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PAULO FERNANDO CARLSTROM

AVALIAÇÃO DA SEGURANÇA DO USO DA PRÓPOLIS VERMELHA BRASILEIRA DURANTE A GRAVIDEZ E NO DESENVOLVIMENTO DA PROLE EM UM MODELO DE CAMUNDONGO

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SAFETY EVALUATION OF BRAZILIAN RED PROPOLIS USES DURING PREGNANCY AND OFFSPRING DEVELOPMENT IN A MOUSE MODEL

Alfenas/MG

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas pela Universidade Federal de Alfenas. Área de concentração: Interação patógeno hospedeiro.

Orientador: Prof. Dr. Masaharu Ikegaki
Coorientador: Prof. Dr. Valdemar Antonio Paffaro Junior

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PAULO FERNANDO CARLSTROM

SECURITY EVALUATION OF BRAZILIAN RED PROPOLIS USES DURING PREGNANCY AND OFFSPRING DEVELOPMENT IN A MOUSE MODEL

A Banca examinadora abaixo-assinada aprova a Dissertação/Tese apresentada como parte dos requisitos para a obtenção do título de Mestra em Ciências Biológicas pela Universidade Federal de Alfenas. Área de concentração: Interação Patógeno-Hospedeiro.

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Prof. Dr. Masaharu Ikegaki
Instituição: Universidade Federal de Alfenas - UNIFAL-MG

Profa. Dra. Évila da Silva Lopes Salles
Instituição: Augusta University

Prof. Dr. Pedro Luiz Rosalen
Instituição: Universidade Federal de Alfenas - UNIFAL-MG

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“Ninguém fica triste quando tem um balão”

(Ursinho Pooh)

RESUMO

Os padrões de saúde e doença são influenciados em diferentes estágios do curso de vida por uma combinação de fatores genéticos, epigenéticos e ambientais. A própolis vermelha brasileira (BRP) é um tipo de própolis com grande potencial na terapia de saúde. No entanto, não há estudos avaliando o uso seguro e a toxicidade do desenvolvimento dessa própolis. O objetivo deste estudo é investigar a segurança do uso de BRP na gravidez e no desenvolvimento da prole e da vida adulta em um modelo animal. Os camundongos prenhes foram divididos em quatro grupos, dois grupos controle (C, Tween 80 1%) e dois grupos BRP (200mg/kg/dia por gavagem), administrados por 10 e 20 dias. Os animais foram submetidos à eutanásia, sendo realizadas análises relacionadas ao ganho de peso, taxa de implantação e natalidade, nível sérico de citocinas (IL-1ß, IL-6 e IL-10), morfológicas e estereológicas do uNK. Os grupos de descendentes (F1C e F1BRP) foram obtidos tratando-se as fêmeas durante a gravidez. Os filhotes foram analisados quanto ao desenvolvimento físico e neurológico por 30 dias após o nascimento e comportamento na idade adulta (atividade locomotora e memória). O consumo de BRP alterou significativamente o número de subtipos de uNK, com diminuição do subtipo III (8,3 células/AT) concomitante a um aumento do subtipo IV (5,31 células/AT) na região 2 do local de implantação no 10º gd no grupo de mães, mas sem alterar o ganho de peso, as taxas de implantação e natalidade e o nível sérico de citocinas. Na geração F1, não foram observadas malformações congênitas, distúrbios nos parâmetros de desenvolvimento físico e neurológico e alterações comportamentais em filhos adultos. Nossos dados indicam que o BRP é seguro para uso durante o período gestacional de camundongos e não causa alterações na prole por meio da programação fetal, apresentando potencial para futuros ensaios pré-clínicos.

Palavras-chave: *Apis mellifera*. Produtos naturais. Célula Natural Killer uterina. Programação fetal.

ABSTRACT

Patterns of health, illness and disease are influenced at different stages of the life course by a combination of genetic, epigenetic and environmental factors. Brazilian red propolis (BRP) is a type of propolis with great potential in health therapy. However, no studies are evaluating the safe use and toxicity of the development of this propolis. The aim of this study is to investigate the safety of using BRP in pregnancy and in the development of offspring and adult life in an animal model. The pregnant mice were divided into four groups, two control groups (C, Tween 80 1%) and two BRP groups (200mg/kg/day by gavage), administered for 10 and 20 days. The animals were euthanized, and analyses related to weight gain, implantation and birth rates, serum level of cytokines (IL-1ß, IL-6 and IL-10), morphological and stereological of the uNK were performed. The groups of offspring (F1C and F1BRP) were obtained by treating females throughout pregnancy. The puppies were analyzed for physical and neurological development for 30 days after birth and behavior in adulthood (locomotor activity and memory). The consumption of BRP significantly changed the number of uNK subtypes, with a decrease in subtype III (8.3 cells / AT) concomitant to an increase in subtype IV (5.31 cells / AT) in region 2 of the implantation site at the 10th gd in the groups of mothers, but without changing weight gain, implantation and birth rates, and the serum level of cytokines. In the F1 generation, no congenital malformations, disturbances in the parameters of physical and neurological development, and behavioral changes in adult offspring were observed. Our datas indicates that BRP is safe to be used during the gestational period of mice and does not cause changes in the offspring through fetal programming, presenting the potential for future pre-clinical trials.

Keywords: *Apis mellifera*. Natural products. uterine Natural Killer cell. Fetal programming.

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LISTA DE ABREVIATURAS E SIGLAS

DBA	lectin Dolichos biflorus aglutinina
F1C	offspring control group
F1BRP	offspring Brazilian red propolis group
GD	gestational day
IL	interleukin
BRP	Brazilian red propolis
PND	post-natal day
uNK	Natural Killer uterine cell

SUMÁRIO

1. INTRODUÇÃO GERAL

A gestação é um período crítico no desenvolvimento de animais, incluindo os seres humanos, por esse motivo, células do feto (células do trofoblasto) e células imunes maternas devem trabalhar em conjunto para assegurar a homeostase gestacional (SOJKA; YANG; YOKOYAMA, 2018). Perturbações neste momento, como agentes microbianos (bactérias, fungos, parasitas e vírus), processos inflamatórios e doenças autoimunes, podem gerar malformações para o feto, complicações durante o parto e em determinadas situações falha gestacional completa (WIRA et al., 2005).

Outra consequência desses distúrbios é a alteração de componentes no sistema imunológico inato das mães, como as células Natural Killer uterinas (uNK) (WIRA et al., 2005). Essas células linfocitárias presentes na fase gestacional de camundongos e humanos (CROY et al., 2000; GUIMOND et al., 1997; GUIMOND; WANG; CROY, 1998; ZHANG; YAMADA; CROY, 2009) são responsáveis por garantir a homeostase gestacional por meio da secreção de citocinas e quimiocinas, como interferon γ (IFN-γ) e fator de crescimento endotelial vascular (VEGF) (BURKE et al., 2010; CROY et al., 2006).

Em camundongos, há duas populações de células uNK que diferem em termos de coloração com ácido periódico de Schiff (PAS) e citoquímica de lectina DBA (aglutinina Dolichos Biflorus) que se liga seletivamente a glicoconjugados contendo N-acetil-D-Galactosamina em células uNK (ZHANG; YAMADA; CROY, 2009). Enquanto as células DBA^- PAS^+ residem no útero, as células DBA^+ PAS^+ chegam pela circulação no início da gravidez (ZHANG; YAMADA; CROY, 2009). Sabe-se que essa população celular sofre alterações morfológicas e funcionais na resposta inflamatória, ocasionando diminuição da reatividade dos grânulos citoplasmáticos e da membrana para a coloração da lectina DBA, sinalizando possíveis riscos à gravidez (LIMA, 2012; ZAVAN et al., 2016).

Em atenção a isso, existe uma busca recorrente por medicamentos indicados para o tratamento de doenças durante a gestação, que não ofereça riscos inerentes a mãe e/ou ao feto (ANDRADE et al., 2004; OSORIO-DE-CASTRO; PAUMGARTTEN; SILVER, 2004), uma vez que é observado que o uso de drogas, fármacos e mesmo alterações nutricionais podem ocasionar modificações permanentes no metabolismo e na fisiologia da prole na fase adulta por meio da programação fetal (SILVEIRA et al., 2007).

Segundo Barker (1997), a hipótese da “programação fetal” está relacionada com as adaptações fisiológicas do feto mediante as alterações no período gestacional, gerando mudanças permanentes no metabolismo e na fisiologia da prole. Os resultados obtidos desse

fenômeno são o aumento do risco de desenvolvimento de doenças crônicas (SEKI et al., 2012) como doenças cardíacas (BARKER, 2007) e obesidade, bem como desordens metabólicas relacionadas à última (BREIER et al., 2001), resultando ainda em possíveis desordens no comportamento na fase adulta (BARKER, 2007).

Dentro deste cenário, a própolis é um produto natural que recebe destaque especial, devido suas propriedades medicinais e aplicação nas indústrias farmacêutica, alimentícia e cosmética, agregando um alto valor comercial (Market Research Future, 2017).

A própolis brasileira é uma substância resinosa não-tóxica, coletada de brotos ou exsudatos de plantas por abelhas da espécie *Apis mellifera*, sendo bastante diversificada quanto a sua composição química, devido a rica biodiversidade (GHISALBERTI, 1979), tornando-se uma importante fonte de novas substâncias bioativas, com função anti-inflamatória (MONTPIED et al., 2003b), imunomoduladora (SFORCIN, 2007), antimicrobiana (CUNHA et al., 2004; MARCUCCI et al., 2001), antineoplásica (ASO et al., 2004; NAGAI et al., 2003; ORŠOLIĆ et al., 2005) e antioxidante (KUMAZAWA; HAMASAKA; NAKAYAMA, 2004; NAGAI et al., 2003).

Um tipo de própolis, chamada própolis vermelha brasileira, por causa de sua cor, foi encontrado no estado de Alagoas (e outros estados da região Nordeste do Brasil) (PARK; ALENCAR; AGUIAR, 2002; SILVA; GONTIJO; NOEL, 2007), atraindo a atenção com uma origem botânica diferenciada, a leguminosa *Dalbergia ecastophyllum* (L.) Taub, popularmente conhecida como rabo-de-bugio, membro da família de plantas rica em isoflavonóides (SILVA; GONTIJO; NOEL, 2007). Após o isolamento de frações desta própolis, foi possível identificar diferentes componentes que caracterizam este novo tipo, entre eles, dois isoflavonóides (vestitol e neovestitol) com potente ação antioxidante e antimicrobiana, revelando grande potencial de aplicação para a indústria alimentícia, como conservantes, e para a indústria farmacêutica na produção de medicamentos e compostos bioativos (OLDONI et al., 2011).

Deste modo, a própolis vermelha brasileira, destaca-se no mercado para ser um produto natural de grande potencial com fins terapêuticos (BUENO-SILVA et al., 2015), inclusive durante períodos críticos como a gestação, mas, entre as preocupações do uso da própolis na medicina, enfatiza-se a toxicidade intrínseca de um produto natural e a escassez de estudos que exploraram os efeitos deste produto no período gestacional em animais e o impacto na vida dos filhos.

