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Effects of α-lipoic acid and myo-inositol supplementation on the oocyte environment of infertile obese women: a preliminary study

Author names and affiliations:

Chiara Novielli[a], Gaia M. Anelli[a], Fabrizia Lisso[a], Anna Marzorati[a], Bina Parrilla[b], Monica Oneta[b], Valeria M. Savasi[a, b], Irene Cetin[a, b] and Chiara Mandò[a*]

* corresponding author

[a]: Università degli Studi di Milano, "Luigi Sacco" Department of Biomedical and Clinical Sciences, Milano, Italy

[b]: ASST Fatebenefratelli Sacco, Unit of Obstetrics and Gynecology, Milano, Italy

Corresponding author:

Correspondence should be addressed to Chiara Mandò;

chiara.mando@unimi.it; fax number +390250319884

Dipartimento di Scienze Biomediche e Cliniche "Luigi Sacco", Università degli Studi di Milano, via GB Grassi 74, 20157 Milano, Italy

ABSTRACT

Obesity is becoming pandemic and is associated with impaired reproductive potential. Oxidative stress, low-grade chronic inflammation and mitochondrial dysfunctions, which characterize obesity, strongly affect oocyte environment and function.

Supplementation with antioxidant and anti-inflammatory compounds has been suggested to improve fertility. Here we evaluated the effect of α-lipoic acid and myo-inositol supplementation on the oocyte environment of infertile obese women.

Nineteen normal-weight and twenty-three obese women, infertile for non-ovarian reasons, were recruited. For two months before ovarian stimulation, all women received 400μg/die folic acid, whereas 15 obese were additionally supplemented with 800mg α-lipoic acid, 2g myo-inositol/die.

Antioxidant capacity was measured in follicular fluid by enzymatic assay; mitochondrial DNA (mtDNA) content and mRNA levels of two respiratory chain subunits were analyzed in granulosa cells by Real-time PCR.

Pregnancy rate was similar between normal-weight and treated obese, and lower in untreated obese patients. Supplemented women showed significantly higher antioxidant levels in follicular fluid compared to the two groups taking only folic acid. Conversely, granulosa cells mtDNA content was decreased in treated and higher in untreated obese patients compared to normal-weight women, suggesting mtDNA increases to compensate for oxidative-stress damages. Reduced expression of respiratory subunits in untreated obese may confirm mitochondria impairment. Interestingly, mtDNA levels inversely correlated to both total and metaphase II oocyte number.

In this preliminary study, combined supplementation of α-lipoic acid and myo-inositol in infertile obese women was associated with amelioration in the oxidative status of the oocyte environment, possibly contributing to a higher pregnancy rate.

KEYWORDS

α-lipoic acid, myo-inositol, infertility, obesity, oxidative status

1. INTRODUCTION

Obesity is nowadays pandemic, representing one of the most important health problems of our society and a major risk factor for several pathologies [1]. It is more prevalent among women and its negative impact on female fertility is widely recognized [2]. Obese women display anovulation, poor oocyte quality with decreased maturation and fertilization rates, delayed time of conception, increased miscarriage rates and pregnancy pathologies [2-4].

Obesity-related malnutrition with micronutrient deficiency can contribute to low-grade systemic chronic inflammation and oxidative stress characterizing obesity. These conditions strongly affect the reproductive trait and the oocyte environment, as well as the systemic and placental milieu [5-8].

Moreover, mitochondrial dysfunctions occur in obesity, possibly impacting oocyte function [9]. Indeed, mitochondria are fundamental organelles for energy production and oocyte developmental competence.

Dietary supplementation with antioxidant and anti-inflammatory compounds gained much attention in recent years in counteracting oxidative stress-related conditions, such as obesity and infertility [10-12]. In particular, α-lipoic acid was reported to decrease body weight and body mass index in obese patients [13] and to reduce risks of pregnancy complications [14]. The insulin-sensitizer myo-inositol was demonstrated to improve fertility in patients with PolyCistic Ovarian Syndrome (PCOS) or metabolic syndromes, and to lower the incidence of Gestational Diabetes in obese women [15-17].

