Impact of the one-carbon metabolism on oocyte maturation, fertilization, embryo quality, and subsequent pregnancy

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Impact of the one-carbon metabolism on oocyte maturation, fertilization, embryo quality, and subsequent pregnancy

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Abstract

Purpose: To investigate impact of the one-carbon metabolism (OCM) on oocyte maturity and embryo development.

Methods: This prospective study analyzed 18 women who agreed to participate. We measured the OCM biomarkers' concentrations including Vitamin B12 (VB12), folic acid (FA), and homocysteine (Hcy) in serum and follicular fluid (FF), and assessed their correlation. We also evaluated the influence of such OCM biomarker concentrations in mono-FF on oocyte maturation, fertilization, embryo quality, and consequent pregnancy after embryo transfers.

Results: All biomarkers showed a high concentration variability in different follicles of each woman, but their mean levels correlated with the serum levels. Among the 106 collected oocytes, 92 were mature, 59 were fertilized, and 16 yielded good-quality embryos. We performed 26 single embryo transfers, and 7 patients achieved clinical pregnancies. VB12 concentration (FF) was significantly lower in fertilized than unfertilized oocytes by univariate analysis. In multivariate logistic analysis, a significant correlation was found between FA concentration (FF) <14.25 ng/mL and good-quality embryos and between Hcy concentration (FF) <4.9 nmol/mL and clinical pregnancy.

Conclusion: OCM in FF may affect fertilization, embryo quality, and clinical pregnancy.

KEYWORDS

clinical pregnancy, embryo quality, folic acid, homocysteine, one-carbon metabolism

1 | INTRODUCTION

One-carbon metabolism (OCM) refers to a metabolic pathway composed of folic acid (FA) and methionine metabolism. Methionine is an essential amino acid required for methylation, DNA synthesis, and protein synthesis, and coenzymes, such as vitamin B6, vitamin B12 (VB12), and FA, are necessary for normal methionine metabolism. Incomplete metabolism and disturbed equilibrium (balance) can result in harmful homocysteine (Hcy) accumulation. Hyperhomocysteinemia affects various stages of reproduction and is related to disorders of morphology, number, and motility of sperm,[1] fetal congenital malformation, abortion, gestational hypertension, gestational diabetes, and low birth weight.[2-4]

FA and VB12 constituting OCM are essential for Hcy remethylation to produce methionine. Therefore, FA and VB12 intake changes caused by nutritional disturbances, such as vegetable insufficiency,

high-fat diet, and processed food, can cause a high-Hcy, low-methionine state, affecting protein and DNA synthesis and methylation regulation.

Although OCM exists ubiquitously in biological tissues, its presence in an embryo consisting of undifferentiated cells remains unconfirmed. In 2010, researchers reported that genes of enzymes involved in OCM are expressed in preimplantation mammalian embryos,[5] and this finding has attracted attention to the OCM effects on embryogenesis.

The ovarian follicle forms the preovulatory microenvironment for oocyte growth/maturation. The follicular fluid (FF), which is partly a serum exudate and partially composed of locally produced substances, closely reflects the metabolism of the oocyte and its surrounding granulosa cells, and provides a vital sample for investigating the intrafollicular microenvironment. Through the in vitro fertilization (IVF) treatment procedure, FF can be obtained readily and noninvasively. If OCM exists in a fertilized oocyte, OCM disturbance appears as the OCM-related biomarkers change in the FF, possibly affecting oocyte maturation, embryo quality, and subsequent pregnancy. Although there have been some studies investigating OCM in FF, there is no consensus about the impact on oocyte quality. Furthermore, studies evaluating the impact of OCM on subsequent pregnancy are few. This study aimed to measure OCM-related biomarkers such as FA, Hcy, and VB12 in serum and FF, to evaluate the variability of their concentrations in different follicles of each woman, and to determine the correlations between serum and FF levels. We also aimed to investigate the relationships between FF concentrations and the quality of oocytes derived from the follicles, embryo quality, and subsequent pregnancy.

