INOSITOL ADMINISTRATION REDUCES OXIDATIVE STRESS IN ERYTHROCYTES
OF PATIENTS WITH POLYCYSTIC OVARY SYNDROME

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**Short title:** Inositol reduces oxidative stress in PCOS
ABSTRACT

Objective: Possibly due to a deficiency of insulin mediators, polycystic ovary syndrome (PCOS) is often associated with insulin resistance and hyperinsulinemia, likely responsible for an elevated production of reactive oxygen species (ROS). 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: 26 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 (OGTT) and Homeostasis Model of Assessment - Insulin Resistance (HOMA-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 glutathione content and increased membrane protein glutathionylation.

MYO treatment significantly improved metabolic and biochemical parameters. Significant reductions were found in insulin resistance, 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 insulin resistance.
**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 inositolphosphoglycan (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-chyro inositol (DCI)-containing IPG (DCI-IPG) (6-8) 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 them. Previous studies have demonstrated that hyperglycemia increases ROS generation from peripheral blood leukocytes (11). The resulting oxidative stress may contribute to a pro-inflammatory state which induces insulin resistance 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 intra-molecular cross linking thus inducing protein degradation (14, 15), clustering (16-18) and enzyme inactivation (18, 19).

We demonstrated recently 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 pre-existing modifications of membrane status, like that observed in glucose-6-phosphate dehydrogenase (G6PD)-deficient patients (18, 21) suffering chronic impairment of antioxidant defenses, or in endometriotic women (22) with systemic inflammation. Diamide, a mild oxidant, affects the sulphydryl groups of cysteines by inducing disulfide bond formation, and triggers band 3 Tyr-P (16-18, 20-22), 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 ΔGSH, 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 inositol effects on erythrocyte membrane functionality and cell redox status, we determined diamide-related band 3 Tyr-P levels and contents of both glutathione and membrane glutathionylated proteins in patients’ erythrocytes.

**Materials and Methods**

**Materials**

Reagents were purchased from Sigma (Milan, Italy), and anti-phospho-tyrosine (P-Tyr) (PY20) mouse monoclonal antibody was obtained from Biosource-Invitrogen (Camarillo, CA, USA). The protease inhibitor cocktail and anti-actin mouse monoclonal antibody came from Calbiochem (Darmstadt, Germany). Anti-mouse secondary antibody conjugated with horseradish peroxidase
(HRP) came from BioRad Laboratories (CA, USA). MYO was purchased from Progine (Firenze, Italy).

Testosterone was measured by Electrochemiluminescence immunoassay (ECLIA method) using commercially available kits (Roche Diagnostics GmbH, Mannheim, Germany); 17 OH Progesterone was measured by a solid phase enzyme-linked immunosorbent assay (ELISA method) using commercially available kits (DRG Instruments GmbH, Germany); Androstenedione was measured by a solid phase, competitive chemiluminescent enzyme immunoassay using commercially available kits (Siemens Healthcare Diagnostics Products Ltd., Llanberis, Gwynedd LL55 4EL, United Kingdom). The intra-assay and inter-assay coefficients of variation were less than 9%.

Serum glucose levels were measured enzymatically with glucose hexokinase kit (Roche Diagnostics, Mannheim, Germany), and serum insulin levels were measured by electrochemiluminescence immunoassay (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 ($\leq$8 menstrual periods in the previous year); clinical and/or biochemical signs of hyperandrogenism; polycystic ovaries; exclusion of other etiologies (25). Exclusion criteria included pregnancy, BMI >25 kg/m$^2$, 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 three 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-hour 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 to perform GSH and GSSP determination.

A 2-hour oral glucose tolerance test (OGTT) with 75 g glucose was carried out. Blood samples were collected after 15, 30, 60, 90 and 120 minutes to determine serum glucose and insulin; values are expressed as the area under the curve (AUC) of glucose and insulin, respectively. A peak of serum insulin value during OGTT > 70 mU/L was considered an IR index.