Oocyte growth and development is supported by the continuous communication with granulosa cells and the surrounding follicular fluid [18]. Therefore, alterations of ovarian environment may also impact on oocyte maturation and reproductive potential. Granulosa cells and follicular fluid can thus account for oocyte state indicators.

In the present preliminary observational study, we aimed at evaluating the effect of α-lipoic acid and myo-inositol supplementation on the oocyte environment of infertile obese women, by analyzing oxidative status and mitochondrial markers in follicular fluid and granulosa cells.

2. MATERIAL AND METHODS

2.1 Population

This was an observational study.

Infertile women were enrolled at the *In Vitro* Fertilization (IVF) center in "Luigi Sacco" Hospital, Milan. Infertility was defined as the failure to conceive after ≥12 months of attempts of natural fertilization [19]. Women underwent a transvaginal ultrasound. Exclusion criteria were infertility for ovarian reasons (ovarian and/or endometriosis cysts), PCOS - defined by any two of the three "cardinal features" (irregular cycles, hyperandrogenism and polycystic ovary morphology [20]), or major medical disorders, such as diabetes or hypertension.

Nineteen normal-weight (18≤ BMI (kg/m^2) ≤25) and twenty-three obese (BMI >28) patients with normal ovarian reserve, defined by antral follicle count and AMH (Anti-Müllerian Hormone) blood levels, were included in the study.

For two months before ovarian stimulation, Folic Acid (FA; 400μg/die) was given to all Normal-Weight (NW-F) and to 9 Obese women (OB-F), whereas 15 Obese women were supplemented with 2 tablets of Sinopol® (OB-S; 400μg FA, 2g myo-inositol, 800mg α-lipoic acid/die; Laborest Italia SRL, Italy).

All patients received ovarian stimulation (FSH+antagonist) and assisted reproductive treatment. On day 2 or 3 of the treatment cycle, ovarian stimulation was started by FSH injection (rFSH, Gonalef®; 150–300 IU/die). In the context of treatment strategies aimed at optimal oocyte retrieval, we used a complete and easy nomogram, based on patient's age and serum day-3 FSH in addition to AMH, to individualize the FSH dose to be administered [21].

A daily 0.25mg dose of a GnRH antagonist (cetrorelix acetate; Cetrotide®) was initiated when the mean diameter of the lead follicle reached 13–14 mm on transvaginal ultrasound; rFSH was continued. When at least two follicles developed to a mean of minimum 18 mm in diameter, hCG (Gonasi®; 5,000 or 10,000 IU) was injected to trigger egg maturation.

Ultrasound-guided transvaginal egg retrieval was performed 34–35 h later. IVF, ICSI, or a combination of both, was performed according to the condition of the sperm.

The study was conducted in accordance with the Declaration of Helsinki, with the subjects' understanding and consent.

2.2 Sample collection and processing

After oocyte retrieval, the collected follicular fluid was centrifuged at 1600 rpm for 10 minutes to separate granulosa cells. Cells were resuspended in 500µl Phosphate Buffered Saline and counted using TC20TM Automated Cell Counter (BioRad). Follicular fluid and granulosa cell samples were immediately stored at -80°C until analyses.

2.3 Total antioxidant capacity in follicular fluid

Total Antioxidant Capacity (TAC) was measured in follicular fluid samples (1:15 dilution) with the Antioxidant Assay kit (Cayman Chemical), following manufacturer's instructions. This assay relies on the ability of anti-oxidants to inhibit oxidation of a chromogen agent. Antioxidant concentration is stated as millimolar Trolox equivalents. Samples were analyzed in duplicate. Values with Coefficient of Variation <10% were considered for statistical analysis.

2.4 Mitochondrial DNA (mtDNA) content and gene expression of respiratory chain complexes in granulosa cells

Total DNA and RNA were respectively isolated from granulosa cells using NucleoSpin Tissue XS kit (Macherey-Nagel) and Purelink RNA Mini kit (Life Technologies), following manufacturer's instructions, and quantified by NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies). mtDNA content was assessed by Real-time PCR (Applied Biosystems, Life Technologies), normalizing levels of the mitochondrial gene *Cytochrome B* to those of *RNaseP*, a single-copy nuclear gene [22].

RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) with random examers. Relative gene expression of *SDHA* (*Succinate dehydrogenase complex, subunit A*) and *COX4I1* (*Cytochrome c oxidase subunit 4 isoform* 1) subunits, respectively belonging to the II and IV respiratory chain complexes, was determined by Real-time PCR with TaqMan assays, according to the $2^{-\Delta\Delta Ct}$ method [23] with *HPRT1* (*Hypoxanthine Phosphoribosyltransferase* 1) as endogenous normalizing gene [24].

2.5 Statistical analysis

Data are presented as mean ± standard error.

Clinical records and *COX4I1* expression values, showing a non-normal distribution, were compared among NW-F, OB-F and OB-S groups by Kruskall-Wallis test, with Mann-Whitney U test performed as post-hoc analysis.

Chi-square test was used to evaluate pregnancy frequencies, with Yates continuity correction.

All other molecular results were examined using one-way ANOVA (ANalysis Of VAriance). Data were also analyzed by ANOVA with planned comparisons in OB-S *versus* the other two groups and by independent-samples t-test among obese women.

Correlation between values was assessed by bivariate Pearson correlation.

Differences and correlations were defined statistically significant when $p < 0.05$.

Analyses were performed using SPSS (IBM SPSS Statistics, v.25).

3. RESULTS

3.1 Clinical data

Table 1 reports clinical data of the study population.

Women age was not significantly different among groups.

As defined by inclusion criteria, body mass index was significantly higher in both obese groups compared to normal-weight women ($p<0.001$); BMI was similar between obese patients supplemented either with only FA or with Sinopol®.

Pregnancy rate, defined as successful implantation rate after IVF procedure, was similar in NW-F women (36.8%) and OB-S patients (33.3%), while it was lower in OB-F patients (11.1%), without reaching statistical significance (Chi-square test).

No significant differences were found in the total number of oocytes retrieved during IVF procedure or in the Metaphase II oocytes number.

3.2 Oxidative status and mitochondrial analysis of oocyte environment

To evaluate the effects of α-lipoic acid and myo-inositol combined supplementation on the oocyte environment of obese infertile women, we first analyzed total antioxidant capacity in follicular fluid. *One-way ANOVA with planned comparisons* showed significantly higher antioxidant levels in obese women supplemented with the compound of α-lipoic acid, myo-inositol and folic acid compared to groups supplemented with only folic acid ($p=0.031$). Within obese patients, TAC levels were higher in OB-S than in OB-F patients (t-test: $p=0.021$; Figure 1).

We then analyzed granulosa cells mitochondria, by evaluating mtDNA content and gene expression of two nuclear-encoded subunits of the respiratory chain.

Mitochondrial DNA content was decreased in OB-S and increased in OB-F granulosa cells, compared to NW-F, though not reaching statistical significance (Figure 2). Interestingly, mtDNA levels in granulosa cells significantly and negatively correlated to the number of both total and metaphase II (mature) oocytes ($r=-0.43$, $p=0.007$ and $r=-0.34$, $p=0.037$ respectively; Figure 3).

On the contrary, mRNA levels of *SDHA* and *COX4I1* subunits were reduced, though not significantly, in granulosa cells of obese women supplemented with only folic acid compared to normal-weight; supplementation with α-lipoic acid and myo-inositol seemed to restore both transcript levels (Figure 4).

4. DISCUSSION

In this very preliminary study we observed the combined effects of α-lipoic acid and myo-inositol in infertile obese women. Alpha-lipoic acid antioxidant properties have been previously reported: this compound counteracts oxidative stress-induced degeneration, chelates metals and reconstitutes antioxidant molecules [25], in addition to inhibiting NfKb and reducing inflammatory responses [26]. In idiopathic recurrent pregnancy-loss women, α-lipoic acid was found to reduce endometrial inflammasome expression and activation [27]. Moreover, it is a cofactor for several mitochondrial enzymes and increases insulin efficiency. Myo-inositol is a polyalcohol that directly or indirectly plays a role in important biological functions, such as increasing mitochondria membrane potential, modulating insulin action, inducing calcium flow into the cytosol and regulating cell growth [28]. Myo-inositol levels are high in the female reproductive tract [29] indicating this as an important element of follicular microenvironment [28, 30].