2 | MATERIALS AND METHODS

This prospective study analyzed 18 women who agreed in our study and received IVF treatment between November 2013 and November 2015 at the University of the Ryukyus Hospital in Okinawa, Japan. The hospital's institutional review board approved this study (February 21, 2013; No. 498). All patients provided informed consents prior to participation.

To examine the follicle-stimulating hormone (FSH) levels, we extracted blood samples from each woman on cycle day 3 before starting the controlled ovarian stimulation. For IVF, we employed the gonadotropin-releasing hormone (GnRH)-agonist short or long protocol, or the GnRH-antagonist protocol for the controlled ovarian stimulation. When the dominant follicles reached >18 mm in diameter, we administered 10 000 IU of human chorionic gonadotropin (hCG) and performed oocyte pickup (OPU) 35 hours later under intravenous anesthesia. We again collected blood samples to determine plasma folate, VB12, and Hcy concentrations on the day of oocyte retrieval. Under transvaginal ultrasonic guidance, we punctured the follicles one by one and collected 2 mL or more of FFs in each follicle. Only FFs containing oocytes were collected. A maximum of 6 FF samples per patient were collected. Furthermore, the VB12, FA, and Hcy levels were measured in each FF. After the obtained oocytes were cultured for 4 hours, IVF or intracytoplasmic sperm injection (ICSI) was performed according to semen quality. Two pronuclei and two polar bodies were noticed 18 hours after insemination or injection.

2.1 | Evaluation outcomes

We investigated the correlation between serum and FF concentrations of VB12, FA, and Hcy and assessed the intraindividual variability of their concentrations in FF. Furthermore, we evaluated the impact of VB12, FA, and Hcy concentrations in FF on oocyte maturation, fertilization, embryo quality, and clinical pregnancy. In this study, oocytes in metaphase II (MII) were defined as mature oocytes, and fertilization was defined as the presence of two prenucleus and two polar bodies. Embryo quality was assessed right before cryopreservation or embryo transfer. Additionally, if cleavage embryos were composed of >4 cells on day 2 or at least 7-8 cells on day 3 and contained <20% of anucleate fragments, they were considered as good quality according to the Veeck classification system; hence, failure to meet these criteria indicated poor quality. According to the Gardner classification system, blastocysts were graded according to size, density, inner cell mass, and trophectoderm development. A grade of "3BB" or greater was defined as good quality, and less than that was defined as poor quality. Moreover, clinical pregnancy was defined as the presence of a gestational sac in the uterine cavity by transvaginal ultrasonography.

2.2 | Statistical evaluation

The relationship between the serum and FF concentrations of VB12, FA, and Hcy was assessed using Pearson correlation. Differences in continuous variables were evaluated using Student's *t* test. The factors independently related to oocyte maturation, fertilization, embryo quality, and clinical pregnancy were determined by logistic regression analysis. A probability (*P*) value of <.05 was considered statistically significant.

3 | RESULTS

This study included 18 women. The mean maternal age, body mass index, and basal FSH were 36 ± 4 (30-44) years, 22 ± 3.1 (17-28) kg/m^2, and 5.5 ± 1.7 (1.5-9.3) IU/mL. The mean infertility duration was 3.1 ± 2.5 years, and infertility was caused by tubal factors, male factors, fertilization failure, and unknown factors in 16.7%, 39%, 22.2%, and 44.4% of patients, respectively. For the controlled ovarian stimulation, the short, antagonist, and long protocol were followed in nine, seven, and two patients. From the 18 patients, 106 oocytes were obtained. Of the 106 obtained oocytes, 92 were mature, and the others were in MI or GV status. We obtained 59

fertilized oocytes in which 16 were good-quality embryos. Then, we performed 26 single embryo transfers, and seven patients achieved clinical pregnancies (Figure 1).

The mean serum VB12, FA, and Hcy concentrations were 384 ± 141 pg/mL, 14.2 ± 7.4 ng/mL, and 6.1 ± 1.7 nmol/mL, respectively, and all were within the normal range (Table 1).