Homeostasis Model of Assessment - Insulin Resistance (HOMA-IR) was also used, according to (26). During the same examination, anthropometric (weight, height, body mass index (BMI)) and clinical (Ferriman-Gallwey score, heart rate, 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 was 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 pre-dosed 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 (post-treatment time, T₁).

**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 x g for 3 min in 5 volumes of Dulbecco’s Phosphate Buffered Saline (D-PBS) to avoid contamination by leucocytes and platelets. Packed cells (50 µl) were resuspended (at 20% hematocrit) in D-PBS and treated at 35°C for 30 min in absence (Basal) or presence (Diamide) of 1.5 mM diamide (dissolved in D-PBS) (22).

Samples were then centrifuged and packed cells were washed in D-PBS and underwent haemolysis in 1.5 ml of hypotonic buffer (5 mM sodium phosphate, pH 8, 0.02 % sodium azide (NaN₃), 30 µM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, and a protease inhibitor cocktail).

Membranes were separated from the cytosol by centrifugation (16100 x g for 20 min in an Eppendorf centrifuge) and washed once in hypotonic buffer. Aliquots of membranes (10 µg) were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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 (T₀) and after (T₁) 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±SD). To express a standardized measure of Tyr-P variation after treatment (defined as the Tyr-P variation index, V_{i(Tyr-P)} ) with respect to baseline value, the following formula was used:

\[ V_{i(Tyr-P)} = 1 - \frac{Tyr-P_{T₁}}{Tyr-P_{T₀}}. \]

**Determination of GSH and glutathione-protein mixed disulfide (GSSP) in erythrocytes**

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

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

$$V_{\delta GSH} = 1 - (\Delta GSH_{T1}/\Delta GSH_{T0})$$

GSPP determination was carried out according to (28). Briefly, membranes, obtained as described above, were deproteinized by adding trichloroacetic acid (TCA) (5% final concentration). After centrifugation for 3 min, a 16100 x $g$ acid-precipitated membrane proteins preparation was resuspended and brought to pH 12, which induces GSH release from proteins via an cysteine reduced/oxidized (SH/SS) exchange. The reaction was stopped by the addition of TCA (5% final concentration); the amount of released GSH was determined enzymatically in the supernatants of centrifuged samples.

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

Also here, a variation index for $\Delta GSPP$ ($V_{\delta GSPP}$) was calculated, according to the following formula:

$$V_{\delta GSPP} = 1 - (\Delta GSPP_{T1}/\Delta GSPP_{T0})$$

**Statistical analysis**

Data are expressed as means ± SD. 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 less than 0.05 was considered statistically significant (two-tailed). Comparison between regression lines was performed with ANOVA on slope and intercept parameters.

**Results**

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

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).

Table 2

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

Table 3

The antioxidant condition of RBC was also evaluated by measuring changes in glutathione cellular content (22). As Table 3 shows, the decrease in GSH (\( \Delta \text{GSH} \)) was significantly less marked (\( p<0.0001 \)) after dietary treatment with MYO; no changes were detected in control patients.

Treatment with dietary MYO also prevented any increase in glutathionylated protein content (\( \Delta \text{GSSP} \)) in erythrocytes from PCOS patients, the variation being even lower at \( T_1 \), compared to \( T_0 \) (\( p<0.0001 \)); conversely, no variation occurred in the placebo group (Table 3).

The variation indices \( V_i \) (see Methods) 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).

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 clarified by figure 1.

Figure 1

Comparison of the two regression lines, at \( T_0 \) and \( T_1 \) respectively, 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, confirmed 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, since part of the women diagnosed with this condition are of normal weight (30). In the present study we remark that insulin resistance can be present in PCOS with normal BMI, confirming that IR is not merely linked to obesity but to a genetic or epigenetic situation (31-34).

