J Assist Reprod Genet (2012) 29:1083–1089
DOI 10.1007/s10815-012-9831-x

ASSISTED REPRODUCTION TECHNOLOGIES

The relationship between pregnancy and oxidative stress markers on patients undergoing ovarian stimulations

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Received: 5 February 2012 / Accepted: 4 July 2012 / Published online: 14 July 2012

Abstract
Purpose We investigated the activities and relevance of a validated panel of antioxidant enzymes, cytokines, specific lipid peroxidation end products and six fatty acids by correlational analyses with peak E_2 levels and pregnancy outcome after ovarian stimulation for IVF or IUI.
Methods Blood samples obtained from 15 patients undergoing ovarian stimulation with rFSH or hMG were divided into two groups. Group-1 was baseline blood collected on day-2-3 of women cycle. Group-2 is blood collected at the end of FSH/hMG injection. Serum was collected and stored in liquid nitrogen at -196 °C until analysis. Standard IVF and IUI procedures were followed. The serum levels of Paraoxonase (PON1), Superoxide Dismutases (SOD), Interleukin-6 (IL-6), Glutathione Peroxidase (GPx), 8-Isoprostane, and fatty acids Arachidic, Palmitic, Stearic, Oleic, Linoleic & Linolenic were measured.
Results With the exception of 8-Isoprostane, results showed a positive correlation between baseline and peak levels of E_2 and that of SOD, GPx, PON1, and IL-6. The PON1, IL-6 and SOD were significantly ($p<0.05$) higher in pregnant than non-pregnant group. Fatty acid levels at baseline and peak E_2 were not different but pregnancy rates were found to be decreasing with higher palmitic, and stearic acid levels.
Conclusions Ovarian stimulation causes a significant increase in serum PON1, SOD, GPx and IL-6 activity in women undergoing IVF or IUI. The high levels of IL-6, SOD, and PON1 and lower levels of palmitic, and stearic acids in the pregnancy positive group indicate that these oxidative stress and nutritional factors may be used as a predictive marker in controlled ovarian stimulation success.

Keywords Oxidative stress · Ovarian stimulation · IVF · IUI · Paraoxonase · Superoxide Dismutases · Interleukin-6 · Glutathione Peroxidase · 8-Isoprostane · Fatty acids · Arachidic · Palmitic · Stearic · Oleic · Linoleic & Linolenic

Introduction

Despite of recent advances in assisted reproductive technologies, the success rates remain low, providing distress both for the individual concerned and for the public socioeconomics of women's health. Investigation of factors that impact outcome of IVF and IUIs may help improve success rates. Oxidative stress in women undergoing gonadotropin stimulation has received little attention. Strong evidence implicated oxidative stress in the pathogenesis of infertility causing diseases in women and has been suggested as one of the most important factors that negatively affect ART outcome (1–4). Antioxidant enzymes such as superoxide dismutase, paraoxonase and glutathione peroxidase and pro-inflammatory cytokines such as Interleukin-6 could be beneficial in enhancing implantation and maintaining pregnancy by antagonizing the harmful oxygen free radicals (5–7). Superoxide dismutase enzyme is believed to play a major role in the first line of antioxidant defense by catalyzing the dismutation of superoxide anion radicals to form hydrogen

