Research Article

Transgenerational effects of maternal circadian melatonin deficiency and melatonin replacement in rats during pregnancy and lactation on the energy metabolism and thermoregulation in the offspring subjected to a high-fat diet

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ABSTRACT

Pineal melatonin participates in the control of numerous biological functions through its immediate effects, which result from its high presence in the systemic circulation during the dark phase of the circadian cycle or, through its prolonged effects, when its level is extremely low during the light phase. At pregnancy, maternal melatonin signals the external photoperiod to the fetus, highlighting its importance not only in synchronizing rhythms, but also in preparing the fetus to adapt the external environments. The fetus and newborns are exclusively dependent on maternal melatonin since their pineal melatonin production only occurs weeks after birth. Thus, maternal hypomelatoninemia stands out as an important factor capable of modulating the physiological systems of their descendants, demonstrating its transgenerational capacity. The present study evaluated the transgenerational influence of maternal melatonin deficiency and replacement during pregnancy on morphometric parameters, thermoregulation and energy metabolism of the offspring submitted to the normal and high-fat diets, respectively. For this, nulliparous Wistar rats at an age of 8 weeks were used and randomized into three groups: CTL (pregnant rats), PINX (pinealectomized pregnant rats), PINX + MEL (pinealectomized pregnant rats submitted to melatonin replacement). After birth, the pups were divided into three groups: (C) pups from control mothers, (P) pups from PINX mothers and (PM) pups from PINX + MEL mothers. One week after weaning, part of the animals was fed a high-fat diet (DH) and rest of them were fed a normal diet (ND) for 12 weeks. Subsequently, the animals were euthanized at ZTs 6 and 18. The results showed that maternal melatonin deficiency disrupted the energy metabolism of the offspring and melatonin replacement normalized the energy metabolism in the offspring submitted to the high-fat diet, enabling them to make functional adaptations such as reduced food consumption and greater thermoregulatory capacity, resulting in reduction in body weight gain white adipose tissue mass.

Key words: High fat diet, melatonin, pregnancy, lactation, energy metabolism, brown adipose tissue.

1. INTRODUCTION

Melatonin is an indolamine produced by almost all species and, therefore, ubiquitously present in nature (1-4). In vertebrates, when produced by the pineal gland, melatonin is able to control numerous biological functions (4) through its immediate effects resulting from its high presence in the systemic circulation during the dark phase of the circadian cycle or through its proximal or distal effects, when its level is extremely low during the light phase (5). Melatonin is also synthesized in different tissues with autocrine and/or paracrine action and, therefore, function locally (functions in respiratory, renal, digestive, cutaneous, retina, placenta systems) (4, 6-10).

In pregnancy, maternal pineal melatonin signals the external photoperiod to the fetus, clearly demonstrating its importance, not only in synchronizing the rhythms of the fetus but also in preparing the fetus to adapt the external environment, represented by day and night as well as seasonal alterations (4, 11-13).

In humans, the same maternal melatonin rhythm is present in the fetus (12, 14, 15). The chronoruption during pregnancy in women with shift-work is associated with a greater risk of miscarriages, premature births, and low birth weight (16-19). In animal study, the results showed that maternal constant light exposure interfered the expression of clock genes in the fetus (20). On the other hand, such events were reversed by the daily melatonin replacement in the maters (12, 21). Thus, it is noted that the fetus and newborns are dependent on maternal melatonin since their pineal melatonin synthesis only occurs weeks after birth(14, 15, 22-25).

During intrauterine development, environmental factors can induce critical changes in the development of the embryos. Therefore, in the prenatal period, the classic insults including alcohol intake, drugs, nutrition, and physical exercises can influence the programming and maturation of physiological systems of fetus (26-30). Maternal hypomelatoninemia has been shown as an important factor capable of programming and modulating the development of the offspring (12, 20, 31-34).

Furthermore, chronoruption in the pregnant females, whether induced by exposure to the constant light or through pinealectomy, can cause changes not only related to the concentration of circulating melatonin, but also in gestational glycemic levels, as well as influencing hormonal aspects (for example corticosteroids), factors of extreme relevance for fetal programming (20, 30).

Considering glycemic homeostasis, scientific evidence demonstrates the positive effects of melatonin administration. In addition to being important in controlling glucose tolerance, insulin sensitivity and modulating adipose mass, it appears to synchronize hepatic gluconeogenesis. These effects, on the contrary, are disturbed after pinealectomy (35). Therefore, the absence of melatonin in animals correlates with diabetogenic characteristics manifested by the glucose intolerance and insulin resistance (36, 37) regarding the cellular influx of glucose through its transport protein GLUT4 (38). On the other hand, these metabolic changes associated with the reduction in plasma melatonin levels can be improved with its replacement (5, 10). Evidence shows that melatonin, when interacting with its receptors, is capable of tyrosine phosphorylating the insulin receptor and triggering subsequent intracellular phosphorylation associated with the biological effects of insulin(36, 39).

In addition to the glucose metabolism, adipose tissue can also be subjected to transgenerational changes, leading to the development of numerous chronic diseases (insulin

resistance, diabetes mellitus, high blood pressure and cardiovascular diseases) These alterations can occur in both white or brown adipose tissue. With the discovery of leptin in 1994, white adipose tissue began to be recognized not only as the main energy reservoir, but also as an important endocrine organ (40-43). On the other hand, brown adipose tissue, despite not having a function directly related to energy reserves, has a role in maintaining energy expenditure, with its main action being the regulation of energy expenditure through the control of body temperature, attributing to the presence of uncoupling protein 1 (UCP1), expressed in the internal membranes of the mitochondria of these adipocytes (44-46).

