Exogenous melatonin Action on hormonal levels, implantation sites and Mel1a receptor expression and PRL-II in ovaries of pinealectomized rats induced hyperprolactinemia

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Abstract
The study evaluated the effect of exogenous melatonin and the induction of hyperprolactinemia on estrogen, prolactin and progesterone levels, implantation sites and expression of Mel1a and PRL-II receptors in pinealectomized rats ovaries, during the third Initial pregnancy. We used 35 rats divided into groups: I-rats Sham-pinealectomized (Sham); II - pinealectomized rats (P); III-pinealectomized rats treated with melatonin (P + Honey); IV-pinealectomized rats treated with domperidone (P + Domp); Pinealectomized V-rats treated with melatonin and domperidone (P + Honey/Domp); VI-rats treated with saline + ethanol (placebo honey) and VII-rats treated with saline solution (placebo domp). Melatonin (200μg/100g) and/or Domperidone (4mg/kg) was applied until the 7th day of gestation. The expression of the MEL1A receptor was low in the ovaries of rats P and P + Mel. The PRL-II receptor did not present differences between the groups as well as in the estrogen levels. Prolactin levels were elevated in rats treated with domperidone, while progesterone showed low levels in rats P and P + Domp. The weight and number of implantation sites were reduced in rats P and P + Mel, with no alteration in the weight of the ovaries. Thus, it is concluded that Hyperprolactinemia promotes an increase in the expression of the Mel1a receptor in the ovaries, at the beginning of pregnancy in pinealectomized rats, however without altering the expression of the PRLII receptor. In these animals, prolactin levels were kept elevated regardless of the presence or absence of melatonin, but there seems to be a synergistic effect when melatonin is associated with hyperprolactinemia and that melatonin is a preponderant factor for maintenance of progesterone levels. Prolactin seems to play an important role during the implantation process.

Background
Prolactin (PRL) is a polypeptide hormone synthesized primarily in the anterior pituitary gland. The actions of this hormone during pregnancy are mediated through specific receptors, the PRLR, located on the cellular surface of the uterus and ovary. The gene for PRLR has been studied in several mammals, including rats, humans, hamsters, sheep and cows, moreover birds, fish and amphibians (STEWART et al., 2000; LEE et al., 2003; TROTT et al., 2007; SHAO et al., 2008).

The PRLRs belong to the superfamily of the receptors of the PRL, among them stands out the PRL II,
sending signals to the nucleus and activating genes involved in cellular processes such as proliferation, differentiation, and cell survival during pregnancy (ROSSI et al., 2002; DEACHAPUNYA et al., 2008). Significant attention has been given to the potential use of the gene that encodes the PRLR as a marker for the performance and maintenance of reproduction (ARIE et al., 2000; LING et al., 2000; TANAKA et al., 2005).

The expression and synthesis of the PRLR gene regulate luteal progesterone concentrations via sub-regulation of 20α-hydroxysteroid dehydrogenase luteal (20α-HSD). In the ovary of rats and mice, 20α-HSD promotes a functional catalysis of the inactive form of progesterone, 20α-hydroxyprogesterone (GROSDEMOUGE et al., 2003).

The prolactin treatment leads to hyperprolactinemia and this is one of the most common hypothalamic-pituitary disorders during reproduction (ROSSI et al., 2010).

In seasonal animals, melatonin, a hormone produced by the pineal, regulates circadian rhythm and prolactin levels (PRL) during pregnancy (LEE et al., 2003). Although it already showed that this hormone regulates the physiological adaptations of reproduction in seasonal mammals in response to changes in the length of the day, its role in the reproduction of non-seasonal mammals is not well established (WOO et al., 2001).

In mammals, melatonin bonding sites have been detected both in the uterus and in the Granulo-lutein cells, whereas melatonin may have a direct effect on the steroidogenesis during pregnancy through a membrane receptor mediated process, where Gonadotropins, LH and FSH, the hormone estrogen and progesterone, play a central role in regulating the function of the ovaries as well as in the blastocyst implantation process (WOO et al., 2001).

