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Combination of Melatonin and Metformin Hydrochloride for Treatment Polycystic Ovarian in Female Rats

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ABSTRACT

***Background*:** Polycystic ovary syndrome (PCOS) is a gynecological endocrine disorder, results in menstrual abnormalities, androgynism and infertility. In the case of women or others animals with PCOS wishing to treat infertility with the aim of becoming pregnant, the most commonly used is metformin hydrochloride. Recent studies have analyzed the combination of metformin hydrochloride with melatonin in oncological treatment but not to treatment of polycystic ovary syndrome (PCOS). The aim of the present study was to analyze the effectiveness of the combination of metformin hydrochloride and melatonin in the treatment of PCOS to improve the fertility of rats and your hormonal alterations.

***Materials, Methods & Results*:** This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Federal Rural of Pernambuco (Permit Number: 23081.009130/2010). A total of 50 albino Wistar rats were used. The animal laboratory of an academic research environment, were randomly separated into five groups consisting of 10 females each. After inducing PCOS, the rats were treated with metformin hydrochloride, and/or melatonin, and the results compared with standard and ultrasound confirmed. The physiological similarities were confirmed by our academic researchers morphological science, and published to the association results of effects syndrome induction through constant lighting in reputable magazine recently. This article was analyzed histological of the implantation sites and ovaries, and the estradiol and progesterone levels on the seventh day of gestation, and the other rats for monitoring pregnancy and morphological identification of possible fetal abnormalities, weight measurement and quantification of offspring. The rats were anaesthetized with intraperitoneal injections of ketamine hydrochloride (80 mg/kg) and xylazine (6 mg/kg) to allow analysis of the reproductive organs. Main outcome measures: The study included histopathology, histochemical and quantitative (of the implantation sites) tests, ultrasound analysis, weight benchmarking and ovarian histology tests, as well as comparison of serum estradiol and progesterone levels, and the morphological assessment of offspring. Results paper shows pharmacological treatment reduced the time needed for pregnancy, increased the plasma progesterone levels, the number and weight of offspring, and reduced plasma estrogen levels and collagen fiber grade, improving blastocyst-endometrium interaction and fetal development.

***Discussion*:** Our team of researchers confirmed in a previous paper; in addition, the main experimental model used in research about PCOS in recent years, and considered appropriate combination of the drugs caused a physiological reaction similar to responses identified in healthy rats without induction of the POS control group. However, the clinical and physiological effectiveness of the combination should be further explored, especially with respect to the possible side effects on offspring. The treatment with a combination of metformin hydrochloride and melatonin was more effective against hormonal alterations produced by PCOS, allowing a normalization of biochemical parameters during pregnancy, than monotherapeutic treatment with these drugs. In conclusion, proposed drug combination is a viable option to treatment of polycystic ovary syndrome and improved fetal development. This article allows suggest that further research should be conducted to examine effects associated with these drugs in the treatment of diseases of the female reproductive system experimentally. Only such treatment later in animals and humans suggest.

Keywords: polycystic ovaries, metformin, melatonin, reproduction, estradiol, progesterone.

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INTRODUCTION

Polycystic ovary syndrome (PCOS) characterized by amenorrhea, hyperandrogenism and/or hyperandrogenemia and follicular cysts, characterizing this syndrome as one of the main causes of female infertility [12,14,15,26,41,]. The treatment of PCOS varies according to the symptoms of the patient and usually takes the form of the administering of antiandrogens and antidiabetic agents such as metformin, often in combination with contraceptive hormones. Metformin is the most widely used drug in clinical cases of the disorder among patients seeking treatment of infertility, and considered safer than the other treatments [13,18].

Some authors have associated the pathogenesis of diseases that cause infertility, such as PCOS, endometriosis and other disorders of the female reproductive system, to oxidative stress. Literature reveals that melatonin is a broad-spectrum antioxidant produced by the pineal gland with a principally hormonal function, controlling cyclicity and reproductive functions. Its levels tend to increase during pregnancy, demonstrating its importance to gestational physiology [2,5,29,33,34].

The combined of metformin and melatonin therapy had better results for levels of lipid peroxidation (LPO) compared with monotherapeutic use of these drugs in rats [1,17,37]. However, there are no reports in literature on the combination of metformin and melatonin for the treatment of ovarian polycystosis and its viability in improving implantation [16,30,31,38]. Therefore, the present study tested the hypothesis that melatonin can enhance the performance of metformin in experimental models induced in the polycystic ovaries, by constant illumination, enhancing conceptive viability.