Thus, the present study evaluated the transgenerational influence of the deficiency of maternal melatonin as well as its replacement on the feeding behavior and morphometric parameters as well as on thermoregulation and energy metabolism of the offspring.

2. MATERIALS AND METHODS

2.1. Chemicals.

Xylazine and ketamine were purchased from Syntec (Santana de Parnaíba, SP, Brazil). Melatonin and primers were purchased from Sigma-Aldrich (St. Louis, MO, USA). Regular insulin was purchased from Lilly (Indianapolis, Indiana, USA). TRIzo, RNase-free water, Turbo DNA-free, ultra-pure water, and SYBR green were purchased from Invitrogen (Waltham, Massachusetts, USA). Improm-II reverse transcriptase and random primers were purchased from Promega Biotecnologia (São Paulo, Brasil).

2.2. Animals.

Nulliparous Wistar rats at 8 weeks old were housed on a 12L:12D light-dark cycle, in a temperature-controlled room (24 ± 2°C). The animals were allowed to access to Standard chow (Nuvilab®, Laborpec Rio Comercial Ltda, Vigário Geral, RJ, Brazil) and water *ad libitum*. After a period of adaptation to the animal facility, the animals were randomized into three experimental groups: CTL (pregnant rats), PINX (pinealectomized pregnant rats), PINX+MEL (pinealectomized pregnant rats submitted to melatonin replacement). Ethics approval was granted by the Committee of Ethics in Animal Experimentation of the Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil (#86/2016/CEUA).

2.3. Maternal pinealectomy.

Before pregnancy, animals belonging to the PINX and PINX-MEL groups were anesthetized with 15 mg/kg of xylazine and 30 mg/kg of ketamine via intraperitoneal injection and subjected to the surgery (47). In brief, anesthetized animal was placed in a stereotaxic apparatus for small animals and a sagittal opening was made in the scalp. The skin and muscles were pushed aside to expose the lambda suture. By means of a circular drill, a disc-shaped bone was drilled over the lambda and gently removed. Thereafter, the pineal gland was extracted. After a brief hemostasis, the skull was closed by returning the disc-shaped bone back and the scalp was sutured with cotton threads(47).

2.4. Melatonin replacement in pinealectomized pregnant and lactating rats.

The PINX-MEL group received 1 mg of melatonin/kg of body weight in drinking water during the dark phase of the light/dark cycle; the CTL and PINX groups received melatonin-free water. The melatonin replacement was started immediately after pinealectomy and

maintained until offspring's weaning. Melatonin solution was daily prepared based on daily nocturnal water intake and the body weight of each rat. The final concentration of melatonin was made available to the animals during the 12 hours of the dark phase of the light-dark lighting cycle. Before the beginning of the period of 12 hours of light, the bottles were replaced by others with melatonin-free water(48, 49).

2.5. Study design and offspring treatment.

Four weeks after pinealectomy, female rats underwent mating with the normal males. After 7 days, the males were removed, and the pregnancy was monitored daily until the offspring were born. Subsequently, the offspring were weaned 21 days after birth. After weaning, only male offspring were used in the study since the objective of this study did not include investigating gender differences.

The offspring were divided into three groups: (C) offspring of control mathers, (P) offspring of PINX mother and (PM) offspring of PINX + MEL mothers.

One week after weaning, some of the animals were fed a high-fat diet (HFD), a food rich in saturated fat (pork fat) with a contribution of 52% of total calories (PragSoluções Biociências, Jaú, SP, Brazil) and the rest of the animals were fed a normal diet (ND), respectively for 12 weeks. HFD is used to induce metabolic disorders (50-52) and enable the investigation of the potential effects of maternal melatonin deficiency and replacement on the offspring under food challenge. During the study period, the animals were weighed weekly. At the end of the protocol, the animals were euthanized at time of ZT18 (6 hours after the start of the dark phase) and ZT6 (6 hours after the start of the light phase)(53).

2.6. Measurements of offspring's glycemic parameters.

The insulin tolerance test (ITT) was performed at ZT10 (two hours before the dark period), one week before the termination of the study. To this end, blood was collected after a cut in the animal's caudal extremity and blood glucose was measured at time zero (before intraperitoneal injection) and at times 5, 10, 15 and 20 minutes after intraperitoneal injection of regular dose of insulin (2 IU/ Kg) (INS HUMULIN R U-100 2X3ML®).

Blood glucose measurement was performed using the Optium Xceed® glucometer and the corresponding reagent strips (Optium Blood Glucose Test Strips, Medisense®, Abbott laboratories, Chicago, Illinois, EUA). The analysis of the glucose decay rate (kITT) was used to estimate insulin tolerance, calculated by the Neperian logarithm [Y=Ln(Y)] of the data and, subsequently, linear regression of the values was performed (37).

2.7. Evaluation of offspring's thermography.

The temperature/thermoregulation evaluation was carried out using type K thermocouples coupled to a four-channel datalogger model RDXL4SD Omega®, relative humidity and temperature datalogger model RHT10 Extech®, in addition to the use of a thermographic camera with infrared display (Flir System® - SC640) (54, 55). Thermocouples and the humidity and temperature datalogger were only used to observe the stability of temperature and humidity in the two experimental rooms and thus improve the thermographic images obtained. These two rooms were maintained at different temperatures with room temperature (24 ºC) and cold (18 ºC), respectively. Prior to the test, the animals were placed in individual cages to control motor activity and under food deprivation. Subsequently, two image captures were taken: 1) 30 minutes after exposure and 2) image recapture after two hours of exposure. This repetition was necessary to evaluate the acclimatization time of the animals and evaluate

the effect of thermal stress. The regions analyzed were: 1) interscapular region (deposit of brown adipose tissue), 2) tail (thermal window to control body temperature) and 3) inguinal region (deposit of beige adipocytes).

