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Review

Dehydroepiandrosterone to induce murine models for the study of polycystic ovary syndrome

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ABSTRACT

During the last decade a battery of animal models used for the study of polycystic ovary syndrome (PCOS) have allowed a focus on different aspects of the pathology. Since dehydroepiandrosterone (DHEA) was found to be one of the most abundant circulating androgens in women with PCOS, a rodent model showing the salient features found in women with PCOS was developed by the injection of DHEA. Although insulin-sensitizing agents, such as biguanides, are clinically used in the treatment of diabetes and PCOS, the complete understanding of their mechanisms of action remains unknown. The present review discusses the molecular mechanisms involved in the development of PCOS by using the DHEA-PCOS murine model and analyzes the role of the biguanide metformin as treatment.

Contents

1. Introduction

The current diagnostic criteria for polycystic ovary syndrome (PCOS) are hyperandrogenism, oligo- or amenorrhea and anovulation [1]. In addition, PCOS is frequently associated with hyperinsulinemia, insulin resistance syndrome, increased cardiovascular risk and diabetes mellitus [1]. Despite its prevalence, little is known about the etiology and pathology of the syndrome. However, during the last decade several clues that may have significant repercussions in the treatment have emerged from human and animal studies. Mahesh and Greenblatt [2] were the first to isolate dehydroepiandrosterone (DHEA) from the ovaries of women with PCOS. After that, Roy et al. [3] produced an animal model for the study of PCOS by injecting DHEA. Subsequent studies established that the DHEA-PCOS murine model exhibits many of the salient features of human PCOS such as hyperandrogenism, insulin resistance, altered steroidogenesis, abnormal maturation of ovarian follicles and anovulation [4–12].

Multiple therapies have been applied in PCOS. Recent studies have investigated the role of a type of insulin-sensitizing agents: the biguanides. The use of *N,N′-dimethyl-biguanide* metformin is becoming increasingly accepted and widespread [[13–16] among others]; however, metformin is being clinically used without a complete understanding of the mechanism involved. This review compiles some of the data on the endocrine and immune aspects that are altered in PCOS by using the DHEA-PCOS murine model and discusses the molecular mechanisms of metformin treatment.

2. Effects of prepubertal hyperandrogenism

In order to assess the effect of hyperandrogenism during the prepubertal stage a PCOS murine model can be obtained by daily

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injection of DHEA (6 mg/kg body weight: 0.75 mg DHEA per mouse) for 20 consecutive days in prepubertal BALB/c mice [9,11]. This dose of DHEA ensures a hyperandrogenized status equivalent to that found in women with PCOS, which is 0.7 mg DHEA in total per day [4–6,9–11]. Histological examination of prepubertal ovaries from the DHEA-treated mice reveals an increase in fat and stromal tissue and enhanced leukocyte infiltration [9]. The ovarian cortex shows an increase in the number of atretic follicles and the formation of no more than two cysts in each ovary. The cysts show a thin layer of theca cells and a compacted formation of granulosa cells with the absence of a vascularized theca interna. As regards the Homeostasis Model Assessment (HOMA) index, prepubertal hyperandrogenism decreases insulin sensitivity [9]. Prepubertal hyperandrogenism alters ovarian functions since DHEA-treated mice show increased serum estradiol and progesterone levels. In addition, the ovarian immunosuppressor prostaglandin E (PGE) is increased when compared with controls [8], which may be a consequence of the altered lipid metabolism, as reported in women with PCOS [17].

