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Does myo-inositol effect on PCOS follicles involve cytoskeleton regulation? ☆

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ABSTRACT

Inositol metabolism is severely impaired in follicles obtained from cystic ovaries, leading to deregulated insulin transduction and steroid synthesis. On the contrary, inositol administration to women suffering from polycystic ovary syndrome (PCOS) has been proven to efficiently counteract most of the clinical hallmarks displayed by PCOS patients, including insulin resistance, hyperandrogenism and oligo-amenorrhea. We have recently observed that myo-inositol induces significant changes in cytoskeletal architecture of breast cancer cells, by modulating different biochemical pathways, eventually modulating the epithelial–mesenchymal transition. We hypothesize that inositol and its monophosphate derivatives, besides their effects on insulin transduction, may efficiently revert histological and functional features of cystic ovary by inducing cytoskeleton rearrangements. We propose an experimental model that could address not only whether inositol modulates cytoskeleton dynamics in both normal and cystic ovary cells, but also whether this effect may interfere with ovarian steroidogenesis. A more compelling understanding of the mechanisms of action of inositol (and its derivatives) would greatly improve its therapeutic utilization, by conferring to current treatments a well-grounded scientific rationale.

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Introduction

Polycystic ovary syndrome (PCOS) is a common metabolic dysfunction and heterogeneous endocrine disorder in women of reproductive age. It is characterized by a clustering of hyperandrogenism, hyperinsulinemia and polycystic ovary, leading to many infertility and pregnancy complications [1]. Impaired oocyte maturation and embryonic developmental competence in PCOS women are likely to be linked with abnormal endocrine and paracrine factors, metabolic dysfunction and alterations in the intra-follicular microenvironment during folliculogenesis. The complex cross-talk between insulin resistance, hyperinsulinemia, and secondary hyperandrogenia leads to a deregulation of several biochemical and epigenetic pathways which, eventually, impair oocyte quality and function [2].

Therefore, a better understanding of how PCOS is related to abnormalities in extra- and intra-ovarian factors [3], and their impact on granulosa cell (GC)–oocyte interactions, oocyte maturation and potential embryonic developmental competence, will be crucial to improving fertility and optimizing clinical stimulation [4].

Myo-Inositol (MYO), D-Chiro-Inositol (DCI) and many other inositol-mono-phosphate derivatives have been thought to participate in the signaling-transduction cascade of insulin [5,6], and in modulating different biochemical pathways within the oocyte [7,8]. In PCOS patients, MYO metabolism is severely deregulated in follicle cells [9,10], while the administration of a proper combination of both MYO and DCI ensures significant clinical outcomes, as demonstrated by an increasing body of clinical

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evidence [11–13]. Indeed, inositol has proven to be an effective treatment for many of the clinical hallmarks displayed by these patients, including insulin resistance, hyperandrogenism, and oligo-amenorrhea [14].

These effects have been deemed a consequence of the modulating activity on insulin, exerted by inositol-phosphoglycans: IPG-P (D-Chiro-Inositol-glycan) and IPG-A (Myo-Inositol-glycan) [15,16]. Yet, it is unlikely that physiological benefits of inositol could only be restricted to its insulin-like activity, given that inositols and their phosphate derivatives display a wide range of pleiotropic effects, including among others, modulation of the PI3K/Akt pathway, calcium release, PKA and PKCδ activity [17–20]. On the other hand, it is worth noting that some reports suggest that MYO may also influence cytoskeleton dynamics.

Cytoskeletal proteins, mainly represented by microfilaments, intermediate filaments and microtubules [21], contribute to the structural integrity of cells and, in addition, participate in cell-to-cell binding, cell proliferation and differentiation [22]. The presence of cytoskeletal proteins is also associated with cellular shape and various cellular functions such as intracellular transport, nucleus-cell-surface interactions and nuclear functions [23].

In oocytes, microtubules make up the mitotic spindle and segregate homologous chromosomes during the first meiotic division and sister chromatids during the second division. Both of these divisions are asymmetric and this feature is ensured by spindle microtubules and actin microfilaments [24].

