Original Article

Association of Follicular Fluid Advanced Glycation End-Products with Oocyte Response and Clinical Pregnancy in Assisted Reproduction Cycles

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Abstract

Context: Advanced glycation end-products (AGEs) are toxic metabolic end-products of lipids, nucleic acids, and proteins. Their accumulation in the ovaries can alter the follicular microenvironment and affect stimulation response. **Aims:** We aimed to study the association of AGEs in follicular fluid (FF AGE) with oocyte response and clinical pregnancy in ART-Assisted Reproductive Technology cycles. **Settings and Design:** This prospective study involved 84 individuals undergoing ART. **Methods and Material:** FF was collected during oocyte retrieval, and the level of AGEs was measured by enzyme-linked immunosorbent assay. Oocyte response was grouped as below target (<7 MII oocytes) or above target response (≥7 MII). **Statistical Analysis Used:** The association of FF AGE with the oocyte response and clinical pregnancy rate was analyzed by Mann–Whitney U-test. The strength of association of FF AGE with the outcome variables was analyzed with receiver operating characteristic (ROC) curve. **Results:** The median FF AGE was 17.6 (8.5) μg/ml. It was significantly higher in the below target than the normal ovarian response group (18.5 [17.8] vs. 16.3 [7.8] μg/ml, $P = 0.046$). Similarly, it was significantly higher in those who did not conceive (19.9 [7.3] vs. 13.5 [5.9] μg/ml, $P < 0.001$). The cutoff of FF AGE obtained by ROC curve analysis was 16.5 μg/ml above which there were significantly lower oocyte response and clinical pregnancy. **Conclusions:** Elevated FF AGE can be a significant negative predictor of clinical pregnancy and ovarian response to stimulation in ART cycles. The FF AGE level above the cutoff value of 16.5 μg/ml was associated with significantly lower oocyte response and clinical pregnancy.

Keywords: *Advanced glycation end-products, assisted reproductive technology, clinical pregnancy, follicular fluid, ovarian response*

Introduction

The success of assisted reproductive technology (ART) depends on many factors. One of the main factors is the ovarian response to stimulation as indicated by the number of oocytes retrieved. Ovarian follicular microenvironment can influence the oocyte quality.[1,2] Advanced glycation end-products (AGEs) are the toxic end-products of metabolism of lipids, nucleic acids, and proteins.[3] They constitute a group of heterogeneous compounds with more than 20 molecules characterized till date.[4-6] These can sometimes get accumulated in biological tissues including the ovaries,

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especially with advanced age, altered glycemic control, oxidative stress, obesity, insulin resistance,[7,8] and with intake of certain food items rich in fat and protein.[9,10] AGEs bind to AGE receptor (RAGE) or directly to extracellular matrix and lead to malfunctioning of key molecules in the extracellular matrix.[2] The interaction of AGEs with RAGE often induces a state of "pro-inflammation" leading to reactive oxygen species generation.[11-14] The accumulation of these in the ovaries, therefore, can alter the ovarian and follicular microenvironment which can affect the ovarian response to stimulation. Elevated level of AGEs was demonstrated in obesity and polycystic ovary syndrome (PCOS).[15-17] The significance of these in non-PCOS individuals undergoing ART has not been addressed much. The role of intraovarian AGE accumulation in causing diminished ovarian response to stimulation in non-PCOS individuals was suggested by Yao *et al*.[18] Their work concentrated chiefly on oocyte response and quality. We tried to extrapolate the same to the embryo quality and clinical pregnancy which would indicate the actual utility as a prognostic test. Our objectives were to identify the association of FF AGE with clinical pregnancy and the oocyte response.

Subjects and Methods

This was a prospective observational study done over a period of 1 year from January 2018 at the department of reproductive medicine of a tertiary care institute. The institutional ethical committee clearance was obtained before the start of the study. Individuals who were undergoing ART at the department were included in the study after obtaining informed written consent.

Our sample size was calculated based on the results of a previous study.[18] With an observed mean difference of 3.8, 80% statistical power, and 95% confidence level, the minimum sample size was calculated to be 52 (26 in each group, i.e., below target [<7 MII] and normal [≥7 MII] oocyte response). However, we included 84 individuals undergoing ART so that we could obtain at least 26 individuals in both the groups.

