

CLINICAL STUDY

Inositol administration reduces oxidative stress in erythrocytes of patients with polycystic ovary syndrome

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Abstract

Objective: Possibly due to a deficiency of insulin mediators, polycystic ovary syndrome (PCOS) is often associated with insulin resistance (IR) and hyperinsulinemia, likely responsible for an elevated production of reactive oxygen species. We investigated oxidative-related alterations in erythrocytes and anti-inflammatory effects of inositol in women with PCOS before and after treatment with myo-inositol (MYO).

Methods: Twenty-six normal-weight PCOS patients were investigated before and after MYO administration (1200 mg/day for 12 weeks; $n=18$) or placebo ($n=8$) by evaluating serum testosterone, serum androstenedione, fasting serum insulin, fasting serum glucose, insulin area under the curve (AUC), and glucose AUC after oral glucose tolerance test and homeostasis model of assessment-IR. In erythrocytes, band 3 tyrosine phosphorylation (Tyr-P) level, glutathione (GSH) content, and glutathionylated proteins (GSSP) were also assessed.

Results: Data show that PCOS patients' erythrocytes underwent oxidative stress as indicated by band 3 Tyr-P values, reduced cytosolic GSH content, and increased membrane protein glutathionylation. MYO treatment significantly improved metabolic and biochemical parameters. Significant reductions were found in IR and serum values of androstenedione and testosterone. A significant association between band 3 Tyr-P levels and insulin AUC was found at baseline but disappeared after MYO treatment, while a correlation between band 3 Tyr-P and testosterone levels was detected both before and after MYO treatment.

Conclusions: PCOS patients suffer from a systemic inflammatory status that induces erythrocyte membrane alterations. Treatment with MYO is effective in reducing hormonal, metabolic, and oxidative abnormalities in PCOS patients by improving IR.

European Journal of Endocrinology 166 703–710

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine–metabolic diseases, affecting 6–10% of women of reproductive age (1). It is characterized by hyperandrogenism, hirsutism, and oligo- or anovulation. Increasing evidence supports the central role of insulin resistance (IR) and compensatory hyperinsulinemia in the pathogenesis of the syndrome (2) and in patients' increased risk of developing dyslipidemia, hypertension, impaired glucose tolerance, type 2 diabetes mellitus, and cardiovascular disease (3). Some actions of insulin may involve low-molecular weight inositol phosphoglycan (IPG) mediators (also known as putative insulin mediators or second messengers) (4, 5) and several pieces of

evidence suggest that a deficiency in a specific D-chiro inositol (DCI)-containing IPG (6, 7, 8) and/or altered DCI metabolism may contribute to IR (9, 10). Previous studies have shown that oral administration of DCI to women with PCOS increases the action of insulin, improving ovulatory function and decreasing blood pressure, and serum androgen and plasma triglyceride concentrations (9, 10).

Oxidative stress (OS) is involved in the pathogenesis and future complications of PCOS. This condition occurs when reactive oxygen species (ROS), which are intermediaries of a normal oxygen metabolism, are produced faster than the endogenous antioxidant defense systems can neutralize. Previous studies have demonstrated that hyperglycemia increases ROS generation from peripheral blood leukocytes (11).

The resulting OS may contribute to a pro-inflammatory state that induces IR and hyperandrogenism in women with this disorder (12) and also increases the risk of cardiovascular disease (13).

Many proteins are susceptible to attack by ROS, especially as sulfhydryl groups are among the most easily oxidized protein residues. Oxidation can lead to inter- and/or intramolecular cross-linking, thus inducing protein degradation (14, 15), clustering (16, 17, 18), and enzyme inactivation (18, 19).

