ORIGINAL RESEARCH ARTICLE

WILEY *Journal of* Cellular Physiology

Myo-inositol and D-chiro-inositol (40:1) reverse histological and functional features of polycystic ovary syndrome in a mouse model

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Abstract

Mice exposed to continuous light undergo functional and histological changes that mimic those of human Polycystic Ovary Syndrome (PCOS). We herein induced the syndrome by exposing 30-day-old females to 10 weeks of permanent light. Ovarian morphology and histology, as well as reproductive parameters (time of observed pregnancy/delivery) were investigated. Ovaries of PCOS-modeled mice showed lack of tertiary follicles and corpora lutea, altered ovarian architecture, and increased thickness of the theca layer. When mice were returned to a normal light-dark regimen for 10 days, a slight, spontaneous improvement occurred, whereas a quick and almost complete recovery from PCOS signs and symptoms was obtained by treating animals with a daily supplementation of 420 mg/kg myo-inositol and D-chiro-inositol (MyoIns/DCIns) in a 40:1 molar ratio. Namely, ovaries from mice treated by this protocol recovered normal histological features and a proper ratio of theca/granulosa cell layer thickness (TGR), suggesting that the androgenic phenotype was efficiently reversed. Indeed, we identified TGR as a useful index of PCOS, as its increase in PCOS-modeled mice correlated linearly with reduced reproductive capability ($r = 0.75$, $p < 0.0001$). Mice treated with a 40:1 formula regained low TGR values and faster recovery of their fertility, with a physiological delivery time after mating. On the other hand, a higher D-chiro-inositol treatment formula, such as MyoIns versus DCIns 5:1, was ineffective or even had a negative effect on clinical-pathological outcomes.

KEYWORDS
continuous light model, D-chiro-inositol, Myo-inositol, PCOS

1 | INTRODUCTION

Polycystic ovary syndrome (PCOS), first described by Stein and Leventhal (1935) as a condition of oligo-amenorrhea and polycystic ovaries accompanied by hirsutism and obesity, is the most common endocrine disorder in reproductive age women. Incidence of PCOS ranges from 5% to 15%, depending on the country and the diagnostic criteria applied (Ehrmann, 2005). The Androgen Excess and PCOS Society recommended in 2006 that PCOS can be identified by presence of hyperandrogenism and/or oligo-ovulation and polycystic ovaries and the exclusion of other related disorders (Azziz et al., 2009).

Besides huge efforts performed to ascertain mechanisms underlying the development of PCOS, its pathogenesis is still a controversial issue

and a comprehensive integrated theory of its pathophysiology is still to be proposed (Tsilchorozidou, Overton, & Conway, 2004). A genetic basis in the etiology of PCOS has been hypothesized by several studies that recorded a higher prevalence of its features in monozygotic than in dizygotic twins (Vink, Sadrzadeh, Lambalk, & Boomsma, 2006). However, evidence gathered to date is weak (Ewens et al., 2010) and the phenotypic heterogeneity observed in siblings points to a contribution of environmental factors, including the presence of endocrine disruptors (Diamanti-Kandarakis, Kandarakis, & Legro, 2006), obesity, and imbalances in diet composition (Carmina, Legro, Stamets, Lowell, & Lobo, 2003). To address this issue and to investigate mechanisms of its development and maintenance, reliable animal models have been proposed but still deserve full validation (Oakley, Lin, Bridges, & Ko, 2011).

Rodents, rats and mice represent the ideal animal species to model human reproductive pathways, for where both normal and abnormal gametogenesis/embryogenesis-related processes are concerned such as in PCOS (Shi & Vine, 2012), including procedures for the treatment of oocytes and preimplantation embryos necessary in cases of infertility (Colazingari, Fiorenza, Carlomagno, Najjar, & Bevilacqua, 2014; Punt-van der Zalm et al., 2009).

To date, more than 20 models have been reported (van Houten & Visser, 2014), half of which do not display sufficient comprehensive traits to represent appropriate reproduction of the PCOS phenotype. Among the remaining ones, some exhibit additional features that are not generally recognized as pertaining to the syndrome (Abbott, Dumesic, Levine, Dunaif, & Padmanabhan, 2006). Incidentally, it is worth noting that PCOS does not occur naturally in rodents (Abbott et al., 2006).

Androgens are considered the main culprit of PCOS, and therefore the most common experimental model makes use of androgen administration to induce symptoms and alterations that represent a good paradigm of hyperandrogenism-dependent PCOS-like syndrome (Paixão, Ramos, Lavarda, Morsh, & Spritzer, 2017).

An alternative experimental model for PCOS induction in rodents is provided by exposing them to a regimen of continuous light. This model is supported by the observation that in humans, women exposed to night light shifts have shortened menstrual cycles, frequently associated with dysmenorrhea (Jain, Jain, Haldar, Singh, & Jain, 2013; Lin, Kripke, Parry, & Berga, 1990), and consistently associated with metabolic syndrome, insulin resistance, and deregulation of glucose metabolism, all of which are recognized risk factors of PCOS (Brum, Filho, Schnorr, Bottega, & Rodrigues, 2015; Sharma et al., 2017).

This maneuver was long ago found to disrupt the estrous cycle (Singh, 1969) by lowering peak levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and progesterone (Takeo, 1984). Disruption of the normal light–dark cycle, which controls the course of the estrous cycle via the suprachiasmatic nucleus of the hypothalamus presumably through modulation of the circadian rhythm of melatonin (Barbacka-Surowiak, Surowiak, & Stoklosowa, 2003), is followed by several changes in endocrine and biochemical patterns (Anisimov et al., 2004), including FSH and LH release (Lowton & Schwartz, 1967).

Exposure of female rats to constant light for even three days unsettles the normal rhythmicity of LH surges, in consequence of the reduction in sensitivities to estradiol and progesterone that are involved with GnRH release from the hypothalamus (Fernando & Rombauts, 2014). Extending the exposure time to 1–6 months results in the appearance of persistent estrus in 24–100% of rats, respectively (Watts & Fink, 1981), the ovaries of which contain follicular cysts and hyperplastic theca-cell layers, but lack corpora lutea (Klochkov & Beliaev, 1977).

PCOS models based on rodents are extremely useful because each model appears appropriate to study some of the complex aspects associated to the different PCOS phenotypes (Singh, 2005). In contrast to hyperandrogenism-based models, the continuous light model may be considered more stressful under a behavioral point of view but less invasive for the lack of pharmacological interventions. Indeed, such a physical mechanism shows advantages in avoiding the off-target effects of hormone inducers, which are likely different from those naturally occurring in human PCOS, whereas producing similar features of PCOS, including irregular cycles, oligo-anovulation, polycystic ovaries, and follicle atresia (Azziz, 2017).

Even though the pathophysiology of PCOS is still unclear, insulin resistance appears to play a central role in its development since patients with PCOS have been shown to suffer from an impairment in intracellular insulin signaling attributed to a defect in the inositolphosphoglycans (IPGs) second messenger pathway that control glucose metabolism (Baillargeon, Nestler, Ostlund, Apridonidze, & Diamanti-Kandarakis, 2008). In the last decades, this finding opened new horizons in the clinical management of PCOS, by increasing tissue availability of inositols, through administration of Myo-Inositol (MyoIns) and D-Chiro-Inositol (DCIns), thus reverting abnormalities observed in the inositol-based pathways, including those based on IPG mediators (Baillargeon et al., 2006). These treatments have recently been shown to be a reliable alternative to conventional hormonal therapies for human PCOS (Colazingari et al., 2014; Dinicola, Chiu, Unfer, Carlomagno, & Bizzarri, 2014; Facchinetti et al., 2015a). Indeed, several lines of evidence suggest that inositol metabolism is severely deregulated in follicle cells of patients with PCOS (Heimark, McAllister, & Larner, 2014), whereas inositol modulates several hormonal pathways and improves both ovary function and oocyte quality (Bevilacqua & Bizzarri, 2016).

Noteworthy, DCIns and MyoIns activity involves different biological mechanisms and both inositol isoforms can be synergistically integrated, by combining them in a ratio corresponding to their physiological plasma relative amount (40:1) (Facchinetti, Dante, & Neri, 2015b). Indeed, several clinical data evidenced that treatments based on this ratio provided promising results in the management of PCOS in humans (Colazingari, Treglia, Najjar, & Bevilacqua, 2013; Dinicola et al., 2014; Facchinetti et al., 2015a).