Reactive oxygen species (ROS), toxic oxygen-derived products, are generated in all aerobic cells and include production of superoxide radical ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) [18]. Nitric oxide (NO) is an essential metabolite involved in vascular function that controls many physiological processes [19,20]. However, when NO reacts with $O_2^{\bullet}$, it produces a nitrogen-derived product (RNOS), peroxynitrite ($ONOO^{\bullet}$), a more aggressive pro-oxidant species than the NO itself [21,22]. Accumulation of ROS and RNOS produce an uncontrolled lipid peroxidation (LPO) of cell membranes, named oxidative stress. As a consequence of the accelerated lipid metabolism, women with PCOS show increases in the circulating oxidative stress markers, which is responsible for the increased impact of cardiovascular diseases [1,23]. In the ovarian tissue, this increased ovarian oxidative stress leads to the loss of gonadotropin receptors and the loss of ovarian functions [24]. Protection against oxidative stress in cells is provided by enzymes, such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), metabolites, such as glutathione (GSH) and antioxidant vitamins [25]. On the other hand, it has been reported that the oxidant–antioxidant balance is endocrine regulated [21,26]. These antecedents led our group to study whether prepubertal hyperandrogenism was able to induce ovarian oxidative stress and whether this stress in turn impairs ovarian functions. We found that hyperandrogenism increases ovarian lipid peroxidation and decreases CAT activity and GSH content [8,9]. The decreased activity of CAT suggests that hyperandrogenism induces accumulation of $O_2^{\bullet}$ thus leading to the accumulation of $ONOO^{\bullet}$, whereas the decreased GSH content suggests that ovarian antioxidant defenses do not respond to hyperandrogenism, thus increasing lipid peroxidation and impairing ovarian function.

It is well known that the immune and endocrine systems are associated during both physiological and pathological processes. The immune system regulates ovarian functions [27,28] and the T lymphocytes are associated with endocrine pathologies [29,30]. In particular, imbalances in the expression of T lymphocytes and altered concentrations of cytokines have been reported in ovaries of women with PCOS [31,32]. In this context, we found that prepubertal hyperandrogenism increases the cell number of T lymphocytes that infiltrate ovarian tissue and retroperitoneal and axillar lymph nodes [8,9]. In addition, hyperandrogenism modifies the CD8+:CD4+ T ratio since it increases the percentage of CD8+ and decreases the percentage of CD4+ T lymphocyte that infiltrate ovarian tissue and retroperitoneal lymph nodes as compared with controls [8,9]. Our data demonstrate that hyperandrogenism increases the percentage of the cytotoxic CD8+ and diminishes that of the helper CD4+ T phenotype and suggest that the increased production of cytotoxic T cells may contribute to the enhanced oxidative stress observed. In agreement with these findings, the hormonal regulation of the T phenotype has been previously described in other pathologies [33,34]. The fact that hyperandrogenism does not affect either the number or the phenotype of the T cell population that infiltrates axillar nodes suggests that a local inflammatory status may be contributing to a selective differentiation of T cells.

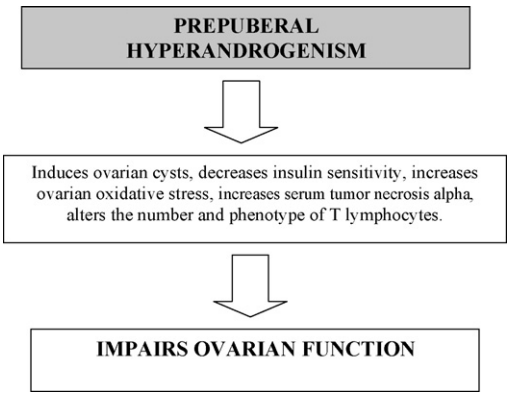

Fig. 1. Effect of hyperandrogenism in prepubertal stage.

In agreement with previous findings [35] we also found that prepubertal hyperandrogenism increases serum tumor necrosis alpha (TNF-α) levels [9]. In fact, a mutation of the TNF receptor associated with hyperandrogenism has been demonstrated [36].

Summarizing, these data indicate that prepubertal hyperandrogenism induces endocrine and immune disturbances that impair ovarian functions (Fig. 1).

