

Experimental splenectomies and malaria in mice¹

Esplenectomias e malária experimental em camundongos

Róbson Miguel de Araújo Negreiros^I, Fabiano Hiromichi Makimoto^{II}, Linda Luciana Oliveira Santana^{II}, Luís Carlos de Lima Ferreira^{III}, Gerson Suguiyama Nakajima^{IV}, Maria Cristina dos Santos^V

^I Master Degree, Professor of Surgery Clinical Department, School of Medicine, UFAM, Amazonas, Brazil.

^{II} Graduate student, School of Medicine, UFAM, Amazonas, Brazil.

^{III} Associate Professor, Pathology Department, School of Medicine, UFAM, Amazonas, Brazil.

^{IV} Associate Professor, Surgery Clinical Department, School of Medicine, UFAM, Amazonas, Brazil.

^V Associate Professor, Parasitology Department, Biological Sciences Institute, UFAM, Amazonas, Brazil.

ABSTRACT

Purpose: To evaluate the importance of spleen in malaric infection in murino model, comparing the parasitemia and the titles of immunoglobulins in the different groups. **Methods:** It was used female mice non-isogenic, in inoculated with *Plasmodium berghei*, cepa ANKA, intraperitoneally. The parasitemia was analyzed in 23rd, 25th, 27th and 32nd day of the experiment, being the stained blood exam colored by Giemsa. The titles of the total serum immunoglobulins IgM and IgG were analyzed by Dot-ELISA technique, at 6th, 22nd and 32nd day, when the animals were sacrificed. **Results:** The parasitemia was gradual in all the inoculated groups. In the end of the experiment, the animals with partial parasitemia present superior parasitemia, but next to the non-splenectomized, while the asplenic present difference bigger than the double. The levels of total serum IgM and IgG didn't have significant changes with the removal partial or total splenic. **Conclusion:** The techniques conservatives in splenic trauma are possible and necessary. The importance of remaining spleen in the clearance of red blood cells parasitized by *Plasmodium berghei* showed being efficient, in order to avoid serious complications resulting of the malaria in mice.

Key words: Splenectomy. Malaria. Sepsis. Infection. Mice.

RESUMO

Objetivo: Avaliar a importância do baço na infecção malárica em modelo murino, comparando a parasitemia e os títulos das imunoglobulinas nos diferentes grupos. **Métodos:** Utilizaram-se camundongos fêmeos não isogênicos, inoculados com *Plasmodium berghei*, cepa ANKA, intraperitoneal. A parasitemia foi analisada no 23º, 25º, 27º e 32º dia do experimento, sendo o exame do esfregaço sanguíneo, corado pelo Giemsa. As titulações das imunoglobulinas totais séricas IgM e IgG foram realizadas pela técnica Dot-ELISA, no 6º, 22º e no 32º dia, quando os animais foram sacrificados. **Resultados:** A parasitemia foi progressiva em todos os grupos inoculados. Ao final do experimento, os animais com esplenectomia parcial apresentaram parasitemia superior, porém próximos as dos não esplenectomizados, enquanto que os asplênicos apresentaram diferença superior a 100%. Os níveis de IgM e IgG totais séricos não foram alterados significativamente com a remoção parcial ou total esplênica. **Conclusão:** As técnicas conservadoras no trauma esplênico são possíveis e necessárias. A importância do remanescente esplênico no clearance das hemácias infectadas pelo *Plasmodium berghei* demonstrou ser eficiente, de modo a evitar sérias complicações decorrentes da malária em camundongos.

Descritores: Esplenectomia. Malária. Sepsis. Infecção. Camundongos.

¹Research performed at the Laboratory of Surgery and Anesthetics, Department of Surgery, Federal University of Amazonas (UFAM), Brazil.

Introduction

Trauma is a severe public health issue across the world, one that has had a major impact on human mortality attributed to external causes. Currently, trauma is the third cause of mortality in the world¹. The epidemiological situation in Brazil is even worse: trauma it is the second leading cause of death, and the leading cause in the first four decades of life;² therefore, it represents a huge and growing challenge to the country in both economic and social terms.