We have herein induced PCOS in mice by exposing animals to continuous light for 10 weeks. Main experimental objectives were: (a) to validate this murine model of PCOS, by considering its macroscopical/histological features and main cellular functions; and (b) to directly evaluate the different therapeutic response of MyoIns and DCIns administration, provided at different molar ratios (5:1, 20:1,

40:1, and 80:1) for 10 days after the end of the continuous-light induction.

2 | MATERIALS AND METHODS

2.1 | Animals

Mice (Charles River Italia, Calco, VA, Italy) were initially housed in a temperature-controlled facility ($22 \pm 1°C$) on a 12/12 hr light/dark cycle (L/D), inside standard cages with unlimited access to food and water. Fluorescent tubes located in the mouse facility produced an intensity of 350 lux to 1 m from the floor.

All experimental protocols and related procedures were approved by the Italian Ministry of Public Health. All efforts were made to minimize animal suffering, according to the European directive 2010/63/EU.

2.2 | PCOS induction

Thirty-day-old C57BL/6N female mice were used for the experimental induction of PCOS or as control animals. Induction of PCOS was obtained by placing 48 mice in the same housing conditions except for the light cycle that was extended to 24 hr (continuous light, L/L), for 10 weeks. A parallel group of nine mice was kept under normal light/dark conditions and used as controls. Animals were weighed weekly during the conditioning period.

At the end of the period, three mice from both the experimental and the control groups were killed for histological analyses, as described below.

2.3 | Animal treatment with myo-inositol and D-chiro-inositol

Remaining experimental mice were then housed under normal light cycle conditions, randomly divided in five groups, four of which were treated with a combination of MyoIns and DCIns for a total of 420 mg/kg in 2 ml drinking H_2O/day at one of the following respective ratios: 5:1; 20:1; 40:1; 80:1. The fifth group received plain drinking water.

At the end of the 10-day treatment, three mice from each group were killed by cervical dislocation. The following organs were rapidly collected and frozen in a mixture of dry ice/70% ethanol: ovaries, uterus, kidneys, liver, heart, and whole brain. Representative uteri were photographed before freezing. Remaining control mice were kept under normal housing conditions.

2.4 | Histological analysis

For histological analysis, one ovary from each of the killed mice was thawed, fixed in 4% paraformaldehyde (PFA) at 4°C overnight, then washed in phosphate-buffered saline (PBS), dehydrated in 30% sucrose in H_2O and embedded in paraffin. Five or 10 μm thick sections were mounted on gelatinized slides, stained with hematoxylin-eosin (H&E), cover-slipped with Eukitt®, and observed under a light transmission microscope (Leica DMLB, Leica Microsystems GmbH, Wetzlar, Germany). Thickness of theca cell and granulosa cell layers of early tertiary follicles from various sections were measured by using the ImageJ software (ImageJ 1.47v, Wayne Rasband, National Institutes of Health; Washington D.C., http://imagej.nih.gov/ij/).

2.5 | Animal mating

Remaining six mice of each group and six control mice were then mated with adult male C57BL/6 mice in trios (one male and two females) and left undisturbed until delivery. The delivery day for all mice was recorded.

2.6 | Chemicals

Where not stated otherwise, chemicals were purchased from Sigma-Aldrich Co. (St. Louis, Missouri). MyoIns and DCIns were kindly provided by Lo. Li. Pharma (Rome, Italy).

2.7 | Data analysis

Data were analyzed by using χ^2 tests and repeated measures analysis of variance (ANOVA) with Tukey Honestly Significant Differences (HSD) post hoc analyses. It is worth noting that the nonlinear character of dose–response relationships (contemporary presence of both positive and negative effects at different doses) and the natural recovery in time (i.e., water treatment) made the most relevant result the pairwise comparison of different doses with L/L and water condition. The relationship between theca/granulosa cell layer thickness ratio (TGR) and pregnancy time was assessed by a bivariate linear regression model, and its significance was calculated by ANOVA. Statistical analyses were performed using R: A language and environment for statistical computing (R development core team, R foundation for statistical computing, ISBN 3–900051-07-0, 2008, Vienna, Austria. http://www.R-project.org).

3 | RESULTS

3.1 | Increase in mouse weights during the light conditioning period and at the end of 10-Day MyoIns/DCIns treatments

During the light conditioning period, the weights of mice exposed to continuous light increased from 16.58 ± 1.28 g (mean ± SD) to 19.0 ± 1.15 and those of control mice increased from 16.54 ± 1.23 g to 20.46 ± 0.99. Induction of PCOS in our model was associated with a significant reduction (7%) in weight gain (ANOVA, $p < 0.05$).

After 70 days of exposure to continuous light, mice were returned to a regular 12/12 hr light/dark cycle and treated with a combination of MyoIns and DCIns in the molar ratios 5:1, 20:1, 40:1, and 80:1 dissolved in drinking water (total Ins concentration: 420 mg/kg in 2 ml water), for 10 days. This period grossly corresponds to three estrous cycles, resembling the inositol treatment of choice for women undergoing in vitro fertilization cycles, which extends for 3 months (Bevilacqua et al., 2015; Colazingari et al., 2013). Control mice received plain water. The goal of this maneuver was to investigate whether PCOS-induced mice
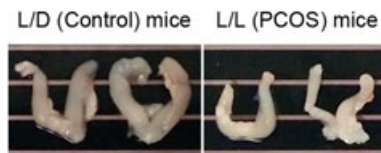
L/D (Control) mice   L/L (PCOS) mice

FIGURE 1 Macroscopic view of mouse uteri at the end of 10 L/L or L/D weeks. Gross morphology of typical uteri from L/L mice (right) and L/D mice (left). Distance between horizontal lines calibrates to 5 mm. Note the thicker appearance of uteri from control mice due to contraction of the tissue compared with the more flaccid appearance of the uteri from PCOS mice. L/L: continuous light; L/D: light/dark cycle; PCOS: polycystic ovary syndrome [Color figure can be viewed at wileyonlinelibrary.com]

initiate a spontaneous recovery trend and MyoIns-DCIns treatments represent a valid rescue approach, as suggested by results obtained in human clinical trials.

During the 10 day period, mouse weights increased as follows: control mice, from 20.46 ± 0.99 g to 21.36 ± 0.96 g (mean $\pm$ SD); mice treated with plain water, from 18.84 ± 1.02 g to 19.52 ± 1.06; mice treated with 5:1 ratio of MyoIns/DCIns, from 18.98 ± 1.12 g to 19.44 ± 1.06; mice treated with 20:1 ratio, from 19.17 ± 1.42 g to 19.55 ± 1.31; mice treated with 40:1 ratio, from 19.02 ± 1.53 g to 19.84 ± 1.27; mice treated with 80:1 ratio, from 18.88 ± 1.01 g to 19.67 ± 1.03. No statistical differences were recorded between mice treated 5:1 and 20:1 MyoIns/DCIns, which increased their weights of approximately 2%. On the contrary, mice belonging to the water, 40:1 and 80:1 groups showed a more pronounced weight recovery of 3.6%, 4.3%, and 4.2%, respectively (ANOVA, $p < 0.05$).