3. Hyperandrogenism in early pregnancy induces embryo resorption

DHEA sulfate (DHEAS), the highest circulating steroid, is the precursor and reservoir of DHEA, which, in turn, produces sex steroids. [37]. During pregnancy, DHEAS is the major source for estrogen formation in the fetoplacental unit [38] and DHEA suppresses the immune reactions by regulating cytokine levels, thus ensuring the development of gestation [39–41]. The decrease in maternal DHEAS or abnormally increased levels of DHEA leads to an imbalance of the ovarian function which results in miscarriage [42]. Moreover, levels of androgens higher than those of normal controls are responsible for the detrimental effect on endometrial function that results in recurrent miscarriages [43]. Taken together, these observations led us to study the effect of hyperandrogenism during the implantation process. We worked with two early-pregnant models in mice, the *peri-implantation* and the *post-implantation* model. The *peri-implantation* model consists of the hyperandrogenization s.c. with DHEA (6 mg/kg body weight) from days 2 to 7 of pregnancy, where day 0 is the day of appearance of a coital plug and day 5 is the day on which implantation occurs. The *post-implantation* model consists of two injections of DHEA on days 6 and 7 of pregnancy. In both models, mice are sacrificed on day 8 of pregnancy. The *peri-implantation* and the *post-implantation* models result in 100% and 40% of embryo resorption respectively [10]. In the study of the mechanisms associated with this embryo resorption, we found that, in both models, the excess of androgen in early pregnant mice decreases serum progesterone levels and increases serum estradiol levels [10]. In view of the role of prostaglandins (PGs) in regulating corpus luteum functions [21], we then evaluated ovarian PGE production. In agreement with previous findings [21], both models showed increased levels of PGE and decreased serum progesterone levels compared with controls [12]. The increase in PGE together with a decreased con-

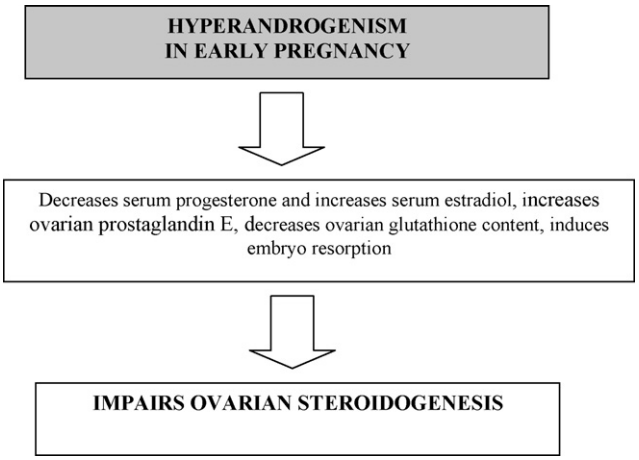

Fig. 2. Effect of hyperandrogenism in early pregnancy.

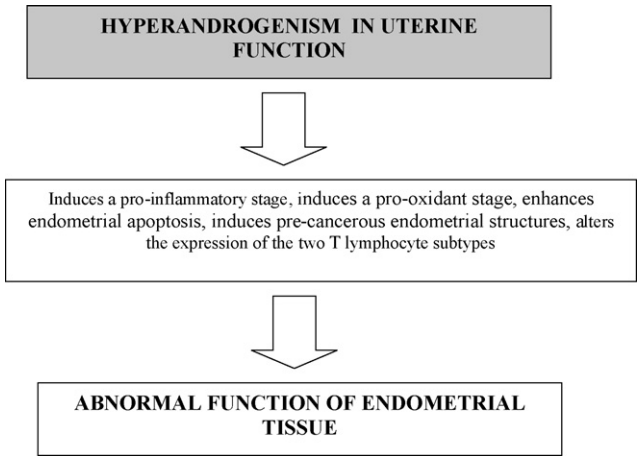

Fig. 3. Effect of hyperandrogenism in uterine function.

tent of the ovarian GSH, the fundamental antioxidant metabolite, suggests that hyperandrogenism during early pregnancy leads to a premature regression of the corpus luteum [12].

Summing up, both the *peri-* and the *post-implantation* models induce embryo resorption and ovarian anomalies that impair steroidogenesis (Fig. 2).

4. Effects of hyperandrogenism in uterine tissue

It has been reported that increased levels of androgens induce detrimental effects on the endometrial response, resulting in miscarriage [42–44], and that women with PCOS have an increased risk to develop endometrial cancer [45]. These data suggest that an excess of androgens alters uterine tissue; however, the potential mechanisms underlying these disorders are complex and await their complete elucidation. For this reason, we designed experiments to study the mechanisms involved in the injurious action of the excess of androgens on uterine functions. We demonstrated that hyperandrogenism induces uterine morphological changes closely related to the development of pre-cancerous structures, concomitantly with an increased incidence of uterine endometrial tissue apoptosis [46]. We also found an enhanced pro-inflammatory stage (shown by increased PGF2α and decreased uterine PGE levels) and a negative feedback mechanism that regulates the expression of uterine cyclooxygenase 2 (COX2) [46,47]. In addition, the excess of androgens promoted a pro-oxidant status since it increased nitric oxide synthase (NOS) activity and decreased SOD and CAT activities and the content of the antioxidant metabolite GSH [46].

