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Noninvasive embryo evaluation method combining time-lapse images with biomarkers in follicular fluid and serum

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ABSTRACT: To increase the success rate of assisted reproductive technologies, embryos with high developmental and implantation potential should be identified and selected. Here, we sought to establish a noninvasive and highly accurate embryo selection method by combining time-lapse monitoring parameters (at S2 and S3) with the following biomarkers in the follicular fluid (FF) and serum samples obtained from study subjects (using 70 eggs, FF, and serum in 58 cycles of conventional in vitro fertilization [c-IVF]): dehydroepiandrosterone sulfate (DHEA-S), insulin-like growth factor 1 (IGF-1), reactive oxygen metabolites (d-ROMs), biological antioxidant potential (BAP), and an oxidative stress index (OSI: d-ROMs/BAP ×100). Our analyses revealed significant positive correlations between some of the biomarkers in FF and serum: DHEA-S ($r=0.88$; $p<0.01$), IGF-1 ($r=0.83$; $p<0.01$), d-ROMs ($r=0.55$; $p<0.01$), and OSI ($r=0.45$; $p<0.01$). The BAP value was higher in FF than in serum in many cases. The follicular OSI in the abnormal fertilization (AF) group tended to be higher than that in the normal-fertilization (NF) group. The FF values of DHEA-S and IGF-1 and the serum DHEA-S tended to be higher in the NF group and blastocyst formation (BF) group. S2 and S3 were shortened in the NF and BF groups. These findings suggest that (1) oxidative stress is related to abnormal fertilization, (2) DHEA-S and IGF-1 are associated with normal fertilization and blastocyst formation, and (3) S2 and S3 are useful timepoints for evaluating embryos.

Key Words: Biomarker, Embryo evaluation, Embryo-synchronous development time, Noninvasive, Time-lapse imaging

Running Head: Embryo evaluation by time-lapse and biomarkers

INTRODUCTION

To increase the success rate of assisted reproductive technologies, it is important to select embryos with high developmental and implantation potential from among the eggs and embryos obtained by egg retrieval, prior to embryo transfer. A morphological evaluation is used in many centers to select the transferred embryos[1–5]. The use of a time-lapse imaging incubator system has allowed us to observe the embryos, and such a system has been used to test the usefulness of dynamic parameters[6,7]. However, there is still room for improvement in the process of selecting high-potential embryos for transfer. We conducted the present study to explore a combined evaluation method with additional parameters. Our main goal is to establish a more accurate embryo evaluation method.

A human egg matures in the follicle, and after ovulation it is taken into the fallopian tube with the follicular fluid (FF), where it is fertilized and divides. In this study, we tested the hypothesis that the composition of the FF is important for oocyte maturation and fertilization, and we attempted to predict the quality of oocytes by measuring biomarkers in the FF in which the oocytes developed. We used dehydroepiandrosterone sulfate

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(DHEA-S) and insulin-like growth factor 1 (IGF-1) as biomarkers of oocyte growth and aging[8–10] . We also measured reactive oxygen metabolite-derived compounds (d-ROMs) and the biological antioxidant potential (BAP) , and we used the oxidative stress index (OSI: d-ROMs/BAP × 100).

It has been reported that with age, the production of free radicals increases and the endogenous defense mechanisms decrease[11] and may affect embryonic development[12,13]. We therefore examined the relationships between the above-cited biomarkers and fertilization and embryo development. We speculated that if these biomarkers are revealed to be useful for selecting high-potential embryos, then evaluations of the quality of oocytes and embryos could be easily accomplished by measuring biomarkers in the patient's serum, which is easier to collect than FF. We therefore determined the correlations between the levels of the biomarkers in both serum and FF. We also determined the correlation between these biomarkers and the patient's age at oocyte pick-up (hereinafter referred to as the 'age').

