Original Article

Comparison of antagonist mild and long agonist protocols in terms of follicular fluid total antioxidant capacity

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ABSTRACT

Objective: A high dose of prolonged gonadotropins can yield higher numbers of oocytes and embryos. The high dose or prolonged regimens can be associated with ovarian hyperstimulation syndrome (OHSS), multiple gestations, emotional stress, economical burden and treatment dropout. In mild stimulation lower doses and shorter duration times of gonadotropin are used in contrast to the conventional long stimulation protocol in IVF. It has been proposed that supraphysiologic levels of hormones may adversely affect endometrium and oocyte/embryo. Also it has been proposed that oxidative stress (OS) may alter ovarian hormone dynamics and could be further affected by additional exogenous hormonal stimulation. Therefore our aim was to compare follicular fluid total antioxidant capacity (TAC) in antagonist mild and long agonist stimulations.
Materials and Methods: Forty patients received antagonist mild stimulation, starting on the 5th day of their cycle and forty patients received long agonist treatment. Seventy-five patients undergoing their first IVF cycle were included in the final analysis. Follicular fluid (FF) samples were analyzed for estradiol (E2), antimullerian hormone (AMH) and TAC.
Results: FF-Total antioxidant capacity (TAC) levels were higher in the long agonist group as opposed to the antagonist group [1.07 ± 0.04 mmol Trolox equivalent/L vs 1 ± 0.13 mmol Trolox equivalent/L] (Fig. 1). Pregnancy rates were not significantly different between the two treatments. The FF-TAC levels were not different among infertility etiologies (Fig. 3). FF-TAC levels did not have a direct correlation with pregnancy but a positive correlation with the total gonadotropin dose was observed.
Conclusion: Patients with good ovarian reserves and under the age of 35 effectively responded to mild stimulation treatment. Using lower amounts of gonadotropin, yielded less FF-TAC levels in patients who underwent antagonist mild protocol. In patients under the age of 35, antagonist mild stimulation is a patient friendly and effective procedure when undergoing their first IVF cycle.

Introduction

The objective of the ovarian stimulation protocol is to stimulate the growth of multiple follicles and oocytes to yield more embryos for selection and transfer.

A high dose of prolonged gonadotropins can yield higher numbers of oocytes and embryos. The long gonadotropin releasing hormone (GnRH) agonist stimulation is one of the most used procedures for in-vitro fertilization (IVF) [1]. The high dose or prolonged regimens can be associated with ovarian hyperstimulation syndrome (OHSS), multiple gestations, emotional stress, economical burden and treatment dropout. Improved understanding of ovarian and follicle development have led to the development of milder, patient-friendly ovulation induction treatments [2]. These milder regimens use lower doses of gonadotropin to yield fewer oocytes in a shorter period of stimulation [3]. These findings have

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helped personalize infertility treatments making them more patient-friendly and accessible.

Oxidative stress (OS) has been linked to many disorders, including infertility. Reactive oxygen species (ROS) in the ovary are created by phagocytic macrophages, parenchymal and endothelial cells [4]. Anti-oxidant enzymes are found in granulosa and theca cells and work to overcome oxidative stress [5]. There is a balance of oxidative and anti-oxidative status in the ovary. Moderate levels of OS stimulate theca cell proliferation but as OS increases it inhibits the proliferation of theca cells [6]. This suggests that OS may alter ovarian hormone dynamics and could be further affected by additional exogenous hormonal stimulation. Further, another study showed that the certain genes in embryos were altered by gonadotropins in a dose-dependent manner [7].

Our hypothesis was to yield favorable pregnancy rates with the usage of fewer amounts of gonadotropins, resulting in less oxidative stress, in patients <35 years of age undergoing their first IVF attempt. Therefore, we compared the follicular fluid total anti oxidative capacity and pregnancy rates using two different ovarian stimulation protocols.

Materials and methods

This prospective study was conducted on a cohort of 80 consecutive patients admitted to the Istanbul University Cerrahpasa Faculty of Medicine, Department of Obstetrics and Gynecology, Division of Reproductive Endocrinology and Infertility Unit between August 2011 and March 2012. Informed consent was obtained from all participants before the study and the ethics committee of the university approved the study protocol. Seventy-five patients were included in the final analysis of data.