Capsule Relationship between Pregnancy & OS markers during Ovarian Stimulations

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peroxide (H_2O_2) and molecular oxygen (8). Glutathione Peroxidase (GPx) is a selenoprotein that reduces lipidic or nonlipidic hydroperoxides as well as H_2O_2 while oxidizing GSH (9). Paraoxonase (PON1) is an antioxidant enzyme on HDL that hydrolyses lipid peroxides in oxidized Lipoproteins and 8-Isoprostane is a prostaglandin -F2-like compound that is produced in vivo by the free radical-catalyzed peroxidation of arachidonic acid (12). Serum 8-Isoprostanes levels have been used as a powerful research tool in the study of neurodegenerative diseases, such as Alzheimer's disease and Down's Syndrome and is considered the most sensitive marker of OS currently available (10, 11). The role of free fatty acids in the development and prevention of cardiovascular diseases and diabetes is well known. But, little attention has been given to investigate their effects on reproductive process. Blood collected from women undergoing ovarian stimulation for IVF or IUI allow a unique opportunity to investigate associations between different oxidative stress metabolites and various events in the reproductive process. In this study we sought to analyze differences in levels of serum Paraoxonase, Superoxide dismutases, Glutathione Peroxidase, Interleukin-6, 8-Isoprostane, and fatty acids (Arachidic, palmitic, stearic, oleic, linoleic & linolenic) with regard to ovarian stimulations and its relation to pregnancy outcome.

Methods and methods

Patients and controlled ovarian cycles

This prospective cohort study was approved by the Institutional review board (IRB) of the Medical Center of Central Georgia-Mercer University School of Medicine. Unselected patients undergoing controlled ovarian stimulation for IVF or IUI were invited with informed consents to participate in the study. A total of 15 patients were enrolled in the study from January 2010 through June 2010. This cohort included couples with PCOS, endometriosis, and unexplained infertility. All IVF patients underwent controlled ovarian hyperstimulation by recombinant human follicle-stimulating hormone (rFSH, Follistim) daily for 7-8 consecutive days and follicular development was monitored by serial transvaginal ultrasonography and serum E2 concentrations. Dosage of gonadotropin had been calculated individually for each patient considering patient's age, body mass index, ovarian pattern, menstrual cycles, basal FSH and E_2 hormones, and response to previous controlled ovarian stimulations. An hCG injection was given to trigger the final stages of oocyte maturation and ultrasound-guided oocyte pick-up was performed 34–36 h later. Standard insemination of oocytes was performed using IVF or intracytoplasmic sperm injection (ICSI) procedures according to indications and sperm quality. Fertilization and cleavage was assessed daily and the embryos were classified according to their morphological appearance. Embryos were transferred at day-3 or 5. All IUI patients were give hMG(Bravelle, Ferring) or rFSH r (Gonal-F) on cycle day 3 of cycle and monitored similar to IVF patients. The dose of gonadotropins was adjusted according to ovarian response. When the largest follicle(s) reached a mean diameter of 18 mm, ovulation was induced with 10,000 U of hCG. A single IUI was performed 36–38 h later with a smith insemination catheter. Clinical pregnancy was defined as a positive serum β-hCG (>25 mIU/mL) on day 14 after insemination and by transvaginal ultrasonographic detection of fetal heart beat(s) 4 weeks. All IVF and IUI procedures were perform by the same physician and one embryologist.

Blood samples

Blood samples were processed immediately by centrifugation at 3,000x rpm for 10 min, and clear serum was used for hormone analysis and then stored frozen at −196 °C. Frozen samples were shipped in dry ice to the Department of Clinical Laboratory and Nutritional Sciences, School of Health and Environment, University of Massachusetts Lowell for the analysis of the fatty acids, oxidative stress and inflammatory markers.

Markers of inflammation and oxidative stress

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless indicated otherwise. Markers kits for human 8-Isoprostane (immunoassay for 8-epi-Prostaglandin F2ά), Interleukin 6, and superoxide dismutase were obtained from Cayman Chemical Company (Ann Arbor, Michigan, USA). Glutathione peroxidase was purchased from Zepto-Metrix Corporation, (Buffalo, New York, USA) . Serum PON1 arylesterase activity was measured using 1 mM ρNPA as substrate. Typically, aliquots of 10 μl of serum were placed in microtiter plate wells in triplicate. The reaction was initiated by the addition of the substrate ρNPA, to yield a final concentration of 1 mM in PBS buffer containing calcium and magnesium. After mixing, the plate was read immediately to establish 0 time values, and the reactions were incubated at 37 °C. Readings were recorded at the end of 30 min. PON1 activity was calculated using the molar absorbitivity (12).