We used time-lapse monitoring to assess early embryos because of the small number of cells, as we and other observers have found that the findings revealed by this monitoring are a stable marker[14,15] . We focused on the three-cell stage (S2: t4–t3) and the stage from five cell to eight cells (S3: t8–t5) to investigate the relationship between fertilization and embryo development.

MATERIALS AND METHODS

The subjects were a retrospective cohort study of 70 eggs, FF, and serum in 58 cycles of assisted reproductive technology (c-IVF) conducted at Koujin Hospital from October 2018 to April 2019.

Blood E2 levels and follicle development were monitored. When the primary follicle was >18 mm, an Ovidrel Syringe 250 μg® or Buserelin Nasal Solution 0.15% "ILS" was administered by nasal spray. Next, 34–35 hr later, the follicles were punctured under transvaginal ultrasound guidance. The FF was then aspirated, and the oocytes were collected. To investigate the environment of the FF and the quality of each oocyte, we targeted only the FF from which the oocyte was collected; no other FF was contaminating.

After confirmation of the second polar body (2PN) at 4–5 hr post-fertilization (c-IVF) , the embryos were cultured in EmbryoScope® (ES+) (Vitrolife, Gothenburg, Sweden) in separate culture wells. When we confirmed blastocyst formation, the cells were frozen by rapid vitrification using a Vitrification Kit and Cryo-top (Kitazato Co. Fuji, Japan) and stored in liquid nitrogen. After the next cycle, thawing was performed using a Vitrification Kit (Kitazato) for 3–5 hr of recovery incubation in natural cycles or hormone replacement cycles. Single-blastocyst transfer was performed by transvaginal ultrasound using an ET catheter (Kitazato).

Pregnancy was determined by blood human chorionic gonadotropin (HCG) levels and transvaginal ultrasound to confirm the fetal sac.

Our further investigation was performed in two parts. In Part 1, we determined the correlation of each biomarker's values between the serum and FF of the respective patient. The correlations between age and biomarker levels was also examined. In Part 2, we performed difference tests between the normal fertilization (NF) group and abnormal fertilization (AF) group, between the blastocyst-forming group and non-forming group, and between the pregnant group and non-pregnant group. In each of these groups, we identified the levels of the biomarkers in both serum and FF (DHEA-S, IGF-1, d-ROMs, BAP, OSI) and evaluated the embryonic development (at S2 and S3) and clinical parameters, i.e., the age and body mass index (BMI) of the patients.

For our examination of the factors that may influence normal fertilization, blastocyst formation and pregnancy, we performed a logistic regression analysis (forward stepwise) to identify variables that met the criteria as influencing factors. The dependent variables were the presence/absence of normal fertilization, the presence/absence of blastocyst formation, and the presence/absence of pregnancy. The covariates in the analysis were the FF biomarker levels, the serum biomarker levels, S2, S3, BMI, and Age.

Collection of FF and serum.

The collected FF was centrifuged (300g for 1 min), and the supernatant was collected and stored in a deep freezer at −70℃. Serum was collected on the day the egg collection was performed and frozen in a deep freezer at −70℃.

Embryo evaluation

All embryos were cultured with ES+, and time-lapse images of each embryo were analyzed. 2PN confirmation was judged as normal fertilization, whereas other nuclei were judged as abnormal fertilization. We conducted a morphological evaluation to calculate the timing of the 2-Blastomere (t2), 3-Blastomere (t3), 4-Blastomere (t4) and 8-Blastomere (t8) division times. We also measured the synchronization of the second (s2: t4–t3) and third cell cycles (s3: t8–t5).

Method for measuring biomarkers in FF and serum

The d-ROMs and BAP values were measured using FREE Carrio Duo® System (Wismerll, Tokyo). The oxidative stress index (OSI) was then calculated as: d-ROMs/BAP ×100. A detailed description of d-ROM and BAP has been reported[16]. DHEA-S was assayed using a fully automated enzyme immunoassay system, the AIA®-2000 (Tosoh Bioscience, Tokyo), and the reagent used was the Ectest Tosoh®II, which is a dedicated reagent for the system. IGF-1 was assayed by the electrochemiluminescence immunoassay (ECLIA) cobas® e 411 (Roche Diagnostics, Indianapolis, IN, USA), and ECLusys® (Roche Diagnostics) was used as the reagent for this system.