Microfilaments play an important role in cortical granules (CGs) motility, being required for cortical CG translocation [25]. GCs are further tethered to the cortex by different CSK proteins. During the germinal vesicle breakdown microtubules are condensed around the chromosomes and begin to migrate to the cortex, whereas microfilaments are densely accumulated in the subcortical region, especially around the meiotic spindle and intermediate filaments are arranged into a cortical meshwork. During the last stage of oocyte maturation (MII stage), microtubules and microfilaments mainly accumulate in the cortical cytoplasmic region, whilst intermediate filaments aggregates dispersedly into multiple small spots, and the previously observed large patches can no longer be identified [26]. Late stages in follicle maturation and early luteal transformation in response to the LH pulse are associated with acute progesterone synthesis, tyrosine phosphorylation of actin, decrease in α-actinin, actin, vinculin, α- and β-tubulin, disappearance of cytokeratins 8 and 18, whereas vimentin remains almost unchanged [27,28]. It could easily be hypothesized that equipped with a moldable and contractile cytoskeleton, the oocyte–granulosa cells complex may become particularly active at the time of the LH surge, participating in the rupture of the surface epithelium and in the discharge of the mature oocyte.

Actually, either disruption or stabilization of cytoskeletal fibers strongly affects oocyte maturation [29] as well as follicular steroid hormone secretion [30]. That latter effect deserve a particularly careful consideration given the changes in steroidogenesis that may take place during cystogenesis [31]. Indeed, microtubules in granulosa cells of preovulatory ovine follicles change in close association with the follicular-luteal (i.e., estradiol-progesterone) shift [32]. Acute steroidogenic stimulation of tropic hormone-sensitive adrenal and gonadal cells maintained *in vitro* has been related to cytoskeletal condensation (cell-rounding) and centripetal clustering of organelles is required for steroidogenesis, presumably because both microfilaments and microtubules bring stores of steroid substrate and operative organelles closely together [33,34]. Inhibition of cytoskeleton remodeling, by inducing microfilaments and microtubule stabilization, suppresses both basal and Chorionic Gonadotropin-stimulated progesterone and 17-β-estradiol synthesis in granulosa cells [35], while depolymerization of both components leads to increased production of progesterone and 20-α-OH-progesterone [36]. This stage of follicle maturation is the step impaired in cystic ovaries, as observed ever since the earliest reports [37].

Some reports have observed distinct patterns of expression of cytoskeletal proteins in ovary cells in animals as well as in PCOS patients. Indeed, cyst development in cow ovaries is associated with simultaneous changes in the expression of cytoskeletal proteins required for the formation of cell contacts and the determination of cell shape [38]. Some of these features, including changes in intermediate filaments were also observed during atresia in normal ovaries [39].

In PCOS patients, granulosa cell layers of cystic follicles display significantly higher levels of vimentin and cytokeratins than normal antral follicles, while E-cadherin and N-cadherin are respectively down- and up-regulated, leading to impaired cell-to-cell junctions. Additionally, the cross-talk between follicular cells and their microenvironment is significantly disturbed, as evidenced by changes in the expression of several extra-cellular matrix (ECM) components [40]. ECM changes, in turn, significantly influence the cytoskeleton as well as the shape acquired by ovary cells [41]. The increase in vimentin recorded in granulosa cells has been related either to increased mitotic activity or with follicular atresia and dedifferentiation associated with loss of cell-to-cell contact [42]. However, given that cell proliferation activity in the granulosa and theca *interna* layers decreases in association with the induction of follicular cysts [43], changes observed in vimentin expression should be considered as an expression of dedifferentiation leading to degenerative modification [44]. However, such a change does not involve the basal layer of granulosa cells where cells lose their vimentin filaments, over-express cadherins, acquire keratin, enhancing the establishment of adherent-junctions and escaping so far the apoptotic process [45]. Indeed, high expression of cytokeratins 8 and 18 confers resistance to apoptosis and inhibits cell remodeling needed for follicle maturation [46].

Overall, these data support the hypothesis according to which granulosa cells from the outer mural layer undergo a transition from the classical adult phenotype to a dedifferentiated epithelial-like phenotype (mesenchymal–epithelial transition, MET) [47]. This finding is of outmost relevance as epithelial–mesenchymal transition (EMT) of granulosa cells is deemed to be a hallmark of proper follicle development [48]. Despite their epithelial origin [49] and the maintenance of some epithelial features, granulosa cells acquire some mesenchymal characteristics along the maturation process: they lose their adherens junctions, express N-cadherin instead of E-cadherin and trigger a cytoskeleton rearrangement enabling cell-to-cell permeability, cell migration and shape remodeling [50]. Even steroidogenesis competence is linked to the loss of epithelial markers, and is fully observed in late-stages of granulosa cells maturation [51]. Coupled with changes in the expression of ECM components (including metalloproteinases), these modifications allow granulosa cells to acquire an 'invasive' phenotype, needed for full oocyte maturation. The EMT occurs at an early stage during follicle development and is only completed at ovulation with the rupture of the basal lamina and subsequent formation of the corpus luteum.