We recently demonstrated that diamide-treated erythrocytes express a well-defined tyrosine phosphorylation (Tyr-P) level of membrane proteins, particularly of band 3 (20). Alterations in band 3 Tyr-P levels represent preexisting modifications of membrane status, like that observed in glucose-6-phosphate dehydrogenase (G6PD)-deficient patients (18, 21) suffering from chronic impairment of antioxidant defenses or in endometriotic women (22) with systemic inflammation. Diamide, a mild oxidant, affects the sulfhydryl groups of cysteines by inducing disulfide bond formation and triggers band 3 Tyr-P (16, 17, 18, 20, 21, 22), which is a useful parameter in the evaluation of oxidation-related damage to cells (18, 21, 22). We also observed that, while diamide treatment reduced total glutathione (GSH) contents detectable in the cytosol of endometriotic patients, normal subjects were unaffected, and the differences between GSH content before and after diamide treatment were defined as Δ GSH, an additional parameter of inflammatory status in endometriosis (22).

The aim of this study was to evaluate any effects on clinical, hormonal, and glucose metabolism parameters in PCOS patients before and after 12 weeks of inositol dietary supplement. We used myo-inositol (MYO) because it is widely distributed in nature (23) and commercially available, whereas DCI, the product of oxidoreductive epimerization of the carbon 1 hydroxyl group of MYO, is relatively rare and present in plants and seeds (24).

In addition, in order to evaluate the inositol effects on erythrocyte membrane functionality and cell redox status, we determined diamide-related band 3 Tyr-P levels and contents of both GSH and membrane glutathionylated proteins in patients' erythrocytes.

Materials and methods

Materials

Reagents were purchased from Sigma, and anti-phosphotyrosine (P-Tyr; PY20) mouse MAB was obtained from Biosource–Invitrogen (Camarillo, CA, USA). The protease inhibitor cocktail and anti-actin mouse MAB came from Calbiochem (Darmstadt, Germany). Antimouse secondary antibody conjugated with HRP came from Bio-Rad Laboratories. MYO was purchased from Progene (Firenze, Italy).

Testosterone was measured by electrochemiluminescence immunoassay (ECLIA) using commercially available kits (Roche Diagnostics GmbH); 17-OH progesterone was measured by a solid-phase ELISA using commercially available kits (DRG Instruments GmbH, Marburg, Germany); androstenedione was measured by a solid-phase, competitive chemiluminescent enzyme immunoassay using commercially available kits (Siemens Healthcare Diagnostics Products Ltd., Llanberis, Gwynedd, UK). The intra-assay and interassay coefficients of variation were <9%.

Serum glucose levels were measured enzymatically with glucose hexokinase kit (Roche Diagnostics), and serum insulin levels were measured by ECLIA (Roche) with Immulite 2000 DPC Siemens Analyzer.

Study protocol

Twenty-six women with PCOS, between the ages of 22 and 30 years, were enrolled at the Department of Medical and Surgical Sciences of the University of Padova, Italy. Diagnosis of PCOS was defined when two of the following criteria were fulfilled: oligo- and/or anovulation (≤ 8 menstrual periods in the previous year), clinical and/or biochemical signs of hyperandrogenism, polycystic ovaries, and exclusion of other etiologies (25). Exclusion criteria included pregnancy, body mass index (BMI) > 25 kg/m², hyperprolactinemia, thyroid dysfunction, Cushing's syndrome, and late-onset adrenal hyperplasia. None of the women had diabetes or had taken oral contraceptives, anti-inflammatory drugs, or other hormonal drugs during the previous 3 months. This study was approved by the Ethics Committee for Research and Clinical Trials of our University and all the patients gave their written informed consent.

At the time of entry into the study, all patients had serum progesterone concentrations below 2.5 ng/ml. On the first day of the study (baseline, T₀), after a 12 h overnight fast, blood samples were collected to measure serum testosterone, androstenedione, glucose, and insulin.

A citrated blood sample was collected from each subject to evaluate Tyr-P levels and assess GSH and glutathionylated proteins (GSSP).

A 2 h oral glucose tolerance test (OGTT) with 75 g glucose was carried out. Blood samples were collected after 15, 30, 60, 90, and 120 min to determine serum glucose and insulin; values are expressed as the area under the curve (AUC) of glucose and insulin. Index of IR was a peak of serum insulin value > 70 mU/l during OGTT.