Fatty acids (FA) methylesterfication and gas chromatography analysis

Methylester of serum lipids was prepared by adding 1 ml of 14 % boron trifluoride (BF3) methanol extracted and dried lipids; the mixture was then placed in water bath at 75 ° C

Table 1 Comparison of pregnant and no-pregnant patients in term of age, infertility period, hormonal levels and oxidative stress markers

	Pregnancy positive Mean±SEM) (n=7)	Pregnancy negative (Mean±SEM) (n=8)	p
Age (years)	33.0 (±3.0)	33.5 (±6.5)	NS
Infertility period (years)	5.2 (±2.9)	6.9 (±3.8)	NS
FSH (mIU/mL) level on 3rd day	7.1 (±1.22)	7.4 (±2.47)	NS
E2 (pg/mL) Baseline level	27 (±15)	29 (±13)	NS
E2 (pg/mL) Peak level on HCG day	1,114 (±579)	726 (±389)	<0.05
P4 (ng/mL) level on HCG day	1.2 (±0.6)	0.8 (±0.3)	NS
SOD (U/mL)	204.1 (±35.9)	156.3 (±14.9)	0.014
GPx mU/mL	589.7 (±34.8)	616.6 (±48.9)	NS
IL-6 (pg/ml)	18.8 (±4.6)	12.5 (±1.2)	0.047
PONI U/L	448.4 (±175)	246 (±78.5)	0.001
8-Isoprostane pg/mL	84.2 (±14.6)	81.0 (±6.0)	NS

for 20 min. A methylestered FA was later extracted in ether and dried under nitrogen gas. Prepared methylesters were then dissolved in hexane and injected in a Sigma-Supelco capillary column (50 m X 0.25 mm, CP 6173); installed in a Varian CP-3380 Gas Chromatography (Varian, Inc USA). The column temperature was kept at 140 ° C for 0.5 min, and then gradually increased up to 225 ° C at the rate of 4 ° C per minute. The injector temperature was kept at 270 ° C and the detector (FID) temperature at 300 ° C (13).

Statistical analysis

The results were expressed as mean±SEM, and the significance of the differences between the means was determined by the Student's t test. The difference was considered statistically significant at $P<.05$. Differences between pregnant and non pregnant groups were analyzed using analysis of variance. Posthoc Bonferroni-Holm test for multiple comparisons of the means was used.

Results

Patients' BMI, Day-3 FSH, and baseline E_2 were within normal ranges of good responders. The mean age of patients was (33.3±3.4 years) and etiology of infertility was endometriosis (n=7), PCOS (n=2), and unexplained (n=6). The mean duration of infertility in the study group was about 5 years. Although not statistically significant, women in the unexplained-infertility group had longer duration of infertility (mean of 6.9 years, including previous failed attempts). The total dose of gonadotropin range from 766 to 1,810 IU and the duration of stimulations were between 6 to 11 days according to patient ovarian response. Patient clinical characteristic, infertility causes and treatment plan were found to be independent of the serum analysis results, thus pooled together and compared on the bases of baseline and peak E_2. Serum levels of PON1, SOD, GPx, IL-6, and fatty acids were positively correlated with E_2 peaks in all patients regardless of treatment plan or infertility etiology. Seven out of fifteen patients achieved clinical pregnancy. Comparison between women who achieved pregnancy after IVF or IUI vs. women who did not are shown in Table 1. There were no statistical differences between the two groups in terms of BMI, duration of infertility, P4, GPx and 8-Isoprostane levels. Women with high serum concentrations of SOD, PON1 and IL-6 at peak E_2 level were found have higher chance of pregnancy compared to others. Data showed that when the larger etiologic groups such as endometriosis and unexplained infertility were unpacked and compared, no differences were observed in the clinical characteristics and the measured serum OS biomarkers. But difference in pregnancy outcome was observed Table 2. Four of the seven endometriosis, two of six unexplained, and one of two PCOS patients achieved clinical pregnancy. The relationship between oxidative stress markers and ovarian stimulations are shown in Fig. 1. There was significant positive correlation between levels of E_2 and the levels of SOD, GPx, PON1 and IL-6 after ovarian stimulation in all patients. The serum levels of 8-Isoprostane was not correlated with peak E_2 levels and was not different in pregnant and non pregnant groups.