The hypothesis

A few but intriguing studies have demonstrated that inositol-monophosphate- and -biphosphate, induce actin synthesis [52] and F-actin ring formation at cell–cell contacts through ROCK-dependent myosin II activation [53]. Even if inositol modulates calcium release from intracellular stores, its action on microfilaments does not depend solely on Ca^{2+} mobilization, as it was previously reported by using Inositol-1,4,5-trisphosphate [54]. Indeed, given

that non-specific protein tyrosine kinase inhibitors nullify the inositol-induced effect on actin-ring formation, it is likely that phosphorylation may play a key role during actin remodeling. Additionally, a very recent report provided evidence of a more complex effect exerted by myo-inositol in modulating MET by down-regulating γ-secretase activity. Indeed, in breast cancer cells, inositol triggers cytoskeleton remodeling, increases focal-adhesion kinases and β-catenin-E-cadherin link underneath the cell membrane, and significantly reduces expression of metalloproteinases (our unpublished observations).

On the basis of data hitherto collected, it can be thereby hypothesized that beneficial effects induced by myo-inositol administration in PCOS patients may also be mediated by an yet unrecognized cytoskeleton-based mechanism, which deserves to be thoroughly investigated.

Evaluation of the hypothesis

Inositol effect on cytoskeleton of oocytes, to our best knowledge, has never been studied until now. To address this issue we propose an experimental approach that would allow this hypothesis to be explored in detail.

A simple and reliable experimental model of cystic ovary is represented by the exposure of mature mice or rats to a constant light. In rodents, the LH surges that trigger ovulation are under the control of a cyclic light–dark photoperiod, on both a daily and seasonal basis [55]. Suppression of this physiological periodicity impairs several endocrine pathways, including estradiol and melatonin-based effects on ovarian tissues. Indeed, continuous light exposure induces LH/FSH and ovary steroid imbalance, eventually leading to cyst formation, characterized by systemic and histological features similar to those observed in PCOS [56,57]. This model is by far the least invasive of all the ones developed till now.

We envisage using female virgin adult rats, 8 weeks old. Before the experiment, the animals will be kept under a controlled cycle of light–darkness (lights on between 6:00 and 20:00), and a temperature of 20–24 °C with free access to water and commercial food. Thirty female virgin adult rats will be used. Twenty of these animals will be placed in the conditions described except for the light cycle, which will be extended to 24 h (continuous light); these conditions will be maintained for 15 weeks. Among this group, ten animal will receive a diet supplemented with both D-chiro-inositol (DCI) and Myo-Inositol (MYO), in the ratio 1:40 (DCI 0.6 mg/kg; MYO 25 mg/kg), given that this is the value expressed in follicular fluid of normal ovaries [10]. A parallel group of 10 animals, under normal environmental conditions, will be used as controls and sacrificed in proestrus to obtain pre-ovulatory antral follicles.

The ovaries will be dissected and fixed in 10% buffered formalin for 6 h at room temperature and washed in phosphate-buffered saline before follicle excision. Confocal and optical investigations, as well as immunohistochemical studies will be performed in order to ascertain differences in the distribution patterns of the diverse components of cytoskeleton: actin, vimentin, α-actinin, phosphorylated-myosin light chain (pMLC), tubulin, and cytokeratins 8/18. E-cadherin and N-cadherin, together with β-catenin, will be investigated and correlated to FAK and cell-to-cell junctions.

