

# *Luffa operculata* (L.) Cogn. Gestational Exposition Induces Anxiety-like Behavior and Interferes with Melatonin and Inflammation in Young Female Rats

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## ABSTRACT

**Background:** *Luffa operculata* (L.) Cogn. (Cucurbitaceae; EBN) dried fruits are abortifacient and showed undesirable effects over male and female adult Wistar rats. **Objectives:** The present study aimed at accessing how the gestational exposition of F1 female Wistar rats (FF1) to EBN interfered with behavior after challenge with stress or Lipopolysaccharide (LPS). **Materials and Methods:** Wistar rat female fetuses were gestationally exposed to a sub-abortifacient dose of EBN, and received stress or LPS challenge at a young adult age. The alterations in behavior, serum hormone levels, cytokines, and liver and kidney histological and biochemical indices were evaluated. **Results:** The gestational administration of EBN and NYM/LPS exposition at a young adult age induced anxiety-like behavior in the FF1, accompanied by an increase in the serum corticosterone, ACTH and melatonin levels. A diminish in the pro-inflammatory cytokines was also observed. No histochemical or biochemical alterations were seen in the liver or kidneys. **Conclusion:** The gestational exposition of EBN led the FF1 to reveal an anxiety-like behavior after being submitted to a stress challenge or LPS exposition at a young adult age and was accompanied by an increase of serum corticosterone and ACTH. A melatonin serum level increase has also been implicated in diminishing the pro-inflammatory cytokines.

**Keywords:** Animal behavior, Cognitive performance, Gestational treatment, Interleukins, Reproduction, Stress.

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**Received:** 22-02-2023;

**Revised:** 16-03-2023;

**Accepted:** 29-04-2023.

## INTRODUCTION

The tea made with the fruits of *Luffa operculata* (L.) Cogn., popularly known in Brazil as *buchinha-do-norte*, is used to minimize sinusitis symptoms by nostril instillation or steam inhalation<sup>[1,2]</sup> and to eliminate the microorganisms related to the respiratory condition.<sup>[3]</sup> The tea of the plant can cause severe nasal irritations and bleeding,<sup>[4]</sup> but the most questionable, inadvertently popular use is to provoke abortion.<sup>[5]</sup> Previous work focused on assessing how the oral administration of 1.0 mg/kg of the EBN in different pregnancy stages could lead to fetal resorption or malformation in rodents.<sup>[6]</sup> The presence of cucurbitacins in *buchinha-do-norte* is well reported,<sup>[7-10]</sup> although

the toxicological effects remain a thought-provoking issue that has challenged our group to perform further studies.

The direct and gestational administration of the tea made with the dried fruit of *Luffa operculata* (L.) Cogn., Cucurbitaceae (EBN)<sup>[11]</sup> affects the behavior of adult male rats,<sup>[12]</sup> female rats,<sup>[13]</sup> and young adult male rats from the F1 generation.<sup>[14]</sup> The gavage administration of EBN to pregnant dams at the gestational days 17 to 21 induced anxiety-like behavior in the dams after they had weaned. The EBN administration interfered with offspring weight, and it has also interfered with the decrease of serum pro-inflammatory cytokine, in the dams.<sup>[13]</sup>

Nonetheless, there are still gaps to be filled, and one of them regards the influence of EBN on the female pups from the F1 generation (FF1) after having received the gestational exposition to EBN. The present study aimed at studying the behavioral changes caused in the FF1 that were gestationally exposed to the dose of 1.0 mg/kg for five consecutive days - from GD17 to GD21 - and after having been submitted to stress or lipopolysaccharide



DOI: 10.5530/pres.15.3.055

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(LPS) challenge at post-natal day 60, a period of life the rats are at a young adult age.

## MATERIALS AND METHODS

### Plant Extract

The aqueous extract obtained from the dried fruits of *L. operculata* (acquired from Santos Flora - BU01/0914, collect on 09/2014, valid until 09/2017, Brazil), was obtained from a decoct that was made according to popular use. Then, 35.0 g, which is approximately equivalent to 15 dried fruits, were added to 4.5 L of boiling water (Milli-q; Millipore, Merck, USA), in the proportion of one fruit to each 300 mL of water, for 10 min, resulting in 14.8 g of the lyophilized (Virtis Co., USA) aqueous extract, EBN (42.7% yield, w/w).

### Animals

Adult male and female *Rattus norvegicus* (Wistar rats) were used in the reproduction technique. Female rats 12 to 14 weeks old, weighing 200 g to 250 g, male rats aged 20 to 23 weeks, weighing approximately 350 g, female rat pups at post-natal day 60 (PND60) weighing 150 to 200 g and male rat pup at PND60 weighing 200 g to 250 g were subjected to the experiments. After birth, female pups were tested, and male pups were directed to other studies.

### Ethics

The UNIP-Ethics Committee approved the current project for Animal Use (ECAU; protocol #043/2016). Good Laboratory Practices and Ethics were adopted to minimize animal suffering and maximize animal well-being by embracing the 3R's concepts and under ethics guides suggested by -CONCEA (*Conselho Nacional de Controle de Experimentação Animal* - National Council for Animal Experimentation Control).

### EBN dose determination

Based on previous work,<sup>[6]</sup> a single dose of 1.0 mg/kg was administered daily to pregnant females at Gestational Days (GD) 17 to 21, by gavage, to the test group named Experimental Group (EG). The treatment was made with the lyophilized tea obtained from a decoct. Also, the Control Group (CG) received water as the vehicle by gavage.

### Mating and Birth Protocol

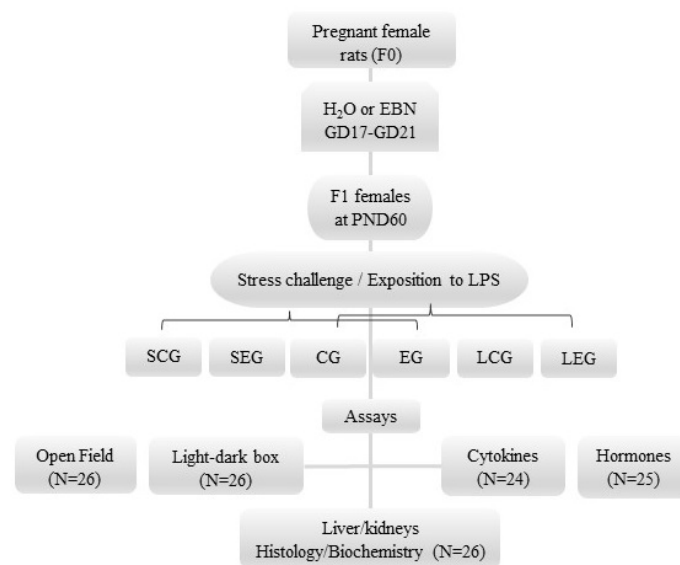
Mating and birth protocols are described elsewhere.<sup>[12,14]</sup> Briefly, sexually inexperienced female rats ( $n_{\text{total}}=26$ ) were accommodated in propylene cages (45.5 X 34.5 X 20 cm), groups of four in each cage, under a controlled environment, temperature of 22°C ± 2°C, relative humidity at 55-65%, artificial lighting (12hr/12hr light/dark regulation, lights on at 7:00 a.m.), to habituation for ten days, before the beginning of the experiments. Sexually experienced male Wistar rats ( $n_{\text{total}}=11$ ) were kept under the same

conditions. The animals were allowed a permanent food and filtered water supply.