Recently, it was demonstrated that melatonin may be interacting with gonadotropins modulating the amplitude of the transduction signal. The ability of this hormone to modulate the function of the ovary and the uterus does not depend only on the levels of circulation of this, but also of the gene expression of its receptors (SOARES JR et al., 2003a). Melatonin receptors show multiple forms, such as Mel1 and Mel2, which are expressed in the uterine and ovarian cells membrane (MASANA; DUBOCOVICH, 2001; CLEMENS et al., 2001). The Mel 1 receptor is directly related to the hormone
melatonin and is subdivided into Mel1a, Mel1b and Mel1c. The MEL1A is related to the reproductive and circadian effects of melatonin, the MEL1B is involved in the sensitivity to light by the retina and the Mel1c is found in amphibians. The Mel 2 receptor is related to N-Acetylserotonin (REPPERT, 1997; DUBOCOVICH et al., 1999). According to Pedreros et al. (2011) The treatment with melatonin in pregnant mares resulted in a normal expression of the receptor for MEL1A in the uterus and ovary and a decrease in progesterone levels.

It is unknown the molecular events that mediate the melatonin actions in these organs, because the receptors are regulated by the Heterotrimeric G protein subunits, including serine/threonine kinase (a family of MAPK kinases – protein kinase Mitogenic Activator) involved in the transduction of signals that regulate the growth, division and differentiation of uterine and ovarian cells, and it is possible that the effects of melatonin in reproduction can be mediated by the signaling of the Cascade MAPK (protein Mitogenic activating kinase) (WOO et al., 2001). However, less than necessary studies have associated the relationship between the circulating levels of melatonin and prolactin to the development of uterine and ovarian morphophysiological alterations in non-seasonal animals (SOARES JR. et al., 2003b). Thus, the present research aimed to investigate the action of exogenous melatonin on hormonal levels, implantation sites and expression of the receptor Mel1a and PRL-II in ovaries of pinealectomized rats and induced to hyperprolactinemia, during the third Initial pregnancy.

Results
The expression of the MEL1A receptor was significantly low in the ovaries of the rats of groups P and P + Mel, when compared to the ovaries of the rats of the groups Placebo, Sham, P + Mel/Domp and P + Domp. However, it was numerically evidenced the expression in the latter Group (Figure 1). For the PRL-II receptor, there were no significant differences in the ovaries of the rats of the studied groups, but the expression of this receptor was numerically elevated in the ovaries of the rats of the group P + Mel (Figure 2).

The Hormonal analysis revealed that there was no difference in serum estrogen levels in the rats of the experimental groups (Table I). However, prolactin levels were elevated in the rats of the P + Domp and P + Mel/Domp groups, being more pronounced in the latter, when compared with the other
experimental groups (Table I). In relation to progesterone, the rats of groups P and P + Domp showed a significant decrease of this when compared with the other rats of the experimental groups (Table I).

**Table I.** Mean ± standard deviation of estrogen, prolactin and progesterone levels of the rats of the various experimental groups (Sham-pinealectomized (Sham); pinealectomized (P); pinealectomized and treated with melatonin (P + Honey); pinealectomized and Induced to Hyperprolactinemia by Domperidone (P + Domp); pinealectomized, treated with melatonin and induced to hyperprolactinemia by Domperidone (P + Mel + Domp); treated with saline solution + ethanol (Placebo honey); Treated with saline solution (Placebo domp).

| Experimental Groups | N  | Estrogen* (pg/mL) | Prolactin* (ng/mL) | Progesterone* (ng/mL) |
|---------------------|----|-------------------|--------------------|-----------------------|
| Sham                | 5  | 534.14 ± 18.80a   | 3.36 ± 0.04a       | 539.24 ± 13.99a       |
| Pineal              |    | 552.56 ± 20.41a   | 2.09 ± 0.06a       | 436.56 ± 18.52b       |
| P+Mel               |    | 523.32 ± 13.82a   | 3.20 ± 0.03a       | 546.66 ± 16.95a       |
| P+Domp              |    | 527.19 ± 24.95a   | 10.15 ± 0.02b      | 422.71 ± 18.55b       |
| P+Mel+Domp          |    | 522.18 ± 25.58a   | 30.90 ± 1.21b      | 537.65 ± 9.62b        |
| Placebo Mel         |    | 532.18 ± 27.06a   | 3.26 ± 0.02a       | 540.97 ± 14.43a       |
| Placebo Domp        |    | 529.67 ± 31.37a   | 3.37 ± 0.05a       | 552.13 ± 14.26a       |

*p = 0.3026

* Averages followed by the same letter do not differ significantly from each other by the Kruskal-Wallis test with post-hoc Dunn (p < 0.05).

