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Case Report

Effects of alprazolam on capture stress-related serum cortisol responses in Korean raccoon dogs (*Nyctereutes procyonoides koreensis*)

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The purpose of this study was to evaluate the effect of alprazolam on the stress that Korean raccoon dogs (*Nyctereutes procyonoides koreensis*) may experience while caught in a live trap by measuring their serum cortisol response. The animals were placed in a live trap with or without being pretreated with oral doses of alprazolam. In both groups, pre-trap blood samples were initially collected without anesthesia before the animals were positioned in the live trap; then post-trap blood samples were collected after the animals had remained in the live trap for 2 h. Changes in cortisol levels were observed using a chemiluminescent immunoassay. The level of cortisol increased in the control group and decreased in the alprazolam-pretreatment group ($p < 0.05$). In this study, we demonstrated that alprazolam pretreatment reduced stress during live trap capture.

Keywords: alprazolam, capture stress, cortisol, Korean raccoon dog, *Nyctereutes procyonoide koreensis*

The capture procedure is necessary for wild animal research and treatment, and the capture period includes the moment of capture by a human or live trap until the moment of release. Unfortunately, an animal that is captured in the wild can experience extreme fear, anxiety, and psychological stress which are often more harmful than physical trauma [9]. It was reported that a wild wolf captured and transported by helicopter exhibited symptoms similar to those of humans with posttraumatic stress disorder [9]. This indicates that extreme fear can induce psychological trauma in animals. Stress can be viewed as the cause of adverse circumstances that induce a wide range of biochemical and behavioral changes [7,9] and even significant oxidative damage in the brain [7]. Stress leads to the increased secretion of cortisol, a glucocorticoid also known as 17-hydroxycorticosterone or, pharmaceutically, hydrocortisone [2,8]. The measurement of cortisol in plasma best reflects the stress response at the time of blood sample collection [8]. The increased secretion of glucocorticoid in response to stress is immediate. Thus, the concentration of cortisol increases within minutes in the bloodstream and is proportional to the severity of stress [4]. The clearance half-life of cortisol is approximately 60 min [4] and can be measured in plasma, urine, or feces [8].

Stress can be reduced by various means. For example, neuroleptic drugs can reduce stress-related injury and mortality in wild animals, and their beneficial effects include general calming, inducing indifference to new and unnatural surroundings, a loss of the fear of people, and a reduction in aggressive behavior [6]. A widely used neuroleptic drug is benzodiazepine, an anxiolytic medication with a rapid onset of action that lasts for several hours and does not induce tolerance to its therapeutic effect. Therefore, it is potentially useful for overcoming any problems involving anxiety, fear, or phobia [1,3,7], especially in small canids [6]. Alprazolam is a benzodiazepine anti-anxiety agent that is frequently used for the treatment of generalized anxiety, panic attacks with or without agoraphobia, and depression in humans as well as dogs; in fact, it is used as part of the treatment protocol for storm phobia in canines [1,3,7]. In mice, alprazolam was effective in ameliorating behavioral alterations due to immobilization and oxidative stress [7]. An important property of benzodiazepines is their wide margin of safety, even in overdose situations; death due to a single dose of benzodiazepines is extremely rare [12]. Additionally, a benzodiazepine antagonist, flumazenil, can be used as an antidote for overdoses and thus ensures patient safety [3,12].

The raccoon dog (*Nyctereutes procyonoides*) is a Canidae family member that traces its origins to from the woodlands of Europe to Eastern Asia, including China, Japan, and Korea. It is a mammal commonly found in Korea and acts as an essential vector of rabies. Rabies is an important public health concern in Korea. For this reason, a great deal of research is being conducted on Korean raccoon dogs (*Nyctereutes procyonoides koreensis*). Therefore, capturing

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Korean raccoon dogs is essential and it is necessary to think about reducing capture-related stress. Therefore, the aim of this study was to measure the capture stress experienced by Korean raccoon dogs in a live trap by assessing the serum cortisol level with and without alprazolam pretreatment.