3.2 | Macroscopic aspects of uteri/ovaries and histological features of mouse ovaries at the end of the light conditioning period

After 70 days of continuous light exposure, gross aspect of mouse uteri and ovaries was inspected visually. Although uteri of control mice displayed a proestrus/estrus-like aspect, typical of sexually mature,
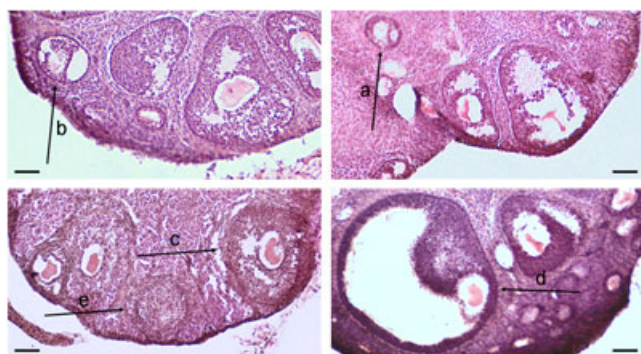

FIGURE 2 Ovarian histology of mice housed under normal L/D cycle conditions for 10 weeks. The ovarian cortex presents primary, secondary and tertiary follicles, as well as corpora lutea. Note the extension of granulosa cell layers in secondary and tertiary follicles. These features are typical of normally cycling mice. (a) primary follicle (b) secondary follicle; (c) tertiary follicle; (d) tertiary antral follicle; (e) corpus luteum [Color figure can be viewed at wileyonlinelibrary.com]
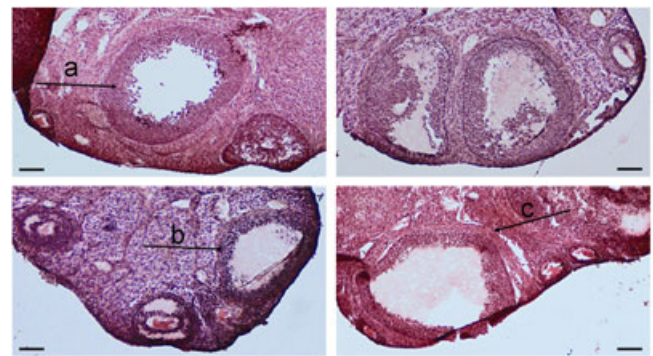

FIGURE 3 Ovarian histology of mice housed under L/L cycle conditions for 10 weeks. The ovarian cortex presents apparently normal primary and secondary follicles, but cystic tertiary follicles devoid of oocytes (a). The granulosa cell compartment (b) in cystic follicles appears reduced when compared with that of control ovaries: as typically observed in human polycystic ovaries. Theca cell layer is grossly hypertrophic (c). Corpora lutea as well as corona radiate are both absent [Color figure can be viewed at wileyonlinelibrary.com]

cycling animals, those of L/L mice had an immature/diestrus-like aspect, typical of noncycling animals (Figure 1). Ovaries from control mice displayed the presence of corpus albicans, products of recent ovulations, and at the histological analysis, showed a normal presence of primary, secondary, and tertiary follicles, as well as of corpora lutea. The thickness of both theca and granulosa cell layers (Figures 2, 4a) was measured and values were used to calculate their respective ratio (TGR) as reported in Table 1. On the contrary, ovaries from L/L mice were smaller and devoid of corpus albicans and, when analyzed histologically, revealed paucity of primary and secondary follicles and the presence of cystic tertiary follicles, strongly resembling those found in human polycystic ovaries (Figure 3). Furthermore, cystic follicles were characterized by the absence of the oocyte and the presence of variable amounts of granulosa cells. It is noticeable that early tertiary follicles containing a living oocyte from these ovaries revealed a hyperplastic theca cell layer and thinner granulosa cell sheet (Figure 4b, Table 1). In addition, nests of luteinized theca cells, resembling features of stromal hyperthecosis (Krug & Berga, 2002), were scattered beneath the ovarian cortex and around cystic follicles.
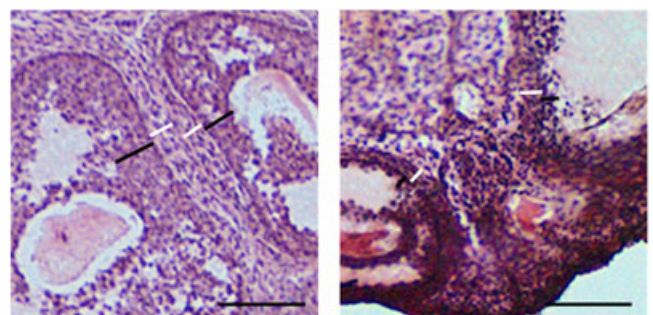

FIGURE 4 Thickness of theca and granulosa cell layers in ovarian follicles from control mice (left panel) and PCOS modeled mice (right panel). The thickness of granulosa (black lines) and theca cell layers (white line) changes abruptly in the two experimental conditions [Color figure can be viewed at wileyonlinelibrary.com]

3.3 | Macroscopic aspects of uteri/ovaries and histological features of light conditioned-mouse ovaries at the end of 10-day MyoIns/DCIns treatments

At the end of the continuous light-exposure period, gross aspects of mouse uteri and ovaries were inspected visually (Figure 5). Uteri of untreated mice and of mice treated with MyoIns/DCIns 5:1 and 20:1 ratios displayed an estrus/diestrus-like aspect suggestive of a still abnormal condition. On the contrary, mice in the MyoIns/DCIns 40:1 ratio treatment group showed complete recovery of the normal uterine morphology and dimension, having full resemblance with that of control animals. Similarly, even in mice treated with the 80:1
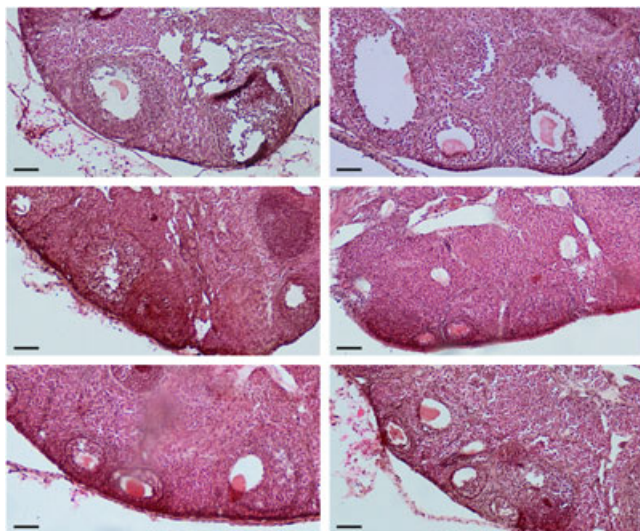
formula, the morphology was almost restored. Uterine features from animals treated with 40:1 (and to a lesser extent from 80:1) MyoIns/ DCIns ratio, were suggestive of animals in which a proper gonadal hormone cycling activity was reestablished.

Macroscopic examination of ovaries allowed detecting further significant details. One control mouse, but none of mice treated with 5:1 and with 20:1 molar ratio MyoIns/DCIns, presented corpora albicantia. Instead, all mice treated with 40:1 molar ratio MyoIns/ DCIns and two of three mice treated with 80:1 molar ratio MyoIns/ DCIns had corpora albicantia in their ovaries (data not shown).

Histological analysis of the ovaries is shown in Figures 6-8.

3.4 | Water-treated PCOS mice

Ovaries from mice that had received plain water (Figure 6 Top) revealed the absence of cystic follicles and the presence of apparently normal secondary and tertiary follicles, although only minimally expanded. The aspect and thickness of theca and granulosa cell compartments (cf. white and black lines in Figure 8 (Control), Table 1) appeared grossly normal, suggesting a partial spontaneous recovery from morphological PCOS signs.

3.5 | MyoIns/DCIns 5:1 group

On the contrary, ovaries from mice that had received MyoIns/DCIns according to the 5:1 ratio displayed reduced cysts but showed an overall atypical and disorganized tissue organization, with scattered primary and secondary follicles and rare tertiary follicles (Figure 6 Center). The stromal compartment was hypertrophic, whereas the theca and granulosa cell interface in rare expanded follicles was coarsely disrupted. In addition, comparison of the aspect and
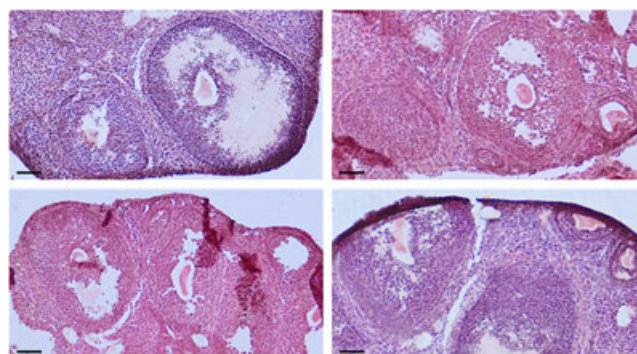