Statistical analysis

The result of the difference test was confirmed by the Levene test, and the two-sample t-test was performed when equal variance was observed. The two-sample t-test as a modification of Welch's test was used when the data were not equally dispersed. The significance of the results was assessed by SPSS Statistics 26 software (IBM, New York) using Student's t-test and the chi-square test. Differences were considered significant at $p<0.05$. Multivariate correlations were calculated using Pearson's moment correlation. We performed a logistic regression analysis using a stepwise variable increase. The logistic regression model was used to calculate the adjusted odds ratio (OR) over the 95% confidence interval (CI) to calculate the risks. The effect size calculation used Cohen's d.

Ethical considerations

This study was explained to the subjects, and their consent was obtained in accordance with the code of ethics of Kojin Hospital. The Graduate School of Kagawa Prefectural University of Health Sciences Ethics Review Committee approved the study (approval no.263).

RESULTS

From 58 cycles, 70 serum samples, FF, and oocytes were collected. Normal fertilization resulted in 56 cells, of which 40 formed blastocysts. A single blastocyst transfer was performed in 28 cycles, and a pregnancy was achieved in 10 cycles, of which seven were births and three were miscarriages. Twelve blastocysts were cryopreserved.

The characteristics of the patients are summarized in **Table 1**. The age at oocyte pick-up (OPU) ranged from 26 to 46 years (mean 39.2 ±5.0 years). All of the biomarker levels (mean ±SD) were lower in the FF than in the serum for each of the patients.

Part 1: Correlations between serum and FF and between age and each biomarker's levels

As illustrated in **Figure 1**, the correlation between the biomarkers levels in the FF and serum was strong and positive for DHEA-S ($r=0.88$, $p<0.01$) and IGF-1 ($r=0.83$, $p<0.01$). The levels of d-ROMs ($r=0.55$, $p<0.01$) and the OSI ($r=0.45$, $p<0.01$) had a moderately positive correlation. However, there was no correlation between the BAP values in the FF and those in the serum ($r=0.22$, $p=0.66$). It is interesting to note that BAP was found in 45.7% (32/70) of the cases, with higher levels in the FF than in the serum. The dotted area in Figure 1D shows the plot where the FF levels were higher than the serum levels.

Table 1 Characteristics of patients

Factor	Mean ± SD	
Total number of patients	58	
Total number of follicles	70	
Age of participants (years old)	39.2 ± 5.0	
BMI (kg/m2)	22.7 ± 3.7	
S2(t4-t3) （h）	2.5 ± 4.5	
S3(t8-t5) （h）	10.0 ± 9.7	
Biomarker	Follicular fluid level	Serum level
DHEA-S(μg/dL)	199.8 ± 102.4	225.9 ± 106.1
IGF-1 (ng/mL)	97.3 ± 27.5	130.1 ± 32.8
d-ROMs (U.CARR)	324.0 ± 59.6	393.9 ± 61.3
BAP (μmol/L)	2057.9 ± 323.1	2098.3 ± 200.9
OSI (%)	16.0 ± 3.2	18.9 ± 3.0