The mating procedure was conducted by introducing one male to a group of three female rats in one cage. The four rats remained together until the identification of pregnancy in a female, verified by spermatozoids in the external vaginal smear. As soon as the pregnant females were identified, they were re-united to their original companions up to GD17 to avoid isolation and loneliness during pregnancy. At GD17, each dam was allocated into individual cages attached to a microisolator system (Tecniplast<sup>®</sup>, Buguggiate, Italy) under the same previous environmental conditions. After mating, the male rats from the F0 generation,<sup>[12]</sup> the male offspring,<sup>[14]</sup> and the dams<sup>[13]</sup> participated in other assays.

### Gestational exposition to EBN, stress challenge and lipopolysaccharide exposition, and experimental design

Adult female rats of the Experimental Group (EG) were given 1.0 mg/kg of EBN by gavage,<sup>[12]</sup> during Gestation Days 17 (GD17) to GD21. The dams from the Control Group (CG) received the vehicle control, distilled water, at a volume corresponding to 1.0 mL/kg, also by gavage. Days GD17 to GD21 have been chosen as it is the period of gestation when the rat embryos reach an essential stage of final maturation of the Central Nervous System (CNS) when external or internal influences regarding mother stress can be more significant to the offspring.<sup>[15]</sup> The dams gave birth, and the offspring were left to be nursed until Post-Natal Day 21 (PND21). Then, at PND21, male and female pups were



**Figure 1:** Experimental design. F1 female pups impact from the gestational exposition to the aqueous extract obtained from the dried fruits of *Luffa operculata* and to a post-natal day 60 challenge with stress (New York Subway Metro stress model) or exposition to Lipopolysaccharide (LPS). EBN=*Buchinha-do-norte* aqueous extract; GD=Gestation day; PND=Post-natal day. N=Total number of animals per analysis.

separated by sex and left to grow up to PND60, when they were considered young adults.<sup>[16]</sup>

The Female Pups (FF1) were divided into groups according to their mother's treatment (CG or EG group), and the stress challenge or LPS exposition they received at PND60 before behavioral, histological, and biochemical evaluations were assessed. The experimental groups SCG (Stress Control Group), SEG (Experimental Stress Group), LCG (LPS Control Group), and LEG (LPS Experimental Group) were obtained, as well as the Control (CG) and Experimental (EG) groups, in which no challenge was made and that were used for both stress challenge or LPS exposition statistical comparisons. Immediately after the stress challenge or LPS exposition, FF1 was submitted to a behavioral evaluation in the Open Field (OF) and Light-Dark Box (LDB) apparatuses, followed by euthanasia. Then, blood, liver, and kidneys were collected for biochemical and histological analyses. The experimental design adopted in the study is pictured in Figure 1. Control groups were used for both NYM and LPS challenges.

The "New York City Subway Stress" was adopted in the present evaluation. The methodology was previously described by two scientists,<sup>[17]</sup> whose laboratory was in New York (NY). The technique received such a name after the resemblance of a subject experience in the moving subway during rush hour, which was mimicked by adapting the classic restraint stress technique and adding some smooth movements. The rats remained under stress exposition for one hour.<sup>[18]</sup>

LPS naturally occurs in the cell membrane of Gram-negative bacteria, such as *Escherichia coli*. Due to its capacity to induce hyperalgesia and increasing corticosterone in rats,<sup>[19]</sup> that has long been used to experimentally mimic bacterial infections by creating a physiological condition that leads to what is known as "sickness behavior". So, LPS was intraperitoneally administered to the rats at a dose of 100 µg/kg.

## Behavior Evaluation

### Open Field Apparatus

Locomotion and anxiety-like behavior were accessed by OF apparatus, an aversion to bright environment model, after rat exposition to EBN.<sup>[20]</sup> Each animal was placed in the central circle of the OF arena, where they remained for three minutes. The following parameters were accessed by filming so no interaction with the observer has been made, except to introduce and remove the animals from the apparatus: frequency of locomotion (units), immobility time (seconds), rearing frequency (units), time of grooming (seconds), time spent in the center and the borders of the apparatus (seconds) and the number of fecal *boli* (units).<sup>[14,21-25]</sup> A 5% ethanol solution was used to remove odors before the following animal was introduced into the apparatus.

### Light-Dark Box Apparatus

Immediately after subjection to OF apparatus, each animal was placed in the LDB to evaluate anxiety. The model is also based on the aversion of rodents to illuminated areas. LDB consists of an acrylic box unequally divided into a smaller dark side and a larger light side, separated by a wall. The wall has a hole through which the animals can cross and explore both sides. LDB promotes a conflictive condition to the animals that must choose between remaining in the dark side's safety or exploring the dangerous light side.

The animals were placed in the dark side of the box and remained under experimental conditions for five minutes. The assay was filmed to register the parameters that were evaluated, such as the latency to transit to the light side (seconds); attempts to cross to the light side (units); the time spent on the dark side, and the light side (seconds); the locomotion frequency in the light and dark sides (units); rearing frequency in light and dark sides (units); the immobility time in both light and dark sides (seconds); side crosses (units), grooming in both sides (seconds); and the number of fecal *boli* in the dark and light sides (units).<sup>[12,26]</sup>

### Hormonal, Histological, and Biochemical Studies

Blood, liver, and kidneys were collected from the F1 females after euthanasia at PND60. Blood was centrifuged for 15 min to remove serum, fractionated into three vials containing approximately 200 µL each, and then stored at -80°C until use. Serum was used in the quantification of the inflammatory cytokines, IL-1α, IL-1β, IL-6, and TNF-α (Magpix RECYTMAG 65K),<sup>[27]</sup> and stress hormones as corticosterone, adrenocorticotrophic hormone (ACTH), and melatonin (Magpix RESHMAG 59K0),<sup>[28]</sup> and aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine in a biochemical analyzer Cobas C111\* (Roche® Diagnostics, Basel, Switzerland),<sup>[29]</sup> using, respectively, Cobas C111\* commercial reagents AST IFCC (code: 04657543190), ALTL (code: 04718569190) and CREJ2 (code: 04718569190), by a colorimetric assay. Liver and kidney samples were fixed in 10% buffered formalin for 24 hr. The histological procedures used conventional paraffin embedding and hematoxylin-eosin (HE) staining methods. An evaluation based on scores was performed considering the following parameters: a) for liver degeneration: nucleus morphology (pyknosis, karyorrhexis, and karyolysis) and cytoplasm vacuolization; b) kidneys: glomerular hypercellularity and tubular vacuolization. All the parameters were scored from 0 to 2, as described as absence (0), moderate (1), and severe (2).<sup>[14]</sup>

### EBN Thin Layer Chromatography

The Thin Layer Chromatography (TLC) analysis of EBN was made to evaluate the presence of cucurbitacins according to a TLC system composed of ethyl acetate, methanol, and water (100:35:10) used in a silica gel GF<sub>254</sub> (Merck) plate. Vanillin

phosphoric acid and plate heat for 10 min at 100°C composed the reagent system (VP41).<sup>[30]</sup>