Mean values of free fatty acid of all patients at baseline and peak E_2 are shown in Fig. 2. There was no significant relationship between fatty acid and E_2 levels during ovarian

Table 2 Pregnancy outcome according to infertility diagnosis

Etiology	% Pregnancy positive (n/n)	% Pregnancy negative (n/n)
Endometriosis	58 % (4/7)	42 % (2/7)
PCOS	50 % (1/2)	50 % (1/2)
Unexplained	33 % (2/6)	64 % (4/6)

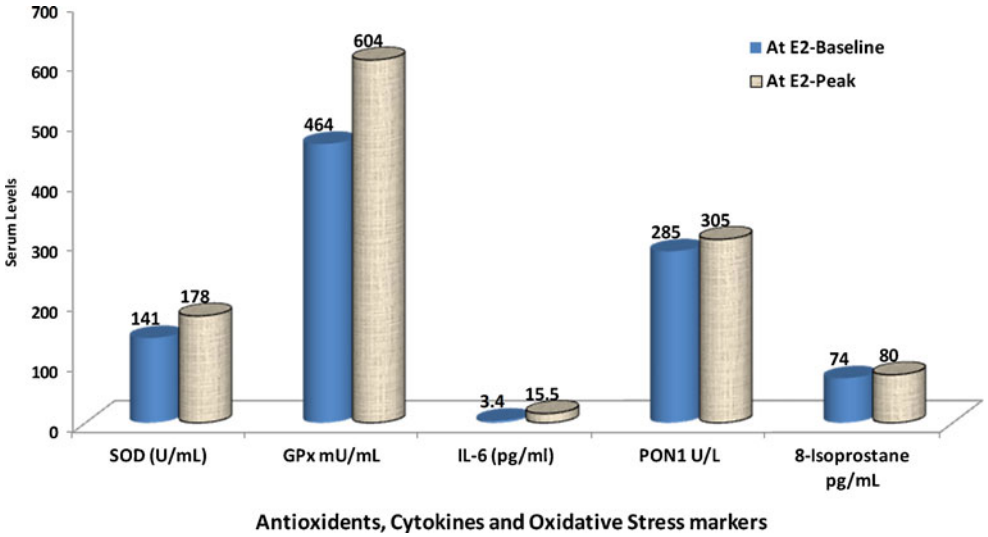

Fig. 1 The levels of serum antioxidant enzymes, cytokines and oxidative stress markers collected at baseline prior to hormonal stimulation and at peak E_2 on the day of hCG injection. PON = Paraoxonase, SOD = Superoxide Dismutases, IL-6 = Interleukin-6, and GPx = Glutathione Peroxidase. Mean values are shown on top of each data point

stimulations. However, differences between baseline and peak values of Palmitic, and Stearic acid were found among pregnant and non-pregnant group (Fig. 3 and 4). The baseline value was higher whereas the peak value was lower ($p<$ 0.05) in pregnant than non-pregnant group (Fig. 3 and 4).

