Effects of Transportation on Antioxidant Status in Cynomolgus Macaques (Macaca fascicularis)

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To evaluate the effects of transportation on oxidative stress in cynomolgus monkeys, we measured serum levels of reduced glutathione (GSH), malondialdehyde, and protein carbonyl (PC) and the activities of total antioxidant capacity (TAOC), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase in cynomolgus macaques before transportation (day 0), on the day of arrival (day 1), and on days 7, 14, and 21 after transportation. Compared with that on day 0, TAOC and catalase activities on days 1, 7, and 14 after transportation were significantly decreased, reached their nadirs on day 7, and increased thereafter to reach their pretransportation levels by day 21 after transportation. Compared with day 0 levels, mean SOD activity and GSH concentration were decreased significantly on day 1; they thereafter increased to reach their pretransportation measures by day 7 after transportation. In contrast, PC and malondialdehyde concentrations in serum and the activity of GSH-Px were increased on day 1 compared with day 0 and thereafter decreased to reach their pretransportation levels by day 14 after transportation. In summary, GSH, TAOC, catalase, and SOD levels decreased and malondialdehyde, PC, and GSH-Px concentrations increased in cynomolgus macaques after transportation. These results suggest that transportation might imbalance oxidant and antioxidant levels to create excess oxidative stress in cynomolgus macaques. Therefore, cynomolgus macaques should have at least 21 d to recover after transportation and regain their healthy status.

Abbreviations: GSH, reduced glutathione; GSH-Px, glutathione peroxidase; PC, protein carbonyl; ROS, reactive oxygen species; SOD, superoxide dismutase; TAOC, total antioxidant capacity

Long- or short-distance transportation of NHP from a vendor to a research facility is inevitable, due to the uneven geographic distribution of NHP (for example, cynomolgus macaques [Macaca fascicularis]). The stress associated with the transportation of NHP in scientific research influences both their physical and mental health. A previous study on transportation-related stress in cynomolgus macaques showed increases in cortisol levels and the neutrophil-lymphocyte ratio on the day of arrival. Stressful conditions can lead to an imbalance between oxidants and antioxidants in favor of oxidants at the cellular or individual level. Alteration of the oxidative balance produces oxidative stress that causes cellular damage and makes the organism sensitive to disease. These alterations are also explained as a resisting mechanism to the negative effects of reactive oxygen species (ROS)—including hydroxyl (HO•), peroxyl (RO2•), alkoxyl (RO•), and superoxide anion (O2•−) radicals and nonradical species, such as hypochlorous acid (HOCl), ozone (O3), and hydrogen peroxide (H2O2)—all of which cause the peroxidation of membrane lipids and protein oxidation. To protect against damage by free radicals, cells have a variety of antioxidant systems in different cellular compartments, some of which are enzyme-based whereas others are not. Main antioxidant enzymes in the body include superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px), and glutathione (GSH) reductase. These antioxidant enzymes react indirectly or directly with ROS.

ROS damage all types of biologic molecules, including protein, lipid, and DNA. Protein carbonyl (PC) groups are produced on protein side chains when they are oxidized. As biomarkers of oxidative stress, the usage of PC groups has some advantages because of their relative early formation and the relative stability of carbonylated proteins. Malondialdehyde, one of the end-products of lipid peroxidation, has been widely applied as the most common biomarker for the assessment of liperoxidation in biologic and medical sciences. Total antioxidant capacity (TAOC) reflects overall response of the nonenzymatic antioxidants and enzymatic antioxidants. To date, few scientific studies have addressed the effects of transportation on oxidative stress in cynomolgus macaques used in the safety evaluation and research of new drugs, chemical agents, vaccines, biologic products, and other substances. Because cynomolgus macaques are widely used in biomedical research and given that transportation from the breed supplier to a research facility is inevitable, it is worthwhile to evaluate the effects of transportation on oxidative stress in cynomolgus macaques. The aim of this study was to evaluate the effects of transportation on antioxidant status (SOD, catalase, GSH-Px, TAOC, and GSH), the production of lipid peroxidation (malondialdehyde), and protein modification (PC) in cynomolgus macaques to identify biomarkers to facilitate improving transportation conditions and to provide useful information for veteraninc and researchers for monitoring animal health and welfare.

