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Reference Intervals for Preprandial and Postprandial Serum Bile Acid in Adult Rhesus Macaques (*Macaca mulatta*)

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The purpose of this study was to determine the 12-h fasting preprandial and 2-h postprandial serum bile acid concentration (SBAC) reference intervals for healthy, adult rhesus macaques (*Macaca mulatta*). We hypothesized that the mean 2-h postprandial SBAC would be significantly higher than the mean preprandial SBAC. We included 40 (24 male, 16 female) macaques after confirming that their health records, physical examinations, CBC, serum chemistry panels, and urinalyses were all within normal limits. In addition, hepatitis A titers were determined, an ultrasound examination of the liver was performed, and two 16-gauge ultrasound guided percutaneous liver biopsies were collected and submitted for histopathology. Macaques were confirmed healthy according to hepatitis A screens and sonographic and histologic evaluation of hepatic tissue. Within 2 wk of the screening procedures, preprandial and postprandial SBACs were measured. Preprandial SBAC (mean ± 1 SD) was 11.1 ± 1.9 µmol/L and postprandial SBAC was 19.7 ± 8.0 µmol/L, which was significantly higher than the preprandial value. Sex and hepatitis titers did not significantly influence preprandial and postprandial SBAC. The current study indicates that the SBAC reference values for rhesus macaques are higher than those reported for humans and companion animals.

Abbreviations: HAV, hepatitis A virus; SBAC, serum bile acid concentration.

Bile acids are amphiphilic organic acids (detergent-like compounds) that are synthesized exclusively in the liver and derived from cholesterol.[5] Their principal functions are to improve intestinal fat assimilation and to eliminate bilirubin and cholesterol.[5] Changes in bile acid metabolism and excretion are a reflection of liver dysfunction.[3] Thus the measurement of total serum bile acid concentration (SBAC) is a sensitive and noninvasive method of identifying hepatic disease.[3,5-7]

The physiology of bile acid synthesis, metabolism, excretion, and enterohepatic circulation is complex and influenced by several factors. Bile salts are synthesized by hepatocytes using cholesterol as a precursor and producing the primary bile acids, cholic acid and chenodeoxylic acid.[15] Secondary bile acids are formed by dehydroxylation during passage through the intestines. In rhesus macaques, the main secondary bile acid is deoxycholic acid. After synthesis, the bile acids are conjugated in the liver, preferentially with taurine, in rhesus macaques.[28] This conjugation process improves their solubility in aqueous solution, where they aggregate to form micelles that can incorporate cholesterol and phospholipids.[3] The bile acids then are transported across the hepatocyte membrane into the biliary canaliculi, through the biliary ducts, and are eventually stored in the gall bladder. When the gall bladder contracts, bile acids are released and pass through the common bile duct into the duodenum. Once the bile acids reach the small intestine, principally the ileum, they are actively transported into the portal circulation, circulated through the sinusoids, and are actively recaptured by the hepatocytes. The enterohepatic circulation of bile acids is 95% efficient.[29]

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In the preprandial fasting state, bile acids are found in low concentration in the peripheral blood and are present due to the leakage of bile acids from the enterohepatic circulation. The SBAC is a measure of this spillover. After the ingestion of a meal, especially one high in fat, the gall bladder contracts. This contraction is mediated by cholecystokinin released in response to food, especially fat, in the upper gastrointestinal system. Subsequently, the concentration of bile acid in the portal vein increases, and this elevation is reflected in the peripheral blood as a postprandial increase in SBAC.

In liver disease, the ability of hepatocytes to recapture bile acids may be decreased, resulting in high postprandial SBAC. An endogenous challenge to the enterohepatic circulation of bile acids is used clinically in small animal and human medicine to evaluate liver function. Preprandial fasting and 2-h postprandial SBAC are used for evaluation.[7] Liver function can be evaluated by other methods as well.[3,27]

The median preprandial and postprandial values can be elevated significantly in dogs with several types of liver disease, including cirrhosis, chronic hepatitis, hepatic necrosis, cholestasis, neoplasia, and portosystemic vascular anomaly.[6] Nonspecific clinical signs often are associated with liver disease (weight loss, lethargy, gastro-intestinal and neurologic abnormalities). SBAC values are especially useful in diagnosing hepatic dysfunction in these cases where there is no icterus and no abnormal (or only mildly increased) liver enzyme activity, such as can be seen with hepatoportal perfusion abnormalities or severely reduced hepatic mass.[6,7] In conjunction with historic data, physical examination, and clinical pathology findings, preprandial and postprandial SBAC provide a convenient noninvasive test that has high sensitivity and variable specificity for hepatic disease in companion animals.[7]

Currently there are no normal reference intervals for preprandial and postprandial SBAC in rhesus macaques. The primary

objective of this study was to establish 12-h fasting preprandial and 2-h postprandial SBAC reference intervals in healthy captive indoor-housed adult rhesus macaques (*Macaca mulatta*). We hypothesize that, as in companion animals, mean postprandial SBAC in rhesus macaques will be significantly higher than mean preprandial SBAC. In addition, a secondary objective was to compare the SBAC of rhesus macaques to defined SBAC references intervals reported for dogs, cats, and humans.

