Relationship between Transportation Stress and Polymorphonuclear Cell Functions of Bottlenose Dolphins, *Tursiops truncatus*

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**ABSTRACT.** Dolphins in a captive environment are exposed to various kinds of stresses. Handling and transportation are stressful events for terrestrial mammals, and such stress may affect immune system function and increase susceptibility to infectious diseases. The same phenomenon could occur in dolphins, however, few studies have reported this in dolphins. The objective of this study was to evaluate the relationship between stress and polymorphonuclear (PMN) cell function of dolphins during transportation. Four bottlenose dolphins (*Tursiops truncatus*) were transported for 6 hr by truck. Serum cortisol levels, leukograms, phagocytosis, and superoxide production of PMN cells were evaluated during handling and transportation compared to resting values. The mean serum cortisol level was significantly increased during handling and transportation (p<0.05) when compared with the resting values. White blood cell (WBC) counts, eosinophil counts, phagocytosis, and superoxide production of PMN cells during handling and transportation stages decreased significantly in comparison with the resting stage (p<0.05). The concentration of serum cortisol was significantly correlated with the results of the WBC counts, eosinophil counts, superoxide production, and phagocytosis (p<0.01, p<0.05, p<0.05, and p<0.001, respectively). The present results indicate that handling and transportation are stressful events for dolphins and could affect their PMN cell functions, thereby leading to the impairment of the immune system.

**KEY WORDS:** dolphin, immune function, transportation stress.

Dolphins in a captive environment are exposed to various kinds of stresses. Studies on other species have demonstrated that such responses are associated with impaired host defenses and thus many predispose to disease [13, 15, 17, 27]. Stress is defined as the response of the body to any threatening situation, and a resource-based trade-off between the immune system and costly behavior characterized by stress-induced immunosuppression [23]. Stress in terrestrial mammals can be monitored by measuring elevations in serum concentrations of adrenal corticosteroids, particularly cortisol [3]. Expression of receptors for the products of the nervous, endocrine, and immune systems and production of hormones in immune cells constitute the basis of immunoneuroendocrine interactions. As a partial effect of increased hormone levels, the total number of circulating neutrophils becomes elevated while lymphocytes and eosinophils decrease [29] and polymorphonuclear (PMN) cell functions change [22].

Bottlenose dolphins, *Tursiops truncatus*, appear to exhibit the same changes, but to a lesser extent than in terrestrial mammals. Some researchers have found that dolphins, which are handled and restrained exhibit eosinopenia and lymphopenia [29]; however, there are currently no published studies on the relationship between stress and PMN cell function.

As a step towards understanding the relationship, we undertook this study to determine the nature of the stress response in bottlenose dolphins. We examined the effects of handling and transportation on cortisol, circulating leukocytes, and on PMN cell functions.

Our aim was to remove bottlenose dolphins from a pool for handling and then transport them for within 6 hr. Their response to handling and transportation was then compared with their blood parameters during resting periods.