Materials and Methods

Animals and transportation. Clinically healthy cynomolgus macaques (n = 16; 8 male and 8 female; age, approximately 4 y; weight, approximately 4 kg) were evaluated in this study. All monkeys were free of Cercopithecine herpes virus type I (B virus), SIV, simian poxvirus, simian T lymphotropic virus type 1, simian retrovirus type D, Mycobacterium tuberculosis, Shigella spp., Salmonella spp., Yersinia enterocolitica, Campylobacter jejuni, pathogenic fungal fungi, ectoparasites, and endoparasites.
The temperature and relative humidity in the animal holding room at our facility (National Shanghai Center for New Drug Safety Evaluation and Research, Shanghai, China) were kept at 18 to 24 °C and 40% to 70%, respectively; these parameters were recorded every hour by a built-in automatic system. Lights were on from 0700 to 1900 on 150 to 500 lx, with 15 to 20 fresh-air changes (100%) hourly. The environmental parameters at Hainan Jingang (Hainan Jingang laboratory animal Co., Ltd., Haikou, Hainan province, China) were kept naturally. Twice each day, macaques were fed high-quality commercial pelleted feed (at the research facility: Beijing Ke'e Xieli Feed, Beijing, China; at Hainan Jingang: Guangzhou Guolong Technology, Guangdong, China) that met or exceeded the nutrient recommendations of the National Research Council; fruits were supplemented once daily at both places. Environmental enrichment consisted of commercial toys. All macaques were housed individually in stainless steel wire cages (800 x 900 x 900 mm) that included a squeeze board and 2 side boards that could be removed to accommodate pair-housed animals. Macaques had free access to fresh deionized drinking water.

The monkeys experienced combined air and road transportation from Hainan to Shanghai. The total transportation time of 8 h was divided as following: 1 h loading, 0.5 h road transportation to the airport, 1 h waiting to board in Hainan, 3 h in airplane, 1 h waiting after landing in Shanghai, 1 h road transportation to the research facility, and 0.5 h unloading. The transportation cages (400 x 240 x 500 mm) were made of plywood, and the macaques could see and hear each other through the center grid wall of the transportation cage. Each set of transportation cages consisted of 5 individual cages. Air-conditioned vans were used in the transportation from the animal vendor facility to the airport at Hainan and from the airport to the final facility in Shanghai. To prevent from causing physical harm, unnecessary discomfort, overheating, excessive cooling, behavioral stress, or trauma, all animals were handled as carefully as possible during the transportation process. The daily minimal and maximal outdoor temperatures were 19 and 22 °C in Hainan and 3 and 13 °C in Shanghai, respectively.

The protocol was reviewed and approved by the IACUC of the National Shanghai Center for New Drug Safety Evaluation and Research, which is AAALAC-accredited.

Preparation of serum. Blood samples (2 mL) were collected from conscious macaques through venipuncture of cephalic vein by the same experienced technician specialist using disposable syringes before transportation (day 0), on the day of arrival (day 1), and at 1 wk (day 7), 2 wk (day 14), and 3 wk (day 21) after transportation. Blood samples were added to EDTA-treated serum separator tubes; serum was obtained after centrifugation at 1000 x g, 4 °C for 10 min and stored at –80 °C until analysis.

Assays of SOD, catalase, GSH-Px, and TAOAC activities and GSH, PC, and malondialdehyde levels. Serum antioxidant enzymes activities (SOD, catalase, GSH-Px, TAOAC) and the levels of GSH, PC, and malondialdehyde in serum were determined by using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. The SOD activity was measured based on the ability of xanthine oxidase and xanthine to generate superoxide radicals, which reacted with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride to form a red formazan dye. The colorimetric absorbance was determined at 550 nm. The SOD activity was calculated by the degree of inhibition of this reaction. One unit of SOD activity was defined as a 50% inhibition of reduction of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride under the condition of the assay.22,47 The SOD activity was expressed in U/mL serum.