The surface temperature was obtained through the analysis of thermographs using the Thermacam 2.9 program (Flir Systems) and Python routines for analyzing regions of interest with an emissivity of 0.98. Statistical analysis was based on the difference between the temperature of the tissue regions (brown and beige) and the ambient temperature, as well as the average temperature of the tail (56).

2.8. The real-time PCR analysis.

Interscapular brown adipose was collected for mRNA analysis. Total RNA was extracted using TRIzol reagent according to the manufacturer's specifications. Extracted RNA was eluted in RNase-free water, treated with Turbo DNA-free, and quantified by spectrophotometry. For mRNA expression analysis, 1 µg of total RNA was reverse transcribed using Improm-II reverse transcriptase and random primers, according to the manufacturer's instructions. Real time PCR was performed (QuantStudio 6 Flex®, Applied Biosystems, USA) in duplicate reactions containing 50 ng cDNA, ultra-pure water, SYBR green mix and specific intron-spanning primers. The cycling parameters were: 95°C for 10 min, followed by 50 cycles of 95 °C for 15s, annealing at 60°C for 30s and extension at 72°C for 30s. Relative gene expression was calculated by the $2^{-\Delta Ct(57)}$ using a housekeeping (*RPL37A*).

2.9. Analysis of mitochondrial enzyme complexes.

Interscapular brown adipose tissue was homogenized in 300 µL phosphate buffer 0.1M pH 7.5 and centrifuged 600 x g 10 min, 4ºC. The supernatant protein concentration was quantified with the Bradford method(58). The enzyme activities of mitochondrial citrate synthase and respiratory chain complexes I, II and IV were determined as described (59) with minor modifications. Assays were performed in 96-well plates in a final reaction volume of 200 µL. Citrate synthase activity was determined by the incubation of 2.5 µg of tissue protein in phosphate buffer (100 mM, pH 7.5) containing 125 µM acetyl-CoA, 100 µM 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), 0.1% (v/v) Triton X-100 and 250 µM oxaloacetate. TNB formation was monitored for 5 min. at 412 nm at 37 °C in a plate reader (FlexStation 3, Molecular Devices).

Complex I activity was determined by incubation of 20 µg of tissue protein in phosphate buffer (50 mM, pH 7.5) containing 3 mg/mL fatty acid-free BSA, 300 µM KCN, 100 µM NADH and 150 µM ubiquinone in the presence or absence of 10 µM rotenone. The oxidation of NADH by complex I was measured by following the decrease of absorbance at 340 nm for 2 min. and complex I-specific activity was calculated by subtracting the NADH oxidation rates obtained in the presence of rotenone from those obtained in the absence of rotenone.

Complex II activity was determined by incubation of 4 µg of tissue protein in phosphate buffer (25 mM, pH 7.5) containing 1 mg/mL fatty acid-free BSA, 300 µM KCN, 20 µM succinate, 0.002175 % (w/v) DCPIP and 50 µM decyl ubiquinone in the presence or absence of 10 mM malonate. The oxidation of succinate by complex II was determined by following the decrease of absorbance at 600 nm for 3min. Complex II-specific activity was calculated by subtracting the values obtained in the presence of malonate from those obtained in the absence of malonate.

Complex IV activity was determined by incubation of 20 µg of tissue protein in phosphate buffer (50 mM, pH 7.5) containing 1 g/mL fatty acid-free BSA and 60 µM reduced cytochrome *c* in the presence or absence of 500 µM KCN. The oxidation of cytochrome *c* by complex IV

was determined by following the decrease of absorbance at 550 nm for 5 min., and complex IV-specific activity was calculated subtracting the values obtained in the presence of KCN from those obtained in the absence of KCN.

2.10. Statistical analysis.

Data were assessed for normality and homogeneity of variance to determine whether to use parametric or non-parametric statistical tests. Data were analyzed using either Kruskal-Wally's test followed by Dunn's multiple comparisons test or Two-way ANOVA followed by Tukey's test, when appropriated. The data were expressed as mean ± SEM, n = 5-20. The acceptable level of significance was 95% ($p \leq 0.05$). Statistical tests were performed using Prism version 8.4.0 (GraphPad, San Diego, CA, USA).

3. RESULTS

3.1. Effects of maternal melatonin deficiency and melatonin replacement on the newborn body weight.

Some morphometric characteristics of the offspring are shown in Figure 1. From the four days of birth (Figure 1a) to the 21st day of lactation (Figure 1b), P animals had lower body weight than those of C and PM animals. Under the HFD challenge, P animals had highest body weight among the other groups and, conversely, PM animals had lowest body weight among the groups (Figure 1c).

