Urinary Excretion of Cortisol from Rhesus Monkeys (*Macaca mulatta*) Habituated to Restraint

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Abstract | Use of monkeys in research has often required that they be restrained in a chair. However, chair restraint can elicit an initial neuroendocrine stress response. Also, inactivity associated with restraint can induce muscular atrophy. We proposed that prior habituation of monkeys to chair restraint would attenuate these neuroendocrine responses without causing substantial muscle wasting. Four rhesus monkeys (*Macaca mulatta*) were trained and habituated to a restraint chair specifically designed for spaceflight. During the study, monkeys were placed in metabolic cages for 7 days (prerestraint, Phase I), placed in a chair restraint for 18 days (Phase II), and then returned to their metabolic cages for 5 days (postrestraint, Phase III). Urine was collected between 0700-1100 daily, and measurements of cortisol, creatinine, and electrolyte concentrations were adjusted for hourly excretion rates. Body weights of the monkeys did not change between start of the prerestraint and postrestraint phases (10.3 ± 0.8 vs. 10.3 ± 0.9 kg, respectively). During the 3 phases, mean excretion rate of cortisol did not change (24.1 ± 10.3, 26.7 ± 7.7, and 19.3 ± 5.8 μg/h, respectively). Mean excretion rate of creatinine (37.3 ± 7.5, 37.5 ± 12.2, and 36.9 ± 17.1 mg/h, respectively), Na^+ (3.3 ± 1.2, 3.2 ± 1.2, 2.2 ± 1.8 mmol/h, respectively), and K^+ (5.3 ± 1.8, 5.4 ± 1.6, and 4.3 ± 2.8 mmol/h, respectively) were also not altered. Lack of an increase in excreted urinary cortisol suggested that prior habituation to chair restraint attenuated neuroendocrine responses reported previously. Also, the chair restraint method used appeared to allow adequate activity, because the monkeys did not have indices of muscle wasting.

Experiments on monkeys are necessary for the development of countermeasures that will mitigate the effects of weightlessness on astronauts (1). Engineering constraints involving the feeding and drinking mechanisms of monkeys and disposal of their excretions as well as the safety and well-being of the monkeys and crew have necessitated the restraint of animals in a chair for the duration of a flight (12 to 14 days) (2-4). Restraining procedures, however, have elicited questions about the welfare of the monkeys (1, 5, 6). Effects of chair restraint on the health and well-being of the monkeys and on experimental measurements have been subjects of concern among researchers (5, 7). Restraint can elicit a neuroendocrine stress response and contribute to muscular atrophy (5, 7-9). It has been suggested that to study the neuroendocrine systems of monkeys, they must be restrained for at least 2 weeks prior to initiation of an experiment so that they can adapt to the restraining procedures (5, 7). Increases in the amounts of creatinine and potassium excreted have been used as indices of muscle wasting (8, 9). An increase in the amount of glucocorticoids excreted, such as 17-hydroxycorticosteroid (17-OHCS) and cortisol, has been used as a means of quantifying neuroendocrine stress responses (10).

During spaceflight experiments, health of the monkeys, determined on the basis of their constant eating and drinking behaviors, was considered to be good (3, 4). However, there is a paucity of published information on the neuroendocrine response in monkeys as a consequence of restraint during spaceflight. Spaceflight studies on monkeys can cause substantial muscular atrophy (4). In ground-based experiments, monkeys restrained in chairs for an 8-week period had increased 17-OHCS and epinephrine excretion rates during the first week, although modifications of body weight or intake behavior was not observed during the study (5, 7).

We proposed that prior habituation of monkeys to restraint procedures would attenuate an increase in urinary cortisol excretion and negate substantial muscular atrophy, conditions normally associated with restraint. Urinary excretion of cortisol, creatinine, and electrolytes, and food and water intake were compared between nonrestraint (pre- and postrestraint phases) and restraint phases to evaluate the effects of chair restraint on the monkeys.

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Materials and Methods

Four male rhesus monkeys (*Macaca mulatta*) (Charles River Primates, FL), intentionally bred for research purposes were used. Monkeys weighed between 9.6 and 11.5 kg and were between 9 and 10 years old. Monkeys were housed at the NASA Ames Research Center Animal Care Facility. All procedures were reviewed and approved by the NASA Ames Research Center's Institutional Animal Care and Use Committee and adhered to National Institutes of Health guidelines for the humane care and use of animals (11).