Homeostasis model of assessment–IR (HOMA-IR) was also used, according to (26). During the same examination, anthropometric (weight, height, and BMI) and clinical (Ferriman–Gallwey score, heart rate, and systolic and diastolic blood pressure) measurements were taken for all subjects.

After baseline evaluations, women were randomized to receive MYO dietary supplement powder 1200 mg/day ($n=18$) or matched placebo powder ($n=8$) for 12 weeks. To avoid selection bias, patients were randomized to each group (treatment or placebo) using a randomly generated list with the Excel 'Rand()' function. In the MYO group, a larger number of patients were set 'a priori' in order to be more confident with the results and to avoid any possible distortion due to the variability in pharmacokinetics of the substance and to the variable ranges of dietary inositol assumption.

Administration was from predosed sachets containing the daily dose; the powder was dissolved in water before oral intake once a day. Subjects were instructed not to change their eating habits, activity level, or lifestyle during the study. At the end of the study, the measurements and tests performed at baseline were repeated (posttreatment time, T_1).

Treatment of erythrocytes

Red blood cells (RBC) were pelleted at 750 *g* for 3 min. After removal of supernatant, packed RBC were washed three times at 750 *g* for 3 min in five volumes of Dulbecco's PBS (D-PBS) to avoid contamination by leucocytes and platelets. Packed cells (50 μ l) were resuspended (at 20% hematocrit) in D-PBS and treated at 35 °C for 30 min in the absence ($_{\text{Basal}}$) or presence ($_{\text{Diamide}}$) of 1.5 mM diamide (dissolved in D-PBS) (22).

Samples were then centrifuged and packed cells were washed in D-PBS, and they underwent hemolysis in 1.5 ml hypotonic buffer (5 mM sodium phosphate, pH 8, 0.02% sodium azide (NaN_3), 30 μ M phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and a protease inhibitor cocktail).

Membranes were separated from the cytosol by centrifugation (16 100 *g* for 20 min in an Eppendorf centrifuge) and washed once in hypotonic buffer. Aliquots of membranes (10 μ g) were suspected to SDS-PAGE (10% gels), transferred to nitrocellulose membranes, and immunostained first with anti-P-Tyr antibody and then with anti-actin sample loading controls.

Band 3 Tyr-P levels were evaluated densitometrically. The Tyr-P value of diamide-treated erythrocytes from PCOS patients before (T_0) and after (T_1) MYO/placebo treatment was calculated as the ratio to the Tyr-P level of diamide-treated erythrocytes obtained in healthy controls (chosen as arbitrary comparison unit, experimentally determined as 0.98 ± 0.065 , mean value \pm s.d.). To express a standardized measure of Tyr-P variation after treatment (defined as the Tyr-P variation index, $V_{i(\text{Tyr-P})}$) with respect to baseline value, the following formula was used:

$$V_{i(\text{Tyr-P})} = 1 - \left(\frac{\text{Tyr-P}_{T_1}}{\text{Tyr-P}_{T_0}} \right).$$

Determination of GSH and GSSP in erythrocytes

Total GSH was determined according to the Tietze method (27). Briefly, 10 μ l cytosol obtained from differently treated erythrocytes were added to 2 ml reaction mixture containing 1.9 ml phosphate 0.1 M/EDTA 0.6 mM buffer, pH 7.4, 30 μ l 5,5'-dithio-bis(2-nitrobenzoic acid) 10 mM, 100 μ l NADPH 5 mM, and 10 μ g glutathione reductase and analyzed spectrophotometrically at 412 nm. The decrease in total GSH content after diamide treatment (ΔGSH) was expressed as $1 - \text{GSH}_{(\text{Diamide})}/\text{GSH}_{(\text{Basal})}$ (22).

