Protein modification as oxidative stress marker in follicular fluid from women with polycystic ovary syndrome: the effect of inositol and metformin

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Abstract

Purpose The purpose of this study was to evaluate the oxidative stress status (OS) of follicular fluid (FF) and the oocyte quality in women with polycystic ovary syndrome (PCOS) undergoing different ovarian stimulation protocols.

Methods FF samples were collected after gonadotropin administration in association or not with metformin or D-chiro-inositol (DCI). OS status was then evaluated by checking the follicular fluid protein oxidation profile after specific labeling of aminoacidic free-SH groups, and two-dimensional electrophoresis followed by qualitative and semiquantitative analysis. Oocyte quality was assessed by international morphological criteria.

Results Our data indicated that both treatments, even if to different extent, recovered a significantly high level of free-SH groups in FF proteins of PCOS women clearly indicating a decrease of OS level with respect to that found in FF samples from gonadotropins alone treated women. A higher number of

good quality MII oocytes was also observed in DCI ($P<0.05$) or metformin ($P<0.05$) study groups in comparison to untreated control group.

Conclusion A natural supplement and a drug both showed a statistically significant positive effect on follicular milieu by decreasing the oxidative damage on FF proteins, as well as in recovering good quality oocytes.

Keywords PCOS · Oxidative stress · D-chiro-inositol · Metformin · Oocyte morphology

Capsule D-Chiro-inositol and metformin both showed positive effects on follicular milieu by decreasing the oxidative damage on follicular fluid proteins and increasing oocyte quality.

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Introduction

Reactive oxygen species (ROS) are products of the cellular metabolic activity and, at physiological concentration, play important roles at local and systemic levels. Indeed, a whole spectrum of reproductive functions are modulated by ROS such as oocyte maturation, ovarian steroidogenesis, corpus luteum functions as well as fertilization, embryo development and pregnancy [1]. An imbalance between ROS production and the antioxidant defense systems, identifies an oxidative stress (OS) status, which may therefore be defined as a pathological condition caused by the displacement of the physiological balance between the production and the elimination of chemical oxidizing species [1].

OS has been also implicated in some conditions associated to infertility such as PCOS [2], a well known syndrome with systemic and reproductive implications characterized by a series of metabolic dysfunctions. Increasing evidence supports the central role of insulin resistance and compensatory hyperinsulinemia in the pathogenesis of the syndrome [3].

Some studies have consistently found increased concentration of OS markers in the follicular fluid (FF) from obese or PCOS patients when compared to controls and a relationship between increased OS level and poor outcome after in vitro fertilization has been reported [4, 5]. It has been extensively

reported that the effective reduction of insulin resistance obtained with metformin restores regular menstrual cycles, ovulation and improves fertility [6–8]. Interestingly, it is been demonstrated that in PCOS women metformin improves insulin action at least in part by increasing insulin-mediated release of D-chiro-inositol phosphoglycan (DCI-IPG) [9]. Previous studies have shown that oral administration of DCI in PCOS women increased insulin activity, improving ovulatory functions, and decreasing blood pressure and serum concentration of androgens and triglyceride [10–14].

Recently, we suggested that a follicular microenvironment compromised by the establishment of an OS status, may lead to a decline in oocyte quality affecting all the following steps of the reproductive process [15 and references therein]. However, since determination of ROS production in cell-free biological fluids is severely hampered by the poor reliability of the available techniques, we proposed that the assessment of the overall OS may be better identified by studying the accumulation of oxidatively modified proteins as reflected by a decrease of free thiol (–SH) groups, which are mainly present on cysteine residues. Free-SH groups labeling with 3-*N*-maleimidopropionyl biocytin (MPB) followed by two dimensional electrophoresis, and computer assisted analysis of the reactive spots may allow a quantitative and qualitative evaluation of the OS level related to the redox state of the follicular microenvironment. Cysteiny residues are highly susceptible to oxidation, and their eventual loss may represent a sensitive and stable marker of an OS status [15, 16].

The objective of this study was therefore to assess the effect of the administration of D-chiro-inositol or metformin on the OS status of the follicular microenvironment by evaluating the level of free –SH groups in FF proteins and its eventual relationship with oocyte quality as assessed by morphological criteria, in PCOS women undergoing ovarian hyperstimulation protocols.