O presente estudo tem, portanto, o objetivo de avaliar os efeitos do uso do extrato de própolis vermelha brasileira durante a gestação sobre o perfil imunológico de citocinas, níveis

do hormônio estrogênico e na morfologia uterina de camundongos prenhes, como também, por meio do estudo da geração F1, investigar a capacidade deste produto natural durante a fase gestacional em induzir o fenômeno de programação fetal, alterando o desenvolvimento físico e neurológico e o comportamento da prole adulta em camundongos. Assim, pretendemos, contribuir para a segurança do consumo natural da própolis vermelha brasileira até a primeira geração em camundongos, para agregar as futuras aplicações deste produto.

2. ARTIGO - Safety evaluation of Brazilian red propolis uses during pregnancy and offspring development in a mouse model

A escrita e estruturação deste artigo seguem as normas descritas para o periódico *"Placenta"*.

Safety evaluation of Brazilian red propolis uses during pregnancy and offspring development in a mouse model

ABSTRACT

Introduction: Patterns of health, illness and disease are influenced at different stages of the life course by a combination of genetic, epigenetic and environmental factors. Brazilian red propolis (BRP) is a type of propolis with great potential in health therapy. However, no studies are evaluating the safe use and toxicity of the development of this propolis. The aim of this study is to investigate the safety of using BRP in pregnancy and in the development of offspring and adult life in an animal model.

Methods: The pregnant mice were divided into four groups, two control groups (C, Tween 80 1%) and two BRP groups (200mg/kg/day by gavage), administered for 10 and 20 days. The animals were euthanized, and analyses related to weight gain, implantation and birth rates, serum level of cytokines (IL-1ß, IL-6 and IL-10), morphological and stereological of the uNK were performed. The groups of offspring (F1C and F1BRP) were obtained by treating females throughout pregnancy. The puppies were analyzed for physical and neurological development for 30 days after birth and behavior in adulthood (locomotor activity and memory).

Results: The consumption of BRP significantly changed the number of uNK subtypes, with a decrease in subtype III (8.3 cells / AT) concomitant to an increase in subtype IV (5.31 cells / AT) in region 2 of the implantation site at the 10th gd in the groups of mothers, but without changing weight gain, implantation and birth rates, and the serum level of cytokines. In the F1 generation, no congenital malformations, disturbances in the parameters of physical and neurological development, and behavioral changes in adult offspring were observed.

Discussion: Our datas indicates that BRP is safe to be used during the gestational period of mice and does not cause changes in the offspring through fetal programming, presenting the potential for future pre-clinical trials.

Keywords: *Apis mellifera*; natural products; uterine natural killer cell; fetal programming.

Abbreviations:
DBA, lectina Dolichos biflorus aglutinina; F1C and F1BRP, offspring groups; GD, gestational day; IL, interleukin; BRP, Brazilian red propolis; PND, post-natal day; uNK, Natural Killer uterine cell.

4. Introduction

Pregnancy is a critical period in the development of animals, including humans (MOORE; PERSAUD; TORCHIA, 2012; VITOLO, 2008), therefore, fetal cells (trophoblast cells) and maternal immune cells must work together to ensure gestational homeostasis (SOJKA; YANG; YOKOYAMA, 2018). Disturbances at this time, such as microbial agents (bacteria, fungi, parasites and viruses), inflammatory processes and autoimmune diseases, can generate malformations for the fetus, complications during childbirth and in certain situations complete gestational failure (WIRA et al., 2005).

Another consequence of these disorders changes in the mothers' innate immune system, such as the uterine Natural Killer cells (uNK) (WIRA et al., 2005). Such lymphocyte cells present in the gestational phase of mice and humans (CROY et al., 2000; GUIMOND et al., 1997; GUIMOND; WANG; CROY, 1998; ZHANG; YAMADA; CROY, 2009) are responsible for ensuring gestational homeostasis through the secretion of cytokines and chemokines, such as interferon γ (IFN-γ) and vascular endothelial growth factor (VEGF) (BURKE et al., 2010; CROY et al., 2006).

In mice, there are two populations of uNK cells that differ in terms of Schiff's periodic acid staining (PAS) and DBA lectin cytochemistry (Dolichos Biflorus agglutinin) that selectively binds glycoconjugates containing N-Acetyl-D-Galactosamine in uNK cells (ZHANG; YAMADA; CROY, 2009). While DBA^- PAS^+ cells are residents in the uterus, DBA^+ PAS^+ arrive through the circulation at the beginning of pregnancy (ZHANG; YAMADA; CROY, 2009). This cell population is known to undergo morphological and functional changes in the inflammatory response, causing a decrease in the reactivity of cytoplasmic granules and the membrane for the staining of DBA lectin, a sign of possible risks to pregnancy (LIMA, 2012; ZAVAN et al., 2016).

In experimental models, the exposure of offspring during pregnancy to environmental stressors resulting from the mother/placenta/fetus relationship can be harmful (MOORE; PERSAUD; TORCHIA, 2012; SILVEIRA et al., 2007) causing chronic diseases and metabolic disorders (BARKER, 1997, 2007; SEKI et al., 2012). Therefore, requiring a recurrent search for drugs indicated for the treatment of diseases during pregnancy, which does not offer risks inherent to the mother and or to the fetus through fetal programming (ANDRADE et al., 2004; OSORIO-DE-CASTRO; PAUMGARTTEN; SILVER, 2004; RAMOS et al., 2008).

In this context, the demand for bee products has increased over time, heating the market for natural products, with a special emphasis on propolis. A non-toxic resinous substance, collected from sprouts or plant exudates by bees of the species *Apis mellifera* (GHISALBERTI, 1979), being quite diverse in terms of its chemical composition, due to its rich biodiversity, becoming an important source of new bioactive substances (KHALIL, 2006; PARK; ALENCAR; AGUIAR, 2002), with anti-inflammatory (MONTPIED et al., 2003a), immunomodulatory (SFORCIN, 2007), antimicrobial (MARCUCCI et al., 2001), antineoplastic (MENDONÇA-MELO et al., 2017; NAGAI et al., 2003; ORSOLIC; BASIC, 2005) and antioxidant (BUENO-SILVA et al., 2013; NAGAI et al., 2003), due to its medicinal properties and applications in the industries (pharmaceutical, food and cosmetics), adding a high commercial value compared to other natural products (Market Research Future 2017).

The most recently discovered type of Brazilian propolis, called Brazilian Red Propolis (BRP), was found in the state of Alagoas and other regions of Northeast Brazil (PARK; ALENCAR; AGUIAR, 2002; SILVA et al., 2008), attracting attention with a differentiated botanical origin, the leguminous *Dalbergia ecastophyllum* (L.) Taub, a member of the isoflavonoid-rich plant family (SILVA et al., 2008). This type of propolis has a chemical composition differentiated from the most common type of propolis in the world (green propolis) (ALENCAR et al., 2007; TRUSHEVA et al., 2006) and after the isolation of fractions of this propolis, it was possible to identify different components that characterize this type, among them, two isoflavonoids (vestitol and neovestitol) with potent antioxidant and antimicrobial action, revealing great potential for application in the food industry, as preservatives, and for the pharmaceutical industry in the production of medicines and bioactive compounds (OLDONI et al., 2011).

Thus, BRP has great potential as a natural product for therapeutic purposes (BUENO-SILVA et al., 2015), possibly during critical periods such as pregnancy. However, among the concerns regarding the use of propolis in medicine, the intrinsic toxicity of a natural product is emphasized and the scarcity of studies on the *in vivo* activity of this type of propolis, as well as its effects on the gestational period and the impact on the lives of children.

This study aimed to evaluate the effects of using the BRP ethanol extract during pregnancy on the uterine morphology of pregnant mice, the immune profile of uNK cells and the level of cytokines, as well as, through the study of the F1 generation, to investigate the ability of this natural product during the gestational phase to induce the phenomenon of fetal programming, altering the physical and neurological development and behavior of adult

offspring in mice and, thus, contributing to the safety of natural consumption of Brazilian red propolis until the first generation in mice, to aggregate the future applications of this product.

5. Methods

BRP solution preparation

Red propolis was collected by scraping the insides of the boxes of *Apis mellifera* bees in the seaside region of Marechal Deodoro (a city in the vicinity of Maceio, capital of Alagoas State, in Northeastern Brazil, wet tropical climate, SL 09.40 and WL 35.41). The chemical composition of the propolis extract used in this research was determined in a previous study by our research group (BUENO-SILVA et al., 2017a), the propolis sample was collected and stored in the same place and period, belonging to the same batch. The chemical profile was performed by Reversed-Phase HPLC (SILVA et al., 2008) and identified phenolic acids and flavonoids: formononetin, vestitol, neovestitol, biochanin A, quercetin, liquiritigenin, isoliquiritigenin and daidzein (BUENO-SILVA et al., 2017a).

The propolis sample (50g) was extracted with absolute ethanol (v/v) (350 ml) in a water bath, at 70°C, for 30 min and then subjected to vacuum filtration to obtaining its ethanolic extract (EEP). The solution was concentrated in a rotary evaporator at 40ºC to obtain the concentrated extract of red propolis (ALENCAR et al., 2007). Subsequently, the samples were diluted in a Tween 80 vehicle solution (1%) with an ultrasound bath at 40°C for 4 hours, for the administration of propolis by oral gavage to the animals.

Experimental animals

Fifty female swiss mice (7 to 8 weeks of age) were mated and the detection of a copulation plug was considered gestation day 1. All mice were kept in the experimentation laboratory at the Laboratory of Integrative Animal Biology (LABAInt) at the Federal University of Alfenas (Unifal-MG, Brazil) and housed under controlled light (12:12 h light-dark cycle) and temperature conditions (25 ± 1°C) with access to food and water *ad libitum*. Among the fifty mice, 40 animals were used to collect the sample for pregnancy (n = 5), and 10 animals were kept during pregnancy until term to obtain offspring (n=5). The puppies obtained were used for developmental analysis and behavioral tests in adulthood (n=10). All procedures for carrying out this work were approved by the Animal Experimentation Ethics Committee of the Federal University of Alfenas (protocol N: 44/2019).

Experimental design and sample collection

Pregnant females on the first day of pregnancy were divided into four treatment groups with the application by gavage of Brazilian Red Propolis (BRP) or vehicle (C, Tween 80 1%): control groups - C 10°gd (vehicle, administered for 10 days), C 1°pnd (vehicle, administered for 20 days); and red propolis groups - BRP 10°gd (200 mg/kg/day, administered for 10 days), BRP 1°pnd (200 mg/kg/day, administered for 20 days). The 200mg/kg concentration used was based on the literature on the efficacy and toxicity of this propolis (DA SILVA et al., 2015).

For the analysis of weight gain during pregnancy, females BRP and C were weighed on the 1st gestational day (gd), 10th gd and 1st postnatal day (pnd). In the 10th gd and 1st pnd, the pregnant females were guillotined and blood samples collected. Subsequently, the embryonic implantation sites (IS) were collected, perfused with a 4% paraformaldehyde fixative (Sigma Chemical Co. USA) and then the embryo developing sites were dissected from the uterus and treated for paraffin embedding. Tissue was cut at 7 μm thickness, mounted on poly-L-lysine coated slides for DBA Lectin labeling.