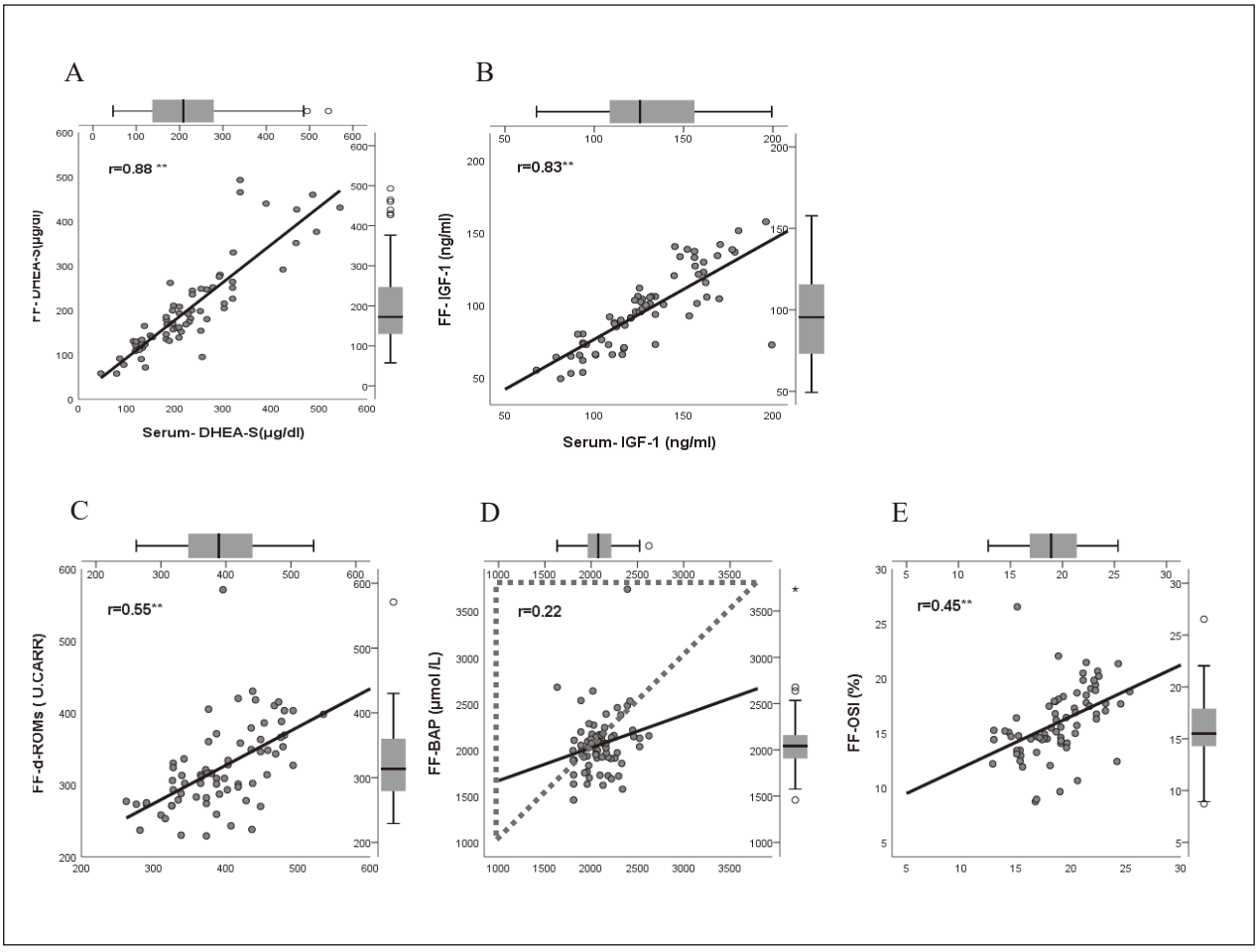

Figure 1 The relationship between the biomarkers' levels in follicular fluid (FF) and serum.

A: DHEAS. B: IGF-1. C: d-ROMs. D: BAP. E: The OSI. The dotted area in panel D is the plot in which the patient's FF level was higher than her serum level. *p<0.05, **p<0.01.

The correlation between age and biomarkers is depicted in **Figure 2**. There was a weak negative correlation between FF-DHEA-S and age ($r=-0.25$, $p<0.05$), but Serum-DHEA-S was not correlated with age ($r=-0.19$, $p=0.10$). There was a moderately negative correlation between FF-IGF-1 and age ($r=-0.43$, $p<0.01$) and between Serum-IGF-1 and age ($r=-0.41$, $p<0.01$). In both the FF and serum, the values of d-ROMs, BAP, and OSI were not correlated with age.

