Hematological reference values and animal welfare parameters of BALB/C-FMABC (Mus musculus) inoculated with Ehrlich tumor kept in the vivarium at ABC Medical School

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Abstract

Background: Biochemical and hematological parameters are important tools for assessing the physiological profile of vital organs, and can be recorded to create reference values used for clinical diagnosis of diseases. Many research laboratories lack the means to establish their own set of reference parameters for use in their research, and while there are articles in the literature that discuss laboratory parameters for healthy BALB/c mice, few studies address the evaluation of these parameters in pathological situations, such as in mice inoculated with Ehrlich tumor.

Method: BALB/c-FMABC mice previously inoculated with Ehrlich tumor were maintained under appropriate conditions. Blood samples were taken for analysis of hematological parameters using automated and semi-automated equipment to create a set of the animal welfare parameters for evaluation.

Result: Results were obtained for all the hematological parameters for all groups analyzed. These showed: statistically significant differences between the initial and final tumor weight; comparable initial tumour volume and weight; an increase in leukocytes in the 7-day group with a characteristic predominance of lymphocytes and neutrophils; statistically significant changes in RDW in the 21-day group and in the welfare parameters in the 28-day group.

Conclusion: The study successfully defined and established reference values for hematological and welfare parameters for all groups analyzed.

Keywords

ehrlich tumor carcinoma, experimental animal model, reference value, welfare
1 | INTRODUCTION

The use of animals in scientific research is extremely important, especially in experimental investigations. Animals are used experimentally to enhance understanding of physiological and metabolic mechanisms and also to establish new treatments and improve existing treatments and drugs used in human and veterinary medicine, thus assisting in scientific development.1

It is thus recognized that animal research has had a major impact on human longevity and has improved human and animal welfare, in particular contributing to advances related to organ transplants, vaccine production, cancer research, cardiovascular disease, and drug production and control.2

In order to guarantee the quality and reliability of the tests applied to, and data obtained from, experimental animals, they must be kept in a strictly controlled environment that conforms with parameters of sanitary and genetic quality established to ensure results are obtained with minimal interference.3

While several species of animals have been used in research, the mouse has been the most widely used for research related to human pathologies because of its favorable characteristics: physiological similarities with humans, small size, short gestation period, and easy maintenance and handling.1

More than 90% of scientific research currently uses mice from isogenic strains. The isogenic mouse is obtained by mating at least 20 inbreeding generations from a single couple, generating an inbreeding coefficient of 98.6%. Isogenic strains have great value because they allow experiments to be carried out that eliminate the factors of genetic variability, and fewer animals are needed to achieve the necessary statistical power.4

A wealth of information about isogenic mice can be found, including genetic mapping, histocompatibility parameters, and physiological, pathological and immunological parameters, which are mainly useful for cancer research and transplantable tumors for therapeutic drug testing. BALB/c mice are isogenic, presenting a low incidence of ovarian cysts and mammary tumors, with zero incidence in male animals, 5% in pregnant females and 1% in virgin females. However, BALB/c mice easily develop other types of cancers, including reticular neoplasms, lung tumors, and kidney tumors.5 6

Ehrlich tumor is a type of transplantable experimental species-specific neoplasm of malignant epithelial origin, corresponding to the female mouse mammary adenocarcinoma. The tumor develops in different strains of animal species in ascitic form when inoculated via the intraperitoneal route, and in solid form when inoculated subcutaneously, and because of its high invasiveness rate, it is frequently used to study the action of physical, chemical and biological compounds in experiments focused on cell pathogenesis, immunology, cytogenetics and even therapy.7 9

Laboratory tests are central to the evaluation of specific changes in the physiological and functional profile of an animal. In experimental procedures where pathological processes are induced, knowledge of normal reference values is indispensable, since some pathologies influence the metabolism and alter the test results, and use of the reference values allows evaluation of the degree of disease effectiveness of treatments.5 10 11

A literature survey of results obtained in several studies using the BALB/c strain of mice revealed great variation in reported biochemical parameters, which depended directly on variable factors such as age, sex, lineage, and genotype, and additionally was influenced by other factors such as age, housing condition, diet, management, and sanitary standards in the vivarium, amongst others.12 15

Thus it is important for each vivarium or research laboratory to establish its own laboratory reference values for the animals used in its research programs, based on the available lineage, age, sex and environmental conditions. In the specific case of BALB/c strain animals infected with Ehrlich tumor, there is a scarcity of reports establishing reference values for laboratory tests associated with the tumor.16

The present work aims to research and establish reference values for hematological laboratory tests and animal welfare parameters for the BALB/c-FMABC strain of mice, highlighting the physiological changes present in animals inoculated with Ehrlich tumor, in order that these parameters can be used to supplement future research involving this experimental model.