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 (33-37) 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 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 ΔGSH, indicating that OS induces structural modifications of membranes (22). Diamide-induced oxidative stress in human erythrocytes causes net membrane rearrangement with band 3 aggregate formation (16, 18) and kinase (38) and phosphatase (39) 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 glutathione 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 ΔGSH ranging from 0.38 ±0.04 (Table 3). By contrast, when membranes were extracted and analyzed for their contents of glutathionylated proteins (GSSP), glutathionylation after diamide treatment increased drastically in PCOS RBC (ΔGSSP 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 glutathione contents (decreased ΔGSH) and the reduction of both band 3 Tyr-P levels and protein glutathionylation (decreased ΔGSSP). The present investigation shows that even a low dose of MYO administered for three months yields therapeutically appreciable effects. In fact, clinical ameliorations were achieved although the posology of 1200 mg/day was almost ¼ of that commonly used for MYO (40, 41) and similar to that for DCI (6). In addition, as anecdotal finding, one of the patients enrolled in the MYO-group continued the assumption 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.

The present study has also evaluated the hormonal and metabolic status of PCOS patients, and compared values at T₀ and T₁, 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 (IP3), is incorporated into activated phosphatidic acid to constitute
membrane phosphatidyl inositols (PI) (41-44). In the second pathway, either myo-inositol or its
epimerase-converted (40) chiro isoform DCI (45, 46), constitutes inositolphosphoglycans (IPG) (4),
which are released from cell membrane glycosylphosphatidylinositols (GPIs) in response to insulin.
Once released, IPGs can affect any tissues and cells implicated in insulin action (46), thus
potentiating insulin effects (47).

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 insulin resistance, 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 oxidative stress induced by the inappropriate hyperinsulinemic response linked to
IR.

Another novel finding is the significant correlation between Tyr-P and testosterone levels at T0 and,
more interestingly, at T1 (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 ΔGSH and ΔGSSP) through a decrease of 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 oxidative stress, with increases in Tyr-P level,
ΔGSH and ΔGSSP parameters, which significantly correlate with testosterone levels. The use of
MYO, even at 1200 mg/day, reduced inflammatory status and insulin resistance-related
hyperinsulinemia (10, 48).
Further study investigating both intracellular mechanism triggered by inositol and 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 since the former can affect insulin target tissues and cells and potentiate insulin effects without the side-effect of metformin.

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The authors have nothing to disclose.

**References**


35. Kriger-Brauer HI & Kather H. Human fat cells possess a plasma membrane-bound H$_2$O$_2$-generating system that is activated by insulin via a mechanism bypassing the receptor kinase. *Journal of Clinical Investigation* 1992 **89** 1006-1013.


Legends

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 significant difference ($p<0.0001$) between intercepts of regression lines in MYO-treated patients.
Figure 1.

190x142mm (300 x 300 DPI)
**TABLE 1.** Baseline clinical characteristics and haematochemical parameters of two groups of PCOS patients.