A variation index for ΔGSH ($V_{i(\Delta\text{GSH})}$) was calculated, as for Tyr-P, according to the following formula:

$$V_{i(\Delta\text{GSH})} = 1 - \left(\frac{\Delta\text{GSH}_{T_1}}{\Delta\text{GSH}_{T_0}} \right).$$

GSSP was determined according to (28). Briefly, membranes, obtained as described above, were deproteinated by adding trichloroacetic acid (TCA; 5% final concentration). After centrifugation for 3 min, a 16 100 *g* acid-precipitated membrane protein preparation was resuspended and brought to pH 12, which induces GSH release from proteins via a cysteine-reduced/oxidized (SH/SS) exchange. The reaction was stopped by adding TCA (5% final concentration); the amount of released GSH was determined enzymatically in the supernatants of centrifuged samples.

The increase in GSH released from membranes of erythrocytes treated with diamide, which was representative of the increase in glutathionylated protein content (ΔGSSP), was expressed as $(\text{GSH}_{(\text{Diamide})}/\text{GSH}_{(\text{Basal})}) - 1$.

Also here, a variation index for ΔGSSP ($V_{i(\Delta\text{GSSP})}$) was calculated, according to the following formula:

$$V_{i(\Delta\text{GSSP})} = 1 - \left(\frac{\Delta\text{GSSP}_{T_1}}{\Delta\text{GSSP}_{T_0}} \right).$$

Statistical analysis

Data are expressed as mean \pm s.d. Comparisons were obtained with Student's *t*-test for paired or unpaired data, as appropriate. Statistical significance was set at $P < 0.05$ (two tailed). Any relationships between pairs of variables were tested by least-squares linear regression. Pearson's correlation coefficient *r* was used to quantify the strength of the relationships. The statistical significance of *r* was determined by ANOVA; a *P* value of < 0.05 was considered statistically significant (two tailed). Comparison between regression lines was performed with ANOVA on slope and intercept parameters.

Table 1 Baseline clinical characteristics and hematochemical parameters of two groups of PCOS patients. Data are mean \pm s.d. Normal values for serum testosterone and androstenedione and insulin and glucose AUC were calculated in a group of healthy women ($n=15$): average age, 23.6 ± 1.5 ; weight, 57.9 ± 4.6 ; and BMI, 21.4 ± 1.2 .

Parameters	Normal range	MYO group ($n=18$)	Placebo group ($n=8$)	P^a
Age (years)	–	23.5 ± 2.1	23.6 ± 1.4	6.1257
Weight (kg)	–	58.9 ± 6.4	58.8 ± 3.5	6.6347
BMI (kg/m^2)	–	21.6 ± 1.9	21.9 ± 0.6	4.3354
Blood pressure (mmHg)				
Systolic	<120	117.2 ± 10.7	112.5 ± 7.1	1.8653
Diastolic	<80	72.2 ± 4.3	71.3 ± 3.5	4.0257
Serum testosterone (nmol/l)	0–8 \pm 0–2	1.77 ± 0.50	1.96 ± 0.25	2.1701
Serum androstenedione (nmol/l)	$7.5 \pm 1-9$	14.99 ± 3.51	14.34 ± 2.13	4.3972
Fasting serum insulin (mU/l)	5–20	7.49 ± 4.44	6.75 ± 1.67	4.5354
Fasting serum glucose (mmol/l)	3.7–6.0	4.79 ± 0.32	4.73 ± 0.38	4.5910
Insulin AUC (mU/l per min)	2467.34 ± 654.45	6989.47 ± 2301.69	7499.38 ± 2047.60	4.1354
Glucose AUC (mmol/l per min)	729.23 ± 37.25	712.06 ± 76.71	700.12 ± 46.61	4.7840
HOMA-IR	<2.5	1.60 ± 0.99	1.40 ± 0.40	4.0625

^aStatistical analysis was obtained with Student's *t*-test between the two groups.

Results

Baseline clinical characteristics and hematochemical parameters determined in the two groups of PCOS showed no significant differences (Table 1).