Discussion

Controlled ovarian stimulation is frequently used with IVF and IUI cycles to obtain multiple oocytes and to improve pregnancy rates. Women undergoing gonadotropin stimulation were always exposed to supra-physiological levels of E_2 and P_4. These high steroid levels may have direct impact on oxidative stress markers and may influence conception (14, 19, 21). The present study demonstrated that ovarian stimulation causes significant increase in serum PON1, SOD, GPx and IL-6 activities in women undergoing IVF or IUI and that the enhanced effects were positively correlated with E_2 peaks. Furthermore, the serum levels of SOD, PON1 and IL-6 was positively associated with pregnancy indicating possible physiologic role in improving implantation. This is in agreement with other investigators who evaluated a variety of oxidative stress biomarkers in follicular fluid and/or serum and found direct relationship between ovarian stimulations and total antioxidant capacity/activity (TAC/TAA) and pregnancy (14–18). The TAC/TAA measured by those investigators showed inverse relationship with ROS levels and pregnancy outcome (20, 22, 26). Some studies even suggested that E_2 by itself has antioxidant property and that adverse oxidative stress is associated with lower E_2 levels (14).

Under physiological conditions, enzymatic antioxidants function to prevent ROS production, scavenge existing free radicals, and promote the repair of ROS-induced damage to cell structures. Some studies found ovarian stimulations altered intrauterine secretions of cytokine, chemokine, and growth factors in women undergoing IVF cycles (27) and showed positive association between IL-6 in the follicles with pregnancy (6). In contrast, other investigators suggested that ovarian stimulation induces the production of

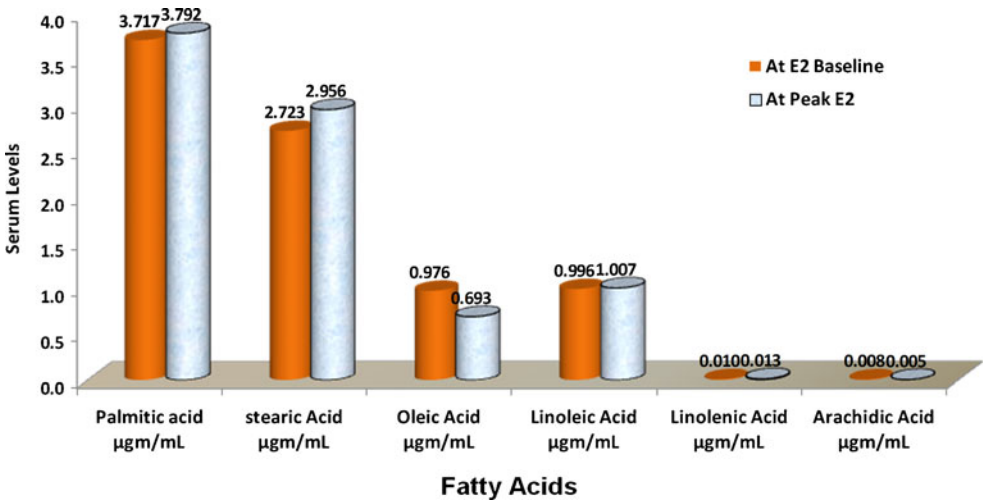

Fig. 2 The levels of serum fatty acids at baseline prior to ovarian hormonal stimulation and at peak E_2 on the day of hCG injection. Mean values are shown on top of each data point