Data are means ±SD.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal range</th>
<th>MYO group (n=18)</th>
<th>Placebo group (n=8)</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>-</td>
<td>23.5 ± 2.1</td>
<td>23.6 ± 1.4</td>
<td>6.1257</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>-</td>
<td>58.9 ± 6.4</td>
<td>58.8 ± 3.5</td>
<td>6.6347</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>-</td>
<td>21.6 ± 1.9</td>
<td>21.9 ± 0.6</td>
<td>4.3354</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>&lt;120</td>
<td>117.2 ± 10.7</td>
<td>112.5 ± 7.1</td>
<td>1.8653</td>
</tr>
<tr>
<td>Diastolic</td>
<td>&lt;80</td>
<td>72.2 ± 4.3</td>
<td>71.3 ± 3.5</td>
<td>4.0257</td>
</tr>
<tr>
<td>Serum Testosterone (nmol/L)</td>
<td>0-8±0-2</td>
<td>1.77 ± 0.50</td>
<td>1.96 ± 0.25</td>
<td>2.1701</td>
</tr>
<tr>
<td>Serum Androstenedione (nmol/L)</td>
<td>7.5±1-9</td>
<td>14.99 ± 3.51</td>
<td>14.34 ± 2.13</td>
<td>4.3972</td>
</tr>
<tr>
<td>Fasting serum insulin (mU/L)</td>
<td>5 - 20</td>
<td>7.49 ± 4.44</td>
<td>6.75 ± 1.67</td>
<td>4.5354</td>
</tr>
<tr>
<td>Fasting serum glucose (mmol/L)</td>
<td>3.7 - 6.0</td>
<td>4.79 ± 0.32</td>
<td>4.73 ± 0.38</td>
<td>4.5910</td>
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<tr>
<td>Insulin AUC (mU/L/min)</td>
<td>2467.34±654.45</td>
<td>6989.47 ± 2301.69</td>
<td>7499.38 ± 2047.60</td>
<td>4.1354</td>
</tr>
<tr>
<td>Glucose AUC (mmol/L/min)</td>
<td>729.23±37.25</td>
<td>712.06 ± 76.71</td>
<td>700.12 ± 46.61</td>
<td>4.7840</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>&lt;2.5</td>
<td>1.60 ± 0.99</td>
<td>1.40 ± 0.40</td>
<td>4.0625</td>
</tr>
</tbody>
</table>
Statistical analysis was obtained with Student’s $t$ test between the two groups.

Normal values for serum testosterone and androstenedione and Insulin and glucose AUC were calculated in a group of health women ($n=15$) average age $23.6\pm1.5$, weight $57.9\pm4.6$, BMI $21.4\pm1.2$. 
TABLE 2. Changes in clinical characteristics and haematochemical parameters after administration of inositol (MYO group) or placebo for 12 weeks in PCOS patients. Data are means ±SD of paired differences between times T₁ and T₀ for each patient. Negative sign indicates a decrease after treatment in comparison with baseline.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MYO group (n=18)</th>
<th>Placebo group (n=8)</th>
<th>p†</th>
<th>effect size‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>-1.83 ± 1.86*</td>
<td>+0.25 ± 0.71</td>
<td>0.0055</td>
<td>1.34</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>-0.69 ± 0.69*</td>
<td>+0.09 ± 0.27</td>
<td>0.0058</td>
<td>1.35</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>-3.3 ± 5.9*</td>
<td>+1.3 ± 6.4</td>
<td>0.0888</td>
<td>0.79</td>
</tr>
<tr>
<td>Diastolic</td>
<td>+0.6 ± 2.4</td>
<td>+2.5 ± 4.6</td>
<td>0.1645</td>
<td>0.62</td>
</tr>
<tr>
<td>Serum Testosterone (nmol/L)</td>
<td>-0.35 ± 0.24*</td>
<td>-0.01 ± 0.29</td>
<td>0.0043</td>
<td>1.38</td>
</tr>
<tr>
<td>Serum Androstenedione (nmol/L)</td>
<td>-3.96 ± 2.16*</td>
<td>+0.28 ± 0.39</td>
<td>&lt;0.0001</td>
<td>2.41</td>
</tr>
<tr>
<td>Fasting serum insulin (mU/L)</td>
<td>-2.33 ± 2.61*</td>
<td>+1.00 ± 0.76*</td>
<td>0.0018</td>
<td>1.55</td>
</tr>
<tr>
<td>Fasting serum glucose (mmol/L)</td>
<td>-0.14 ± 0.31</td>
<td>+0.06 ± 0.13</td>
<td>0.0816</td>
<td>0.77</td>
</tr>
<tr>
<td>Insulin AUC (mU/L/min)</td>
<td>-1668.08 ± 1388.52*</td>
<td>+347.38 ± 314.98*</td>
<td>0.0005</td>
<td>1.77</td>
</tr>
<tr>
<td>Glucose AUC (mmol/L/min)</td>
<td>-13.42 ± 65.52</td>
<td>+17.06 ± 28.64</td>
<td>0.2224</td>
<td>0.55</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>-0.54 ± 0.62*</td>
<td>+0.26 ± 0.16*</td>
<td>0.0015</td>
<td>1.57</td>
</tr>
</tbody>
</table>

*Statistically significant variation between times T₁ and T₀ within patient groups (p<0.05 as significance limit); statistical analysis with paired Student’s t-test.