Females with a diagnosis of PCOS were excluded because previous publications consistently showed elevated AGE concentration (serum or follicular fluid [FF]) in PCOS patients and the bias that would have arisen if these patients were included could be overcome by this criterion. After recruitment, the demographic and baseline endocrine parameters were recorded. Baseline hormone analysis was done on day 2 or 3 of the menstrual cycle before initiating any form of treatment. The routine hormonal assay as part of our department protocol included basal follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), thyroid-stimulating hormone (TSH), prolactin (PRL), and anti-Mullerian hormone (AMH). Further, baseline transvaginal sonography (TVS) was done as a routine to assess the antral follicle count (AFC) and to identify any other significant findings that might alter the treatment plan.

The individuals underwent ART for which we utilized the standard long protocol (downregulation with injection LUPRIDE DEPOT 3.75 mg, SUN PHARMA, INDIA) or the antagonistic (fixed) protocol (injection CETROTIDE 0.25 mg, Merck, Germany). Ovarian stimulation was done using recombinant FSH (injection GONAL-F, Merck Serono, Switzerland) or human menopausal gonadotropin (IVF-M, LG Life sciences). Monitoring was done using TVS and hormonal assays. The criteria for monitoring and the final trigger were as per the standard practice. The final trigger before retrieval was done using human chorionic gonadotropin (hCG) (10,000 IU of urinary hCG intramuscularly – injection OVUNAL SC, INTAS/250 μg of recombinant hCG – injection Ovitrelle, Merck Serono) or GnRH agonist (injection DECAPEPTYL 0.2 mg s/c, Ferring Pharmaceuticals). Assay of serum E2 and progesterone was done on the trigger day.

TVS-guided oocyte retrieval was done at 35–36 h post and trigger. FF was collected during the retrieval and was processed. Thereafter, the total number of oocytes obtained and the number of good quality oocytes (MII) were noted. The oocyte response of each individual as assessed by the number of MII oocytes retrieved was grouped as below target response (<7 MII oocytes) or above target (normal) (≥7 MII oocytes). Intracytoplasmic sperm injection (ICSI) or IVF was done as per standard protocol. Fertilization check was done at 18 h post and insemination. Embryo transfer (ET) was done on day 2 or day 3 (cleavage stage transfer) as per the institutional protocol. The total number of embryos and Grade A embryos was noted. We excluded those who did not have any oocytes retrieved and those in whom we could not perform a fresh ET, and the FF of these individuals was not analyzed further. Luteal support was administered with micronized progesterone (injection HALD 100 mg IM [INTAS] or CRINONE 8% vaginal gel [MERCK SERONO] once daily). Pregnancy test (serum β-hCG) was done 2 weeks later, and a value of more than 50 IU/L was taken as positive. TVS was done 2 weeks later to identify the presence of a gestational sac to define clinical pregnancy. Another scan was done at 12 weeks of gestation for the identification of ongoing pregnancy.

FF was collected from all follicles during oocyte retrieval. Fluid from the first and the last tube was

discarded considering the possibility of it getting mixed with the flushing medium. Blood-stained fluid was also discarded. The remaining FF was centrifuged at 3000 rpm for 15 min. Supernatant was transported to the laboratory for assessing level of AGEs. If assay was not done immediately, then the fluid was stored in special containers at −180°C in liquid nitrogen and assay was done within 4 weeks. AGE assay was done employing the quantitative sandwich enzyme immunoassay technique using enzyme-linked immunosorbent assay (ELISA) kit (Cusabio Inc., USA). The detection range was 0.78 µg/ml–50 µg/ml, and the kit had high sensitivity and excellent specificity for detection of human AGEs. The intra-assay and inter-assay coefficients of variation were <8% and <10%, respectively.

FSH, LH, E2, progesterone, and PRL assays were done using chemiluminescence immunoassay (SIEMENS ADVIA CENTAUR XP, GERMANY). AMH assay was by ELISA (STAT FAX 4200). AFC (follicles of diameter 2–9 mm) was assessed by TVS performed with LOGIQ P5 machine (GE Inc., USA) using TVS probe of frequency 6–8 MHz. Oocyte response was defined based on the number of MII oocytes obtained as below target response (<7) or normal response (≥7). The clinical pregnancy (defined as the presence of intrauterine gestational sac) was identified by TVS 2 weeks after positive β-hCG. Ongoing pregnancy was defined as those with fetal cardiac activity at 12 weeks of gestation.