**MATERIALS AND METHODS**

*Animals and collection of blood:* Four female bottlenose dolphins (body weight of 200–250 kg) were examined. All of them had been kept for over 5 years in the same dolphinarium, and maintained in a pool in Wakayama, Japan. It was presumed that they were already sexually matured at the point of this study. All of the dolphins were considered normal as a result of prior physical examination and did not show abnormal parameters in blood examinations (complete blood counts and blood biochemical examinations) before the current research commenced. All dolphins had been trained to raise their tail flukes to have blood drawn from the superficial fluke veins (husbandry training) and we collected the samples before handling and transportation to obtain a resting period value. These samples were obtained at 09:00 between October 2001 and February 2002, and the ambient temperature was from 5 to 21°C. The dolphins were transported from Wakayama to Osaka in March 2002, with the maximum and minimum climatic temperatures on the day of transport as 16 and 8°C, respectively. To transport the dolphins, water was first drained from their pools (taking 2 or 3 hr). Then the dolphins were removed from the
pool on stretchers and immediately suspended in transport units. Blood samples were collected at this time (about 07:00) for the handling stage. The units were carried into trucks where air and water temperature were maintained below 20°C, and the back and flukes of dolphins were kept wet using a watering pot during transportation and were covered with a sponge cushion to prevent scratches from the wall of the unit. The dolphins were transported for about 6 hr at an average speed of 50 km/hr. The distance was about 250 km. After the truck arrived at the receiving facility, further blood samples were collected immediately in the units for transport stage samples (about 13:00) and the dolphins were released into the pool. Peripheral blood samples were drawn from a superficial blood vessel on the ventral aspect of the tail fluke. Blood samples for PMN cell functions and hematology were collected in tubes containing heparin. Blood for serum cortisol was collected in tubes without anticoagulant. Samples were placed on ice until they were analyzed, usually within 6 hr. Serum was separated and frozen before being analyzed.

Separation and measurements of functions of PMN cells: For isolation of PMN cells and measurements of PMN cell function in the dolphins, we used methods previously reported by Noda et al. [16].

The blood sample was diluted 1:1 with Hank’s balanced salt solution without Ca²⁺ (HBSS: Nissui Pharmaceutical Co., Ltd., Tokyo). The diluted blood was layered on lymphocyte isolate solution (d=1.077 g/m³) obtained after centrifugation at 400 × g for 20 min. After centrifuging, the supernatant containing mononuclear cells was aspirated and the bottom layer containing PMN cells and erythrocytes was collected. Erythrocytes were lysed using NH₄-Tris solution. The cell pellet obtained after centrifugation at 400 × g for 20 min at 4°C was washed twice with HBSS, and then suspended in HBSS. Viable cells were determined by Trypan Blue exclusion and counted with a Bürker-Türk counting chamber. The viability of PMN cells was over 95%. The final cell concentrations were adjusted to 5.0 × 10⁶ viable cells/ml of HBSS.

For the evaluation of phagocytosis by PMN cells, polystyrene latex beads (diameter=1.0 mm; Polysciences, Inc., PA) were used, as described previously in Noda et al. [16]. Briefly, after pre-incubating PMN cell suspension and autologous serum, 0.1% non-opsonized polystyrene latex beads solution was added to the cell suspension. Then, the mixtures were incubated at 37°C and 0°C for 12 hr, respectively. After the incubation, the reaction was terminated. After washing off the superficially attached beads, the PMN cells were smeared on three slide glasses using Cytospin (Shandon Co., PA). The smears were stained with Giemsa solution, and the number of cells ingesting beads per 600 PMN cells was counted under a microscope. Phagocytic activity was expressed by the percentage of PMN cells phagocytosing three or more particles.

To evaluate superoxide production, nitroblue tetrazolium (NBT) reduction by PMN cells was evaluated using methods previously described in Noda et al. [16]. The NBT reduction test was conducted in duplicate in 15 × 105 mm silicon-coated glass tubes. 0.5 ml of cell suspension (5.0 × 10⁶/ml) was mixed with 0.4 ml of the NBT solution (1 ml/mg/ml) and 0.1 ml of zymosan A suspension (10 mg/ml) opsonized by dolphin serum, and incubated at 37°C for 30 min. After incubation, the reaction was terminated by 0.5 M HCl. The mixture was then centrifuged and the supernatant was discarded. The precipitate was dissolved with 3 ml of dimethyl sulfoxide (DMSO), heated in boiling water for 5 min and then allowed to cool. After the mixture was clarified by centrifuging at 500 × g for 5 min, the optical density at 565 nm was determined immediately using a spectrophotometer (Shimadzu Co., Kyoto), and using a DMSO blank. The reduction of NBT by resting cells was determined in a similar way but without opsonized zymosan A.