The malondialdehyde level in serum was measured based on the reaction of malondialdehyde and thiobarbituric acid to produce a pink-colored complex, which was detected at 532 nm by using colorimetric absorbance.46,44 The malondialdehyde level was expressed in nmol/mL serum. The kit for PC used the reaction of 2,4-dinitrophenylhydrazine with the carbonyl groups in proteins to produce a compound that was quantified by colorimetry at 370 nm.9,44 The PC level was expressed in nmol/mg protein. The GSH-Px activity assay was based on the fact that GSH-Px catalyzes peroxide and reduces glutathione to produce water and oxidized glutathione. GSH-Px activity can be represented by its enzymatic reaction rate and can be calculated by measuring GSH consumption rate in this enzymatic reaction. These 2 substrates (GSH and peroxide) can undergo redox reaction without enzyme (noinzymatic reaction). Therefore, the GSH consumption caused by nonenzymatic reaction was deducted during the calculation of GSH-Px activity. GSH reacts with dithiobisnitrobenzoic acid to produce 5-thio-dinitrobenzoic acid anions, which has a relatively stable yellow color that can be detected at 412 nm to estimate the change in the GSH level.16,22,34 In a reaction at 37 °C for 5 min, a decrease of 1 μmol/L GSH concentration (less the effect of the nonenzymatic reaction) per 0.1 mL serum was considered as 1 unit of GSH-Px activity.

GSH (deproteinization) is a tripeptide composed of glutamic acid, glycine, and cysteine and is the main nonprotein sulfhydryl compound. Dithiobisnitrobenzoic acid reacts with sulfhydryl compounds to produce a yellow compound, which can be quantitatively estimated by colorimetry at 420 nm. The GSH concentration was expressed in mg/L. Catalase activity was determined based on the H2O2 substrate, and the enzymatic reaction was stopped by the addition of ammonium molybdate. The intensity of the yellow complex formed by molybdate and H2O2 was measured at 405 nm.56,57 Catalase activity was defined such that 1 μmol H2O2 decomposition per mL serum per second was considered as 1 unit of activity.

TAOC activity was measured by its ferric reducing ability of the supernatant. The stable color of the Fe3+-o-phenanthroline complex (produced by the reducing agents in serum reducing Fe3+ to Fe2+, which reacted with the substrate o-phenanthroline) was detected at 520 nm. TAOC was expressed in U/mL serum where 1 U was defined as an increase in absorbance (520 nm) of 0.01/min/mL serum at 37 °C.47

Statistical analysis. Data were analyzed using SPSS Statistics 21.0 (IBM, Armonk, NY). All parameters including TAOC, catalase, SOD, GSH, PC, GSH-Px, and malondialdehyde were analyzed for statistical significances before and after transportation by homogeneity of variance according to the Levene test. When group variances were homogeneous, oneway ANOVA was used to determine whether any statistically significant differences existed between the pre- and posttransportation groups. If the analysis of variance gave a significant result, the Dunnett test was performed to detect any significant differences between the pre- and posttransportation groups. For all tests, a P value of 0.05 (2 tailed) was used as the criterion for significance. Percentage change and principal components analysis were applied to analyze the relationship of TAOC, catalase, SOD, GSH, PC, GSH-Px, and malondialdehyde in cynomolgus macaques after transportation.

Results

The TAOC, catalase, SOD, malondialdehyde, GSH, GSH-Px, and PC activities or levels at pretransportation and different
time point after transportation are presented in Figure 1. TAOC activity on days 1, 7, and 14 after transportation showed a significant decrease ($P < 0.001$) compared with that on day 0, reached its lowest activity on day 7, and increased thereafter to reach its pretransportation activity by day 21 after transportation. Catalase activity on day 1 ($P < 0.001$), day 7 ($P < 0.001$), and day 14 ($P < 0.01$) after transportation showed a significant decrease compared with that on day 0, reached its lowest activity on day 7 posttransportation, and increased thereafter to reach its pretransportation activity by day 21. Significant decreases in the mean SOD activity ($P < 0.001$) and GSH level ($P < 0.05$) were noted on day 1 after transportation and returned to their pretransportation levels by day 7. The mean levels of PC ($P < 0.001$), GSH-Px ($P < 0.001$), and malondialdehyde ($P < 0.01$) showed a significant increase on day 1 as compared with those before transportation and thereafter decreased to their pretransportation levels by day 14. The PC level and GSH-Px activity on days 1 and 7 after transportation showed a significant increase as compared with those on day 0. By day 14, both PC and GSH-Px had returned to normal levels, but a higher GSH-Px activity was noted on day 21 ($P < 0.05$). There was no statistically significant difference between male and female animals.