Following the 12-week treatment, the studied parameters in the MYO group showed significant decreases with respect to baseline (Table 2). Conversely, in the placebo group, no significant change was found at the end of the treatment, except for an increase in insulin-related metabolic parameters (Table 2).

This slight worsening of insulin AUC in the placebo group is probably due to the fact that patients were not treated for a few months before and during the study. This is a further demonstration that inositol improves metabolic patterns even after 3 months.

Comparing the two groups, significant differences were observed in BMI, serum testosterone, serum

androstenedione, fasting serum insulin, insulin AUC after OGTT, and HOMA-IR (Table 2).

To examine RBC membrane status, diamide-related band 3 Tyr-P levels were determined in patients' erythrocytes. Although in the absence of diamide stimulation Tyr-P could not be detected in erythrocytes from either patients or controls (data not shown), when PCOS RBC were incubated with diamide, membranes showed higher band 3 Tyr-P levels in comparison with RBC from healthy subjects (Table 3, see footnote). Comparing RBC from PCOS patients at the two time points T_0 (baseline) and T_1 (following MYO treatment) shows that Tyr-P levels were significantly affected by MYO treatment (Table 3, $P < 0.0001$). No variations were found in the placebo-treated group (Table 3).

The antioxidant condition of RBC was also evaluated by measuring changes in GSH cellular content (22). As shown in Table 3, the decrease in GSH (Δ GSH) was

Table 2 Changes in clinical characteristics and hematochemical parameters after administration of MYO or placebo for 12 weeks in PCOS patients. Data are mean \pm s.d. of paired differences between times T_1 and T_0 for each patient. Negative sign indicates a decrease after treatment in comparison with baseline.

Parameters	MYO group ($n=18$)	Placebo group ($n=8$)	P^a	Effect size ^b
Weight (kg)	-1.83 ± 1.86^c	$+0.25 \pm 0.71$	0.0055	1.34
BMI (kg/m^2)	-0.69 ± 0.69^c	$+0.09 \pm 0.27$	0.0058	1.35
Blood pressure (mmHg)				
Systolic	-3.3 ± 5.9^c	$+1.3 \pm 6.4$	0.0888	0.79
Diastolic	$+0.6 \pm 2.4$	$+2.5 \pm 4.6$	0.1645	0.62
Serum testosterone (nmol/l)	-0.35 ± 0.24^c	-0.01 ± 0.29	0.0043	1.38
Serum androstenedione (nmol/l)	-3.96 ± 2.16^c	$+0.28 \pm 0.39$	<0.0001	2.41
Fasting serum insulin (mU/l)	-2.33 ± 2.61^c	$+1.00 \pm 0.76^c$	0.0018	1.55
Fasting serum glucose (mmol/l)	-0.14 ± 0.31	$+0.06 \pm 0.13$	0.0816	0.077
Insulin AUC (mU/l per min)	-1668.08 ± 1388.52^c	$+347.38 \pm 314.98^c$	0.0005	1.77
Glucose AUC (mmol/l per min)	-13.42 ± 65.52	$+17.06 \pm 28.64$	0.2224	0.55
HOMA-IR	-0.54 ± 0.62^c	$+0.26 \pm 0.16^c$	0.0015	1.57

^aComparison between groups; statistical analysis with Student's *t*-test.

^bThe effect size was calculated *post-hoc* according to Cohen's *d* formula (48), using pooled s.d.s. According to Cohen's *d*, an effect size of 0.2–0.3 is considered a 'small' effect; around 0.5, a 'medium' effect; and >0.8, a 'large' effect.

^cStatistically significant variation between times T_1 and T_0 within patient groups ($P < 0.05$ as significance limit); statistical analysis with paired Student's *t*-test.

Table 3 Comparison between the parameters investigated in erythrocytes from PCOS patients in MYO and placebo groups. Data are mean ± s.d. calculated according to the formula in the Materials and methods section. Tyr-P value of diamide-treated erythrocytes from PCOS patients before (T₀) and after (T₁) MYO/placebo treatment, calculated as ratio to Tyr-P level of diamide-treated erythrocytes obtained in healthy controls (chosen as arbitrary comparison unit, experimentally determined as 0.98 ± 0.065, mean value ± s.d.).