The inclusion criteria for this study included; patients who were undergoing their first IVF cycle, younger than 35 years old with a basal follicle stimulating hormone (FSH) level <10 mIU/mL, basal metabolic index (BMI) of 19–30 kg/m^2, and no current or past diseases affecting the ovaries. Poor responders and endometriosis cases were excluded. Diagnosis of polycystic ovary syndrome (PCOS) was performed according to the Rotterdam criteria [8]. Eligible patients were assigned sequentially to one of the IVF treatments, except patients with PCOS who were assigned to the antagonist protocol exclusively.

Forty patients received antagonist mild ovarian stimulation with the GnRH antagonist cetrorelix (*Cetrotide®*, Serono, Turkey) and recombinant FSH (Gonal F®, Serono, Turkey). The rFSH cycle was started on the fifth day with a dose of 150 IU if the antral follicle count (AFC) was >10 or 225 IU if the AFC was <10. A subcutaneous (s.c) dose of 0.25 mg/day cetrorelix was administered when the largest follicle reached a diameter of 14 mm. Thirty-five patients were included in the final analysis, five patients dropped out of the study, as they could not be contacted after the initial treatment. Forty patients received the conventional long GnRH agonist treatment, leuprolide acetate 1 mg/day s.c (Lucrin®, Abbot, Turkey) beginning on day 21 of the previous cycle. On the 3rd day of the cycle leuprolide dose was reduced to 0.5 mg/day, and a daily dose of rFSH was initiated at 225–300 or 375 IU depending on age and BMI.

Patients were followed up by transvaginal ultrasound scans, and when more than 2 follicles >18 mm were seen, 250 μg human chorionic gonadotropin (hCG; Ovitrelle® 250 mcg, Serono, Turkey) was injected to induce final oocyte maturation. After 36 h, ovum pick-up (OPU) was performed. Single embryo transfer (SET) was performed after 3 days according to the Turkish Ministry of Health's legislation. The luteal phase was supported with 8% vaginal progesterone (Crinone gel®, Serono, Turkey) until the pregnancy test 12 days after embryo transfer. Clinical pregnancy was defined as observation of fetal heartbeats at 7–8 weeks of gestation by ultrasound. Classification of oocyte maturity and embryo grading were performed according to Veeck et al. [9].

Before the IVF treatment, serum anti-Müllerian hormone (AMH), follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), thyroid-stimulating hormone (TSH), and estradiol (E2) levels were measured on cycle day 3 in all patients. Serum AMH concentrations were measured with an enzymatically amplified two-sided immunoassay (DSL-10-14400 Active Müllerian Inhibiting Substance/AMH enzyme-linked immunosorbent assay [ELISA] kit, Diagnostic Systems Laboratories [DSL], Webster, TX). The theoretical sensitivity of the method was 0.006 ng/ml, with an intra-assay coefficient of variation for high values of 3.3% and an interassay coefficient of variation for high values of 6.7%. Serum FSH, E2, and LH were measured using a chemiluminescent microparticle immunoassay (Architect Abbott Lab, IL, USA).

Follicular fluid (FF) of mature follicles >17 mm was aspirated and pooled for each patient. Follicle aspirates that were not clear or were contaminated with blood were discarded. After collecting the oocytes, FF was centrifuged at 2000×*g* for 10 min to separate erythrocytes, leukocytes, and granulosa cells. The samples were frozen at −80 °C until assayed. TAC is the measurement of total antioxidant capacity against free radicals. TAC was measured using the total antioxidant status kit (TAC assay kit, RL0017, Rel Assay Diagnostics, Gaziantep, Turkey). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a vitamin E analog, was used as a standard and the assay results are expressed in mmol Trolox equivalent L^{-1} in reference to a standard curve. Antioxidants in the sample reduce the dark blue-green-colored ABTS radical to a colorless reduced ABTS form. The change of absorbance at 660 nm is related with the total antioxidant level in the sample (TAC assay kit, RL0017, Rel Assay Diagnostics, Gaziantep, Turkey). Follicular fluid samples were analyzed by spectrophotometer at 450 nm for AMH and E2 while for TAC 660 nm was used.

The primary outcome of the study was the difference of FF-TAC levels among the groups. A secondary outcome of our study was the difference in pregnancy rates among the groups.

