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Original Research

Hematology and Culture Assessment of Cranially Implanted Rhesus Macaques (*Macaca mulatta*)

Jacob H Theil,[1,5*] Jennifer L Johns,[2] Poyin Chen,[3,4] David M Theil,[6] and Megan A Albertelli[5]

The use of percutaneous cranial implants in rhesus macaques (*Macaca mulatta*) has long been a valuable tool for neuroscience research. However, when treating and assessing these animals, veterinarians are required to make assumptions about diagnostic results due to a lack of research into how these implants affect physiology. Microbial cultures of cranial implant sites show an abundance of colonizing bacteria, but whether these microbes affect animal health and wellbeing is poorly understood. In addition, microbial antibiotic resistance can present significant health concerns for both the animals and the researchers. To help elucidate the relationship between percutaneous cranial implants and blood parameters, complete blood cell counts and serum chemistry results were assessed on 57 nonhuman primates at our institution from September 2001 to March 2017. Generalized estimating equations were used to compare the results before and after an animal's first implant surgery. This modelling showed that cranial implants were a significant predictor of alterations in the number of neutrophils, lymphocytes, and red blood cells, and in the concentration of hemoglobin, alkaline phosphatase, creatinine, calcium, phosphorus, total protein, albumin, and globulin. Anaerobic and aerobic bacterial cultures were performed to identify bacteria associated with cranial implants. *Staphylococcus spp.*, *Streptococcus spp.*, and *Corynebacterium spp.* comprised the majority of the aerobic bacterial isolates, while *Fusobacterium spp.*, *Peptostreptococcus spp.* and *Bacterioides fragilis* comprised the majority of anaerobic bacterial isolates. Using a *Pearson r* correlation for statistical analysis, we assessed whether any of these bacterial isolates developed antibiotic resistances over time. Cefazolin, the most frequently used antibiotic in monkeys in this study, was the only antimicrobial out of 41 agents tested to which bacteria developed resistance over time. These results indicate that percutaneous implants are associated with a generalized inflammatory state, multiple bacterial species are present at the implant site, and these bacteria may contribute to the inflammatory response.

Abbreviations: CBC, complete blood cell count; CHEM, serum chemistry; WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean cell volume; MCHC, mean cell hemoglobin concentration; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyl transferase; BUN, blood urea nitrogen; CK, creatine kinase; GEE, generalized estimating equation; AID, anemia of inflammatory disease

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Neuroscience research often employs in vivo analysis of neuronal distribution, activity, and function, all of which require visualization and manipulation of the brain in living animals. To achieve this, cortical structures are accessed by the placement of percutaneous implants that are anchored to the skull and outfitted with transcranial ports. Studies of the auditory system,[72,78] visual cortex,[20,22,46,54] motor cortex,[23,44] perception,[70] and optogenetics[23,80] have all benefitted from the use of these implants in multiple laboratory species. However, few studies have investigated the effects of these implants on the general physiology of research animals.[32]

Due to human similarities in neuroanatomy, physiology, social behavior, and cognition, rhesus macaques (*Macaca mulatta*) have proven invaluable in translational studies of cortical structures. This species has long been the recipient of percutaneous cranial implants[29] and therefore, has been the subject of many refinements in their construction to improve research outcomes.[3,11,40,43,60] Traditionally, these implants are constructed out of titanium or titanium alloy hardware that is anchored in place with acrylic or bone cement. Recording chambers provide access points through craniotomy sites where devices are implanted into deeper structures of the brain. Titanium alloys have been shown to provide a good bone/implant interface through the development of titanium oxides;[17,32,59] however, these implants may leach ions into surrounding tissues, with unknown health or research implications.[32] Acrylics provide a moldable and easily repairable substrate, but have poor biocompatibility.[5,53] Their addition to implants increases exposed tissue surface area, creating ideal environments for infectious agents to thrive.[2,43]

Cranial implants can develop complications, including inflammation and infections at skin margins and the bone/implant interface.[32] Multiple species of bacteria, including *Staphylococcus* spp., *Corynebacterium* spp., *Enterococcus* spp., and *Providencia rettgeri*, have been recovered from the chambers and skin edges of cranial implants in rhesus macaques.[10,43,88]

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[1]Campus Veterinary Services, University of California, Davis, Davis, California; [2]Department of Biomedical Sciences, Oregon State University Carlson College of Veterinary Medicine, Corvallis, Oregon; [3]Division of Infectious Disease, Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts; [4]Department of Microbiology and Immunology, Harvard University, Boston, Massachusetts; [5]Department of Comparative Medicine, Stanford University, Stanford, California; [6]Independent researcher
[*]Corresponding author. Email: jhtheil@ucdavis.edu

These infections may be due to manipulation and contamination of implants by the animals themselves. Chronicity of infections is also likely due to the inability to apply therapeutic agents to deeper regions of tissue shielded by the implant. Chronic pathology is evident in deeper structures at the neural-implant interface.[67] For example, sustained glial responses and neural degeneration have been identified at sites where electrodes and arrays contact brain tissue.[12,74]

Chronic inflammation has well-established effects on hematologic values, such as red and white blood cell counts, platelet numbers, and total protein measurements. Few studies are currently available that link chronic percutaneous implants to changes in hematology in rhesus macaques, requiring clinicians to use established reference intervals of nonimplanted animals when analyzing bloodwork. This requires assumptions about effects of implants on overall health; these assumptions may or may not be correct, but are the necessary basis for assessments and therapies. In this study, systemic physiologic changes were identified in clinically healthy, implanted animals to provide a framework for analysis of complete blood cell counts (CBC) and serum chemistry (CHEM) values for clinicians working with rhesus macaques with percutaneous implants. We also assess bacterial cultures taken from these implants to identify common contaminants and their antibiotic resistance profiles. We hypothesize that clinically healthy rhesus macaques with percutaneous implants have significantly different hematology profiles than those without implants, despite a lack of clinical signs. By understanding how implants and associated bacteria affect routine blood test results, observed changes can be better characterized as normal or abnormal, so that veterinary care can be tailored as needed. This important step will allow improved care and welfare of animals used in neuroscience research.