## Statistical Analysis

Data were obtained based on independence, randomness, and normality. Outliers were identified by the ROUT test (Q=5%) and submitted to the Shapiro-Wilk normality test. After that, one-way ANOVA and Tukey's *post hoc* test, or Kruskal-Wallis and Dunn's *post hoc* test, were adopted to compare means/medians resulting from the experiments describing the influence of the gestational exposition to the Aqueous Extract (EG) and of Water (CG) to FF1 after being submitted to stress challenge or LPS exposition at a young adult age, or PND60. The effect size was calculated to some parameter factors, considering a test power of 80%. Estimation of a group size of  $n=5$  ( $n=4C*(s/d)^2$ ),  $C=(Z^{\alpha/2}+z\beta)^2$ , confidence

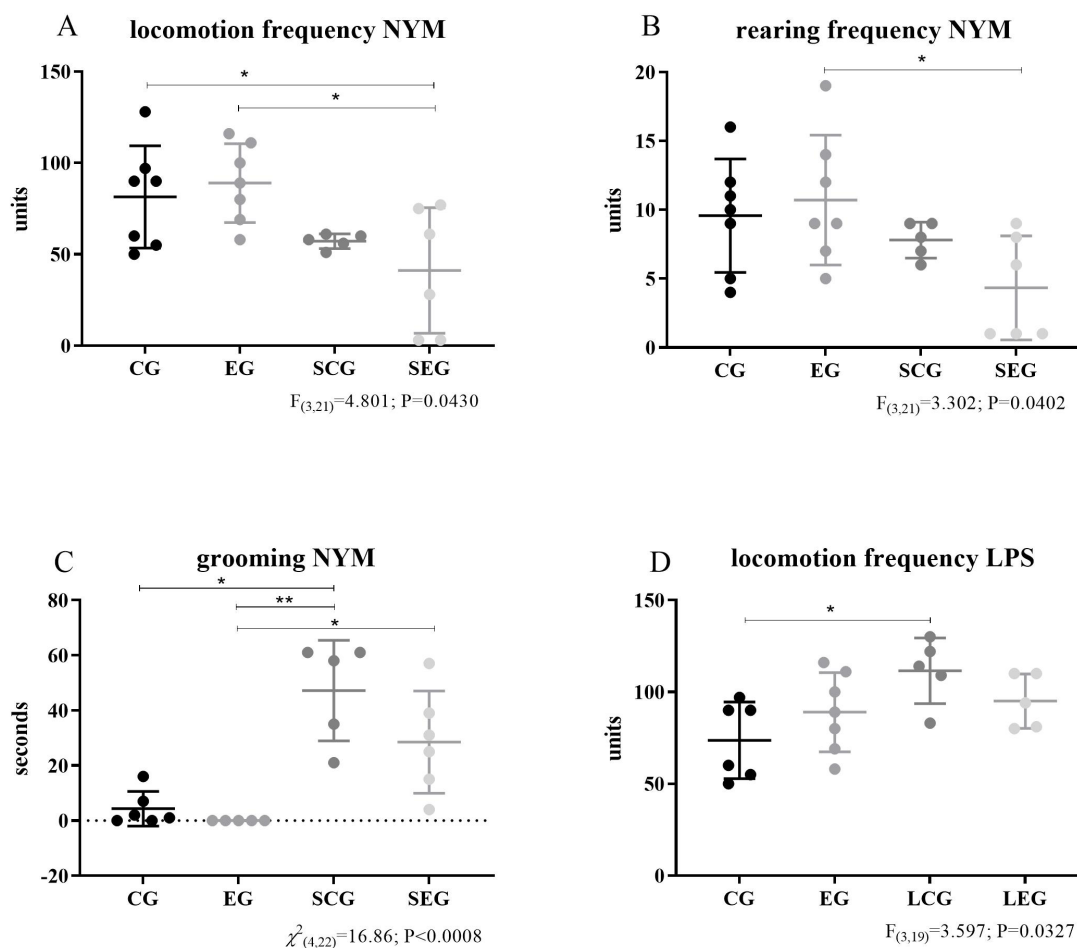
interval 0.95/2 (0.475),  $z=1.96$ , test power of 80% ( $z\beta=0.842$ ), max dev. 20%, difference between groups 50%;<sup>[31]</sup> the resource equation method was also used.<sup>[32]</sup> All tests were made in Excel or GraphPad Prism7.0, and significances were considered as  $\alpha=0.05$  for all analyses.<sup>[33]</sup>

## RESULTS

### Behavior Analyses in the Open Field and Light-Dark Box Apparatuses

Figures 2 and 3 report findings obtained from the OF and LDB apparatuses. Data related to the Shapiro-Wilk normality test, non-parametric Kruskal-Wallis, or parametric ANOVA analyses can be assessed in Table 1S (Supplementary Material) as medians and maximum/minimum limits (non-parametric data) or means and standard error (parametric data).

### Open field New York Metro or LPS challenges



**Figure 2:** F1 female rats' behavior evaluation performed in the open field apparatus. The rats were gestationally exposed to 1.0 mg/kg of *Luffa operculata* Aqueous Extract (EBN) and challenged with New York Metro stress (NYM) or Lipopolysaccharide (LPS) at Post-Natal Day 60 (PND60). A. Locomotion frequency after stress challenge; B. Rearing frequency after stress challenge; C. Grooming after stress challenge; D. Locomotion frequency after LPS exposition. CG=Control Group; EG=Experimental Group; SCG=NYM control group; SEG=NYM experimental group; LCG=LPS control group; LEG=LPS experimental group.

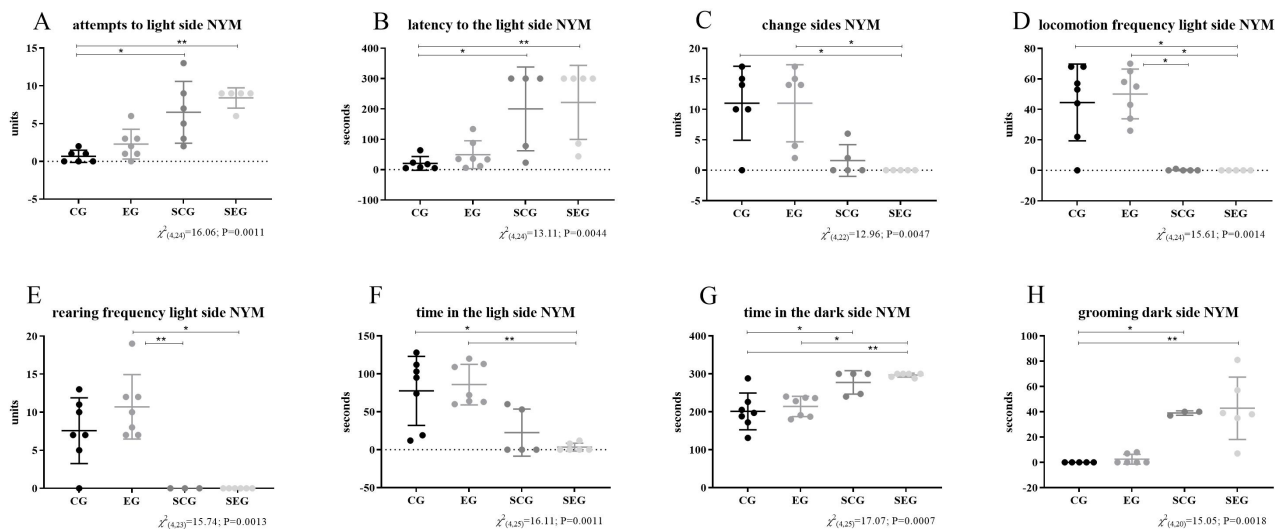
Concerning the OF assay (Figure 2), the results obtained after the challenge with the NYM stress factor pointed that the FF1 rats from the SEG group showed a diminished locomotion frequency in relation to EG ( $p=0.0134$ ) and CG ( $p=0.0430$ ; Figure 2A) groups. Rearing frequency was reduced in SEG in relation to EG ( $p=0.0341$ ; Figure 2B). In Figure 2C, grooming was augmented in SCG in relation to CG ( $p=0.0359$ ) and EG ( $p=0.0015$ ), and SEG was increased in relation to EG ( $p=0.0268$ ). Locomotion frequency was augmented in LCG in relation to CG ( $p=0.0205$ ; Figure 2D) after the challenge with LPS.