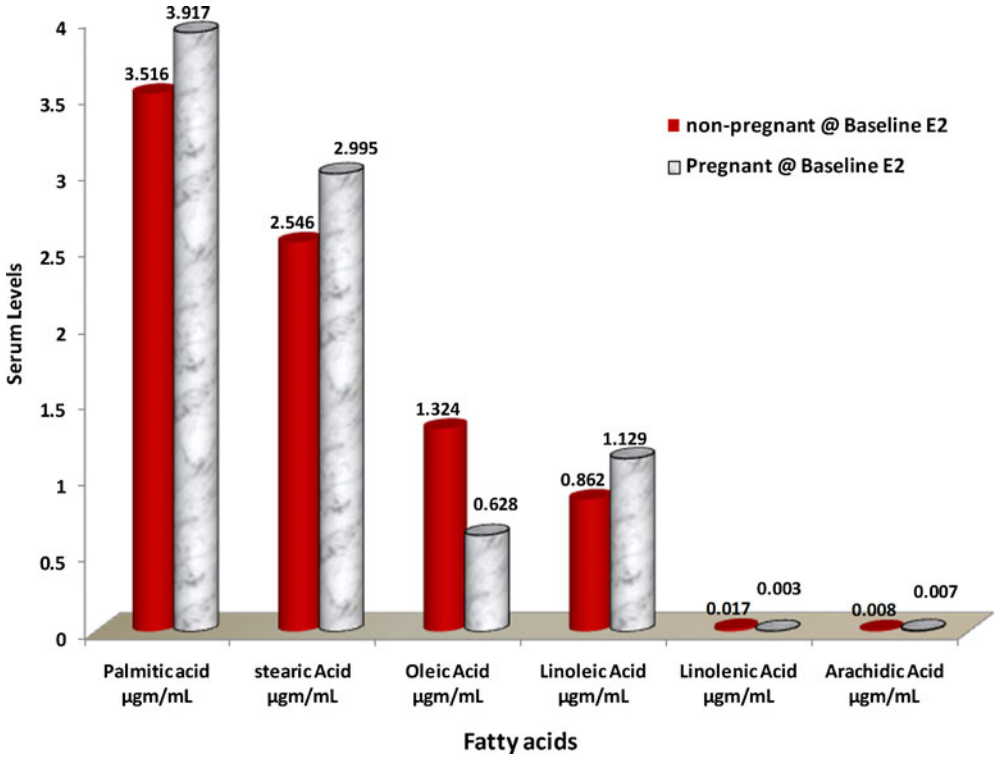

Fig. 3 Difference between pregnant and non-pregnant patients on the levels of serum fatty acids at baseline prior to ovarian hormonal stimulation. Mean values are shown on top of each data point

ROS and perturbation in the oxidant-antioxidant balance (15, 19). The disagreement of these findings with ours may reflect differences in methodology or variation in patient populations.

In this study we also saw no associations between serum 8-Isoprostane and the free fatty acids with ovarian stimulation and peak E_2 levels. While 8-isoprostane level was not affected by pregnancy outcome, there was a negative association between palmitic and stearic acid levels and pregnancy rates. The 8-isoprostane finding was in agreement with other investigators (11) who reported that 8-Isoprostane levels in follicular fluid were similar to that in plasma and were not affected