Three of the captive Korean raccoon dogs (BW: 6.7 ± 1.0 kg) used for this study were at the Seoul Zoo (Korea) and eight (BW: 4.7 ± 0.9 kg) were at the Gyeonggi Wild Animal Rescue Center (Korea). All animals were clinically healthy. Our study was conducted as a cross-over experiment; the eleven Korean raccoon dogs were initially used as controls and then again used for the alprazolam pretreatment study 2 weeks later. The control group did not receive alprazolam pretreatment while the alprazolam group was given oral doses of alprazolam prior to being placed in the live trap. For the alprazolam pretreatment, the drug (1 mg; Myung In, Korea) was dissolved in distilled water and administered orally with a syringe prior to the animals being placed in the live trap. For both groups, "pre-trap" indicated the time before the animals were left in the live trap and "post-trap" was the period after animals had been in the live trap for 2 h. Pre-trap blood samples were collected without anesthesia from the cephalic vein in both groups. The animals then were kept in a double-door live trap (91 × 25 × 30 cm; Havahart, USA) for 2 h. After the animals spent 2 h in the live traps, post-trap blood samples were collected. Immediately following collection, the blood was placed in a bottle containing EDTA (Sewon Medical, Korea) and serum collection tubes with serum separator gel and clotting activator (Vacuette; Greiner Bio-One, Austria). The blood samples were centrifuged in 6,000 rpm at room temperature for 5 min and the plasma was stored at −20°C. The cortisol analyses were conducted using a chemiluminescent immunoassay (Immulite-1000; Diagnostic Products, USA). The red blood cell and white blood cell counts were determined manually and the serum samples were processed using a Dri-Chem 3500i (Fujifilm, Japan) to determine the serum biochemistry. The hematological and biochemical panels are described in Table 1. All analyses were conducted using statistical software (PASW Statistics v.18; SPSS, USA). The differences in values between the pre-trap and post-trap levels were compared using paired samples *t*-tests. *P*-values of less than 0.05 were considered to indicate statistically significant differences.

Table 1. Serum cortisol levels, hematology, and serum biochemistry in the control and alprazolam pretreatment groups

	Control group		Alprazolam group	
	Pre-trap	Post-trap	Pre-trap	Post-trap
Cortisol (μg/dL)	2.21 ± 1.05	3.10 ± 1.09*	3.30 ± 0.94	1.78 ± 1.00*
WBC ($\times10^3$/μL)	13.45 ± 1.59	13.91 ± 2.34	12.92 ± 2.59	11.86 ± 2.59
Lymphocyte ($\times10^3$/μL)	4.41 ± 1.16	3.08 ± 0.65*	3.84 ± 0.58	3.56±1.05
Monocyte ($\times10^3$/μL)	1.24 ± 0.21	1.49 ± 0.45	1.06 ± 1.12	1.00±0.27
Eosinophil ($\times10^3$/μL)	0.09 ± 0.04	0.03 ± 0.05	0.14 ± 0.07	0.13±0.04
Basophil ($\times10^3$/μL)	0.16 ± 0.05	0.28 ± 0.34	0.15 ± 0.05	0.11±0.04
RBC ($\times1{,}000^6$/μL)	7.19 ± 0.69	7.20 ± 0.51	6.77 ± 0.64	6.71 ± 0.83
Hb (g/dL)	11.26 ± 2.70	12.17 ± 1.89	9.82 ± 5.60	9.65 ± 6.49
Hct (%)	34.27 ± 2.06	34.78 ± 2.30	32.61 ± 4.01	33.08 ± 4.37
PLT ($\times10^3$/μL)	322.64 ± 103.11	297.55 ± 83.87	368.73 ± 104.10	328.09 ± 69.60
TP (g/dL)	7.1 ± 0.3	7.1 ± 0.4	7.2 ± 0.4	7.1 ± 0.5
Alb (g/dL)	3.9 ± 0.3	3.9 ± 0.3	3.8 ± 0.4	3.7 ± 0.2
TB (mg/dL)	0.8 ± 0.2	0.9 ± 0.4	0.8 ± 0.1	0.8 ± 0.4
GGT (U/L)	16.3 ± 10.1	18.5 ± 14.9	7.4 ± 2.8	8.8 ± 5.2
AST (U/L)	113.1 ± 86.8	198.9 ± 210.0	63.6 ± 11.4	80.3 ± 29.3
ALT (U/L)	98.1 ± 61.8	121.3 ± 94.7	70.4 ± 29.4	76.7 ± 31.7
ALP U/L)	175 ± 111.5	163.1 ± 99.0	124.8 ± 48.3	122.8 ± 51.3
CK (U/L)	565.2 ± 384.5	1008.7 ± 590.9*	380.6 ± 129.0	722.1 ± 382.8*
TG (mg/dL)	133.1 ± 105.5	193.9 ± 187.1	66.7 ± 20.0	62.0 ± 17.8
TC (mg/dL)	186.0 ± 32.8	185.7 ± 32.1	171.6 ± 31.7	160.6 ± 21.4
Glu (mg/dL)	67.9 ± 28.6	63.9 ± 20.3	55.9 ± 42.5	52.6 ± 15.9
Crea (mg/dL)	0.7 ± 0.1	0.7 ± 0.1	0.73 ± 0.2	0.6 ± 0.1
BUN (mg/dL)	34.3 ± 11.2	35.1 ± 13.0	31.9 ± 7.9	31.5 ± 9.7
IP (mg/dL)	7.1 ± 2.1	5.8 ± 1.7	5.4 ± 0.7	5.5 ± .12
Ca (mg/dL)	10.3 ± 0.5	10.1 ± 2.3	11.1 ± 2.2	10.2 ± 0.4