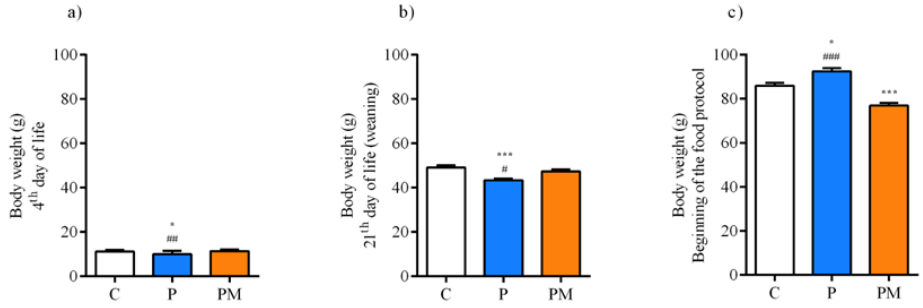

Fig. 1. Effects of maternal melatonin deficiency and melatonin replacement on the neonatal morphometric parameters.

a). Body weight on the fourth day of birth. b). Body weight at weaning (21 days of life). c). Body weight under the HFD challenge. C: control, P: pinealectomy, PM: pinealectomy plus melatonin replacement. Kruskal-Wally's test. n=20 rats/group. $^{*}p < 0.05$ *vs C;* $^{***}p < 0.001$ *vs C;* $^{\#}p < 0.05$ *vs PM;* $^{\#\#}p < 0.01$ *vs PM;* $^{\#\#\#}p < 0.001$ *vs PM.*

3.2. Effects of maternal melatonin deficiency and melatonin replacement during pregnancy and lactation on the food consumption, high fat diet associated body weight gain and fat accumulation in offspring.

During the experimental period, PM offspring showed lower food intake on both normal and high fat diet feedings (Figure 2a and 2b). This eating behavior had an impact on body weight gain (Figure. 2c and 2d), and the difference between the PM group in relation to the other groups was more evident in the high-fat dietary treatment (Figure 2d).

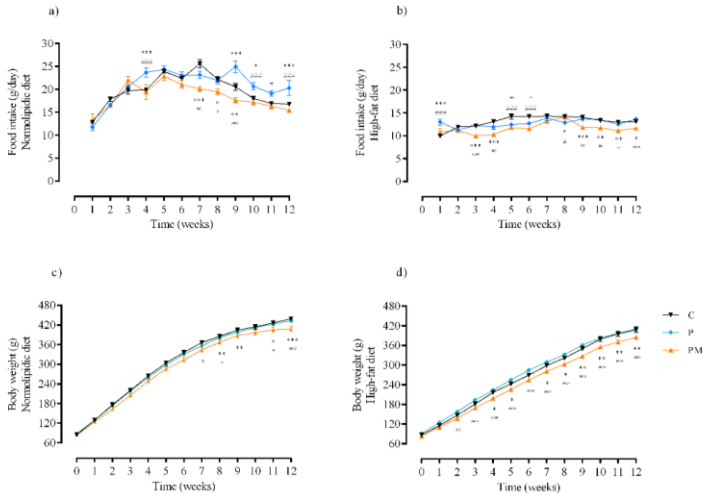

Fig. 2. Effects of maternal melatonin deficiency and melatonin replacement during pregnancy and lactation on food consumption and HFD associated body weight gain.

a). Normal diet consumption. b). High-fat diet consumption. c). Body weight gain under normal diet. c). Body weight gain under high-fat diet. C: control, P: pinealectomy, PM: pinealectomy plus melatonin replacement.Results are expressed as mean ± SEM (n= 15-20 rats/group). Two-way ANOVA followed by Tukey's test. $^{*}p < 0.05$ *vs C;* $^{**}p < 0.01$ *vs C;* $^{***}p < 0.001$ *vs C;* $^{o}p < 0.05$ *vs P;* $^{oo}p < 0.01$ *vs P;* $^{ooo}p < 0.001$ *vs P;* $^{\#}p < 0.05$ *vs PM;* $^{\#\#}p < 0.01$ *vs PM;* $^{\#\#\#}p < 0.001$ *vs PM.*

The maternal pinealectomy and high-fat diet consumption had a significant impact on adipose tissue content of the offspring. The high-fat diet consumption caused a significant increase of the adipose volume compared to the normal diet fed offspring in all groups (Figure. 3a, b). The results also showed that in the perigonadal as well as retroperitoneal regions, the P group with HFD had a significant great fat deposit while the offspring of PM group with HFD showed a significantly reduced perigonadal and retroperitoneal fat deposit compared to other groups (Figure 3a, b). Considering the interscapular brown adipose tissue the P-HFD group showed significant reduction compared to other groups under both normal diet or HFD conditions (Figure 3c).

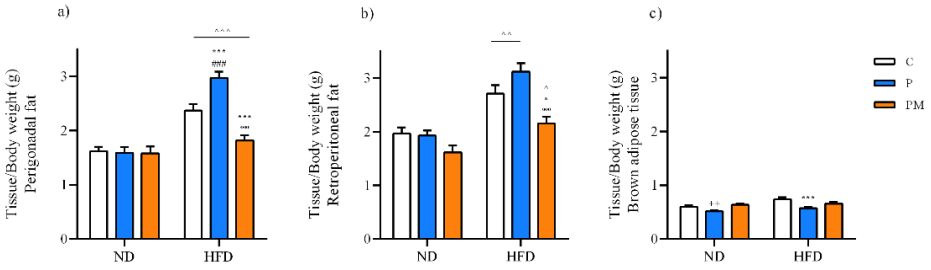

Fig. 3. Effects of maternal melatonin deficiency and melatonin replacement during pregnancy and lactation on fat accumulation of offspring under different dietary protocols.

a). Perigonadal adipose tissue content relative to body weight. b). Retroperitoneal adipose tissue content relative to body weight and c). Interscapular brown adipose tissue content relative to body weight. C: control, P: pinealectomy, PM: pinealectomy plus melatonin replacement, ND: normal diet, HFD: high fat diet. Results are expressed as mean ± SEM, n= 20 rats/group. Two-way ANOVA followed by Tukey's test. $^{*}p < 0.05$ *vs C;* $^{***}p < 0.001$ *vs C;* $^{ooo}p < 0.001$ *vs P;* $^{\#\#\#}p < 0.001$ *vs PM;* $^{\wedge}p < 0.05$ *vs ND group;* $^{\wedge\wedge}p < 0.01$ *vs ND group;* $^{\wedge\wedge\wedge}p < 0.001$ *vs ND group; ++ p < 0.01 vs HFD group.*

3.3. Effects of maternal melatonin deficiency and melatonin replacement during pregnancy and lactation on glycemia and insulin sensitivity in the offspring.