FIGURE 6 Ovarian histology of PCOS-modeled mice after ten-day natural recovery of treatments with MyoIns and DCIns in the 5:1 and 20:1 formulations. (Top) Ovarian sections from mice subjected to no treatment, showing primary: secondary: and tertiary follicles: suggesting partial recovery from PCOS. (Center) Sections from mice subjected to 10-day treatment with MyoIns and DCIns (5:1), showing presence of rare primary follicles, several of which contain atretic oocytes and the absence of both tertiary and cystic follicles, suggesting a reduction in PCOS features but a persisting impairment in folliculogenesis. (Bottom) Sections from mice subjected to 10-day treatment with MyoIns and DCIns (20:1), showing the presence of primary and secondary follicles. Primary follicles were only minimally developed [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 7 Ovarian histology of PCOS-modeled mice after ten-day treatments with MyoIns and DCIns in the 40:1 and 80:1 formulations. (Top) Ovarian sections from mice subjected to 10-day treatment with MyoIns and DCIns (40:1), showing presence of primary, secondary and tertiary follicles, including expanded ones, and corpora lutea. These features suggest a much stronger recovery from PCOS with respect to untreated mice. (Bottom) Sections from mice subjected to 10-day treatment with MyoIns and DCIns (80:1). Although primary, secondary and tertiary follicles are present in these ovaries, the recovery of folliculogenesis is less pronounced with respect to the 40:1 dosage [Color figure can be viewed at wileyonlinelibrary.com]
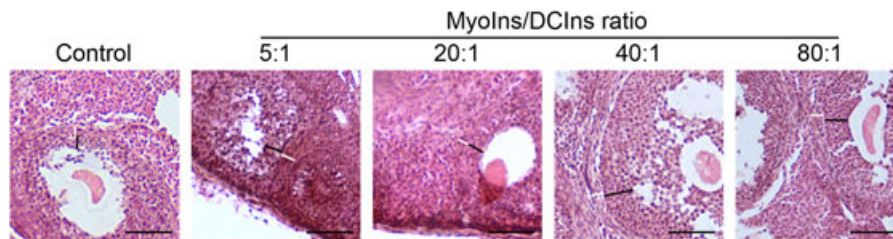
FIGURE 8 Thickness of theca and granulosa cell layers in ovarian follicles from mice after ten-day natural recovery (Control) or treatments with MyoIns and DCIns. (5:1, 20:1, 40:1 or 80:1) The thickness of granulosa (black lines) and theca cell layers (white line) changes depending on administration of various MyoIns and DCIns formulations [Color figure can be viewed at wileyonlinelibrary.com]

thickness of theca and granulosa cell compartments of these follicles, probably all of residual cystic nature, showed a theca layer wider than that observed in normal ovaries (Figure 8 (5:1), Table 1). These findings, when compared with ovarian features of untreated mice, suggested that the specific treatment with the 5:1 formula induces a detrimental effect on PCOS-modeled mice.

3.6 | MyoIns/DCIns 20:1 group

Ovaries from mice that had received MyoIns/DCIns in the 20:1 ratio had scattered developing follicles that reached the initial tertiary stage (Figure 7 Bottom). No cystic follicles were visible in these ovaries. The theca/granulosa layer ratio, even if reduced in respect to values observed in the previous arm, was still higher than normal (Figure 8 (20:1), Table 1).

3.7 | MyoIns/DCIns 40:1 group

Ovaries from mice that had received MyoIns/DCIns with the 40:1 ratio were reminiscent of those of control, normal mice, with follicles

at all stage of development and enlarged Graafian follicles. The thickness of theca and granulosa cell layers was fully restored in these ovaries (Figure 8 (40:1), Table 1).

3.8 | MyoIns/DCIns 80:1 group

Finally, ovaries from mice that had received MyoIns/DCIns with the 80:1 ratio displayed primary, secondary, and tertiary follicle as well, but these were all less expanded and numerous than those observed with the previous formula. Proper theca/granulosa cell layer ratio was partially normalized with this formulation (Figure 8 (80:1), Table 1).

Overall, observations on theca and granulosa cell layers may be summarized as follows: as shown in Figure 9, theca cell layer was significantly enlarged in PCOS ovaries, as expected in an androgenic committed phenotype, whereas granulosa layer was pointedly reduced. Only minor changes (albeit significant) were observed in water-treated group, whereas no changes were noticed in the MyoIns/DCIns 5:1 treated group. Instead, an impressive recovery of normal values in the theca/granulosa ratios was recorded in 40:1 and 80:1 groups. This suggests that formulations with a MyoIns/

TABLE 1 Thickness of theca (TC) and granulosa cell (GC) layers in control, PCOS, and PCOS-treated mice. Six representative early tertiary follicles from mice of different experimental treatments were measured using the ImageJ software

				PCOS mice treated with									
L/D (control) mice		L/L (PCOS) mice		Water		5:1		20:1		40:1		80:1	
TC	GC	TC	GC	TC	GC	TC	GC	TC	GC	TC	GC	TC	GC
0.144	0.256	0.112	0.096	0.096	0.144	0.224	0.192	0.128	0.16	0.16	0.24	0.144	0.256
0.112	0.208	0.16	0.112	0.144	0.176	0.144	0.16	0.16	0.16	0.176	0.32	0.16	0.256
0.096	0.144	0.192	0.16	0.176	0.192	0.208	0.224	0.16	0.128	0.144	0.304	0.16	0.272
0.128	0.24	0.176	0.192	0.16	0.224	0.192	0.192	0.144	0.176	0.112	0.224	0.128	0.24
0.112	0.24	0.208	0.16	0.128	0.144	0.24	0.176	0.176	0.144	0.128	0.224	0.176	0.24
0.160	0.288	0.176	0.16	0.112	0.16	0.208	0.24	0.144	0.16	0.144	0.272	0.144	0.24
TGR													
Mean	0.553		1.185		0.7841*		1.037		0.999		0.548**, ****		0.607**, ***
St. Dev.	0.064		0.174		0.1053		0.192		0.197		0.067		0.069

Note. Values were then converted in μm according to a micrometer eyepiece. The TC/GC Ratio (TGR) (mean ± SD) is reported below. *$p < 0.05$ versus L/L; **$p < 0.001$ versus L/L; ***$p < 0.05$ versus water; ****$p < 0.001$ versus water.
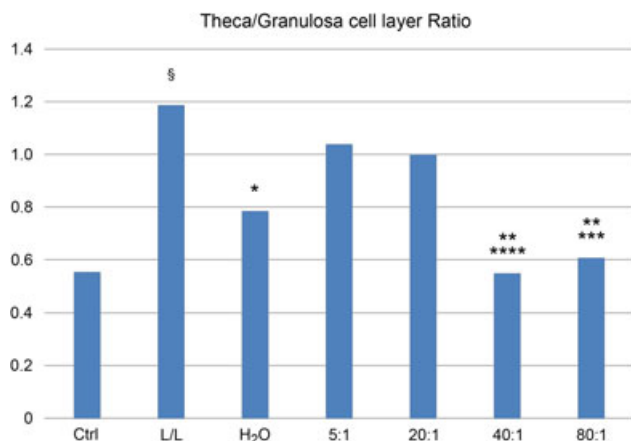
FIGURE 9 Ratio of the Theca-Granulosa cells layer thickness. Ctrl, control; L/L, light-induced PCOS; H_2O: water-treated PCOS mice; PCOS mice treated with MyoIns/DCIns at the indicated formulas: 5:1: 20:1: 40:1 and 80:1. Objects indicate significant differences between groups: $^§p < 0.001$ versus Ctrl; $*p < 0.05$ versus L/L; $**p < 0.001$ versus L/L; $***p < 0.05$ versus H_2O; $****p < 0.001$ versus H_2O [Color figure can be viewed at wileyonlinelibrary.com]

DCIns ratio higher than 20:1 significantly ameliorate PCOS histological features, and pinpoint the 40:1 one as the optimal one for the morphological recovery from PCOS.

3.9 | Pregnancy and delivery of light conditioned-mice mated at the end of 10-Day MyoIns/DCIns treatments

After light conditioning and subsequent 10 day treatments, mice were housed with fertility-proven males for mating and left undisturbed until delivery. The delivery day was finally recorded (Figure 10). In the plain water group, one mouse delivered at day + 32, two mice at day + 38, one mouse at day + 41, one mouse at day + 44, and one mouse at day + 46.

In the 5:1 molar ratio MyoIns/DCIns group, one mouse delivered at day + 48, one mice at day + 52, one mouse at day + 56, two mice at day + 61, and one mouse did not deliver by day + 65.

In the 20:1 molar ratio MyoIns/DCIns group, one mouse delivered at day + 43, one mouse at day + 45, one mouse at day + 49, one mouse at day + 51, one mouse at day + 52, and one mouse did not deliver by day + 65.