Materials and methods

Patients

The study was performed at the Center for Couple Sterility of Obstetrics and Gynecology Unit at the University Hospital of Siena and Modena. A total of 68 patients with PCOS undergoing IVF/ICSI treatment for female factor of infertility were included in the study. The diagnosis of PCOS was based on the Rotterdam criteria [17]. Other disorders in which hirsutism and menstrual irregularities are prominent, such as congenital adrenal hyperplasia, Cushing's syndrome, and androgen-secreting tumors were excluded. The physical and demographic characteristics of all recruited women are shown in Table 1. Patients were randomly allocated into three study

groups: PCOS treated with DCI (DCI group), treated with Metformin (metformin group) and treated only with gonadotropin (untreated PCOS group). Randomization was computer generated.

Ethical approval

The study was approved by local ethic committees of Siena and Modena University Hospital; each patient signed a written informed consent for the inclusion in the study and the analysis of follicular fluids.

Pre-treatment protocol

Starting 3 months before ovarian stimulation protocol, a group of 26 women were treated with D-chiro-inositol (Chirofol, LJ Pharma, 500 mg bid) (DCI-PCOS, *n*=26). A second group of 20 PCOS women were treated during the same period with metformin (Met-PCOS) (850 mg twice a day). Finally, a total of 22 untreated PCOS patients were included in the control group (uPCOS).

Ovulation induction

Ovarian hyperstimulation was performed administering recombinant gonadotropins (Gonal F - Merck Serono, Italy and Puregon, MSD, Italy) at a dose of 100–200 IU per day from the 2nd or 3rd day of spontaneous or induced menstruation. The dose of gonadotropins was then adjusted according to ovarian response as detected by ultrasound examination. As soon as the dominant follicle reached 14 mm in diameter, a GnRH antagonist was administered daily (Orgalutran, MSD and Cetrotide, Merck Serono), until the day of ovulation triggering which was obtained by HCG injection (Gonasi 10,000 IU, IBSA Italy) when at least three follicles of size >16 mm were present in the ovaries. The oocyte pick-up was performed 34–36 h after the HCG injection. After oocyte recovering, the FF was immediately centrifuged at 2700 rpm for 10 min, the pellet was discarded, and the supernatant was frozen at –80 °C until use. Only follicular fluid samples with no macroscopic evidence of blood were selected, corresponding to at least 2–5 follicles from each patients.

Oocyte morphology

Oocyte morphology was evaluated according to Alpha Scientist in Reproductive Medicine and ESHRE Special Interest Group of Embryology [18]. Dimorphisms were subdivided into intracytoplasmatic and extracytoplasmatic. In the first case we evaluated the presence of incorporations, refractile bodies, vacuoles, aggregation of the smooth endoplasmatic reticulum and dense granulation. The assessment of extracytoplasmatic dimorphism was based on the first

Table 1 Physical and demographic characteristics of PCOS woman recruited in the study and outcome of the different ovarian hyperstimulation protocols

	DCI+Gonadotropin	Metformin+Gonadotropin	Gonadotropin
N of patients	26	20	22
Age	32.6±4.2	31.9±4.6	33.5±3.4
BMI	24.2±5.1	25.1±4.4	25.2±4.1
Glycemia (mg/dL)	101.5±10	99±10	103±8
Insulin level (mU/l)	12.8±2.2	14±3.1	13.5±2.8
HOMA index	3.20±0.3	3.42±0.5	3.43±0.2
Smokers (%)	22	28	18
Total gonadotropin dosage (IU)	1850±400	1850±400	2050±450
Retrieved oocytes (n)	10.3±2.8	9.8±3.1	9.6±2.4
MII oocytes (n)	8.5±2.5	8.8±2.2	7.2±1.5
High quality oocytes (n)	5.9±1.7 ^a	6.8±1.6 ^b	2.9±0.6 ^{a,b}
Low quality oocytes (n)	2.6±0.7	2.0±0.6	4.3±0.9
Inseminated oocytes (n)	7.5±1.5	7.0±1.8	4.7±1.1
Fertilized oocytes (n)	6.9±1.2	6.3±1.0	4.5±1.3
Evolutionary embryos (n)	5.8±1.3	5.5±2.1	4.2±1.1

Data are presented as Mean± standard deviation. Differences were considered significant when *P*-value <0.005

^{a,b} *P*-value <0.05

polar body morphology, perivitelline space size and granularity, zona pellucida defects and shape anomalies. We considered high quality oocytes these cells without any alterations previously described. All the other were classified as low quality oocytes. The evaluation was carried out by three different embryologists, blinded to the study protocol.