Parameters	MYO group (n=18)	Placebo group (n=8)	P ^a	Effect size ^b
Tyr-P				
T ₀	2.06 ± 0.28	2.00 ± 0.24	0.5819	0.23
T ₁	1.36 ± 0.30 ^c	2.01 ± 0.27	<0.0001	2.32
V _i (Tyr-P)	0.34 ± 0.10	-0.01 ± 0.05	<0.0001	4.12
ΔGSH				
T ₀	0.38 ± 0.04	0.39 ± 0.03	0.5290	0.28
T ₁	0.23 ± 0.06 ^c	0.40 ± 0.02	<0.0001	3.43
V _i (ΔGSH)	0.40 ± 0.14	-0.02 ± 0.05	<0.0001	3.62
ΔGSSP				
T ₀	0.33 ± 0.03	0.32 ± 0.02	0.3695	0.38
T ₁	0.17 ± 0.04 ^c	0.31 ± 0.03	<0.0001	3.90
V _i (ΔGSSP)	0.48 ± 0.11	0.02 ± 0.07	<0.0001	4.79

ΔGSH, decrease in cytosol glutathione content after diamide treatment, expressed as 1 - GSH_(Diamide)/GSH_(Basal); ΔGSSP, increase in glutathionylated proteins after diamide treatment, expressed as the increase in glutathione released from membrane proteins or (GSH_(Diamide)/GSH_(Basal)) - 1.

^aP value following Student's *t*-test, comparison between MYO and placebo groups.

^bThe effect size was calculated *post-hoc* according to Cohen's *d* formula (48), using pooled s.d.s. According to Cohen's *d*, an effect size of 0.2-0.3 is considered a 'small' effect; around 0.5, a 'medium' effect; and >0.8, a 'large' effect.

^cP<0.0001, paired Student's *t*-test, comparison between T₁ and T₀ within each group.

significantly less (P<0.0001) after dietary treatment with MYO; no changes were detected in control patients.

Treatment with dietary MYO also prevented any increase in glutathionylated protein content (ΔGSSP) in erythrocytes from PCOS patients, the variation being even lower at T₁ compared with T₀ (P<0.0001); conversely, no variation occurred in the placebo group (Table 3).

The variation indices V_i (see Materials and methods section) of the three parameters of RBC membrane-oxidative status allow for a direct unbiased comparison of Tyr-P, GSH, and GSSP changes occurring in the two groups of patients (Table 3). All three parameters were significantly different in MYO and placebo groups (P<0.0001).

To assess interrelationships between RBC parameters and hormonal/metabolic data obtained in blood from patients at the two time points, before and after MYO treatment, a linear regression was performed and the strength of correlation was assessed by Pearson's correlation coefficient *r* (Table 4).

The main result obtained was the significant correlation between serum testosterone and Tyr-P levels

(P<0.05). The link between Tyr-P and testosterone levels before and after MYO treatment is shown in Fig. 1.

Comparison of the two regression lines, at T₀ and T₁, revealed a significant difference between the intercepts in MYO-treated patients (P<0.0001); the slope remained the same, indicating that, although phosphorylation kinetic mechanisms were unaffected by MYO administration, they were quantitatively reduced. The same analysis applied to the placebo-treated group confirming the significant correlation between erythrocyte Tyr-P and serum testosterone levels at baseline (P=0.0032; data not shown).

A significant correlation was also detected between insulin AUC and Tyr-P, but only before treatment (Table 4). After MYO treatment, the concomitant reduction found for Tyr-P and insulin AUC values produced a clustering of data that abolished the regression of the two variables.

Discussion

A common misconception related to PCOS is that every patient is overweight, but this is not always the case, because some of the women diagnosed with this condition are of normal weight (29). In this study, we show that IR can be present in PCOS with normal BMI, confirming that IR is not merely linked to obesity but also to a genetic or epigenetic situation (30, 31, 32, 33).