After 12 weeks of the dietary protocol, the groups fed with HFD showed significantly high glycemia compared to those fed with a normal diet (Figure 4a). The P offspring showed higher glycemia in both dietary conditions than those of other groups (Figure 4a). On the other hand, when analyzing insulin sensitivity, the PM-ND and PM-HFD groups presented a better index when compared to the other groups under the same dietary conditions (Figure 4b).

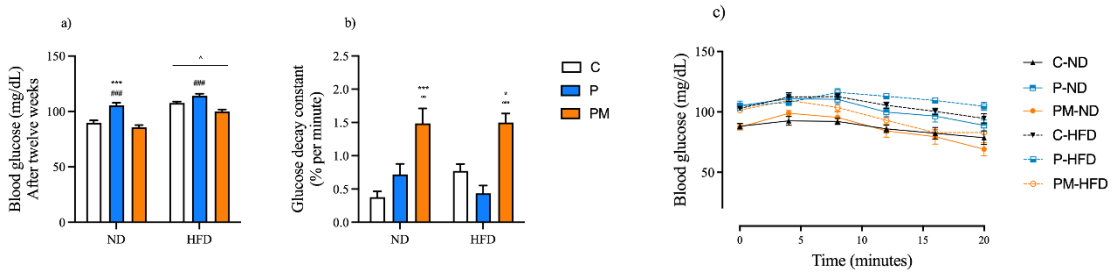

Fig. 4. Effects of maternal melatonin deficiency and melatonin replacement during pregnancy and lactation on glycemic profile of offspring.

*a). Blood glucose (n=15 rats/group). b). Glucose decay constant. c). Blood glucose levels. C: control, P: pinealectomy, PM: pinealectomy plus melatonin replacement, ND: normal diet, HFD: high fat diet. Results are expressed as mean ± SEM, n=9 rats/group. Two-way ANOVA followed by Tukey's test. * $p < 0.05$ vs C; *** $p < 0.001$ vs C; ^{oo} $p < 0.01$ vs P; ^{ooo} $p < 0.001$ vs P; ### $p < 0.001$ vs PM; ^ $p < 0.05$ vs ND group.*

3.4. Effects of maternal melatonin deficiency and melatonin replacement during pregnancy and lactation as well as diet challenge on brown and beige adipose tissue distributions and thermogenesis in offspring.

Figure 5 showed thermoregulation by the interscapular brown, inguinal beige fats and caudal tissues in animals subjected to room temperature (24°C). The PM-HFD group had a significantly high temperature in the interscapular tissue compared to the same group on a normal diet (PM-ND) and compared to the other groups under the same dietary conditions (Figure 5a). Under a normal or high fat diet, pups from pinealectomized mothers had a higher temperature in the inguinal region compared to pups from C and PM mothers kept under the same feeding conditions (Figure 5b). In addition to heat production, heat loss through the tail is important for maintaining thermoregulation. As observed, the P offspring were able to increase tail heat loss, regardless of the type of diet consumed (Figure 5c). In sub-neutrality, P-HFD offspring showed lower functional capacity of the interscapular brown adipose tissue, as well as lower thermoregulatory capacity through the tail than other groups.

Figure 6 describes the characterization of thermoregulation by the interscapular brown, inguinal beige fats and tail tissues in animals subjected to sub neutrality. Although the interscapular brown adipose tissue in C and PM groups responded to the temperature and HFD challenge; however, P group did not show this response, and the HFD induced a lower temperature than those in the C-HFD and PM-HFD groups (Figure 6a). P Group on HFD lost more heat through the tail than the other groups, indicating impaired thermoregulation in this group (Figure 6c). Interestingly neither group was recruited the fat in the inguinal region (Figure 6b).

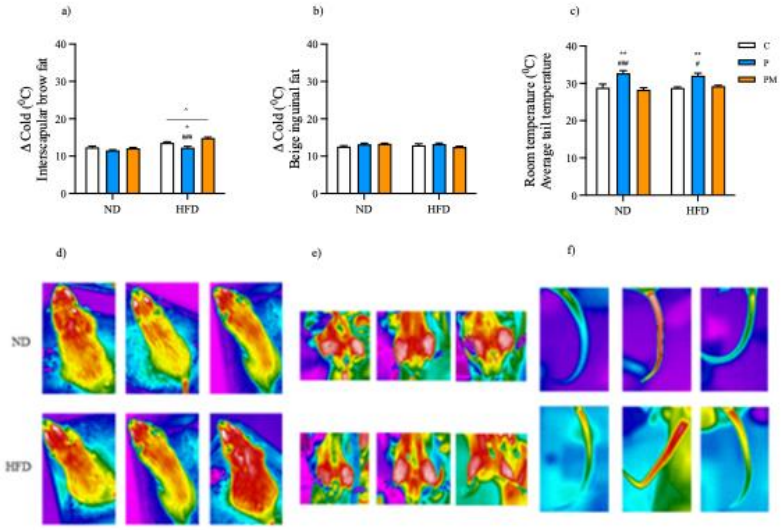

Fig. 5. Effects of maternal melatonin deficiency and melatonin replacement during pregnancy and lactation on thermography of interscapular brown, inguinal beige fats and tail adipose tissue in thermoneutrality after 12 weeks of the dietary protocol.