The statistical analysis related to weight and number of implantation sites had a significant reduction of these parameters in the rats of groups P and P + Mel when compared with the other groups studied. However, there were no statistical differences in the weight parameter of the ovaries (Table II).

**Table II:** Mean ± standard deviation of the number of implantation sites, weight of the uterus and ovaries of the rats of the various experimental groups (Sham-pinealectomized (Sham); pinealectomized (P); pinealectomized and treated with melatonin (P + Honey); Pinealectomized and induced to Hyperprolactinemia by Domperidone (P + Domp); pinealectomized, treated with melatonin and induced to hyperprolactinemia by Domperidone (P + Mel + Domp); treated with saline + ethanol (Placebo honey); Treated with saline solution (Placebo domp).
| Experimental Groups | N | Number of deployment Sites* | Uterus Weight (g)* | Ovarian Weight (g)* |
|---------------------|---|-----------------------------|-------------------|-------------------|
| Sham                | 5 | 10.80 ± 3.27<sup>a</sup>    | 0.75± 0.14<sup>a</sup> | 0.26 ± 0.03<sup>a</sup> |
| P                   | 5 | 5.20 ± 1.92<sup>b</sup>     | 0.31±0.20<sup>b</sup> | 0.12 ± 0.03<sup>a</sup> |
| P + Mel             | 5 | 5.80 ± 1.30<sup>b</sup>     | 0.33±0.13<sup>b</sup> | 0.09 ± 0.009<sup>a</sup> |
| P + Domp            | 5 | 9.60 ± 1.14<sup>a</sup>     | 0.75 ± 0.27<sup>a</sup> | 0.15 ± 0.03<sup>a</sup> |
| P + Mel/Domp        | 5 | 9.40 ± 1.14<sup>a</sup>     | 0.73 ± 0.10<sup>a</sup> | 0.13 ± 0.05<sup>a</sup> |
| Placebo Mel         | 5 | 9.40 ± 2.19<sup>a</sup>     | 1.00±0.11<sup>a</sup> | 0.26 ± 0.03<sup>a</sup> |
| Placebo Domp        | 5 | 10.20 ± 1.30<sup>a</sup> p<sup>-0.05</sup> | 1.05±0.20<sup>a</sup> p<sup>-0.05</sup> | 0.12 ± 0.03<sup>a</sup> p = 0.3165 |

* Averages followed by the same letter do not differ significantly from each other by the Kruskal-Wallis test with post-hoc Dunn (p < 0.05).

The histological analysis of the sites of implantation of rats from the experimental groups showed that they were fully adhered to the uterine wall (Figure 3A). However, P and P + Mel groups presented a possible delay in the development of these implantation sites (Figure 3B). Histologically, trophoblasts were observed at different developmental stages with mitotic activity (Figure 3C). Cytotrophoblasts with Polyploidia were also observed. However, in the animals of the P + Domp Group, these cells were more lumping when compared with the other experimental groups (Figure 3D).

The ovaries of all rats in the experimental groups externally presented a layer of connective tissue referring to the albugineous tunic, and the cortical and medullary layer well delimited and defined (Figure 4A). The Group P + Mel/Domp was similar to the placebo groups with the well preserved and differentiated follicles, besides the presence of Corpus Luteum (Figure 4B). However, the P + Domp Group presented a greater amount of corpus luteum (Figure 4C), when compared with group P and P + Mel (Figure 4D).