Experimental Procedures: Prior to initiation of the study, all monkeys were trained to chair restraint procedures. Monkeys were trained in 2 restraint chairs, the Primate Products (PPR; Primate Products, Redwood City, CA) restraint system and the flight chair restraint (FCR) system. In the PPR system, a monkey was restrained by a collar while seated on its callosities, but they were allowed full range of motion of their hind limbs. The yoke that held the collar was set at a height such that the monkey had a slight curve in its spine, mimicking a normal unrestrained sitting posture. The monkey was able to rotate 360° in the chair. The FCR system used a jacket with a soft waist divider as the main form of restraint when a monkey was seated on its callosities with its hind limbs extended. Shoulder straps on the jacket assisted with restraint; however, the straps did not prevent the monkey from leaning forward. The jacket had an additional piece of material at the waist into which a solid horseshoe-shaped frame was inserted. The material with the frame was slid into a groove in the chair and locked in place. This prevented the monkey from removing the penis sheath which was used to collect urine. The monkey could rotate the upper part of its torso, but could not rotate the lower part of its body. A bar was placed across the upper portion of the thighs to prevent the monkey from pulling its hind limbs in close and removing the urine collection system.

Both restraint chairs were similar in terms of the range of motion allowed to the monkey and the positioning of the restraining device. Two types of chairs were used, because there was a greater availability of PPR system chairs initially; therefore, we could accommodate more monkeys to training and handling during a shorter period. The FCR system was in a constant stage of development during the time in which we were conducting PPR system training; thus, only a few systems were available at a

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time. Therefore, all preliminary training was done in the PPR chair and more-advanced training was done in the FCR chair.

Monkeys were trained to restraint procedures during a 4-year period. Initial adaptation to the PPR system consisted of restraint for 4 to 8 h. Monkeys were progressively trained to be restrained for up to 20 days. They were then maintained on 10-day restraint periods alternated with periods of nonrestraint throughout the remainder of their training. Monkeys were given a minimum of twice the time out of restraint for their time in restraint (i.e., 4 h in restraint resulted in a minimum of 8 h of nonrestraint. During training, monkeys were offered fruit as rewards. Monkeys were fed a pelleted feed via a psychomotor test system, a joystick-based automated computer training system (12). Monkeys in the FCR system could move and shift sufficiently to use the psychomotor test system computer. Once monkeys were habituated to the PPR system, they were trained to the FCR system used in this study for periods of restraint of up to 20 days.

Approximately 5 weeks prior to the start of the experiment, monkeys were surgically instrumented for other experiments being conducted simultaneously. Surgical procedures consisted of implantation of electrodes for electromyographic recordings in their hind limbs, and muscle, bone, and lymph node biopsies. Surgery was performed under general anesthesia, using sterile techniques. Monkeys were evaluated by the veterinary staff and found to be in good health prior to initiation our study.

When monkeys were not restrained, they were placed in separate metabolic cages (27"x 30"x 36") that did not restrict movement. Initially, monkeys were placed in their metabolic cages for 7 days (prerestraint, phase I). Food and water consumption were monitored daily. A urine sample was collected throughout the 4-h period between 0700 and 1100 h. If a sample was not obtained, the collection period was continued for an additional 4 h. Urine samples were collected in containers attached to the bottom of the metabolic cages. Urine volumes were recorded, and an aliquot was frozen for subsequent analysis. On the eighth day, monkeys were placed in the FCR system for the restraint phase of the study (phase II). At that time, they were placed in the jacket and fitted with a penis sheath for collection of subsequent urine samples. Sedation of monkeys was not required, because they cooperated with their placement in the FCR system. Monkeys remained in the FCR system for 18 days before being returned to their metabolic cages for an additional 5 days (postrestraint, phase III).

Sample analyses: After extraction of urinary cortisol by dichloromethane, concentrations were measured by use of a radioimmunoassay kit (DPC, Los Angeles, CA) with a sensitivity of 0.2 µg/dl. All samples for a specific monkey were measured in the same assay. Excretion rates were calculated as the volume of urine collected over time multiplied by urine concentration of cortisol, and expressed as an hourly excretion rate. Samples were also analyzed for sodium, potassium, and creatinine concentration on a Cobas auto-analyzer system using electrochemical detectors (Roche Diagnostics, Somerville, NJ).

