

Replacement of Animals Used in the Maintenance of *Aedes Aegypti* by Alternative Methods. Refinement of Procedures That Minimize the Discomfort of Insect Maintenance by Swiss Webster Mice

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ABSTRACT

One of the most critical points in Laboratory Animal Science (CAL) is the use of some species as a blood supplier for food (Blood Meal) and breeding of hematophagous insects, vectors of serious and (often) neglected diseases. In summary, the main objectives of the project are:

a) Replacement of the use of mice (*Mus musculus*) for the blood meal of *Aedes aegypti* in the laboratory.

b) In case of failure to replace the animal, that is, in all phases of the life cycle of *Aedes aegypti*, we will test the application of two refinements in the procedure: The first is based on the use of Erythropoietin, increasing the number of and we hope to speed up the time to food satiety for the mosquito and the second to use an analgesic, an opiate with minimal adverse effects. Complementing, in the case of successful use of Erythropoietin. We will apply the association of the recombinant hormone with the analgesic for mice during the *Aedes aegypti* Blood Meal in the laboratory aiming, firstly, to avoid the use of the animal. However, if it is not possible to apply it in the maintenance routine of *Aedes aegypti* to minimize the stress and discomfort of this animal during feeding and creation of hematophagous vectors, in our case, the mosquito.

KEYWORDS: Animal; Blood meal; Replace; Refine system

INTRODUCTION

Sérgio Arouca, Medical Doctor at the National School of Public Health (ENSP/FIOCRUZ), then Federal Deputy, formulated a bill that regulated the use of animals in laboratories throughout Brazil. In 2008, Law No. 11,794 of October 2008 was voted and approved, deservedly called the Arouca Law, which regulated the use of animals for didactic and scientific purposes in teaching and research institutions [1]. Visionary Dr. Sérgio Arouca makes it clear in Law No. 11,794 that the use of animals in didactic practices or scientific use should only be carried out if there is

no alternative method that can be used to replace animals [2]. In general, the respective law it is based on the "Ethical Principles for Animal Experimentation" formulated by Burch & Russell in 1959, which applied a simple and brilliant idea: The Principle of the 3Rs, which are replacement, reduction and refinement. It would mean replacing the use of animals, reducing the number to the minimum possible for statistically reliable results and refining the techniques and procedures for handling and experimenting with animals, whether of any species, sex or age [3].

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Guided by the principle of the 3Rs, the Arouca Law, the CONCEA and the CEUAs, we developed a protocol to address a sensitive, yet compelling topic in the science of laboratory animals. Our goal is to replace animals in the feeding of blood-sucking insects and disease vectors (Blood Meal) with a simpler, more efficient and accessible method through the use of an electronic device called Hemotek® (Think Up Themes Ltd, UK) with four membranes from different materials: cellulose, silicone, vegetables and collagen [4]

This is a complex and difficult topic. Many variables are involved, especially the study of serious and neglected diseases such as Leishmaniasis, Chagas disease and Dengue in their experimental forms [5]. However, the blood meal of mosquitoes in live animals is still a frequent practice in the laboratory. However, in many circumstances it is not possible to use it, due to the lack of adequate facilities for the animals, for example, the absence of a regulatory structure or the non-permission for the use of animals, which may restrict the blood supply. Furthermore, the principles of "3Rs" in scientific practice with live animals also need to be considered when feeding autogenous mosquitoes. In contrast, artificial feeders can replace live animals as blood sources for insects. In addition, we emphasize that Hemotek® (artificial feeder) can also be used in addition to artificial feeder for *A. aegypti* but have the accuracy of ingestion of the viral load titration for mosquitoes. However, some younger stages of the mosquito are unable to feed due to the fragility of the proboscis to pierce the membrane used in Hemotek® [6].

***Aedes Aegypti*: Characteristics and Behavior**

Considered a cosmopolitan insect, present in tropical and subtropical areas of the planet, *A. aegypti* is originally from Africa. It was probably introduced in Brazil during the colonial period, possibly during the slave trade [7]. Male and female need a sugary solution such as nectar and sap for survival, but only female mosquitoes feed on blood. In general, the females, as soon as they pose on the host, carefully select the site of the bite with the sensory organs and with a pair of buccal stylets, introduce them to the skin of the host for hematophagy. Sucking blood is an essential act to obtain the nutrients necessary for the development of the ovaries and the maturation of the eggs, therefore, it is a fundamental physiological process in the life cycle of the species [9]. Often, in order to have a satisfactory blood meal, female *A. aegypti* feed on more than one human host until engorgement is complete. However, the act of sucking blood gives females the ability to transmit arboviruses, such as Dengue, and other pathogens from an infected vertebrate [8].