*a) and d). The temperature response of interscapular brown adipose tissue on different diets, b) and e). The temperature response of the inguinal beige adipose tissue. c) and f). Average tail temperature on different diet. C: control, P: pinealectomy, PM: pinealectomy plus melatonin replacement, ND: normal diet, HFD: high fat diet. Results are expressed as mean ± SEM. Two-way ANOVA followed by Tukey's test. n=6 rats/groups. * p < 0.05 vs C; ** p < 0.01 vs C; *** p < 0.001 vs C; °° p < 0.01 vs P; # p < 0.05 vs PM; ### p < 0.001 vs PM; ^^^ p < 0.001 vs ND group.*

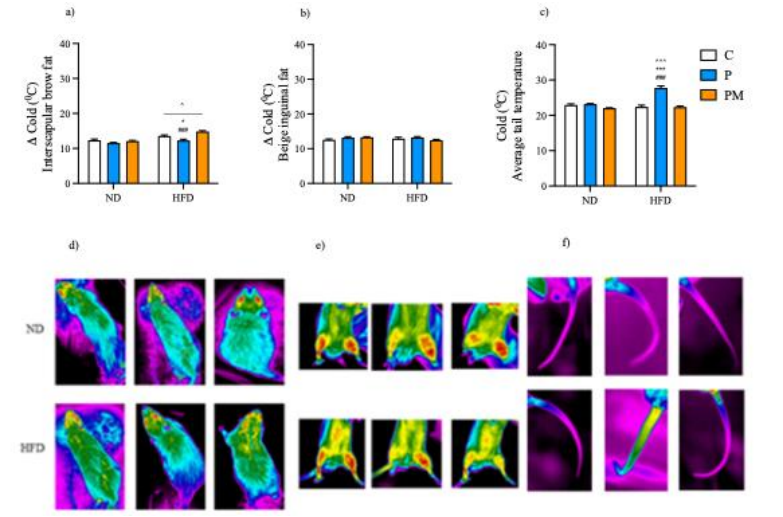

Fig. 6. Effects of maternal melatonin absence and melatonin replacement during pregnancy and lactation on thermography of brown interscapular, beige inguinal and tail adipose tissue in sub-thermoneutrality, after 12 weeks of the dietary protocol.

*a) and d) The temperature response of interscapular brown adipose tissue on different diets, b) and e) The temperature response of the inguinal beige adipose tissue, c) and f) Average tail temperature on different diet. C: control, P: pinealectomy, PM: pinealectomy plus melatonin replacement, ND: normal diet, HFD: high fat diet. Results are expressed as mean ± SEM. Two-way ANOVA followed by Tukey's test. a) n=6 rats/group. * p < 0.05 vs C; *** p < 0.001 vs C; ### p < 0.001 vs PM; ^ p < 0.05 vs ND group; ^^^ p < 0.001 vs ND group.*

3.5. Effects of zeitgeber time on activity of complex IV and Ucp1 expression under different diet challenges.

Due to the reduced response of brown adipose tissue observed above, it was important to investigate whether this would be associated with a lower functional performance of the tissue. Although the results showed no significant alterations in mitochondrial complexes I and II among groups (Figure 7a and b), zeitgeber time influenced activity of mitochondrial complex IV in all experimental groups, independent of dietary treatment (both in ND and HFD groups), The complex IV activity was higher at ZT 18 than other time (Figure 7c). In addition, at ZT6, the HFD was able to induce an increase in the expression of the Ucp1 mRNA expression in the P group; however, its expression in the PM-HFD group was lower than that in other HFD groups at ZT18 (Figure 7d).

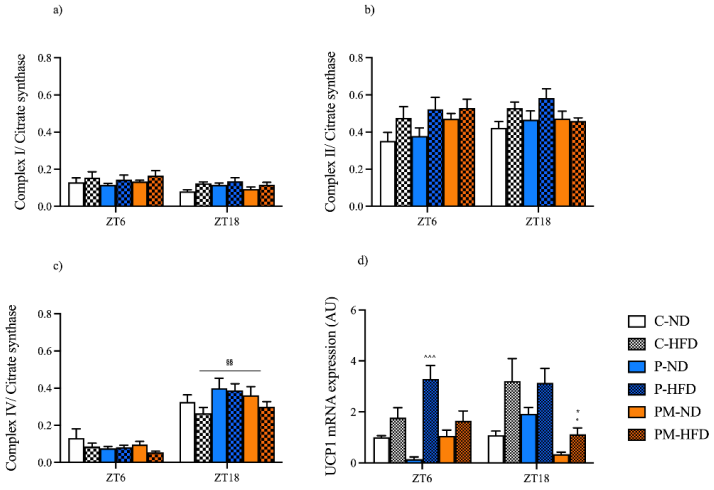

Fig. 7. Effects of zeitgeber time on activity of complex IV and Ucp1 expression after 12 weeks of the dietary protocol.