**Discussion**

The Literature reports that the pinealectomy in sheep (seasonal animal) promotes a decrease in the expression of the Mel1a receptor in the reproductive organs, especially in the ovaries (PANDI-PERUMAL et al., 2008). Although, Okatani et al. (2001), studying non-seasonal pinealectomized animals and treated with melatonin at a dose of 0, 4mg/kg reported a small expression in the Mel1a
receptor in the ovary, but when the same author used exogenous melatonin at the dose of 4mg/kg
There was an increase in the expression of this receptor. Thus, the lower expression of this receptor
in the ovaries of rats in group P + Mel similar to group P verified in this study may be related to the
dose of melatonin (200μg/100g), suggesting a dose-dependent effect of this hormone in the
expression of this receptor.
In addition, Lee et al. (2003) reported that the presence of endogenous prolactin in sheep is capable
of stimulating the expression of the Mel1a melatonin receptor, and that pinealectomized ewes
submitted to Hyperprolactinemia, presented regulation of the Gonoidal functions, as well as increased
expression of this receptor in the ovaries (NISWENDER et al., 2000; PERKS et al., 2003),
The expression of the Mel1a receptor due to hyperprolactinemia is due to a possible stimulus in the
production of P450SCC protein, which activates the dissociation of G proteins in α and βγ dimers.
Interacting with several effector molecules involved in the transmission of Cell signaling, stimulating
the expression of the Mel1a receptor (MASANA et al., 2001; PANDI-PERUMAL et al., 2008; TAMURA et
al., 2008). Explaining the numerical increase of the Mel1a receptor expression in the ovaries of rats in
the P + Domp group.
The PRL-II receptor did not present significant differences in the ovaries of the rats between the
experimental groups, but the expression of this receptor was numerically elevated in the ovaries of
the rats of the group P + Mel. According to Lee et al. (2003) Pinealectomized Rats submitted to
hyperprolactinemia present numerically low expression of the receptor PRL-II during the beginning of
gestation, reaching a greater expression when the placenta is formed. Reese et al. (2000) Studying
mice (females) submitted to hyperprolactinemia report that the receptor PRL-II is expressed in the
uterus. In addition, Jabbour; Critchley (2001) studying pinealectomized and melatonin-treated rats
demonstrated that there was a greater expression of the PRL II receptor in the uterus of these
animals, and that when they were still submitted to hyperprolactinemia the expression of the PRL II
receptor It decreased, which may explain the values observed for the experimental group P + Domp
and P + Mel + Domp.
The Hormonal analysis of serum estrogen levels in the rats of the experimental groups confirmed the
results already cited in the literature, because according to Torres-Farfan et al. (2003) pinealectomized rats treated with melatonin and/or subjected to Hyperprolactinemia does not present alterations in the production of estrogen in the early stages of gestation.

It is established that oral administration of melatonin at a dose of 4 mg/kg in pinealectomized rats causes an abrupt increase in the concentration of prolactin after 30 min of application (DIAZ et al., 1999). Studies conducted with pregnant ewes, treated daily with melatonin at a dose of 2mg/kg showed that this hormone was able to elevate the plasma concentration of PRL (DICKS, 2000). In addition, studies with goats reported that the treatment with exogenous melatonin is able to delay the fall time of the PRL levels, keeping them elevated (SANTIAGO-MORENO et al., 2004). However, the elevated levels of prolactin observed in the present study were only seen in the rats of the P + Domp and P + Mel/Domp groups, being more pronounced in the latter. As there was no increase in the PRL levels in the rats of the P + Mel groups, we can suggest that the amount of melatonin administered in the present study at the beginning of pregnancy was not sufficient to maintain elevated PRL levels and that there seems to be a synergistic effect of Melatonin and Domperidone.

The levels of progesterone in rats of groups P and P + Domp showed a significant decrease of this when compared with the other rats of the experimental groups. According to Grasselli et al. (2008) Ewes treated daily with 2, 5mg/Kg of melatonin promoted the resumption of ovarian activity, stimulated by the increase of progesterone. In Addition, Coelho et al. (2006) reported that pinealectomized lambs and treated with melatonin at a dose of 0, 8mg/kg showed an increase in the plasma concentration of progesterone. Bonnefond et al. (2006) also reported that the increase in progesterone may be influenced by the presence of melatonin. And McConneli and Hinds (1995) reported that high levels of prolactin during pregnancy may cause a negative feedback on the increase in progesterone. This suggests a regulating effect of melatonin on the production of progesterone during pregnancy, with or without prolactin.