An increase in the generation of ROS by peripheral blood leucocytes in response to acute hyperglycemia has been previously demonstrated in PCOS patients (11), and the finding that physiological and pharmacological insulin infusions *in vivo* or *in vitro* revealed the oxidative effect of insulin (32, 33, 34, 35, 36) raised the hypothesis of systemic redox impairment in such patients.

Our results show that this redox impairment is effectively systemic, as indicated by oxidative-related alterations in the erythrocytes of all patients. In addition, insulin AUC significantly correlated with RBC alterations in patients, rather than fasting glucose and fasting insulin or glucose AUC. The values of the last

Table 4 Correlation between hormonal/metabolic parameters and Tyr-P levels in erythrocytes before (T₀) and after (T₁) myo-inositol administration (n=18).

Tyr-P vs	Pearson's correlation coefficient <i>r</i>	
	T ₀	T ₁
Serum testosterone (nmol/l)	0.5698*	0.4943*
Serum androstenedione (nmol/l)	0.2028	-0.1447
Fasting serum insulin (mU/l)	0.3264	0.2756
Fasting serum glucose (mmol/l)	-0.2193	0.0320
Insulin AUC (mU/l per min)	0.5032*	0.1673
Glucose AUC (mmol/l per min)	-0.2076	-0.3208
HOMA-IR	0.2968	0.2913

*P<0.05; statistical analysis of regression by ANOVA.

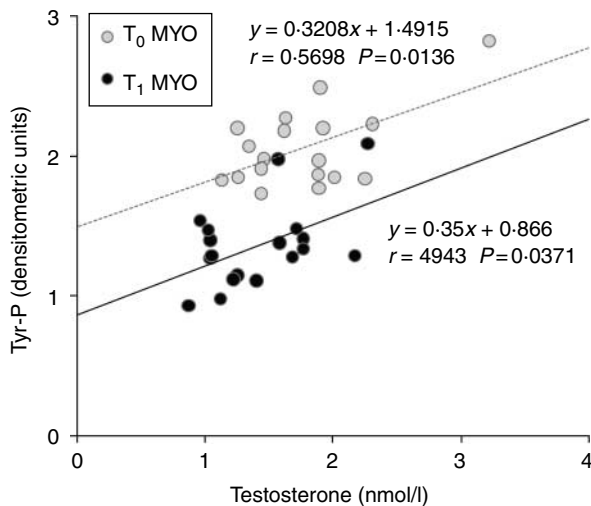


Figure 1 Linear regression between erythrocyte Tyr-P values and testosterone serum levels in MYO group before (T_0) and after (T_1) treatment. ANOVA indicated a significant difference ($P < 0.0001$) between intercepts of regression lines in MYO-treated patients.

three parameters were comparable with those of normal healthy women, thus corroborating the oxidative effect of insulin rather than that of hyperglycemia (Table 1).

We have recently demonstrated that enhanced ROS production in endometriotic patients is correlated with both increased diamide-induced erythrocyte band 3 Tyr-P level and high Δ GSH, indicating that OS induces structural modifications of membranes (22). Diamide-induced OS in human erythrocytes causes net membrane rearrangement with band 3 aggregate formation (16, 18) and kinase (37) and phosphatase (38) recruitments, all leading to a triggering of band 3 Tyr-P levels (20). In PCOS, RBC are much more sensitive to diamide treatment and, consequently, band 3 Tyr-P reaches levels two or three times higher than those of controls, probably due to an altered redox system, predisposing membrane proteins to be more markedly oxidized. This was further confirmed by the observation that total cell GSH did not differ from that of healthy controls (data not shown) but that, once RBC are incubated with diamide, patients' GSH contents are far lower, with Δ GSH ranging from 0.38 ± 0.04 (Table 3). By contrast, when membranes were extracted and analyzed for their contents of glutathionylated proteins (GSSP), glutathylation after diamide treatment increased drastically in PCOS RBC (Δ GSSP, 0.33 ± 0.03 ; Table 3) but not in healthy controls (data not shown). Taken together, these results show that PCOS RBC are subjected to OS, which induces membrane oxidative status alterations.