Regarding the LDB assay (Figure 3), the results obtained from the analysis after the NYM stress challenge were the following. Concerning the attempts to the light side (Figure 3A), SCG and SEG attempted more than CG ( $p=0.0217$  and  $p=0.0024$ , respectively) to enter the light side. SCG and SEG showed higher latency to cross to the light side than CG ( $p=0.0446$  and  $p=0.0081$ , respectively; Figure 3B). Nonetheless, SEG crossed between light and dark sides less than CG and EG ( $p=0.0382$

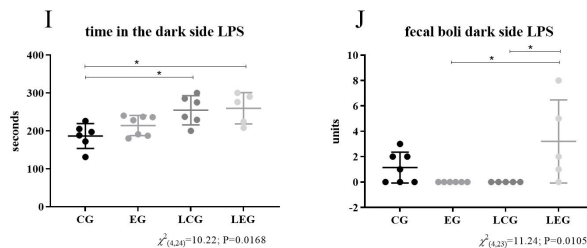
and  $p=0.0178$ , respectively; Figure 3C). Figure 3D shows that EG locomoted more than SCG and SEG on the light side ( $p=0.0286$  and  $p=0.0117$ , respectively), while CG locomoted more than SEG ( $p=0.0384$ ). Rearing frequency was higher in EG in relation to SCG and SEG on the light side ( $p=0.0360$  and  $p=0.0039$ , respectively; Figure 3E). Figure 3F shows that SEG spent less time on the light side than CG ( $p=0.0155$ ) and EG ( $p=0.0085$ ). Figure 3G shows that SEG spent more time on the dark side than CG ( $p=0.0052$ ) and EG ( $p=0.0195$ ) and that SCG spent more time in the dark than CG ( $p=0.0298$ ). Figure 3H shows that on the dark side, SCG ( $p=0.0355$ ) and SEG ( $p=0.0116$ ) performed more grooming than CG.

Concerning the observations made with the groups challenged with LPS, Figure 1 shows that CG spent less time on the dark side than LCG ( $p=0.0422$ ) and LEG ( $p=0.0497$ ). It was observed (Figure J) that the LEG group made more fecal boli than EG and LCG ( $p=0.0324$  and  $p=0.0464$ , respectively).

### LDB New York Metro challenge



### LDB LPS challenge



**Figure 3:** Light-dark box apparatus behavior evaluation of female pups at post-natal day 60 gestationally exposed to 1.0 mg/kg of *Luffa operculata* aqueous extract (EBN) and challenged with New York Metro (NYM) stress (figures A to H) or LPS (figures I and J) at post-natal day 60 (PND60). A. Attempts to the light side; B. Latency to the light side; C. Change sides; D. Locomotion frequency in the light side; E. Rearing frequency in the light side; F. Time spent in the light side; G. Time spent on the dark side; H. grooming in the dark side; I. Time spent in the dark side; J. Fecal boli in the dark side. CG=control group; EG=experimental group; SCG=stress control group; SEG=stress experimental group; CG=control group; EG=experimental group; SCG=NYM stress control group; SEG=NYM stress experimental group; LCG=LPS control group; LEG=LPS experimental group.

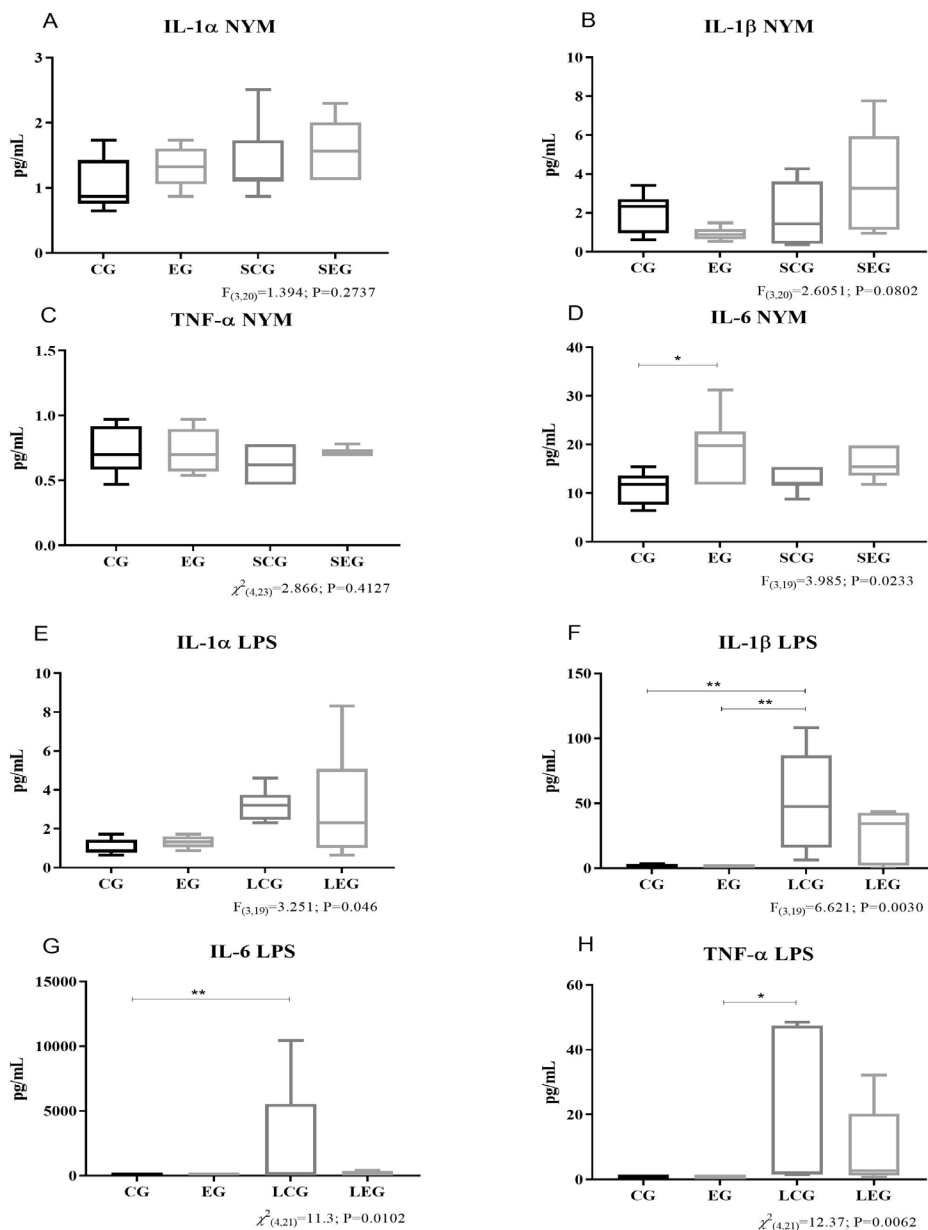
## Serological Studies on the Female Pups at Post-natal Day 60

### Pro-inflammatory Cytokines

Figures 4A, 4B, 4C, and 4D show the alterations in the serum concentrations of the pro-inflammatory cytokines after the NYM stress challenge. In contrast, Figures 4E, 4F, 4G, and 4H show the differences in the level of cytokines after the LPS challenge.