Meanwhile, the mean VB12, FA, and Hcy concentrations in FF were 290 ± 98 pg/mL, 17.9 ± 9.6 ng/mL, and 4.8 ± 1.3 nmol/mL, respectively. All biomarkers in FF had a high concentration variability in different follicles of each woman, but their mean levels positively correlated with the serum levels ($r^2 = .65$, $P < .0001$; $r^2 = .65$, $P < .0001$; $r^2 = .66$, $P < .0001$, respectively) (Figures 2 and 3).

We evaluated the relationship of the VB12, FA, and Hcy concentrations in each FF with oocyte maturation, fertilization, and embryo quality by univariate analysis. Among the 106 obtained oocytes, 92 were mature, with no relationship between the concentration of these parameters and oocyte maturation. Of the 92 matured oocytes, 59 were fertilized. VB12 concentration (FF) was significantly lower in fertilized than unfertilized oocytes (267 pg/mL vs 314 pg/mL, $P = .029$). The FA and Hcy concentrations (FF) did not correlate with fertilization rate. Of the 59 fertilized oocytes, 16 had a good embryo quality, whereas 43 had a poor embryo quality. The parameters (FF) had no relationship with embryo quality (Table 2).

The oocyte maturation, fertilization, embryo quality, and clinical pregnancy in the samples, which were divided into two subgroups using the VB12, FA, and Hcy levels in the FF according to their respective median levels, were examined by multivariate logistic analysis. Significant associations were found between FA (FF) <14.25 ng/mL and good-quality embryos (OR, 24; 95% CI, 3.6-263, $P = .0005$) and between Hcy (FF) <4.9 nmol/mL and clinical pregnancy (OR, 19.8; 95% CI, 1.6-693, $P = .018$); however, these FF concentrations had no correlation with oocyte maturation and fertilization (Table 3).

4 | DISCUSSION

In this study, the serum concentrations of VB12, FA, and Hcy constituting the OCM strongly correlated with FF concentrations. Moreover, all biomarkers had a high concentration variability in different follicles of each woman, but their mean levels correlated with serum levels. The investigation of relationships of VB12, FA, and Hcy concentrations in FF with oocyte maturation, fertilization, and embryo quality showed that the VB12 (FF) level was significantly decreased for fertilized oocytes. According to the multivariate logistic analysis, significant associations were found between FA (FF) <14.25 ng/mL and good-quality embryos and between Hcy (FF) <4.9 nmol/mL and clinical pregnancy.

Consistent with other reports,[6,7] this study demonstrated strong positive correlations between the serum and FF levels of VB12, FA, and Hcy constituting the OCM; hence, serum levels have a strong impact on FF levels. VB12 and FA are vitamins obtained from food, and changes in their serum levels caused by insufficient dietary intake may affect the intrafollicular microenvironment. Although the intra-individual variability in the FF concentration of OCM biomarkers has