The effect of the combined treatment of α-lipoic acid, myo-inositol and folic acid on obese women fertility has never been investigated before. Interestingly, it has been tested on subfertile men [31]. Indeed, spermatozoa are particularly vulnerable to oxidative damage, and a 3-months supplementation of this compound in normal-weight subfertile patients resulted in improved sperm parameters. Authors hypothesized that the combination of α-lipoic acid, myo-inositol and folic acid may decrease inflammation, protect sperm mitochondria from excessive Reactive Oxygen Species (ROS) and raise their membrane potential, improving semen quality. Moreover, the two components are likely to have a synergistic effect, since they allow the improvement of sperm quality with a lower dosage than when administered individually [31].

Combined administration of α-lipoic acid and myo-inositol was also used in women affected by PCOS. Two recent studies report normalization of menstrual cycle and amelioration of insulin, hormonal and metabolic aspects in PCOS women supplemented with this compound [32-33]. Moreover, normal-weight PCOS patients undergoing IVF who did not achieve pregnancy with myo-inositol treatment alone were

given both α-lipoic acid and myo-inositol [34]. Authors found a decrease in insulin levels, BMI and ovarian volume, and a trend to higher pregnancy rates was reported.

Previous studies in women with PCOS also showed positive effects of the administration of myo-inositol alone on endometrial cells, fertilization rate and embryo quality, as well as changes in granulosa cells gene expression leading to the improvement of oocyte development and competence [15, 35]. On the contrary, the specific effect of alpha-lipoic acid on the oocyte environment and competence has never been investigated. Its antioxidant and anti-inflammatory properties have been reported in human male fertility and in mice models [36-37].

In this preliminary observational study we could not evaluate separated effects of alpha-lipoic acid and myo-inositol in our cohort of obese women without co-morbidities. To our knowledge, no studies have been reported about their specific activity in populations with these characteristics.

We measured oxidative status and mitochondrial markers in the oocyte environment of obese and normal-weight infertile women undergoing IVF, after two months of administration of a compound of α-lipoic acid, myo-inositol and folic acid *versus* folic acid only. We excluded patients presenting ovarian pathologies or with specific diet prescription, in order to avoid potential confounding factors.

Pregnancy rate was lower in obese patients supplemented with folic acid, while it was similar between NW-F and obese women supplemented with α-lipoic acid and myo-inositol.

Follicular fluid and granulosa cells obtained during oocyte retrieval were analyzed for total antioxidant capacity and mitochondrial DNA content, respectively. Indeed, mtDNA levels have been shown to compensatory increase with oxidative stress [38-40]. Moreover, gene expression of two nuclear-encoded subunits of Complex II and Complex IV of the respiratory chain was assessed in granulosa cells.

The obese group taking Sinopol® showed significantly increased antioxidant levels in follicular fluid compared to the other two groups. Mitochondrial DNA content tended to decrease in OB-S and increase in OB-F granulosa cells, compared to NW-F, while both *SDHA* and *COX4I1* transcripts showed an opposite trend, being lower in OB-F and slightly restored in obese women supplemented with α-lipoic

231 acid and myo-inositol. Mitochondrial DNA levels were negatively associated with both total number of
232 oocytes and the number of metaphase II oocytes retrieved for IVF.
233
234 Oxidative-stress products, such as ROS, are physiologically contrasted by endogenous and exogenous
235 antioxidants, which are jointly defined as the Total Antioxidant Capacity of cells. Oxidative stress in
236 follicular fluid can be promoted by obesity [41] and has been reported to predict reduced pregnancy
237 outcome [42] and affect embryonic development [43]. Vice versa, antioxidant capacity in follicular fluid
238 was found to positively correlate with oocyte competence [41], in terms of fertilization rate and
239 developmental ability [43]. In our population of obese infertile women, combined supplementation with
240 α-lipoic acid, myo-inositol and folic acid increased the Total Antioxidant Capacity of follicular fluid
241 compared to supplementation with folic acid alone. This result is consistent with data in mouse oocytes,
242 showing that α-lipoic acid reduces ROS levels and improves TAC [44]. Moreover, supplementation with
243 myo-inositol and active antioxidants (glutathione, selenium, C and E vitamins, zinc) in PCOS women
244 increased glutathione activity in follicular fluid [45].
245 As of today, few studies investigated mitochondria in the ovarian environment, with conflicting results.
246 Mice fed an obesogenic diet, showing oocytes with delayed maturation and decreased developmental
247 competence, presented abnormalities of oocyte mitochondrial morphology, distribution, metabolism and
248 spindle formation [46]. High mtDNA copy number in blastocysts was found indicative of lower embryo
249 viability and implantation [47-48]. Accordingly, our data showed decreased oocyte number with
250 increasing mitochondrial DNA content in granulosa cells. Moreover, mtDNA levels tended to increase in
251 obese patients supplemented with only folic acid compared to equally treated normal-weight women. This
252 result is consistent with previous data showing increased mtDNA content in placentas and peripheral
253 blood of obese pregnant women [22, 49]. Furthermore, higher mtDNA copy number was reported in
254 oocytes of obese mice, with lower citrate levels and increased mitochondrial biogenesis and fission [50-
255 51].