The external causes are on the rise in Brazil. The program that addressed emergency and trauma of the Ministry of Health³

reported, at the time, 100,000 deaths per year³. At the end of the 20th century, around 124,000 deaths were attributed to trauma⁴ and this rose to 150,000 in 2004². Hundreds of thousands of those who suffer trauma have permanent sequelae and represent a socially marginalized group.

The spleen is one of the organs most frequently injured in closed abdominal traumas, especially in children. Spleen injuries frequently result from penetrating wounds on the left side of the body, of the transition thoracic-abdominal and of the back. The most common cause of splenic injury is the closed trauma produced by an automobile accident; such injuries have predominant

conduct in the lacerations the total splenic ablation⁵. Asplenic patients are more susceptible to severe infections by encapsulated bacteria⁵ and malaria⁶.

Despite significant technological and scientific developments, malaria remains a major public health problem. According to the World Health Organization, it is the more concerning tropical disease because of the high rate of mortality of the illness; in addition, it is a cause of many socio-economic problems. It is estimated that 40% of the world's population live in areas at risk of malaria transmission in more than 100 countries⁷. According to the Department of Health Surveillance of the Ministry of Health (2003), in Brazil, there are 600,000 cases of malaria per year, mainly in the Amazon Basin, where 99.5% of the cases are recorded⁸.

It is remarkable that a person can survive after an important body organ like the spleen is removed. A full splenectomy generally does not involve severe complications, suggesting the presence of compensatory mechanisms for the loss of several splenic functions⁹. However, many effects of total ablation of the spleen are reported, mostly related to the functioning of the secondary lymphoid organ that is responsible for immune response to the stock antigen circulate¹⁰. A total splenectomy was reported to reduce the levels of immunoglobulins in serum, especially the IgM¹¹. The removal of the spleen had effects on other organs, particularly the liver⁹. It appeared that after the total ablation of spleen, the patients die in greater numbers and earlier than the expected age of the general population, not only as a result of severe infections, but also due to pulmonary embolism, acute myocardial infarction¹² and atherosclerotic phenomena¹³.

Partial splenectomies have been done since the beginning of the nineteenth century, but only after the studies by Campos Christo¹⁴ was the surgical procedure done based on segmental and terminal vascularization. The preservation of the upper pole's spleen, after ligation of the vascular pedicle was proposed by Petroianu¹⁷. This has been studied both clinically and experimentally in several works, and has been done successfully since 1984, because it provides the spleen with twice the area of irrigation¹¹.

In the present study, we assessed the effects of partial and full splenectomies in mice infected by *Plasmodium berghei*.

Methods

This was an experimental prospective study with a longitudinal cohort design in mice (*Mus domesticus domesticus*), a Swiss house mouse, to evaluate the action of *Plasmodium berghei* in splenectomized (full and partial) and non-splenectomized animals. The study was conducted according to the Ethical Principles of the Brazilian College of Animal Experiments - COBEA and was approved by the Ethics Committee on the Use of Animals (CEUA) of the School of Medicine of Federal University of Amazonas, on 16 April 2008.

Twenty female mice were used; the mice were non-isogenic, age four to five weeks, and weighed between 15 to 20 grams. The animals were provided by the vivarium of Physiological Sciences Department at the Biological Sciences Institute, Federal University of Amazonas (UFAM). The animals went through an acclimation period of seven days in polypropylene cages with wood shavings, mineral water and ration Labina® autoclaved, at room temperature (25°C) with light-dark cycles of 12 hours.

The animals were separated into four groups of five and procedures performed with each group as described below:

Group I: No intervention.

Group II: Anesthesia, laparotomy, inoculation of contaminated blood.

Group III: Anesthesia, laparotomy, partial splenectomy, inoculation of contaminated blood.

Group IV: Anesthesia, laparotomy, full splenectomy, inoculation of contaminated blood.

