisol.

Acknowledgments

The authors thank Erik McArthur, David Taylor, Lee Martson, and Guillermo Garcia for technical assistance with data collection. Dianne Lattemann and Carolyn Crockett provided valuable insights. Special thanks to Dr Orville Smith for making the baboon experiment possible.

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