Materials and Methods

Animals. The animals in this study were unrelated male and female rhesus macaques (*Macaca mulatta*) housed at Stanford University during various intervals between September 2001 to March 2017. All subjects were housed and maintained in accordance with *The Guide for the Care and Use of Laboratory Animals*[39] in an AAALAC-accredited facility. In general, monkeys were maintained on a 12:12 light:dark cycle at temperatures between 64 °F and 84 °F and humidity between 30% and 70%. The rhesus macaques were housed in stainless steel caging, in pairs or singly housed based on research or veterinary exemptions. Subjects received daily rations of a commercially available primate diet (Teklad 2050, Envigo, Madison, WI) as well as various fruits and vegetables. In addition, dry goods were presented in toys and devices for environmental enrichment. All macaques were tested biannually for *Mycobacterium tuberculosis* and annually for *Macacine herpesvirus 1* (Herpes B), *Simian betaretrovirus* (SRV), and *Simian T-cell lymphotrophic virus* (STLV) via serology.

In this retrospective study, all rhesus macaques were enrolled in neuroscience research protocols studying various cortical structures under one of 3 research groups. In addition to cranial implant surgery, experimental procedures included behavioral tasks with touch screen interaction, fluid regulation, and liquid rewards. Animal procurement and stock, surgical procedures, implant maintenance, experimental tasks, housing, and environmental and food enrichment varied by research group. However, due to the archival nature of the data, much of the specific information regarding these variables was incomplete or not available. All research was approved by Stanford University's Institutional Animal Care and Use Committee.

Surgical Procedures. With the exception of 5 animals, all rhesus macaques underwent 1 or more cranial implant surgeries focused on the dorsal cranium and necessary for accomplishing project-related goals or implant management. Surgical goals included placement of head restraint posts, craniotomies, placement of craniotomy chambers (used as a barrier and access point for craniotomy sites), placement of electrodes and electrode arrays, placement of cooling loops (a device for cooling cortical structures), placement of cranial windows, viral vector injections, implant repairs, and explants. Surgeries were performed by the research groups and associated surgeons, and materials included titanium hardware and biologically compatible epoxy. For the purposes of this study, only the first cranial implant surgery date was used for data analysis. Implants and craniotomy sites were regularly cleaned and maintained by the research groups based on need. Various antibiotics were used topically for disinfection after cleaning of implants and craniotomies, including neomycin/polymyxin/bacitracin ointment, neomycin/polymyxin/gramicidin solution, or cefazolin solution.

Sample Collection and Analysis. Blood was collected from the femoral vein of animals under sedation with ketamine and an α2-receptor agonist (medetomidine or dexmedetomidine) given intramuscularly at veterinarian-approved dosages (exact dosage information was unavailable). Blood for CBC was placed in potassium EDTA collection tubes while blood for CHEM analysis was placed in anticoagulant-free collection tubes. Collected blood for CHEM was allowed to clot at room temperature for approximately one hour and was then centrifuged for 6.5 min at 18,187 × g (Eppendorf Centrifuge 5415R, Hamburg, Germany). Hematologic analysis was performed within 4 h of sample collection, with storage of blood at 4 °C prior to analysis. CHEM analysis was performed within 24 h of sample collection, with storage of serum at 4 °C prior to analysis. Automated hematology analysis was performed inhouse on either a Sysmex XT-2000-iV(Sysmex Corp., Kobe, Japan) or a Cell-Dyn 3500 (Abbott Diagnostics, Chicago, IL) analyzer. CBC results were confirmed via blood smear examination and manual verification of automated differentials by a medical technologist trained in nonhuman primate hematology. In January of 2013, the analyzer for CBC analysis was changed from the Cell-Dyn 3500 to the Sysmex XT-2000iV. Verification of instrument performance and correlation between hematology analyzers was performed prior to this change. CHEM analysis was performed on a Siemens Dimension Xpand Plus Integrated Chemistry System (Siemens, NY, NY). All animals underwent multiple blood collection procedures during the study period.

Aerobic and anaerobic bacterial samples were collected via sterile swab of the implant margins or chambers. Swabs were cultured using appropriate media and interpreted at the Stanford Animal Diagnostic Laboratory or a nearby diagnostic laboratory. Due to the archival nature of this study, precise information on media types, temperatures, and laboratories was unavailable. Antibiotic sensitivity profiles were assessed for all bacterial isolates and included a subset of the following compounds (based on appropriateness for the bacterial species and availability of the test): amikacin, amoxicillin/clavulanic acid, amoxicillin, ampicillin, cefazolin, cefotaxime, cefovecin, cefoxitin, cefpodoxime, ceftazidime, ceftiofur, cephalexin, cephalothin, chloramphenicol, ciprofloxacin, clindamycin, doxycycline, enrofloxacin, erythromycin, gentamicin, imipenem, marbofloxacin, methicillin, metronidazole, minocycline, neomycin, nitrofurantoin, orbifloxacin, oxacillin, penicillin, piperacillin/tazobactam, polymyxin b, pradofloxacin, rifampin, streptomycin, tetracycline, ticarcillin/clavulanic acid, ticarcillin, trimethoprim sulfamethoxazole, tribrissen, and vancomycin.

Data Management. Historic CBC, CHEM, and aerobic and anaerobic culture information was stored using a DOS based program and transferred manually to Microsoft Excel (Microsoft Office 2016, Microsoft Corporation, Redmond, WA). The data was organized using Microsoft Excel and Microsoft Access (Microsoft Office 2016, Microsoft Corporation, Redmond, WA). Birthdates and surgery data were maintained in hard copy records and manually transferred to Microsoft Excel. Only rhesus macaques with available birthdate information were included in the study. Animals with only birth year information were given an estimated birthdate of July 2.