In the 40:1 molar ratio MyoIns/DCIns group, two mice delivered at day + 28, one mouse at day + 30, one mouse at day + 33, one mouse at day + 34, and one mouse at day + 37.

In the 80:1 molar ratio MyoIns/DCIns group, one mice delivered at day + 31, one mouse at day + 34, one mouse at day + 37, two mice at day + 40, and one mouse at day + 42.

In the control group, one mouse delivered at day + 23, two mice at day + 25, one mouse at day + 27, one mouse at day + 28, two mice at day + 30, one mouse at day + 31, and one mouse did not deliver by day + 65.
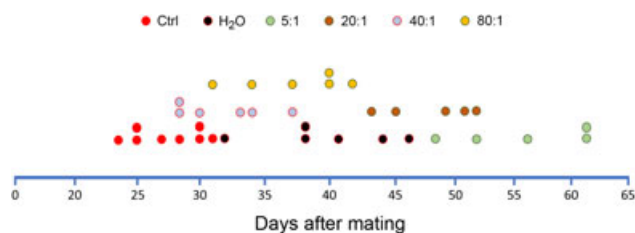

FIGURE 10 Pregnancy in PCOS mice. Occurrence of pregnancy in control and PCOS-modeled mice, both untreated and treated with different MyoIns/DCIns formulations. Pregnancies occurred during a physiological interval time after mating only in control and 40:1 treated groups. Each marble is relative to a single mouse [Color figure can be viewed at wileyonlinelibrary.com]

It is noteworthy that delivery of mice in the 40:1 group was shifted towards the pattern typically observed in control mice ($p < 0.001$ in 40:1 group vs. water [spontaneous recovery], Figure 10). On the contrary, delivery in all other groups was significantly delayed with respect to the water-treated group ($p < 0.001$, in 5:1 and 20:1 group vs. water-treated mice, Figure 10).

Analysis of the two variables by a bivariate linear regression model allowed us to obtain a relationship between TGR and pregnancy time. The two variables were highly correlated (Pearson $r = 0,7511$, $p < 0.0001$), thus evidencing how recovery of the reproductive capability of PCOS-mice is linearly dependent on the normalization of the androgenic phenotype, as epitomized by the TGR index. Figure 11 shows the correlation between the TGR values measured for each group (and, as such representative of a wider, random sample), and time of delivery recorded within similar clusters (normal controls, water-treated, and inositol-treated mice, according to different ratios) of animals. It is worth of note the linear juxtaposition of different experimental groups along the regression
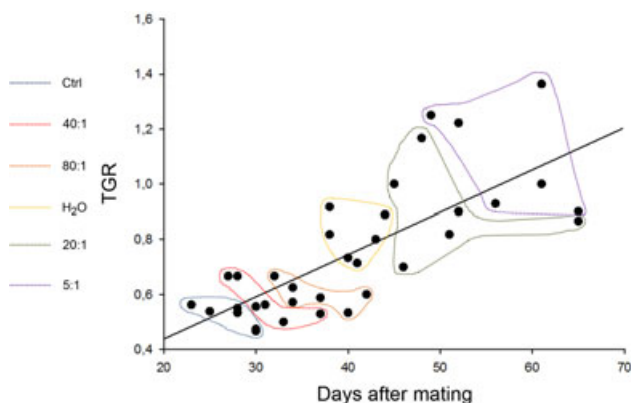

FIGURE 11 Correlation between the Theca/Granulosa cell layer thickness Ratio (TGR) and pregnancy time in PCOS mice. Occurrence of pregnancy in control and PCOS-modeled mice, both untreated and treated with different MyoIns/DCIns formulas, were plotted against TGR values calculated in each cluster (Pearson $r = 0:7511: p < 0.0001$) [Color figure can be viewed at wileyonlinelibrary.com]

line, which provides a proof-of-concept of the relevance of TGR index as a marker of reproductive capability.

4 | DISCUSSION

Results here reported constitute the clinical body of a study on the murine model of PCOS, which includes analysis of cellular and molecular aspects of ovaries and major organs that will be described elsewhere.

Major conclusions of experiments here presented may be summarized as follows.

First, exposure of mice to continuous light represents a simple and reliable procedure to obtain a useful experimental model of human PCOS. Under these conditions, in fact, mice develop ovaries with morphological features typical of human PCOS and the macroscopic aspect of their uteri, resembling those of noncycling animals, strongly suggests that functional gonad activity, in terms of normal steroidogenesis, is also impaired.

Despite the observation that obesity and overweight are common signs of human in PCOS (Norman et al., 2004), mice modeled by the continuous light paradigm displayed a body weight gain significantly reduced with respect to same-aged controls. A similar reduction has been observed in other mouse models (Amini, Tehranian, Movahedin, Tehrani, & Soltanghoraee, 2016). The reduced weight gain may be due to the lipolytic effects of augmented circulation of glucocorticoids or changes in activity of hypothalamic paraventricular nuclei that can prevent food intake in continuous light exposed mice. In any case, women suffering from PCOS are not all overweight or obese and many differences in body weight can be observed among different PCOS populations (Norman et al., 2004).

Second, according to the aforementioned model, PCOS mice spontaneously reverse to a "physiological" cycling condition when returned to a regular light-dark regimen. In our experimental conditions, when their ovaries were observed 10 days after recovery, they displayed presence of developing follicles and if tested for fertility by natural mating, they delivered offspring as early as approximately 40 days after the end of the L/L PCOS induction. Previous observations on the rat PCOS model obtained by continuous light exposure also describe a natural recovery from the syndrome. Takahashi, Ford, Yoshinaga, and Greep (1977) reported the occurrence of ovulation 48 hr after the end of the exposure to continuous light for 8 weeks. On the other hand, Salvetti, Canal, Gimeno, and Ortega (2004) showed that the reproductive ability of rats in which PCOS had been induced through continuous (15 weeks) light exposition, were partially recovered if assessed after 15 weeks of re-exposure to a normal light cycle. In that paper, however, there is no description of a timed pattern of natural recovery from PCOS. Therefore, we do not know how early after the end of the light-conditioning period, the reversion of PCOS symptoms is first observed in rats. As of now, no data of this kind are available for mice. Therefore, our observations represent the first report on this issue. Yet, the time

interval (40 days) we observed is significantly delayed with respect to that typical of age-matched control mice (25 days). This may be the simple result of the physiological recovery of several pathways needed to restore ovarian function and proper fertility after induction of PCOS. Since mice subjected to the complete procedure were almost four-month-old at the time of mating, and therefore close to the time of natural reduction in their fertility (Nagy, Gertsenstein, Vintersten, & Behringer, 2003), this observation would alternatively support the notion that PCOS itself and/or the continuous light-model used for its induction foster reproductive ageing in mice (Anisimov, Vinogradova, Panchenko, Popovich, & Zabezhinski, 2012).

Third, mice suffering from PCOS show a faster recovery of physiological ovarian phenotype as well as a normal reproductive function when treated with a combination of MyoIns + DCIns in the ratio of 40:1 with respect to water alone or other MyoIns + DCIns formulations. A positive, although less pronounced effect, was obtained with the 80:1 MyoIns + DCIns formula. Mice treated accordingly also showed a faster increase in body weight, which correlates with physiological features. In addition, it is impressive how the ratio of theca/granulosa cell layer thickness was fully restored in both the 40:1 and 80:1 MyoIns/DCIns treated animals (Figure 8, Table 1). Yet, the 40:1 formula resulted the most effective in restoring a normal uterus, regular structure of ovaries and follicles, and fertility observed as time of delivery after mating, confirming previous clinical trials in which this ratio has been proven to efficiently rescue women from PCOS major symptoms (Monastra, Unfer, Harrath, & Bizzarri, 2017).

As for the extension of theca and granulosa cell layers in ovarian follicles observed in the various experimental conditions, it is unfortunate that it has been so rarely quantified in histological studies, since it represents a reliable estimation of the shift towards an androgenic-like phenotype occurring in PCOS ovaries (Caldwell et al., 2014), the hypertrophy of the theca cell layer, a hallmark of PCOS, being strictly associated to a greater production of androgens (Gilling-Smith, Willis, Beard, & Franks, 1994).

Our data show that an impressive normalization of TGR was only obtained by treating animals with the 40:1 formula, and outline that quantitative assessment of the TGR may represent a useful parameter for estimating PCOS, and its responsiveness to treatment in experimental models.

