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The association between plasma and follicular fluid folate levels and the pregnancy rate in women enrolled in ICSI cycles

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Abstract

Background: Among the environmental factors, diet has received much attention because of growing bulk of data that discloses significant and substantial role for dietary elements, particularly, micronutrient in fertility pathophysiology. One of the major dietary elements that have been recently under focus is dietary folate. A number of vital cellular events, such as transfer RNA, synthesis of DNA, methionine and cysteine, require folate as an essential molecular participant. A number of previous studies have documented that folate supplementation can improve fertility outcome; whereas, other studies have denied such an association between folate supplementation and fertility outcomes.

Aim of the study: to study a possible correlation between plasma and follicular fluid folate levels and the pregnancy rate in women enrolled in ICSI cycles.

Patients and methods: The present study was done on 65 infertile couples who were chosen from those attended the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies who were subjected to an intracytoplasmic sperm injection (ICSI) cycles. Their ages ranged from 18 to 42 years. Both primary and secondary types of infertility were involved, with heterogeneous causes. From each woman, venous blood sample and follicular fluid sample was obtained for folate concentration determination using ELISA technique.

Results: The biochemical pregnancy rate was 32.3 %. There was significant association between plasma folate level and positive pregnancy outcome ($P < 0.05$), when comparing the pregnancy rate of deficient group, lower normal group and high group with that of normal group. The highest pregnancy rate was observed in women with normal plasma folate and it equals 56.7 %, therefore it was considered a reference group for purpose of comparison. Pregnancy rate of deficient folate group was significantly lower than that of normal folate group, 11.1 % versus 56.7 % ($P = 0.043$). In addition, pregnancy rate of lower normal folate group was significantly lower than that of normal folate group, 10.0 % versus 56.7 % ($P = 0.028$). Moreover, pregnancy rate of high folate group was significantly lower than that of normal group, 12.2 % versus 56.7 % ($P = 0.004$).

Conclusion: Both high and low plasma folate are associated with low pregnancy rate; therefore, it should be recommended that serum folate should be monitored when prescribing folate to infertile women and that it should be discontinued once its level reaches the maximum normal plasma range.

Keywords: pregnancy outcome, plasma and follicular fluid folate, ICSI cycles

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INTRODUCTION

The problem of infertility is a common health issue that is seen daily during clinical practice all over the world including Iraq (Cetorelli, 2014; Vander Borght M, Wyns, 2018; Elhussein et al. 2019; Gopalakrishnan, et al. 2016).Couples are going to be considered infertile when they fail to have a child following one year of unprotected natural intercourse (Ebisch et al. 2007); however, the exact definition of infertility differs among

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various fertility institutes globally (Gurunath et al. 2011). Couples complaining of infertility are going to seek medical advice in order to have a child and when medical intervention, such as ovulation induction medications, fails, they often accept to undergo assisted reproductive technology cycles including in vitro fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI); however, these techniques usually do not treat the exact cause of infertility, but try to overcome such causes (Ebisch et al. 2007).

Indeed, the list of causes of infertility is so long, nevertheless, fertility specialists used to group them into 4 major categories: male factors, female factors, combined male and female factors and unexplained infertility (Öztekin et al. 2019). Some causes of infertility are genetic in origin (Zorrilla and Yatsenko, 2013, Venkatesh et al. 2014) and others are environmental in origin (Hruska et al. 2000; Gaskins and Chavarro, 2018). Among the environmental causes, diet has received much attention because of growing bulk of data that discloses significant and substantial role for dietary elements, particularly, micronutrient in fertility pathophysiology (Gaskins and Chavarro, 2018). One of the major dietary elements that have been recently under focus is dietary folate (Gaskins et al. 2014).

A number of vital cellular events, such as transfer RNA, synthesis of DNA, methionine and cysteine, require folate as an essential molecular participant (Ebisch et al. 2007). This implies that folate is essential during period of rapid cell division, multiplication and growth including the events occurring during the physiological process of cleavage, embryo formation and growth, embryo implantation and subsequent embryo growth and development (Forges et al. 2007). The principal reason for folate recommendation early in pregnancy is the current medical truth that folate can prevent neural tube defect in the growing embryo; however, research work has documented a role for folate in improving fertility outcome in women undergoing IFV/ICSI cycles (Gaskins et al. 2014).

A number of previous studies have documented that folate supplementation can improve fertility outcome (Szymanski and Kazdepka-Zieminska, 2003, Boxmeer et al. 2009); whereas, other studies have denied such an association between folate supplementation and fertility outcomes (Haggarty et al. 2006). The presence of such controversy and the poverty of Iraqi literatures dealing with the association between pregnancy outcome and maternal plasma and follicular fluid folate in women undergoing IFV/ICSI cycles have justified the planning and conduction of present study aiming at finding the effect of plasma and follicular fluid level on pregnancy outcome in infertile women undergoing assisted reproductive technologies.