Statistical analysis

Statistical analysis was done using IBM SPSS Statistics, Version 22.0 (Armonk, NY: IBM Corp.). The continuous variables were not normally distributed as assessed by the Shapiro–Wilk test and are therefore expressed as median (interquartile range). The primary outcome (the relationship of the FF AGE concentration with the oocyte response and clinical pregnancy) was calculated using Mann–Whitney *U*-test. The correlation of the continuous variables with FF AGE was assessed using the Spearman's correlation coefficient analysis, and the association of FF AGE with the multiple categorical variables was done using Kruskal–Wallis test. $P < 0.05$ was taken as statistically significant. Receiver operating characteristic (ROC) curve analysis was done to identify the strength of association between FF AGE and oocyte response and clinical pregnancy.

Results

We recruited 89 individuals during the study period. Of these, three individuals did not have any oocytes retrieved, and another two did not have any transferable embryos. The final number of individuals for the analysis was 84. The distribution of study participants based on their baseline and cycle-related variables is depicted in Tables 1 and 2.

The median age was 32 (5) yrs and the main indication for undergoing ART was male factor (25%) and Diminished Ovarian Reserve (DOR) (22%). The median FF AGE level was 17.6 (8.5) µg/ml in the individuals. The median number of MII oocytes obtained was 11 (9). Biochemical and clinical pregnancies were 42.9% and 33.3%, respectively. Kruskal–Wallis test was employed to analyze the distribution of FF AGE across the different indications for ART. There was no significant difference in the FF AGE between the groups ($P = 0.796$). There was no significant difference in the distribution of FF AGE in those with and without POR ($P = 0.814$), endometriosis ($P = 0.382$), male factor (0.473), tubal factor ($P = 0.657$), and unexplained infertility ($P = 0.838$) as analyzed using Mann–Whitney *U*-test.

FF AGE showed a significant negative correlation with total oocytes retrieved, number of MII oocytes, and total number of embryos and Grade A embryos obtained [Table 3]. The association of FF AGE with oocyte response and clinical pregnancy is shown in Table 4. The individuals with below target (<7 MII

Table 1: Distribution of study participants based on their baseline and cycle-related variables

Variable	Median	IQR
Age (years)	32	5
BMI (kg/m^2)	24.3	4.5
Duration of infertility (years)	6.5	4
Basal FSH (IU/L)	5.9	3.1
Basal LH (IU/L)	3.9	2.5
Basal estradiol (pg/ml)	48.8	29.5
TSH (IU/L)	2.0	1.1
Prolactin (ng/ml)	9.6	9.1
AMH (ng/ml)	2.8	4.4
AFC	11	9.8
Total FSH (IU/L)	2738	2464
Gonadotropin duration (days)	13	2
Peak estradiol (pg/ml)	3218	1835
Peak progesterone (ng/ml)	1.5	1.1
Endometrial thickness (mm)	9.3	2.1
FF AGE (µg/ml)	17.6	8.5
Oocytes	13	8
MII oocytes	11	9
Total embryos	8	7
Total Grade A embryos formed	4	5
Embryos transferred	3	1

AMH=Anti-Mullerian hormone, AFC=Antral follicular count, FF AGE=Follicular fluid advanced glycation end-product concentration, BMI=Body mass index, FSH=Follicle-stimulating hormone, LH=Luteinizing hormone, TSH=Thyroid-stimulating hormone, IQR=Interquartile range, MII=Metaphase II

Table 2: Frequency distribution of study participants based on their baseline data, cycle details, and cycle outcome

Variable	Category	*n* (%)
Occupation	Homemaker	42 (50.0)
	Semi-professional	22 (26.2)
	Professional	20 (23.8)
Socioeconomic status	High	55 (65.5)
	Low	4 (4.8)
	Middle	25 (29.8)
Chief indication for ART	Azoospermia	4 (4.8)
	OAT	17 (20.2)
	Endometriosis	17 (20.2)
	Diminished ovarian reserve	19 (22.6)
	Tubal factor	15 (17.9)
	Unexplained	12 (14.3)
Protocol	Antagonist protocol	15 (17.9)
	Long protocol	69 (82.1)
Oocyte response (MII oocytes)	Below target (<7)	26 (31)
	Normal response (≥7)	58 (69)
Pregnancy	Biochemical	36 (42.9)
	Clinical	28 (33.3)
	Ongoing	25 (29.8)