*a). Activity of complex I between ZTs 6 and 18 on different diets, b). Activity of complex II between ZTs 6 and 18 on different diets. c). Activity of complex IV between ZTs 6 and 18 on different diets. d). Ucp1 expression between ZTs 6 and 18 on different diets. C: control, P: pinealectomy, PM: pinealectomy plus melatonin replacement, ND: normal diet, HFD: high fat diet. Results are expressed as mean ± SEM. Two-way ANOVA followed by Tukey's test. n=5-7 rats/group. p < 0.05, §§ ZT6 vs ZT18, ^^^ZT6: P-HFD vs ND groups and *ZT18: PM-HFD vs C-HFD and P-HFD.*

4. DISCUSSION

Studies have suggested the potential correlation between maternal health and characteristics of offspring throughout their development (29, 30). The impact of the deficiency of maternal melatonin on the development of the offspring has been increasingly explored. The blood melatonin level starts to increase in the first weeks of pregnancy, reaching its highest values between 32 and 36 weeks. This elevated melatonin level is essential for maintaining a healthy pregnancy and fetal development (49). However, industrialization and night shifted work for pregnant women have had a negative impact on the gestational period as well as on the secretion of melatonin to maintain healthy pregnancy. The exposure to light at night significantly reduces endogenous melatonin production. It has estimated that one in five workers perform night shift work and this condition promotes health threat to them including metabolic disorders resulting from inefficient body weight control, intestinal malfunction, and

imbalance of the metabolic rate (31, 32). The effects of melatonin on energy homeostasis have already been evidenced by its action in increasing energy expenditure, reducing food consumption and improving leptin signaling (4, 5, 10, 60). Melatonin is one of the few substances capable of crossing the placental barrier with ease. Maternal melatonin transduces a temporal circadian and seasonal signal to the fetus to prime their central nervous system properly to adapt the environmental day/night fluctuation after birth (4, 11, 13, 23, 25). It is confirmed that the breast milk (15, 61-63) plays an essential role for neuroprotection and neurodevelopment of the neonates (34, 64). Our study showed that maternal melatonin alterations due to pinealectomy (24) or its replacement during pregnancy and lactation was able to transgenerationally modulate energy metabolism (65). Based on the results, we specifically addressed the following issues, i.e., the effects of the maternal melatonin deficiency caused by pinealectomy and maternal melatonin replacement on the metabolic parameters of offspring under the normal or high fat diet (HFD) challenge. Considering the transgenerational effects of melatonin, it is important to emphasize that, although the influence of pinealectomy or melatonin replacement was not directly to act on the offspring, the impact of the maternal condition may sufficiently trigger biological response in the offspring. Both pregnancy and lactation are considered the periods of susceptibility to influence the characteristics of offspring from their childhood until to aging. This issue has been widely discussed as the concepts of fetal and neonatal development and programming and epigenetic influences on the origin of health and disease (DOHaD) based on the importance of environmental insults in periods of fetus susceptibility and its impact on the development of diseases, especially non-communicable ones (29). An example would be gestational diabetes *mellitus*, which is considered a relevant metabolic problem, influencing not only maternal health, but also the health of offspring in both the short, medium, and long term (66, 67).

In the experimental design of this work, it is proposed that, in addition to the known direct effects of melatonin on fetal development and programming(4), it becomes relevant to reflect on a possible influence of pinealectomy on the development of insulin resistance in the mater (5, 37) which is similar to type II diabetes *mellitus* and which possibly influenced the metabolic features of the offspring. We observed that in the neonatal period of the male offspring of pinealectomy mothers had the less weight gain compared to others but this was just a transitory characteristic since in the food introduction phase this phenomenon was reversed and had no difference with the C offspring over time. However, the HFD became an important challenge for the metabolic development of the offspring of pinealectomy mother. They showed a significant increase in white adipose volume under the HFD challenge, even without the altered eating behavior. These findings are in line with evidence about the importance of melatonin in controlling body weight through mechanisms not associated with food consumption (68). Even though specific biochemical signals related to eating behavior were not measured, some changes in the food consumption of PM offspring were identified. Evidence has emerged that melatonin inhibits adipogenesis (69) and its visceral storage (70) in addition to its anti-inflammatory effect in adipogenesis (10). We previously demonstrated that hyperglycemia significantly reduced the pineal melatonin synthesis and secretion (71). In the current study, we found that the melatonin deficiency mother with pinealectomy promoted the hyperglycemia of their offspring. All of these results suggest the transgenerational influence of melatonin on energy metabolism. Regarding glucose metabolism, the offspring of pinealectomy mother exhibited significantly high blood glucose level compared to the controls. In contrast, the offspring of mother with melatonin replacement, no matter under normal or high fat diet, the PM-ND and the PM-HFD groups showed great sensitivity to insulin indicated by the rapid glucose decay constant compared to other groups. Considering the role of melatonin on carbohydrate metabolism and glycemic homeostasis, the described features installed in the offspring of pinealectomy mother may aggravatingly cause the glucose metabolic imbalances

and forms a vicious cycle from the beginning of HFD consumption. In a comparative analysis between anthropometric, insulin sensitivity and lipid deposition data (72), it concluded that the white adipose tissue helps to clean approximately10% of plasma glucose. The greater the fat mass, the more the glucose uptake will be and it can uptake up to 40% of plasma glucose (73). However, if insulin resistance occurs in this tissue, the glucose uptake function of the adipose tissue will be compromised and even exacerbates the hyperglycemic state and it will also impair the melatonin production, starting a new vicious cycle of environmental challenges.

In relation to brown adipose tissue, P-ND and P-HFD animals had lower content relative to body weight than other groups. However, this alteration is not sufficient to infer the functional performance of the tissue. Therefore, the thermography and molecular analysis data were collected to further explore this phenomenon. It is well known about the action of melatonin on thermogenesis of brown adipose tissue (68, 74-78). For example, pregnant sheep were exposed to constant light to reduce their melatonin production, and the thermogenesis of their offspring was analyzed under exposure to both thermoneutrality (24ºC) and the sub neutral environment (4ºC). Despite the increased expression of the activity markers of brown adipose tissue (Ucp1, Pparg, Ppargc1a), the function of this tissue was less active than the offspring of normal light cycle exposed mothers (79). Another recent study evaluated the influence of the HFD fed mice during their lactation period on the thermogenesis of their offspring (80). The authors showed a reduction in thermogenesis by brown adipose tissue, and this was associated with changes in the composition of breast milk and to the development of adiposity and metabolic syndrome in puppies.