Surgical procedures

On the eighth day of the trial, groups II, III and IV were submitted to pre-anesthesia with atropine sulphate (0.045mg/kg to 0.025%, intramuscular) fifteen minutes before anesthesia. The animals were anesthetized with a combination of ketamine hydrochloride (60mg/kg to 5%, intramuscular) and xylazine hydrochloride (16mg/kg to 2%, intramuscular). This was done with anti-sepsis and sterilized, median laparotomy and spleen exposure. At the end of surgery, the abdominal wall was closed.

In Group III, after exposure of the spleen, the ligation of corresponding vessels was done for the segment to be resected; the spleen tissue was sutured with U-shaped stitches and 50% of the spleen was removed. In Group IV, the ligation of the main pedicle and of the main spleen-gastric vessel was held and organ was subject to total ablation.

At the end of surgery, the mice received subcutaneous hydration with 2mL of sodium chloride 0.9% in the areas of the dorsolateral neck. The animals were then placed on Mayo's tables covered with double fields and incandescent surgical lamps to raise the temperature to around 30°C during post-anesthetic recovery. The animals were evaluated twice at the end of surgery, after 30 and 60 minutes.

Infection by *plasmodium berghei*

On the 20th day of the experiment, 10⁶ parasitized red blood cells were inoculated with *Plasmodium berghei*, the ANKA strain. The inoculation was done intraperitoneally in the left lower quadrant for the animals in groups II, III and IV.

Tracking the parasitemia level

The evolution of the parasitemia level in the mice was done using a hemoscopic survey (blood smear) on the 23rd, 25th, 27th, 29th and 32nd day. After taking the smear, it was fixed with 100% methanol and stained using the Giemsa method. The hemoscopic examination was performed using a microscope optical, with 1000x increases and analysis of ten fields. The level of parasitemia was determined using the ratio of red blood parasitized cells to the total number of red blood cells counted.

Dosage of immunoglobulins

On the 6th, 22nd and 32nd day, blood samples of 200µL were collected from the venous plexus retro-eye of all the mice in the study. The samples were centrifuged on low and serum was stored at a temperature of -20°C. The titles of total serum immunoglobulin class M and G were titrated using the Dot-ELISA

technique described by Towbin and Gordon¹⁵. The technique consists in two micro-liters of each serial dilution of sera (1:16 until 1:262144) applied at membranes of nitrocellulose 0,45µm. After drying at room temperature, the membranes were immersed in blocking solution containing 5% of skimmed milk dissolved in buffer Tris-Saline (TBS) pH 7.5 for two hours at room temperature. After the blocking, the membranes were placed in the presence of conjugated: anti-IgG Fc mouse region marked with peroxidasis or an anti-IgM chain of µ marked with peroxidasis, diluted 1:1000 in TBS pH 7,5 for one hour, at room temperature. Soon after, the membranes were washed four times with saline phosphate buffer (PBS) pH 7.2 and the antibody-conjugated reactions revealed with five milligrams of the substance chromogen 3'3' Diaminobenzidine diluted in 30 mL of TBS of pH 7,5, in the presence of the peroxidasis substrate, the hydrogen peroxide PA, to 0.015%. The reaction of

coloring by precipitation of DAB was interrupted by adding distilled water and the membranes were then placed between two sheets of filter paper for drying. The immunoglobulin titles present in the sera of mice were obtained by the visual reading of end point reactions. For statistical analysis, the titles of immunoglobulins were presented using a neperian logarithm.

Animal euthanization

The animals were euthanized by applying three times the dosage of anesthesia through intramuscular injections.

Statistical analysis

The correlation of the data and the statistical analysis were performed (or done) by taking the averages of the groups and adding or subtracting the standard deviation of the average number of experiments. The results were analyzed by the statistical method considered by ANOVA and Student's t-test, using the program GraphPad Prism® 3.0. Differences were considered significant when the probability was less than 5% (p <0.05).