Statistical analysis: Comparisons were made among the 3 phases. Values for the nonrestraint phases (Phase I and III) was used as baseline control values to which the restraint phase values were compared for determine of significant increases in the measured variables. Results were expressed as mean ± 1 SD. Significant differences were determined, using a repeated-measures analysis of variance (ANOVA). When differences in means were detected over time, a Neuman-Keuls test was performed. Differences were considered significant at $P \leq 0.05$.

Results

The experiment was terminated in one monkey after 23 days (16 days of restraint) because of an ulcer on its right callosity. Values for this monkey during the 23-day period were not significantly different from those for the other monkeys. Data analysis after the twenty-third day was continued with the 3 remaining monkeys.

A difference in mean urine flow rates was not observed between phases I and II (96.4 ± 28.0 ml/h vs 120.6 ± 35.3 ml/h, respectively) (Figure 1). However, the rate for phase III (74.9 ± 30.0 ml/h) was significantly ($P = 0.01$) less than phase II. During

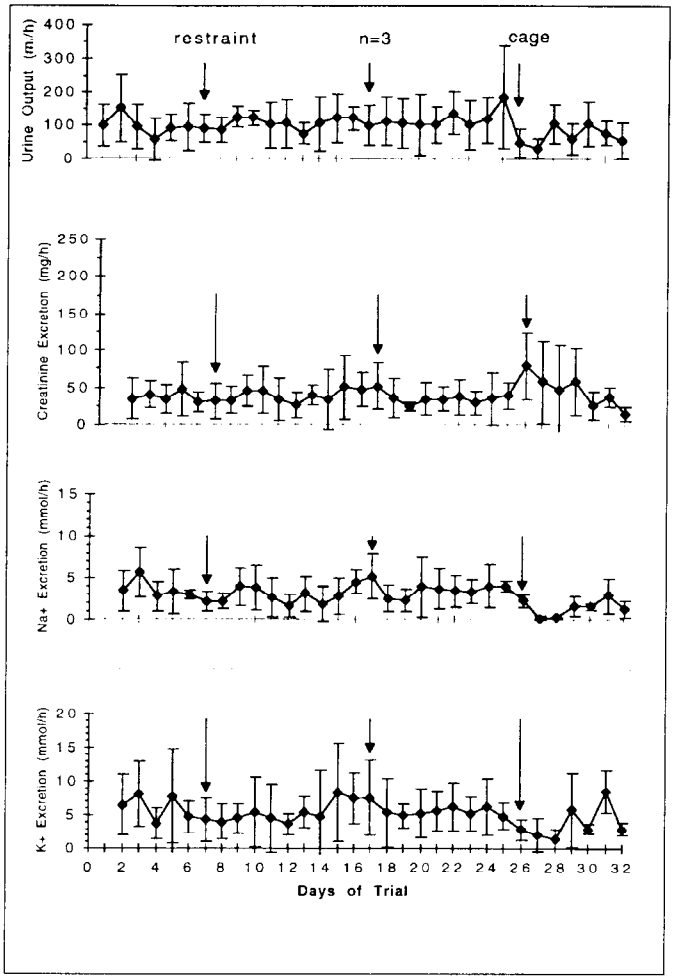

FIG. 1. Results of analysis of urine samples obtained from Rhesus monkeys used in a restraint study. A significant ($P = 0.01$) decrease was observed in urine output during the postrestraint phase. "Restraint" denotes the day monkeys were placed in their restraining chair; "n=3" denotes the day 1 monkey was removed from the study and means were calculated from the remaining 3 monkeys; and "cage" denotes the first full day monkeys spent in their metabolic cages after being restrained. Values represent mean ± 1 SD.