The percentages of change in TAOC, catalase, SOD, malondialdehyde, GSH, GSH-Px, and PC are shown in Figure 2. Generally, elevated parameters were malondialdehyde, PC, and GSH-Px and decreased parameters were GSH, catalase, TAOC, and SOD. Results of principal components analysis are shown in Figure 3. The 7 parameters of antioxidative and oxidative statuses were classified into 3 groups by using the 2-factor loading plots: (1) SOD and GSH; (2) TAOC and catalase; and (3) PC, GSH-Px, and malondialdehyde. Parameters within the same group were correlated with each other.

**Discussion**

Reactive species are produced by body tissues and fluids. Imbalance between reactive species production and endogenous antioxidants can result in oxidative stress, which disrupts or damages essential cellular functions. The present study investigated the effects of transportation on oxidative stress damage. The transportation was able to change serum antioxidant enzyme activities and contents of production of oxidative stress. Here, increased levels of malondialdehyde, PC and activity of GSH-Px, and decreased activities of SOD,
are important in the control of whole-body homeostasis and the organism’s response to stress.

Carbonic (CO) groups (for example, in aldehydes and ketones) are produced on protein side chains (especially of Pro, Arg, Lys, and Thr) when they are oxidized.\(^{25}\) CO groups, which are normally absent from cellular proteins, are thought to be formed after free-radical attack on amino acid residues. CO groups are only byproduct that results from oxidative attack on proteins, but because the frequency at which the production of carbonyl residues on a typical protein alters its function is unknown, they may signal the occurrence of significant damage.\(^{10,32}\) In the present study, the significantly increased level of PC in cynomolgus macaques on day 1 compared with that before transportation showed that the proteins in serum were attacked by free radicals and that antioxidant imbalance was present. The increase in both malondialdehyde and CO concentrations further suggested that the oxidative status was favored over the antioxidant status in cynomolgus macaques after transportation.

As the major defense against superoxide radicals, SOD plays an important role in defense mechanism of biologic cells exposed to oxygen. SOD is the first line of defense against oxidative stress and represents a group of enzymes that catalyze the dismutation of superoxide anion radical (\(O_2^-\)) and the formation of hydrogen peroxide (\(H_2O_2\)).\(^{30}\) Our results were consistent with the findings that the mean activity of SOD significantly decreased during and after transportation in goats and horses.\(^{23,35}\) The decrease in SOD activity could be due to the excessive production of ROS during transportation-induced stress, which eventually exceeds the body’s antioxidant capacity and therefore reduces SOD activity.

Catalase is one of the major antioxidant enzymes in cells, particularly cell structures that use oxygen-producing \(H_2O_2\). Catalase catalyzes \(H_2O_2\) into water and oxygen. Here, the decrease in catalase activity could be due to the excessive production of \(H_2O_2\) during transportation, thus consuming catalase. Catalase activities were decreased in the liver and stomach of rats experiencing immobilization stress.\(^{41}\)

GSH-Px exists in cell cytosol and mitochondria and has the ability to transform \(H_2O_2\) into water. This reaction uses GSH and transforms it into oxidized glutathione. GSH-Px and catalase have the same action on \(H_2O_2\), but GSH-Px has a higher affinity at high ROS concentration, and catalase has an important action with lower \(H_2O_2\) concentration. To protect an organism against oxidative damage, GSH-Px catalyzes the reduction of \(H_2O_2\) and lipid hydroperoxides to \(H_2O\) and their corresponding alcohols, respectively. A previous study showed that GSH-Px activity increased when an oxidative stress situation occurred, such as hyperglycemia or lipid oxidative damage.\(^{17,25,40}\) Here, the increased GSH-Px activity of serum could be interpreted as a healthy defensive response against oxidative injury during oxidative stress in cynomolgus macaques.

Tripeptide GSH (γ-L-glutamyl-L-cysteinyl-glycine) is found in almost every cell at levels from 10 to 100-folds higher than its oxidized form under physiologic conditions. Due to its high concentration under physiologic conditions and its central role in maintaining the cell’s redox state, GSH is the most important cellular antioxidant. Mixed-disulfide GSH is produced mainly by the catalysis of GSH-Px as well as through the direct interaction of GSH with electrophilic compounds, for example, radical species.\(^{4}\) Here, cynomolgus macaques showed significantly decreased concentrations of serum GSH after transportation. This decrease indicated that our cynomolgus macaques experienced considerable oxidative stress during transportation. In addition, the decrease in GSH levels were in accordance with

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**Figure 2.** Percentage change of markers of oxidation status in cynomolgus macaques after transportation. (A) Increased level or activity of malondialdehyde, protein carbonyl, and glutathione peroxidase after transportation. (B) Decreased level or activity of glutathione, superoxide dismutase, total antioxidant capacity, and catalase after transportation.
the increased activities of GSH-Px, given that GSH-Px can cause reduced GSH to revert to oxidative GSH.