Differentiation of healthy implanted and nonimplanted animals was based on whether bloodwork was performed for clinical or routine management reasons. Routine bloodwork was performed during biannual exams and for cross matching. Blood that was collected to assess morbidity was considered clinical and removed from the study. Routine bloodwork was further differentiated based on whether it was performed before or after the animal's first implant surgery. Because animals in this study had bloodwork performed more than once, blood samples were numbered for each macaque from 1 to the maximum number of blood draws performed on that animal.

Culture swabs collected from either the implant margins or the implant chambers were pooled for data analysis. Antibiotic sensitivity was scored from 1 to 3 with 1 being susceptible, 2 being intermediate susceptibility, and 3 being resistant.

Statistical Methodology. CBC and CHEM results were analyzed with a repeated-measures generalized estimating equation (GEE) using IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, NY). The subject variable was monkey, and the within-subject variable was bloodwork number. Individual equations were developed for each dependent variable of interest. For CBCs, this included white blood cell concentration (WBC), red blood cell concentration (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin concentration (MCHC), absolute neutrophil concentration, absolute lymphocyte concentration, absolute monocyte concentration, absolute eosinophil concentration, and absolute basophil concentration. Platelet, band neutrophil, and reticulocyte concentration information was incomplete or missing, so was not included. For CHEM analysis, the dependent variables included concentrations of glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transferase (GGT), total bilirubin, direct bilirubin, indirect bilirubin, cholesterol, blood urea nitrogen (BUN), creatinine, calcium, phosphorus, total protein, albumin, globulin, creatine kinase (CK), sodium, potassium, and chloride. Factors for analysis were 1) whether the blood was collected prior to or after a monkey's first cranial implant surgery and 2) the research group in which the monkey was being studied. Whether the blood was collected prior to or after January 1st, 2013 was added to CBC equations to assess whether the hematology analyzer affected bloodwork results. Due to incomplete records, this date was estimated with regard to the change from one hematology analyzer to the other. The age of the monkey at the time of the bloodwork was added to all equations as a covariate. Gender was not included in the analysis because too few females fit the study criteria. Factors and covariates were included in all models because of clinical relevance, regardless of significance.

Posthoc analysis was not performed for differences between research groups because research goals, surgical techniques, implant care, and animal care were institution-specific, complex, and beyond the scope of this study; such distinctions would be arbitrary and not useful for other institutions. This is also true of differences between hematology analyzers. These institutionally specific differences are likely not of clinical significance for other institutions. Therefore, significant differences between research laboratories and hematology analyzers were used for model assessment but the magnitude and direction of these differences were ignored.

To correct for multiple comparisons, we used a modification of the False Discovery Rate procedure:[8,65]

$$P = a / \sum_{i=1}^{k} (1 / \mathrm{i})$$

where α is 0.05 and k is hypotheses tested. This allowed for a conservative estimate of significance without assuming independence of variables.[65] For CBC equations, significance was set at P of 0.017. For CHEM equations, significance was set at P of 0.014.

To assess changes in antibiotic resistance over time, a Pearson r was computed for the relationship between antibiotic sensitivity score and the year the culture was collected. This was performed on all isolates cultured and all antibiotics tested. To correct for multiple comparisons, a conservative estimate of significance of $P < 0.0005$ was used assuming independence of a large number of variables.

Results

Animals. Of the 100 rhesus macaques with hematology data during the study period, 57 met the birthdate criterion for study inclusion. 55 of the 57 were male and by the end of the study time period, 52 of the macaques had undergone at least one cranial implant surgery. The average age of the animal at first surgery was 6.0 y (SD = 1.5, min = 3.7, max= 10.3). A breakdown of monkeys by research group can be seen in Table 1.

Blood Sampling. 343 routine blood samples were used from the animals included in this study. Of these 343 samples, 115 were collected prior to an animal's first implant surgery and 228 were collected afterwards. The mean age of animals at blood collection prior to surgery was 5.3 y (SD = 1.4, min = 3.0, max = 10.2) and the mean age of animals at blood collection after the first surgery was 9.8 y (SD = 3.1, min = 4.1, max = 20.7).

Of the 343 samples collected, a CBC was performed in 318. 108 CBCs were performed prior to first surgery and 210 CBC after first surgery. Partial or complete CHEM reports were included for 340 out of 343 samples collected. 114 CHEM assays occurred prior to first surgery and 226 CHEM assays occurred after first surgery.

Complete Blood Cell Count. The only significant predictor of total WBC concentration was research group ($\chi^2 = 9.53$, $P = 0.009$). However, total neutrophil concentrations were significantly higher ($\chi^2 = 14.73$, $P < 0.001$) and lymphocyte concentrations were significantly lower ($\chi^2 = 50.07$, $P < 0.001$) after the first surgery as compared with before the first surgery. Age was also a significant predictor of lymphocyte concentration ($\chi^2 = 6.93$, $P = 0.009$) as lymphocyte concentrations steadily decreased over time. Only research group was predictive of absolute monocyte concentration ($\chi^2 = 9.640$, $P = 0.008$), and only blood analyzer had predictive power of absolute eosinophil concentration (χ^2 11.54, $P = 0.001$). None of the factors studied were predictive of absolute basophil concentration.

For RBC parameters, research group was a significant predictor of total RBC concentration ($\chi^2 = 27.38$, $P < 0.001$), and RBC concentrations were significantly lower after an animal's first surgery ($\chi^2 = 8.20$, $P = 0.004$). Research group ($\chi^2 = 12.54$,

Table 1. Age, sex, and first surgery age statistics of *Rhesus macaques* used for research by 3 different neuroscience groups.