Infiltrating as well as resident leukocytes play important roles in the cyclic tissue remodeling events [48]. It is well known that reproductive events that involve uterine tissue, such as menstruation and implantation, are associated with changes in the immune response. In the uterus, decidual CD8+ T cells display a cytolytic activity that regulates the invasion of extravillous trophoblasts, a crucial process for normal uteroplacental development [49]. In the study of the immune response during the hyperandrogenized condition, we demonstrated that hyperandrogenism increases CD4+ and decreases CD8+ T lymphocytes [46,47]. These data suggest that hyperandrogenism during the implantation period generates a deficient expression of CD8+ T lymphocytes that prevents trophoblast invasion [49].

Summing up, the excess of androgens induces endocrine and immune alterations in the uterine tissue and potentially induces pre-cancerous structures (Fig. 3).

5. Role of metformin in the treatment of hyperandrogenism: molecular mechanisms involved

Insulin-sensitizing agents, such as biguanides are used in the treatment of PCOS, without a complete understanding by which metformin increases peripheral insulin. It has been reported that metformin reduces insulin resistance by restoring insulin sensitivity [50–52] and that it regulates ovarian steroidogenesis either directly or indirectly [53,54]. However, controversial results have been reported with regards to metformin and its relationship with the immune system. In patients with type-2 diabetes, this biguanide enhances the tyrosine kinase activity of the insulin receptor by modulating the plasma cell differentiation antigen (PC-1) [55]. Nevertheless, Ruat et al. [56] have failed to demonstrate any relationship between metformin and proliferation of T cells of lymph nodes (Fig. 4).

By using the different DHEA-PCOS murine models, we have studied the efficacy of metformin treatment to reverse the endocrine and immune abnormalities induced by an excess of androgens during different stages of the reproductive age [11]. We found that the oral administration of metformin (50 mg/100 g body weight; a dose equivalent to that used in PCOS patients) to hyperandrogenized BALB/c mice decreases serum insulin (and consequently the HOMA index), progesterone, estradiol and ovarian PGE values to those of control values (Fig. 4). Although metformin is usually used for the treatment of chronic obese, insulin-resistant type-2 diabetic and PCOS patients [50–52], little is known about the role of this drug during conditions of normal glucose. We demonstrated the mechanism by which metformin is able to increase peripheral insulin sensitivity during a normal-glucose condition

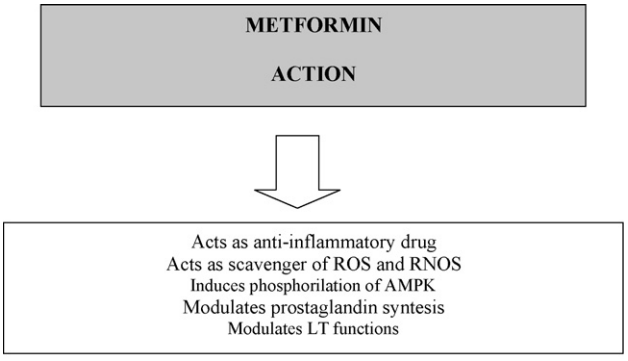

Fig. 4. Molecular mechanisms of metformin action, AMPK: AMP-dependent kinase; ROS: reactive oxygen species; RNOS: reactive nitrogen species; LT: T lymphocytes.

[11], in agreement with reports that described beneficial actions of metformin in non-diabetic women with PCOS without a complete understanding of the mechanism involved [57–59]. Because metformin modulates insulin concentration, which, in turn, controls ovarian steroidogenesis, it was first suggested that metformin acted indirectly on the steroidogenic activity of theca and granulose cells [53]. However, later, Mansfield et al. [54] demonstrated that metformin exerts a direct effect on cultured ovarian cells.