Part 2: Test for differences and factor analysis of influence between each group

Table 2 summarizes the results of our comparison of the biomarker levels in FF and serum between the NF and AF groups. The normal fertilization rate was 80.0% (56/70). There was no significant difference in any of the biomarkers or in embryonic development between the NF and AF groups. However, the values of the following markers tended to be higher in the NF group compared to the AF group: FF-DHEA-S (204.7 ±109.9 vs. 179.9±63.8), Serum-DHEA-S (229.5±113.7 vs. 211.6 ±69.1), FF-IGF-1 (99.5 ±26.1 vs. 88.7±31.9), and Serum-IGF-1 (133.5±33.3 vs. 116.5±27.8, respectively).

The d-ROMs values and the OSI tended to be lower in the NF group compared to the AF group. The results of the logistic regression analysis demonstrated that none of the factors that we examined affected the presence or absence of normal fertilization.

Table 3 summarizes the results of our comparison of the biomarker levels in FF and serum between the blastocyst-forming group and the non-forming group. The blastocyst formation rate was 71.4% (40/56). There was no significant between-group difference in any of the biomarkers, but the values of the following markers tended to be higher in the blastocyst-forming group compared to the non-forming group: FF-DHEA-S (217.0 ±115.2 vs. 174.1±91.6), Serum -DHEA-S (242.1±118.2 vs. 198.1 ±98.0), and FF-IGF-1 (100.5 ±26.3 vs. 97.0 ±26.2, respectively). The patients were younger in the blastocyst-forming group than in the non-forming group.

S2 and S3 were delayed in the non-forming group compared to the blastocyst-forming group, but not significantly. The logistic regression analysis revealed that the two factors with significant ORs (95%CI) in the final model were age (OR 0.72, 95%CI: 0.54–0.95) and S3 (OR 0.889, 95%CI: 0.81–0.98).

Table 4 summarizes the results of our comparison of biomarker levels in FF and serum between the pregnant and non-pregnant groups. None of the biomarker levels and neither S2 nor S3 differed significantly between these groups. The pregnant group was significantly younger than the non-pregnant group (34.6±3.9 vs. 40.1±3.8; $p<0.05$). The results of the logistic regression analysis showed that age was a significant factor in the final model (OR 0.67. 95%CI: 0.49–0.92).

DISCUSSION

Many embryo evaluation methods using time-lapse imaging have been reported[17–19], and we have used such imaging as one of the methods for evaluating embryos. In this study, we focused on the synchronization of S2 and S3, as they are stages at which it is easy to determine cell division stably with small errors among observers. Our observations revealed that S2 was shortened in the normal-fertilization group, blastocyst-formation group and pregnancy group, although there was no significant difference. S3 was also shorter in the blastocyst-formation group than in the non-formation group. It has been suggested that a shorter S2 has a higher blastocyst formation rate[20], and that embryos that divide properly at the optimal time are good-quality embryos[6, 21]. Our present findings also indicate that evaluations of S2 and S3 is an effective method for embryo selection. Since the goal of this study was to develop a method to select more high-potential embryos, the combined method that we used was designed to evaluate a composite of embryos with the addition of biomarkers as new parameters.

Our results did not confirm the validity of the addition of five biomarkers for the assessment of fertilization, blastocyst formation, or pregnancy establishment. However, two of the biomarkers, i.e. ,