To obtain the offspring, the pregnant females were treated similarly to that described above, and from the first day of the offspring's life, the pups were analyzed for physical and neurological development on alternate days from the 1st to the 30th day after birth (pnd), with offspring being standardized in 10 or 8 offspring (number of females equal to the number of males) per mother and the fostering procedure was carried out to exclude the effects that breastfeeding and maternal behavior could have in the formation of the young. Thus, the offspring of control mothers (F1C) were adopted by other control mothers and the offspring of mothers treated with red propolis (F1BRP) were adopted by control mothers.

DBA lectin histochemistry

Histological sections were deparaffinized, hydrated, and treated with 1% hydrogen peroxide (Sigma Chemical Co. USA) for 30 min. After washing with 50 mM PBS, sections were incubated with 1% bovine serum albumin (BSA) (Sigma Chemical Co. USA) in PBS for 30 min, followed by incubation with biotinylated DBA lectin (Sigma Chemical Co. USA) diluted 1:300 in 1% PBS/BSA or with Biotinylated DBA lectin +1% GalNac (negative control) for overnight at 4 °C. After washing with PBS, sections were incubated with streptavidin-peroxidase (Sigma Chemical Co. USA) for 1h at room temperature and visualized with 3,3-diaminobenzidine (Sigma Chemical Co. USA) in 50 mM TBS containing 0.1% hydrogen peroxide. Sections were counterstained with Harris’s hematoxylin, mounted

with Entellan (Merck, Darmstadt, Germany) and observed by light microscopy (Nikon Eclipse 80i, Tokyo, Japan).

Stereological and morphometric study

From histological mid-sagittal sections subjected to DBA lectin, three sections of three implantation sites of five animals each of the experimental group were used for stereology based on the previously described method (Paffaro 2003). This provided density profiles (QA) of the four morphological subtypes of uNK DBA^{+} cells classified by size, chromatin condensation and N-acetyl-galactosamine expression on the cell surface and granules, located in the 3 regions of the pregnant uterus of mice. The delimitation of these 3 regions followed the variations resulting from the uterine growth in the 10th gd. The test area (TA, 200µm²) was delimited using a quadratic test system which has two exclusion lines, only the cells in each TA with an observed nucleus were counted. We also analyzed the presence of altered uNK cells (uNK DBA^{low}) in the 3 regions of the pregnant uterus of mice (LIMA, 2012; ZAVAN et al., 2016).

Cytokines levels

After collecting blood from control mothers and treated with red propolis, all samples (n=5) were centrifuged at 1500 rpm for 15 min. The serum was collected and stored in Eppendorf tubes at -80ºC. The measurement of cytokines IL-1ß, IL-6 and IL-10 was performed using the ELISA immunoassay kit, following the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). The concentration of each analytical was expressed in pg/µl and samples were evaluated, the mean values obtained were used to calculate the concentrations of each marker.

Physical and neurological development of offspring

From the second day of life, after standardization and fostering of the offspring, the puppies were submitted to analysis of physical and neurological parameters during a period of thirty days. The tests were carried out every two days at the same time and for such observations, the puppies were marked daily with a permanent pen, to be identified. The evaluated parameters were: ear detachment (ED), hair appearance (HA), eye-opening (EO), rupture of the upper incisor (RUI), rupture of the lower incisor (RLI), the opening of the vagina in females (OV) and descent of testicles in males (DT), straightening reflex (SR), negative geotaxis (GN), palmar reflex (PR) and auditory startle (AS). The presence or absence

of each parameter observed in the animal was recorded until all the pups had positive aspects, according to the methodology previously described (CASTRO; CHIORATO; PINTO, 2000). The growth of the animal was also recorded, with weight measurements and crown-rump length.

Adult offspring behavior: Open field and Memory of spontaneous object recognition

Sixty days after the birth of the offspring, they were submitted to the open field test and the object recognition test, in search of possible behavioral changes caused by exposure during the gestational phase to BRP. Behavioral analyzes in all groups were performed in the morning in a room with a constant temperature of 25°C and controlled light. The behavior tests were recorded using a previously installed video camera and analyzed later.

The Open Field test was used to assess the locomotor activity of the animals. We have used the apparatus and evaluated the same behavioral parameters previously described by Olivier et. al. (OLIVIER et al., 2011), considering the total number of crossings and the immobility time. The object recognition test was used to assess the short-term memory of animals exposed to red propolis during the gestational period, assessing the ability to discriminate between new and familiar objects. This test has performed the model described by Abe et. al. (ABE; ISHIDA; IWASAKI, 2004).

Statistical analysis

The statistical analysis was performed using the Graphpad Prism 8.0 employing the two-way analysis of variance (ANOVA) (Two-Way) and by Student's t-test, followed by the means comparison test. P values < 0.05 were indicators of significance.

6. Results

Effects on maternal parameters and pregnancy outcomes

The analysis of the difference in weight of pregnant females, the 1st and 10th gestation day (gd) and on the 1st and 20th gd (Figure 1 a-b), did not reveal statistically significant differences between the mothers of the control groups and those who consumed BRP.

In the BRP group, the average weight gain on the 10th gd was 2.86g and 28.62g for the 20th gd and these values were not statistically different from the weight gain of control animals (C) on the same days of gestation, 2.78g and 28.49g, respectively.

The implantation sites of animals in groups C and BRP were analyzed macroscopically and counted to assess gestational viability (figure 1 c-e). The number of implantation sites was averaged, and in all animals analyzed, embryonic implantation sites were observed in the uterus on the 10th day of pregnancy, after laparotomy, and sites with hemorrhagic foci or in the process of resorption were not identified. The groups had an average of 14.5 (C) and 15.2 (BRP) implantation sites, with no statistical differences between groups (Figure 1c). For the number of puppies born, the analysis did not indicate differences for the average values of the total number of puppies (12.2 and 12.8) and the number of females (6 and 6) and males (6.2 and 6.8) between groups C and BRP, respectively (Figure 1d).

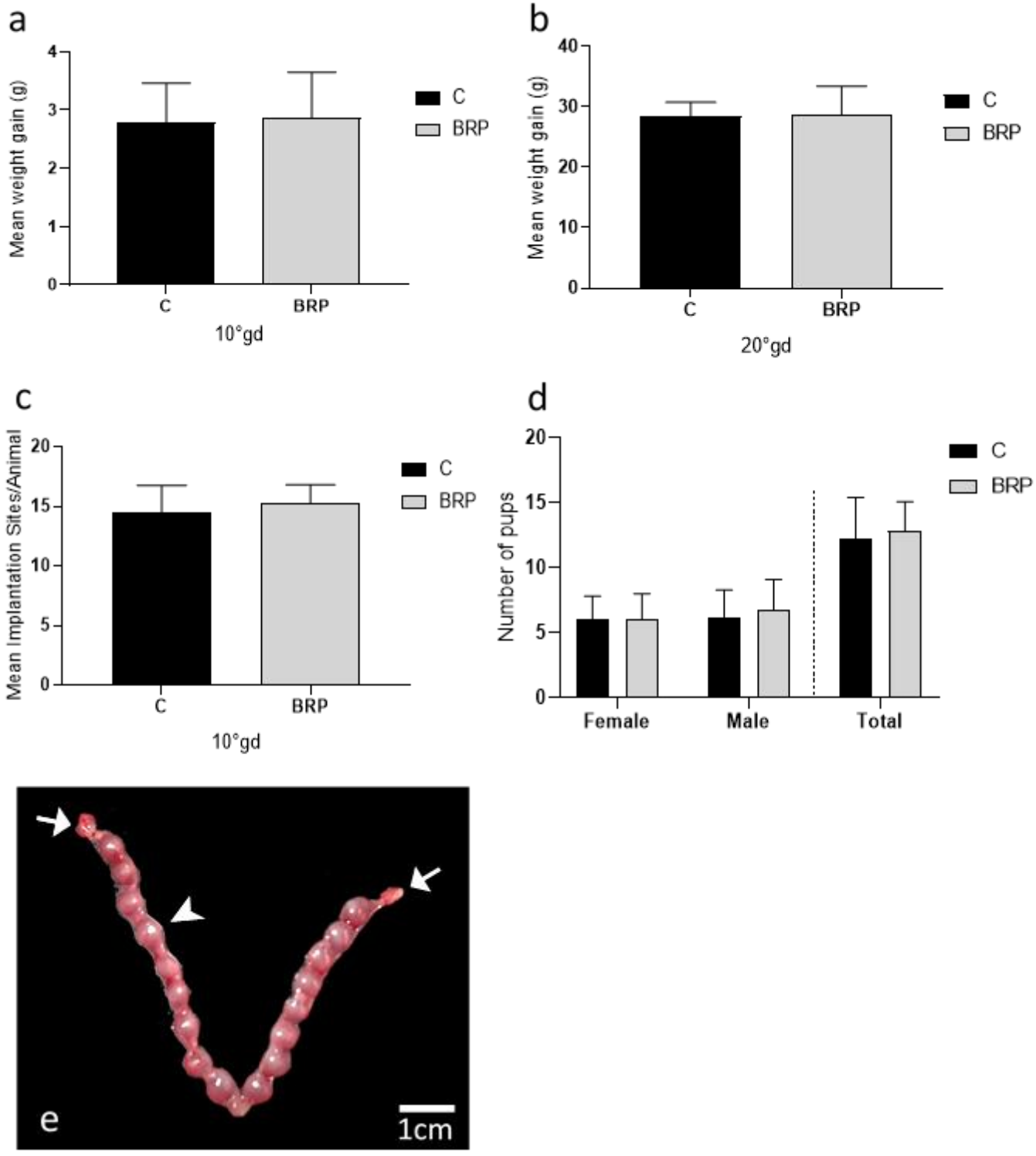

Fig. 1. Weight gain of pregnant control females (C) and treated with red propolis (BRP) at 10th (a) and 20th (b) gestation day. Number of implantation sites (c) and puppies born (d). Macroscopic image of the pregnant uterus 10th gestation day (e), ovaries (arrows); Implantation sites (arrowheads). Test t--Student and two-way ANOVA (mean and SD).

Differential distribution and quantification of DBA^{+} uNK cells

In our studies, reactive cells for DBA lectin (uNK DBA +) distributed in three regions located on the mesometrial side of the embryonic implantation sites of C and BRP mice (Figure 2) were observed, thus, in these animals it was possible to identify all 4 subtypes described in the literature (PAFFARO et al., 2003).

Through the stereological study, it was observed that subtype I and II uNK cells were preferentially distributed in region 1 (R1), with no statistically significant differences between groups C (10.8 and 10.4 cells / AT) and BRP (11.8 and 10.28 cells / AT), respectively (Figure 2 a-b).