In order to correlate the microscopic and histological findings with biochemical pathways, rat oocytes will be cultured in a three-dimensional setting. 3D-oocyte culture models have been proven to be reliable and reproducible tools for studying the dynamical cross-talk among the different cell types participating in the follicle architecture (oocyte, cumulus cells, granulosa cells, internal and external theca cells), as well as ECM and other stroma components [58]. This model may likely allow to investigate cytoskeleton changes altogether with selected pathway deemed to be involved during epithelial–mesenchymal transition (presenilin-1, NF-kB, Notch-1, SNAI, α-sma). Isolation of theca and granulosa cells from normal and PCOS ovary (from both inositol-treated and untreated animals) will allow exploring in depth how steroidogenesis behaves under different conditions. By using specific cytoskeleton inhibitors (colchicines, nocodazole), and/or inducers/inhibitors of inositol-phosphate synthesis (phorbol-myristate/spermine) or other inositol inhibitors, we will attempt to ascertain if cytoskeleton changes could be attributed to inositol and to inositol-induced mechanisms.

Additionally, some questions must be carefully addressed in order to provide a clear-cut mechanistic basis in support of the observed clinical benefit ensured by inositol-based treatments:

- Does the deregulation of inositol metabolism influence cytoskeleton rearrangement in ovary cells from PCOS patients? To answer this question we will investigate whether inositol supplementation triggers epithelial–mesenchymal transition in granulosa cells and/or eventually any other effects on oocyte and theca cells.
- How could this action actually be ascribed to different inositol isoforms (DCI vs MYO), and/or to specific inositol-mono/bis-phosphate derivatives? Biochemical assay using both DCI and MYO at different concentration, as well as dynamical test using selected inhibitors/promoters of inositol phosphokinases, will be warranted to evidence the role different inositol-derivatives are surmised to play. How may the inositol-dependent cytoskeletal changes entail specific gene-based and biochemical pathways (NF-kB, PI3K/Akt, presenilin-1, Notch, GDF-9 and other TGF-β family components)?
- These changes should be eventually correlated to the steroidogenic pathway taking place in both theca and granulosa cells. Particularly, inhibition of inositol-monophosphatases by Lithium chloride or other specific agents (like bisphosphonate L-690,330), is likely to be a highly valuable approach in focusing on the role sustained by inositol-mono, -bis, and tris–phosphate in modulating LH transduction and steroidogenic metabolism [59,60].
- Besides the well-known recognized effects on insulin transduction and insulin-dependent changes in steroidogenesis, the inositol interference on other pathways (PI3K, PKA, GSK3β, Calcium release through Inositol-1,4,5-P) [61,62] is also worth of detailed investigation.

Consequences of the hypothesis and discussion

The proposed study is thought to address whether inositol modulates cytoskeleton dynamics in both normal and cystic ovarian cells, and if such an effect could modify steroid synthesis. Assessment of inositol effects will have huge implications for human fertility given that PCOS is deemed to be a relevant cause of anovulatory infertility. Even if lower than previously recognized [63], PCOS is still associated with high recurrent spontaneous miscarriage rate, indeed [64]. Inositol supplementation has already been proven to ensure a significant clinical benefit: in fact, inositol improves spontaneous ovulation rate and regular menstrual cycles, as well as increases the production of progesterone in the luteal phase of infertile PCOS patients [65]. Yet, a more compelling understanding of its mechanisms of action would greatly improve its therapeutic utilization, by conferring a well-grounded scientific rationale. Moreover, the aforementioned hypothesis, by linking cytoskeleton modifications to oocyte physiology, may shed light into the complex array of cellular effects triggered by inositol

and its mono-phosphate derivatives in other pathological conditions, like cancer, neurological and metabolic diseases [66].

Testing this hypothesis would allow to establish a proper formulation of inositol-based drug to overcome uncertainties and paradoxical results observed with empirical-based compositions or by using only DCI at high doses [67,68]. By no doubt, this purpose, if confirmed, would have a great impact on human reproductive medicine, opening a fertile new investigation into the benefits of inositol supplementation. There is an urgent requirement for new, reliable e low-cost drugs. As aptly stigmatized by D.F. Horrobin "substantial parts of the pharmaceutical industry are failing to innovate at a rate which is needed for their health or for the health of the general public" and "research management needs to be rethought with a [...] greater openness to the information gained from clinical studies" [69]. This "openness" should entail an in depth rethinking of the potential usefulness of natural product. Natural products and their derivatives have historically been invaluable as a source of therapeutic agents. However, in the past decade, research into natural products has declined, due to the emphasis on synthetic and computer-aided drug design. However, failure and high costs encompassed by several class of the new engineered drugs, have led to a renewed interest in natural products in drug discovery [70]. May this be also the case for inositol and its derivatives?

Conflict of interest

All authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence their work.

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