	N	Males: Females	Surgery not performed	Mean age at first surgery (years)	Age range at first surgery (years)
Group A	20	20:0	2	5.93	4.62–7.86
Group B	12	12:0	0	5.96	3.91–8.13
Group C	25	23:2	3	6.09	3.66–10.31
Total/Mean/Range	57	55:2	5	6.00	3.66–10.31

$P = 0.002$) and surgical status ($\chi^2 = 7.656$, $P = 0.006$) were both significant predictors of HGB, with lower total HGB seen after the first surgery. Research group was the only significant predictor of HCT ($\chi^2 = 15.72$, $P < 0.001$), and blood analyzer was the only significant predictor of MCV ($\chi^2 = 8.83$, $P = 0.003$) and MCHC ($\chi^2 = 9.74$, $P = 0.002$) (Figure 1).

Serum Chemistry Panel. For CHEM parameters, ALP ($\chi^2 = 11.28$, $P = 0.001$) was significantly lower after the first surgery. However, none of the factors in the models predicted glucose, AST, ALT, GGT, total bilirubin, direct bilirubin, or indirect bilirubin. Only research group was a significant predictor of cholesterol ($\chi^2 = 16.10$, $P < 0.001$).

Research group was a significant predictor of BUN ($\chi^2 = 10.56$, $P = 0.005$). Creatinine was not significantly associated with research group but was significantly higher after surgery ($\chi^2 = 7.29$, $P = 0.007$). No factors in the model were significant predictors of CK.

Calcium ($\chi^2 = 6.92$, $P = 0.009$) and phosphorus ($\chi^2 = 9.94$, $P = 0.002$) both decreased after the first surgery, while research group and age were not significant predictors of these variables. Surgery and research group were not significant predictors of sodium, potassium, or chloride, but potassium significantly increased with age ($\chi^2 = 13.52$, $P < 0.001$).

Surgery and age were significant predictors of total protein, albumin, and globulin. Total protein ($\chi^2 = 12.40$, $P < 0.001$) and globulin ($\chi^2 = 42.74$, $P < 0.001$) were significantly higher after surgery. Total protein ($\chi^2 = 11.80$, $P = 0.001$) and globulin ($\chi^2 = 58.22$, $P < 0.001$) also increased with age. Research group was a marginally significant predictor of total protein ($\chi^2 = 9.02$, $P = 0.011$). Conversely, albumin decreased after surgery ($\chi^2 = 11.17$, $P = 0.001$) and with age ($\chi^2 = 39.05$, $P < 0.001$) (Figure 2).

Bacterial Culture Results. Ninety-three samples were taken from the implant margins or chambers of 28 monkeys during the study period and submitted for aerobic culture. Twenty of the 93 samples were concurrently submitted for anaerobic culture. One hundred eighty-four isolates were cultured (145 aerobic, 39 anaerobic); no bacteria were isolated from 16 samples and a nonspecific culture designation was applied to 4 samples. Bacteria isolated represented 12 aerobic genera and 6 anaerobic genera, as summarized in Tables 2 and 3.

Of all the antibiotics tested for a correlation between susceptibility and time, only Cefazolin showed a significant increase in resistance over the years of the study period (Pearson r: $n = 97$, $r = 0.37$, $P < 0.0005$).

Discussion

Using GEE modeling, we were able to identify the predictive effects of cranial implants, age, research group, and blood analyzer on CBC parameters in rhesus macaques. Cranial implants were significant predictors of neutrophil concentration, lymphocyte concentration, RBC concentration, and HGB. Research group was a significant predictor of total WBC concentration, monocyte concentration, RBC concentration, HGB, and HCT. Age of the animal was a significant predictor of lymphocyte concentration, and blood analyzer predicted the parameters of eosinophil concentration, MCV, and MCHC. None of the factors studied was predictive of basophil concentrations.

Cranial implants, age, and research group were also predictive of multiple CHEM parameters. Cranial implants were significant predictors of ALP, creatinine, calcium, phosphorus, total protein, albumin, and globulin. Age was a significant predictor of potassium, total protein, albumin, and globulin, while research group was a significant predictor of BUN, cholesterol, and total protein. None of the factors in this study were predictive of glucose, AST, ALT, GGT, total bilirubin, direct bilirubin, indirect bilirubin, CK, sodium, or chloride concentrations.

The use of percutaneous cranial implants in primates has proven invaluable in the study of neuroscience; however, the effects that these implants have on normal physiologic function has been poorly studied. Through analysis of past data, we were able to determine that significant changes to hematologic values occur in instrumented animals that may be overlooked because they fall within established reference intervals. Most of the mean CBC and CHEM values presented in this study fall within the reference intervals of adult indoor-housed rhesus macaques published in *Laboratory Animal Medicine Third Edition*.[56] Some values fall marginally outside of these intervals, such as RBCs and monocyte concentrations, but some variability must be attributed to differences in collection technique, analysis of samples, and population. For example, reference intervals reported by our institution's diagnostic lab, as well as other studies on hematology values in rhesus macaques, were substantially different from those reported by others.[18,32,45,71,89] Hematologic and biochemical reference intervals are laboratory- and instrument-specific. These differences underscore the need to evaluate all hematologic parameters on a case-by-case and facility-by-facility basis, as many changes in blood values that are within reference intervals may actually have clinical significance.