Subtype III, considered the mature uNK cell subtype, was preferably located in region 2 (R2) of the implantation site, however, statistical analysis showed a decrease in cell density in this region for the BRP group (8.3 cells/AT) compared to group C (9.8 cells/AT) for the same region (Figure 2c). No changes were identified in the other regions of the implantation site for this subtype. Concomitantly, it was found that subtype IV, considered the morphologically senescent type and predominantly in region 3 (R3), had an increase in the density of this subtype in region 2 in the group treated with Brazilian red propolis (5.31 cells/TA) compared to the control group (2.9 cells/TA) (Figure 2d), there were no changes in the other regions.

There were no statistically significant differences, in the total number of cells for each region and the number of uNK DBA^{low} cells, the fifth subtype that showed a decrease in reactivity of cytoplasmic granules and membrane for lectin DBA and also granules of empty aspect, between groups C and BRP (data not shown).

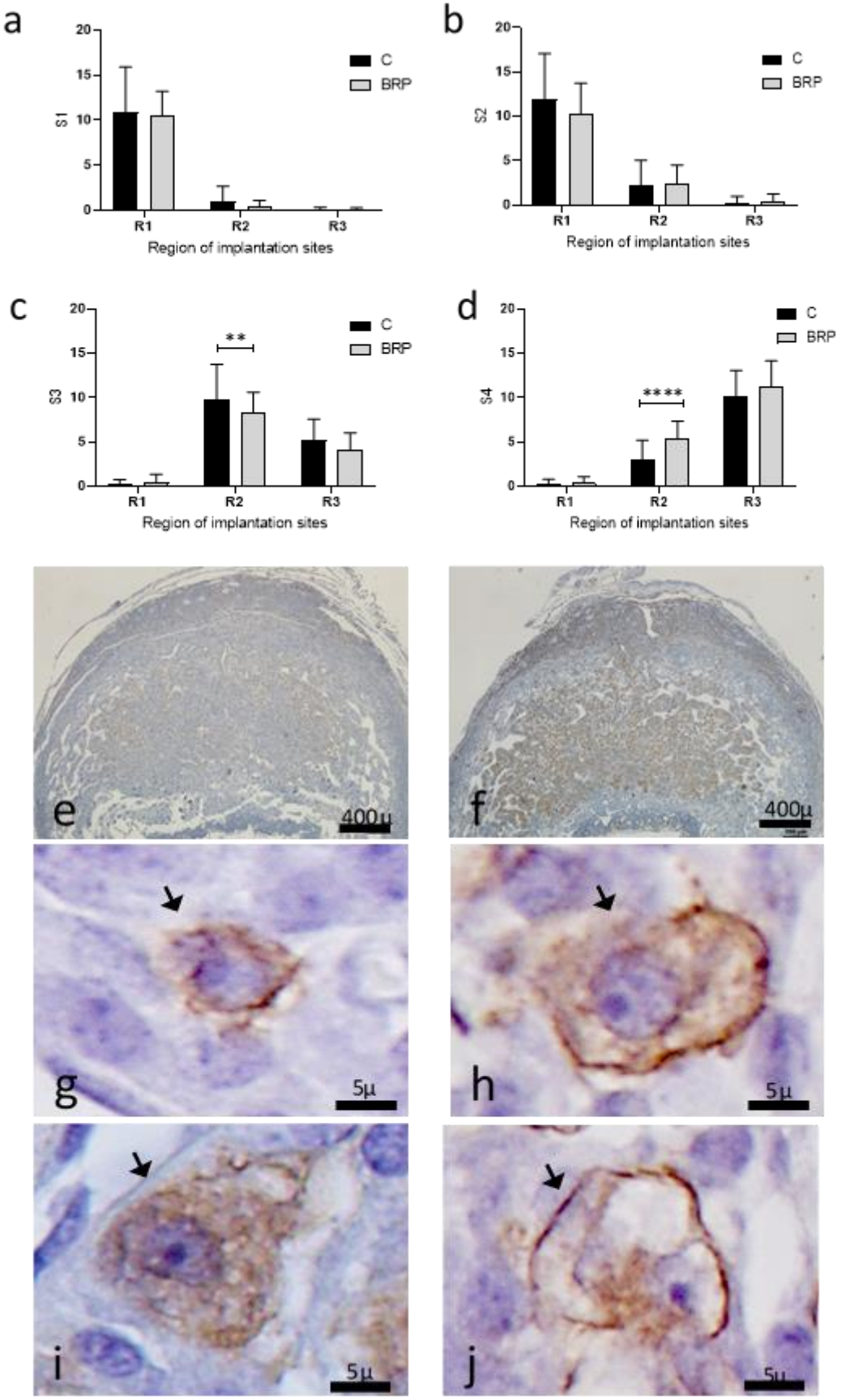

Fig. 2. Representative photomicrographs from histological sections of mouse implantation sites at 10th gd after DBA lectin histochemistry and quantification of 4 DBAþ uNK cell subtypes (S1-4) in each of the three regions of the 10th gd embryo implantation site from each experimental group: C (control) and BRP (red propolis) (a-d). Mesometrial region of the implantation site (e-f). The reaction allowed the identification of 4 subtypes of DBA^+ uNK cells (g-j)(arrows; uNK DBA^+). DBA Lectin/DAB-peroxidase/Harris Hematoxylin. ** p=0,057; **** p<0,0001.

Serum cytokines levels

The plasma concentration of the pro-inflammatory cytokines IL-1β and IL-6, and of the anti-inflammatory cytokine IL-10, were measured after treatment with red propolis during 10 days of gestation (C 10th gd and BRP 10th gd) and 20 days of gestation with blood collection being performed after delivery (C 1st pnd and BRP 1st pnd), Figure 3.

We observed that the serum levels of the analyzed cytokines were not statistically different about their respective controls and the Naive group (non-pregnant and untreated animals). However, we point out that a trend of decreasing levels of IL-6 was observed for both groups, C and BRP in the 1st pnd, compared to their respective groups in the 10th gd (Figure 3b). However, due to the large value of the standard deviation found in these analyzes, significance was not found.

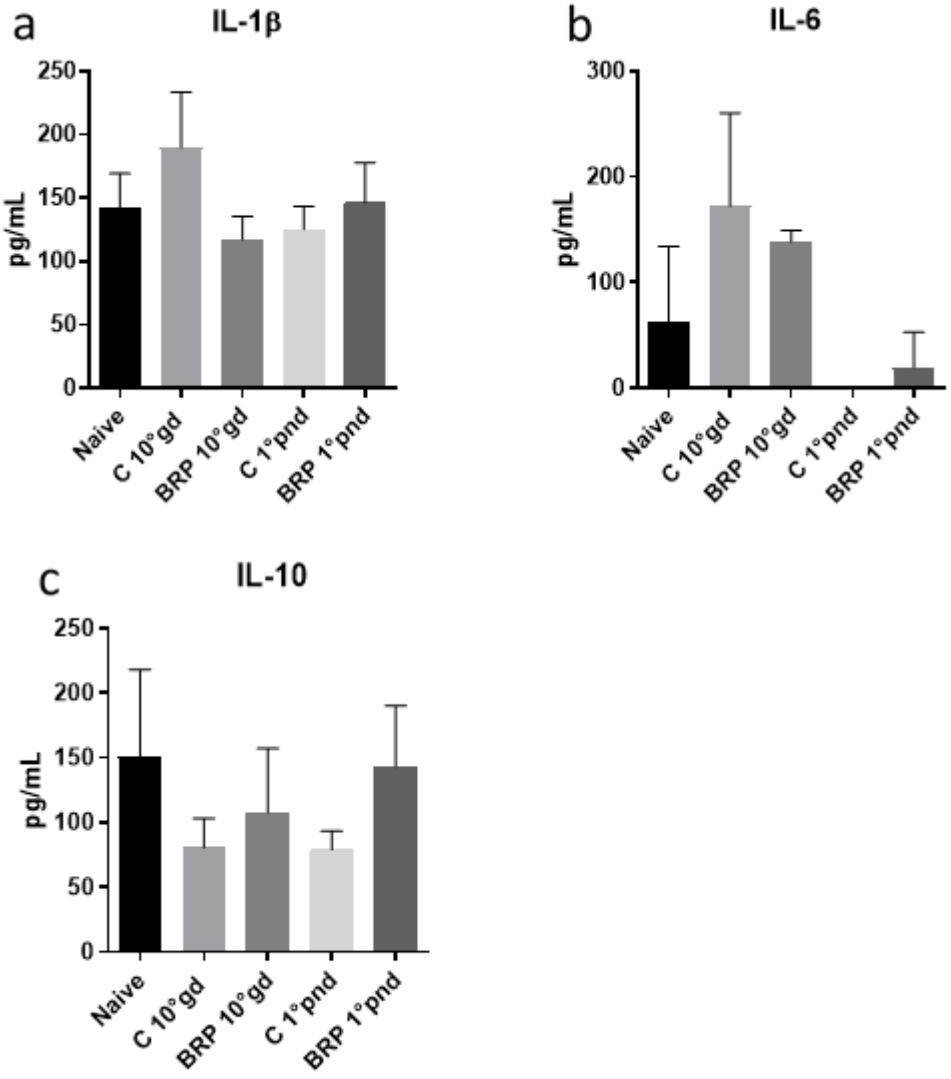

Fig. 3. Serum levels of cytokines: IL-1ß (a), IL-6 (b) and IL-10 (c). Naive: non-pregnant and untreated control; C: control; BRP: propolis red; gd: gestation day; pnd: post-natal day.

Offspring development and adulthood behavior

After giving birth, the puppies (female and male) had their weight and size (crown-rump length) analyzed on alternate days, from the 2nd to the 30th day after birth (PND). For both sexes, the analysis showed no statistical difference from the average weight gain and growth of the animals at any specific time over the analyzed time (figure 4), when comparing the control group (F1C) and the puppies from which the mothers were treated with red propolis during pregnancy (F1BRP).

The other physical parameters evaluated in the offspring, such as ear detachment (ED), hair appearance (HA), eye-opening (EO), rupture of the upper incisor (RUI), rupture of the lower incisor (RLI), the opening of the vagina in females (OV) and descent of testicles in males (DT), showed no significant differences in the tests performed (Figures 5a and 5c). Regarding neurological development tests: straightening reflex (SR), negative geotaxis (GN), palmar reflex (PR) and auditory startle (AS) no differences were found between the F1C and F1BRP groups, for puppies of both sexes (Figure 5b and 5d).

The analysis of behavior related to locomotor activity and similar to anxiety (open field; Figure 6 a-d), and of memory (spontaneous object recognition; Figure 6 e-g), indicated that there were no behavioral changes detectable by tests performed with adult males and females. in the group in which the mothers were treated with red propolis during pregnancy (F1BRP) compared to the control group (F1C).