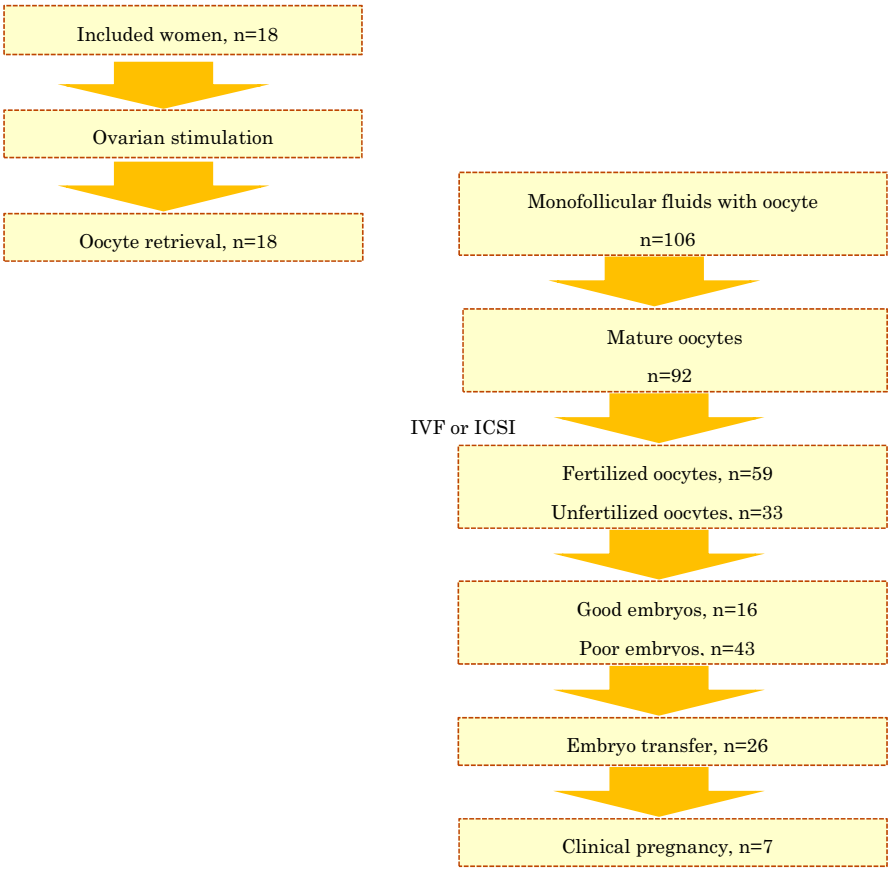

FIGURE 1 Patient flow chart. This prospective study analyzed 18 women who agreed to participate. Among the 106 collected oocytes, 92 were mature, 59 were fertilized, and 16 yielded good-quality embryos. We performed 26 single embryo transfers, and 7 patients achieved clinical pregnancies

TABLE 1 Serum concentrations of vitamin B12 (VB12), folic acid (FA), and homocysteine (Hcy)

	Serum VB12 (pg/mL)	Serum FA (ng/mL)	Serum Hcy (nmol/mL)
Mean ± SD	386.5 ± 140.7	14.2 ± 7.4	6.1 ± 1.6
Median (range)	376 (228-792)	13.5 (5.6-32.1)	6.1 (3.1-9.0)
Normal range	180-914	4.0<	3.7-13.5

Note: Normal range refers to the value obtained from SRL, Inc, the company which had conducted inspection.
Abbreviation: SD, standard deviation.

been seldom studied, Kralikova et al[8] have demonstrated that a high concentration variability of Hcy was found in different follicles of each woman, and this variability was extremely high in some women. They also revealed that VB12, FA, and Hcy concentrations are greatly varied in different follicles in each women, suggesting that not only serum concentrations but also the OCM in the intrafollicular microenvironment affect their concentrations in each FF. Considering that the intrafollicular microenvironment is formed by metabolism and interactions of oocytes and granulosa cells, changes in FF levels may affect subsequent oocyte maturation and embryogenesis.

Since Steegers et al[9] confirmed the presence of Hcy, FA, and VB12 in the FF, the effects of Hcy in the FF on oocyte maturation, fertilization, and embryo quality have been investigated. Regarding oocyte maturation, the percentage of oocyte maturation reportedly increases as the Hcy concentration in the FF decreases,[9,10] and high FF Hcy levels negatively correlate with oocyte quality.[11] Regarding fertilization, the fertilization rate decreases as the Hcy concentration in the FF increases[11,12]; in addition, the Hcy concentration in the FF negatively correlate with the follicular diameter, suggesting that a high-Hcy concentration in the FF inhibits folliculogenesis and may affect oocyte maturation and fertilization.[13] In the present study, the FF levels of VB12, FA, or Hcy had no correlation with oocyte maturation, but the VB12 level in the FF was significantly decreased for fertilized oocytes. VB12 is a nutrient necessary for Hcy remethylation to produce methionine in the OCM. In previous studies, no link was found between the FF VB12 level and the fertilization rate; however, the absence of association likely reveals that VB12 was properly used to maintain intrafollicular concentrations of physiologically required Hcy and methionine and to keep the subsequent S-adenosylmethionine synthesis optimal. Regarding embryo quality, some studies have evaluated the