2 | MATERIALS AND METHODS

2.1 | Animals and ethical aspects

All experimental procedures described in this study were approved by the Animal Experimentation Ethics Committee of the ABC University Health Center in accordance with Law 11.794/2008 (Arouca Law) approved in 2019 under protocol 05/2019.

All procedures used are in accordance with the Brazilian Directive for the Care and Use of Animals for Scientific and Didactic Purposes of National Council for Animal Experimentation Control (CONCEA).

2.2 | Description of subjects

This experimental study was conducted at the vivarium in the ABC University Health Center and used 45 male albino mice of the BALB/c strain from the vivarium, with an average weight of 30 ± 5 g. During the experiment, the animals were maintained for 28 days in a 12 hour light/ dark photoperiodic cycle with controlled ventilation (20 air changes/hour) and temperature (20 ± 2ºC), and relative humidity between 45% and 65%, and were fed with filtered water and Nuvilab® CR-1 (Nuvital) feed offered ad libitum. The animals were handled quickly and carefully through the base of the tail, following the protocol mandated for good animal care practice at the ABC University Health Center vivarium.

2.3 | Experimental design

To obtain a solid tumor, tumor cells were obtained from a 7-day-old Ehrlich ascites tumor. The number of total cells subsequently
implanted was determined by counting under optical microscopy using a Neubauer chamber. Ascitic fluid was diluted ten times with saline and aliquoted. A cell viability test was performed with Trypan-Blue® dye, and only suspensions with cell viability greater than 95%, assessed according to the protocol of the Clinical Analysis Laboratory at the ABC University Health Center, were used.

The dorsal region of each mouse was shaved and the cell suspension was injected at a concentration of $2 \times 10^5$ cells/mL into the lateral region of the back using a 24-gauge needle (Figure 1).

Seven days after inoculation, daily observations of the following parameters began: tumor progression by size ($\text{cm}^2$), animal weight, welfare analysis and survival of the animal. The observations were maintained for a maximum period of 28 days. The animals were divided into three groups – 7-, 21- and 28-day groups – for analysis of the results. This allowed assessment of laboratory parameters not only after 28 days but throughout the period of tumor development, to evaluate the progression of the disease and provide reference parameters for future research on the tumor in its acute and chronic phases (Figure 2).

### 2.4 Blood collection procedure

After 7, 21 and 28 days, the mice were euthanized by an intraperitoneal Cristália™ sodium thiopental anesthetic overdose (100 mg/kg). After establishing anesthesia, a laparotomy was performed, followed by caudal vena cava puncture to remove the maximum possible amount of blood. The blood was homogenized and prepared for analysis in pediatric plastic tubes containing the anticoagulant BD™ K$_2$EDTA. The blood was then analyzed using automated hematological analysis equipment.

### 2.5 Hematological analysis

Hematological analysis was performed by flow cytometry method, using Sysmex XN-1000™ equipment and the Sysmex™ reagents. Erythograms, leukograms and platelet parameters were generated, and microscopic analysis of a blood smear was conducted to assess hematological cell differentiation, following the rules of good practice in clinical laboratory analysis.

### 2.6 Statistical analysis

For qualitative variables, absolute and relative values were used. Median, 95% confidence interval and 25 and 75 percentiles were used to express the non-normal quantitative data (Shapiro-Wilk < 0.05). For the data representing normality (Shapiro-Wilk > 0.05), mean, standard deviation, and minimum and maximum values were used. To test differences between and within groups for the parameters recorded, the chi-square, Kruskal-Wallis and

![Figure 1](https://example.com/figure1.png)

**Figure 1** Ehrlich tumor macroscopy and microphotography. A, Solid Ehrlich tumor, inoculated in the dorsolateral region (tip) and onset of tumor ulceration. B, Inflammatory infiltrate (arrow). C, Blood vessel (v) and cells with loss of nucleus-cytoplasm relationship (zoom) (Hematoxylin & Eosin stain). D, Ehrlich ascitic tumor with binucleation (N), evident nucleoli (nu) and presence of lymphocytes (L), stained by Leishman. Source: Prepared by the author.
ANOVA tests were performed. For all analyses a 95% confidence level was used. The statistical program used was GraphPad Prism® version 7.0.