ART=Assisted reproductive technique, OAT=Oligoasthenoteratozo ospermia, MII=Metaphase II

Table 3: Correlation of follicular fluid advanced glycation end-product concentration (μg/ml) with various parameters

Variable	Spearman's correlation coefficient	*P*
Age (years)	−0.032	0.78
BMI (kg/m^2)	0.096	0.39
Duration of infertility (years)	−0.008	0.94
Basal FSH (IU/L)	0.108	0.33
Basal LH (IU/L)	0.15	0.17
Basal estradiol (pg/ml)	0.08	0.48
TSH (IU/L)	0.11	0.34
Prolactin (ng/ml)	0.24	0.03*
AMH (ng/ml)	−0.03	0.76
AFC	−0.21	0.05
Total FSH (IU/L)	0.06	0.59
Gonadotropin duration (days)	−0.09	0.97
Peak estradiol (pg/ml)	−0.03	0.97
Peak progesterone (ng/ml)	0.21	0.55
Endometrial thickness (mm)	−0.11	0.32
Total oocytes	−0.22	0.048*
MII oocytes	−0.215	0.049*
Total embryos	−0.37	<0.001*
Grade A embryos	−0.36	0.001*

FSH=Follicle-stimulating hormone, LH=Luteinizing hormone, TSH=Thyroid-stimulating hormone, AMH=Anti-Mullerian hormone, AFC=Antral follicular count, MII=Metaphase II, BMI=Body mass index, *=statistically significant

oocytes) response had significantly higher FF AGE level than those with normal response (18.5 [17.8] vs. 16.3 [7.8], $P = 0.046$). Similarly, FF AGE was significantly lower in those who had clinical pregnancy as opposed to those who did not have (13.5 [5.9] vs. 19.9 [7.3], $P < 0.001$).

The strength of association of FF AGE, serum AMH, and AFC with oocyte response and clinical pregnancy was assessed using ROC curve analysis [Figures 1 and 2]. The AUC for predicting clinical pregnancy was 0.82 ($P < 0.001$), 0.675 ($P = 0.009$), and 0.604 ($P = 0.12$) for FF AGE, AFC, and serum AMH, respectively. The corresponding values in prediction of a normal oocyte response were 0.638 ($P = 0.046$), 0.827 ($P < 0.001$), and 0.746 ($P < 0.001$). Based on the ROC curve, FF AGE cutoff was obtained as 16.5 μg/ml above which the absence of clinical pregnancy and a below target oocyte response could be predicted with sensitivity and specificity of 85.7%, 75% and 76%, 54%, respectively.

Discussion

This study examined the relationship between levels of AGEs in FF with oocyte response and clinical pregnancy rate in patients undergoing ART. FF AGE was significantly higher in those with below target oocyte response (<7 MII oocytes) and in those who did not become pregnant. This suggests a detrimental effect of oxidative stress as evidenced by elevated FF AGE in affecting ovarian response and pregnancy in ART cycles. Our findings are in agreement with the previous reports of association of AGEs with adverse reproductive outcome.[2,18,19] The exact mechanisms underlying the effect of AGEs in reproduction need to be elucidated. Many theories are proposed by previous investigators including malfunction of key molecules of the extracellular matrix.[2]

We analyzed FF AGE of 84 individuals, all of whom had fresh ET. The main indications for undergoing ART were male factor and DOR. There was no significant difference in the level of FF AGE across the various indications for ART. We had excluded patients with polycystic ovarian syndrome as this group was already proved to be having elevated level of FF AGE.[15-17] No difference was found in FF AGE in those with endometriosis or POR as compared with those couples having male factor, tubal factor, or unexplained infertility. Similar outcome was reported by Yao *et al*. in 2018.[18] This suggests that increased oxidative stress might not exist in these conditions. Since we had only 84 subjects and the individual indications had relatively less number of subjects, a definite conclusion cannot be made.