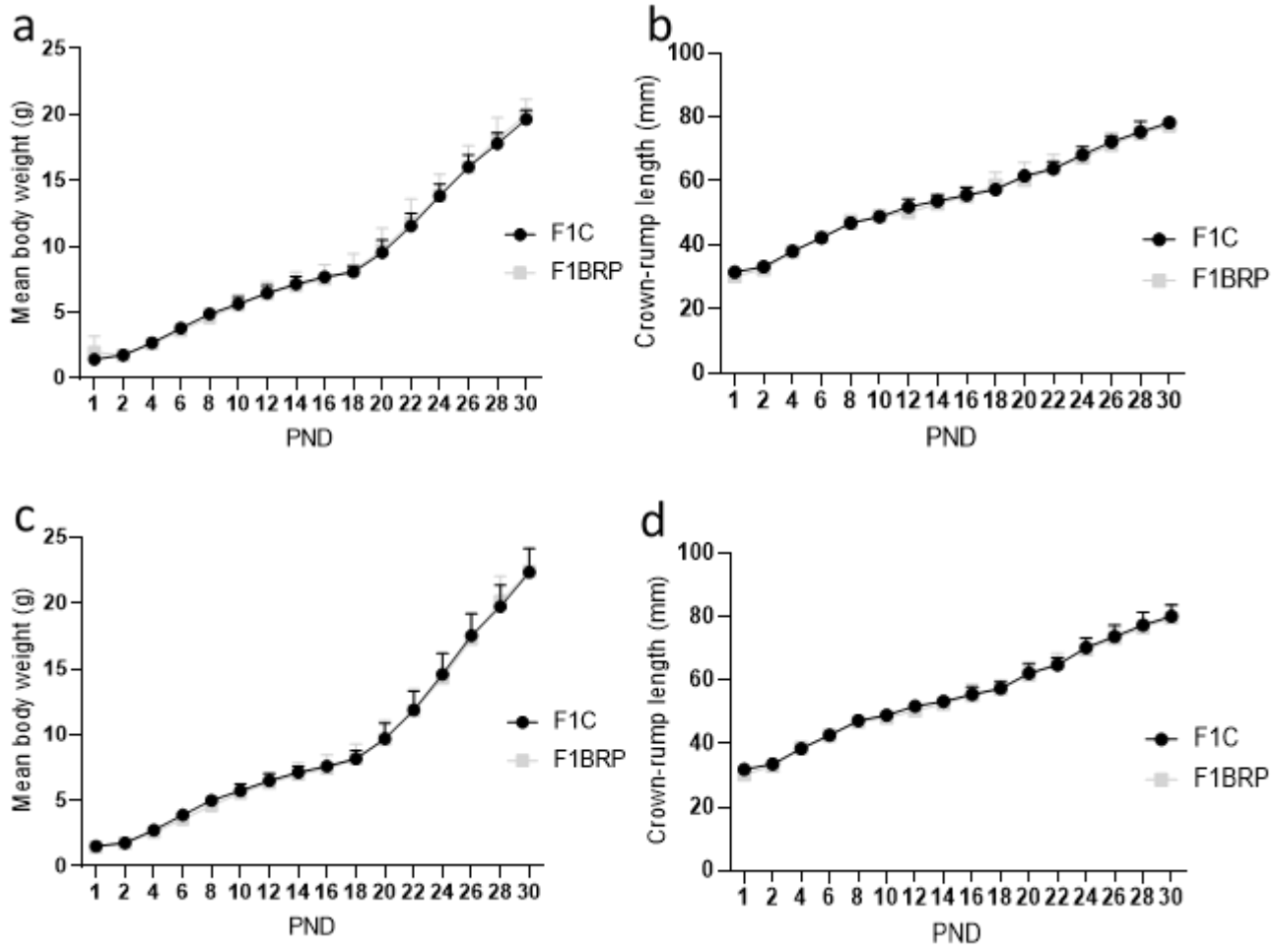

Fig. 4. Mean weight gain and offspring growth in the first month of life, females (a-b) and males (c-d). Control offspring group (F1C) and red propolis offspring group (F1BRP). Two-way ANOVA (mean and SD).

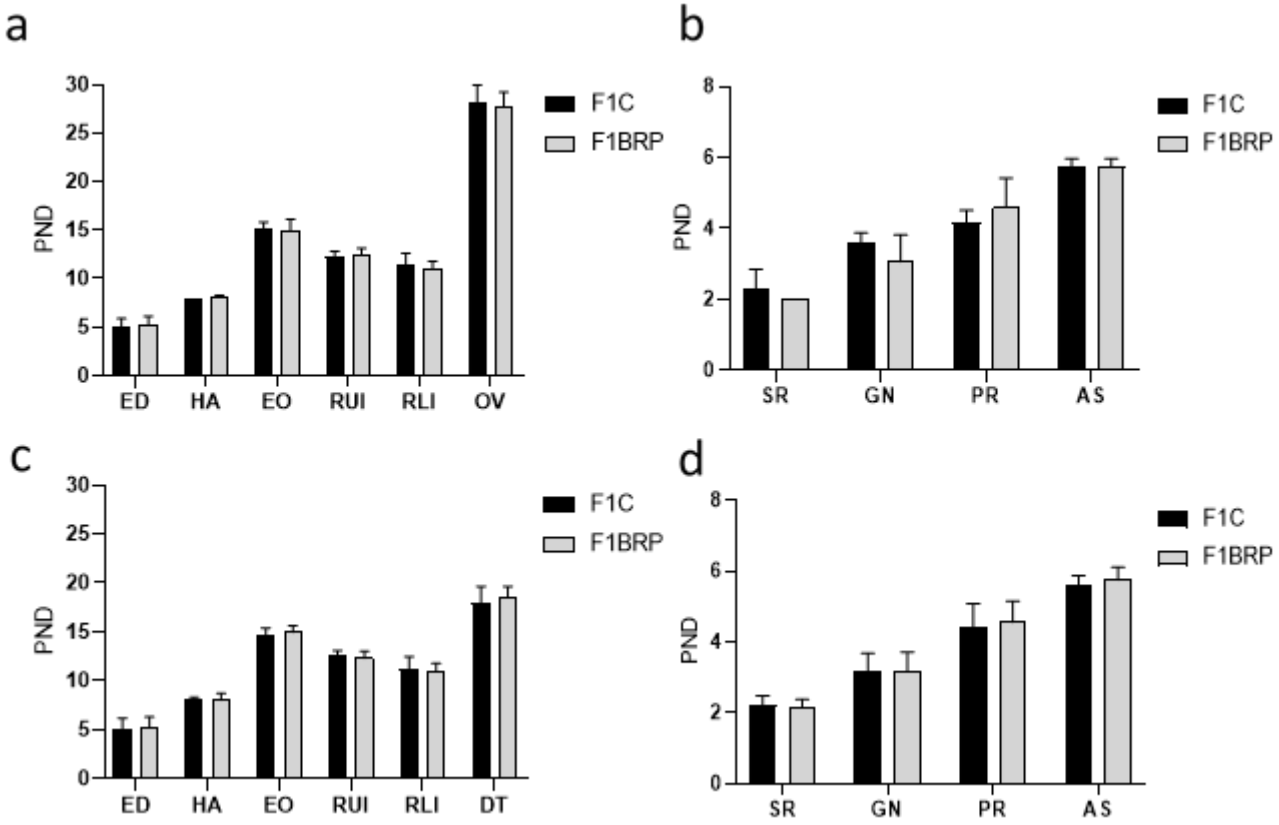

Fig. 5. Physical and neurological parameters of offspring, females (a-b) and males (c-d). Ear detachment (ED); hair appearance (HA); eye-opening (EO); rupture of the upper incisor (RUI); rupture of the lower incisor (RLI); the opening of the vagina (OV); descent of testicles in males (DT); straightening reflex (SR); negative geotaxis (GN); palmar reflex (PR); auditory startle (AS). Control offspring group (F1C) and red propolis offspring group (F1BRP). Test t-Student (mean and SD).

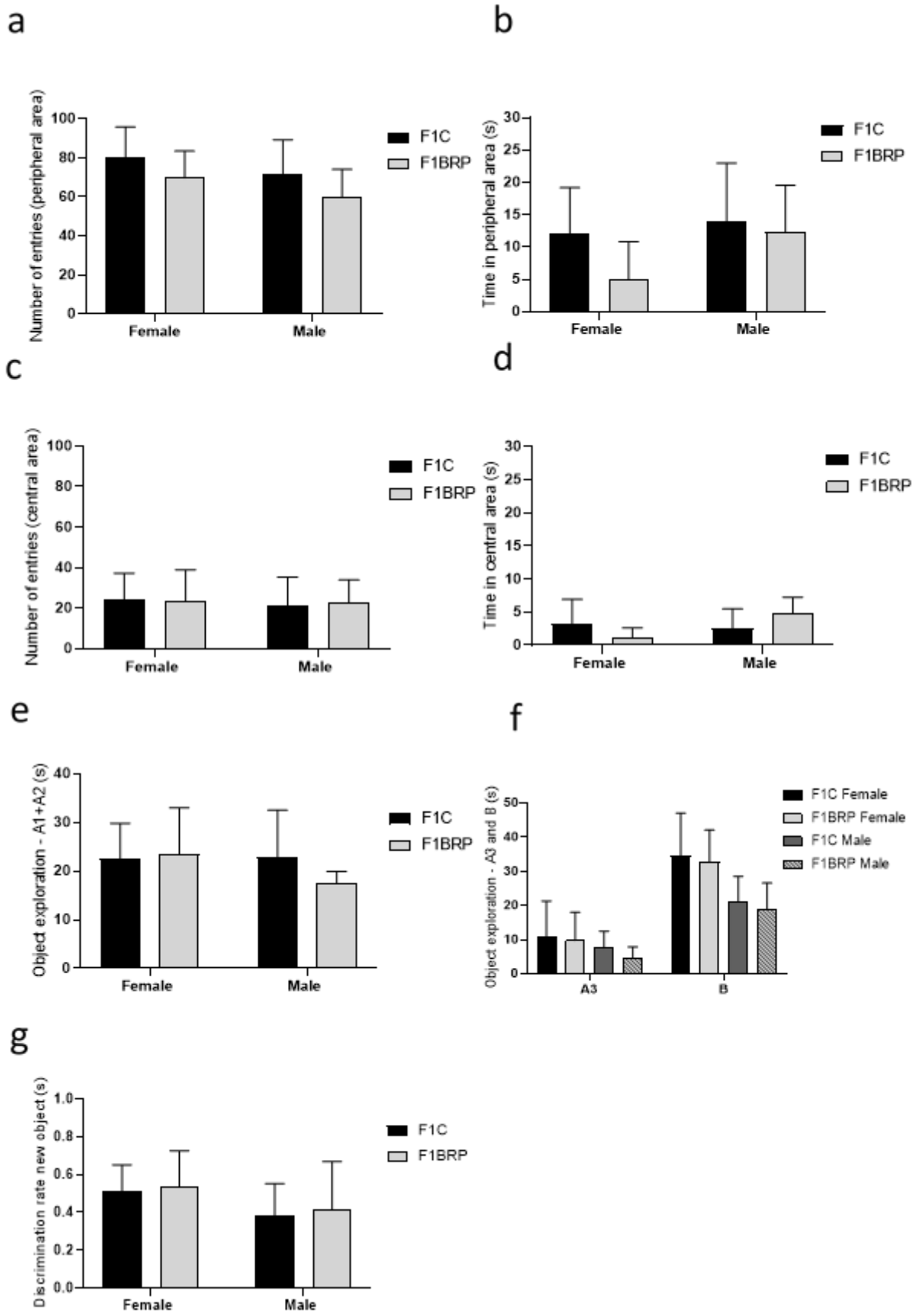

Fig. 6. Adulthood behavior: open-field (a-d) and memory (e-g). Number of entries, peripheral area (a), central area (c); Time spent, peripheral area (b), central area (d). Time spent in object recognition (e-f), discrimination rate new object (g). Control offspring group (F1C) and red propolis offspring group (F1BRP). Two-way ANOVA (mean and SD).