MATERIALS AND METHODS

Installation of bioassay

The experiment was performed in the Histology Laboratory of the Department of Animal Morphology and Physiology of the Universidad Federal Rural of Pernambuco. A total of 50 albino (*Rattus norvegicus albinus*) Wistar rats, aged 90 days and weighing approximately 200 ± 30 g, were used. The rats were taken from the biotery of the department.

The rats kept in cages and supplied with food and water *ad libitum*. Temperature of 22 ± 1ºC and under artificial lighting, establishing a photoperiod of 12 h of light and 12 h of darkness, with the light period being from 06:00 to 18:00.

After an adjustment period, vaginal smears were collected to determine the estrous cycle. Female rats who had undergone three regular estrous cycles were randomly separated into five groups, each consisting of 10 animals. Five for the purposes of histological analysis of the implantation sites and ovaries, and the plasma estradiol and progesterone levels on the seventh day of gestation, and the other rats for monitoring pregnancy and morphological identification of possible fetal abnormalities, weight measurement and quantification of offspring.

Group I - rats without ovarian polycystosis were kept in a 12/12 h light/dark cycle for 100 days and then mated (control);

Group II - rats with induced ovarian polycystosis and then mated (PCOS + Placebo);

Group III - rats with induced ovarian polycystosis treated with melatonin for 20 days and then mated (PCOS + Mel);

Group IV – rats with induced ovarian polycystosis treated with metformin for 20 days and then mated (PCOS + Met);

Group V - rats with induced ovarian polycystosis treated with melatonin and metformin for 20 days and then mated (PCOS + Mel + Met).

Induction of Polycystic Ovary Syndrome

For the induction of PCOS, the rats of groups II, III, IV and V subjected to constant light stimulation, achieved using a wooden box with an area of 0.5 m³, with slits for ventilation, containing two fluorescent PHILLIPS 40W lamps, which provided sufficient brightness of around 400 lux. These lamps remained continuously lit for a period of 100 days, enough time for the development of the clinical features of PCOS [20,35]. The presence of PCOS was confirmed by ultrasound. After the period of continuous lighting change androgenic hormone function, contributes to the induction of PCO disease of female rats. Irregularity androgens result from a reduction in sensitivities to estradiol and progesterone that are involved with GnRH release from hypothalamic centers. Consistent hormonal changes have been reported among various studies utilizing constant light rat PCO models. Compared to control values, plasma luteinizing hormone levels were normal, follicle-stimulating hormone and estradiol levels were elevated. Thus, the constant light rat model presents absence of *corpus luteum*, which is an indication of chronic anovulation, electron microscopic studies of PCO ovaries showed thickening in the tunica

albuginea and abnormalities of the stroma and of granulosa and theca cells relative to control [27].

Ultrasound of ovaries

The analysis of ultrasounds was performed in the Department of Veterinary Medicine of UFRPE. The images were in double blind obtained before and after the treatment of rats, by using the ESAOTE / MyLab GOLD™ 30-ultrasound machine with a 10MHz frequency linear transducer. The rats were intramuscularly anesthetized with ketamine hydrochloride (80 mg/kg) and xylazine (6 mg/kg) and manually immobilized. The abdomens of the rats were moistened with 70% alcohol to enable ultrasounds to be performed, and the position and size of the ovaries was identified, taking as a reference the position of the kidneys and the evaluating the echotexture and echogenicity of the organ.

Treatment with Metformin Hydrochloride

Metformin hydrochloride (Glifage®)[1] was administered by gavage at a dose of 50 mg/100 g b.w. diluted in 0.5 mL of distilled water for twenty consecutive days. The dose of metformin administered was equivalent to that used in the treatment of women with PCOS, that is 500 mg/day, and that keep the pharmacodynamics [10,28].

Treatment with Melatonin

After the rats were weighed, rats from group III and group V were treated with melatonin in accordance with the methodology described by Prata-Lima *et al*. [25]. (Melatonin)[2] was administered at a dose of 200 µg/100 g b.w. by subcutaneous injections in the evening (18:00) for twenty consecutive days. According to Drobnik *et al*. [9], doses higher than 60 and below 300 µg/100 g b.w. expressed an equivalent effect to endogenously secreted pineal indoleamine. The melatonin dissolved in ethanol (0.02 mL) and diluted with 0.2 mL of sodium chloride (0.9% NaCl).