The median FF AGE level was 17.6 (8.5) in the study group. We did not find any correlation of FF AGE level

Table 4: Association of study variables with oocyte response and clinical pregnancy

Variables	Oocyte response (MII oocyte number)				Clinical pregnancy			
	MII	Mean (SD)	Median (IQR)	*P*	Category	Mean (SD)	Median (IQR)	*P*
B FSH (IU/L)	<7	7.2 (3.1)	7.4 (4.9)	0.027*	Yes	5.6 (1.9)	5.9 (3.3)	0.259
	≥7	5.8 (2.1)	5.5 (2.6)		No	6.5 (2.7)	5.9 (3.5)	
B E2 (pg/ml)	<7	56.5 (33.9)	51.8 (35.3)	0.580	Yes	53.5 (25.9)	47.7 (37.1)	0.924
	≥7	53.8 (28.7)	43.6 (30.0)		No	55.2 (32.3)	48.9 (26.5)	
AMH (ng/ml)	<7	1.99 (1.7)	1.2 (1.8)	<0.001*	Yes	4.6 (4.2)	2.9 (4.4)	0.122
	≥7	4.8 (4.1)	3.6 (4.3)		No	3.6 (3.5)	2.8 (3.8)	
AFC	<7	7.0 (3.3)	6.0 (4.3)	<0.001*	Yes	13.2 (4.5)	12.5 (6.8)	0.009*
	≥7	12.9 (5)	13.0 (8.6)		No	10.1 (5.4)	9 (9.8)	
FF AGE (µg/ml)	<7	22.4 (11.3)	18.5 (17.8)	0.046	Yes	14.6 (9.9)	13.5 (5.9)	<0.001*
	≥7	17.2 (7.53)	16.3 (7.8)		No	20.8 (7.9)	19.9 (7.3)	

MII=Metaphase II, B FSH=Basal follicle-stimulating hormone, B E2=Basal estradiol, AMH=Anti-Mullerian hormone, AFC=Antral follicular count, FF AGE=Follicular fluid advanced glycation end-product concentration, IQR=Interquartile range, SD=Standard deviation, *=statistically significant

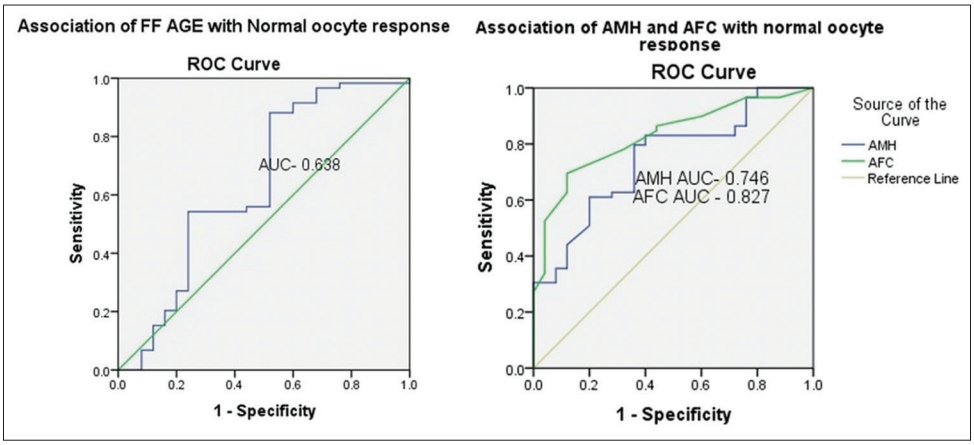

Figure 1: Receiver operator characteristic curve showing the association between oocyte response with follicular fluid advanced glycation end-product, serum anti-Mullerian hormone, and antral follicle count

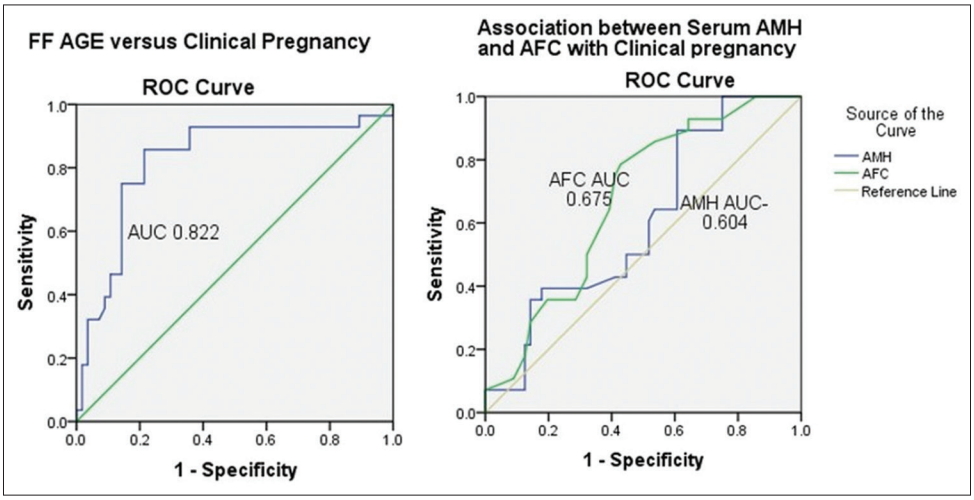

Figure 2: Receiver operator characteristic curve showing the association between clinical pregnancy with follicular fluid advanced glycation end-product, serum anti-Mullerian hormone, and antral follicle count