Follicular fluid oxidative stress analysis

Free -SH groups labeling with MPB

This assay was carried out according to a modified protocol as described by de Lamirande and Gagnon [19]. A solution of 3-N-maleimidopropionyl biocytin (MPB; Sigma Aldrich) 1 mM in Tris-HCl 15 mM pH 6.8 was added to equivalent amount of FF proteins, in a final volume of 100 µl. The protein concentration was determined by Bicinchoninic Acid Kit (Sigma-Aldrich, St Louis, MO, USA) following the manufacturer's instruction. The mixture was then heated at 95 °C for 5 min and immediately processed for 2D electrophoresis.

SDS-PAGE

MPB labeled proteins (10 µg) were suspended in sample buffer 4X containing 20 % (w/v) glycerol, 240 mM Tris-HCl, pH 6.8, 8 % SDS (w/v), 8 % (w/v) β-mercaptoethanol and 0.02 % (w/v) bromophenol blue and separated by electrophoresis on polyacrylamide gel according to the method described by Laemmli [20]. The separation gel for the cell Mini Protean (BioRad Microsciences) was prepared with a 6–16 % gradient of acrylamide/bisacrylamide.

Two-dimensional electrophoresis

Two-dimensional electrophoresis was carried out as described below. Briefly, 30 µg of total protein were resuspended in the rehydration solution (REID: 8 M urea, 4 % CHAPS (w/v) bromophenol blue 0.005 % (m/v) mixed with 0.2 % (w/v) immobilized pH gradient (IPG) buffer with a 3–11 nonlinear pH range (IPG buffer, GE Healthcare, Uppsala, Sweden) to a final volume of 140 µl. Samples were loaded onto Immobiline DryStrips (7 cm) with immobilized nonlinear pH gradient, ranging from pH 3 to 11 (GE Healthcare). Isoelectric focusing was performed as described by Görg et al. [21] and Bjellqvist et al. [22]. Isoelectric focused strips were equilibrated for 15 min with 50 mM Tris-HCl pH 6.8 containing 30 % v/v glycerol, 6 M urea, and 2 % w/v sodium dodecyl sulfate (SDS) and for an additional 5 min in the same solution containing 2.5 % w/v iodoacetamide (IAA) and 0.1 % w/v bromophenol blue, and then placed on a 6–16 % polyacrylamide linear gradient SDS gel and subjected to electrophoresis at 25 mA/gel according to Laemmli [20].

Western blotting

For western blotting analysis, proteins were electroblotted from polyacrylamide gels to nitrocellulose (Bio-Rad Laboratories, Hercules, CA, USA) overnight at 180 mA and 500 V in 25 mM Tris-HCl pH 8.3, containing 192 mM glycine, and 20 % v/v methanol according to Towbin et al. [23]. Transferred proteins were reversibly stained with 0.1 % w/v Ponceau S in 3 % v/v acetic acid to check protein transfer. Membranes were blocked with 3 % w/v non-fat dried milk in 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.1 % v/v (TBS) and then incubated with streptavidin conjugated to

peroxidase in TTBS (TBS containing 0.2 % Tween 20) containing 1 % non-fat dry milk (w/v). After washings, reactivity was detected using an Immuno-Star HRP Chemiluminescent kit (Bio-Rad Microsciences) following the manufacturer's instructions and revealed with an XRS instrument ChemiDoc (Bio-Rad Microsciences, Hemel Hempstead, UK). Images were then processed using the Quantity One® 4.5.7 and PDQuest™ 7.4.0 softwares (Bio-Rad Microsciences) for spots identification and quantification as pixel/mm².

Statistical analysis

Statistical analysis was performed with the software Graph pad Prism 4. Parametric or non parametric tests were used when appropriate. Analysis of variance between groups was performed by ONE WAY ANOVA test. P-value levels less than 0.05 were considered statistically significant.

Results

The baseline characteristics of the patients included in the study are reported in Table 1. No differences were found in mean age, body mass index (BMI), insulin levels, HOMA index and smoking status among the study groups. Moreover, the intermediate outcome of IVF cycles were similar in treated or untreated women.

The side effects of the two treatments were significantly different: metformin-treated patients frequently reported nausea, vomiting and diarrhea whereas DCI treated women did not report any adverse effects.

For the analysis of the oxidative stress level equivalent amounts of FF proteins were labeled with MPB, separated by SDS-PAGE and transferred to nitrocellulose in order to highlight the spots related to free-SH protein groups.