A primary change observed before and after implantation was attributed to chronic inflammation that developed in instrumented animals. While total WBC concentrations did not change, neutrophil concentrations increased after the first surgery. Differentials for neutrophilia are extensive and include disease processes such as inflammation, paraneoplastic syndromes, autoimmune disease, leukemia, and genetic disorders. However, for animals assessed in this study, the presence of an established inflammatory response and a cortisol-mediated physiologic response are the most likely. An inflammatory response is commonly characterized by increased neutrophil production and release from the bone marrow, induced by proinflammatory mediators such as cytokines, interleukins, and growth factors.[76,83] Low-grade inflammation is expected when healing is impeded by a foreign body such as an implant, with mild erythema, purulent discharge, and thickening observed at all implant margins. This was confirmed in a study[32] that found histopathologic evidence of chronic inflammation at cranial implant margin sites. In a mild chronic inflammatory state, bone marrow neutrophil production can likely adequately meet peripheral demand, resulting in mature neutrophilia (rather than neutropenia and/or left shift).[84] In addition, an acute inflammatory response can cause lymphocytes to be sequestered in regional lymph nodes, decreasing circulating lymphocytes, as

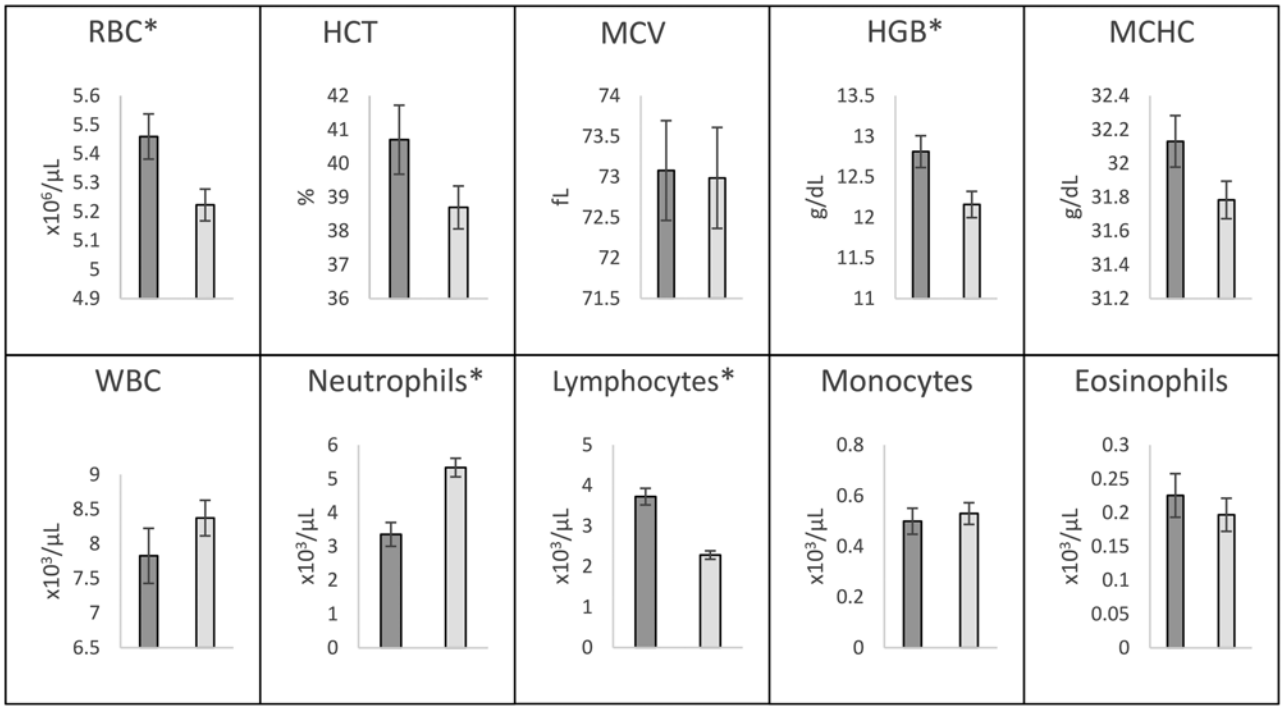

Figure 1. Estimated marginal means and standard error intervals of complete blood cell counts before (dark) and after (light) the first cranial implant surgery. *Significance set at $P = 0.017$

was noted in this study. Unfortunately, the absence of data on immature neutrophils makes the assessment of a left shift and confirmation of inflammation difficult based on hematologic analysis alone. However, the increases in globulin and the evidence put forth by others[32] make these results suggestive of an inflammatory response, although the response could be acute or chronic.

In addition to inflammation, another cause of increased circulating neutrophils is a physiologic response caused by stress or excitement and subsequent release of epinephrine.[84] In this study, evidence does not support an epinephrine response for 3 reasons: standardization of blood collection procedures across all blood draws, the use of ketamine for blood collection, and lymphopenia. All animals were chemically restrained for blood draws, whether implanted or not, making it unlikely that an acute stress response due to restraint was the cause of neutrophilia only in implanted animals. All macaques also received ketamine hydrochloride prior to blood draw procedures, a practice that has been shown to decrease the stress response associated with physical restraint.[9] Finally, lymphopenia does not support an acute epinephrine-mediated response and is more consistent with hypercortisolemia, which causes the redistribution and sequestration of circulating lymphocytes in acute forms and cytolysis in chronic cases. One possible reason for potential cortisol release was the chronic disease process experienced by the implanted macaques (that is infection, and the long-term presence of foreign material). In addition, the animals may have experienced a chronic excitatory condition after implantation related to prolonged or more frequent handling, training, and restraint.

Differences in red blood cell parameters before and after implantation provide further evidence of an inflammatory response to instrumentation. The normocytic normochromic anemia, characterized by lower RBC and HGB concentrations after implantation, is consistent with anemia of inflammatory disease (AID). In AID, downregulation of iron metabolism and absorption, decreased erythropoiesis, and shortened red blood cell life span reduce RBC numbers.[85] Although not statistically significant, HCT trended lower after the first surgery, supporting the possibility of AID (Figure 1). While these parameters may be attributable to blood loss during surgery, almost all of the bloodwork was performed during routine exams either before or significantly after surgery, making this unlikely.