3 | RESULTS

3.1 | Animal weight and tumor volume

Regarding the weight of the animals, statistical variance was observed when data obtained at the beginning were compared with data obtained at the end of the experiment in the 7- and 21-day groups, with an increase in the 7-day weight ($P = .0140$) and a decrease in 21-day weight ($P = .0010$); however, no statistical difference was observed in 28-day animals, as shown in Figure 3.

Tumor volume was also evaluated by measuring the tumors using caliper rulers on the first and last experimental days in the analyzed groups. Tumor volume was also assessed after euthanasia, when a surgical excision of the tumor was performed and the volume compared with the initial and final tumor volumes. Final tumor volume was significantly different in the 21- and 28-day groups ($P < .0001$), as shown in Figure 4A, and a significant difference in the weight of the isolated tumor ($P < .0001$) after 28 days was also observed (Figure 4B).

The tumor growth curve showed a continuous increase with an accelerated increase after the 21-day period, corroborating the results obtained in other analyses (Figure 5).

3.2 | Hematological parameters

There were no statistical differences over time in most parameters measured in the hematological analysis, except for red blood cell distribution width (RDW) and absolute white blood cell count ($P < .0001$). The RDW in the 21-day group showed a statistically significant difference ($P = .0108$) compared with the other groups and the absolute leukocyte count showed a considerable increase in the first 7 days.

The distribution of red blood cells (RDW) is calculated by the size of the red blood cells distributed up to 20% above the base of the automated histogram from the red blood cell count, determining the size of all evaluated red blood cells, thus showing the size variation between the erythrocytes in percentage.\textsuperscript{10, 11}

The degree of anisocytosis should be confirmed by visual microscopic analysis. Even though RDW data showed statistically significant differences, no anisocytosis was found in the slides of the animals studied.\textsuperscript{10-12}

A predominance of lymphocytes and neutrophils was observed in the differential leukocyte count, and the concentrations of these cells in the 7-day group were significantly different, as shown in Table 1.

3.3 | Animal welfare parameters

Since its control directly influences the results, animal welfare was evaluated throughout the experimentation period, with a final overall score calculated at the end of each period. The 7- and 21-day groups did not show any significant statistical differences, but the 28-day group differed significantly ($P = .0008$) from the 7-day group, as shown in Table 2.
3.4 | The Neutrophil/Lymphocyte Ratio (NLR)

Using data from Table 1 (hematological parameters), the neutrophil/lymphocyte ratio (NLR) was calculated and compared with the score obtained by averaging the total animal welfare score at the end of each period analyzed. The NLR increase was compatible with the hematological data in the first 7 days and as a result it was possible to observe significant differences compared with the 21- (P = .0028) and 28-day periods (P = .0015).

The mean of the final score increased over the analysis periods, but the difference was only statistically significant in the 28-day group compared with the 7-day group (P = .0008), as shown in Figure 6.

4 | DISCUSSION

Hematological, biochemical and immunological parameters are faithful indicators of the physiological response of the animal to pathogenic stimulation and endogenous alterations, and are widely used as diagnostic biomarkers. Regular monitoring of laboratory parameters is extremely important for keeping animals in breeding systems, and a change in laboratory parameters may suggest organ or tissue damage, environmental changes and even infections.

Pérez et al report that, when correctly determined, these parameters can accurately show the animal’s conditions at the time of blood collection and can also be used to assess its housing quality, and several authors describe the importance of laboratory parameters for different strains of experimental mice. However, there is a lack of established parameters for mice with Ehrlich carcinoma in conditions where there is no treatment.

In view of this, this work established hematological and welfare parameters in BALB/c albino mice produced in the vivarium at ABC University Health Center and inoculated with the transplantable Ehrlich tumor.

Muriithi et al report that variations in hematological parameters can be observed both between mouse strains and in sex and age. In this study, we did not differentiate between animal sex in our analyzes, choosing rather to work with a heterogeneous population to characterize broader values that may be used in different experimental situations.

Statistical differences in the weight of the animals (Figure 3) between the 7- and 21-day groups were observed. The 7-day group presented an average weight increase of about 10% of the value initially observed, possibly linked to tumor growth; in the 21-day group, a mean decrease of around 12% was observed, which could be attributed to angiogenesis factors and nutritional needs of the tumor. Lack of interest in food (inappetence) is common in cancer patients, resulting in low food intake and depletion of muscle and adipose tissue, favoring cachexia.