Interestingly, MYO treatment positively affected the oxidative status of RBC, as shown by the partial restoration of GSH contents (decreased Δ GSH) and the reduction of both band 3 Tyr-P levels and protein glutathionylation (decreased Δ GSSP). The present

investigation shows that even a low dose of MYO administered for 3 months yields therapeutically appreciable effects. In fact, clinical ameliorations were achieved although the posology of 1200 mg/day was almost one-fourth of that commonly used for MYO (39, 40) and similar to that for DCI (6). In addition, as an anecdotal finding, one of the patients enrolled in the MYO group continued the intake of half a dose of MYO (i.e. 600 mg/day) after the end of the study period, and the clinical symptoms appeared again (data not shown). This would suggest that the dose used in our study may represent the lowest range of a posology aimed at obtaining an appreciable effect in PCOS patients.

This study has also evaluated the hormonal and metabolic status of PCOS patients, and compared values at T_0 and T_1 , to analyze the effect of MYO treatment on clinical parameters.

Our findings confirm that oral MYO administration significantly decreases testosterone, androstenedione, fasting serum insulin, insulin AUC levels, and HOMA-IR.

Two different pathways may depend on MYO: phosphoinositide-related signal transduction and insulin-related metabolic response. In the first pathway, MYO, synthesized from glucose 6-phosphate, carried into the cell from plasma or obtained by a recycling of intracellular inositol 1,4,5-triphosphate, is incorporated into activated phosphatidic acid to constitute membrane phosphatidylinositols (PI) (40, 41, 42, 43). In the second pathway, either MYO or its epimerase-converted (39) *chiro* isoform DCI (44, 45) constitutes IPGs (4), which are released from cell membrane glycosylphosphatidylinositols in response to insulin. Once released, IPGs can affect any tissues and cells implicated in insulin action (45), thus potentiating insulin effects (46).

The significant reduction of Tyr-P levels in RBC found after MYO treatment and the reduction in insulin AUC confirm that this kind of therapy can improve IR, one of the typical features of PCOS (10). In fact, dietary MYO uptake can affect membrane composition both directly, by increasing intracellular PI contents and/or PI-related pathways, and indirectly, by modulating the OS induced by the inappropriate hyperinsulinemic response linked to IR.

Another novel finding is the significant correlation between Tyr-P and testosterone levels at T_0 and, more interestingly, at T_1 (Table 4), as shown by a comparison of the two regression lines (Fig. 1). The significant difference between the intercepts of the two lines in MYO-treated patients ($P < 0.0001$) suggests that alteration of the phosphorylation process of erythrocyte band 3 is mediated by higher testosterone levels in serum. We hypothesize that administration of MYO (but not of placebo) improves band 3 Tyr-P (and related Δ GSH and Δ GSSP) through a decrease in testosterone levels, thus supporting the MYO-related insulin messenger pathway.

In conclusion, PCOS patients suffer from a systemic inflammatory status, as shown by the increased erythrocyte response to diamide-induced OS, with

increases in Tyr-P level, Δ GSH, and Δ GSSP parameters, which significantly correlate with testosterone levels. The use of MYO, even at 1200 mg/day, reduced inflammatory status and IR-related hyperinsulinemia (10, 47).

Further studies investigating both the intracellular mechanism triggered by inositol and the extracellular inositol-related IPG pathways are warranted to better define how MYO could improve insulin response and oxidative status in patients with PCOS. The Tyr-P process could be very useful in monitoring patients' conditions and in choosing an adequate therapy for IR. In addition, MYO could be proposed as an alternative to metformin treatment because the former can affect insulin target tissues and cells and potentiate insulin effects without the side effects of metformin.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Italian Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST).

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Received 29 September 2011

Revised version received 23 December 2011

Accepted 5 January 2012