7. Discussion

Few studies in the literature relate the consumption of propolis to the bodyweight of the animal models used, especially studies with pregnancy models. However, a study observed the relationship between weight and body score of Santa Inês ewes in the flushing period with Brazilian red propolis as a feed additive and similar to the results of this study, the consumption of red propolis did not change the animals' body weight (Figure 1) (MORSY et al., 2013). Another study that used two varieties of Brazilian propolis (green and brown) as an additive in the feeding of calves, demonstrated that the bodyweight is not altered by the consumption of propolis, but can change the feed conversion rates (ÍTAVO et al., 2011).

In contrast, a study with a model of diabetes mellitus in pregnant rats, pointed out the applicability of propolis (variety from Malaysia) in reversing the loss of maternal weight and fetal body weight of diabetic animals, in addition to reducing blood glucose levels, implantation and oxidative stress markers (USMAN; ABU BAKAR; MOHAMED, 2018). In this sense, for the time being, we can suggest that the propolis varieties can prevent weight loss in pregnant model animals when they are subjected to some type of stress (environmental or congenital), but in optimal conditions (normal), the bodyweight parameter does not change due to the consumption of propolis, in particular red propolis.

The BRP in the dose used did not cause changes in the pregnancy of mice, maintaining the gestational viability and birth rate similar to the control animals. Equivalent results have been described for the use of two extracts of propolis (variety from Indonesia), consumed throughout the gestational period of mice, and did not affect birth rates and the number of implantation sites, reporting the absence of sites in the process of resorption (FIKRI et al., 2019).

Again, the work with previously mentioned diabetic pregnant rats shows the potential that the propolis varieties have to contain the changes caused by metabolic disorders or chronic inflammatory conditions since the diabetes model used caused high rates of implantation loss (42%) and a reduced number of puppies born (>50%), but propolis treatment reduced these rates to similar values in the group treated with insulin (reference drug) and the offspring did not present congenital anomalies (USMAN; ABU BAKAR; MOHAMED, 2018).

After demonstrating the safety of using propolis for the health of the females and the pregnancy itself, we analyzed the effects of using propolis for uterine Natural Killer cells. These findings suggest an acceleration in the process of differentiation and maturation of

UNK DBA^{+} cells, due to the decrease in subtype III in region 2 of the implantation site and concomitant to the increase in subtype IV in the same region. Differences in the distribution of uNK cell subtypes may indicate signaling gradients for maintaining natural killer cells in the uterus (PAFFARO et al., 2003).

Together with these data, studies show that inflammatory response conditions with possible risks to pregnancy (intraperitoneal injection of LPS and embryonic euthanasia protocols) are associated with the reduction of one or more subtypes of uNK cells and the appearance of uNK DBA^{low} cells (morphology amended) (LIMA, 2012; ZAVAN et al., 2016). Thus, unlike these studies, changes in the observed subtypes are not associated with the appearance of uNK DBA^{low} cells, indicating that the administered red propolis did not result in an inflammatory condition or represents some type of stress for pregnancy and is the first indication of safety for their use, regarding the health of the offspring.

The physiological and molecular mechanisms responsible for regulating the differentiation, activation and functions of uNK cells are only partially known (CROY et al., 2006), with a proposal that estrogenic hormones and progesterone exert regulatory effects on uNK indirectly cytokines, such as IL-15 (VERMA et al., 2000) and prolactin (GUBBAY et al., 2002), since estrogen and progesterone receptors are not found in uterine Natural Killer (CROY et al., 2006). A study demonstrated that uNK has receptors for prolactin, which through phosphorylation of the Jak/STAT pathway (signaling pathway involved in immunity, division and cell death) stimulates the MAPK/ERK pathway, responsible for activating regulatory differentiation and transcription factors cell proliferation (GUBBAY et al., 2002).

Among the factors that regulate the production of the hormone prolactin, are the estrogenic hormones, mainly estradiol and estriol (only in high concentrations), stimulating the production of the hormone by the maternal pituitary (DEL DIAZ et al., 1989; JOHNSON; CROWLEY, 1983; STANTON; KOEPPEN, 2009). However, not only hormonal compounds but also flavonoids can exert estrogenic effects (agonists and antagonists) in the body of humans and other animals, binding to estrogen receptors and binding sites at different locations in the body (DANCIU et al., 2018; HAVSTEEN, 2002; MANACH et al., 2004). Given our results, we postulate that red propolis or some of its flavonoid components such as, isosativan, medicarpin, formononetin, quercetin, biochanin A, liquiritigenin, isoliquiritigenin, daidzein, vestitol and neovestitol (BUENO-SILVA et al., 2015; OLDONI et al., 2011; TRUSHEVA et al., 2006), accelerate the process of differentiation/maturation of the uNK, based on the hypothesis that they could play a phytoestrogen function in the pregnancy of mice, increasing the levels of prolactin that consequently affect the differentiation processes

of the uNK. However, there are still no publications that indicate that all of these components described for BRP perform a similar function to estrogens *in vivo* and *in vitro*, requiring further analysis of these issues.

Another important variable to be considered for a healthy pregnancy are well-coordinated immune responses, in which cytokines and IFNs are critical mediators, due to their ability to dramatically alter cell function, migration, proliferation and gene expression, with complications in pregnancy and poor formations observed during childbirth are closely related to inadequate levels and unregulated expression of these mediators (YOCKEY; IWASAKI, 2018).

The different stages of pregnancy, from implantation to delivery, are related to specific profiles performed by the immune system (YOCKEY; IWASAKI, 2018). The implantation period is marked by a highly conserved inflammatory reaction in mammals (placental and marsupial), with positive regulation of inflammatory cytokines, including IL-1 and IL-6 (GRIFFITH et al., 2017), similar to our results (Figures 5a and 5b). Childbirth is another important event in pregnancy that uses the immune system and is associated with a pro-inflammatory environment, with an over-regulation of inflammatory cytokines (IL-1β and IL-8) in maternal-fetal tissues (CHRISTIAENS et al., 2008). Studies demonstrate that IL-1β can directly induce contraction of smooth muscle in the uterus, through calcium influx in myometrial smooth muscle, phosphodiesterase activity and production of prostaglandin F2a, to which everyone can contribute to essential muscle contraction in childbirth (OGER et al., 2002; TRIBE et al., 2003).

Disorders in the levels of these cytokines include, neuronal damage due to inflammatory premature birth in mouse models (LEITNER et al., 2014) and induction of premature labor in non-human primate models (SADOWSKY et al., 2006), for IL -1ß, and induces an autism-like phenotype and abnormal brain development in mouse models for IL-6 (SMITH et al., 2007). In contrast, the immunomodulatory cytokine IL-10 is closely related to uterine Natural Killer cells and plays a protective role in preventing fetal resorption in inflammatory conditions such as after intraperitoneal injection of LPS or CpG, a TLR9 agonist (MURPHY et al., 2005; THAXTON; ROMERO; SHARMA, 2009).

In this scenario, *in vitro* and *ex vivo* studies with red propolis and its components, vestitol and neovestitol, demonstrated the potential to regulate these cytokines, from stimuli in arthritis models and exposure of macrophages and neutrophils to LPS; reducing the levels of IL-1ß, IL-6 and the release of other pro-inflammatory factors, as the levels of IL-10 increased by activated macrophages. (BUENO-SILVA et al., 2015, 2017a, 2017b; FRANCHIN et al.,

2016). However, in two studies that carried out treatment groups with propolis separately, the results show that red propolis only exhibited its immunomodulatory property of these cytokines when there was an inflammatory stimulus by LPS (BUENO-SILVA et al., 2015, 2020). results, in which we seek to understand how PV can influence a normal and “perfect” pregnancy, we believe that these data provide safety for its use during pregnancy without disturbing these cytokines and we assume that Brazilian red propolis can be used for treatments inflammatory conditions in the gestational period to reverse or reduce the impacts caused, this being a hypothesis for future studies.

Animal development assessments include observations regarding the appearance of physical signs and aspects related to neurobehavioral development, frequently used to identify changes caused by pre and/or postnatal exposure to toxic compounds (CASTRO; CHIORATO; PINTO, 2000), as pesticides, which in certain doses do not affect the mother and birth rates, and do not cause congenital malformations, but impair the embryo-fetal maturation process, delaying the development of the puppies' physical and neurological abilities (CASTRO; CHIORATO; PINTO, 2000; CASTRO, 2006).

Fetal weight is the main parameter to assess levels of exposure to developmental toxicity (FOOD AND DRUG ADMINISTRATION (FDA), 2005; HOBERMAN; LEWIS, 2017), with crown-rump length being another parameter of great relevance (HOBERMAN; LEWIS, 2017). Similar to our results, propolis varieties from Indonesia when administered in low doses (380mg/kg) did not show differences in the fetal weight and crown-buttock length of the treated mice when compared to the control group (FIKRI et al., 2019). In an unprecedented way, our study provides assessments of offspring development using a variety of propolis, in which, we consider that the physical and neurological parameters evaluated, were not directly influenced by maternal treatment with BRP.

In the open field test, the animals' preference for peripheral areas of the apparatus was found, considered normal behavior for the model animal, in which the preference for peripheral areas can be considered an indication of hesitation (CAROLA et al., 2002). However, the absence of variations in immobility rates in the two areas of the apparatus, indicates that the use of red propolis during pregnancy does not affect the locomotor activity and does not result in a state of anxiety/hyperactivity in the offspring. When observing the results obtained in the spontaneous object recognition test, it can be stated that there was no change in the short-term memory retention in the animals, that is, there was no significant difference in the discrimination rates. The spontaneous object recognition experiment has been widely used as an important tool for investigating the effects of drugs on short and long-

term memory and also to investigate the mechanisms by which these effects happen (BAKER; KIM, 2002; ROSA et al., 2003; TANG et al., 1999), which in our study points out that red propolis does not cause the event of fetal programming.

The results obtained in our physical, morphological, histochemical, immunological and gestational viability studies, allow us to conclude that the Brazilian Red Propolis does not cause adverse effects in pregnant females and does not pose risks to normal pregnancy. However, the administration of red propolis promoted small changes in the density of uNK cell subtypes, although they were not associated with negative effects, these results have not been completely elucidated by our work. In the F1 generation, propolis did not cause physical, neurological and behavioral changes in adulthood (locomotor activity and short-term memory retention), in the offspring exposed during the gestational period. Therefore, this was a prospective study, which showed that red propolis is safe to be used during the gestational period of mice and does not cause changes in the offspring through fetal programming, presenting great potential as a treatment in future pre-clinical trials.