with age of the patients or body mass index (BMI). This is contrary to the earlier reports of elevated AGEs in those with advanced age and in those with obesity or high BMI.[7,8,18] We did not observe any significant correlation of FF AGE with basal FSH, serum AMH, AFC, total FSH dosage, and peak E2. The work published by Yao *et al*. in 2018[18] reported significant correlations of FF AGE with all of these. Since there are

no further reported studies addressing this association, we cannot come to a definite conclusion. Based on our findings, the basal demographic and hormonal factors do not have any effect on intraovarian oxidative stress as evidenced by FF AGE levels.

There was a significant negative correlation between FF AGE and total number of oocytes retrieved, total number of MII oocytes (depicting oocyte response), total number of embryos formed, and the number of good quality embryos. Other investigators also have reported poor oocyte response[1,2,18] and reduced embryo number and quality[18] in those with elevated FF AGE levels.

The ROC curve dealing with oocyte response suggested an AUC of 0.638 for FF AGE in predicting a below target oocyte response. This was lower than that obtained for AMH (AUC – 0.746) and AFC (0.827). Even though all these were statistically significant, the strength of association was less for FF AGE and maximum for AFC in predicting oocyte response. Yao *et al*.[18] obtained similar observations, but AMH was the best predictor in their study than AFC and FF AGE. This suggests that it would be prudent to continue with the current markers such as AFC and AMH for predicting oocyte response and FF AGE may still be considered in research settings only until proved otherwise. However, in the prediction of clinical pregnancy, FF AGE had a higher strength of association (AUC – 0.822) than AFC (AUC – 0.657) and AMH (AUC – 0.604). This finding might be attributed to some ultrastructural changes in the oocyte induced by oxidative stress which can alter the embryo quality and development. We also need to consider whether this is part of a systemic oxidative stress or any effect on endometrium and implantation. Measurement of serum AGE levels concurrently would have helped to get a glimpse into the systemic oxidative stress levels and its correlation with FF AGE levels.

We obtained a cutoff level of FF AGE as 16.5 μg/ml (higher value implies negative outcome) for predicting oocyte response (sensitivity 76% and specificity 54%) and clinical pregnancy (sensitivity 85.7% and specificity 75%). Yao *et al*.[18] reported a cutoff of 15.3 μg/ml with 84.6% sensitivity and 55.5% specificity in predicting a below target oocyte response. However, they did not extrapolate their results to analyze the association with clinical pregnancy.

Our main methodological strength was proper collection of FF by avoiding possible alterations arising out of contamination with blood and flushing medium. An ideal scenario would be to collect FF from a single follicle, assess that particular oocyte, and transfer that embryo only. However, this is not practical in most cases and can probably be attempted in animal models only. FF AGE cannot be utilized before the start of ovarian stimulation and cannot predict the cycle outcome beforehand. It also will not be predictive of the number of oocytes retrieved. FF AGE measurement cannot be routinely employed due to practicality issues, and also, it might not be cost-effective. However, it will be useful in future research settings. Serum AGE measurement, if standardized and well correlating with FF AGE, might be a tool for earlier prediction, including the number of oocytes obtained.

Conclusions

Objective evidence of oxidative stress as shown by elevated FF AGE levels can be a significant negative predictor of clinical pregnancy and ovarian response to stimulation in ART cycles. A cutoff value of 16.5 μg/ml of FF AGE was obtained above which oocyte response and clinical pregnancy were significantly lower. However, further studies are required to elucidate the molecular mechanism of the AGE–RAGE axis in the ovarian follicular microenvironment that affects the development of competent follicles. Moreover, some possible therapeutic interventions need to be identified in the context of elevated FF AGE.

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Conflicts of interest

There are no conflicts of interest.

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