Alterations in IL-1 $\alpha$  ( $p=0.2737$ ), IL-1 $\beta$  ( $p=0.0802$ ), and TNF- $\alpha$  ( $p=0.4127$ ) were not observed, while IL-6 was highly expressed in EG in relation to CG ( $p=0.0325$ ).

After the LPS challenge, the level of IL-1 $\alpha$  was statistically similar among groups ( $p=0.0446$ ). IL-1 $\beta$  was augmented in LCG in relation to CG ( $p=0.0063$ ) and EG ( $p=0.0052$ ). IL-6 was increased in LCG in relation to CG ( $p=0.0075$ ). TNF- $\alpha$  was augmented in LCG in relation to EG ( $p=0.0276$ ).



**Figure 4:** F1 female pups' serum inflammatory cytokine levels at post-natal day 60. The animals were gestationally exposed to 1.0 mg/kg of *Luffa operculata* fruits and challenged with New York Metro stress (NYM) Lipopolysaccharide (LPS) at PND60. A. IL-1 $\alpha$  and NYM; B. IL-1 $\beta$  and NYM; C. IL-6 and NYM; D. TNF- $\alpha$  and NYM; E. IL-1 $\alpha$  and LPS; F. IL-1 $\beta$  and LPS; G. IL-6 and LPS; H. TNF- $\alpha$  and LPS Results are expressed as ANOVA/Kruskal-Wallis tests and *post hoc* tests Tukey/Dunn's, applied according to the normality test analyses. Significance was considered as  $\alpha < 0.05$  in the analyses. CG=Control Group; EG=Experimental Group; SCG=stress control group; SEG=stress experimental group; LCG=LPS control group; LEG=LPS experimental group.

## Hormones

The amounts of corticosterone, ACTH, and melatonin were assessed from the blood sera after rat exposition to stress or LPS and are shown in Figure 5. Higher amounts in the serum levels of corticosterone (Figure 5A) were observed in the SEG in relation to CG ( $p=0.0391$ ; Figure 5A). ACTH (Figure 5B) was higher in SEG in relation to CG ( $p=0.0329$ ) and EG ( $p=0.0160$ ). Melatonin (Figure 5C) was diminished in EG in relation to SCG ( $p=0.0290$ ) and SEG ( $p=0.0211$ ). For the rats submitted to the LPS exposition, it was observed that the levels of serum corticosterone (Figure 5D) were elevated in LEG in relation to LCG ( $p=0.0311$ ), and melatonin (Figure 5F) was higher in LEG than CG ( $p=0.0049$ ) and EG ( $p=0.0022$ ). ACTH was not altered in the experiment ( $p=0.4506$ ; Figure 5E).

## Histology and Biochemistry of Liver and Kidneys of FF1

### Kidneys and Liver histological analyses

No histological differences were observed in any of the liver or kidney parameters of the female pups at PND60. Figure 6 shows the lack of differences among groups.

## Biochemical Parameters of Kidneys and Liver

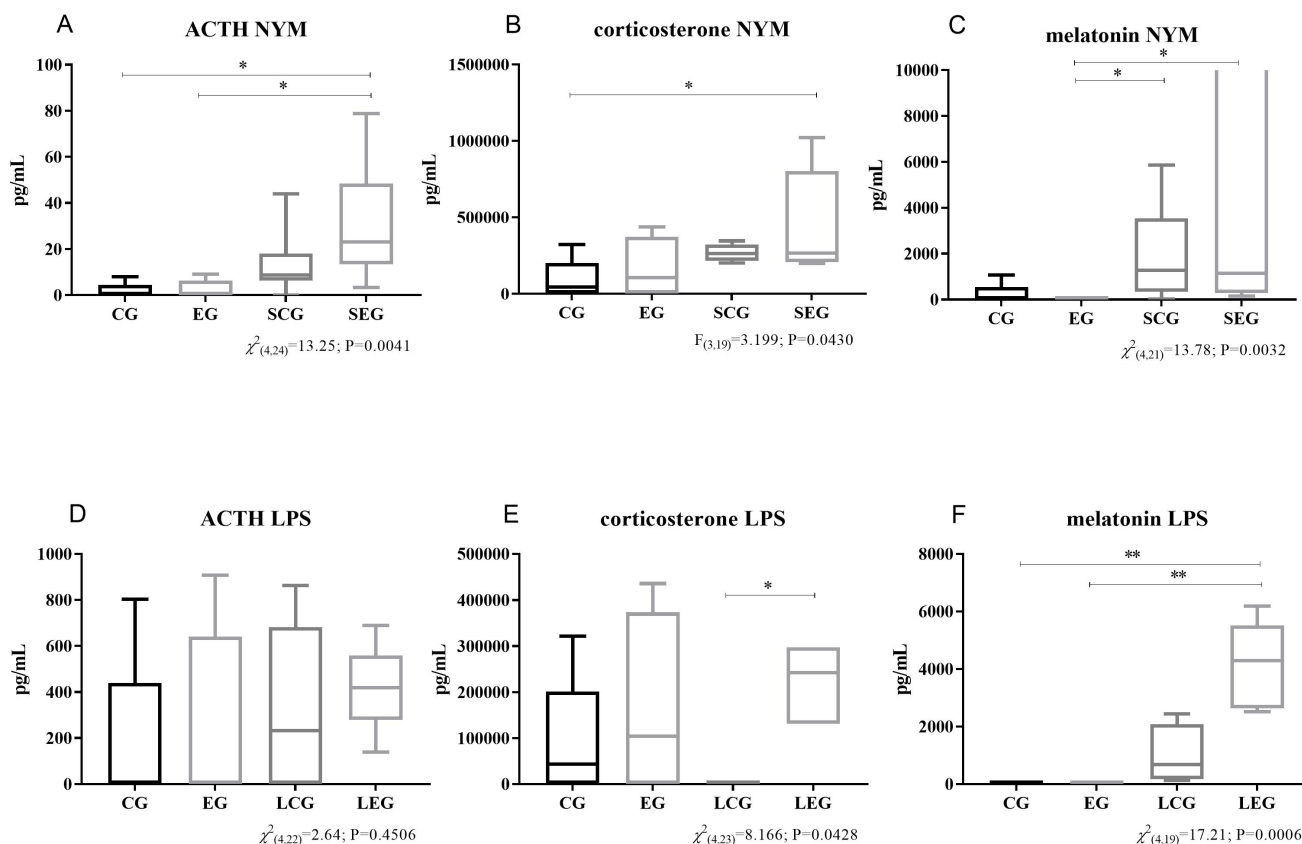
No liver biochemical alterations were observed, based on AST ( $p=0.7333$  and  $p=0.8921$ , respectively, for NYM and LPS challenge), ALT ( $p=0.6660$  and  $p=0.8946$ , respectively for NYM and LPS challenge), or creatinine ( $p=0.0833$  and  $p=0.2404$ , respectively for NYM and LPS challenge) levels in the female pups at PND60 (Figure 7).