Hb: hemoglobin, Hct: hematocrit, PLT: platelet, TP: total protein, Alb: albumin, TB: total bilirubin, GGT: gamma-glutamyl transferase, AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, CK: creatinine kinase, TG: triglyceride, TC: total cholesterol, Glu: glucose, Crea: creatinine, BUN: blood urea nitrogen, IP: inorganic phosphate, Ca: calcium. All data are represented as the mean ± SD. *$p < 0.05$.

Many researchers have suggested that serum cortisol levels are a reliable indicator of stress responses in animals [2,4-6,8,10]. Morton *et al.* [10] reported that the cortisol levels appear to rise after capture in 672 individual animals of 17 different wildlife species. Additionally, it was reported that immobilization induces a two- to three-fold increase in plasma cortisol levels among sheep [5]. Increased cortisol levels have also been linked with anxiety-like behavior and stressful situations in cattle [2].

In the control group, the mean serum cortisol levels pre-trap and post-trap were 2.21 ± 1.05 μg/dL and 3.10 ± 1.09 μg/dL, respectively; thus, the mean cortisol level significantly increased after 2 h of capture in a live trap ($p < 0.05$). In the alprazolam pretreatment group, the mean serum cortisol levels of pre-trap and post-trap were 3.30 ± 0.94 μg/dL and 1.78 ± 1.00 μg/dL, respectively. Alprazolam treatment clearly led to a significant decrease in the serum cortisol levels after 2 h of capture in a live trap ($p < 0.05$). However, the pre-trap cortisol levels in the control and alprazolam pretreatment groups were different. These levels might be

influenced by environmental factors such as temperature, weather or season [4]. Since this was a cross-over study, the animals may have remembered the control experiment as an aversive event [9], and this could have influenced the subsequent experimental study. We found that the pre-trap cortisol levels of the alprazolam pretreatment group and the post-trap cortisol levels of the control group were similar. If the Korean raccoon dogs remembered the control study as an aversive event, they could have become more anxious and fearful when we captured them and collected blood samples during the second trial. Hence, these factors could have influenced the results of the study.

The hematological and serum biochemical values of the Korean raccoon dogs pre-trap and post-trap are provided in Table 1. There were no statistically significant differences in hematology and serum biochemistry between the pre-trap and post-trap samples from both groups. Lymphopenia is known as an indicator of stress responses [11], and the number of lymphocytes significantly decreased in the control group compared to the alprazolam pretreatment group. This result demonstrates that the control group experienced more stress in the live trap. Creatinine kinase is an enzyme located in the cytoplasm of muscle cells that leaks from these cells when they are damaged [11]. In this study, the mean values of creatinine kinase were statistically higher in the post-trap samples compared to the pre-trap samples in both the control and alprazolam pretreatment groups ($p < 0.05$). This may be explained by the fact that the control group had more self-inflicted injuries [11]; two Korean raccoon dogs lost toenails and seven animals were bleeding from the teeth because they tried to chew their way out of the trap. On the other hand, although the Korean raccoon dogs in the alprazolam pretreatment group tried to escape immediately after being placed in the trap, they calmed down quickly. Thus, none of these animals injured themselves, which is an important aspect for wild animal research. In this study, some pre-trap biochemistry results were significantly different between the control and alprazolam groups. The serum biochemistry of the animals can be affected by various factors, but one possible reason for the differences we observed could have been due to dietary changes that influence liver function [11]. Following the post-trap blood sample collection, the alprazolam pretreated animals did not show any symptoms of sedation when released from the trap. Consequently, they were able to escape and run away, which is also beneficial for conducting field work.

Bait dosed with an appropriate quantity of alprazolam may be useful for future studies. However, during the capture procedure in a field study, it is impossible to measure the body weight of an animal before its capture. Based on our results, we recommend a dosage of 1 mg for a Korean raccoon dog regardless of body weight due to the safety and convenience of the drug. In conclusion, the results from this study are relevant to improving animal welfare and applicable to performing field studies using bait dosed with alprazolam along with translocation procedures for the purpose of wildlife conservation and research.

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