Mating of animals and confirmation of copulation

After treatment the females placed were for mating daily, in the evening (18:00), at a ratio of one male to two females. Cervical cytology was performed to confirm and monitor the mating receptivity of the females, using the presence of spermatozoa in vaginal smears as a parameter. This was considered as the first day of gestation. Rods fitted at the ends with cotton balls soaked in saline were used to collect smears. Immediately after sample collection, the cells were transferred to histological slides through rotational movements of the rod. These slides were immediately immersed in a solution of ether alcohol in equal parts, and were then stained using the Harris-Shorr method. Recorded the time that the females of all groups needed to mate.

Blood collection for determination of plasma estradiol and progesterone levels

The rats were immobilized in wooden boxes and blood was collected by puncturing the lateral tail vein with a catheter (24G) on the seventh day of gestation. After centrifugation, chilled plasmas were placed in 1.5 mL microcentrifuge tubes and frozen at -20°C until dosage [11,36]. The plasma levels of estradiol and progesterone were assayed using the Enzyme Linked Immunosorbet Assay (ELISA) using commercial kits (ADI 900-100 lot n.: 05121109) and (ADI 900-011 lot n.: 10191116B) respectively, both manufactured by *Enzo LifeSciencePRO*.

Histology and morphometry of uterus collagen fibers

On the seventh day of gestation, five females from each group were anesthetized intramuscularly with ketamine hydrochloride (80 mg/kg) and xylazine (6 mg/kg). The uterine horns containing the implantation sites and the ovaries were collected. The ovaries were weighed on an analytical scale and the implantation sites were quantified with the aid of a magnifying glass. All samples were then immediately immersed in 10% buffered formalin. The samples were subsequently processed for inclusion in paraffin and the sections underwent hematoxylin-eosin (HE) staining. These were examined using an Olympus BX-49 light microscope and photographed with an Olympus BX-50 photomicroscope. For morphometric, the implantation sites were stained with sirius-red/fast-green (RG). Gimp2.6 software (GNU Image Manipulation Program, UNIX platforms) used to quantify the changes in the pixels of the stained material. The results recorded underwent statistical testing, and a comparison chart of the groups was created.

Monitoring of copulation

The rats were monitored to determine time of mating. When mating was confirmed they were weighed daily until the end of pregnancy. During this period number of abortions and the weight loss of the rats was monitored. After birth, the offspring weigh, were quantified and analyzed macroscopically for the presence of visible malformations in the head, trunk or limbs.

Statistical analysis

Nonparametric Kruskal-Wallis analysis was used. Where necessary, means were compared using the Wilcoxon-Mann-Whitney test with a 95% confidence interval.

RESULTS

Ultrasounds of ovaries

The statistical analysis of the ovarian dimensions revealed by the ultrasounds showed a significant increase in the dimensions of the ovaries of the females of all groups induced with PCOS. However, after treatment there was a significant reduction in the ovaries size in the female treated with melatonin, metformin and a combination of these drugs (Table 1).

Weight of the female rats during treatment and pregnancy

There was no statistically significant difference in the weight of the rats during treatment, however, during pregnancy the rats from the group PCOS + Placebo displayed reduced weight gain. This was the only experimental group to differ from the control group (in which PCOS was not induced) and PCOS + Mel (in which PCOS was induced and treated with melatonin). In other words, all the groups treated with single drugs or combinations of drugs had improved gestational weight gain balance (Table 1).

Analysis of the time required for copulation and fertility

The number of days required for confirmation of pregnant, was significantly higher in the PCOS + Placebo group. Furthermore, only 30% of the females in this group enabled copulation. Replacement of the placebo with melatonin did not result in significant differences in relation to the control group and the PCOS + Placebo group. On the other hand, treatment with metformin or with a metformin and melatonin combination significantly reduced of the confirmation of pregnant compared to the PCOS + Placebo group (Figure 1A). The PCOS + Placebo group presented a statistically significant reduction in the number of implantation sites on the seventh day of gestation, and offspring (Figure 1B). On the seventh day of gestation, none of the experimental groups displayed evidence of abortion reabsorption into the uterine horns. There were no stillbirths, alteration in number of offspring (Figure 1C) or morphological identification of malformations, but it has been found a statistically significant reduction in the weight of offspring from the PCOS + Placebo group (Figure 1D).

