A double-edge sword: the role of D-chiro-inositol in oocyte and embryo quality

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ABSTRACT — *D-chiro-inositol is one of the nine stereoisomers of inositol, a water-soluble cyclic carbohydrate with six hydroxyl groups. Inositol has an essential function in the activation of crucial enzymes in glucose metabolism. In fact, D-chiro-inositol shortage can lead to insulin resistance development. D-chiro-inositol administration in pathologies where insulin resistance is an important factor, i.e. diabetes, has given satisfying results; however in other disorders, i.e. Polycystic ovary syndrome, this stereoisomer can be detrimental for pregnancy. Indeed, high follicular D-chiro-inositol levels in presence of reduced myo-Inositol values are associated with worsened oocyte quality. In conclusion, some recent studies and researches suggest that restoring the physiologic ratio between myo-inositol and D-chiro-inositol is mandatory to protect the efficiency of the reproductive system in women.*

KEYWORDS
Aromatase, D-chiro-inositol, Epimerase, Follicular fluid, Insulin resistance, Myo-inositol, Oocyte, PCOS, Pregnancy, r-FSH, T2DM.

INTRODUCTION

The term "Inositol(s)" defines a family of nine natural stereoisomers that play pivotal roles in cell functions[1]. Chemically they are water-soluble cyclic carbohydrates with six hydroxyl groups. D-chiro-inositol (DCI) belongs to this family of molecules, while myo-inositol (MI) is the most significant and widespread compound among them. Inositol(s) are found in numerous foods, mainly in fruits, cereals, nuts, and animal tissues. In plants, they are normally contained as hexakisphosphate (i.e., phytic acid or phytate)[2]; on the other hand, the human body is able to synthetize MI from glucose 6-phosphate, meanwhile DCI can be obtained from MI by means of an insulin-dependent epimerase, especially in liver, muscle and blood, where the highest conversion rate (from 7% to about 9%) have been recorded[3]. Inside cells, MI and DCI have been detected in their free form or bound to other molecules (Phosphoinositides), giving rise to inositol phosphates and two different inositol phosphoglycans (IPGs), containing either MI or DCI (MI-IPG and DCI-IPG). IPGs enter the cells and influence the metabolism, by activating key enzymes involved in oxidative and non-oxidative metabolism of glucose[4]. The control and regulation of cell processes such as proliferation, fertilization, contraction, metabolism, vesicle and fluid secretion are under MI control through different mechanisms, including the IP3/Ca2+ pathway[5]. The activation of glucose transporters and glucose utilization is influenced by MI, whereas DCI participates in modulating glycogen synthesis[6, 7]. On the other hand, in the ovary, MI regulates glucose uptake and follicle-stimulating hormone (FSH) signaling, while DCI modulates insulin-induced androgen synthesis[8]. In many cell types, MI is the preponderant part of inositol(s) content (more than 99%) and DCI is the remaining one. A considerable variability in MI and DCI concentrations has been highlighted in muscle, fat and liver and it is due to the different activity ratio of the epimerase depending on the specific functions played by each tissue through the two stereoisomers.

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THE DUAL ROLE OF D-CHIRO-INOSITOL IN PCOS

DCI plays a crucial role in the activation of key enzymes in glucose metabolism as DCI deficiency can lead to insulin resistance (IR) development. This was demonstrated in a study that correlated DCI decrease in urinary excretion in patients with reduced glucose tolerance[9] or with type 2 diabetes mellitus (T2DM)[10]. Moreover, DCI amount in skeletal muscles was lower in T2DM patients compared to the healthy ones[10]. *In vivo* studies have supported how DCI administration, in T2DM mice, improves glucose tolerance, and decreases insulin secretion in monkeys with variable degrees of IR[11]. As summarized by Larner[4], DCI administration to T2DM animals (rats, Rhesus monkeys) and later to humans was able to enhance glucose disposal and to sensitize insulin action. The cause underlying such pathology was a defect (reduction) in the epimerization of MI to DCI in insulin sensitive tissues, as shown in vivo the GK type 2 diabetic rats. Thus, it was stated that DCI administration can counteract this metabolic anomaly that ends causing a significant depletion of DCI associated with IR and at least partially restores insulin sensitivity and glucose disposal[4]. Therefore, DCI was seen as a fundamental mediator of insulin signaling, able to improve the sensitivity to this hormone in IR subjects. For such reason, its use was reputed to be recommended for PCOS women, in consideration of the beneficial effects that DCI could have on this pathology[12].