Cachexia in cancer is a syndrome that combines physiological, metabolic and psychological factors. The main signs manifested are progressive and involuntary weight loss, fatigue, anemia, excessive energy expenditure and tissue loss. This syndrome can manifest at any stage of the tumor process, and other studies report that solid tumors are more likely to cause tissue loss than hematologic cancers.

The weight loss observed in the 21-day group of animals in this study was also noted in da Silva et al in a control group of animals with Synadenium umbellatum receiving no treatment. This group demonstrated a decrease in muscle mass and adipose tissue, and behavioral changes were also observed, where the animal adopted hypoactive behaviors, possibly related to fatigue.

The tumors isolated after excision presented differences in the final weight parameter, with significance notable only at 28 days (Figure 4A); tumor volume showed a significant statistical difference
in the 21- and 28-day groups (Figure 4B). Comparing these data, proportional compatibility between the increases in tumor weight and volume could be seen over time.

It was possible to construct a tumor growth curve using data obtained by measuring tumor area (in cm\(^2\)) over the period of observation (Figure 5); from 7 to 14 days, there was a 30.12% increase in average tumor growth; from 14 to 21 days there was a slight increase of 22.52%; and over the last period from 21 to 28 days the largest increase occurred, with a 66.91% increase in total area.

These results for tumor growth are compatible with the study by Silva,\(^9\) who reported that the proliferation peak in Ehrlich ascitic tumor occurs at 7 days and the growth peak for the solid form occurs after 14 days. In our study the peak occurred after day 21, progressively increasing until the end of the experiment.

While the hematological data for most of the parameters - erythrocyte count, hemoglobin, hematocrit, MCV and MCH levels - were normal and did not differ significantly (Table 1), RDW was significantly different. However, data from the hemogram suggested that the difference in RDW was not clinically relevant for the animals and the platelet values were stable throughout the experimental periods, with no relevant statistical variance.

In the leukogram, the mean absolute white blood cell count (Table 1) showed a considerable increase over the first 7 days, resulting in a statistically significant difference when compared to the other periods, and the differential leukocyte count showed a high presence of lymphocytes (71.89%) and neutrophils (23.70%) and a low prevalence of monocytes (4.55%), suggesting an intense inflammatory response in the early stage of tumor development. When

| TABLE 1 Hematological parameters obtained by flow cytometry |
|-----------------------------------------------------------|
| Parameters                                      | Unit | 7 days (mean ± 2 SD) | 21 days (mean ± 2 SD) | 28 days (mean ± 2 SD) |
|-----------------------------------------------------------|
| Erythrogram                                                  |      |                     |                      |                      |
| Erythrocytes                    | \(10^9/\text{mm}^3\) | 10.2 (10.98; 9.42) | 10.1 (11.19; 9.00) | 10.52 (11.46; 9.57) |
| Hemoglobin                     | g/dL  | 14.89 (16.23; 13.54) | 14.88 (13.4; 16.36) | 14.96 (16.12; 13.8) |
| Hematocrit                      | %     | 52.59 (57.59; 47.59) | 51.58 (58.18; 44.98) | 52.58 (57.78; 47.38) |
| MCV                            | fL    | 51.63 (58.03; 45.23) | 51.08 (52.5; 49.66) | 49.98 (51.38; 48.58) |
| MCH                            | pg    | 14.62 (16.34; 12.9)  | 14.73 (16.63; 12.83) | 14.24 (14.56; 13.88) |
| RDW                            | %     | 25.26 (27.02; 23.5)  | 24.1* (25.86; 22.34) | 25.51 (26.83; 24.19) |
| Leukogram                       |       |                     |                      |                      |
| Total Leukocytes                | \(10^9/\text{mm}^3\) | 12.74* (16.01; 9.47) | 3.01 (5.45; 0.57) | 4.37 (6.59; 2.15) |
| Neutrophils                     | /mm\(^3\) | 3.02* (6.74; 0) | 0.12 (0.3; 0) | 0.42 (0.84; 0) |
| Lymphocytes                     | /mm\(^3\) | 9.16* (13.9; 4.42) | 2.86 (5.2; 0.52) | 3.91 (5; 2.8) |
| Monocytes                       | /mm\(^3\) | 0.58 (1.68; 0) | 0.00 | 0.01 (0.04; 0) |
| Platelets                       | /mm\(^3\) | 982.1 (1309.3; 654.9) | 870.8 (1241; 500.6) | 975.2 (1294.6; 655.8) |

Note: The mean and standard deviation were calculated, with statistical variance observed in the final score in the 28-day group.