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8. CONSIDERAÇÕES FINAIS

Os resultados obtidos em nossos estudos físicos, morfológicos, histoquímicos, imunológicos e de viabilidade gestacional, nos permitem concluir que a Própolis Vermelha Brasileira não causa efeitos adversos em fêmeas prenhes e não traz riscos a gestação normal.

Porém, a administração de própolis vermelha promoveu pequenas alterações na densidade dos subtipos de células uNK, embora, não foram associados a efeitos negativos, esses resultados não foram completamente elucidados por nosso trabalho.

Na geração F1, a própolis não ocasionou alterações físicas, neurológicas e comportamentais na fase adulta (atividade locomotora e retenção de memória a curto prazo), na prole exposta durante o período gestacional.

Portanto, esse foi um estudo prospectivo, que mostrou que a própolis vermelha é segura para ser utilizada durante o período gestacional de camundongos e não provoca alterações na prole por meio da programação fetal, apresentando grande potencial como tratamento em ensaios pré-clínicos futuros.

Referências

ABE, H.; ISHIDA, Y.; IWASAKI, T. Perirhinal N-methyl-D-aspartate and muscarinic systems participate in object recognition in rats. **Neuroscience Letters**, v. 356, n. 3, p. 191–194, 2004.

ALENCAR, S. M. et al. Chemical composition and biological activity of a new type of Brazilian propolis : Red propolis. **Journal f Ethnopharmacology**. v. 113, p. 278–283, 2007.

ANDRADE, S. E. et al. Prescription drug use in pregnancy. **American Journal of Obstetrics and Gynecology**, v. 191, n. 2, p. 398–407, 2004.

ASO, K. et al. Inhibitory Effect of Propolis on the Growth of Human Leukemia U937. **Biological & Pharmaceutical Bulletin**, v. 27, n. 5, p. 727–730, 2004.

BAKER, K. B.; KIM, J. J. Effects of stress and hippocampal NMDA receptor antagonism on recognition memory in rats. **Learning and Memory**, v. 9, n. 2, p. 58–65, 2002.

BARKER, D. J. P. **Maternal nutrition, fetal nutrition, and disease in later life.Nutrition**, 1997.

BARKER, D. J. P. **The origins of the developmental origins theory**. Journal of Internal Medicine. **Anais**...2007

BREIER, B. H. et al. Fetal programming of appetite and obesity. **Molecular and Cellular Endocrinology**, v. 185, n. 1–2, p. 73–79, 2001.

BUENO-SILVA, B. et al. Effect of neovestitol-vestitol containing Brazilian red propolis on accumulation of biofilm in vitro and development of dental caries in vivo. **Biofouling**, v. 29, n. 10, p. 1233–1242, 2013.

BUENO-SILVA, B. et al. Brazilian red propolis attenuates inflammatory signaling cascade in lpsactivated macrophages. **PLoS ONE**, v. 10, n. 12, p. 1–14, 2015.

BUENO-SILVA, B. et al. Brazilian red propolis effects on peritoneal macrophage activity: Nitric oxide, cell viability, pro-inflammatory cytokines and gene expression. **Journal of Ethnopharmacology**, v. 207, p. 100–107, 2017a.

BUENO-SILVA, B. et al. Anti-inflammatory mechanisms of neovestitol from Brazilian red propolis in LPS-activated macrophages. **Journal of Functional Foods**, v. 36, p. 440–447, 2017b.

BUENO-SILVA, B. et al. Vestitol drives LPS-activated macrophages into M2 phenotype through modulation of NF-κB pathway. **International Immunopharmacology**, v. 82, n. February, p. 106329, 2020.

BURKE, S. D. et al. Uterine NK Cells, Spiral Artery Modification and the Regulation of Blood Pressure During Mouse Pregnancy. **American Journal of Reproductive**

Immunology, v. 63, n. 6, p. 472–481, 2010.

CAROLA, V. et al. Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. **Behavioural Brain Research**, v. 134, n. 1–2, p. 49–57, 2002.

CASTRO, V. L. DE; CHIORATO, S. H.; PINTO, N. F. Relevance of developmental testing of exposure to methamidophos during gestation to its toxicology evaluation. **Toxicology Letters**, v. 118, n. 1–2, p. 93–102, 2000.

CASTRO, V. L. Estudo Experimental em Ratos da Interação Mãe-Filhote Expostos e Agroquímicos. **Embrapa**, p. 1–7, 2006.

CHRISTIAENS, I. et al. Inflammatory processes in preterm and term parturition. **Journal of Reproductive Immunology**, v. 79, n. 1, p. 50–57, 2008.

CROY, B. A. et al. Transplantation into genetically alymphoid mice as an approach to dissect the roles of uterine natural killer cells during pregnancy - A review. **Placenta**, v. 21, n. SUPPL.1, p. 1996–1999, 2000.

CROY, B. A. et al. Uterine natural killer cells: a specialized differentiation regulated by ovarian hormones. **Immunological Reviews**, v. 214, p. 161–185, 2006.

CUNHA, I. B. DA S. et al. Antitrypanosomal Activity of Brazilian Propolis from Apis mellifera. **Chem. Pharm. Bull.**, v. 52, n. 5, p. 602–604, 2004.

DA SILVA, R. O. et al. Acute and sub-acute oral toxicity of Brazilian red propolis in rats. **JOURNAL OF ETHNOPHARMACOLOGY**, v. 170, p. 66–71, jul. 2015.

DANCIU, C. et al. Main Isoflavones Found in Dietary Sources as Natural Anti-inflammatory Agents. **Current Drug Targets**, v. 19, n. 7, p. 841–853, 2018.

DEL DIAZ, M. C. et al. Estriol affects prolactin and LH secretion in rats. **Journal of Endocrinological Investigation**, v. 12, n. 1, p. 1–7, 1989.

FIKRI, A. M. et al. The effect of propolis administration on fetal development. **Heliyon**, v. 5, n. 10, p. e02672, 2019.

FOOD AND DRUG ADMINISTRATION (FDA). Food and Drug Administration (FDA) - Detection of Toxicity to Reproduction for Medicinical Products & Toxicity to Male Fertility, (ICH) S5 (R2). **ICH Guidelines**, v. 5, n. June 1993, p. 1–24, 2005.

FRANCHIN, M. et al. Vestitol Isolated from Brazilian Red Propolis Inhibits Neutrophils Migration in the Inflammatory Process: Elucidation of the Mechanism of Action. **Journal of Natural Products**, v. 79, n. 4, p. 954–960, 2016.

GHISALBERTI, E. L. Propolis : A Review. **Bee World**, n. July, p. 59–84, 1979.

GRIFFITH, O. W. et al. Embryo implantation evolved from an ancestral inflammatory

attachment reaction. **Proceedings of the National Academy of Sciences of the United States of America**, v. 114, n. 32, p. E6566–E6575, 2017.

GUBBAY, O. et al. Prolactin induces ERK phosphorylation in epithelial and CD56+ natural killer cells of the human endometrium. **Journal of Clinical Endocrinology and Metabolism**, v. 87, n. 5, p. 2329–2335, 2002.

GUIMOND, M.-J. et al. Absence of Natural Killer Cells during Murine Pregnancy is Associated with Reproductive Compromise in TgE26 Mice1. **Biology of Reproduction**, v. 56, n. 1, p. 169–179, 1997.

GUIMOND, M.-J.; WANG, B.; CROY, B. A. Engraftment of Bone Marrow from Severe Combined Deficits in Natural Killer Cell – deficient tg ♪ 26 Mice. **Journal of Experimental Medicine**, v. 187, n. 2, p. 217–223, 1998.

HAVSTEEN, B. H. **The biochemistry and medical significance of the flavonoids**. [s.l: s.n.]. v. 96

HOBERMAN, A. M.; LEWIS, E. M. **Juvenile toxicology testing**. [s.l.] Elsevier Inc., 2017.

ÍTAVO, C. C. B. F. et al. Addition of propolis or monensin in the diet: Behavior and productivity of lambs in feedlot. **Animal Feed Science and Technology**, v. 165, n. 3–4, p. 161–166, 2011.

JOHNSON, M. D.; CROWLEY, W. R. Acute effects of estradiol on circulating luteinizing hormone and prolactin concentrations and on serotonin turnover in individual brain nuclei. **Endocrinology**, v. 113, n. 6, p. 1935–1941, 1983.

KHALIL, M. L. Biological Activity of Bee Propolis in Health and Disease. **Asian Pacific Journal of Cancer Prevention.**, v. 7, p. 22–31, 2006.

KUMAZAWA, S.; HAMASAKA, T.; NAKAYAMA, T. Antioxidant activity of propolis of various geographic origins. **Food Chemistry**, v. 84, n. 3, p. 329–339, 2004.

LEITNER, K. et al. IL-1 receptor blockade prevents fetal cortical brain injury but not preterm birth in a mouse model of inflammation-induced preterm birth and perinatal brain injury. **American Journal of Reproductive Immunology**, v. 71, n. 5, p. 418–426, 2014.

LIMA, P. D. ET AL. Heterogeneity in composition of mouse uterine natural killer cell granules. **J Leukoc Biol**, v. 92, p. 195–204, 2012.

MANACH, C. et al. Polyphenols : food sources and bioavailability . Am J Clin Nutr. **American Journal of Clinical Nutrition**, v. 79, p. 727–747, 2004.

MARCUCCI, M. C. et al. Phenolic compounds from Brazilian propolis with pharmacological activities. **Journal of ethnopharmacology**, v. 74, n. 2, p. 105–12, 2001.

MENDONÇA-MELO, L. et al. Similitud química y genética entre propóleos de Dalbergia ecastaphyllum y rojos del Noreste de Brasil. **Journal of Apicultural Research**, v. 56, n. 1, p. 32–39, 2017.

MONTPIED, P. et al. Caffeic acid phenethyl ester (CAPE) prevents inflammatory stress in organotypic hippocampal slice cultures. **Molecular Brain Research**, v. 115, n. 2, p. 111–120, 2003a.

MONTPIED, P. et al. Caffeic acid phenethyl ester (CAPE) prevents inflammatory stress in organotypic hippocampal slice cultures. **Molecular Brain Research**, v. 115, n. 2, p. 111–120, 23 jul. 2003b.

MOORE, K. L.; PERSAUD, T. V. N.; TORCHIA, M. G. **Embriologia Clínica**. Rio de Janeiro: Elsevier, 2012.

MORSY, A. S. et al. Effect of Brazilian red propolis administration on hematological, biochemical variables and parasitic response of Santa Inês ewes during and after flushing period. **Tropical Animal Health and Production**, v. 45, n. 7, p. 1609–1618, 2013.

MURPHY, S. P. et al. Uterine NK Cells Mediate Inflammation-Induced Fetal Demise in IL-10-Null Mice. **The Journal of Immunology**, v. 175, n. 6, p. 4084–4090, 2005.