Artificial Feeding of *A. aegypti*

So, in order to study these serious diseases transmitted by hematophagous vectors in a laboratory environment, it was necessary to carry out maintenance, as in our case, of the mosquitoes that transmit Dengue and part of this is to keep them fed. In summary, this feeding is often performed using live animals under anesthesia (however, in some cases not anesthetized) and they remain for 30 to 40 minutes inside the "Mosquito Cage". Thus, in this protocol we will explore all possibilities for the replacement of the animal. We will use the animal model, the mouse (*Mus musculus*). We will explore the capacity of the equipment, the Hemotek®, which makes it possible to offer blood to all forms of the female life cycle, especially the young forms of the *A. aegypti* mosquito, without interfering with the researchers' experimental protocol.

In addition, we plan in case of failure to replace the animals.

We will use Refinement. This protocol is based on the formulation of new methodologies that use animals. But we start from the principle: less time and in less number. However, maintaining the number of insects created and capable of being used for scientific purposes and/or reducing the time the animal is exposed to insect bites, minimizing its discomfort. Our experimental design uses analgesics in the animals during the meal and we will evaluate the interference in the behavior, biology and biochemistry of *A. aegypti*.

Replacement of Mice

The biggest problem at work is having to use animals because for the maintenance of the *A. aegypti* mosquito, for its Blood Meal, young females can only feed on animals. Insect feeding for years has been done in this way in several species, mainly in guinea pigs. Live animals are anesthetized or immobilized, on or inside the cage for 30 to 40 minutes [9]. Our main work problem is to replace, or free, any animal species from the discomfort of being incessantly bitten by insects, maintaining the necessary maintenance of hematophagous insects and vectors of serious diseases, such as those transmitted by *A. aegypti* for example.

Several studies demonstrate the neurobiological, physiological and behavioral changes in animals that have been in this situation, such as: stress, suffering, anguish, fear, anxiety or depression-like that affect the physical and mental health of animals. Therefore, we hope that at the end of the execution of these protocols, no animal needs to be used for feeding insects, or rather, be forced to have this bloody and uncomfortable experience.

Refinement of Techniques

Most of the times, the blood diet for *A. aegypti* females is performed using live animals such as: rats (*Rattus norvegicus*) hamsters (*Mesocricetus auratus*), which after anesthesia are placed inside the cage for a period of 30 to 40 minutes or with guinea pigs for approximately 3 hours depending on the procedure [11]. Mice (*Mus musculus*) are also used in insect feeding. Barros 2012 in their studies proves that many bites of the mosquito *A. aegypti* and the use of salivary gland extract of females of this insect, developed a strong pulmonary allergic reaction in mice [10].

Despite the difficulty in measuring pain and suffering, the current blood meal protocol offers indisputable indications that the animals are under intense stress, discomfort and distress. Preventing this from happening would already be the justification for this project. Furthermore, studies carried out in recent decades have expanded the understanding of animal sentience, indicating that the potential of animals to suffer harm is greater than was evaluated and that it is currently recognized that animals can feel distress, pain, and that even with handling delicate, they can show marked changes in hormonal and physiological stress markers. It is also known that animals demonstrate coordinated pain responses similar to humans, sharing neurophysiological, genetic and physiological similarities with humans [11].

So our Refinement Techniques Application Protocol is Based on:

- 1) Create or make changes to the artificial feeding device, called Hemotek®, which allows all evolutionary forms of mosquitoes to be able to feed, that is: to suck blood from the membrane of the device without the need to use the animal
- 2) Use of Human Recombinant Erythropoietin, increasing the count of the number of red blood cells (Number of red blood

cells per ml - hematimetry), thus seeking to accelerate the satiety state of the insects. Consequently, the reduction in the animal's time for the Blood Meal and the decrease in the repetition of the Blood Meal

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METHODOLOGY

Breeding of Mosquitoes

For the development of the protocol, the Laboratory of Parasitic Diseases/IOC will supply the female *Aedes aegypti* mosquitoes of the Rockefeller lineage. The mosquitoes will be raised in the insectary of the Laboratory of Parasitic Diseases, IOC/Fiocruz, which operates at $26^{\circ}\pm 2^{\circ}\text{C}$, with humidity of $80\% \pm 10\%$ and natural photoperiod. For each experiment, about 3000 eggs will be hatched in plastic vats containing 3.0 litres of filtered water and 0.5 grams of fish feed in granules, to feed the larvae. After hatching, 500 larvae will be created per vat, forming a total of 4 vats. The larvae will remain in the vats until the pupal stage, at this time, they will be separated by age, transferred to cardboard cages in 100 ml cups for adult emergencies. After emergence, adults will receive a 10% sugar solution as a food source [12].