MATERIALS AND METHODS

Patients and Methods

This prospective study was done in the High Institute of Infertility diagnosis and Assisted Reproductive Technologies at Al-Nahrain University Baghdad/Iraq) during the period from September 2019 until February 2020. The study was officially approved by the Local Medical Ethical Committee of the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University, a written Informed consent hadbeen taken from each patient who was involved in this study.

The present study was done on 65 infertile women who were chosen from those attended the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies who were subjected to an intra-cytoplasmic sperm injection (ICSI) cycles. Their ages ranged from 18 to 42years, both primary and secondary types of infertility were involved, with different causes.

All patients involved in the study fulfilled the following criteria:

Inclusion Criteria

- All patients had been chosen with the use of (similar protocol) GnRH antagonist protocol.
- Age ranged from 18 to 42years.
- Fasting 6 -8 hours on day of blood sampling (day of oocyte pick up).
- Absence of endocrine disorders (hyperprolactinemia, diabetes mellitus, Cushing syndrome, congenital adrenal hyperplasia), thyroid dysfunction
- Couples with unexplained infertility, and other various causes except sever endometriosis (stage 3 and stage 4, confirmed laparoscopically).
- No history of ovarian surgery.
- All women received folic acid supplement with different doses ranged from (0.4 mg -0.8mg) for 1-2 months.

Exclusion Criteria

- Patients with coeliac disease , malabsorption syndrome and other chronic diseases.
- Male partner with testicular biopsy sperm sample (azospermia).
- Patients with antifolate drugs (omeprazole, methprim, metformin, anti-convalsant drugs).
- Chronic smoker (passive and active).

All participants passed through the routine ICSI procedure including clinical evaluation (history, examination and investigation), controlled ovarian stimulation, triggering of ovulation, oocyte retrieval under general anesthesia, blood and follicular fluid collection for later assessment of the concentration of folate, oocyte denudation, oocyte maturation evaluation, intracytoplasmic sperm injection of mature (MII) oocytes,

evaluation of fertilization and cleavage and embryo grading, embryo selection and embryo transfer, luteal phase support, beta hCG determination (to document biochemical pregnancy).

All patients were subjected to antagonist protocol. In this which ovarian stimulation was commenced by (rFSH) and (HMG) was added accordingly, by daily subcutaneous injection in a dose of 150-225 IU were taking place from the second day of cycle till the day of trigger by hCG injection while the GnRH antagonist injection (Cetrorelix) was added in a dose of 0.25 mg daily once the growing follicles reached (12-14 mm) in their diameter by ultrasound monitoring. The antagonist was given together with the gonadotropin stimulation till a good response is obtained and Ovulation induction was induced by the administration of recombinant hCG (rhCG 6500 IU, Ovitrelle®; Merck, Italy) subcutaneously.Oocyte pick up under general anesthesia with the guidance of transvaginal ultrasound thirty-four to thirty-six hours following the hCG injection with sperm collection from the women partners on oocyte retrieval day then subjected to ICSI. Fertilization check was performed on day 1 of insemination by ICSI.

Sperm processing has been carried after oocyte denudation. Oocyte and embryo quality were assessed depending on microscopic morphological criteria. Samples of plasma and follicular fluid were taken on day of oocytes pick up, frozen and were analyzed for folate levels using ELISA technique within a single analytical run for all patients at the end of study.

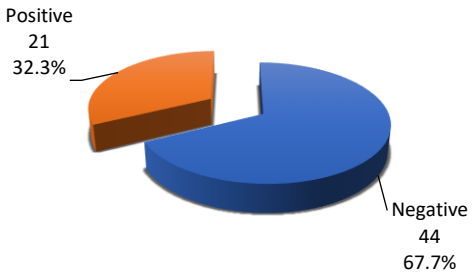

Fig. 1. Pie chart showing the frequency distribution of infertile women according to pregnancy test results

RESULTS

The current prospective study included 65 infertile women of whom 21 got positive biochemical pregnancy results at the end of the study making the overall rate of pregnancy (32.3 %), as shown in **Fig. 1**. The demographic characteristics of infertile women participating in this study are shown in **Table 1**. There was no significant difference in any of these characteristics between pregnant and non-pregnant women.