Considering that our findings indicate that hyperandrogenism induces increased oxidative stress [8–12,46,47,60], we were first interested in studying whether metformin decreased the ovarian oxidative stress induced by the excess of androgens and lead it to control values [8]. In the literature, there is no agreement about the relationship between metformin and oxidative stress. It has been reported that metformin is effective in improving antioxidant defenses [61], antioxidant activities in red blood cells from high fructose-fed rats [62,63], hepatic antioxidant levels in rats [64] and in decreasing xanthine oxidase activity in type-2 diabetic patients [65,67]. On the other hand, it has also been described that metformin fails to decrease serum lipid peroxidation in lean patients with PCOS [68] and that it does not scavenge the $O_2^{\bullet}$ and H_2O_2 generated by stimulated human leucocytes [69]. Bonnefont-Rousselot et al. [69] proposed that the efficacy of metformin in scavenging ROS depends on the ROS generator, since they found that neither $O_2^{\bullet}$ nor H_2O_2 can be scavenged by metformin. These findings, together with the fact that our results show that neither the activity of ovarian catalase (CAT) – the enzyme that neutralizes H_2O_2 – nor lipid peroxidation can be modulated by metformin led us to speculate that $O_2^{\bullet}$ and H_2O_2 might be the most abundant oxidant species generated by hyperandrogenized ovaries. We confirmed this hypothesis in a second study where we demonstrated that $O_2^{\bullet}$ and H_2O_2 are the two principal ROS produced by the hyperandrogenized ovary [60] (Fig. 4).

Regarding the role of metformin in regulating RNOS, we found that metformin reverses the increased inducible NOS expression generated by hyperandrogenization with DHEA in the ovary [8]. A cross-talk mechanism between metformin and NO has been described in diabetic rats [70], bovine aortic cells [71] and hepatic cells [72]. However, our data provide novel evidence that metformin regulates the ovarian NO/NOS pathway during the hyperandrogenized condition in ovaries.

Unlike with ROS, there is agreement regarding the modulation of GSH by metformin. It has been reported that metformin regulates GSH levels during *in vitro* maturation of oocytes [73] in the liver of diabetic rats [74], pancreatic islets from type-2 diabetic patients [75,76], erythrocytes from type-2 diabetic patients [66] and during carbon tetrachloride hepatotoxicity in mice [76]. However, our data represent the first evidence that metformin regulates the ovarian GSH content in hyperandrogenized ovaries.

With respect to the immune system, our data constitute the first evidence that metformin acts either directly or indirectly on its regulation. In fact metformin restores the percentages of CD4+ and CD8+ T lymphocyte populations from both ovarian tissue and retroperitoneal lymph nodes [11]. These findings suggest that the restoration of the endocrine status could lead to the restitution of the immune system; however, we have later demonstrated a direct effect of DHEA and metformin in cultured T lymphocytes [77].

A positive correlation has been demonstrated between hyperandrogenism and serum TNF-α in patients with PCOS [28,29]. In agreement with these findings, we found that animals treated with DHEA have higher serum TNF-α levels than controls. It has been reported that TNF-α modulates steroidogenesis of both granulosa and theca-interstitial cells by a mechanism independent of those induced by insulin and insulin-like growth factor-I (IGF-I) [78]. For this reason, we suggest that the increased serum TNF-α levels found in hyperandrogenized mice would be an additional alteration, ahead from hyperinsulinemia, that impairs ovarian steroidogenesis. In agreement with previous findings [79–82], we found that metformin restores TNF-α levels to control levels, thus allowing the animals to recover normal ovarian steroidogenesis and ovulation [11].

It has been reported that metformin activates the AMP-dependent kinase α (AMPK-α) pathway to decrease glucose production, increase fatty acid oxidation and promote the uptake of glucose by cells [71,83,84]. Furthermore, metformin activates the AMPK pathway during states of stress where ATP is depleted [85,86]. In addition, it has been recently demonstrated that metformin activates AMPK via the production of RNOS [87] and a direct cross-talk mechanism between AMPK, NO and metformin has been recently described in bovine aortic endothelial cells [87]. In agreement with these findings, we found that RNOS, ROS, antioxidant defenses and the PG system are involved in metformin action [8]. It is important to point out that NO and the PG system share a number of pathways [88–90]. In fact, it has been demonstrated that NO modulates cyclooxygenase (COX) activity by combining with the heme group of COX [88], and we demonstrated, for the first time, that the aminoguanidine-like activity of metformin both modulates both NOS and COX2 expression [8], possibly by interacting with the heme group of both NOS and COX as it has been previously reported [91]. In agreement with previous findings [69,71,72,83,84,92–95], we demonstrated that oxidative stress and antioxidant defenses are related to the phosphorylation of AMPK [8]. Moreover, we also demonstrated a direct relationship between ROS and metformin in cultured T cells [77].