TAOC has been described as a critical tool for assessing redox status. TAOC is a reliable and sensitive biomarker to monitor changes of in vivo oxidative stress, which might not be measurable through the detection of single, more specific, antioxidants. TAOC depends mostly on the concentrations of albumin and uric acid, whereas the remaining biomarkers depend on other molecules such as low-molecular-weight antioxidants, macromolecules, and different types of enzymes, such as catalase, SOD, GSH-Px and so on. In the present study, the decrease in TAOC activity in cynomolgus macaques after transportation is consistent with previous reports that transportation stress significantly decreased the mean serum TAOC activity of beef cattle. In addition, the decrease in TAOC activity is in accordance with the decreases in the main enzymatic compounds, such as catalase and SOD activities and GSH levels, noted in the current study.

All of these antioxidant enzymes not only synergistically prevent the damage caused by ROS, but they also protect against each other. The superoxide anion can inactivate catalase and GSH-Px, but SOD can catalyze the dismutation of superoxide anion into H$_2$O$_2$. H$_2$O$_2$ inactivates SOD and catalase, but GSH-Px catalyzes H$_2$O$_2$ into H$_2$O and O$_2$ (Figure 4). Therefore, once a member in this mutual protection system is abolished or decreased, the entire enzymatic protection system might collapse, leading to irreversible cell damage and, potentially, clinical symptoms including decreased appetite, activity, immunity, and so on.

The antioxidative parameters of SOD and GSH, of TAOC and catalase, and of the oxidative parameters PC and malondialdehyde were positively correlated with each other in cynomolgus macaques after transportation (Figures 2 and 3). In addition, GSH-Px was positively correlated with PC and malondialdehyde. Perhaps GSH-Px had higher affinity with H$_2$O$_2$ at high ROS concentration, and catalase had an important action at lower H$_2$O$_2$ concentration. However, GSH-Px and catalase had the same action upon H$_2$O$_2$. However, a few limitations in the present study might have influenced the results. Blood collection might be a potential stressor in conscious animals. Therefore, to minimize this effect, the procedure was conducted by the same experienced technician specialist. Repeated blood collection might be another factor to cause stress in our animals. In this study, only a minimal amount of blood (2 mL) was collected each time point. A previous study indicated that removal of 7.5% of the total blood caused no disturbance to the normal physiology of cynomolgus macaques. In addition, the removal of as much as 40% of a rat’s total blood volume over 24 h and again 2 wk later caused no gross ill effects. Withdrawing 2 mL blood (that is, 0.6% to 0.8% of the total blood volume) from each macaque weekly for 3 wk likely did not affect the transportation-related effects noted in the current study.

A small sample size poses challenges to any statistical analysis and leads to decreased predictive potential when compared with models developed with more events. As sample size increases, accuracy typically increases also, until the maximal potential accuracy is achieved, thereby reaching an asymptote. Having an appropriate number of animals for each experiment is crucial: too many animals can lead to unnecessary use, and too few animals might require repetition of the entire experiment due to difficulties in analysis of the results, thus wasting animals. A previous study on transportation-related stress used 6 animals per group, which met the requirement of biostatistics. In the current study, we used 8 animals per sex per group to assess the effects on oxidative stress in cynomolgus macaques after transportation, and we noted statistically significant changes in all parameters examined. In addition, to minimize interanimal
variability and to reduce animal use, each macaque served as its own control.46

In conclusion, our results showed that increases in the activity or level of malondialdehyde, PC, and GSH-Px and decreases in GSH, SOD, catalase, and TACO occurred after transportation of cynomolgus macaques (Figure 4). These parameters returned to basal levels after approximately 21 d of recovery. Our findings indicate that transportation caused excessive oxidative stress and resulted in the imbalance of the oxidant and antioxidant systems in cynomolgus macaques. Veterinarians and researchers should be aware that these transportation-induced changes might affect animal health and research results. Therefore, cynomolgus macaques should be allowed to recover for at least 21 d to regain their healthy status after transportation.

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References