The plasma folate and follicular fluid folate of infertile women according to pregnancy outcome is shown in

Table 1. Demographic characteristics of infertile women according to pregnancy outcome

Characteristic	Total *n* = 65	Positive pregnancy *n* = 21	Negative pregnancy *n* = 44	*P*
Age (years)				
Mean ±SD	30.82 ±5.83	30.05 ±6.38	31.18 ±5.59	0.467†
Range	19 -42	19 -42	19 -40	NS
< 35, *n* (%)	48 (73.8 %)	15 (71.4 %)	33 (75.0 %)	0.579
≥ 35, *n* (%)	17 (26.2 %)	6 (28.6 %)	11 (25.0 %)	YNS
BMI (kg/m2)				
Mean ±SD	29.20 ±4.76	28.43 ±5.61	29.57 ±4.32	0.373†
Range	18-42.01	18 -42.01	22.03 -41.74	NS
Underweight, *n* (%)	1 (1.5 %)	1 (4.8 %)	0 (0.0 %)	
Normal, *n* (%)	12 (18.5 %)	5 (23.8 %)	7 (15.9 %)	
Overweight, *n* (%)	26 (40.0 %)	8 (38.1%)	18 (40.9 %)	
Class I obesity, *n* (%)	18 (27.7 %)	4 (19.0 %)	14 (31.8 %)	
Class II obesity, *n* (%)	5 (7.7 %)	2 (9.5 %)	3 (6.8 %)	
Class III obesity, *n* (%)	3 (4.6 %)	1 (4.8 %)	2 (4.5 %)	
Duration of infertility (years)				
Mean ±SD	7.03 ±3.58	6.95 ±3.84	7.07 ±3.50	0.907†
Range	2 -19	2 -17	2 -19	NS
Type of infertility				
Primary, *n* (%)	42 (64.6 %)	16 (76.2 %)	26 (59.1 %)	0.178
Secondary, *n* (%)	23 (35.4 %)	5 (23.8 %)	18 (40.9 %)	¥NS
Causes of infertility				
Male, *n* (%)	32 (49.2 %)	9 (42.9 %)	23 (52.3 %)	0.311
Female, *n* (%)	17 (26.2 %)	8 (38.1 %)	9 (20.5 %)	¥NS
Unexplained, *n* (%)	16 (24.6 %)	4 (19.0 %)	12 (27.3 %)	
History of ART				
0, *n* (%)	40 (61.5 %)	13 (61.9 %)	27 (61.4 %)	
1, *n* (%)	12 (18.5 %)	3 (14.3 %)	9 (20.5 %)	0.585
2, *n* (%)	10 (15.4 %)	3 (14.3 %)	7 (15.9 %)	¥ NS
3, *n* (%)	3 (4.6 %)	2 (9.5 %)	1 (2.3 %)	
Parity				
0, *n* (%)	52 (80.0 %)	18 (85.7 %)	34 (77.3 %)	
1, *n* (%)	7 (10.8 %)	3 (14.3 %)	4 (9.1 %)	0.188 ¥
2, *n* (%)	6 (9.2 %)	0 (0.0 %)	6 (13.6%)	NS

n: number of cases; SD: standard deviation; BMI: body mass index; ART: assisted reproductive technologies; †: Independent samples t-test; ¥: Chi-square test; Y: Yates correction; NS: not significant at $P > 0.05$

Table 2. Plasma folate and follicular fluid folate of infertile women according to pregnancy outcome

Characteristic	Total *n* = 65	Positive pregnancy *n* = 18	Negative pregnancy *n* = 47	*P*
		Plasma folate		
Mean ±SD	12.71 ±6.52	11.60 ±5.57	11.74 ±8.80	0.950 †
Range	2.30 -25.10	2.3 -22.1	2.3 -25.1	NS
		Follicular fluid folate		
Mean ±SD	8.00 ±5.39	7.84 ±4.68	8.39 ±6.19	0.719 †
Range	0.60 -23.20	0.6 -18.5	1.1 -23.2	NS

n: number of cases; SD: standard deviation; †: Independent samples t-test; NS: not significant at $P > 0.05$

Table 3. The frequency distribution of infertile women according to plasma folate level and pregnancy outcome

Plasma folate	Total *n* =65	Positive pregnancy *n* = 21		Negative pregnancy *n* = 44		*P*
		n	%	N	%	
Deficient (< 3ng/ml)	9	1	11.1	8	88.9	0.043 YS
Lower normal (3-5.9 ng/ml)	10	1	10.0	9	90.0	0.028 YS
Normal (6-20 ng/ml)	30	17	56.7	13	43.3	Reference
High (> 20 ng/ml)	16	2	12.5	14	87.5	0.004 YHS

n: number of cases; **Y**: Yates correction for continuity; **S**: significant at $P \leq 0.05$; **HS**: highly significant at $P \leq 0.01$

Table 2. Neither plasma folate nor follicular fluid folate showed significant variation with respect to pregnancy outcome ($P > 0.05$).