As well-known, Polycystic ovary syndrome (PCOS) is a multifactorial disease that affects 10-15% of women in reproductive age and is the most common cause of infertility, ovarian dysfunction and menstrual irregularity[13]. A direct association was displayed between PCOS and IR development[14], that has been established in about 80% of obese women with PCOS and in about 40% of lean women with PCOS, even if the causes are not completely clear yet[15]. In physiological signaling, insulin acts in synergic way with luteinizing hormone (LH), improving the androgens production in ovarian theca cells[16]. Several studies have highlighted that the insulin signal transduction would seem altered in PCOS women, favoring IR in all the peripheral tissues. However, other studies have displayed that PCOS ovarian cells are even more sensitive to insulin, inducing abnormal ovarian steroidogenesis[17] and increasing the ovarian androgens production[18].

The insulin pathway involves several secondary messengers. Some insulin actions involve the activation of low molecular weight inositol phosphoglycan mediators. The binding of insulin to the receptor activates the hydrolysis of the glycophosphatidylinositol lipids located at the outer leaflet of the cell membrane, causing the release of these mediators, then internalized [12]. Therefore, ovarian hyperinsulinemia increases the theca cells stimulation, by means of the production of inositol phosphoglycan mediators.

The data acquired on the importance due to this molecule prompted to carry out the first clinical trial during which were administered DCI dose of 1200 mg or placebo to PCOS women for 8 weeks. It was seen an increase in the ovulation of 86% in women treated with DCI compared to 27% in women treated with placebo [12]. Then, a large multicenter trial was started by increasing DCI dose to 2400 mg per day. Even if the results of the trial have never been published, a note from the pharmaceutical company, that had started the study, explained how it had not reached a statistical significance of the data [19]. As stated by Nestler and Unfer[20], the higher dose of DCI failed to replicate the findings of previous studies, at least in terms of improving ovulatory frequency, and the lack of efficacy in the latter trial may have been related to the high amount of DCI administered.

Insights on DCI administration in PCOS women have displayed how it can negatively influence the quality of the oocyte. In fact, the progressive increase in DCI dose was found to be directly proportional to the worsening of oocyte quality, and the ovarian response of PCOS women. In particular, compared to the placebo group, oocytes recovered from women treated with high doses were in greater proportion immature oocytes.

As mentioned previously, each tissue has its own MI/DCI ratio reflecting the specific functions. Recently, the hypothesis that the reduced energy metabolism in the ovarian tissue of PCOS women goes to affect the oocyte quality and FSH signaling was confirmed in a study, where the ovarian MI and DCI action was compared. For the first time, it was displayed how MI administration improves the energy metabolism at the ovarian level, as it is responsible for the glucose uptake by the cells. In fact, an important data obtained was the improvement of oocyte quality, consistent with the initial hypothesis. In T2DM patients, the epimerase activity, the enzyme that converts MI to DCI, is greatly reduced, decreasing effectively DCI synthesis[21]. On the other hand, unlike other tissues, such as muscle and liver, the ovary never becomes insulin-resistant, but on the contrary remains insulin sensitive. Therefore, it was speculated that PCOS patients with hyperinsulinemia likely present an enhanced MI to DCI epimerization in the ovary[22], altering the physiological MI/DCI ratio in that tissue and consequently producing MI deficiency and DCI excess. It leads to a decreased MI/DCI ratio (i.e., overproduction of DCI), with a MI deficiency in the ovary, responsible for the poor oocyte quality observed in PCOS women. Furthermore, because MI supplementation reduces the rFSH IU administrated during *in vitro* fertilization (IVF) cycles, it is likely that the putative MI deficiency in the ovary would also impair the FSH signaling, resulting in an increased risk of ovarian hyperstimulation syndrome for PCOS patients. This hypothesis was named as "the D-chiro-inositol paradox in the ovary" [22]. The epimerase upregulation

really induces a greater DCI incorporation, due to its high concentration, into the GPI-phospholipid precursor and/or the GPI-protein precursor, that could be split into INS-2, which has been displayed to enhance insulin sensitivity[23].