Results

The parasitemia of animals was progressive in all groups inoculated by *Plasmodium berghei* (Figure 1). At the end of the experiment (on day 32nd), the parasitemia of the group of non-splenectomized animals (Group II) was 31.04%; the group of

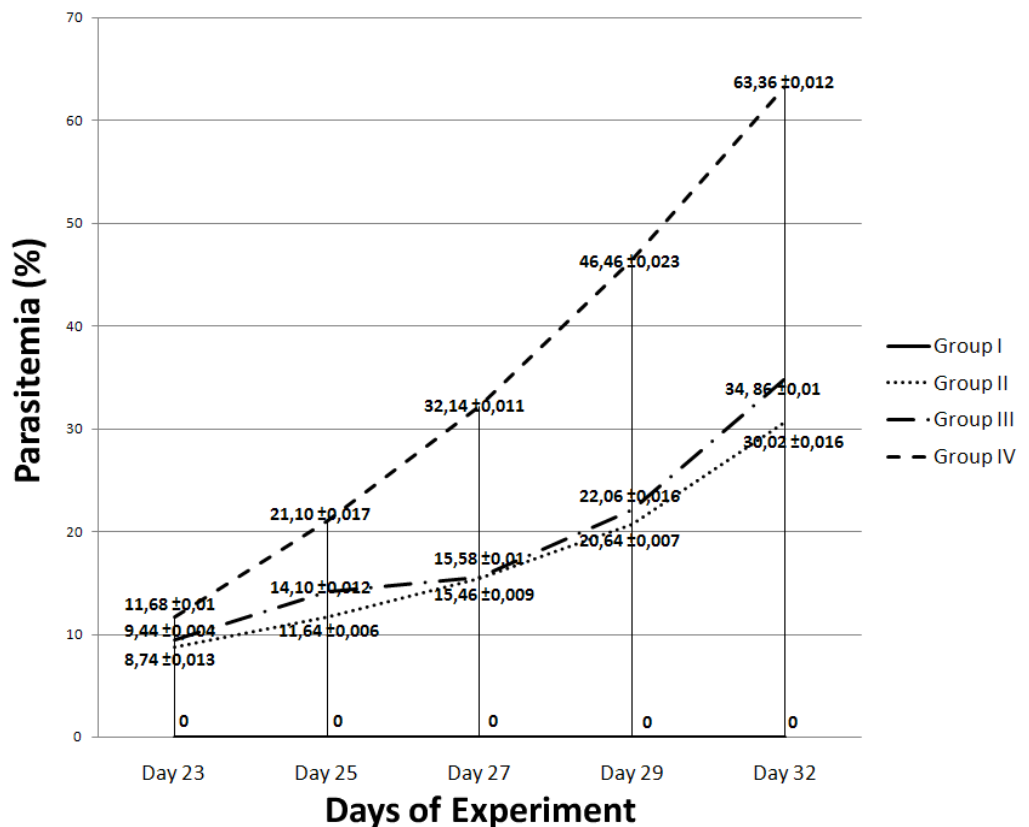


FIGURE 1 - Parasitemia's curve of the different groups during the experiment

P.S.: Student's t-test, paired by day:
 Group 2 X Group 3: p= 0,0183;
 Group 2 X Group 4: p= 0,0155;
 Group 3 X Group 4: p= 0,0170

animals treated with partial splenectomy (Group III), 35.01%, and 63.22% (p<0.05) for the group of completely splenectomized animals (Group IV). The average parasitemia among the groups was statistically significant (G3xG4: p = 0.0183; G3xG5: p = 0.0155 and G4xG5: p = 0.0170, paired by day Student's t-test).

There was no difference among the titles of immunoglobulin of the total serum of classes M and G (p>0.05) among groups in the study during the days upon which blood was collected (Figures 2 and 3).