Due to the limited data on follicular fluid levels of TAC in different stimulation protocols, *a priori* sample size calculation was not performed. However, a post-hoc power analysis (Wilcoxon–Mann–Whitney test) revealed that the present sample size was adequate to evaluate the observed differences in follicular fluid TAC levels between mild protocol and long agonist protocol at 0.05 significance level and 86.9% power. Statistical power analysis was performed using the G*Power software.

The Shapiro–Wilk test was used to demonstrate a normal distribution. Data are expressed as means ± standard deviation (SD), median (IQR), or frequencies and percentages. For independent samples, the Mann–Whitney *U*-test was used to analyze non-normally distributed data, and the independent-sample *t*-test was used for normally distributed data. Categorical characteristics of patients were compared using χ^2 with Yates correction. Pearson correlation was used to explore the relationship between FF-TAC and total dose of gonadotropins. Logistic regression analysis was used for the review of categorical and continuous data. The discriminatory abilities of FF-TAC, FF-AMH and FF-E2 on pregnancy were assessed by receiver operating characteristics (ROC) curve analysis. ROC curve results were given by area under the curve (AUC) and 95% confidence interval (CI). All analyses were performed using STATA version 14.0 (StataCorp LP, Texas, USA) and GraphPad Prism 7.0 (GraphPad Software, California, USA). A *p*-value of <0.05 was considered statistically significant.

Results

Demographic data is given in Table 1. The starting dose of gonadotropin, total dose, and days of stimulation were significantly lower in the antagonist mild group. In the follicular fluid the median AMH and mean E2 levels were not significantly different. However, mean TAC levels were higher in the long agonist group than in the antagonist group (Fig. 1). There were no significant differences between the groups in terms of oocyte and embryo quality (Table 2). The number of pregnant patients was 10 in the antagonist mild group (10/35, 28.5%) and it was 18 in the long agonist group (18/40, 45%). The fertilization and pregnancy rates were not significant among the groups (Table 2).

There was no difference in terms of follicular fluid parameters among the pregnancy subgroups (Table 3). Pregnant patients were followed up at our clinic or contacted by phone. In the antagonist mild group, nine of ten patients were contacted, three pregnancies resulted in early pregnancy loss (<12 gestational week) and six resulted in a term live birth. In the long agonist group, 15 patients were contacted, resulting in three early pregnancy losses and twelve full-term live births *(NS)*.

We also compared the FF-TAC levels between pregnant and non-pregnant patients for each group. This comparison did not yield significant differences. FF-TAC levels were not different among infertility etiologies and as well as PCOS/nonPCOS patients (Fig. 3). Logistic regression analysis did not find a significant relation between FF-TAC and pregnancy ($p = 0.56$). The area under the curve (AUC) is calculated for FF-TAC, FF-AMH and FF-E2 in the prediction of pregnancy. AUC for FF-TAC was 0.57 [95% CI, 0.44–0.71], for FF-AMH was 0.57 [95% CI, 0.44–0.70] and for FF-E2 was 0.45 [95% CI, 0.31–0.59]. None of the AUC values were significant in the prediction of pregnancy (Fig. 2).

Further, no correlation was detected between FF-TAC and FF-AMH and FF-E2 levels. FF-AMH also did not correlate with FF-E2 and pregnancy. We did however, find a weak positive relationship between FF-TAC and total gonadotropin dose ($r = 0.241$, $p = 0.03$).

Discussion

Our results yielded similar oocyte quality, pregnancy and live birth rates in the antagonist mild group as compared to the long
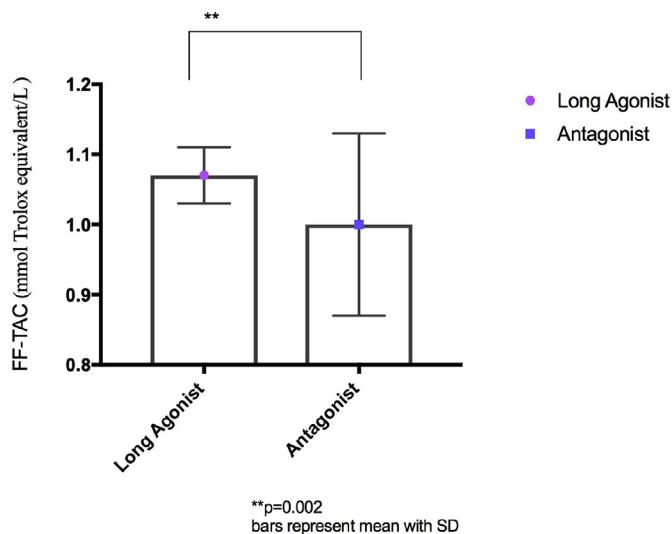