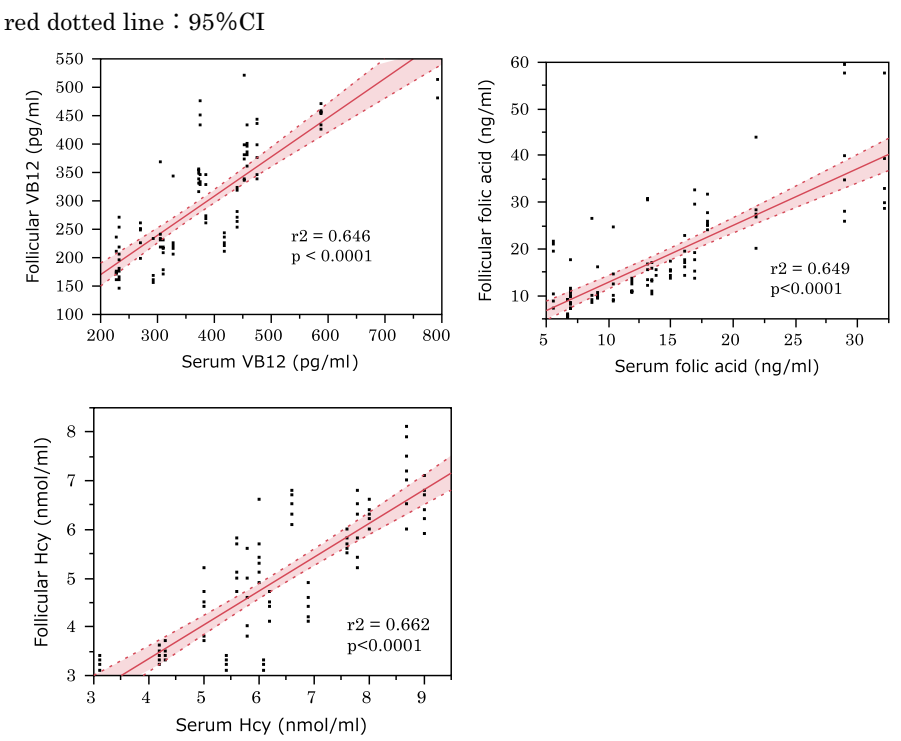

FIGURE 2 Correlation between serum and follicular fluid concentrations of vitamin B12 (VB12), folic acid (FA), and homocysteine (Hcy). The mean concentrations of VB12, FA, and Hcy in follicular fluid were correlated with the serum levels

TABLE 2 Correlation of VB12, FA, and Hcy concentrations in follicular fluid with oocyte maturation, fertilization, and embryo quality (univariate analysis)

	Mature (n = 92)	Immature (n = 14)	p value	Fertilized (n = 59)	Unfertilized (n = 33)	*P* value	Good embryo (n = 16)	Poor embryo (n = 43)	*P* value
VB12 (pg/mL)	284.3 ± 10.3	300.1 ± 26.4	0.577	267.5 ± 12.7	314.4 ± 16	.0294	250 ± 25.3	274 ± 15.4	.42
FA (ng/mL)	17.3 ± 1.2	21.4 ± 3.0	0.199	16.7 ± 1.45	18.4 ± 1.9	.477	20.3 ± 2.56	15.3 ± 1.56	.099
Hcy (nmol/mL)	4.9 ± 0.14	4.8 ± 0.37	0.66	5.1 ± 0.18	4.7 ± 0.25	.217	4.9 ± 0.35	5.14 ± 0.21	.56