Given that obtaining a rapid pregnancy is the ultimate end point of every infertility treatment, this result is of utmost relevance to evaluate the benefit and efficiency of any PCOS treatment. At the same time, it is worth noting that while in both control and 80:1 MyoIns/DCIns groups, intermediate degrees of recovery from PCOS signs at both the morphological and functional levels were observed, no improvement at all was shown by animals treated with MyoIns/DCIns concentrations below the threshold of 40:1.

Even though specific features of mice and humans do not allow translating these results immediately into clinical practice, nevertheless some conclusions can be drawn. (a) MyoIns supplementation of

PCOS-modeled mice for a period grossly corresponding to three ovulatory cycles has a positive effect on ovarian structure and function. This confirms an increasing number of observations obtained both in humans and other species. A positive correlation has been demonstrated in humans between MyoIns content in the serum or follicular fluid, oocyte quality, and pregnancy outcome (Chiu & Tam, 1992; Chiu et al., 2002). Furthermore, clinical trials performed on PCOS patients undergoing in vitro fertilization (IVF), have shown that MyoIns pretreatment beginning three months before the onset of ovarian stimulation produces significant improvements in hormonal responses, reduces the amount of FSH necessary for optimal follicle development, and ameliorates quality of oocytes and resulting embryos (Ciotta et al., 2011; Papaleo et al., 2009).

At the ovarian level, it is established that MyoIns is essential to ensure proper oocyte maturation by modulating the intracellular calcium ion concentration in response to the action of gonadotropins (Matsuda et al., 2009). Supplementation of culture medium with MyoIns increases meiotic maturation of mouse oocytes whereas intracellular depletion of MyoIns desensitizes inositol-dependent transductions pathways and reduces meiotic maturation (Chiu, Rogers, Briton-Jones, & Haines, 2003). In addition, implantation rate and postimplantation viability of embryos resulting from oocytes matured in the presence of MyoIns and fertilized in vitro is also increased (Chiu et al., 2003). More recently, our group has also shown that MyoIns supplementation of in vitro produced and cultured mouse embryos enhances their ability to complete preimplantation development (Colazingari et al., 2014) and to develop to term normally (Kuşcu, Bizzarri, & Bevilacqua, 2016). This mainly occurs by increasing their cleavage rate via serine-473 phosphorylation of PKB/Akt, an enzyme inherited from the oocyte and directly involved in the promotion of blastomere proliferation under control of the PI3K pathway during mid-to-late preimplantation stages (Fiorenza et al., 2008; Luconi et al., 2005). These data may suggest a similar effect of MyoIns on follicle cells, which however warrants further investigation; (b) DCIns administration at high concentration is very likely detrimental in PCOS. Indeed, mice treated with an inositols ratio < 40:1 displayed worse structural and functional outcomes, the severity of which was directly proportional to the DCIns molar ratio administered. These results are apparently at odds with those obtained in the water-treated PCOS group, in which physiological ovarian structures were restored more rapidly and animal delivery was observed with a much shorter delay. In fact, our finding suggests that formulations with high DCIns content, particularly the 5:1 MyoIns/DCIns ratio, are not only ineffective in PCOS, but may be toxic for the ovarian organization and physiology. It is worth noting that similar conclusions have been drawn at the clinical level (Isabella & Raffone, 2012). On the other hand, it is very impressive that all animals treated with a 40:1 ratio showed the most rapid reversion of PCOS, according to both histological and clinical results; (c) administration of DCIns at the proper dose is, however, required. Indeed, the 80:1 MyoIns/DCIns formulation that contained the minimal amount of DCIns was less effective than the 40:1 ratio in terms of both ovarian structure and clinical outcome.

Results herein reported with various MyoIns/DCIns ratios deserve further discussion regarding the specific functions of the two isomers. Although the intracellular inositol pool in the whole body is almost totally represented by MyoIns, the balance in the MyoIns/DCIns ratio, which is maintained by activity of a nicotinamide adenine dinucleotide (NAD), NADH-dependent epimerase that converts MyoIns into DCIns (Bizzarri, Fuso, Dinicola, Cucina, & Bevilacqua, 2016b), is strictly tissue-dependent and reflects specific tissue-related functions. Indeed, MyoIns and DCIns have different physiological roles since the former increases cellular glucose uptake, whereas the latter is crucial for glycogen synthesis (Huang, Fonteles, Houston, Zhang, & Larner, 1993). Consequently, DCIns is present at high levels in glycogen-storage tissues, such as fat, liver, and muscle, but at very low levels in tissues with high glucose utilization, such as brain and heart (Larner, 2002). Accordingly, the ovary does not require high doses of DCIns for its function and the follicular fluid of healthy women contains the two isomers at a ratio of approximately 100 MyoIns:1 DCIns (Unfer et al., 2014).

Interestingly, it has been shown that in the ovaries of patients with PCOS, MyoIns is continuously transformed into DCIns by hyperactivation of epimerase under insulin control (Bizzarri et al., 2016b), that leads to an unbalanced, severely decreased ratio of approximately 0.2 MyoIns:1 DCIns in the follicular fluid (Unfer et al., 2014).

The insulin-dependent increased conversion of MyoIns into DCIns in PCOS ovaries is therefore responsible for MyoIns deficiency that, in turn, would impair oocyte quality. Under these circumstances, an excess of MyoIns can compensate such deficiency, whereas an excess of DCIns not only is ineffective, but also actually worsens PCOS symptoms (Nestler, Jakubowicz, Reamer, Gunn, & Allan, 1999). Indeed, whereas MyoIns exerts numerous actions on cell biochemistry, DCIns is present only within the phosphoinositide pool (as phosphatidyl-DCInositol-phosphate) and it is released as free messenger in the form of inositol-phosphoglycan (IPG). To date, the only recognized function of IPG is to enhance insulin transduction downstream of the insulin interactions with its receptor. Consequently, even if DCIns improves insulin activity at the systemic level (thus reducing insulin values and counteracting insulin resistance), in the ovary DCIns mostly serves as the signal transduction system for insulin's stimulation of human thecal testosterone biosynthesis (Nestler et al., 1998). Furthermore, it has been shown that DCIns treatment at high doses impairs estradiol synthesis, mostly due to the sharp inhibition of aromatase (Sacchi et al., 2016).

Overall, these data suggest that DCIns fosters the androgenic phenotype commitment by inhibiting estrogen synthesis and thus increasing androgen levels.

Finally, it has already been reported that exposure to constant light deregulates, via disruption of the rhythmic melatonin release, the suprachiasmatic nucleus of the hypothalamus, leading to and endocrine imbalance observed in rodent PCOS models (Barbacka-Surowiak et al., 2003). It is unlikely that inositol may antagonize the hormonal imbalance directly by acting on the hypothalamus–pituitary–ovary axis. Inositol are more likely to exert their effect(s)

at the ovarian level. These effects involve modulation of FSH and aromatase activity, steroid hormone synthesis, insulin-mimetic activity, as well as remodeling of cytoskeleton components of both somatic and germ cells (follicles, theca, and granulosa cells; Bevilacqua et al., 2015; Bizzarri et al., 2016a; Morgante, Orvieto, Di Sabatino, Musacchio, & De Leo, 2011).

Although broader investigation is warranted to understand the rescuing activity of other molecules, including melatonin (Takahashi et al., 1977), in the PCOS treatment, further specific studies are needed to investigate how treatment with inositols in the ratio of 40:1 may interfere with steroidogenesis in PCOS animals. This issue is matter of ongoing investigations in our laboratory.

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REFERENCES

Abbott, D. H., Dumesic, D. A., Levine, J. E., Dunaif, A., & Padmanabhan, V. (2006). Azziz, R., Nestler, J. E., & Dewailly, D. (Eds.), *Androgen Excess Disorders in Women* (2nd edn, pp. 259–272). Humana Press.

Amini, L., Tehranian, N., Movahedin, M., Ramezani tehrani, F., & Soltanghoraee, H. (2016). Polycystic ovary morphology (PCOM) in estradiol valerate treated mouse model. *International Journal of Women's Health and Reproduction Sciences*, 4(1), 13–17.

Anisimov, V. N., Baturin, D. A., Popovich, I. G., Zabezhinski, M. A., Manton, K. G., Semenchenko, A. V., & Yashin, A. I. (2004). Effect of exposure to light-at-night on life span and spontaneous carcinogenesis in female CBA mice. *International Journal of Cancer*, 111, 475–479.