Fig. 1. FF-TAC levels among groups.

agonist group, which had less gonadotropin and fewer stimulation days. Follicular fluid TAC levels were higher in the long agonist group compared to the antagonist group (Fig. 1). Moreover, we found a positive relationship between FF-TAC and total gonadotropin dose. These findings support our hypothesis of an increase in oxidative stress due to increased amounts of exogenous gonadotropin. On the other hand, FF-TAC levels were not significant among infertility etiologies (PCOS, tubal, unexplained and male) (Fig. 3) and pregnant subgroups.

Mild ovarian protocols were developed to extend the FSH gate through the administration of exogenous FSH during the mid-to-late follicular phase [10]. The strategy of administering low doses of FSH in this period resulted in fewer oocytes with compatible pregnancy rates. A few trials have compared the long agonist stimulation with the mild stimulation in patients with normal ovarian reserve. Hohmann et al. compared three protocols: a long agonist protocol (group A, $n = 45$), a mild protocol that began rFSH on cycle day 2 (group B, $n = 48$) and another mild protocol that

Table 1
Demographic data of the study groups.

	Mild stimulation group (n:35)	Long agonist group (n:40)	p value
Age (year)	28.53 ± 3.56	30.05 ± 3.21	0.07
BMI (kg/m^2)	23.69 ± 3.06	24.42 ± 3.24	0.326
Number of smokers (%)	7 (21%)	9 (22%)	0.842
Primary infertility (%)	32 (94%)	34 (85%)	0.19
Secondary infertility (%)	2 (6%)	6 (15%)	0.19
Infertility duration (years)	5.05 (2–6)	6 (3.5–8)	0.02*
Infertility Etiology			
PCOS	16 (45%)	0	–
Male	11 (31%)	18 (45%)	0.21
Tubal	3 (8.5%)	5 (12%)	0.62
Unexplained	5 (14%)	17 (42%)	0.008*
Day 3 serum FSH (mIU/ml)	4.84 (3.88–6)	5.74 (4.47–6.91)	0.03*
Day 3 serum LH (mIU/ml)	4.95 (3.5–6.5)	3.62 (2.82–4.91)	0.003*
Day 3 serum E2 (pg/ml)	43.78 ± 16.15	39.59 ± 18.22	0.07
Day 3 serum TSH (mIU/ml)	1.65 ± 0.77	1.93 ± 1.06	0.34
Day 3 serum PRL (ng/ml)	15.6 (11.2–23.9)	17 (13.1–25.5)	0.39
Day 3 serum AMH (ng/ml)	4.84 (3.23–8.63)	2.37 (1.3–2.95)	0.001*
Antral follicle count (AFC)	9 (7–12)	5 (4–6.5)	0.001*
Gonadotropin starting dose (IU)	183.4 ± 50.3	301.8 ± 68.5	0.001*
Total gonadotropin dose (rFSH) (IU)	1458.82 ± 453.56	2757.36 ± 877.61	0.000*
Duration of stimulation (days)	8.03 ± 1.22	9.26 ± 1.48	0.000*

AMH: anti-müllerian hormone; BMI: body-mass index; E2: estradiol; FSH: follicle stimulating hormone; LH: luteinizing hormone; TSH: thyroid stimulating hormone; PRL: prolactin; PCOS: polycystic ovary syndrome. Data presented as means ± standard deviation, median (IQR) or number (%). *$p < 0.05$ is significant.

Table 2
Comparison of the laboratory and embryologic data between groups.