It is known that the reduction of circulating melatonin caused by pinealectomy induces an increase in oxidative stress, but does not inhibit the implantation of the blastocyst in the endometrial epithelium. Pinealectomized rats present a reduction in the number of implanted sites. Whereas, pinealectomized
rats subjected to doses of 10mg/kg of exogenous melatonin for 3 weeks present an increase in the rate of blastocyst implantation, by stimulating the production of gonadotrophic hormones, with consequent stimulus of the production of Progesterone and greater development of Corpus luteum (KOCH et al., 2010). Sandyk et al. (2000) also reported that when non-pinealectomized pregnant rats are treated with melatonin there is a stimulus in the production of progesterone, which prevents the immunological rejection of trophoblasts, thus facilitating the implantation, and stimulating the greater Corpus luteum Development. This fact was evidenced in the results presented in the present study, because the rats of the experimental groups P and P + Mel presented the lowest means of implanted sites and less development of them, while the rats of the experimental groups P + Domp and P + Mel/Domp exhibited a greater number of implantation sites and a greater amount of corpus lutees. Thus, it seems that although the literature reports that melatonin administration increases the implantation rate, the dosage of 200μg/100g of melatonin was not sufficient to stimulate such increase.

In relation to cytotrophoblasts cells It was evidenced that these in the P + Domp group were more voluminous when compared to the other groups studied. According to Natale et al. (2003) Rats when submitted to pinealectomy presents greater action of estrogenic and androgenic hormones, producing morphological and quantitative alterations in the trophoblasts cells, and Sutherland (2003) reports even though the Presence of estrogen stimulates morphological and functional differentiation of the trophoblast during pregnancy. Dair et al. (2008) report that in pinealectomized rodents the circulating estrogen level is not altered during the initial stage of pregnancy. According to Gomes et al. (2009) Mice submitted to hyperprolactinemia present proliferation of trophoblastic cells, with an increase in the mitotic index as well as its volume. Rossi et al. (2002) showed that mice submitted to hyperprolactinemia had more proliferated trophoblastic cells and with a higher volume, being morphologically more developed, in the pregnancy phase.

Thus, it is concluded that Hyperprolactinemia promotes an increase in the expression of the Mel1a receptor in the ovaries, at the beginning of pregnancy in pinealectomized rats, however without altering the expression of the PRLII receptor. In These animals, prolactin levels were kept elevated
regardless of the presence or absence of melatonin, but there seems to be a synergistic effect when melatonin is associated with hyperprolactinemia and that melatonin is a preponderant factor for Maintenance of progesterone levels. Prolactin seems to play an important role during the implantation process. Furthermore, treatment with melatonin seems to retard the implantation process in the uterine wall as well as provoke a lower development of corpus luteum in the ovary, having treatment with domperidone contrarian effects in non-seasonal animals.

Conclusions
In conclusion, the prolactin levels were kept elevated regardless of the presence or absence of melatonin, but there seems to be a synergistic effect when melatonin is associated with hyperprolactinemia and that melatonin is a preponderant factor for Maintenance of progesterone levels. Prolactin seems to play an important role during the implantation process.

Methods
We used 35 albino rats (*Rattus norvegicus Albinus*) of the Wistar lineage, 90-days-old and weighing around 200 ± 30g, from the Animal of the Department of Morphology and Physiology of the Federal Rural University of Pernambuco. These animals were kept in cages, with food and water ad libitum, at the temperature of 22 ± 1 ºC and artificial illumination that established a photoperiod of 12 hours of light and 12 hours dark, considering the light period of 06:00 to 18:00HS

The females were randomly divided into seven groups, each consisting of 5 animals, namely:

Group I-Sham-pinealectomized rats (Sham);
Group II – pinealectomized rats (P);
Group III-pinealectomized rats treated with melatonin (P + Honey);
Group IV-pinealectomized and hyperprolactinemia-induced rats by domperidone (P + Domp);
Group V-rats pinealectomized, treated with melatonin and induced to hyperprolactinemia by Domperidone (P + Mel/Domp);
Group VI-rats treated with saline solution + ethanol (honey placebo);
Group VII-rats treated with saline solution (placebo domp).

After the formation of the respective groups, the rats were treated for 7 days. The Experimental Protocol was approved by the institutional Ethics of Committee to Universidade Federal Rural de Pernambuco n º. 23082.009629/2010.