Discussion

The spleen performed a fundamental role in the clearance of *Plasmodium berghei*¹², through the removal of red blood cells infected with the parasite¹⁴. The maintenance of the portion of spleen tissue was satisfactory in controlling parasitemia, because the mice subjected to partial splenectomy presented a level of parasites similar to that of non-splenectomized mice, while the animals subjected to full splenectomy had twice the amount of circulating parasites (Figure 1). This corroborated previous studies indicating that the asplenia hampers control of parasitemia¹¹.

The animals without spleens also present deficiencies in the production of IgM, due to sharp decrease of B residents lymphocytes, especially those in the area surrounding the spleen¹⁶. In the samples collected on the 6th, 22nd and 32nd days of the trial, no significant changes (p>0.05) were observed for the titles of IgM in any of the four groups. The titles of IgM detected on the last day of the trial (day 32nd) show that the mice inoculated and

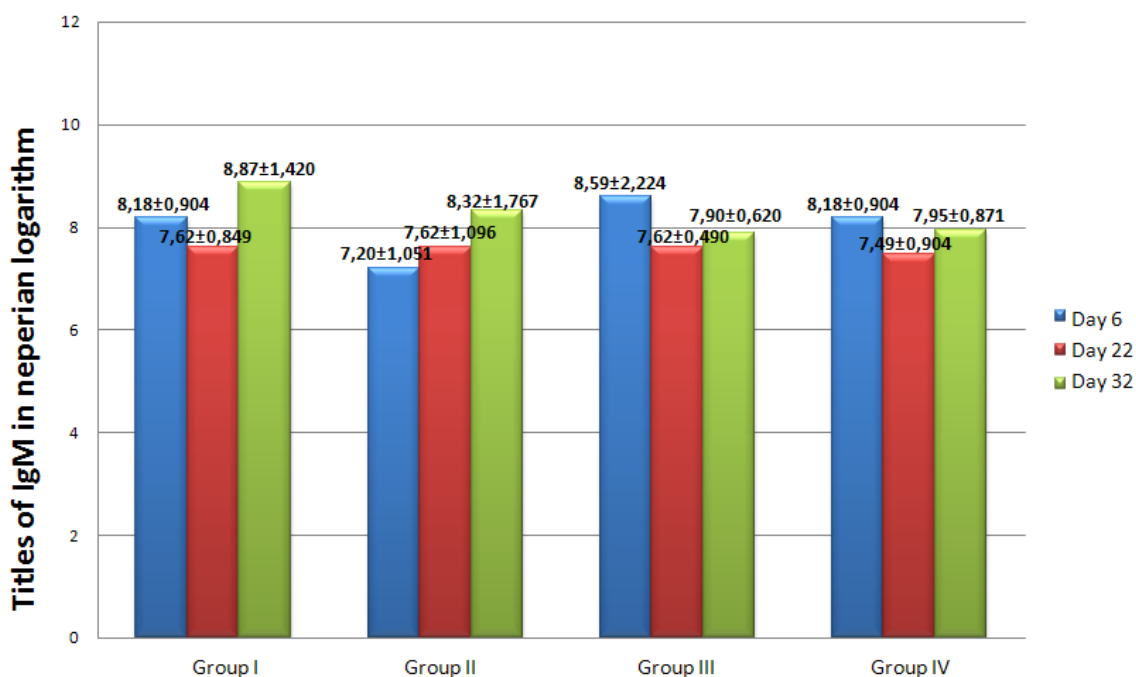


FIGURE 2 - Titles of total M Immunoglobulins in different groups

P.S.: Variance's Analysis by ANOVA, one way:

Group 1, 2, 3 e 4: p=0,5993

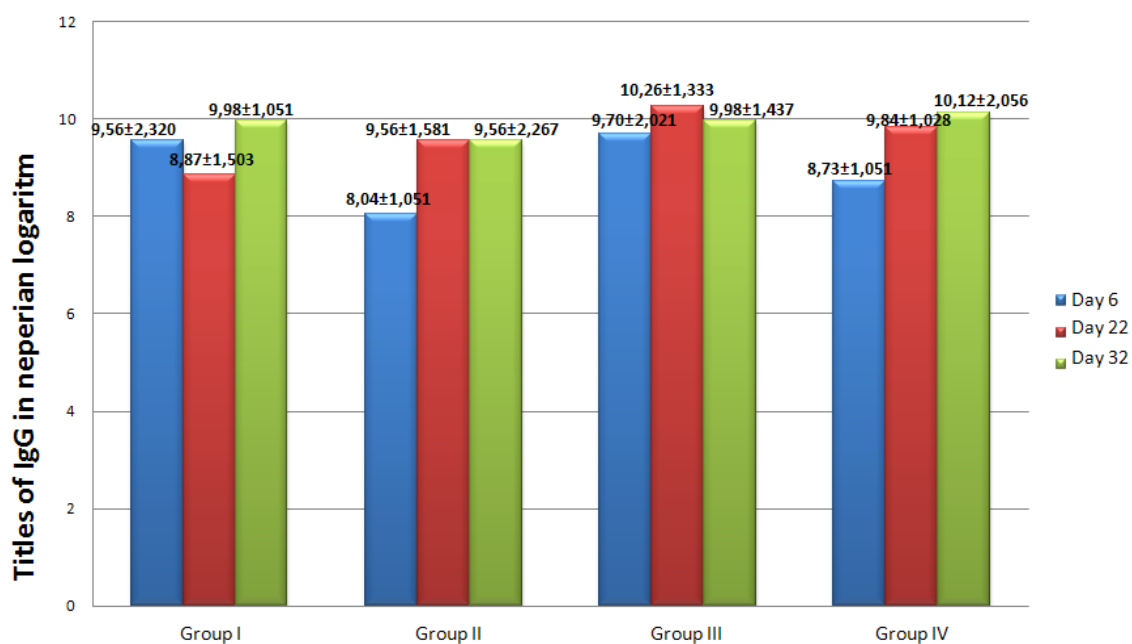


FIGURE 3 - Titles of total G Immunoglobulins in different groups

P.S.: Variance's Analysis by ANOVA, one way:

Group 1, 2, 3 e 4: p=0,1341.

not subjected to splenectomy (Group II) displayed a decrease of title of IgM when compared to the individuals of control group (Group I), but this difference was not significant ($p>0.05$). In mice inoculated and subjected to partial splenectomy (Group III), despite the fact that the level of parasites had been similar to that of Group II, the levels of immunoglobulins were lower on the 32nd

day ($p>0.05$), probably due to the decrease of B lymphocytes as described by WARDEMANN *et al.*¹⁶. However, on the 6th day the titles of IgM were higher in the animals of Group III ($p>0.05$). In the mice inoculated and subjected to full splenectomy (Group IV), the parasitemia was exacerbated, indicating the inability to fully eliminate parasites. However, although the difference is not

significant ($p > 0.05$), a small increase of the titles IgM was observed on the 32nd day for Group III, and, on the same day, the titles of IgG were increased in relation the other groups. It is worth noting that the immunoglobulins present in the plasma of Group IV animals were not specific to *Plasmodium berghei*, because they were not effective in neutralizing the circulating parasites. The results show that levels of total serum IgM and IgG did not change with partial or total removal of the spleen. These levels may be reached by secondary lymphoid organs or even by the liver which would again perform the immune function, as it does in the fetal stage.

Conclusion

The use of conservative techniques aimed at partial or full preservation of the spleen should always be sought given the importance of the spleen in clearing red blood cells parasitized by *Plasmodium berghei*. This could prove key to preventing serious infections arising from malaria.