In the current study, the thermographic analyzes were carried out in a thermoneutral and cold environment. At 24 ºC, respectively, both the P-ND and P-HFD groups did not activate the interscapular brown adipose tissue but showed greater recruitment of beige adipose tissue from the inguinal region and greater heat loss through the tail compared to other groups. It is possible that these animals reached the maximum capacity of their brown adipose tissue activation already; therefore, they could only recruit, for example, the inguinal beige adipose tissue, where the browning phenomenon occurs (81, 82). This adipose tissue browning phenomenon has been observed in both animals and humans (83), originating beige cells (84) with multilocular morphology, rich in mitochondria and that express Ucp1 and other proteins related to thermogenesis (85) and melatonin also promoted this process (86). There is evidence that both brown adipose tissue and beige cells can assist in the non-shivering thermogenesis in mammals. (87-94). At the molecular level, the higher mRNA expression of Ucp1 in the P-HFD group did not correlate with the functional activity of thermoregulation in the cold condition. Furthermore, these animals lost more heat through the tail and the thermogenesis activated via beige adipocytes did not appear efficient to compensate the heat loss, demonstrating a significant functional change in the thermoregulation system of these offspring. These findings indicate that maternal pinealectomy is an important factor to impact the thermoregulation of their offspring, as already suggested in other studies (14, 15, 22).

Our data also showed that the offspring of the pinealectomy mothers receiving daily melatonin replacement and submitted to a high-fat diet (PM-HFD) had a protective and anticipatory effect on weight gain control after the 2nd week of feeding protocol compared to PM-ND puppies, whose significant body weight reduction was observed only after the 7th week. The feeding behavior of PM-DH puppies corroborated this characterization, since this group showed lower food consumption and was reflected in other morphometric parameters, such as body weight gain. Additionally, it is worth highlighting that maternal melatonin replacement therapy prevented the accumulation of white adipose tissue in the offspring and did not alter interscapular brown adipose tissue, as observed in the other groups. This feature, combined with the great thermoregulation capacity of the tail, makes recruitment of the inguinal beige adipose tissue unnecessary. The greater activity of the interscapular brow

adipose tissue in these animals, independent of the cold challenge, was an important modulating/protective factor of energy storage and exacerbated body weight gain.

The ability of brown adipose tissue to assist in energy expenditure has already been described in humans, with an estimated participation of about 15% of total energy expenditure. It is estimated that this tissue is responsible for a caloric expenditure equivalent to 4.1 kg per year in adulthood (95). Excessive calorie consumption is one of the main drivers of this regulation, however, the inability to activate thermogenesis can trigger an imbalance in body weight control and facilitate the development of obesity (96-98). Thermoregulatory capacity may not be efficient if HFD consumption is chronic or if excess calories are maintained for a significant period (96, 99-101). In the present study, maternal melatonin replacement programmed this activity so that the offspring could maintain energy homeostasis, even when faced with a HFD, especially in relation to food consumption, which resulted in the reduced body weight and white adipose tissue accumulation, the improved insulin sensitivity, glycemic homeostasis and, additionally, greater thermoregulatory capacity.

The tail is an important thermal window for the animal, as it is responsible for controlling its loss of body heat. Melatonin appears to induce greater vascular constriction in this region, which promotes heat retention in rats. In the mean tail temperature analyses, it was observed that, under basal conditions, P animals, regardless of the type of food consumed, presented a higher mean tail temperature in relation to C and PM animals, which indicates that maternal pinealectomy induced thermoregulatory loss of this territory in the offspring, making them more susceptible to heat loss. Our group has already demonstrated communication between the brown and inguinal tissues in aged animals, i.e., when one tissue has reduced activity, the other is recruited for the compensation purpose (56). In the present study, it was found that the male offspring of pinealcetomized mothers during pregnancy and lactation significantly recruited inguinal adipose tissue under basal temperature condition, regardless of the type of feed consumed. This may indicate that, not only during aging, but also at earlier stage of life, this signaling between interscapular, and inguinal brown adipose tissue can occur and be triggered by signal such as melatonin during the gestational period. This profile remained in the cold condition, especially after HFD. The P-HFD group acutely exposed to subneutral condition had a higher mean tail temperature than that in the other groups, once again, when the pinealectomized mothers had received melatonin replacement, this condition was reversed to level of the control females (C-HFD). Based on these results, it is observed that maternal pinealectomy promotes morphometric adjustments in their offspring, as well as interfering with the ability to modulate energy homeostasis through molecular, enzymatic, and functional aspects. On the other hand, maternal melatonin replacement anticipated and potentiated changes in offspring submitted to HFD, enabling them to make functional adaptations such as reduced food consumption and greater thermoregulation capacity, resulting in the reduction of weight gain and white adipose tissue mass accumulation.

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AUTHORSHIP

BLT and CNJ contributed to the experimental design. BLT, GPRL, GG, MC, da CFM, MF, MTLC, AFG provided thechnical help. BLT, GPRL and CNJ contributed with analytic tools,

data analysis, performed the collation of data and writing assistance. CNJ provided acquisition of funding and support. All authors read and approved the manuscript's final format.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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