Pinealectomy

The Pinealectomy was performed in animals previously anesthetized with pentobarbital 40mg/kg
intraperitoneal route (LIMA et al., 2005; LACERDA et al., 2007). Afterwards, the trichotomy and asepsis of the dorsal area of the head were performed. An incision was made on the dorsal midline of the head with a low-speed micromotor and a dentist drill No. 05, a circular fragment of the cap was removed. This fragment was placed in saline solution at 0.9%. After removal of the bone fragment, venous sinus ligation was performed for the removal of the pineal (KUSZAK; RODIN, 1977). Next, the bone fragment was replaced and the skin was sutured. For Post-surgical pain prevention, buprenorphine (Tengesic®) was administered at a dose of 0.05 mg/kg, by subcutaneous route every 12 hours and 30mg of Ampicillin intramuscularly (MI), both for a period of five days, such as pain prevention and encephalopathy Resulting from surgical procedures (i.e., pinealectomy and Sham-pinealectomy) (GUVENAL et al., 2001; KREIMER et al., 2005; YAMATOGI et al., 2005).

Colpocytological Examination

For the confirmation of mating, we collected a vaginal secretion in which cotton stems moistened with saline solution were used. Shortly after harvesting, a smear was made on histological slides through a rotational movement of the stem. These slides were immediately immersed in a mixture of ethanol-ether in equal parts and then stained with the Shorr-Harris method and analyzed in light microscope with the presence of spermatozoa in the slides as a parameter.

Melatonin Treatment

The Treatment with melatonin (Sigma, St. Louis, MO, USA) was performed according to the methodology proposed by Prata Lima et al. (2004). 200μg of melatonin per each 100g of the animal's body weight was administered through subcutaneous injections at the beginning of the night (18:00h). Melatonin was dissolved in a volume of ethanol (0, 02mL) and diluted in saline solution 0.9% (NaCl 0.9%). The animals in the placebo group received, respectively, 0.9% NaCl solution and 0, 02mL of ethanol.

Treatment with Domperidone (DOMP)

The induction to Hyperprolactinemia was obtained with the subcutaneous injection of domperidone at a dose of 4mg per kilo of daily body weight, always at the time of 11:00 hours in the morning. DOMP was dissolved in 10mL of saline solution. The animals in the placebo group received only saline
Histological Analysis

Five Females of each group were euthanized after 7 days of treatment. For This purpose, they were anesthetized with ketamine hydrochloride (80mg/kg) and xyazine (6mg/kg), by intramuscular route.

Next, the uterus was removed containing the implantation sites, and the ovaries, which were immersed in Boüin's liquid, remaining in the same for 48 hours. After This procedure, the ovaries and the uterus were cleaved and submitted to the histological technique of paraffin inclusion. Then, the blocks were cut, stained with Hematoxylin and Eosin (H.E.) and analyzed under light microscope.

Hormone Dosage

One mL of blood was collected by cardiac puncture (RICHTER et al., 2009), being packed in heparinized tubes. Afterwards, the samples were centrifuged under refrigeration and the supernatant was packaged in Eppendorfs microtubes, always in duplicate, and frozen at-20ºc until the time of hormonal dosages (TEIXEIRA et al., 2004, 1). The samples were collected in triplicate at 7 days of gestation in five rats of each group. The levels of the estrogen, prolactin and progesterone hormones were measured using the Enzyme Linked Immunosorbent Assay (ELISA) method, through commercial KIT's...

Weight of the Ovaries and Count and Weight of the Implantation Sites

The ovaries and uterus were weighed on a precision analytical scale. Subsequently, the implantation sites were counted.

RNA Extraction

The Samples of the ovaries, duly identified, were packaged in a freezer-80 ºC for posterior RNA extraction. At the time of extraction, the sample was transferred to a N2-cooled mortar to undergo a maceration process. After This step, the material was transferred to an Eppendorf tube, receiving as a complement of Trizol 1000uL. RNA was extracted under the conditions recommended by the manufacturer.

The extracted RNA was quantified in Spectrophotometer (Bionate3 of Thermoscientific) and analyzed in agarose gel at 1.5%, (Pronadisa) stained with Syber Green II (RNA-LCG Biotecnolia), analyzed in
ultraviolet light and photographed (Olympus – Digital Chamber – C-7070 Wide) to check its quality.