References

- Geyer PG, Albarran CB, Breigeiron R. Trauma renal. Acta Med. 2007;18:291-7.
- Trauma e violência. Projeto de Atenção Nacional ao Trauma. Sociedade Brasileira de Atendimento Integrado ao Traumatizado. Disponível em: <<http://www.s bait.org.br/projeto.php>> Acesso em: 04 jul.2008.
- Brasil. Ministério da Saúde. Secretária de Vigilância e Saúde. Programa de Enfrentamento às Emergências e Traumas. Projeto 1 – Prevenção. Projeto 2 – Atendimento Pré-hospitalar. Projeto 3 – Atendimento hospitalar. Projeto 4 – Reabilitação. Brasília, 1990.
- Brasil. Conselho Nacional de Saúde. Aprova a proposta do Ministério da Saúde de Política Nacional de Redução de Morbimortalidade por Acidente e Violência. Resolução n. 309, de 08 de março de 2001. In: Diário Oficial da União, Brasília, mar. 2001.
- Marques RG, Petroianu A. Infecção fulminante pós-esplenectomia. Arq. Gastroenterol. 2003;14(1):47-54.
- Demar M, Legrand E, Hommel D, Esterre P, Carme B. *Plasmodium falciparum* malaria in splenectomized patients: two case reports in french guiana and a literature review. Am J Trop Med Hyg. 2004;71(3):290-3.
- Engwerda CR, Beattie L, Amante FH. The importance of spleen in malaria. Trends Parasitol. 2005;21(2):75-80.
- Brasil. Ministério da Saúde. Secretária de Vigilância e Saúde. Programa Nacional de Controle da Malária; 2003.
- Bucher GA. The role of the spleen and immunization against malaria. Trends Parasitol. 2005;21(8):356-7.
- Karlsson MCI, Guinamard R, Bolland S, Sankala M, Steinman RM, Ravetch JV. Macrophages control the retention and trafficking of B lymphocytes in the splenic marginal zone. J Exp Med. 2003;198(2):333-40.
- Petroianu A. Avaliação da função fagocitária em remanescentes de esplenectomia subtotal e auto-implante esplênico autógeno. Rev Bras Hematol Hemoter. 2003;25(1):25-30.
- King H, Shumacker Jr. H.B. Susceptibility to infection after splenectomy performed in infancy. Ann Surg. 1952;136(2):239-42.
- Petroianu A. Mortalidade após esplenectomia. Rev Brasil Hematol Hemoter. 2007;29(2):103-5.
- Campos-Christo M. Esplenectomias parciais regradas: nota prévia sobre os três primeiros casos operados. Hospital. 1959; 56:645-50.
- Towbin H, Gordon J. Immunoblotting and dot immunobinding-current status and outlook. J Immunol Methods. 1984;72(2):313-40.
- Wardemann H, Boehm T, Dear N, Carsetti R. B1-a B Cells that link the innate and adaptive immune responses are lacking in the absence of the spleen. J Exp Med. 2002;195(6):771-80.
- Petroianu A. Esplenectomia subtotal e anastomose esplenorrenal proximal para o tratamento da hipertensão portal. Rev Bras Cir. 1983;73(2):101-4.

Acknowledgements

The author wishes thank to Dr. Roberto Sena Rocha, from Oswaldo Cruz Foundation in Amazon, and the Dr. Luiza Helena Carvalho, of the Research Center René Rachou in Minas Gerais, for the cession of the *Plasmodium berghei*'s cepa. Also he wishes thank to Prof. Fábio Tonissi Moroni and Prof. Dr. José Fernando Marques Barcellos, from Biological Sciences Institute of UFAM, for yield mouse and materials for the accomplishment of the project.

Conflict of interest: none

Financial source: none

Correspondence:

Róbson Miguel de Araújo Negreiros
Surgery Clinical Department, School of Medicine, UFAM
R. Afonso Pena, 1053
69020-160 Manaus – AM Brazil
Phone: (55 92)3621-6590 / 9982-0927
negreirosrobson@hotmail.com

Received: April 15, 2009

Review: June 09, 2009

Accepted: July 14, 2009

How to cite this article

Negreiros RMA, Makimoto FH, Santana LLO, Ferreira LCL, Nakajima GS, Dos-Santos MC. Experimental splenectomies and malaria in mice. Acta Cir Bras. [serial on the Internet] 2009 Nov-Dec;24(6). Available from URL: <http://www.scielo.br/acb>