Ovarian weight, estradiol and progesterone levels and immunohistochemistry uterus

The ovarian weights of the females of all group in treatment with metformin hydrochloride, melatonin or combination of both drug, reduced the weight of the ovaries. None of the treatments reduced the values to resemble the group uninduced to PCOS. Of these groups, the rats receiving only metformin hydrochloride showed values similar to the group that had PCOS + Placebo, these last groups showed the highest values measured weights of ovaries (Figure 2A).

Levels of estradiol in the plasma of rats in which polycystic ovary syndrome was induced, and which received only a placebo were statistically higher than those observed in other groups. The PCOS + Mel, PCOS + Met and PCOS + Met + Mel had the highest averages in relation to the control PCOS + Placebo groups, with these being more significant in the latter group. In turn, the PCOS + Placebo group had lower levels of progesterone and was statistically different from the other experimental groups (Figures 2B and 2C).

In terms of the presence of collagen fibers, the group induced with PCOS and treated only with a placebo revealed greater intensity, identified through picro-sirius and fast green dye, compared to the other groups. The groups that were treated with melatonin or metformin had statistically significant collagen marking when compared to the PCOS + Placebo group. However, the combination of metformin and melatonin produced a greater reduction in collagen labeling compared to PCOS + Placebo, but was not statistically different from the control group (Figure 2D).

Histological analysis

The implantation sites of the female rats in all the experimental groups were well-preserved and fully inserted in the uterus wall (Figures 3A-3E). The ovaries of the control rats revealed the presence of corpus luteum with well-preserved luteal cells, and the presence of follicles at different stages of development (Figure 4A). In the PCOS + Placebo group were many atresics follicles with a discreet presence of corpus luteum (Figure 4B). The experimental groups that were treated with metformin or a combination of metformin and melatonin had histologic features similar to the control group (Figures 4C-4E).

Table 1. Mean and standard deviation of experimental data of rats subjected to the experimental model of polycystic ovary syndrome.

Grupo	Control	PCOS + Placebo	PCOS + Mel	PCOS + Met	PCOS + Mel + Met	F[p]
US1(cm²)	0,08 ± 0,01a	0,46 ± 0,10b	0,57 ± 0,12b	0,65 ± 0,17b	0,52 ± 0,13b	12,530,001
US2(cm²)	0,11 ± 0,02a	0,36 ± 0,04b	0,24 ± 0,05a	0,26 ± 0,19a	0,27 ± 0,86a	22,940,001
WT(g)	228,80 ± 22,12a	240,76 ± 17,67a	229,80 ± 17,56a	226,86 ± 12,65a	226,86 ± 12,65a	0,40750,8011
WP(g)	245,88 ± 14,16a	255,17 ± 20,21a	242,91 ± 15,75a	237,22 ± 27,74a	244,37 ± 24,75a	0,47240,7554
WGP(g)	122,6 ± 19,56a	62,8 ± 15,75b	110,8 ± 10,43a	98,6 ± 15,33a	98,2 ± 11,56a	3,93680,0162

Ovarian diameter measured by ultrasound after induction of polycystic ovary syndrome (US1), measurement of the diameter of the ovary after treatment (US2), weight of rats during treatment (WT), weight of rats during pregnancy (WP) and weight gain during pregnancy (WGP). Means followed by the same letter in the parameters analyzed did not differ significantly, according to the Wilcoxon-Mann-Whitney test ($P > 0.05$).