Elevations in total protein and globulin concentrations observed after the first implant procedure are also consistent with an inflammatory state. Increases in globulin and total protein occur with chronic infection and inflammation due to chronic antigenic stimulation.[36,49] Albumin, a negative acute-phase protein, was lower after surgery, which is also an indication of ongoing inflammation.[36] These changes in globulins and albumin in instrumented animals have likewise been reported by others.[32]

Albumin is the main calcium binding protein found in the blood, and decreases in albumin correspond with postoperative decreases in calcium.[30] Others have also reported lower serum calcium concentrations in implanted rhesus macaques, as compared with nonimplanted macaques.[32] Phosphorous concentrations were lower in macaques after their first surgery, possibly due to the consumption of large amounts of food enrichment and treats during testing periods after the first surgery. Dietary imbalance can result in vitamin D deficiency and/or decreased dietary phosphorous, with subsequent changes in blood calcium and phosphorus levels.[30] Therefore, food enrichment could have significant effects on research and health outcomes. Careful consideration of food enrichment regimens is recommended in long-term studies of instrumented animals.

In addition to variable food enrichment, subjects in this study generally underwent research-driven water restriction that increased in frequency and duration after implantation to promote task participation, with fluid provision used as a positive reinforcement. Water restriction, while heavily monitored, carries the risk of dehydration and hypovolemia. After the first

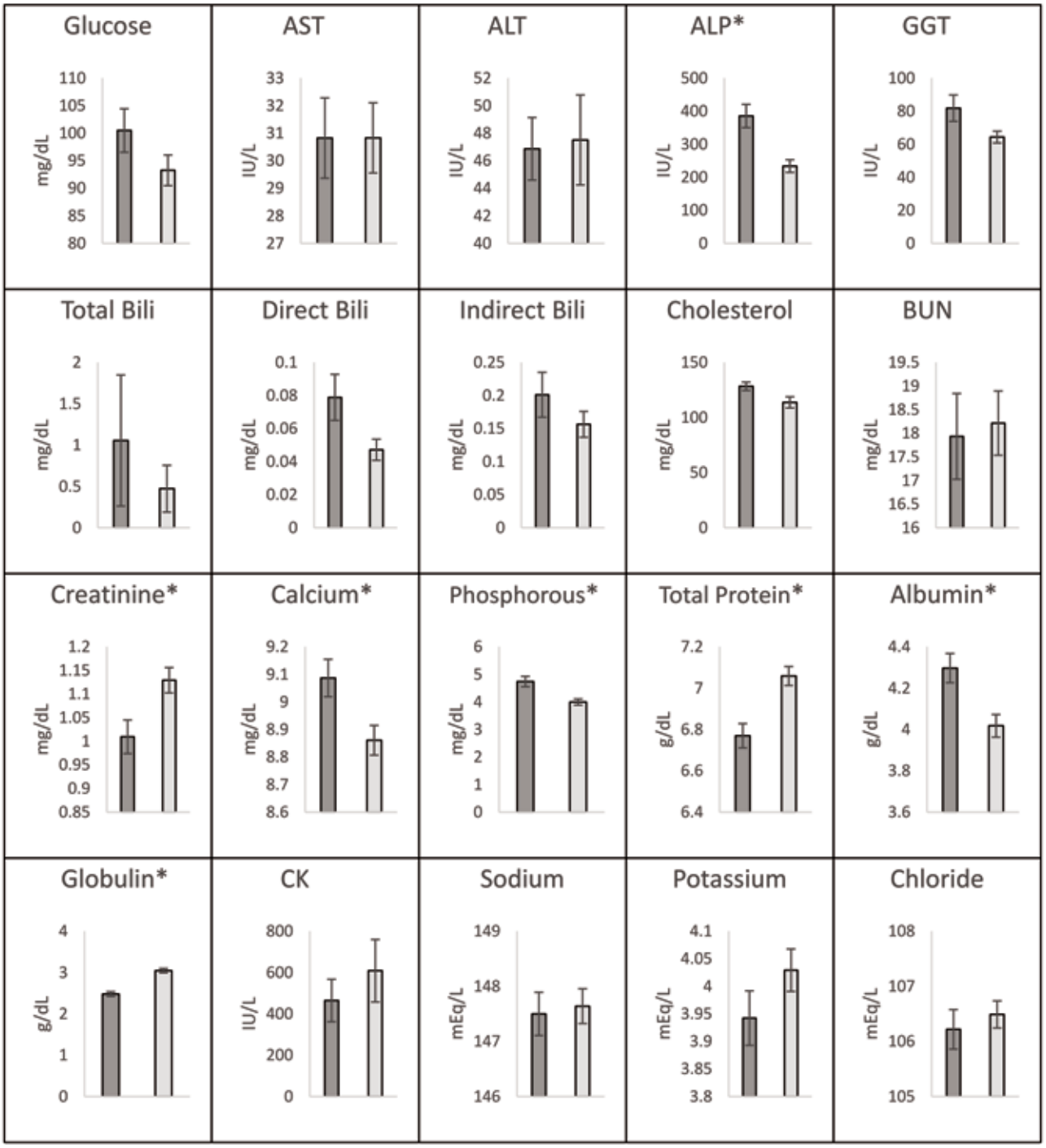

Figure 2. Estimated marginal means and standard error intervals of serum chemistry results before (dark) and after (light) the first cranial implant surgery. *Significance set at $P = 0.014$

surgery, CHEM analysis showed higher serum creatinine concentrations, which is often the result of dehydration and hypovolemia.[31] However, in prerenal azotemia, increases in both BUN and creatinine are generally observed unless the patient has decreased muscle mass.[31] Increased creatinine alone can be seen in macaques with sudden severe cachexia.[31] However, all blood analyzed in the current study was collected from apparently healthy animals. A dehydrated animal can present with a normal BUN, with the dehydration masking decreases in total BUN, in animals with severe liver disease.[50] However, many of the primary markers associated with hepatic damage, biliary hyperplasia, or cholestasis appeared unaffected by the surgery, making hepatic insufficiency unlikely. Alkaline phosphatase, a membrane bound hepatic enzyme, was decreased after the first surgery, but this enzyme is a poor indicator of hepatic and biliary disease in monkeys.[50] To summarize, dehydration and hypovolemia were unlikely, but the reason for serum creatinine rises remains unknown.