## TLC Analysis

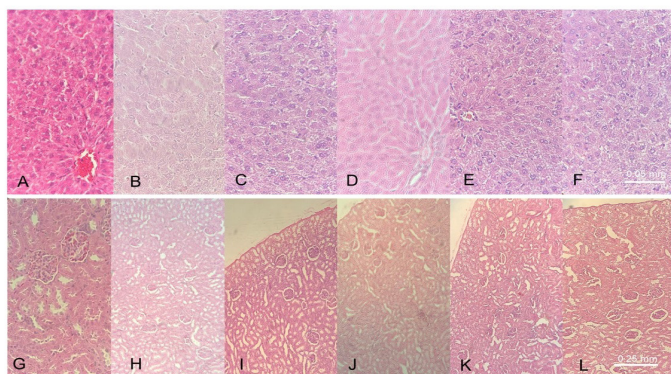
Figure 8 shows the TLC evaluation of EBN, and pale-yellow spots at the front line,  $R_f=0.96$ , and yellow-brown spots at the application point,  $R_f=0.01$ , and a pale spot at  $R_f=0.51$  were observed, which suggests the presence of aglycones and glycosides derived from the 23,23-dihydrocucurbitacins, respectively, according to Wagner and Bladt.<sup>[30]</sup>

## DISCUSSION

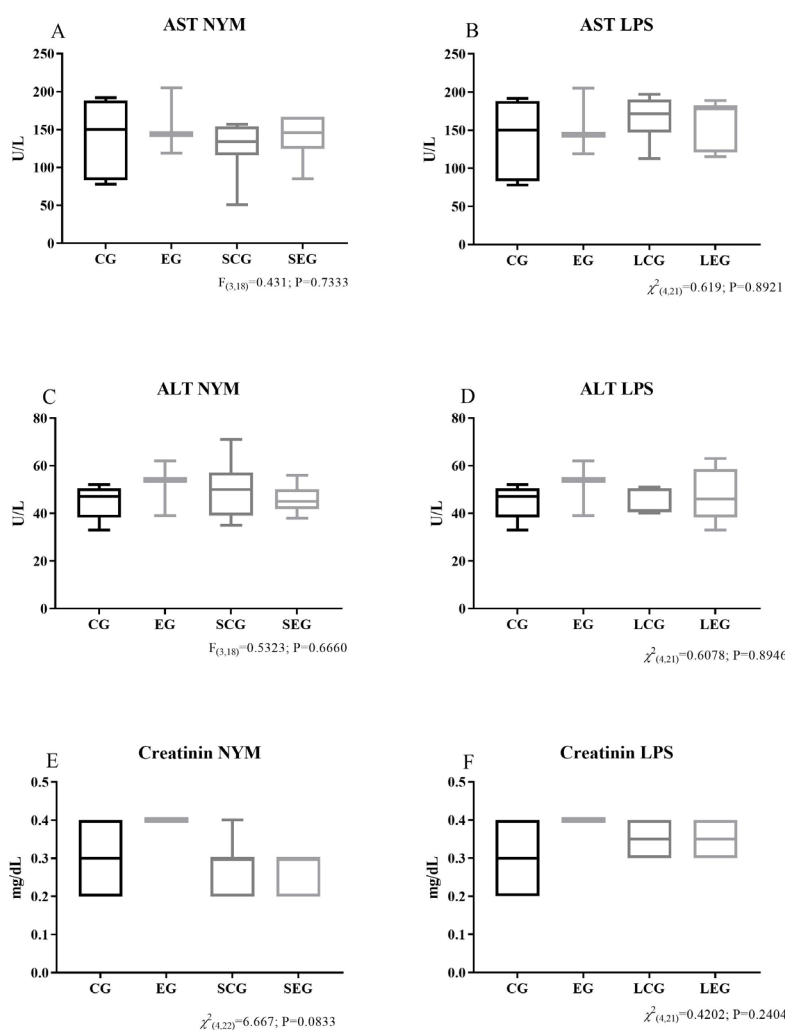
The present work aimed at assessing the influence of the gestational administration of the EBN sub-abortive dose of 1.0 g/kg during GDs 17 to 21 over female Wistar rat pups after a stress



**Figure 5:** Hormone levels in the serum of female pups at post-natal day 60. Quantifications are given in pg/mL for all parameters. A. Corticosterone levels after New York Metro (NYM) stress challenge; B. Adrenocorticotrophic hormone levels after NYM challenge; C. Melatonin levels after NYM challenge. D. Corticosterone after LPS challenge; E. Adrenocorticotrophic hormone after LPS exposition; F. Melatonin after LPS exposition. Data are described as mean/median  $\pm$  SE/maximum and minimum limits for all measurements and were calculated by ANOVA or Kruskal-Wallis tests. Tukey's or Dunn's *post hoc* tests were adopted for multiple comparisons, considering significant differences at  $\alpha < 0.05$ . \*= $p < 0.05$ . CG=control group; EG=experimental group; SCG=stress control group; SEG=stress experimental group; LCG=LPS control group; LEG=LPS experimental group.



**Figure 6:** Histological evaluation of liver (A-F) and kidneys (G-L) observed in the control group (CG), experimental group (EG), stress control group (SCG), experimental control group (SEG), LPS control group (LCG) and LPS experimental group (LEG). Control groups received water as the vehicle, and test groups received EBN (aqueous extract of *Luffa operculata*). Bars reported a magnification of 40X for the liver and 10X for the kidneys.

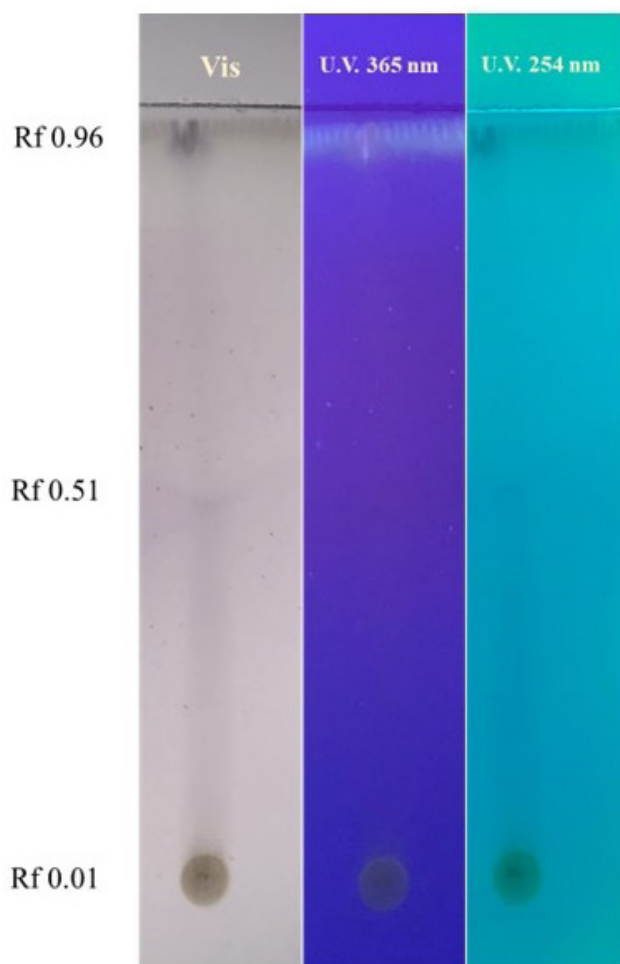


**Figure 7:** AST (aspartate aminotransaminase), ALT (alanine aminotransaminase), and creatinine levels in the serum of female pups at post-natal day 60. Quantifications are given in pg/mL for all parameters. A. AST levels after New York Metro (NYM) stress challenge; B. AST levels after the LPS challenge; C. ALT levels after the NYM challenge. D. ALT after LPS challenge; E. Creatinin levels after NYM exposition; F. Creatinin levels after LPS exposition. Data are described as mean/median  $\pm$  SE/maximum and minimum limits for all measurements and were submitted to ANOVA or Kruskal-Wallis tests. Tukey's or Dunn's *post hoc* tests were adopted for multiple comparisons, considering significant differences at  $\alpha < 0.05$ .  $*=p < 0.05$ . CG=control group; EG=experimental group; SCG=stress control group; SEG=stress experimental group; LCG=LPS control group; LEG=LPS experimental group.

challenge or LPS exposition at PND60, a period that the rats are considered young adults.