White blood cell count, differential counts of leukocytes, and serum cortisol: Leukocytes were counted using a Cell-tac-α clinical auto-analyzer (Nihonkoden, Tokyo) [16]. Leukocyte differentiation was estimated by a blood smear stained with Giemsa solution. Serum cortisol concentration was determined by the electrochemiluminescence immunoassay (ECL-IA) method [19] using rabbit antibodies (Rosh Diagnostics Co., Ltd. Tokyo).

Analytical procedures: Data were presented as mean ± standard deviation (SD) and were analyzed for significant differences between resting stage and handling and transport stages by ANOVA using Microsoft Excel® (Microsoft Co., Washington, DC, U.S.A.). A P value < 0.05 was considered statistically significant. Pearson correlation coefficients were calculated using Microsoft Excel® to investigate linear relationships between cortisol and PMN cell functions and differential counts of leukocytes.

RESULTS

The leukogram of the dolphins is shown in Table 1. WBC counts decreased significantly with handling and transportation (5725 ± 806 and 4800 ± 744/µl, respectively) compared with resting stage (7225 ± 250/µl). There was a significant decrease in eosinophils at the transport stage (91 ± 114/µl) in comparison with the resting stage (1008 ± 128/µl).

Serum cortisol concentrations of dolphins significantly increased during handling and transportation (5.6 ± 2.4 and 6.2 ± 2.4 µg/dl, respectively) compared with the resting stage (1.2 ± 0.2 µg/dl); however, there was no difference between handling and transportation.

NBT reduction was lower during the handling and transportation stages (0.047 ± 0.025 and 0.050 ± 0.016, respectively) compared with the resting stage (0.078 ± 0.013). In particular, the optical density during the transport stage was significantly lower than that at the resting stage (p<0.05).

In addition, the phagocytic activity was also significantly lower during the handling and transport stages (39.9 ± 6.5% and 38.4 ± 2.8%, p<0.05, respectively) compared with resting stage (57.4 ± 6.3%). However, there was no significant
The concentration of serum cortisol was significantly correlated with the results of the NBT reduction test \(r = -0.77, \text{N}=4, P<0.05\) and phagocytosis \(r = -0.82, \text{N}=4, P<0.001\). In addition, a correlation between serum cortisol and WBC \(r = -0.87, \text{N}=4, P<0.01\) and eosinophil counts \(r = -0.70, \text{N}=4, P<0.05\) was observed.

**DISCUSSION**

The effects of capture and transport-induced stress have been reported in various mammals including dolphins [14, 15, 19, 20]. Stressful conditions interfere with the immune response [4, 24]. In many animals, transportation has been associated with hematological, biochemical, metabolic, and endocrine changes that may increase susceptibility to diseases [12, 19, 21]. Immunosuppression caused by stress has been mainly ascribed to adrenal secretions of corticosteroids [9, 19]. In these reports, an elevation of serum cortisol is reported to be a good indicator of a stress response [14, 15, 19, 20]. It has been reported that even 10 min after handling, the serum cortisol concentration had increased and peaked after 1.5 hr in bottlenose dolphins [29]. In the current study, the cortisol level of dolphins during handling and transportation was significantly higher than the level recorded at the resting stage. However, an elevated cortisol level may have occurred prior to handling since it took about 3 hr to remove the water from the pool, and the dolphins were exposed to continued stress before the removal.

Suzuki et al. reported a diurnal changes in serum cortisol levels in the absence of stress effects in Indo-Pacific bottlenose dolphins and killer whales [28]. This report showed that the serum cortisol concentration peaked at 09:00 and gradually decreased during the evening and night. In our research, regardless of the time of blood sampling for handling and transport stages at 07:00 and 13:00, respectively, the serum cortisol levels of these stages were higher than those at the resting stage (09:00). Therefore, it is considered that the stress of handling and transportation affects these changes. In addition to increased cortisol levels, terrestrial mammals show leukopenia, neutrophilia, and eosinopenia when under stress [2, 22]. Our results showed that the WBC and eosinophil counts decreased during the handling and transport stages. Thus, handling and transportation are stressful events for bottlenose dolphins.