Reverse Transcription (RT-PCR)

The first cDNA tape synthesized by reverse transcriptase was binded to the RT-PCR system using an adapter 3’-Oligo (dT) 18 (Quiagen), 1 M g total RNA and 1U reverse transcriptase (Promega, Madison, WI) under the conditions Recommended by the manufacturer, using 0.5 M g of random primers (Roch, Nonnenwald, Germany). The cDNA amplifications of the Mel1a and PRL-II genes were carried in the Rotor-Gene 3000 operated with the software version 6.0.19 (Corbett Research, Mortlake, 2137 NSW, Australia; www.cobettlifescience.com). According to the protocol described in the next section.

Amplification Reaction (PCR) Real Time

The amplifications were made in the Rotor-Geneq (Quiagen) as a platform, consisting of a denaturation of the initial DNA at 95 °C (3min), followed by 60 cycles with 95 °C (3SEC), 60 °C for 30 minutes of the ring temperature of Probe primers (at specific temperatures for each pair of primers established depending on the region to be amplified) and 45 °C of extension for 40 seconds. The final extension was 45 °C for 40 seconds. After Cycling, a suitable amplification curve was established for each amplified gene for the establishment of nonspecific amplifications (LEE et al., 2003).

Standardization Curve of the gene expression

For the relative quantification of MEL1A and PRL-II receptors three pairs of primers were used separately: a pair of primers to amplify each receptor Mel1a and the PRL-II, and the other for an endogenous control, β-actin, which served as a comparison pattern (LEE et. A l., 2003). The analysis of the expression was by comparing the difference in the values of the thresholds curves (Ct) between the analyzed samples and the basal gene.

Statistical Analysis

The statistical analysis of the weight of the ovaries, weight and site number of implantations, hormonal levels and expression of Mel1a and PRL II receptors was analyzed in a software InStat®, where data were evaluated by means of non-parametric tests of Kruskal-Wallis with Dunn post-hoc (p < 0.05). The MEL1A and PRL-II genes were normalized from B-actin (2-ΔΔCT) (LIVAK; SCHMITTGEN,
Declarations

- Ethical Approval and Consent to participate

The experimental protocol was approved by the Institutional Ethics Committee, number 23082.009629/2010.

- Consent for publication

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- Authors' contributions

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Figures
Expression of the Mel1a receptor in the ovaries of the rats of the different experimental groups presenting mean ± standard deviation (P = 0.0028) (Sham-pinealectomized (Sham); pinealectomized (P); pinealectomized and treated with melatonin (P + Honey); Pinealectomized and induced to Hyperprolactinemia by Domperidone (P + Domp); pinealectomized, treated with melatonin and induced to hyperprolactinemia by Domperidone (P + Mel + Domp); treated with saline + ethanol (placebo honey); Treated with saline solution (placebo domp). * Averages followed by the same letter do not differ significantly from each other by the Kruskal-Wallis test with post-hoc Dunn (p < 0.05).
Expression of the receptor PRL-II in the ovaries of the rats of the different experimental groups presenting mean ± standard deviation (P = 0.7601) (Sham-pinealectomized (Sham); pinealectomized (P); pinealectomized and treated with melatonin (P + Honey);
Pinealectomized and induced to Hyperprolactinemia by Domperidone (P + Domp); pinealectomized, treated with melatonin and induced to hyperprolactinemia by Domperidone (P + Mel + Domp); treated with saline + ethanol (placebo honey); Treated with saline solution (placebo domp)). * Averages followed by the same letter do not differ significantly from each other by the Kruskal-Wallis test with post-hoc Dunn (p < 0.05).
Figure 3

Implantation Sites of the rats of the experimental groups. (A) – Sham-pinealectomized Group with implantation site fully adhered to the uterus wall (S). (B)-Group P + Mel observe poorly developed implantation sites (S). (C) – Group P + Mel/Domp implantation sites with trophoblasts at different developmental stages (short arrows) and some with mitotic activity (arrow). (D)-Group P + Domp volous trophoblasts (arrows). H-E.
Ovaries of Rats of the experimental groups. (A)-Placebo Group. Observe the albugineous Tunic (arrow), cortical (C) and medullary (M) region. (B)-Group P + Mel + Domp similar to the placebo group with well-preserved and differentiated follicles (arrows) Besides the presence of Corpus Luteos (CL). (C)-Group P + Domp with a greater amount of corpus luteum (CL). (D) – Group P + Mel notice less quantity of Corpus lutetes. H-E.