Materials and Methods

The animal care and use program at University of California–Davis is AAALAC-accredited and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*[16] and IACUC-approved protocols.

Animals and housing. Randomly selected adult male (n = 24; age, 10.9 ± 2.2 y; weight, 12.2 ± 1.5 kg) and female (n = 16; age, 8.8 ± 3.1 y; weight, 6.6 ± 1.8 kg) rhesus macaques (*Macaca mulatta*) were used in the study. These animals were from the conventional colony and therefore considered to be positive for B virus. Most (39 of 40) macaques were screened for simian retrovirus, simian T lymphotrophic virus 1, and SIV also; all screened animals were negative for these pathogens with the exception of one macaque that was positive for simian T lymphotrophic virus 1. The macaques were housed indoors at the California National Primate Research Center in stainless steel cages (Lab Products, Seaford, DE) at 22 °C, with ambient humidity between 30% to 70%, on a 12:12-h light:dark cycle. Air changes were 10 to 15 per hour. Tap water was available ad libidum, and the macaques were fed commercial chow (High Protein Diet, Ralston Purina, St Louis, MO) and fruit. Compatible animals were paired for 8 h daily.

Inclusion criteria. Prior to their incorporation in the study, all macaques were screened to assess their health status. The medical record of each animal was reviewed and evaluated for weight loss, diarrhea, and any clinical signs associated with liver dysfunction. Animals were included in the study only if they were clinically healthy and had a stable weight over the previous 3 mo.

The physical exam screenings was performed under ketamine (10 mg/kg IM; Ketaject, Phoenix Pharmaceutical, St Joseph, MO) after a 12-h fast. The screening included a complete physical exam, hematology, serum chemistry panel, urinalysis, hepatitis A titer, and a coagulation panel. A CBC count with differential (ABX Pentra 60+, ABX Diagnostic, Irvine, CA) was performed at the California National Primate Research Center clinical laboratory on an EDTA-treated blood sample. A manual differential was performed on WBC; erythrocyte morphology was evaluated on the blood smear also. Total plasma protein and fibrinogen levels were determined by using a refractometer (T/S Meter, Reichert, Depew, NY).

Serum chemistry panels were performed on a Hitachi 917 (Roche Biomedical, Indianapolis, IN) at the University of California–Davis Veterinary Medical Teaching Hospital's clinical laboratory. Urine was collected by cystocentesis and evaluated at the California National Primate Research Center clinical laboratory for specific gravity by using a refractometer (Reichert T/S meter) and a reagent strip (Bili-Labitix, Bayer, Elkhart, IN). Urine was centrifuged and microscopic sediments were cytologically evaluated for the presence of cells and crystals.

Each selected macaque then underwent further evaluation within 2 wk of completing the primary selection process. Hepatitis A antibody titers (IgG and IgM), inhouse APTT coagulation test, ultrasonograms, and liver biopsies were performed under ketamine sedation (10 mg/kg IM; Ketaject, Phoenix Pharmaceutical).

Hepatitis A titers. Blood (3 mL) was collected from each macaque to determine antibody titers against hepatitis A virus (HAV). Total (antiHAV IgG and IgM) antibody level against HAV was measured by enzyme immunoassay at the University of California Davis Medical Center (Sacramento, CA). When the result showed reactivity, antiHAV IgM levels were determined by immunoassay.

Coagulation panel. Whole blood (1 drop) was applied to the sample well of an APTT test cartridge (Coagcheck Plus System, Roche Diagnostics), and the result was displayed on the Coagucheck system.

Liver ultrasonography and biopsy. The liver was evaluated sonographically for any gross abnormalities and changes in echogenicity. The cranial abdomen was surgically prepped, and a sterile gel was applied to the skin. Two percutaneous ultrasound-guided 16-gauge tru-cut biopsies (EZ Core Single Action Biopsy Needle, Products Group International, Lyons, CO) were collected from the liver parenchyma at 2 distinct sites and submitted for histopathology in 10% buffered formalin. The liver was rescanned to verify the absence of clinically significant hemorrhage after the biopsy.

Histopathology of liver biopsies. Liver biopsies were fixed in 10% buffered formalin, embedded in paraffin blocks, sectioned at 5 µm, and stained with hematoxylin and eosin. Histopathologic analysis was performed by a board-certified veterinary pathologist at the California National Primate Research Center.