	Mild stimulation group (n:35)	Long agonist group (n:40)	p value
Serum E2 level on the day of HCG (pg/ml)	1394 (880—1772)	1339 (824—1904)	0.67
Follicular fluid AMH (ng/ml)	1.37 (0.76—1.62)	1.72 (1.12—1.97)	0.06
Follicular fluid E2 (pg/ml)	483.9 ± 146.9	463.7 ± 117.8	0.51
Follicular fluid TAC (mmol Trolox equivalent/l)	1 ± 0.13	1.07 ± 0.04	0.002*
Number of oocyte retrieved	10.24 ± 4.15	9.15 ± 2.92	0.32
Number of MII oocytes	7.7 ± 3.59	7.25 ± 2.93	0.57
Number of ICSI performed oocytes	7.56 ± 3.29	7.53 ± 2.67	0.96
Number of oocytes with normal polar body	7.93 ± 3.52	7.47 ± 2.84	0.56
Number of oocytes with normal zona pellucida	7.83 ± 3.65	7.13 ± 3.15	0.41
Number of oocytes with normal size	7.93 ± 3.52	7.52 ± 2.76	0.60
Number of pronuclear (PN) zygote 2	4.97 ± 2.98	4.84 ± 2.35	0.83
Number of grade 1 embryo on day 3	3.22 ± 1.64	1.80 ± 1.13	0.06
Number of grade 2 embryo on day 3	1.90 ± 0.99	1.83 ± 1.11	0.88
Fertilization rate (%)	65.7%	64.3%	0.95
Pregnancy rate (%)	28.5%	45%	0.14

E2: estradiol; AMH: anti-müllerian hormone; TAC: Total antioxidant capacity; PN: pronuclear zygote. Data presented as means ± standard deviation, median (IQR) or number (%). *$p < 0.05$ is significant.

Table 3
Comparison of the pregnant subgroups.

	Pregnant patients of the mild stimulation group (n:10)	Pregnant patients of the long agonist group (n:18)	p value
Age (years)	29 ± 3.53	29.6 ± 3.58	0.67
BMI (kg/m^2)	23.28 ± 2.19	24.39 ± 2.8	0.31
Infertility duration (years)	5.5 (3.5—6)	6 (4—7)	0.65
Day 3 serum FSH (mIU/ml)	4.96 ± 1.18	6.16 ± 2.27	0.15
Day 3 serum E2 (pg/ml)	40.22 ± 16.2	32.92 ± 18.68	0.32
Day 3 serum AMH (ng/ml)	4.62 (3.2—6.43)	2.4 (1.63—3.56)	0.14
Antral follicle count (AFC)	10.5 (8—12)	6 (5—8)	0.13
Serum E2 level on the day of HCG (pg/ml)	1464 (1324—2081)	1250 (659—1913)	0.55
Total gonadotropin dose (rFSH) (IU)	1491 ± 404.66	2313 ± 766.86	0.004*
Duration of stimulation (days)	8 (7—9)	9 (8—10)	0.32
Follicular fluid AMH (ng/ml)	1.61 (1.37—1.78)	1.57 (1.11—1.81)	0.76
Follicular fluid E2 (pg/ml)	488.5 ± 183.1	452.71 ± 136.5	0.96
Follicular fluid TAC (mmol Trolox equivalent/l)	1.07 (0.99—1.08)	1.08 (1.06—1.09)	0.34
Number of oocyte retrieved	10.4 ± 3.08	9.72 ± 2.69	0.53
Number of ICSI performed oocytes	8.33 ± 2.44	8.44 ± 2.28	0.90
Number of pronuclear (PN) zygote 2	7 ± 1.2	6.3 ± 1.6	0.71
Number of grade 1 embryo on day 3	3.33 ± 1.52	2.12 ± 0.99	0.14
Number of grade 2 embryo on day 3	2 ± 0.77	2.11 ± 1.05	0.83
Number of early pregnancy loss (≤12 gestational week)	3	3	0.43
Number of live birth at term	6	12	0.75
Live birth rate (%)	60%	66.6%	0.86

AMH: anti-müllerian hormone; TAC: Total antioxidant capacity; BMI: body-mass index; E2: estradiol; FSH: follicle stimulating hormone; PN: pronuclear zygote. Data presented as means ± standard deviation, median (IQR) or number (%). *$p < 0.05$ is significant.

began rFSH on cycle day 5 (group C, $n = 49$). In group C, shorter stimulation and lower total dose of gonadotropin were reported with no difference in the pregnancy rates [11]. In another study, mild stimulation started with 150 IU/day rFSH beginning on day 5 of the cycle and the other group received long agonist stimulation starting with 225 IU/day rFSH [12]. The ongoing pregnancy rate per cycle was not significant among the groups [12]. In our study the groups were significantly different in terms of stimulation period (days) and total gonadotropin dose (rFSH) however, the pregnancy and fertilization rates were similar between the groups, which aligns with the literature. We used fresh single embryo transfer and there were no significant differences among the groups in terms of number of retrieved oocytes as well as the number and quality of oocytes/embryos. Casano et al. reported similar pregnancy rates with the mild stimulation protocol in both fresh and thaw IVF cycles [13].