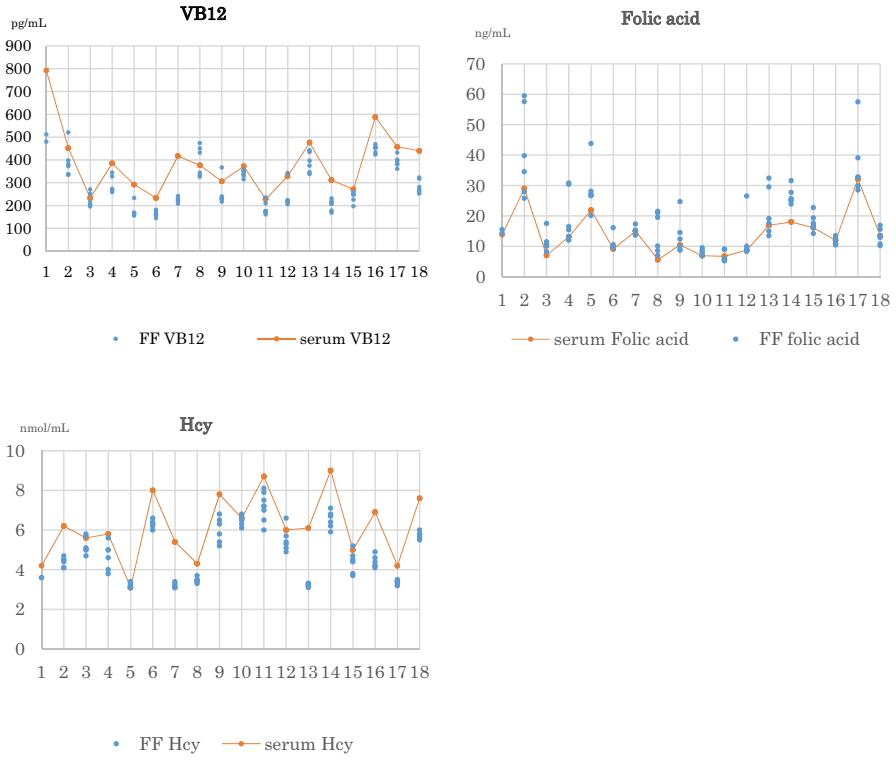

FIGURE 3 Intraindividual variability of vitamin B12 (VB12), folic acid, and homocysteine (Hcy) concentrations in follicular fluid (FF). All biomarkers had a high concentration variability in different follicles of each woman, but their mean levels correlated with the serum levels

association with the Hcy concentration in FF, but the results were different, thereby consensus remains unachieved. Ebisch et al and Berker et al[6,11] reported that the embryo quality decreases as the Hcy concentration in the FF increases. However, Ocal et al[14] stated that the Hcy concentration is unrelated to embryo quality. Meanwhile, Booxmer et al[15] discussed that an increased Hcy level in the FF contributes to an embryo quality improvement. The main adverse action of Hcy is generation of reactive oxygen species (ROS), causing decreased cell division, production of inflammatory cytokines, altered nitric oxide metabolism, increased oxidative stress, increased apoptosis, and disordered methylation.[16-19]

TABLE 3 Correlation of VB12, FA, and Hcy concentrations in follicular fluid with embryonic developmental capacity (oocyte maturation, fertilization, embryo quality, and clinical pregnancy); Dividing into two groups by the median concentrations of VB12, FA, and Hcy in follicular fluid. (Multiple regression analysis)

	OR	95% CI	*P* value
Embryo quality			
VB12 (<256 pg/mL)	0.518	0.111-2.168	.372
FA (<14.25 ng/mL)	24	3.601-262.6	.0005
Hcy (<4.9 nmol/mL)	3.818	0.668-31.55	.136
Clinical pregnancy			
VB12 (<256 pg/mL)	2.938	0.151-128.1	.482
FA (<14.25 ng/mL)	0.341	0.011-6.464	.47
Hcy (<4.9 nmol/mL)	19.8	1.622-692.7	.0182

Note: No significance in maturation and fertilization.
Abbreviations: CI, confidence interval; OR, odds ratio.