| TABLE 2 Animal welfare parameters |
|-----------------------------------|
| Parameters | Groups | 7 days | 21 days | 28 days | Mean (± SD) |
|------------|--------|--------|---------|---------|-------------|
| Ulcer      |        | 0      | 9       | 22      | 10.3 (±11.00) |
| Coat       |        | 4      | 16      | 18      | 12.66 (±7.57) |
| Movement   |        | 0      | 4       | 1       | 1.6 (±2.00) |
| Posture    |        | 0      | 2       | 3       | 1.6 (±1.52) |
| Tail       |        | 0      | 0       | 0       | 0            |
| Eyes       |        | 0      | 0       | 6       | 2.0 (±3.40) |
| Vibrissae  |        | 0      | 5       | 0       | 1.6 (±2.80) |
| Final Score|        | 4      | 36      | 50*     | 30 (±19.25) |

Note: The mean and standard deviation were calculated, with statistical variance observed in the final score in the 28-day group.

| FIGURE 6 A, Neutrophil-lymphocyte ratio (NLR). Statistically different values labeled by asterisk (*) | B, Final score obtained from daily analysis scores assigned to each welfare parameter, with statistically different value labeled by asterisk (*) in the 28-day group |
comparing the absolute and differential counts with the other periods (21 and 28 days), there was a non-significant decrease in the average values, suggesting a decrease in the inflammatory response; the tumor may play a fundamental role in inhibiting the response through the release of anti-inflammatory cytokines.

The welfare parameters were evaluated over the 7-, 21- and 28-day periods in order to characterize the main clinical signs during the disease development in the animals. During the observed periods, only the animals of the 28-day group presented higher scores that together were statistically different from the other groups. This was because during the course of tumor development these signs became increasingly evident (Table 2), with the development of skin ulcerations in the dorsolateral region of the animal (where the solid tumor is located) predominant among them. Ulceration became much more frequent after the 21-day period, and worsened until the end of the experiment at 28 days. Another parameter that contributed to the high final score was the appearance of the animals’ coat; due to the increasing stress and pain resulting from the evolution of the tumor, the animals ceased to groom their coats, leaving them bristled and spiculated, with a feathery appearance.

The posture and movement of the animals were also evaluated, since the tumor, by invading cavities as it increases in size, can compromise organs and the spine, and interfere with physiological functions and movement and even with respiration via lung compression. Other parameters analyzed were considered to be less affected, with no change in scoring during the observed periods.

Although the animals had a high score at the end of the 28-day period, no deaths occurred in any of the groups analyzed. The NLR is a value obtained by calculating the number of neutrophils in relation to the number of lymphocytes, and by evaluating the result obtained it was possible to show a statistical difference between the 7-day group and the others, with NLR elevation evident in the first 7 days (Figure 6A).

The value of NLR increases during inflammatory reactions, providing data regarding the inflammatory state, which may play a fundamental role in tumor growth, progression, invasion and metastasis. NLR is therefore an important parameter in tumor evaluation and is used as a predictive factor in cancer, with high NLR results indicating a worse prognosis in most cases. Compared to other invasive examinations (such as biopsies), the NLR value is easily obtained through the results available in the hemogram, and is thus a valid and inexpensive option, offering less risk to the patient. The increase observed in the NLR data over the 7-day period in this study is consistent with the results obtained in the absolute and differential leukocyte count, since the NLR result is derived from these values.

The decrease in NLR observed in the 28-day group is still clinically relevant for the evaluated animals, since the tumor, as it develops and increases in volume, is able to cause immune suppression. This may have been a determining factor in the decrease in lymphocytes and neutrophils. The immune suppression would lead to a decrease in NLR (Figure 6A) while the animal welfare parameter as observed in the final score results in the 28-day group indicated a worsening prognosis (Figure 6B).

The results of this research confirm that laboratory reference values and welfare parameters were successfully obtained for BALB/c-FMABC mice inoculated with Ehrlich tumor. These results can supplement future research in this model, providing greater reliability and accuracy of the results, and promoting scientific integrity.

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CONFLICT OF INTEREST
None.

AUTHOR CONTRIBUTIONS
PGSP and EBDS designed the study, acquired and analyzed the data, wrote the initial manuscript and approved the final version to be published. PRKS, CDSC and DSN wrote and critically revised the final manuscript. GP helped to design the study and provided the animals used in this work and worked to ensure the biosafety and ethics were used accordingly. FLAF and EGS helped to design the study and to critically revise its contents. All the authors proofread every submission and approved the manuscript.

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