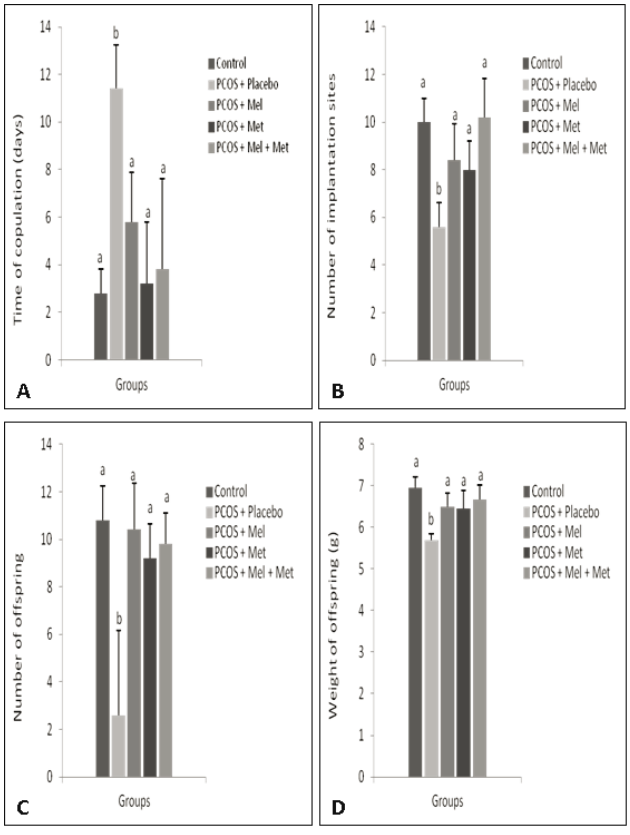

Figure 1. Note significant increase in the time required for copulation (A) and reduction in the number of implantation sites (B), and of number (C) and weight of the offspring (D) in PCOS + placebo group compared to the other experimental groups. Means followed by the same letter in the parameters analyzed did not differ significantly, according to the Wilcoxon-Mann-Whitney test ($P < 0.05$).

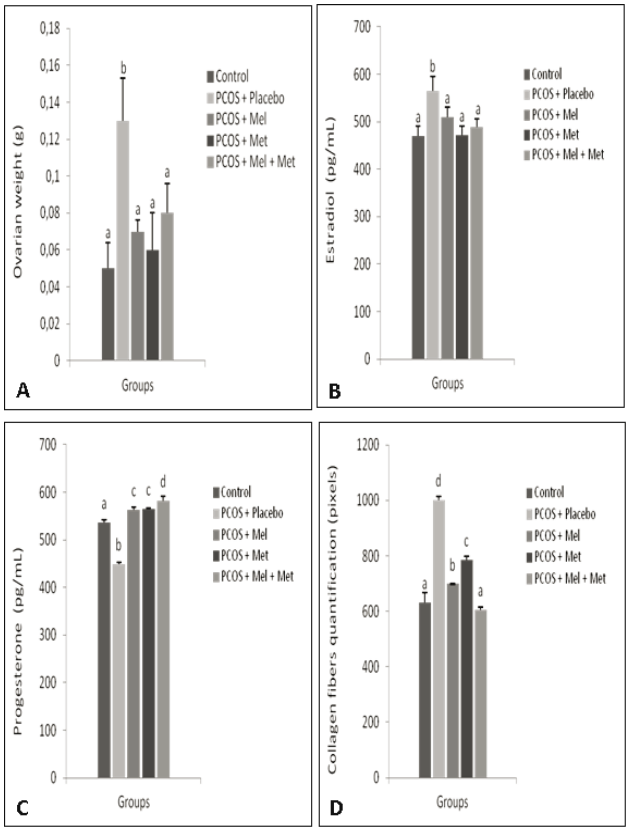

Figure 2. Note values of ovarian weight (A) and estradiol levels (B) similar to the control after treatment with Mel, Met and Mel + Met. (C) Check significant increase in progesterone levels, compared to control, being more significant in the group PCOS Mel + Met. (D) Reduction in content of collagen fibers after treatment with Mel, Met e Mel + Met, when compared with group PCOS + placebo. In treatment with combination of drugs the content of collagen fibers was similar to Group control. Means followed by the same letter in the parameters analyzed did not differ significantly, according to the Wilcoxon-Mann-Whitney test ($P < 0.05$).