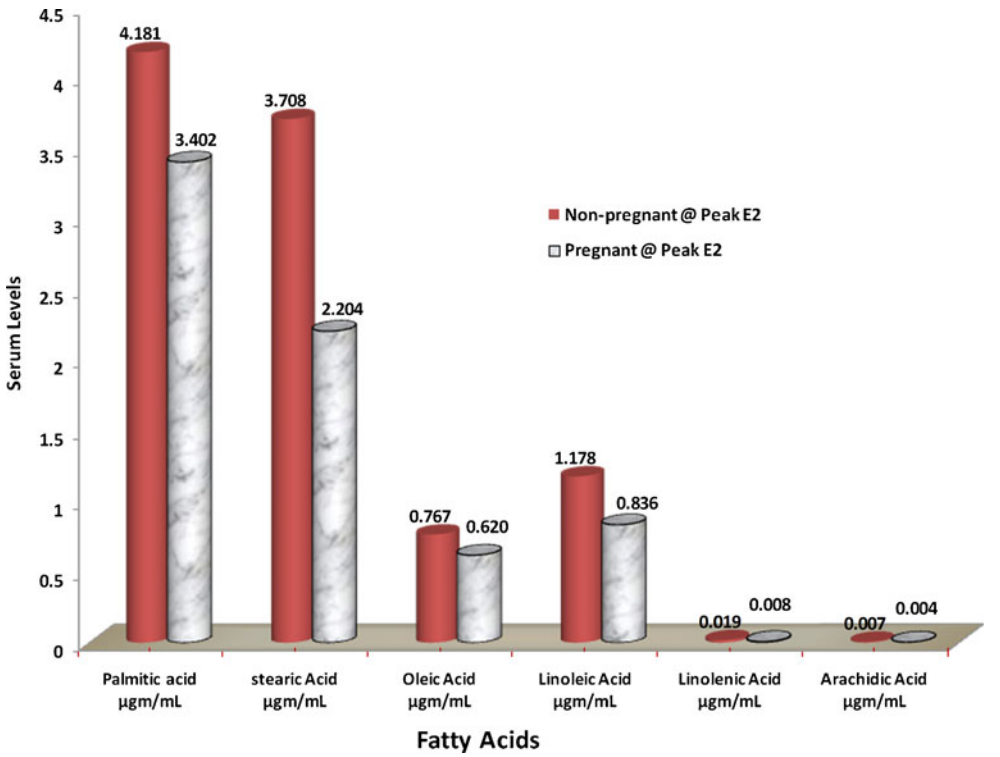

Fig. 4 Difference between pregnant and non-pregnant patients on the levels of serum fatty acids at peak E_2 level after ovarian hormonal stimulation. Mean values are shown on top of each data point

by patient's age, infertility diagnosis, and pregnancy outcome (11). Our free fatty acid data is comparable with the study of Jungheim et al. (28), which showed no associations between free fatty acid and ovarian response to gonadotropin in women undergoing IVF (28). These investigators demonstrated that major free fatty acid in the ovarian follicle were oleic, palmitic, stearic, and linolei acids and also found associations between elevations in total follicular fatty acids and poorer oocyte quality suggesting that excess fatty acid adversely influence ovarian follicular function. In another study by the same group (29), elevated levels of alpha-linolenic acid in serum or follicular fluid were negatively associated with embryo implantation rates and occurrence of clinical pregnancy (29). It is important to note that those investigators collected serum from fasting patients on the morning of oocyte retrieval, whereas, in our study bloods were drawn from non-fasting patients prior to/and at the end of ovarian stimulation with gonadotropin. Regardless of fasting or not, our finding of the negative association of serum palmitic and stearic acids levels with pregnancy outcome suggest possible role of these fatty acids on embryo development and raise questions for future research that worth exploring. Palmitic and stearic acids have been shown to cause adverse effects on human granulosa cells (24) and oocytes (23). Moreover, high levels of both palmitic and stearic acids in follicular fluid were recently implicated as marker of infertility in dairy cows and heifers (25).

There are some limitations to our study that need to be taken into consideration. First, while the finding regarding association between pregnancy and high levels of IL-6, SOD, and PON1 and lower levels of palmitic and stearic acids are significant, the number of women in the study is small. In addition, we do not know whether the increased levels of serum PON1, SOD, GPx, IL-6, and free fatty acids were the result of increased nutritional consumption or indicative of variations in metabolism, or both. Therefore these results cannot be extrapolated to all infertility patients before further future studies with larger number of patients has been conducted. Additional research question raised by the study was whether the type of food and multivitamin intakes during ovarian stimulations alters serum fatty acids and OS biomarker which influence IVF-IUI outcomes. Finally, when the larger etiologic groups such as endometriosis and unexplained infertility were unpacked and compared, more than half of the women who achieved pregnancies were known to have endometriosis. That result was important and interesting, but the number of women was small to justify an statistical conclusion, therefore we suggest further study with larger numbers of patients to see possible association between endometriosis, PCOS or unexplained infertility with these OS markers. The high levels of IL-6, SOD, and PON1 and lower levels of palmitic, and stearic acids in pregnancy positive group indicate that these oxidative stress and nutritional factors may be used as a predictive marker in controlled ovarian stimulation success.

Acknowledgement This work was partially supported by a faculty startup grant from the University of Massachusetts Lowell to M.G.

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