Serum ALP was significantly lower after implantation as compared with pre-surgical values. ALP levels become elevated in animals experiencing significant bone remodeling or lysis.[81] Bone remodeling was often observed in monkeys with cranial implants, and often required surgical intervention in the form of explants, implant repairs, and craniotomy repairs. Moreover,

Table 2. Aerobic bacteria cultured from 93 percutaneous cranial implants swabs of 28 rhesus monkeys, including the total number of isolates cultured, percentage breakdown of isolates, percentage of monkeys that cultured positive, and the percentage of swabs that cultured the organism.

Genus	Species	Total isolates	Isolates (%)	Monkey (%)	Cultures (%)
Staphylococcus		68	41.2	85.7	63.4
	aureus	56	33.9	75.0	52.7
	unspeciated	12	7.3	39.3	11.8
Streptococcus		21	12.7	39.3	20.4
	viridens	5	3.0	10.7	5.4
	agalactiae	4	2.4	7.1	4.3
	bovis/equinus	4	2.4	14.3	4.3
	equisimilis	1	0.6	3.6	1.1
	unspeciated	7	4.2	25.0	7.5
Corynebacterium		19	11.5	50.0	20.4
	ulcerans	3	1.8	10.7	3.2
	pseudotuberculosis	1	0.6	3.6	1.1
	renale	1	0.6	3.6	1.1
	unspeciated	14	8.5	39.3	15.1
Enterococcus		13	7.9	39.3	14.0
	faecalis	6	3.6	21.4	6.5
	avium	3	1.8	7.1	3.2
	faecium	1	0.6	3.6	1.1
	unspeciated	3	1.8	10.7	3.2
Serratia	*marcescens*	8	4.8	14.3	7.5
Proteus		4	2.4	10.7	4.3
	mirabilis	3	1.8	7.1	3.2
	vulgaris	1	0.6	3.6	1.1
Pseudomonas		4	2.4	14.3	4.3
	aeruginosa	2	1.2	7.1	2.2
	fluorescens	1	0.6	3.6	1.1
	unspeciated	1	0.6	3.6	1.1
Enterobacter		3	1.8	3.6	3.2
	cloacae	2	1.2	3.6	2.2
	unspeciated	1	0.6	3.6	1.1
Actinomyces		2	1.2	7.1	2.2
	neuii	1	0.6	3.6	1.1
	unspeciated	1	0.6	3.6	1.1
Morganella	*morganii*	2	1.2	3.6	2.2
Escherichia	*coli*	1	0.6	3.6	1.1
Pasteurella	*unspeciated*	1	0.6	3.6	1.1
Nonspecific		3	1.8	10.7	3.2
No aerobic bacteria cultured		16	9.7	17.9	17.2

multiple animals with cranial implants required phenobarbital for medical management of seizures; this drug is known to induce cholestasis and increase ALP in multiple species.[34,55] The combination of bony changes and phenobarbital treatment suggests that ALP would be higher, not lower, in postsurgical animals. However, ALP appears to have a protective role in inflammation through the dephosphorylation of proinflammatory molecules such as endotoxin and certain clotting factors.[47,69] In a chronic inflammatory state such as what occurs in implanted rhesus macaques, ALP may be metabolized faster than in physiologically normal animals, resulting in decreased serum concentrations despite bone remodeling and phenobarbital treatment.

The research groups using the macaques were also a source of variation in multiple hematologic values assessed in this study, likely due to differences in research goals, postsurgical testing protocols, housing environment, enrichment, and study population. Differences between groups in surgical technique and implant type likely affected the amount of blood loss and inflammation, affecting parameters such as WBCs, absolute monocyte concentration, RBCs, and HCT. Variations between groups in fluid restriction protocols may have resulted in differences in dehydration parameters, such as HCT, BUN, creatinine, and total protein. Finally, differences in genetics or feeding regimens between groups likely influenced multiple parameters under study; however, analysis of these factors was beyond the scope of this study.

Age was a significant predictor of lymphocyte, total protein, albumin, globulin, and potassium concentrations. Decreases in lymphocyte populations with age have been documented in both rhesus macaques[45,71,89] and people.[28] Decreases in albumin and increases in total protein and globulin have also been reported with increasing age,[45,71] as also found in our study.

Table 3. Anaerobic bacteria cultured from 20 percutaneous cranial implant swabs of 14 rhesus monkeys, including the total number of isolates cultured, percentage breakdown of isolates, percentage of monkeys that cultured positive, and the percentage of swabs that cultured the organism.

Genus	Species	Total isolates	Isolates (%)	Monkeys (%)	Cultures (%)
Fusobacterium		12	30.8	71.4	55.0
	nucleatum	7	17.9	42.9	35.0
	necrophorum	4	10.3	28.6	20.0
	unspeciated	1	2.6	7.1	5.0
Peptostreptococcus		9	23.1	50.0	45.0
	anaerobius	7	17.9	35.7	35.0
	unspeciated	2	5.1	14.3	10.0
Bacteroidies	*fragilis*	8	20.5	28.6	40.0
Prevotella		5	12.8	35.7	25.0
	intermedia	3	7.7	21.4	15.0
	unspeciated	2	5.1	14.3	10.0
Porphyromonas	*unspeciated*	3	7.7	14.3	15.0
Eubacterium	*unspeciated*	1	2.6	7.1	5.0
Nonspecific		1	2.6	7.1	5.0

Elevated potassium (hyperkalemia) was observed in older animals in this study, but a relationship between age and serum potassium has not been reported in rhesus macaques. In people, hyperkalemia is associated with the aging kidney, secondary to decreased glomerular filtration rate and aldosterone levels.[13,27,82] Similarly, renal excretion of serum potassium is diminished in older rats.[7] Overall, age-related changes in specific blood parameters are consistent with existing literature on aging animals and further support the validity of the models presented.