Measurement of preprandial and postprandial SBAC. Macaques were fasted for 12 h, and sedated with ketamine (10 mg/kg IM; Ketaject, Phoenix Pharmaceuticals). Blood (3 mL) was collected for the preprandial SBAC. An orogastric tube was placed, and each macaque was gavaged with 20 mL/kg of blended Purina chow (High Protein Diet, Ralston Purina) mixed with 237 mL Boost Nutritional Energy drink (Nestle Health Care Nutrition, Florham Park, NJ). Two hours later, macaques were resedated with ketamine (10 mg/kg IM; Ketaject, Phoenix Pharmaceuticals), and 3 mL blood was collected for the postprandial SBAC. The serum was tested for SBAC by using a liquid stable enzymatic colorimetric assay on a Hitachi 900 (Randox Laboratories, Crumlin, UK). In this assay, serum bile acid molecules are oxidized and reduced by 3-α hydroxyl steroid dehydrogenase with a concomitant accumulation of reduced coenzyme thio-NADH. The formed thio-NADH is proportional to the amount of total bile acid in the sample and can be detected at a wavelength of 405 nm.[25]

Reported SBAC values for other species. A literature search was performed to find SBAC reference values for dogs, cats, and humans. All reported SBAC were determined by enzymatic methods, and postprandial samples were collected 2 h after a meal. For dogs, the mean preprandial SBAC is 1.7 ± 0.73 µmol//L (n = 26),[13] and the mean postprandial SBAC reference value is 6.8 ± 1.0 µmol/L (n = 36).[13] For cats, the mean preprandial SBAC reference is 1.7± 0.3 µmol/L (n = 20),[8] and the mean postprandial SBAC is 8.3 ± 0.8 µmol/L (n = 20).[8] In humans, the mean preprandial SBAC is 6.6 ± 0.7 µmol/L (n = 28),[24] and the mean postprandial SBAC reference is 7.6 ± 0.7 µmol/L (n = 28).[24]

Statistical analysis. The JMP 10 (SAS Institute, Cary, NC) statistical tool was used to calculate the mean and standard deviation of the preprandial and postprandial SBAC in rhesus macaques. A t test for paired samples was used to compare the preprandial and postprandial SBAC and the effect of sex and hepatitis A titer. The level of significance was set at a P value of

less than 0.05. Graphics were generated by using Prism software (GraphPad Software, La Jolla, CA).

Results

Overall, preprandial SBAC (mean ± 1 SD) in our 24 adult male and 16 female rhesus macaques was 11.1 ± 1.9 µmol/L, and postprandial SBAC was 19.7 ± 8.0 µmol/L. Therefore preprandial and postprandial SBAC reference intervals (that is, mean ± 2 SD) for rhesus macaques are 7.3 to 14.9 µmol/L and 3.7 to 35.7 µmol/L, respectively. The postprandial SBAC level was significantly ($P < 0.0001$) higher than the preprandial value. There were no significant differences detected when macaques were assessed separately by sex. The preprandial and postprandial SBAC data were normally distributed. The goodness-of-fit (Shapiro–Wilk *W*) test was used to assess the normality of the data; the data were not transformed.

The complete hematology, serum chemistry, and urinalysis values of all macaques in the study were within reference intervals (data not shown).

Hepatitis A titers. Five macaques (3 male, 2 female) were positive for HAV according to combined IgG and IgM antiHAV titers but were negative according to antiHAV IgM titers only. Preprandial and postprandial SBAC did not differ significantly between the macaques that were HAV-positive according to combined IgG and IgM titers and those that were negative.

Liver ultrasonography. Ultrasonography of the liver did not reveal any significant abnormalities in 39 of the 40 study animals. One male macaque had mild hepatomegaly with diffuse, mildly increased echogenicity of the liver parenchyma. This finding was not considered to be clinically significant.

Histopathology of the liver biopsy. No histopathologic lesions were found in any of the animals in this study. A mean of 13 portal areas were examined in all animals.

Discussion

In the current study, we determined the preprandial and postprandial SBAC reference intervals for rhesus macaques to be 7.3 to 14.9 µmol/L and 3.7 to 35.7 µmol/L, respectively.

The validity of any reference interval is dependent on the following: the appropriate selection of subjects that accurately represent the population of interest, the acquisition of samples under clearly stated criteria, and the assessment of the analytical variation in the production of the reference values.[26] The minimal sample size required for the estimation of the 2.5 and 97.5 percentiles is 40 values, but a sample of 120 values is preferable for reliable estimates.[4] As a convention, a reference interval is defined as the central 95% interval bounded by the 2.5 and 97.5 percentiles.[4] In the current study, we selected 40 healthy rhesus macaques with thorough hepatic screening, including histopathology, to provide a dependable reference interval for preprandial and postprandial SBAC.