Animals

The most used models in this practice are Guinea Pigs and Hamsters. However, the Swiss Webster mouse is the most common animal in all animal facilities in Brazil. Therefore, we decided to carry out the tests in male and adult Swiss Webster mice (body weight between 40 and 50 grams)

Filming

All tests will be filmed, evaluated and archived by an IP Robot Camera 3 Antennas, Wireless Wifi Wireless Hd with Night Vision (Infrared). Camera with motion alert system, infrared, where you can watch night images quietly. WiFi connection, with total practicality and comfort, allowing you to monitor through the application on your cell phone, tablet, computer and notebook in high resolution. This camera will be inside the Feeding Cage, allowing the recording of the work and making it possible to carry out the Blood Meal requirements that we will use.

Hematophagic Parameters

For the experiments the females will be seven days old, grouped in cardboard cages for food (100 females/cages) and will be without any sugary food for 48 hours to increase blood avidity.

Animal Replacement Protocols

First is the replacement of the pig intestine epithelium (commonly used) by artificial membranes. For this we will use the Hemotek® device, an electronic device that allows blood temperature regulation according to the desired range. This device has a flexible cable connection and extension for a small aluminium plate, 1.3" thick. cm and 3.7 cm in diameter that stores blood. This aluminium plate uses a thin membrane capable of penetration and heat resistance [13]. We structured the following experimental groups, calculated so that the result is statistically significant ($p \leq$

0.05). Being composed of:

G1: Blood meal with an anesthetized animal according to the Protocol described by Herculano in 2000.

G2: Use of Collagen Membrane for Blood meal

G3: Use of Cellulose Membrane for Blood meal

G4: Use of Silicone Membrane for Blood meal

G5: Use of Vegetable Membrane for Blood meal

Blood Meal Parameters: We will film around 30 to 40 minutes which is the standard time of the Blood Meal. Through the filming, they will be the basis for the quantification and qualification of the Blood Meal as it is routinely done today. We will quantify through the filming:

- Number of females who performed the Blood meal.
- Typification of the evolutionary form of the mosquito in its cycle performing the meal.
- Total time of each evolutionary stage performing the meal.
- Survival and mortality of insects and of each evolutionary stage in each group.
- Each female that takes a complete blood meal is counted, separated and confined for oviposition.
- The eggs obtained will be placed to dry at room temperature and then counted, identified and stored in a humid chamber for later hatching and viability assessment. On-site observations will be noted and will be considered subjective results.

Refinement Techniques

We will carry out the following protocols:

1) Application of Human Recombinate Erythropoietin (rhEPO) one dose (90m IU/0.5ml) subcutaneously and daily evaluate the hematimetry of these animals until finding the time in which there is a maximum number of red blood cells in circulation.

2) Test the efficient dose of Fentanyl Citrate that promotes analgesia in the animal. We will start from 0.015 mg/kg and test by caudal and foot reflex the presence of pain sensation and monitoring of electrocardiographic tracings, pain signals, or the range of insensitivity time. Finding the ideal dose, stipulate the time of onset of action of the drug and its duration, ideal ≥ 30 minutes.

Assay: G1: Blood meal with an anesthetized animal according to the Protocol described by Herculano in 2000.

G2: Animal model with Erythropoietin

G3: Animal model with Fentanyl Citrate

G4: Animal model with Erythropoietin associated with Fentanyl Citrate

Evaluated Parameters: We will film around 30 to 40 minutes, which is the standard time for the Blood Meal. Through the filming, they will be the basis for the quantification and qualification of the Blood Meal as it is routinely done today. We will quantify through the footage

- Number of females who performed the Blood meal.

- b. Typification of the evolutionary form of the mosquito in its cycle performing the Repasto.
- c. Total time of each evolutionary stage performing the meal.
- d. Survival and mortality of insects and of each evolutionary stage in each group.
- e. Each female that takes a complete blood meal is counted, separated and confined for oviposition.
- f. The eggs obtained will be placed to dry at room temperature and then counted, identified and stored in a humid chamber for later hatching and viability assessment. On-site observations will be noted and will be considered subjective results.

CONCLUSION

Our main expected result is to be able to apply a Conclusion: Our main expected result is to be able to apply an artificial membrane to Hemotek® that allows the blood feeding of all evolutionary forms of the *A. aegypti* mosquito in mice. In this way, replacing the use of animals and maintaining the reliability and reproducibility of the results of the study projects of the experimental arbovirus, Dengue. If this attempt fails, refine. Seek to use the animal in the most refined way possible. Perform trichotomy on the animal's back (exposing an area of easy access to mosquitoes). Increase the number of red blood cells and perform analgesia of the animal, reducing the time of the blood meal for all evolutionary forms of the mosquito, consequently reducing the time of suffering of the animal. Finally, we hope to demonstrate that any method tested, replacing the membrane in the artificial feeder is feasible for younger forms of the mosquito without causing a decrease in blood meal in this population. If we are not successful, apply refinement methodology, reducing the mealtime, blocking the pain and discomfort of successive mosquito bites in mice.

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