Another advantage of reduced stimulation is the avoidance of adverse effects on endometrial receptivity and embryo quality [2]. It has been proposed that supraphysiological levels of progesterone and estrogen in the luteal phase of IVF cycles may adversely affect endometrium and oocyte/embryo [14—17]. Increased morphological anomalies have been observed in oocytes when they are exposed to high gonadotropin doses during *in vitro* maturation [18,19]. In another study mild stimulation resulted in fewer oocytes and a decreased proportion of aneuploid embryos [12]. Further, different ovarian stimulation protocols resulted in different rates of mosaicism in good-quality embryos [20]. Another issue affecting the reproductive system is oxidative stress (OS). Oxidative Stress (OS) is defined as an imbalance between reactive oxygen species (ROS) and antioxidants. OS contributes many physiological and pathological conditions in the ovary [21,22]. Oxidative stress was shown to increase in repeated ovarian stimulation leading to mitochondrial DNA mutation and a decrease in oocyte quality [23]. Further, Velker et al. suggested that the methylation of imprinted genes in embryos was altered by gonadotropins in a dose-dependent manner [7]. Those findings lead us to investigate the follicular fluid anti oxidative capacity in two different IVF treatments.
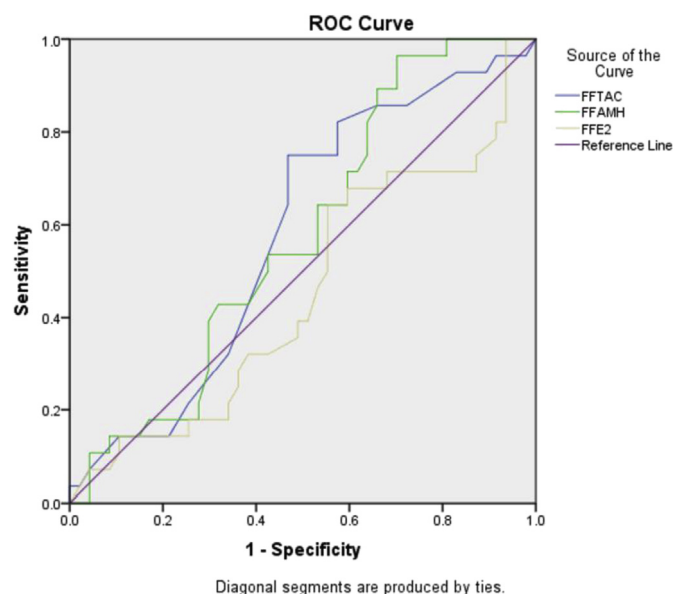
Fig. 2. ROC curves for FF-TAC, FF-AMH and FF-E2.

Fig. 3. Infertility etiology and FF-TAC/PCOS-nonPCOS and FF-TAC.

Appasamy et al. evaluated the relationship between FF oxidative stress and ovarian hormones in IVF cycles using long agonist stimulation [24]. No significant relationship was observed between FF-TAC and infertility etiology or pregnancy outcomes. Another study using antagonist treatment found that the FF total antioxidant response (TAR) was not significantly different between pregnant and non-pregnant groups [25]. They also reported lower FF-TAR levels in endometriosis cases however, no differences were observed in PCOS or tubal-factors compared with male factors [25]. Further, a recent study showed no difference between PCOS and non-PCOS in terms of FF-TAC levels [26]. In our study, we excluded endometriosis cases. The mean FF-TAC levels in the long agonist group were higher than those levels found in the antagonist mild group. This difference might be related to the gonadotropin dose, as higher exogenous gonadotropins can cause increased oxidative stress, and more antioxidant capacity is needed to overcome this response. We also found a positive correlation between FF-TAC and total gonadotropin dose. On the other hand, FF-TAC levels were not significant among different infertility etiologies (PCOS, tubal, unexplained and male), pregnant subgroups as well as PCOS/nonPCOS patients (Fig. 3).