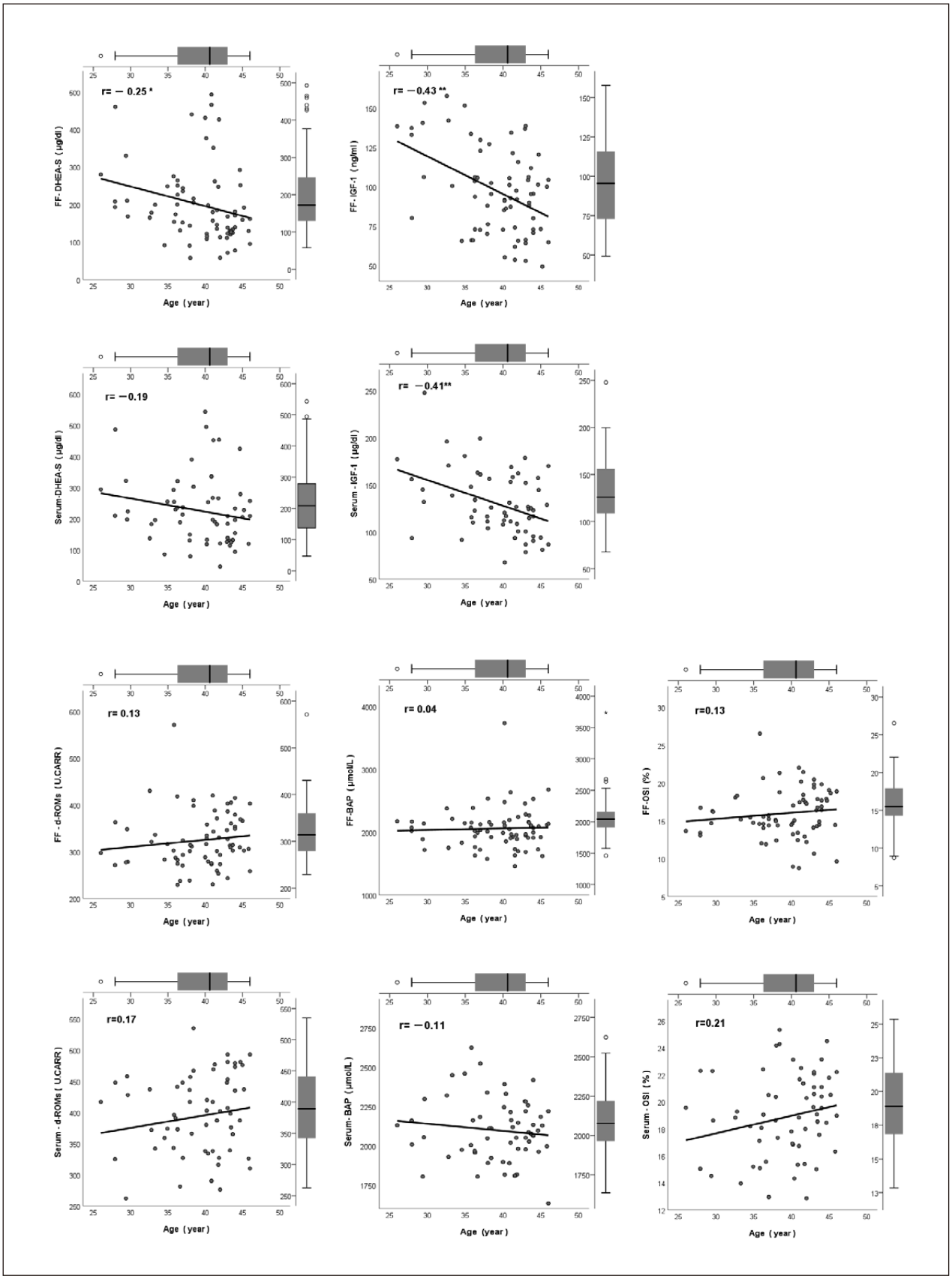

Figure 2 The relationship between age and the biomarkers' levels in the follicular fluid and serum.

The values of DHEA-S, IGF-1, d-ROMs, BAP, and the OSI are shown. The x-axis shows the patient's age at oocyte pick-up. *p<0.05, **p<0.01.