The behavioral analyses obtained from the stress-challenged groups indicated diminished ground and spatial exploratory parameters, such as locomotion and rearing, respectively, and improved time spent performing grooming in the open field apparatus. Also, the FF1 spent more time on the dark side and less time on the light side of the LDB and showed fear in spending more time attempting to cross to the light side or crossing less between sides in LDB, which indicated a behavior typically adopted by rats undergoing an anxiolytic-like behavior.

The administration of EBN in gestation days 17 to 21, corresponding to the maturation of the central nervous system (CNS),<sup>[15]</sup> has worked as a chemical stressor to the uterine environment where the fetuses developed. Pre-natal stressors



**Figure 8:** Thin layer chromatography analyses of EBN. Vis=visible light; U.V.=ultraviolet light. R<sub>f</sub>=retention factor.

can imprint alterations in the CNS that may induce behavioral or cognitive abnormalities in the offspring likely to be expressed after birth at any age.<sup>[34]</sup> The imprint may ease a forthcoming disruption in physical or mental health,<sup>[35]</sup> which can be expressed as behavioral alterations such as anxiety, sleep disturbance, alteration in locomotion, retardation in cognition, some degree of memory impairment,<sup>[15]</sup> or even psychiatric diseases as schizophrenic episodes, minor depression, neurotic symptoms, alcoholism or criminal behavior.<sup>[36]</sup> A stressor is a well-known and widely used tool to study behavior alterations during a rat's lifetime.<sup>[37]</sup> Using a second stressor, also known as a "challenge", at a defined age of the rat offspring can ease the observation of subtle behavioral/cognitive alterations, as a first stress event has previously sensitized the rat.

In the present study, two stressors were adopted to reveal the influence of EBN over FF1 better. The first stressor was the EBN gestational exposition during GD17 up to GD21, and the second was a restraint stress challenge or the LPS exposition at PND60 when the female rats were considered young adults.<sup>[38]</sup> Some authors consider PND60 the final period of rat "adolescence", which begins at weaning.<sup>[39]</sup> PND21 to PND60 is when the CNS

development earns dramatic maturation and rearrangement of neurotransmitter pathways in some areas related to cognition, emotion, motivation, and reaction to stress.<sup>[40]</sup> The present work focused on the post-pubertal period because the significant developmental changes occur in a lower path, aiming for adulthood.

It seems that the first stressor primed FF1 systems yet in the *uterus* and predisposed them to alterations in behavior. The second stressor highlighted the behavioral, hormonal, and immune responses undergone by FF1. An interesting theory named the two-hit hypothesis was first proposed to rat models used to study schizophrenia.<sup>[41,42]</sup> According to the idea, two subsequent events, or hits, need to occur to develop the neuronal sickness: The first is related to a genetic predisposition that can be familial or acquired. A second event or hit with environmental causes, such as stress or a sickness condition. Although the present report does not focus on schizophrenia, the two-hit theory fits the sequential procedures adopted in the experimental design to evaluate behavioral changes. Indeed, in the current work, the first stressor may not be related to a genetically inherited predisposition but to an epigenetic alteration induced by the EBN administration, yet to be further investigated.

It was observed that both challenges proposed to the F1 females, NYM stress or LPS, have highlighted the behavioral alterations caused by the gestational administration of EBN to FF1. Previous data indicated that the EBN gestational administration at the end of pregnancy has also interfered with the stress-related behavioral phenotype response in the male pups by having dysregulated the hypothalamus-pituitary-adrenal axis, HPAA.<sup>[14]</sup>

The elevated concentrations of both corticosterone and ACTH support the anxiety-like behavior observed mainly in the LDB apparatus. Corticosterone is long known to be produced in the cortex of the adrenal glands in response to a stressor activity or stressful condition.<sup>[43,44]</sup> Also, corticosterone synthesis can be stimulated by ACTH via the melanocortin-2-receptor, or MC2.<sup>[45]</sup> Present findings show that corticosterone concentration in the SCG group was not elevated as was in the SEG, indicating that the animals that were gestationally exposed to EBN and the stress challenge showed more predisposition to an anxiety-like state induced by stress, which is also supported by the elevated blood concentration of ACTH in the same group of animals. Although high concentrations of IL-1 $\beta$  would be expected, no improvement in pro-inflammatory cytokines was observed. According to our findings, the melatonin influence in diminishing inflammation signs can be further explored, as it was also augmented.

LPS can induce hyperalgesia and increase corticosterone in rats,<sup>[19]</sup> which experimentally mimics bacterial infections that lead to "sickness behavior". Under such conditions, the animal will likely reveal any subtle behavioral changes. The most prominent result from OF was that LEG locomoted less, suggesting a direct

influence over ground exploration. In the LDB apparatus, the LEG group remained longer on the dark side, and produced more fecal boli, which indicated an anxiety-like behavior, and presumable alteration in emotionality, evidenced by the increased number of fecal boli in the secure environment. Again, corticosterone was augmented in this group, as well as melatonin.

The two-hit hypothesis theory fits our findings on cytokine expression in the groups receiving NYM stress or LPS as the second events, SGC, LCG, SEG, and LEG. In the groups receiving NYM stress, none of the groups showed alteration in the cytokine concentrations. On the other hand, all pro-inflammatory cytokines tested were augmented in the LCG group, which received LPS as a challenge. A previous report<sup>[46]</sup> showed an increased cytokine expression in two LPS administrations, where the first was executed during gestation and the second at a late period of the offspring development. Also, in the LPS groups, a sickness condition was observed as an elevation in the IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, and TNF- $\alpha$  concentrations in the LCG group, which did not receive the gestational exposition to EBN but were exposed to LPS at PND60. Meanwhile, the blood cytokine concentrations in the LEG group were diminished due to the administration of EBN as the first event, summed to the LPS exposition as the second event.

The two-hit hypothesis and the elevated melatonin may explain the reduction of the pro-inflammatory cytokines in the LEG group. Among various physiological functions, melatonin is also an efficacious anti-inflammatory and antioxidant substance<sup>[47,48]</sup> that can exert local and systemic activities.