The first demonstration of an amplified insulin sensitivity in ovarian cells in PCOS women occurred in 2013. *In vitro* study, ovarian theca cells from healthy patients and PCOS patients were used, comparing both MI and DCI levels, present in follicular fluid (FF), both the speed of the epimerization from MI to DCI. The results obviously confirmed that there is a higher rate of epimerization from MI to DCI in PCOS, given by the stimulation of insulin signaling, in hyperinsulinemia conditions, and by the increased response of the ovarian theca cells caused by the reduced MI/DCI ratio[24]. Such altered conversion caused a rise in the response to insulin signaling, as it acts as a second messenger by reducing cellular hyperglycemia[25]. Interestingly, a clinical trial had displayed how MI administration is able to increase the frequency of spontaneous menstrual cycles and pregnancies in PCOS women[26].

Finally, it was crucial to determine MI and DCI pathological and physiological concentrations[27]. The results identified a MI/DCI ratio in FF of about 0.2:1 in PCOS women and 100:1 in healthy women. In this values range, a 40:1 ratio was identified as probable optimal clinical dose[27, 28].

D-CHIRO-INOSITOL EFFECTS ON OOCYTE AND EMBRYO QUALITY: NEW INSIGHTS

The advance of *in vitro* fertilization methods, Intracytoplasmatic Sperm Injection (ICSI) and IVF, and the more and more widespread use of this technology have allowed the intensification of in-depth studies on the evaluation of oocyte quality. Therefore, the selection of the best oocyte to be seeded is becoming a duty to increase the fertilization success.

For some time, various parameters have been proposed and used for the evaluation of oocyte quality. The morphological parameters, such as the cumulus-oocyte complex, cytosol and polar body, meiotic spindle, ovary and follicle, provide pre-selective information for the identification of high-quality oocytes. Furthermore, cellular and molecular parameters, such as mitochondrial status, oxidative stress in FF and granulosa cells, and other intrinsic factors, provide critical information to establish objective criteria of oocyte quality and increase the efficiency of *in vitro* maturation systems[29].

Evaluating these parameters, a score is assigned to each oocyte, from grade 1 to grade 4[30]. The score allows to establish a sort of qualitative scale, indicating the probability of the oocyte to generate a functional and healthy blastocyst, which can allow and support the implant and the embryonic development.

Among all these factors, FF is very important in oocyte maturation, constituting the micro-environment in which the oocytes grow and develop, and therefore plays a fundamental role in the processes of fertilization and embryogenesis, directly influencing the oocyte quality. For this reason, FF may play a critical role in the oocyte quality. In fact, the concentration of the different components in the FF can increase or decrease the quality of the follicle, probably reflecting the oocyte quality[31]. The chemical constituents of FF can be grouped into different categories, such as hormones, growth factors, Reacting Oxygen Species (ROS), proteins, sugars, anti-apoptotic factors and prostanoids. In these years, recent research has focused on the analysis of these different components of FF by looking for a biochemical marker that can accurately predict the quality of the oocyte. Interesting results were obtained by studying inositol(s) amount, in particular MI, contained into FF. The presence of high inositol concentrations was correlated with elevated levels of estradiol (E2). This correlation was found in the increase of embryo quality, and therefore in the corresponding oocytes[32].

Considering the recent studies, DCI importance for oocyte quality and PCOS therapy has been gradually decreasing. Instead, MI is acquiring an increasingly crucial role.

A recent paper demonstrated that in a mouse model, PCOS histopathological and functional features were almost completely reversed by treating animals with a 40:1 MI:DCI formula, while no effects (or eventually negative results) were observed in mouse treated with highest DCI content[33].