Table 2 Comparison between normal fertilization group and abnormal fertilization group: levels in follicular fluid and serum

Factor	normal(n=56)	abnormal(n=14)	P-value	effect size d
Age (years old)	39.2 ± 4.6	39.1 ± 6.6	0.949	0.02
BMI (kg/m2)	22.4 ± 3.7	23.7 ± 3.3	0.236	0.36
S2(t4-t3) (h)	2.1 ± 4.2	4.3 ± 5.7	0.251	0.49
S3(t8-t5) (h)	9.6 ± 9.7	12.4 ± 10.1	0.414	0.29

	Follicular fluid level (Mean ± SD)				Serum level (Mean ± SD)			
Biomarker	normal(n=56)	abnormal(n=14)	P-value	effect size d	normal(n=56)	abnormal(n=14)	P-value	effect size d
DHEA-S(μg/dL)	204.7 ± 109.9	179.9 ± 63.8	0.278	0.24	229.5 ± 113.7	211.6 ± 69.1	0.575	0.17
IGF-1 (ng/mL)	99.5 ± 26.1	88.7 ± 31.9	0.191	0.40	133.5 ± 33.3	116.5 ± 27.8	0.083	0.53
d-ROMs (U.CARR)	320.6 ± 53.1	337.4 ± 81.9	0.349	0.28	390.8 ± 59.0	406.2 ± 70.8	0.404	0.25
BAP (μmol/L)	2082.2 ± 338.8	1960.6 ± 235.9	0.210	0.38	2094.4 ± 203.5	2113.7 ± 196.6	0.750	0.10
OSI (%)	15.7 ± 3.0	17.3 ± 3.7	0.094	0.51	18.8 ± 2.9	19.3 ± 3.7	0.534	0.16

Effect size calculation used Cohen's d.

Table 3 Comparison between blastocyst forming group and non forming group: levels in follicular fluid and serum

Factor	formation(n=40)	non formation(n=16)	P-value	effect size d	OR	95% Cl	P-value
Age (years old)	38.5 ± 4.6	41.0 ± 4.0	0.074	0.56	0.72	0.54-0.95	0.021
BMI (kg/m2)	22.0 ± 3.5	23.4 ± 4.2	0.236	0.38			
S2(t4-t3) (h)	1.7 ± 2.9	3.3 ± 6.3	0.206	0.39			
S3(t8-t5) (h)	7.5 ± 6.3	16.0 ± 14.8	0.066	0.90	0.89	0.81-0.98	0.018

	Follicular fluid level (Mean ± SD)				Serum level (Mean ± SD)			
Biomarker	formation(n=40)	non formation(n=16)	P-value	effect size d	formation(n=40)	non formation(n=16)	P-value	effect size d
DHEA-S(μg/dL)	217.0 ± 115.2	174.1 ± 91.6	0.190	0.39	242.1 ± 118.2	198.1 ± 98.0	0.193	0.39
IGF-1 (ng/mL)	100.5 ± 26.3	97.0 ± 26.2	0.659	0.13	132.7 ± 30.6	135.6 ± 40.5	0.767	0.09
d-ROMs (U.CARR)	322.3 ± 49.1	316.4 ± 63.5	0.708	0.11	390.4 ± 58.6	392.0 ± 62.1	0.929	0.03
BAP (μmol/L)	2063.4 ± 222.0	2129.3 ± 538.2	0.642	0.19	2088.0 ± 209.2	2110.6 ± 194.4	0.711	0.11
OSI (%)	15.8 ± 2.7	15.4 ± 3.7	0.696	0.13	18.8 ± 3.0	18.6 ± 2.5	0.780	0.07

Effect size calculation used Cohen's d.