NAGAI, T. et al. Preparation and antioxidant properties of water extract of propolis. **Food Chemistry**, v. 80, n. 1, p. 29–33, 2003.

OGER, S. et al. Interleukin-1β induces phosphodiesterase 4B2 expression in human myometrial cells through a prostaglandin E2- and cyclic adenosine 3′,5′-monophosphate-dependent pathway. **Journal of Clinical Endocrinology and Metabolism**, v. 87, n. 12, p. 5524–5531, 2002.

OLDONI, T. L. C. et al. Isolation and analysis of bioactive isoflavonoids and chalcone from a new type of Brazilian propolis. **Separation and Purification Technology**, v. 77, n. 2, p. 208–213, 2011.

OLIVIER, J. D. A. et al. Fluoxetine administration to pregnant rats increases anxiety-related behavior in the offspring. **Psychopharmacology**, v. 217, n. 3, p. 419–432, 2011.

ORŠOLIĆ, N. et al. Effects of Local Administration of Propolis and Its Polyphenolic Compounds on Tumor Formation and Growth. **Biological & Pharmaceutical Bulletin**, v. 28, n. 10, p. 1928–1933, 2005.

ORSOLIC, N.; BASIC, I. Antitumor, hematostimulative and radioprotective action of water-soluble derivative of propolis (WSDP). **BIOMEDICINE & PHARMACOTHERAPY**, v. 59, n. 10, p. 561–570, 2005.

OSORIO-DE-CASTRO, C. G. S.; PAUMGARTTEN, F. J. R.; SILVER, L. D. O uso de

medicamentos na gravidez [The use of drugs in pregnancy]. **Ciência & Saúde Coletiva**, v. 9, n. 4, p. 987–996, 2004.

PAFFARO, V. A. et al. Subset classification of mouse uterine natural killer cells by DBA lectin reactivity. **Placenta**, v. 24, n. 5, p. 479–488, 2003.

PARK, Y. K.; ALENCAR, S. M.; AGUIAR, C. L. **Botanical Origin and Chemical Composition of Brazilian Propolis**. [s.l: s.n.].

RAMOS, W. L. P. et al. Análise Do Uso De Medicamentos Durante a Gestação em Mães de Pacientes Portadores de Malformações Fetais. **Revista Saúde e Pesquisa**, v. 1, n. 1, p. 56–64, 2008.

ROSA, R. M. et al. Facilitation of long-term object recognition memory by pretraining administration of diphenyl diselenide in mice. **Neuroscience Letters**, v. 341, n. 3, p. 217–220, 2003.

SADOWSKY, D. W. et al. Preterm labor is induced by intraamniotic infusions of interleukin-1β and tumor necrosis factor-α but not by interleukin-6 or interleukin-8 in a nonhuman primate model. **American Journal of Obstetrics and Gynecology**, v. 195, n. 6, p. 1578–1589, 2006.

SEKI, Y. et al. **Minireview: Epigenetic programming of diabetes and obesity: Animal models.Endocrinology**, 2012.

SFORCIN, J. M. **Propolis and the immune system: a reviewJournal of Ethnopharmacology**, 2007.

SILVA, B. B. et al. Chemical composition and botanical origin of red propolis, a new type of Brazilian propolis. **Evidence-based Complementary and Alternative Medicine**, v. 5, n. 3, p. 313–316, 2008.

SILVA, C. L. M.; GONTIJO, L. S.; NOEL, F. Noradrenaline-induced contraction of mice aorta is enhanced in schistosomiasis. **VASCULAR PHARMACOLOGY**, v. 46, n. 2, p. 122–128, 2007.

SILVEIRA, P. P. et al. **Developmental origins of health and disease.J Pediatr (Rio J)**, 2007.

SMITH, S. E. P. et al. Maternal immune activation alters fetal brain development through interleukin-6. **Journal of Neuroscience**, v. 27, n. 40, p. 10695–10702, 2007.

SOJKA, D. K.; YANG, L.; YOKOYAMA, W. M. Uterine natural killer cells: To protect and to nurture. **Birth Defects Research**, v. 110, n. 20, p. 1531–1538, 2018.

STANTON, B. A.; KOEPPEN, B. M. (EDS.). **Berne & Levy - Fisiologia**. 6. ed. Rio de Janeiro: Elsevier, 2009.

TANG, Y. P. et al. Genetic enhancement of learning and memory in mice. **Nature**, v. 401, n. 6748, p. 63–69, 1999.

THAXTON, J. E.; ROMERO, R.; SHARMA, S. TLR9 Activation Coupled to IL-10 Deficiency Induces Adverse Pregnancy Outcomes. **The Journal of Immunology**, v. 183, n. 2, p. 1144–1154, 2009.

TRIBE, R. M. et al. Interleukin-1β induces calcium transients and enhances basal and store operated calcium entry in human myometrial smooth muscle. **Biology of Reproduction**, v. 68, n. 5, p. 1842–1849, 2003.

TRUSHEVA, B. et al. Bioactive Constituents of Brazilian Red Propolis. **Evidence-Based Complementary and Alternative Medicine**, v. 3, n. 2, p. 249–254, 2006.

USMAN, U. Z.; ABU BAKAR, A. B.; MOHAMED, M. Propolis improves pregnancy outcomes and placental oxidative stress status in streptozotocin-induced diabetic rats. **BMC COMPLEMENTARY AND ALTERNATIVE MEDICINE**, v. 18, 2018.

VERMA, S. et al. Human decidual natural killer cells express the receptor for and respond to the cytokine interleukin 15. **Biology of Reproduction**, v. 62, n. 4, p. 959–968, 2000.

VITOLO, M. R. **Nutrição: da gestação ao envelhecimento**. Rio de Janeiro: Rubio, 2008.

WIRA, C. R. et al. **Innate and adaptive immunity in female genital tract: Cellular responses and interactions.Immunological Reviews**, 2005.

YOCKEY, L. J.; IWASAKI, A. Role of interferons and cytokines in pregnancy and fetal development. **Immunity**, v. 49, n. 3, p. 397–412, 2018.

ZAVAN, B. et al. COX-2 plays a role in angiogenic DBA+ uNK cell subsets activation and pregnancy protection in LPS-exposed mice. **Placenta**, v. 44, p. 34–45, 2016.

ZHANG, J. H.; YAMADA, A. T.; CROY, B. A. DBA-lectin Reactivity Defines Natural Killer Cells that have Homed to Mouse Decidua. **Placenta**, v. 30, n. 11, p. 968–973, 2009.

3. ANEXO A

MINISTÉRIO DA EDUCAÇÃO
Universidade Federal de Alfenas. Unifal-MG
Rua Gabriel Monteiro da Silva, 700. Alfenas/MG. CEP 37130-000
Fone: (35) 3299-1000. Fax: (35) 3299-1063

Comissão de Ética no Uso de Animais – CEUA/UNIFAL

CERTIFICADO

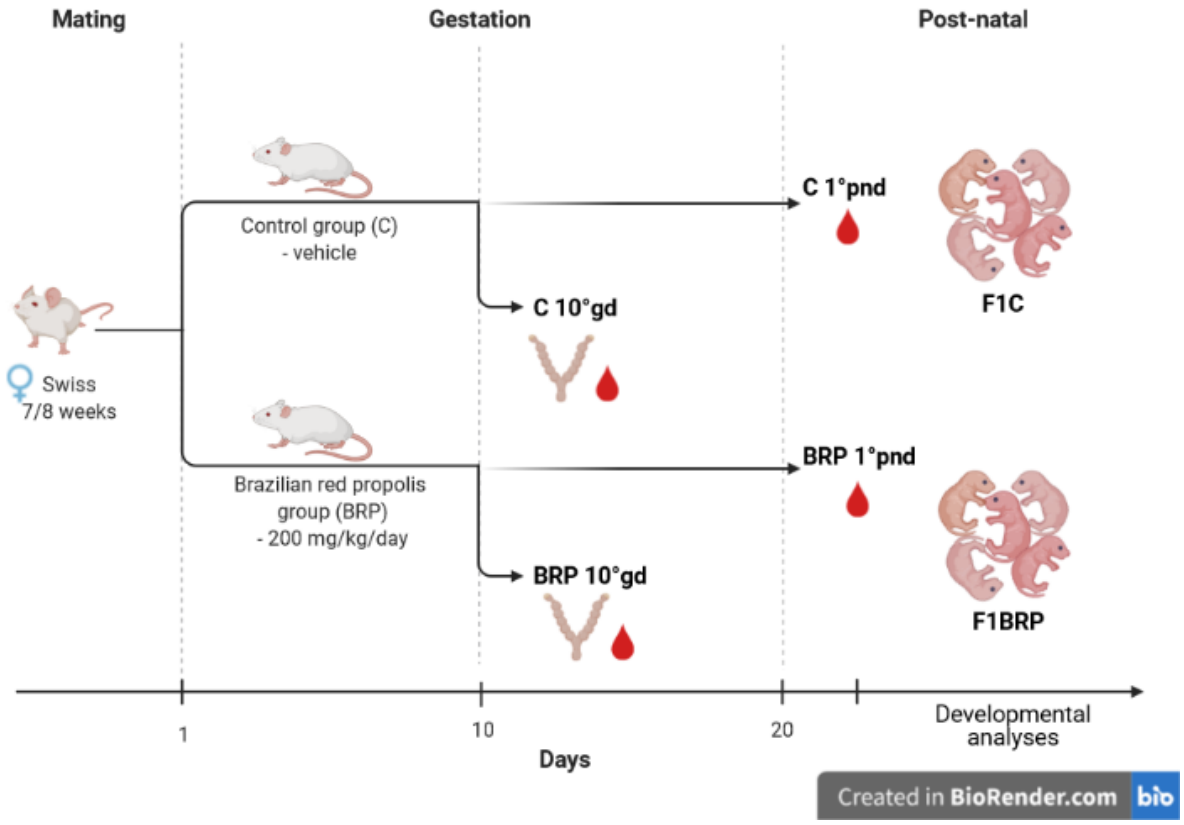
Certificamos que a proposta intitulada "Avaliação dos efeitos da própolis vermelha brasileira durante a gestação de camundongos e no desenvolvimento da prole", registrada com o nº 44/2019, sob a responsabilidade de Masaharu Ikegaki, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA-UNIFAL) DA UNIVERSIDADE FEDERAL DE ALFENAS.

Finalidade	( ) Ensino (X) Pesquisa científica
Vigência da autorização	De 14/11/2019 a 01/02/2021
Espécie/linhagem/raça	Camundongo Swiss / heterogênico
Nº de animais	118
Sexo	33 - Machos 85 - Fêmeas
Origem	Biotério Central da UNIFAL

Alfenas, 14 de Novembro de 2019.

Prof. Dr. Leonardo Augusto de Almeida
Coordenador do CEUA/UNIFAL-MG

ANEXO B



ANEXO C - Registro no SisGen

For access to the Propolis sample, this research was registered under the number A305815, in the Brazilian National System Management of Genetic Heritage and Associated Traditional Knowledge (SisGen), according to Brazilian Law nº 13,123/2015.