A very interesting result in this sense was given by a recent clinical trial. The study included pretreatment with MI or DCI in PCOS patients who subsequently underwent IVF. The first difference was obtained for the total units of recombinant FSH (r-FSH) used and the days of stimulation significantly reduced in MI group. Even if the total number of oocytes recovered did not differ in both groups, the number of mature oocytes recovered was significantly higher in MI group, and significantly reduced the number of immature oocytes obtained. Finally, the number of grade 1 embryos, and then of pregnancies obtained, was significantly higher with the integration of MI[34].

The decrease in the number of r-FSH units required for ovarian stimulation, with MI supplementation during IVF protocols, had already been observed in other studies[35, 36].

Furthermore, a preclinical study in mice demonstrated that higher concentrations of MI in the germline vesicle significantly increased the percentage of normal post-implantation embryos. The meiotic maturation and the potential for subsequent development correlate with the direct bioavailability of MI. Such evidence was obtained by observing the spon-

taneous intracellular Ca^{2+} fluctuations, which are more frequent in mice subjected to high MI concentration[37]. It is interesting to note that Ca^{2+} plays a key role in the mammalian oocytes maturation [38]. This is because MI is a precursor of phosphoinositide, and the latter activates a primary importance pathway for the mobilization of Ca^{2+} within cells [5].

Further investigations were made on the quality of the blastocyst. A recent study confirmed that a high rate of MI/DCI ratio in FF is related to qualitatively good grade 3 - 4 blastocysts, with satisfying results in IVF [39]. In fact, MI has induced a favorable effect on all parameters analyzed, whereas DCI augmentation showed a detrimental impact. The authors were able to identify a threshold (MI/DCI content in FF = 70:1) that correlates with blastocysts quality. Indeed, values between 70:1 and 100:1 correspond to good quality blastocysts fit to get a successful IVF. In this regard, it is noteworthy that a quite recent study demonstrated that DCI decreases in dose–response manner the expression of the aromatase gene, named CYP19A1[40]. Aromatase is an enzyme involved in the transformation of androgens to estrogens, hence the partial inhibition of its activity ends up causing an increase of androgen levels, which can help to explain the worsening of oocyte and blastocyst quality observed with high DCI levels.

This finding suggests that FF MI/DCI ratio might serve as a promising functional indicator. Based on these studies, the pretreatment with MI in women undergoing *in vitro* fertilization improved the quality of the oocyte and the outcome of Assisted Reproductive Technology (ART) itself. Therefore, MI can be defined as a 'high quality' oocyte biomarker, whereas DCI represents a 'low quality' biomarker.

CONCLUSIONS

Inositol(s) levels in FF can be considered a very important biochemical marker of oocyte quality. Moreover, in these studies it is clear that at the base of some clinical features of PCOS there would be a disequilibrium of MI/DCI ratio. This disequilibrium worsens oocyte quality and compromises FSH signaling. So, the studies that display the positive effects on oocyte quality of DCI should be revisited under this new information. As mentioned previously, the physiological MI/DCI ratio changes according to the considered tissue. In fact, the ovarian tissue is metabolically active, and exploits the energy obtained from the glucose metabolism. Therefore, it requires a high concentration of bioavailable MI and it does not tend to accumulate glycogen by means of DCI action. The ratio identified as optimal is between 70:1 and 100:1 in FF[39] and 40:1 in the circulation of healthy women[41], with unavoidable consequences for a suitable therapy in PCOS[28].

Within the ovary, MI acts as the second messenger about FSH signaling, the glucose activation transporters and its utilization[42], while DCI acts on androgens production insulin-dependent[7]. The ovary is unable to acquire IR, as several indirect studies have displayed, and the elegant direct study obtained by analyzing ovarian theca cells[24].

DCI ovarian toxicity was demonstrated in different studies. DCI administration significantly decreases the oocyte quality, MI on the contrary increases the oocyte quality, FSH sensitivity, the blastocyst quality and the pregnancies number, in addition to partially restoring some clinical PCOS effects.

All these new observations will have, and will give, in the next years, a change of course to the evaluation of oocyte quality. Therefore, they will have many therapeutic repercussions for PCOS treatment. The innovative concept that MI/DCI ratio is a crucial factor in restoring the oocyte quality and ovarian function of these patients to a condition that is close to the physiological one.

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