Pinealocytes produce melatonin in the pineal gland<sup>[49,50]</sup> via the absence of light stimulus in the suprachiasmatic nucleus,<sup>[51]</sup> thus regulating the circadian cycle.<sup>[52]</sup> It is produced in higher concentrations at night. However, it is produced in much lower concentrations during sunrise<sup>[53]</sup> when preparing the body to waken, enabling the increase of cortisol or corticosterone physiological amounts.<sup>[54]</sup> It is an indispensable regulatory agent of the sleep cycle. It can be synthesized by tissues other than the pineal gland, such as placental tissue, bone marrow, brain, retina, lens, cochlea, Harderian gland, airway epithelium, skin, gastrointestinal tract, liver, kidney, thyroid, pancreas, thymus, spleen, immune system cells, carotid body, reproductive tract, and endothelial cells.<sup>[51]</sup> It is also involved in various physiological functions related to the neuro-immuno-endocrine system.<sup>[55]</sup>

Although melatonin regulates many brain functions, it can be clinically used in neuropsychiatric and/or sleep disorders,<sup>[56]</sup> with limitations. Previous studies have reported how the stress caused by sleep suppression during pregnancy-induced stress behavior in the offspring.<sup>[53]</sup> Disruption in the balance of melatonin can lead to depressive or anxiety-like behavior in rats.<sup>[57]</sup> Among the wide range of physiological functions, the melatonin anti-inflammatory<sup>[47,48]</sup> and immunomodulatory activities<sup>[58]</sup> are

distinguished and are expressed after organism exposition to sickness conditions leading to diseases<sup>[59]</sup> or stress.<sup>[48,60]</sup> Besides melatonin's capacity to detoxify free radicals generated during photosynthesis and metabolism<sup>[61]</sup> during organism evolution, it became a pleiotropic molecule that resists oxidation-related stress and suppressed inflammation.<sup>[62]</sup>

The wide range of melatonin activity across the body can be mediated by the melatonin receptors MT1 and MT2 in different tissues and executed without a receptor. The melatonin concentrations in each site are also critical to its physiological function and support the hormone's endocrine, paracrine, and autocrine actions.<sup>[51]</sup> There is complex crosstalk among the pineal gland, the adrenal gland, the immune system, and the hypothalamus, which is intermediated by a truncate system involving hormones (melatonin, corticosterone, ACTH), cytokines (TNF- $\alpha$ , IL-2, IL1, and IL-6, as examples), the so-called "clock" genes (PER1, PER2 or BMAL1) and their proteins, membrane receptors as the glucocorticoid receptor,  $\beta$ -adrenergic,  $\alpha$ 1-adrenergic receptors, and the presence or absence of a normal or a sickness condition.

Some authors have reported the presence of MT1 receptors in the cortex of the adrenal gland<sup>[63]</sup> and how melatonin inhibits or stimulates the cortisol response to ACTH in humans<sup>[64]</sup> or rats<sup>[65]</sup> by interfering with (rat) PER1, PER2 or BMAL1 clock genes and their proteins which are involved in the day/light cycle regulation.<sup>[65]</sup> Also, some authors describe the melatonin-corticosterone crosstalk that regulates the organism's recovery from an inflammatory condition, mediated by immunological signs,<sup>[54]</sup> particularly by IL-2.<sup>[66]</sup> The crosstalk between the immune system and melatonin synthesis is known as the immune-pineal axis, which has been described since the late 1980s<sup>[67]</sup> until more recently.<sup>[68]</sup>

The immune-pineal axis involves the endocrine melatonin synthesis in the pineal gland by a nocturne stimulus of the suprachiasmatic nucleus that stimulates the CNS production of IL-2 by T helper lymphocytes, blocking the leucocyte transmigration through the endothelial cell during a normal homeostatic condition. Under a local or systemic pathological condition, the axis automatically adjusts to an alert state due to the organism's need to preserve energy to heal and search for a cure. To establish the awake state, the pathological condition induces the production of TNF- $\alpha$  by T helper lymphocytes, which reaches the hypothalamus and inhibits the synthesis of melatonin by blocking AA-NAT, the enzyme that catalyzes serotonin into N-acetylserotonin, an intermediate in the synthesis of melatonin.

At that point, melatonin's absence facilitates immune cells' transmigration to the pathological site, whatever tissue or organ. The immune cells automatically release higher cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6,<sup>[69]</sup> which induces the inflammatory condition.<sup>[66]</sup> Also, these cells produce NF- $\kappa$ B,<sup>[70]</sup> which locally

induces the local production of melatonin, which helps to defeat the aggressor, as a potent anti-inflammatory and antioxidant agent, by diminishing inflammatory signs. The immune-pineal axis and its relationship to the HPA axis support the comprehension of disturbances in hormones or receptors other than those immediately related to melatonin synthesis, which can contribute to the final expression of a physiological condition.

Present findings describe that EBN administration to FF1 induces anxiety-like behavior, improved serum amounts of corticosterone, ACTH, and melatonin, and decreased pro-inflammatory cytokines. Compared to a previous report,<sup>[13]</sup> sex-experienced female rats that received EBN also showed an induction of anxiety-like behavior and diminished pro-inflammatory cytokines. The female rats can be resilient to the EBN effects, a condition yet to be further assessed.

Present findings report the elevated amount of melatonin in the SEG and LEG groups. The LEG group showed diminished cytokine concentrations, which means that the inflammatory process was impaired due to systemic melatonin and the sensitization caused by the gestational exposition to EBN, as the LCG group showed an elevated amount of pro-inflammatory cytokines. The gestational administration of EBN has eased the systemic anti-inflammatory performance of melatonin in a way yet to be tracked. Besides the potential pharmacological use of some chemicals produced by the plant, it is essential to state that the aqueous extracts made with the fruits of *L. operculata* are abortive to women<sup>[5]</sup> by inducing hemorrhage. Also, it disrupts behavior in young and adult male and female rats<sup>[12-14]</sup> and degenerates seminiferous tubules in sex-experienced adult male rats.<sup>[12]</sup>

## CONCLUSION

The gestational exposition of EBN led the FF1 young female rats to reveal fear and anxiety-like behavior after being submitted to a stress challenge or LPS exposition at a young adult age and were accompanied by an increase of serum corticosterone and ACTH. An increase in the melatonin serum levels was also observed and implicated in diminishing the pro-inflammatory cytokines.

## ACKNOWLEDGEMENT

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP, São Paulo, SP, Brazil [grants # 2017/03470-9 and 2019/12202-3] and CNPq Productivity Scholarship #304699/2018-7 awarded to IBS; and by Universidade Paulista - UNIP [Scholarships awarded to CSA, HVE, and MSCA].

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**ACTH:** Adrenocorticotrophic hormone; **ALT:** Alanine aminotransaminase; **AST:** Spartate aminotransaminase; **CG:** Control group; **CNS:** Central nervous system; **EBN:** *Buchinha-do-norte* dried fruit aqueous extract; **EG:** Experimental group; **F0:** Parental rats; **FF1:** Female rats of the F1 generation; **GD:** Gestation day; **HPAA:** Hypothalamus-pituitary-adrenal axis; **IL:** Interleukin; **LCG:** LPS control group; **LDB:** Light-dark box; **LEG:** LPS experimental group; **LPS:** Lipopolysaccharide; **NYM:** New York Metro stress; **OF:** Open field; **PND:** Post-natal day; **SCG:** Stress control group; **SEG:** Stress experimental group; **TNF- $\alpha$ :** Tumor necrosis factor alpha.

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**Cite this article:** Alves CS, Frias HV, Bonamin LV, Bondan EF, Coelho CP, Martins MFM, et al. *Luffa operculata* (L.) Cogn. Gestational Exposition Induces Anxiety-like Behavior and Interferes with Melatonin and Inflammation in Young Female Rats. *Pharmacog Res.* 2023;15(3):524-36.