As shown in Fig. 1 (Panel A and B), after one-dimensional electrophoresis a similar pattern of protein distribution was detected both in metformin or DCI treated groups in comparison with the uPCOS group, indicating that any protein was lost after our preparation protocol. After two dimensional electrophoresis, avidin-blotting of labeled free-SH residues in FF proteins of DCI-PCOS was carried out. By this procedure we detected numerous spots distributed in a pH range of 4–7 with an apparent molecular weight between 15 and 75 kDa (Fig. 2a–1). The same qualitative analysis of labeled free-SH groups performed in FF proteins of Met-PCOS (Fig. 2a–2), also showed various spots distributed between pH 4 and 7, but with a wide molecular weight ranging from 60 to 200 kDa approximately.

On the other hand, in 2D blot of FF from uPCOS (Fig. 2a–3), a clearly smaller amount of spots related to labeled free-SH residues was detected in comparison to the

Fig 1 Western blotting of MPB labeled follicular fluid proteins from PCOS women treated with DCI (DCI-PCOS), metformin (Met-PCOS) or untreated (uPCOS), after SDS-PAGE electrophoresis

previous analyzed samples (DCI-PCOS and Met-PCOS) with an apparent molecular weight ranging between 37 and 75 kDa approximately. In this experiment, the absence of some of the previously detected spots was due to a loss of free thiol groups on FF proteins, clearly indicating an increased level of oxidative stress.

In order to quantify the decrease in the protein free-SH groups, we used a computer-assisted image analysis of the resulting blots. In each comparison, the evaluation was carried out by assigning a value of 100 % to the sample with the highest total spot intensity originally determined as pixel/mm².

By this approach we established that the total intensity of the spots found in the 2D blot from uPCOS was only 22.2 % of the total intensity found in DCI-PCOS (100 %) and decreased to 10.13 % when the total intensity was calculated respect to Met-PCOS (100 %) samples (Fig. 2b). When we

Fig 2 A: Western blotting of MPB labeled follicular fluid proteins from PCOS women treated with DCI (panel A), metformin (panel B) or untreated (panel C), after two-dimensional electrophoresis. B: In column we reported the Total labeling intensity of protein free-SH groups in the

FF: Comparison between uPCOS and DCI-PCOS or uPCOS and Met-PCOS samples. In each comparison, a 100 % value was assigned to the sample with the highest labeling intensity

compared both treatments with untreated samples, the results clearly indicated that metformin lead to a significant increase ($P < 0.001$) of labeled FF proteins, in comparison with DCI treatment (Fig. 3). Finally, oocytes quality was investigated. No statistically significant differences among the three study groups were found in the mean number of retrieved MII oocytes, although in DCI or metformin treated patients a slightly greater number of mature oocytes was recovered. MII oocyte quality was classified as previously described in “high” and “low”. As reported in Table 1, a significant higher number of “high quality” oocytes was obtained from women treated with gonadotropin and DCI in comparison with patients treated with gonadotropin alone ($P < 0.05$). In patients treated with gonadotropin and metformin, a significant higher number of top quality oocyte ($P < 0.05$) was also found.

Discussion

The present study demonstrated at molecular level the antioxidant action of DCI in follicular fluid of women

Fig. 3 Total labeling intensity of protein free-SH groups in the FF: comparison among uPCOS, DCI-PCOS and Met-PCOS samples

with PCOS and that this activity is comparable to that induced by metformin, an oral antidiabetic drug of the biguanide group.

It is well known that oxidative damage can lead to alteration and impairment of several cellular macromolecules, resulting in cell death [24]. In this context the cellular damage seems to be directly correlated to cellular ROS concentration. Usually, cells are able to cope with ROS through different protective mechanisms that prevent the generation of reactive species, or remove them before they can fatally damage cells [25]. The main target of free radicals are lipids, nucleic acids, carbohydrates and proteins. As a result of oxidative reactions, proteins undergo proteolysis, structural changes, aberrant aggregation and oxidation of the side chains of amino acids [26, 27]. Indeed, modifications of protein molecular weight due to the formation of intramolecular bonds and their cleavage into peptides have been consistently reported [26, 27]. Sulphur-containing amino acids such as cysteine and methionine are more susceptible to ROS attack [28]. Thiols, in fact, have the characteristic to be oxidized in an almost irreversible manner and this identifies them as key components in the mechanism involved in redox balance. In humans, research on ROS and oxidative stress is hampered by methodological difficulties in assessing the levels of markers of oxidative stress in biological samples [29, 30]. In particular, the direct evaluation of the level of oxidative stress in cell-free biological fluids is very difficult since they lack ROS producing cells. The effect of ROS, however, can be demonstrated indirectly, for example through the evaluation of the oxidative damage of proteins, as we previously did in human seminal plasma through the analysis of free -SH groups [31] thus confirming that this methodology has to be considered as a reliable method for the indirect evaluation of the OS status [15, 16]. Recently, we also