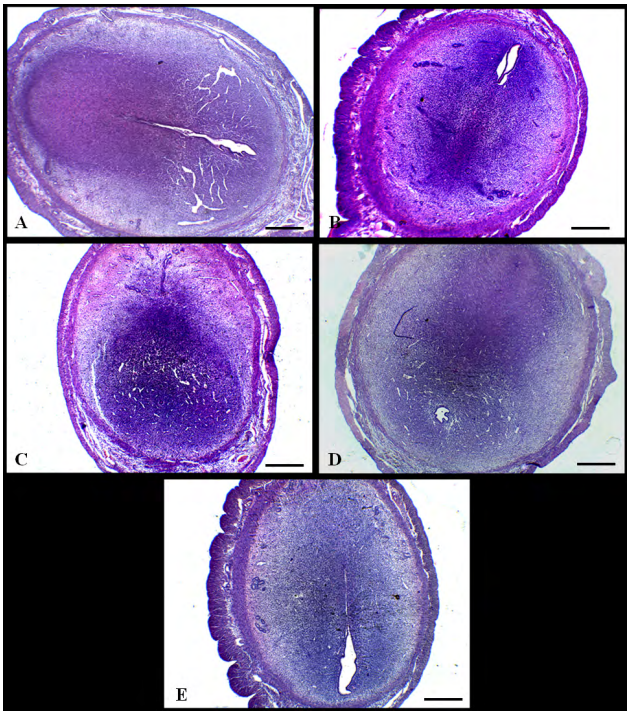

Figure 3. Sites of implantation. (A) control, (B) PCOS + Placebo, (C) PCOS + Mel, (D) PCOS + Met and (E) PCOS + Mel + Met. H.E. [Bars = 100 µm].

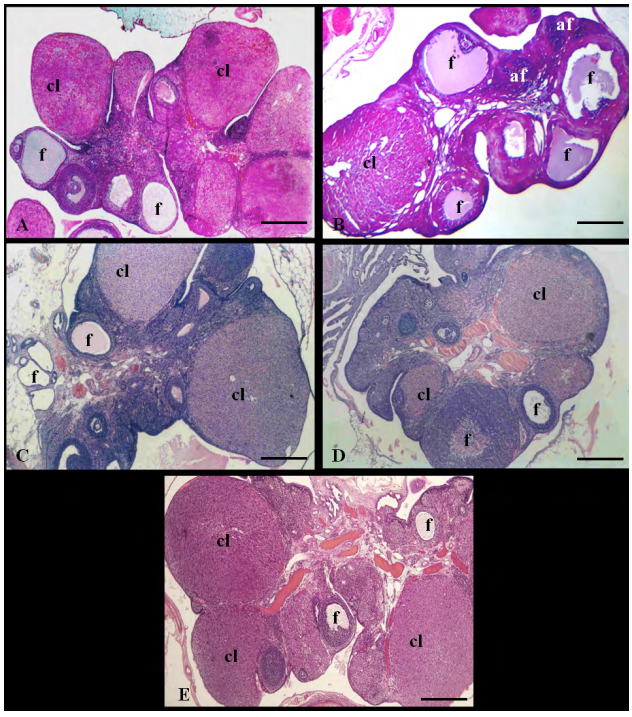

Figure 4. Ovaries. (A) control, (B) PCOS + Placebo, (C) PCOS + Mel, (D) PCOS + Met and (E) PCOS + Mel + Met. H.E. [Bars = 200 µm]. cl: *corpus luteum*; f: follicles; af: atresic follicles.

DISCUSSION

Our team of scientists has been working for many years with the female reproductive system and after long-term research, recently published on the physiological similarities of symptoms of polycystic ovary syndrome compared to the experimental model. This research team found that there is benefit in linking drug in the treatment of polycystic ovary syndrome. Female rats from the PCOS + Placebo group had lower weight gain during pregnancy, while those animals treated with melatonin, had weight gains similar to those of the control group. Additionally, there was a reduction in the number of implantation sites observed in rats from the PCOS + Placebo group, resulting in a reduced number of offspring. There was also a histhochemical increase in collagen content in the uterine horns, which may have hindered blastocyst-endometrial interaction. On the other hand, rats that received treatment with either a combination of drugs or a single drug had a similar number of implantation sites and offspring to the control group. With respect to collagen fiber, content only the PCOS + Mel + Met group had similar values to the control group, while the PCOS + Mel and PCOS + Met groups revealed a significant reduction in collagen fiber content in relation to the PCOS + Placebo group. Endocrinologically, plasma progesterone levels were higher in the groups in which rats were treated with a combination of drugs or a single drug, with the difference being more statistically significant where treatment involved a combination of drugs, suggesting that such treatment has a synergistic effect on the release of progesterone. This may have occurred due to the function of melatonin as a protector of the granulosa cells, which are precursors of the corpus luteal as a producer of progesterone, when 100 µg/100g animal body weight was administered orally to rats for 60 days [32]. There was also an antioxidant effect on human follicle cell incubation experiments, and regulating progesterone receptors at a concentration of 100 mg/mL [6]. In the same way metformin administered in a dose of 500 mg/day for 35 days resulted in an increase in plasma progesterone levels in 90% of women undergoing treatment of PCOS, who quickly began to ovulate again [20,23]. In cell culture it was shown that metformin leads to the restoration of progesterone and improves its genetic markers [40]. This explains the occurrence of increased levels of progesterone when treated with melatonin combined with other drugs is used, proving the synergy of the effects of such treatment.