Nisr and colleagues hypothesized that cells initially respond to nutrient-excess damages by increasing mitochondrial content; when this overload becomes sustained and chronic, it overwhelms cell compensation capacity and highly compromises mitochondria [52]. The increase of mtDNA in obesity might thus represent a compensatory mechanism to replace oxidative stress-damaged mitochondria. Indeed, mitochondrial dysfunction was found in muscle and adipose tissue in obese-related conditions, with reduction in respiratory chain complexes expression and activity, ATP deficiency and excessive mitochondrial ROS production [52-55]. The trend to decreased expression that we found in two subunits of the respiratory chain, *SDHA* and *COX4I1*, might confirm a scenario characterized by mitochondria impairment.

The administration of the α-lipoic acid, myo-inositol and folic acid compound might therefore reduce mtDNA levels and slightly restore *SDHA* and *COX4I1* expression in granulosa cells of our population of obese women.

5. CONCLUSIONS

This study supports the evidence that preconceptional health is fundamental for fertility and a successful conception [3, 56]. Preconception lifestyle and diet, properly reinforced with supplementation when inadequate, are needed to improve health of mothers and their offspring, reducing the burden of non-communicable diseases [57]. Given the ongoing obesity pandemic and its effect on reproduction, the research of possible interventions for infertility treatment of obese women has become mandatory in nowadays society.

To our knowledge this is the first pilot study analyzing the effect of combined α-lipoic acid, myo-inositol and folic acid supplementation on the oocyte environment of infertile obese women. Alpha-lipoic acid and myo-inositol were chosen for their antioxidant properties, and for their role in improving insulin action and mitochondrial functionality. The combined supplementation of α-lipoic acid, myo-inositol and folic acid in our population of infertile obese women showed a possible amelioration in the oxidative

status of oocyte environment. This possibly contributed to ovarian improvement, which might have led to higher pregnancy rates in this group of women. Larger studies with longer duration of supplementation are needed to confirm these results and give strength to this hypothesis.

Declaration of interest:

none.

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Author contribution statement

CN performed experiments, statistically analyzed data and drafted the manuscript. GMA, FL and AM performed experiments and analyzed data. BP and MO performed IVF and collected clinical data. VMS recruited and followed-up patients, performed egg retrieval, and revised the manuscript. IC conceived the study and revised the manuscript. CM conceived the study, interpreted data and drafted the manuscript. All authors revised and approved the final manuscript.

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	NW-F	OB-F	OB-S
Age (years)	36.7 ± 0.6	37.6 ± 1.7	35.9 ± 1.1
BMI (kg/m^2)	20.8 ± 0.4	30.2 ± 0.7**	32.7 ± 1.1**
Total oocytes n°	6.9 ± 1.0	7.3 ± 2.1	4.7 ± 0.8
Metaphase II oocytes n°	3.8 ± 0.6	3.6 ± 1.2	2.1 ± 0.7

TABLE 1. Clinical data of population, compared among groups using Kruskall-Wallis test. BMI: Body Mass Index. **p=0.000 *versus* NW-F, Mann-Whitney U test (post-hoc analysis)