There was a significant difference between the resting stage and handling and/or transport stage in both NBT reduction and phagocytosis. The observed changes in

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<th>Resting stage</th>
<th>Handling stage</th>
<th>Transport stage</th>
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<tbody>
<tr>
<td>WBC count (/µl)</td>
<td>7225 ± 250</td>
<td>5725 ± 806*</td>
<td>4800 ± 744*</td>
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<tr>
<td>Stab (/µl)</td>
<td>0</td>
<td>52 ± 61</td>
<td>52 ± 61</td>
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<tr>
<td>Segments (/µl)</td>
<td>4625 ± 752</td>
<td>3604 ± 918</td>
<td>3655 ± 686</td>
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<tr>
<td>Lymphocytes (/µl)</td>
<td>1620 ± 818</td>
<td>1374 ± 388</td>
<td>885 ± 177</td>
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<tr>
<td>Monocytes (/µl)</td>
<td>126 ± 124</td>
<td>106 ± 77</td>
<td>48 ± 7</td>
</tr>
<tr>
<td>Eosinophils (/µl)</td>
<td>1008 ± 128</td>
<td>641 ± 365</td>
<td>91 ± 114*</td>
</tr>
<tr>
<td>Basophils (/µl)</td>
<td>0</td>
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Values are the mean ± SD. * Significant difference from the resting stage (P<0.05).
phagocytosis and NBT reduction were correlated with alterations in circulating concentrations of cortisol induced by the stress of handling and transportation. Elevated serum cortisol concentrations and changes in immune systems have been reported associated with the transportation of terrestrial mammals [22, 27, 30]. There are few reports on the effects of stress on PMN cell functions, however, Dixit et al. [7] reported that stress was correlated with neutrophil functions. In most species, corticosteroids reduce or have no effect on phagocytosis and tend to impair oxidative functions in a dose-dependent manner [1]. However, hydrocortisone has been shown to enhance the chemiluminescence response of human neutrophils [10]. The effect of stress on neutrophil functions differs between experiments. Hormone secretion such as aldosterone and corticosteroids is rapidly when animals receive stress, and then the hormones cause redistribution, lysis, or impaired communication between immunocompetent cells [6, 9, 18] and thus interfere with a limiting step of immune system reactions. Although some researchers have shown that beluga whales and bottlenose dolphins had phagocytosis and oxygen radical generation by peripheral blood leukocytes like terrestrial animals [5, 11], the overall effect of these changes on the immunocompetence of transported dolphins is unclear. In the current study, the concentration of serum cortisol was significantly correlated with the results of WBC counts, eosinophil counts, NBT reduction, and phagocytosis. As a result, the stress of handling and transportation not only affects the leukograms of dolphins but also could reduce the PMN cell functions. We did not evaluate the changes of cortical, leukograms, and PMN cell functions after transportation. In terrestrial mammals, these parameters are recovered to a level before transportation within a week at most [15, 22, 27]. These reports showed that immunological uncertainty following transportation would enhance the potential risk of infectious disease in susceptible individuals. Some reports showed that the increase in cortisol under stress continued for 7 hr and decreased within a few days in dolphins [8, 26, 29]. However, no studies have reported changes in the PMN cell functions after transportation. We must complete further research to examine whether PMN cell functions recover to resting values accompanied by recovery of the cortisol level as in terrestrial mammals. Then we can discuss whether the dolphin has increased susceptibility for infectious diseases after transportation as with some domestic animals.

In conclusion, handling and transportation are stressful events for bottlenose dolphins and this is clearly indicated in serum cortisol levels and leukograms. In addition, the handling and transportation stresses suppress PMN cell functions. We must further study the relationship between stresses and other immune system functions in dolphins, and investigate ways to prevent stress-related immune system suppression.

REFERENCES