The role of AMH in FF has been investigated in various studies of oocyte quality and pregnancy rates. In a study comparing agonist and antagonist treatments, both protocols exerted similar effects on FF levels of AMH [27]. In our study, FF-AMH was not significantly different either among protocols or within pregnancy subgroups.

The strength of our study was that we compared both pregnancy rates and the follicular fluid TAC levels in two different IVF protocols. So far, the studies on antagonist mild stimulations only compared the pregnancy rates with other protocols not involving follicular fluid TAC. Further, the studies on FF-TAC levels conducted on patients undergoing only one IVF protocol. To the best of our knowledge, our study is the first to compare the follicular fluid anti oxidative status between the antagonist mild stimulation and the long agonist stimulation. The main limitation of our study occurred during the follow-up phase. We contacted a limited number of patients to document the number of early pregnancy loss and live births.

Most studies on mild stimulation were conducted on patients less than 38 years of age with good ovarian reserves and our results are similar to those found i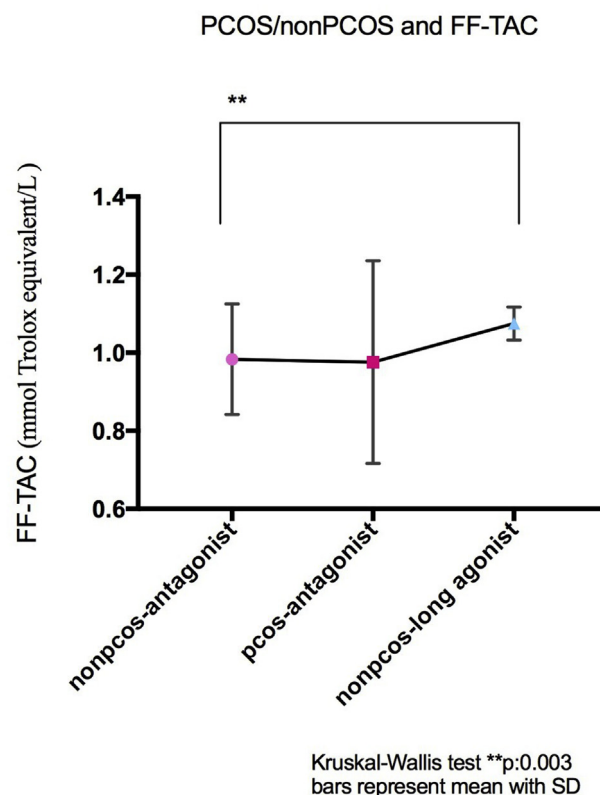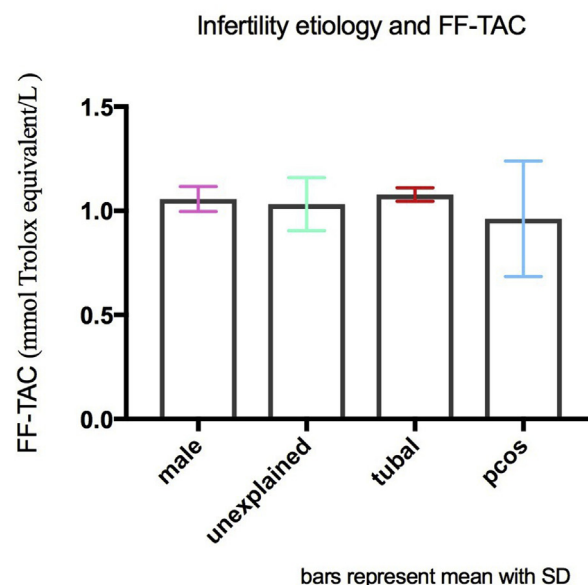n the literature. Moreover, mild ovarian stimulation was found to be more cost effective than the conventional protocol in poor responders [28].

In conclusion, patients with good ovarian reserves and under the age of 35 effectively responded to antagonist mild treatment. Using lower amounts of gonadotropin, yielded less FF-TAC levels in patients who underwent antagonist mild protocol. In patients under the age of 35, mild stimulation is a patient friendly and effective procedure when undergoing their first IVF cycle. Further studies are needed to evaluate the affects of different stimulation protocols and gonadotropin doses on follicular fluid oxidative status.

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Conflict of interest

All authors have nothing to disclose.

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