FIGURES

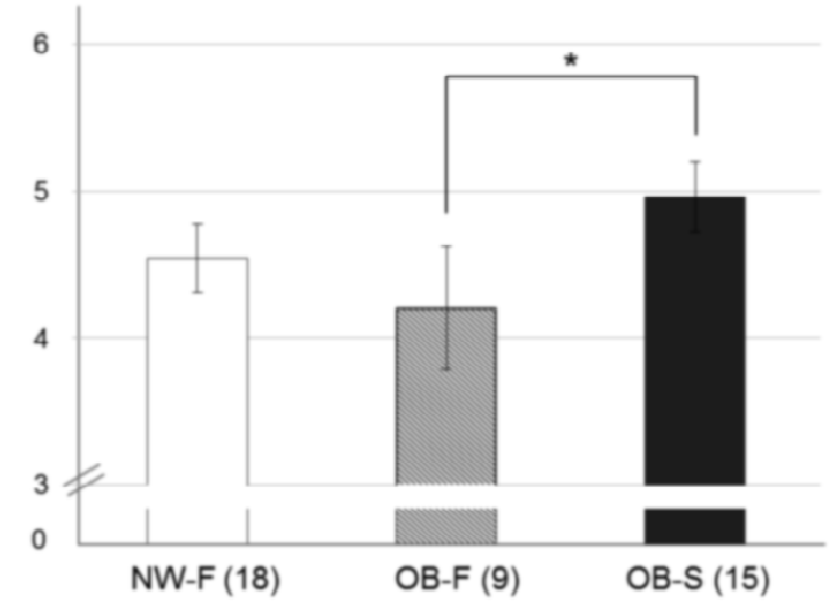

Figure 1. Total antioxidant capacity, indicated as Millimolar Trolox equivalents, in follicular fluid. Values are presented as mean ± standard error; patients numbers are indicated in brackets. *p<0.05 OB-S *versus* OB-F, t-test

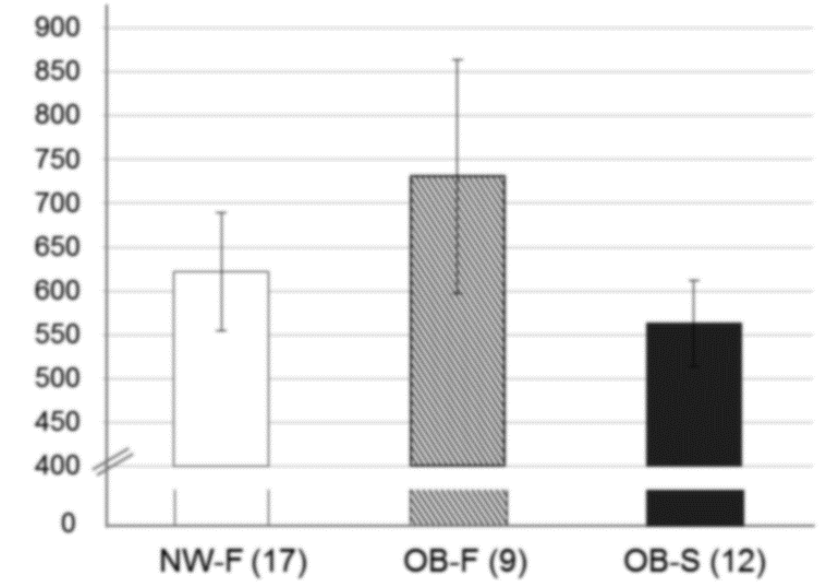

Figure 2. Mitochondrial DNA content in granulosa cells, assessed with Real-time PCR by normalizing the levels of a mitochondrial gene (*Cytochrome B*) to those of a single-copy nuclear gene (*RNaseP*). Values are presented as mean ± standard error; patients numbers are indicated in brackets.