Anisimov, V. N., Vinogradova, I. A., Panchenko, A. V., Popovich, I. G., & Zabezhinski, M. A. (2012). Light-at-night-induced circadian disruption, cancer and aging. *Current Aging Science*, 5(3), 170–177.

Azziz, R. (2017). PCOS: Animal models for PCOS - not the real thing. *Nature Reviews Endocrinology*, 13(7), 382–384.

Azziz, R., Carmina, E., Dewailly, D., Diamanti-Kandarakis, E., Escobar-Morreale, H. F., Futterweit, W., ... Witchel, S. F. (2009). The androgen excess and PCOS Society criteria for the polycystic ovary syndrome: The complete task force report. *Fertility and Sterility*, 91, 456–488.

Baillargeon, J. P., Diamanti-Kandarakis, E., Ostlund, R. E., Jr, Apridonidze, T., Iuorno, M. J., & Nestler, J. E. (2006). Altered D-chiro-inositol urinary clearance in women with polycys- 359 tic ovary syndrome. *Diabetes Care*, 29, 300–305.

Baillargeon, J. P., Nestler, J. E., Ostlund, R. E., Apridonidze, T., & Diamanti-Kandarakis, E. (2008). Greek hyperinsulinemic women, with or without polycystic ovary syndrome, display altered inositols metabolism. *Human Reproduction*, 23, 1439–1446.

Barbacka-Surowiak, G., Surowiak, J., & Stokłosowa, S. (2003). The involvement of the suprachiasmatic nuclei in the regulation of estrous cycles in rodents. *Reproductive Biology.*, 3, 99–129.

Bevilacqua, A., & Bizzarri, M. (2016). Physiological role and clinical utility of inositols in polycystic ovary syndrome. *Best Practice & Research Clinical Obstetrics & Gynaecology*, 37, 129–139.

Bevilacqua, A., Carlomagno, G., Gerli, S., Montanino Oliva, M., Devroey, P., Lanzone, A., ... Kamenov, Z. A. (2015). Results from the international consensus conference on myo-inositol and D-chiro-inositol in obstetrics and gynecology—assisted reproduction technology,. *Gynecological Endocrinology*, 31(6), 441–446.

Bizzarri, M., Cucina, A., Dinicola, S., Harrath, A. H., Alwasel, S. H., Unfer, V., & Bevilacqua, A. (2016a). Does myo-inositol effect on PCOS follicles involve cytoskeleton regulation? *Medical Hypotheses*, 91, 1–5.

Bizzarri, M., Fuso, A., Dinicola, S., Cucina, A., & Bevilacqua, A. (2016b). Pharmacodynamics and pharmacokinetics of inositol(s) in health and disease. *Expert Opinion on Drug Metabolism & Toxicology*, 12(10), 1181–1196.

Brum, M. C. B., Filho, F. F. D., Schnorr, C. C., Bottega, G. B., & Rodrigues, T. C. (2015). Shift work and its association with metabolic disorders. *Diabetology & Metabolic Syndrome*, 7, 45.

Caldwell, A. S. L., Middleton, L. J., Jimenez, M., Desai, R., McMahon, A. C., Allan, C. M., ... Walters, K. A. (2014). Characterization of reproductive, metabolic, and endocrine features of polycystic ovary syndrome in female hyperandrogenic mouse models. *Endocrinology*, 155(8), 3146–3159.

Carmina, E., Legro, R. S., Stamets, K., Lowell, J., & Lobo, R. A. (2003). Difference in body weight between American and Italian women with polycystic ovary syndrome: Influence of the diet. *Human Reproduction*, 18(11), 2289–2293.

Chiu, T. T., Rogers, M. S., Briton-Jones, C., & Haines, C. (2003). Effects of myo-inositol on the in-vitro maturation and subsequent development of mouse oocytes. *Human Reproduction*, 18, 408–416.

Chiu, T. T. Y., Rogers, M. S., Law, E. L. K., Briton-Jones, C. M., Cheung, L. P., & Haines, C. J. (2002). Follicular fluid and serum concentrations of myo-inositol in patients undergoing IVF: Relationship with oocyte quality. *Human Reproduction*, 17, 1591–1596.

Chiu, T. T. Y., & Tam, P. P. L. (1992). A correlation of the outcome of clinical in vitro fertilization with the inositol content and embryotrophic properties of human serum. *Journal Of Assisted Reproduction And Genetics*, 9, 524–530.

Ciotta, L., Stracquadanio, M., Pagano, I., Carbonaro, A., Palumbo, M., & Gulino, F. (2011). Effects of myo-inositol supplementation on oocyte's quality in PCOS patients: A double blind trial. *European Review for Medical and Pharmacological Sciences*, 15, 509–514.

Colazingari, S., Fiorenza, M. T., Carlomagno, G., Najjar, R., & Bevilacqua, A. (2014). Improvement of mouse embryo quality by myo-inositol supplementation of IVF media. *Journal Of Assisted Reproduction And Genetics*, 31, 463–469.

Colazingari, S., Treglia, M., Najjar, R., & Bevilacqua, A. (2013). The combined therapy myo-inositol plus D-chiro-inositol, rather than D-chiroinositol, is able to improve IVF outcomes: Results from a randomized controlled trial. *Archives of Gynecology and Obstetrics*, 288, 1405–1411.

Diamanti-Kandarakis, E., Kandarakis, H., & Legro, R. S. (2006). The role of genes and environment in the etiology of PCOS. *Endocrine*, 30, 19–26.

Dinicola, S., Chiu, T. T. Y., Unfer, V., Carlomagno, G., & Bizzarri, M. (2014). The rationale of the myo-inositol and D-chiro-inositol combined treatment for polycystic ovary syndrome. *Journal of Clinical Pharmacology*, 54(10), 1079–1092.

Ehrmann, D. A. (2005). Polycystic ovary syndrome. *New England Journal of Medicine*, 352, 1223–1236.

Ewens, K. G., Stewart, D. R., Ankener, W., Urbanek, M., McAllister, J. M., Chen, C., ... Spielman, R. S. (2010). Family-based analysis of candidate

genes for polycystic ovary syndrome. *Journal of Clinical Endocrinology and Metabolism*, *95*, 2306–2315.

Facchinetti, F., Bizzarri, M., Benvenga, S., D'anna, R., Lanzone, A., Soulage, C., ... Devroey, P. (2015a). Results from the International Consensus Conference on Myo-inositol and d-chiro-inositol in Obstetrics and Gynecology: The link between metabolic syndrome and PCOS. *European Journal of Obstetrics, Gynecology and Reproductive Biology*, *195*, 72–76.

Facchinetti, F., Dante, G., & Neri, I. (2015b). The ratio of MI to DCI and its impact in the treatment of polycystic ovary syndrome: Experimental and literature evidences. *Front Gynecological Endocrinology.*, *3*, 103–109.

Fernando, S., & Rombauts, L. (2014). Melatonin: Shedding light on infertility?--A review of the recent literature. *Journal of Ovarian Research*, *7*, 98.

Fiorenza, M. T., Torcia, S., Canterini, S., Bevilacqua, A., Narducci, M. G., Ragone, G., ... Mangia, F. (2008). TCL1 promotes blastomere proliferation through nuclear transfer, but not direct phosphorylation, of AKT/PKB in early mouse embryos. *Cell Death and Differentiation*, *15*(2), 420–422.

Gilling-Smith, C., Willis, D. S., Beard, R. W., & Franks, S. (1994). Hypersecretion of androstenedione by isolated thecal cells from polycystic ovaries. *Journal of Clinical Endocrinology and Metabolism*, *79*, 1158–1165.

Heimark, D., McAllister, J., & Larner, J. (2014). Decreased myo-inositol to chiro-inositol (M/C) ratios and increased M/C epimerase activity in PCOS theca cells demonstrated increased insulin sensitivity compared to controls. *Endocrine Journal (Kyoto Japan)*, *61*, 111–117.

Huang, L. C., Fonteles, M. C., Houston, D. B., Zhang, C., & Larner, J. (1993). Chiroinositol deficiency and insulin resistance. III. Acute glycogenic and hypoglycemic effects of two inositol phosphoglycan insulin mediators in normal and streptozotocin-diabetic rats in vivo. *Endocrinology*, *132*, 652–657.