ROS have bipolar actions on reproduction; excess ROS are associated with increased embryonic fragmentation, decreased embryo cleavage, and decreased rate of blastocyst formation.[20] In a physiological concentration range, ROS are necessary for oocyte maturation, ovulation, luteinization, progesterone production by corpus luteum, and monofolliculogenesis.[16,21] Embryogenesis may also require a certain level of Hcy, and harmful effects may be prevented at an optimal follicular Hcy level. In the present study, no significant differences in Hcy concentrations in the FF were found between good- and poor-quality embryos, and no association was observed for VB12 or FA concentration.

In multivariate logistic analysis, a significant correlation was found between FA concentration (FF) < 14.25 ng/mL and good-quality embryos and between Hcy concentration (FF) <4.9 nmol/mL and clinical pregnancy.

In an animal study, folate metabolism cycle inhibition and FA deficiency result in ovulation inhibition and blastocyst development suppression.[22] Meanwhile, a human study reported that the FA level in the FF negatively correlates with follicular diameter and that a high FA level may adversely affect follicular development.[13] In addition, follicular development requires certain levels of inflammation and oxidative stress, and increased inflammation marker levels in the FF are reportedly indicators of follicle activity.[23] However, Twiht et al[24] mentioned that the expression levels of inflammatory proteins in the FF were suppressed in the FA intake group, suggesting that excessive FA intake suppresses inflammation, reduces the follicular activation, and results in oocyte/embryo quality reduction. Identifying the optimal FF level of FA at which not only neural tube defects are prevented but also

improve the oocyte/embryo quality, is necessary. Furthermore, the effects of OCM-related biomarker concentrations in the FF on pregnancy have been seldom investigated, providing a limited basis of understanding. Booxmer et al[21] reported that the chances of biochemical pregnancy increase with a high FA level in the FF. Regarding Hcy, Ocal et al[20] demonstrated that the Hcy level in the FF was significantly low in pregnant women, and Boyama et al[25] reported that low Hcy levels in culture media are involved in increased pregnancy rates. Hcy < 4.9 may be a useful indicator of clinical pregnancy; however, considering the paucity of cases, further examinations in a larger number of cases are necessary.

As a limitation, this study has a small sample size. In addition, IVF treatment-related factors, such as the fertilization methods (insemination/ICSI) and the stage to evaluate embryo quality (cleavage/blastocyst), might have affected the outcomes. Conversely, the strength of this study is the use of a prospective design. Few studies have evaluated the impact of OCM on subsequent pregnancy, but our study results revealed that it may be a useful predictor of IVF outcome.

In summary, the serum levels of VB12, FA, and Hcy constituting OCM strongly correlated with their respective FF levels. Although large variations existed in the VB12, FA, and Hcy levels in FF samples in each woman, the mean levels correlated well with the serum levels. The analysis of relationships of VB12, FA, and Hcy levels in the FF with oocyte maturation, fertilization, and embryo quality showed that the VB12 level in the FF was significantly low in fertilized oocytes. Moreover, the multivariate logistic analysis revealed that FA < 14.25 ng/mL and Hcy < 4.9 nmol/mL were potential indicators of good-quality embryos and clinical pregnancy, respectively. Our results suggest that changes in serum levels of OCM biomarkers should be strongly reflected in FF when the balance of methionine metabolism is disturbed by dietary habits. Therefore, maintaining a balanced methionine metabolism is essential. However, because all biomarkers had a high concentration variability in different follicles, each follicle is considered to have its own OCM and reflects it. Therefore, by analyzing the OCM biomarkers in each FF, it may be possible to estimate oocyte quality, embryo quality, and subsequent pregnancy of the obtained oocyte derived from each follicle.

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DISCLOSURES

Conflicts of Interest: Kozue Akamine, Keiko Mekaru, Keiya Gibo, Chinatsu Nagata, Rie Nakamura, Sugiko Oishi, Maho Miyagi, Chiaki Heshiki, and Yoichi Aoki declare that they have no conflict of interest. *Human rights statements and informed consent*: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all patients for being included in the study. This article does not contain any studies with animal subjects performed by the any of the authors. *Approval by Ethics Committee*: This retrospective study was approved by the Institutional Review Board of the University of the Ryukyus Hospital (February 21, 2013; No. 498).

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