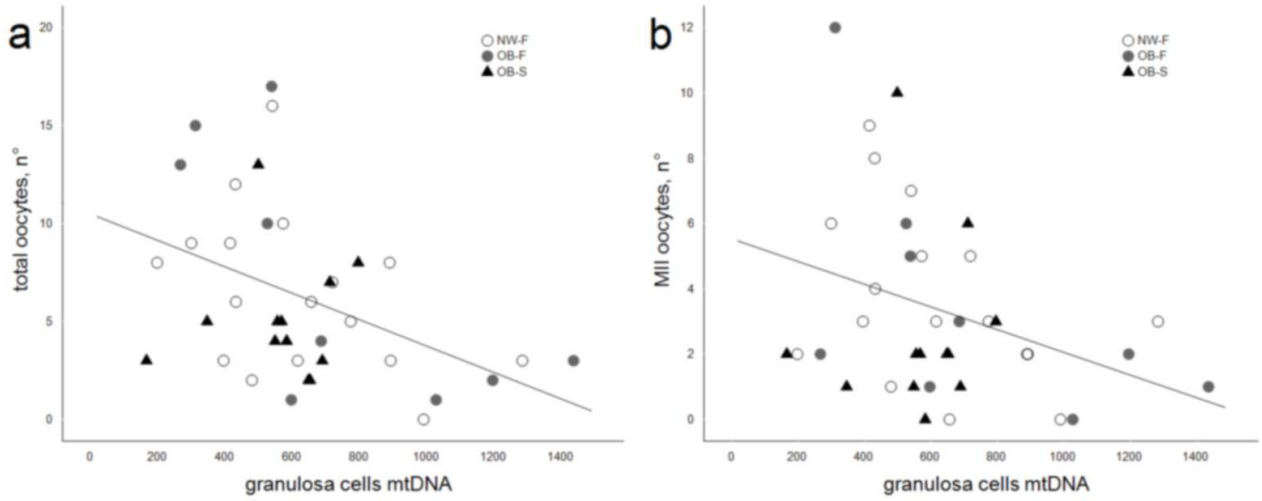

Figure 3. Significant inverse correlations between mtDNA content in granulosa cells and total oocyte number (**a**) (r=-0.43, p=0.007) or Metaphase II oocyte number (**b**) (r=-0.34, p=0.037).

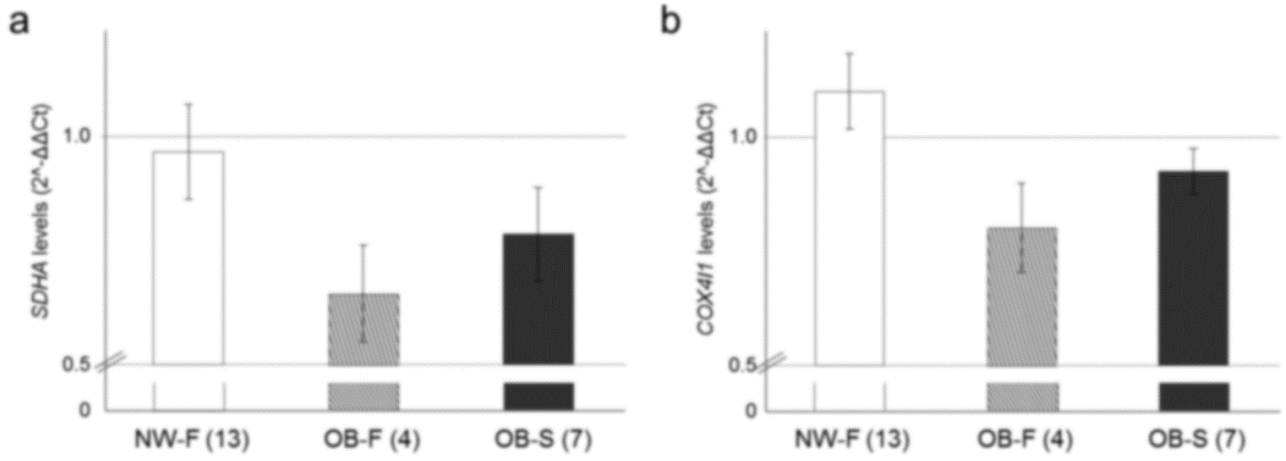

Figure 4. *SDHA* (**a**) and *COX4I1* (**b**) gene expression levels in granulosa cells, determined by Real-time PCR according to the $2^{-\Delta\Delta Ct}$ method with *HPRT1* as normalizing gene. Values are presented as mean ± standard error; patients numbers are indicated in brackets.