After the bloodwork assessment, we investigated microbial populations as potential contributors to chronic inflammation observed in healthy implanted macaques through assessment of aerobic and anaerobic cultures taken from implant sites. Of the bacteria cultured, *Staphylococcus aureus* was the most common aerobic isolate cultured from head implant wells and margins, followed by *Streptococcus spp.*, *Corynebacterium spp.*, and *Enterococcus spp.* These bacteria are considered normal flora in rhesus macaques and are easily cultured from skin, nasal, oral, rectal, and/or vaginal swabs[19,24,73,77] and swabs of cranial implant margins.[10,51,52,88] *Streptococcus equisimilis,* isolated from one subject, is rarely reported in rhesus macaques[24] and appears to be transmitted to animals by people.[33] In one study, *C. ulcerans* was collected from the oropharyngeal region of a rhesus macaque, but as this animal was cranially implanted, it was doubtful that this was normal flora.[10]

Colonization of rhesus macaques by aerobic bacteria such as *Actinomyces neuii*, *Enterococcus avium, Pseudomonas aeruginosa,* and *Serratia marcescens* has not been well studied. *A. neuii* is a Gram-positive rod that differs from other *Actinomyces* species in that it is aerobic. It has been cultured from multiple infection sites in people, including prostheses and implants, and is not considered to be a component of healthy human flora.[79] *E. avium* can colonize the gastrointestinal tract of healthy chickens, dogs, pigs, and people[66] but is also associated with human brain abscesses and breast implant infections.[1,61] *P. aeruginosa* is an environmental opportunistic pathogen in people, and although it is not considered to eb normal flora, it has been found in the feces of healthy individuals.[14] However, reports of implant-associated infections are numerous.[4,15,25,37,75] While rhesus macaques have been used as a model for chronic bronchitis induced by *P. aeruginosa*,[21] reports of this species as a cranial implant associated pathogen are absent from the current literature. Finally, like *P. aeruginosa*, *Serratia marcescens* is an opportunistic pathogen found in the environment and an important cause of nosocomial infections in human hospitals,[38] but its significance as a pathogen in rhesus macaques has yet to be determined.

In contrast to aerobic flora, normal anaerobic flora in rhesus macaques are less defined. *Bacterioides fragilis, Peptostreptococcus anaerobius, and Porphyromonas spp.* have been cultured from vaginal swabs in healthy rhesus females[24] and have also been isolated from the gastrointestinal tract of healthy people.[62,63,86] *Eubacterium spp.* are considered commensal gastrointestinal flora in rhesus macaques, but *Eubacterium* can cause bacteremia in human patients.[16] Similarly, *Prevotella intermedia* is considered to be normal gingival flora in humans, but has also been implicated as a cause of periodontal and endodontal infection.[35] In rhesus macaques, one study reported *P. intermedia* cultured from gangrenous polymicrobial infection of the thigh that was likely caused by repeated morphine sulfate injections.[58]

Fusobacterium nucleatum and *F. necrophorum* are identified as commensals in people and animals,[26,48] but can be associated with severe disease. *F. nucleatum* colonizes subgingival sulci of healthy rhesus macaques and is often isolated in both rhesus and human periodontal disease.[26,68] *F. necrophorum* is a leading cause of foot rot, hepatic abscesses, and respiratory tract infections in cattle and other animals.[42,64] In people, *F. necrophorum* causes Lemierre syndrome, an acute oropharyngeal infection accompanied by internal jugular thrombophlebitis.[41] Overall, determining the significance of culturing any of these bacteria is difficult due to a dearth of literature on their pathogenicity in rhesus macaques. This underscores the need for increased reporting of infectious disease in rhesus macaques in order to better inform treatment decisions when potential pathogens are cultured.

Control of implant-associated bacterial infections was generally performed by the research groups and involved cleaning and disinfecting implant margins and craniotomy sites, as well as administration of antimicrobial therapy. In many cases, cefazolin was administered topically to implant and craniotomy sites after disinfection to reduce bacterial load. Cefazolin was one of the most frequently used perioperative antibiotics for this purpose within the study period and was the only antibiotic against which resistance developed. Although the Association of Primate Veterinarians advises against the topical use of cephalosporins in recording chambers,[6] topical application of cefazolin has been used in studies of fresh or completely

clean wounds.[57,87] As regards the use of cefazolin in this study, its administration to craniotomy sites likely neither adequately reached infections extending beyond the immediate implant site, nor was treatment performed at a frequency that maintained local and systemic therapeutic concentrations. Altogether, these factors likely contributed to the formation of an ideal environment for the development of resistance.

Due to the archival nature of this study, many factors were not available for analyses because of incomplete or absent records. Surgical techniques, cleaning regimens, treatment data, and husbandry information would have all provided a more granular understanding of blood value changes or specific culture results. Details on changes in personnel, sample collection, and specimen analysis over the years may have provided greater model fit. The absence of data highlights the importance of detailed and complete recordkeeping for derivation of a complete picture from experimental and epidemiologic research, particularly when live animals are used. Maximizing data collection from the lowest necessary number of animals is a core tenant of laboratory animal use, so record keeping must be complete and fastidious.

In conclusion, our use of historical data has improved our understanding of how percutaneous cranial implants affect systemic health in rhesus macaques by provoking an inflammatory response and perhaps a coinciding cortisol stress response. Numerous bacterial species may contribute to inflammation, yet the degree to which these bacteria are the source of infection and inflammation or are merely members of the normal skin microbiome is unknown. In addition, age and experimental techniques also influence blood values. These factors should be considered when assessing animal health and welfare in a laboratory setting.

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