Isabella, R., & Raffone, E. (2012). Does ovary need D-chiro-inositol? *Journal of Ovarian Research*, *5*, 14.

Jain, P., Jain, M., Haldar, C., Singh, T. B., & Jain, S. (2013). Melatonin and its correlation with testosterone in polycystic ovarian syndrome. *Journal of Human Reproductive Sciences*, *6*, 253–258.

Klochkov, D. V., & Beliaev, D. K. (1977). Effect of continuous illumination on the reproductive function in rats. *Aftereffects. Ontogenez.*, *8*, 487–496.

Krug, E., & Berga, S. L. (2002). Postmenopausal hyperthecosis: Functional dysregulation of androgenesis in climacteric ovary. *Obstetrics & Gynecology*, *99*(5 Pt 2), 893–897.

Kuşcu, N., Bizzarri, M., & Bevilacqua, A. (2016). Myo-inositol safety in pregnancy: From preimplantation development to newborn animals. *International Journal of Endocrinology*, *2016*, 2413857.

Larner, J. (2002). D-chiro-inositol – its functional role in insulin action and its deficit in insulin resistance. *International Journal of Experimental Diabetes Research*, *3*, 47–60.

Lawton, I. E., & Schwartz, N. B. (1967). Pituitary-ovarian function in rats exposed to constant light: A chronological study. *Endocrinology*, *81*, 497–508.

Lin, M. C., Kripke, D. F., Perry, B. L., & Berga, S. L. (1990). Night light alters menstrual cycles. *Psychiatry Research*, *33*, 135–138.

Luconi, M., Torcia, S., Grillo, D., Fiorenza, M. T., Forti, G., Mangia, F., & Baldi, E. (2005). Enhancement of mouse sperm motility by the PI3-kinase inhibitor LY294002 does not result in toxic effects on preimplantation embryo development. *Human Reproduction*, *20*(12), 3500–3504.

Matsuda, M., Tsutsumi, K., Kanematsu, T., Fukami, K., Terada, Y., Takenawa, T., ... Hirata, M. (2009). Involvement of phospholipase C-related inactive protein in the mouse reproductive system through the regulation of gonadotropin levels. *Biology of Reproduction*, *81*, 681–689.

Monastra, G., Unfer, V., Harrath, A. H., & Bizzarri, M. (2017). Combining treatment with myo-inositol and D-chiro-inositol (40:1) is effective in restoring ovary function and metabolic balance in PCOS patients. *Gynecological Endocrinology*, *33*(1), 1–9.

Morgante, G., Orvieto, R., Di Sabatino, A., Musacchio, M. C., & De Leo, V. (2011). The role of inositol supplementation in patients with polycystic ovary syndrome, with insulin resistance, undergoing the low-dose gonadotropin ovulation induction regimen. *Fertility and Sterility*, *95*, 2642–2644.

Nagy, A., Gertsenstein, M., Vintersten, K., & Behringer, R. (2003). *Manipulating the Mouse Embryo: A Laboratory Manual* (3rd ed.). Cold Spring Harbor Laboratory Press

Nestler, J. E., Jakubowicz, D. J., Reamer, P., Gunn, R. D., & Allan, G. (1999). Ovulatory and metabolic effects of D-chiro-inositol in the polycystic ovary syndrome. *New England Journal of Medicine*, *340*, 1314–1320.

Nestler, J. E., Jakubowicz, D. J., de Vargas, A. F., Brik, C., Quintero, N., & Medina, F. J. (1998). Insulin stimulates testosterone biosynthesis by human thecal cells from women with polycystic ovary syndrome by activating its own receptor and using inositolglycan mediators as the signal transduction system. *Clinics in Endocrinology and Metabolism*, *83*(6), 2001–2005.

Norman, R. J., Noakes, M., Wu, R., Davies, M. J., Moran, L., & Wang, J. X. (2004). Improving reproductive performance in overweight/obese women with effective weight management. *Human Reproduction Update (Oxford)*, *10*(3), 267–280.

Oakley, O., Lin, P. C., Bridges, P., & Ko, C. (2011). Animal models for the study of polycystic ovarian syndrome. *Endocrinology and Metabolism*, *26*(3), 193–202.

Paixão, L., Ramos, R. B., Lavarda, A., Morsh, D. M., & Spritzer, P. M. (2017). Animal models of hyperandrogenism and ovarian morphology changes as features of polycystic ovary syndrome: A systematic review. *Reproductive Biology and Endocrinology*, *15*(1), 12.

Papaleo, E., Unfer, V., Baillargeon, J. P., Fusi, F., Occhi, F., & De Santis, L. (2009). Myo-inositol may improve oocyte quality in intracytoplasmic sperm injection cycles. A prospective, controlled, randomized trial. *Fertility and Sterility*, *91*, 1750–1754.

Punt-van der Zalm, J. P., Hendriks, J. C., Westphal, J. R., Kremer, J. A., Teerenstra, S., & Wetzels, A. M. (2009). Toxicity testing of human assisted reproduction devices using the mouse embryo assay. *Reproductive BioMedicine Online*, *4*, 529–535.

Sacchi, S., Marinaro, F., Tondelli, D., Lui, J., Xella, S., Marsella, T., ... La Marca, A. (2016). Modulation of gonadotrophin induced steroidogenic enzymes in granulosa cells by d-chiroinositol. *Reproductive Biology and Endocrinology*, *14*(1), 52.

Salvetti, N. R., Canal, A. M., Gimeno, E. J., & Ortega, H. H. (2004). Polycystic ovarian syndrome: Temporal characterization of the induction and reversion process in an experimental model. *Brazilian Journal of Veterinary Research and Animal Science*, *41*(6), 389–395.

Sharma, A., Laurenti, M. C., Dalla Man, C., Varghese, R. T., Cobelli, C., Rizza, R. A., ... Vella, A. (2017). Glucose metabolism during rotational shift-work in healthcare workers. *Diabetologia*, *60*(8), 1483–1490.

Shi, D., & Vine, D. F. (2012). Animal models of polycystic ovary syndrome: A focused review of rodent models in relationship to clinical phenotypes and cardiometabolic risk. *Fertility and Sterility*, *98*(1), 185–193.

Singh, K. B. (1969). Induction of polycystic ovarian disease in rats by continuous light. I. The reproductive cycle, organ weights and histology of the ovaries. *American Journal of Obstetrics & Gynecology*, *103*, 1078–1083.

Singh, K. B. (2005). Persistent estrus rat models of polycystic ovary disease: An update. *Fertility and Sterility*, *84*(Suppl 2), 1228–1234.

Stein, I. F., & Leventhal, M. L. (1935). Amenorrhea associated with bilateral polycystic ovaries. *American Journal of Obstetrics and Gynecology*, *29*, 181–191.

Takahashi, M., Ford, J. J., Yoshinaga, K., & Greep, R. O. (1977). Ovulation in light-estrous rats induced by darkness. *Endocrinologia Japonica, 24,* 89–96.

Takeo, Y. (1984). Influence of continuous illumination on estrus cycle of rats: Time course of changes in levels of gonadotropins and ovarian steroids until occurrence of persistent estrus. *Neuroendocrinol, 39,* 97–104.

Tsilchorozidou, T., Overton, C., & Conway, G. S. (2004). The pathophysiology of polycystic ovary syndrome. *Clinical Endocrinology, 60,* 1–17.

Unfer, V., Carlomagno, G., Papaleo, E., Vailati, S., Candiani, M., & Baillargeon, J. P. (2014). Hyperinsulinemia alters myo-inositol to D-chiro-inositol ratio in the follicular fluid of PCOS patients. *Reproductive Sciences, 21,* 854–858.

van Houten, E. L. A. F., & Visser, J. A. (2014). Mouse models to study polycystic ovary syndrome: A possible link between metabolism and ovarian function? *Reproductive Biology, 14*(1), 32–43.

Vink, J. M., Sadrzadeh, S., Lambalk, C. B., & Boomsma, D. I. (2006). Heritability of polycystic ovary syndrome in a Dutch twin-family study. *Journal of Clinical Endocrinology and Metabolism, 91,* 2100–2104.

Watts, A. G., & Fink, G. (1981). Effects of short-term constant light on the-proestrous luteinizing hormone surge and pituitary responsiveness in the female rat. *Neuroendocrinol, 33,* 176–180.