† comparison between groups; statistical analysis with Student’s t test.

‡ the effect size was calculated post-hoc according to Cohen’s d formula (29), using pooled standard deviations. 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.
Table 3. Comparison between the parameters investigated in erythrocytes from PCOS patients in MYO and placebo groups. Data are means ±SD calculated according to formula in Methods.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>MYO group (n=18)</th>
<th>Placebo group (n=8)</th>
<th>p †</th>
<th>effect size‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr-P</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₀</td>
<td>2.06±0.28</td>
<td>2.00±0.24</td>
<td>0.5819</td>
<td>0.23</td>
</tr>
<tr>
<td>T₁</td>
<td>1.36±0.30*</td>
<td>2.01±0.27</td>
<td>&lt;0.0001</td>
<td>2.32</td>
</tr>
<tr>
<td>Vᵢ(Tyr-P)</td>
<td>0.34±0.10</td>
<td>-0.01±0.05</td>
<td>&lt;0.0001</td>
<td>4.12</td>
</tr>
<tr>
<td>ΔGSH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₀</td>
<td>0.38±0.04</td>
<td>0.39±0.03</td>
<td>0.5290</td>
<td>0.28</td>
</tr>
<tr>
<td>T₁</td>
<td>0.23±0.06*</td>
<td>0.40±0.02</td>
<td>&lt;0.0001</td>
<td>3.43</td>
</tr>
<tr>
<td>Vᵢ(ΔGSH)</td>
<td>0.40±0.14</td>
<td>-0.02±0.05</td>
<td>&lt;0.0001</td>
<td>3.62</td>
</tr>
<tr>
<td>ΔGSSP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₀</td>
<td>0.33±0.03</td>
<td>0.32±0.02</td>
<td>0.3695</td>
<td>0.38</td>
</tr>
<tr>
<td>T₁</td>
<td>0.17±0.04*</td>
<td>0.31±0.03</td>
<td>&lt;0.0001</td>
<td>3.90</td>
</tr>
<tr>
<td>Vᵢ(ΔGSSP)</td>
<td>0.48±0.11</td>
<td>0.02±0.07</td>
<td>&lt;0.0001</td>
<td>4.79</td>
</tr>
</tbody>
</table>

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±SD).

(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\textsubscript{(Diamide)}/GSH\textsubscript{(Basal)})-1.

† p-value following Student’s t-test, comparison between MYO and placebo groups.

* p<0.0001, paired Student’s t test, comparison between T\textsubscript{1} and T\textsubscript{0} within each group.

‡ the effect size was calculated post-hoc according to Cohen’s d formula [29], using pooled standard deviations. 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.
TABLE 4. Correlation between hormonal/metabolic parameters and Tyr-P levels in erythrocytes before (T₀) and after (T₁) MYO administration (n=18).

<table>
<thead>
<tr>
<th>Tyre-P VS</th>
<th>Pearson’s correlation coefficient r</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T₀</td>
</tr>
<tr>
<td>Serum Testosterone (nmol/L)</td>
<td>0.5698 †</td>
</tr>
<tr>
<td>Serum Androstenedione (nmol/L)</td>
<td>0.2028</td>
</tr>
<tr>
<td>Fasting serum insulin (mU/L)</td>
<td>0.3264</td>
</tr>
<tr>
<td>Fasting serum glucose (mmol/L)</td>
<td>-0.2193</td>
</tr>
<tr>
<td>Insulin AUC (mU/L/min)</td>
<td>0.5032 †</td>
</tr>
<tr>
<td>Glucose AUC (mmol/L/min)</td>
<td>-0.2076</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.2968</td>
</tr>
</tbody>
</table>

†p<0.05; statistical analysis of regression by ANOVA