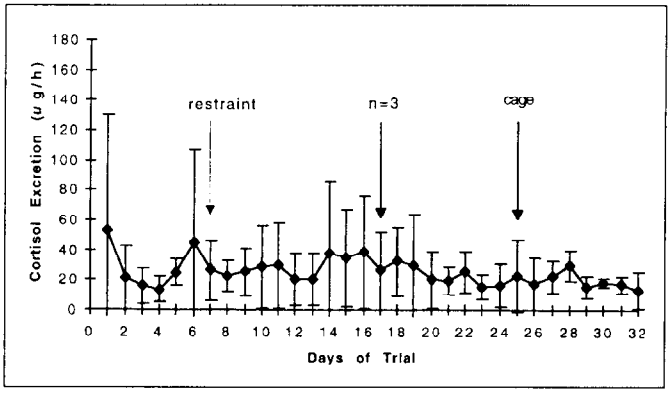

FIG. 2. Results of analysis of urinary cortisol excretion in Rhesus monkeys during a restraint study. Significant differences in cortisol excretion were not detected among the 3 phases (prerestraint, restraint, and postrestraint) of the study. Values represent mean ± 1 SD. See Figure 1 for key.

these 3 phases, mean excretion rates of creatinine (37.3 ± 7.5, 37.5 ± 12.2, and 36.9 ± 17.1 mg/h, respectively), sodium (3.3 ± 1.2, 3.2 ± 1.2, and 2.2 ± 1.8 mmol/h, respectively), and potassium (5.3 ± 1.8, 5.4 ± 1.6, and 4.3 ± 2.8 mmol/h, respectively) were not significantly altered. Mean excretion rate of cortisol during the 3 phases also did not change significantly (24.1 ± 10.3, 26.7 ± 7.7, and 19.3 ± 5.8 µg/h, respectively) (Figure 2).

Weight of monkeys did not change significantly between the start of the prerestraint and the start of the postrestraint phases (10.3 ± 0.8 vs 10.3 ± 0.9 kg, respectively). Daily water intake was not altered between phases I and II (1,502.1 ± 101.0 vs. 1,485.4 ± 184.2 ml/day, respectively). Mechanical problems with the feeders during phase I of the study resulted in nonquantitative values for food consumption. Therefore, values for food consumption during phase II were compared with values for phase III. Again, daily food intake remained constant throughout the course of the restraint phase and was similar to the postrestraint phase (290.4 ± 47.7 vs. 234.2 ± 56.7 g/d, respectively).

Discussion

Rhesus monkeys have been used in spaceflight experiments to quantify the effects of microgravity so that researchers can develop countermeasures to alleviate the consequences of weightlessness (1). However, concerns have arisen regarding the health and well-being of the monkeys during spaceflight, partly due to the fact that the monkeys must remain restrained in their chairs throughout the duration of the flight (1, 6).

In the study reported here in which the monkeys were habituated to the method of restraint, there did not appear to be a neuroendocrine stress response induced by chair restraint as indicated by a lack of increase in cortisol excretion during the restraint phase. Also, substantial muscular atrophy was not apparent as indicated by a lack of increase in creatinine and potassium excretion during the restraint phase. Daily food and fluid intake were also not altered, providing evidence that the monkeys were in good health.

Glucocorticoid excretion values from phase 1 of our study (24.1 ± 10.3 µg/h) were similar to those during a 4-week prerestraint period in another study (18.5 ± 2.9 µg/h) (5). However, in that study, glucocorticoid values during chair restraint were increased threefold above baseline values for the first 3 days of restraint. The increase of 17-OHCS excretion led those authors to suggest that 2 to 4 weeks of chair adaptation be used prior to initiation of a study. The lack of an increase in urinary excretion of cortisol in our study suggested that habituation to restraint attenuated the neuroendocrine response reported in previous studies.

Restraint of monkeys for extended periods can result in substantial muscular atrophy (13-16). This atrophy, associated with cellular wasting, results in an increased excretion of creatinine and potassium (15, 16). In the study reported here, in which food intake and body weight were not altered, we did not observe a change in creatinine or potassium excretion. This suggested that pathologic alteration in muscle mass did not develop under the study conditions.

Although definitive conclusions cannot be drawn from such a small sample, analysis of the data indicated that habituating monkeys to chair restraint would result in minimal effects on neuroendocrine responses, muscular atrophy, and daily food and water intake. Lack of a change in urinary excretion of cortisol suggested that the monkeys were well trained to the restraint procedures. Therefore, an acclimation period of restraint prior to the initiation of experiments would not be warranted for previously habituated monkeys. Furthermore, the restraint used in this study appeared to allow adequate activity, because the monkeys did not exhibit indices of muscle wasting. Training and restraint procedures used in this study would appear to have minimal impact on scientific studies performed to evaluate the pituitary-adrenal axis.

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