Estrogen levels in the PCOS + Placebo group were high. This was most likely due to the physiological response to this syndrome leading to irregularities in the production of the hormone, or because non-treatment of the syndrome may lead to conversion of androgens to estrogen hormones. In some cases, endometrial cancer can occur because of excessive estrogen, as well as leading to increased oxidative stress and consequently, increased activation of cytokines in the body or fibroids in the uterus [19,22]. This growth hormone also explains the increase in collagen fiber content in rats in the PCOS + Placebo groups, as according to Batista *et al*. [4], fibroblasts, the cells responsible for collagen synthesis, are estrogen dependent, and increase their synthesis in situations with high estrogen levels. Therefore, due to the return of plasma estrogen levels to values similar to the control group, in females treated with a combination of drugs or a single drug, there was a significant reduction in collagen fiber content, mainly in the PCOS + Mel + Met group. In addition, the restoration of estrogen levels to values similar to the control group reduced the time required for pregnancy when compared to the PCOS + Placebo group, which had a longer period. This can be explained by the fact that when rats are subjected to constant illumination for the induction of PCOS, there is a break in estrous cyclicity, establishing an estrous framework with high estrogen levels, necessitating a longer period for the resumption of cyclicity after interruption by light stimulus [27].

Treatment with drugs associated or not, led to a reduction in ovarian size and also to reduce the weight of the ovaries, although they have not reached similar values to those without PCOS. Among the treatments, only the rats receiving metformin hydrochloride remained with the average weight similar to the weight of the ovaries of rats induced the PCOS group that received only placebo. Probably these findings are related to the activities indirectly the metformin hydrochloride in ovarian cells. This is associated with the ability of melatonin to regulate the hypothalamic axis, inhibiting the release of hormones such as follicle stimulating hormone, which aids in the reduction of cysts. Additionally, has anti-inflammatory and antitoxic effects, acts against free radicals, and reduces cytokines that are part of the activation process of the synthesis by fibroblasts [24,25]. Another function of melatonin is to regulate the gonadotropin releasing hormone (GnRH) through its MT1 and MT2 receptors, which are members of receptors coupled to the G 7-transmembrane protein in the granulosa luteal cells, that inhibit GnRH receptor expression and preserve the corpus lutea, and consequently increase progesterone production [21,39]. Thus, the luteal cells remain active and help to reduce ovarian weight by decreasing the content of ovarian polycystic. Although normal reproductive functions are restored, ovarian weight most likely increases because the kinetics of metformin hydrochloride characteristically act directly on cells that secrete ovarian theca. Their modulation involves activation of the AMPK (adenosine monophosphate-activated protein kinase) enzyme, which is an enzyme that acts on the ovaries, promoting the inhibition of CYP17 activity (enzyme 17a hydroxylase-17, 20-lyase). CYP17 is responsible for reducing the conversion of androgens and stabilizing the production of steroid hormones, in addition to promoting increased progesterone production [3,7,8]. The benefits of membership of the drugs were also evident in reducing oxidative stress control and molecular proinflammation factors, as reported by some scientific letters [16,17]. Thus, there was improvement that is more significant in the group with PCOS and treated with melatonin and the group treated with the combination of drugs, showing that the combination of these drugs improves the physiological state of the rats, when compared to rats of the group that received only hydrochloride metformin.

CONCLUSION

Concluded that the proposed drug combination is a viable option for the recovery of cyclicity, reduction of plasma estrogen levels to normal, leading to a reduction in bonding fiber content in the endometrium, and promoting a significant increase in progesterone, making implantation possible. In addition, reducing time required for pregnancy, thereby improving blastocyst-endometrium interaction and resulting in improved fetal development.

MANUFACTURERS

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Ethical approval. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Federal Rural of Pernambuco (Permit Number: 23081.009130/2010).

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***Declaration of interests*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

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