Table 4 Comparison between pregnant group and non pregnant group: levels in follicular fluid and serum

Factor	pregnant (n=10)	non pregnant(n=18)	P-value	effect size d	OR	95% Cl	P-value
Age (years old)	34.6 ± 3.9	40.1 ± 3.8	0.001 *	1.43	0.67	0.49-0.92	0.013
BMI (kg/m2)	21.4 ± 3.1	21.6 ± 3.1	0.460	0.06			
S2(t4-t3) (h)	0.7 ± 0.7	2.1 ± 3.7	0.145	0.46			
S3(t8-t5) (h)	7.4 ± 6.4	6.7 ± 6.7	0.879	0.11			

	Follicular fluid level (Mean ± SD)				Serum level (Mean ± SD)			
Biomarker	pregnant (n=10)	non pregnant(n=18)	P-value	effect size d	pregnant (n=10)	non pregnant(n=18)	P-value	effect size d
DHEA-S(μg/dL)	216.5 ± 108.4	224.6 ± 119.2	0.860	0.07	226.2 ± 110.3	258.9 ± 134.3	0.518	0.26
IGF-1 (ng/mL)	98.2 ± 33.1	105.4 ± 23.8	0.508	0.26	126.4 ± 38.3	136.5 ± 25.0	0.409	0.33
d-ROMs (U.CARR)	321.3 ± 29.2	315.8 ± 55.7	0.774	0.11	395.3 ± 44.1	375.3 ± 55.4	0.336	0.39
BAP (μmol/L)	2130.0 ± 141.9	2124.8 ± 218.4	0.951	0.03	2162.2 ± 237.0	2075.4 ± 212.2	0.329	0.39
OSI (%)	15.2 ± 1.5	15.0 ± 2.9	0.891	0.08	18.6 ± 3.6	18.1 ± 2.4	0.685	0.17

Differences were considered s,giuficant when * P values < 0 05
Effect size calculation used Cohen's d.

DHEA-S and IGF-1 have been reported to be factors involved in follicle development, oocyte maturation, and embryo quality[22] . We observed strong positive correlations of DHEA-S and IGF-1 between serum and FF levels in the present investigation, and we therefore speculate that the measurement of serum levels could be used to estimate FF levels and could serve as a measure of egg maturation and embryo evaluation.

Terao et al.[23] reported that d-ROMs, BAP, and the OSI in the follicular fluid were involved in fertilization and cell division. Our present analyses demonstrated that the values of d-ROMs, BAP, and the OSI were not affected by age factors. We thus speculate that if d-ROMs, BAP, and the OSI are involved in fertilization and cell division, measuring them could be useful for predicting embryo quality without the use of the age factor.

It is interesting to note that among the biomarkers examined in this study, only BAP was not correlated between the serum and FF. Locally generated reactive oxygen species (ROS) have been reported to play an important role in follicle development, egg maturation, and ovulation[24], but it has been reported that high levels of ROS can cause oxidative stress in the oocytes and granulosa cells in the follicle, which may lead to a decrease in the quality of the oocytes[25] . On the other hand, antioxidants have been reported to act as scavengers to protect DNA and cellular components[26]. We observed herein that BAP was higher in the FF than in the serum in most cases, which suggests that there are more antioxidants in FF than in serum, and that the oocytes in the follicles may be protected by antioxidant effects. These findings suggest that oxidative stress is related to abnormal fertilization. Further research is needed to determine whether this phenomenon (i.e. , the higher BAP values in FF) is related to egg quality and embryonic development.

Our present findings do not support a noninvasive, simple and accurate embryo selection method by combining biomarkers with parameters from time-lapse monitoring. However, our sample size was small because the study was limited to c-IVF in order to eliminate the differences in the speed of division caused by sperm quality. In addition, because we examined minimum ovarian stimulation cycles and natural OPU cycles, there was no room to choose a single embryo to be transferred in each cycle. These limitations may have affected our inability to identify useful biomarkers as indicators for embryo evaluation. In the future, the ovarian stimulation protocol should be used and larger sample sizes should be tested.

If an evaluation method for selecting embryos based on embryonic developmental dynamics and biological biomarkers can be confirmed, the method could become a promising tool for noninvasively predicting blastocyst formation and successful pregnancy.

Conflicts of interest

The authors report no conflicts of interest in this study.

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