In our rhesus macaques, the 2-h postprandial SBAC level was significantly ($P < 0.001$) higher than the 12-h fasting preprandial SBAC, confirming that endogenous challenge of the enterohepatic circulation can be used to evaluate liver function in this species. When macaques were assessed individually, 97.5% (39 of 40 animals) had a postprandial SBAC that was equal to or higher than their preprandial SBAC. The variability in the postprandial SBAC was higher than we expected. A possible physiologic explanation may be a delay in the contraction of the gall bladder in some subjects. The use of ketamine for orogastric tubing may have caused a delay in the normal gastrointestinal transit of food and affected the timing of gall bladder contraction and released of SBA in some macaques. This delay may explain the large range and standard deviation in the postprandial SBAC values in our study.

We assessed HAV titers in our study animals. Humans and nonhuman primates, including great apes, are natural hosts of HAV.[21,22] The disease caused by HAV is usually more severe in humans than in nonhuman primates, which typically manifest a mild, often subclinical infection followed by complete recovery.[1] In our study, 5 macaques were considered to be HAV-positive after screening by using a test that detected the presence of combined IgM and IgG to HAV, indicating previous or current infection or immunity to HAV. However, an IgM antiHAV titer is needed to detect the presence of a recent infection,[10] and all 5 macaques that were positive according to the combined test were negative according to IgM antiHAV titer, confirming no active or recent infection with HAV. We also confirmed that the presence of these circulating antibodies did not significantly affect preprandial and postprandial SBAC in rhesus macaques.

Liver biopsy typically is used to establish a definite etiologic diagnosis and the prognosis of hepatobiliary disease.[18,19] In humans, 15 portal triads should be evaluated to accurately define acinar involvement in liver disease.[11] In our study, the pathologist evaluated a mean of 13 portal triads per macaque. The selection of a 16-gauge tru-cut needle biopsy and the collection of 2 biopsies per animal were elected to obtain a representative morphologic and diagnostic liver biopsy sample. Some studies have shown an overall discordance between tru-cut needle biopsy and wedge biopsy of the liver, suggesting that wedge liver biopsy is still the 'gold standard' for accurate diagnosis of hepatobiliary disease.[11,19,23] We used history, physical exam, clinical data, serology, and ultrasound evaluation of the liver with ultrasound-guided tru-cut needle biopsy to establish the lack of active hepatobiliary disease. This thorough evaluation should circumvent any disadvantage of tru-cut biopsy compared with wedge biopsy of the liver. In making our choice for tru-cut biopsy, we also considered the invasiveness of the wedge liver biopsy and assessed the risk:benefit ratio.

Several methodologies can be used to determine the total serum bile-acid level. These techniques include gas–liquid chromatography, HPLC, enzymatic assays, and enzyme cycling assays. In clinical laboratories, the enzymatic cycling assay is now the most widely used method for the detection of total serum bile acids.[25] This liquid-stable technique can be used for all types of automated chemistry analyzers.[25] The enzymatic cycling assay had several advantages over conventional methods, including minimal interference with lipemic or hemolytic samples, high detection sensitivity, and the requirement of only a small aliquot of serum.

The mean values of preprandial and postprandial SBAC obtained for healthy rhesus macaques are higher than those reported for both humans and companion animals. In our rhesus macaque population, the mean preprandial SBAC was 11.1 ± 1.9 µmol/L compared with values of less than 5 µmol/L for dogs and cats and less than 10 µmol/L in humans.[2,5,14,17,20,24] The mean postprandial SBAC for rhesus macaques was 19.7 ± 8.0µmol/L, which similarly is higher than the normal postprandial values for dogs, cats, and humans. Normal postprandial SBAC values are less than 15 µmol/L in dogs,[5,9] less than 10 µmol/L in cats,[5] and less than 20 µmol/L in humans.[14,20,24] The only reference in the literature for SBAC in nonhuman primates was obtained from an unknown number of fed animals by using a radioimmunoassay technique, which yielded reference ranges of 0 to 7 µmol/L for rhesus macaques and 0 to 97 µmol/L for squirrel monkeys.[12]

The current study showed that postprandial SBAC was significantly higher than the preprandial SBAC value in rhesus macaques and established normal reference values for healthy adult rhesus macaques. The reference interval for preprandial SBAC in healthy adult animals is 7.3 to 14.9 µmol/L, and the postprandial SBAC is 3.7 to 35.7 µmol/L. Additional studies should be performed in rhesus macaques with hepatobiliary disease to confirm the diagnostic value of the SBAC value.

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