reported preliminary results on the quantitative analysis of free-SH groups of proteins in human FF [32].

Whereas the role of ROS in the reproductive male function has been widely studied [33–37], only in last few years attention of researchers has been also focused on the female reproductive function. It is believed that ROS over-production in the reproductive tract may significantly contribute to woman infertility mainly because of the ability of these molecules to exert a toxic effect decreasing oocyte quality [2, 38, 39]. The follicular fluid may be seen as a “biological window” which reflects all metabolic processes and hormonal microenvironment in which the oocyte matured [40]. In the follicular microenvironment, besides granulosa cells, leukocytes and macrophages can also be found and all these cell types are able to produce ROS [41]. Furthermore, the expression of antioxidant enzymes in cells that produce steroids (granulosa, thecal and luteal cells) was also reported [42, 43]. This suggests that the oocyte is physiologically exposed to oxidative stress and to antioxidant activity both present in the FF [44].

Several studies have shown that hyperglycemia increases ROS production by peripheral blood leukocytes [45–47]. The resulting oxidative stress can contribute to a pro-inflammatory status that may enhance insulin resistance and hyperandrogenism in women with PCOS [48–50].

In the present study, we analyzed the effect of DCI and metformin, two well known insulin-sensitizing agents, on the oxidative stress status in follicular fluid of women with PCOS. Since –SH groups are highly sensitive to oxidative attack the molecular damages induced by ROS were investigated by labeling the free thiol groups of proteins with MPB followed by one- and two dimensional electrophoresis, avidin-blotting and computer assisted image analysis. The present results revealed a significant increase in protein free-SH groups in the follicular fluid of women treated with DCI or metformin when compared to untreated PCOS women. This study for the first time detected, at molecular level, the activity of metformin and DCI on OS reduction of follicular fluid in PCOS women. The protein free-SH groups resulted significantly increased after both treatments, with more possibility to obtain high quality oocytes. Even if a natural supplement as DCI and a synthetic drug as metformin have a similar effect on OS, our data show that metformin induces a more significant increase of the level of free-SH group in FF proteins. Nevertheless, these results are really interesting because it is well known that many patients treated with metformin referred serious side effect such as nausea, vomiting, gastric pain leading to interruption of therapy, whereas DCI did not have any adverse consequence. Moreover, the choice of a natural substance is much more accepted by patients and clinicians, who consider metformin only an antidiabetic drug and other uses are considered “off label”.

A compromised follicular microenvironment due to an increased OS status, may thus lead to a decline in oocyte quality, as frequently reported in women with PCOS. In this study, we tried to assess this assumption by also evaluating the oocyte characteristics in PCOS women according to Alpha Scientist in Reproductive Medicine and ESHRE Special Interest Group of Embryology [18]. The administration of DCI as well as metformin seems able to restore a physiological OS status, and improve oocyte quality.

As regard to DCI, our results could be explained by the role of this IPG containing-molecule as a substrate of phosphatidylinositol 3-kinases (PI3Ks) an enzyme involved in fundamental pathways for regulation of survival and activation of primordial follicles, proliferation and differentiation of granulosa cells in response to gonadotropins and resumption of meiotic process [51 and references therein]. Indeed, DCI by contributing to granulosa cell health, may improve oocyte maturation and quality.

Conclusions

The present study was conducted to evaluate OS of follicular fluid and oocyte quality in PCOS women undergoing different ovarian stimulations with gonadotropins in association or not with metformin or DCI. Our results show that both treatment induce a significantly high level of free-SH groups in FF protein of PCOS women and clearly indicating a strong significant decrease of OS level with respect to that found in FF samples from gonadotropins alone treated women. In conclusion DCI and metformin, both treatments together gonadotropins showed a similar positive effect on follicular fluid by decreasing the oxidative damage on FF protein, as well as in recovering good quality oocytes.

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