In summary, our findings show that metformin modulates ovarian oxidative stress (including RNOS) and the PG pathway, and then, the phosphorylation of AMPK (Fig. 4).

A significant number of women with PCOS become pregnant after metformin treatment [50,51,72,59]; however, its use is being conducted without a complete understanding of the mechanisms involved. By using the post-implantation DHEA-injected model previously described, we found that 50 mg/kg body weight of metformin, a dose equivalent to that used in the treatment of PCOS patients [96–99], restores endocrine parameters such as serum estradiol and progesterone levels, circulating glucose and insulin levels and the ovarian oxidative balance by decreasing lipid peroxidation and increasing ovarian antioxidant defences GSH and CAT activity [12]. Estradiol is essential during the implantation process [100], because it induces and maintains the expression of progesterone receptor [101], stimulates endometrial tissue and initiates microvascular permeability and angiogenesis [102]. We also found that metformin restores the increased serum TNF-α level in post-implantation DHEA-treated mice [11]. It is important to note that this cytokine regulates steroidogenesis by acting both on granulosa and theca-interstitial cells by an insulin-independent mechanism [78]. Concomitantly with the embryo resorption induced by hyperandrogenism, we found that uterine NOS activity is decreased and that metformin restores NOS activity to control values [11]. It has been described that NO plays fundamental roles during critical steps of the development of pregnancy, such as, implantation, decidualization, vasodilatation of deciduas, placental and uterine vessels and myometrial relaxation [103,104]. In addition, NO participates in vascular invasion of the trophoblast [105] and controls infection processes during pregnancy [106]. Although it has been previously reported that the relationship between metformin and the NO system restores vascular function [107] and that metformin activates the phosphorylation of AMP-activated protein kinase via the NO pathway [71,72,84], our results represent the first evidence regarding the action of metformin in regulating NOS activity during pregnancy, and in ensuring uterine relaxation.

In the study of metformin's intracellular mechanisms in preventing embryo resorption, we demonstrated for the first time that

hyperandrogenism during early pregnancy prevents the expression of progesterone-induced blocking factor (PIBF) in the implantation sites [108]. We also demonstrated the immunolocalization of PIBF in decidua and trophoblast cells, thus, providing the first evidence that other cell types different from lymphocytes produce PIBF [108]. In that study we also showed that, as a consequence of the decrease in progesterone levels, the expression of PIBF disappears [108]. In fact, it has been previously reported that the neutralization of PIBF results in pregnancy loss [109,110] and, in agreement with those findings, we found a direct correlation between embryo resorption and the lack of uterine PIBF expression. Concomitantly with the restoration of uterine PIBF expression, metformin treatment prevents the reduction of interleukin 6 (IL-6) [108]. It is important to point out that IL-6 is a Th2 type cytokine which induces the synthesis of asymmetric antibodies in a protective mechanism developed during pregnancy [111,112]. Therefore, the failure in the production of IL-6 by trophoblast and decidual cells is fundamentally related to abortions [112]. We also analyzed the cascade of regulations downstream upon the production of PIBF and found that PIBF inhibits the release of arachidonic acid [113] that is converted to PGs by the enzyme COX2. In this context we found a direct effect of hyperandrogenism and metformin in the localization and expression of COX2 (evaluated by immunohistochemical assays and Western Blotting respectively) in the implantation sites. Thus, the implantation sites from hyperandrogenized-pregnant mice show increased expression of COX2 localized both in trophoblasts and in the inner side of lacunae. This effect is prevented by metformin administration.

To sum up, we demonstrated that post-implantation hyperandrogenism results in embryo resorption by: (i) the lack of PIBF production due to a reduced availability of progesterone; (ii) a diminished IL-6 production due to the lack of PIBF; (iii) an increased expression of COX2 and an imbalance in PG production [108].

6. Conclusions

The present study describes the mechanisms by which an excess of androgens impairs the endocrine and immune systems and induces metabolic alterations. The investigations were carried out during different stages of the reproductive period and aim to gain insights into the molecular mechanisms involved in each abnormal condition. We also demonstrated the role of metformin treatment in reversing the alterations induced by the excess of androgens.

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