The plasma folate level was converted into a categorical variable in which deficient level was considered at < 3ng/ml, lower normal level was considered at 3-5.9 ng/ml, normal level was considered at 6-20 ng/ml and high level was considered at > 20 ng/ml, as shown in **Table 3** and **Fig. 2**, according to (WHO, 2012). Accordingly, there was significant association between plasma folate level and positive pregnancy outcome ($P < 0.05$), when comparing the pregnancy rate of deficient group, lower normal group and high group with that of normal group. The highest pregnancy rate was observed in women with normal plasma folate and it equals 56.7 %, therefore it was considered a reference group for purpose of comparison. Pregnancy rate of deficient folate group was significantly lower than that of normal folate group, 11.1 % versus 56.7 % ($P = 0.043$). In addition, pregnancy rate of lower normal folate group was significantly lower than that of normal folate group, 10.0 % versus 56.7 % ($P = 0.028$). Moreover, pregnancy rate of high folate group was significantly lower than that of normal group, 12.2 % versus 56.7 % ($P = 0.004$), **Table 3** and **Fig. 2**.

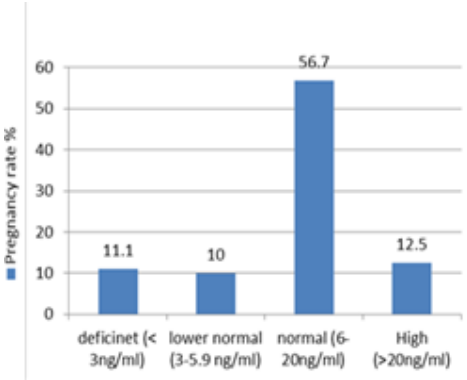

Fig. 2. Bar chart showing the pregnancy rate according to plasma folate level of women enrolled in the current study

Plasma folate was insignificantly correlated to maturity index, fertilization rate and cleavage rate ($P > 0.05$), **Table 4**. However, comparison of maturity index, fertilization rate and cleavage rate between women with normal plasma folate and other women has shown that fertilization rate in women with normal plasma folate was significantly higher ($P = 0.048$) than other women, 74.01 ±24.74 % versus 65.90 ±26.32, respectively, as shown in **Table 5**.

Follicular fluid folate showed highly significant positive correlation to plasma folate level ($r = 870$; $P <$

Table 4. Correlation of plasma folate to maturity index, fertilization rate and cleavage rate

Characteristic	Plasma folate	*n*	Mean	SD	*P*
Maturity index	Deficient	9	67.54	21.67	0.808 † NS
	Lower normal	10	57.61	14.93	
	Normal	30	63.17	23.90	
	High	16	63.43	22.76	
	Total	65	62.98	21.86	
Fertilization rate	Deficient	9	61.98	20.70	0.537 † NS
	Lower normal	10	70.97	22.52	
	Normal	30	74.01	24.74	
	High	16	64.94	31.78	
	Total	65	69.64	25.73	
Cleavage rate %	Deficient	9	83.33	35.36	0.945 † NS
	Lower normal	10	81.00	32.81	
	Normal	30	85.08	29.17	
	High	16	79.27	35.93	
	Total	65	82.78	31.65	

†: One way ANOVA test; NS: not significant at $P > 0.05$

Table 5. Comparison of maturity index, fertilization rate and cleavage rate between women with normal plasma folate and other women

Characteristic	Total *n* = 65	Normal *n* = 30	Other *n* = 35	*P*
Maturity inde	62.98 ±21.86	63.17 ±23.90	62.82 ±20.30	0.950 †NS
Fertilization rate	69.64 ±25.73	74.01 ±24.74	65.90 ±26.32	0.048 †S
Cleavage rate %	82.78 ±31.65	85.08 ±29.17	80.81 ±33.93	0.591 †NS

Data were expressed as mean and standard deviation (SD); n: number of cases; †: independent samples t-test; NS: not significant at $P > 0.05$; S: significant at $P \leq 0.05$.

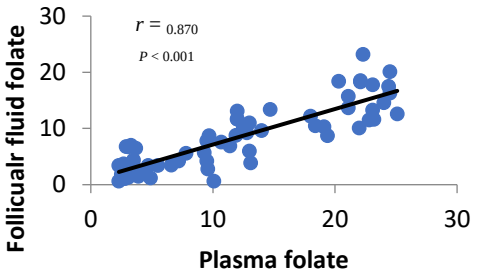

Fig. 3. Scatter plot showing the correlation between plasma folate level and follicular fluid folate level

0.001), as shown in **Fig. 3**, which indicate that increment in plasma folate causes proportional increment in follicular fluid folate.

DISCUSSION

In the present study, there was no significant difference in mean plasma folate and follicular fluid folate between pregnant and non-pregnant women; however, when women were categorized into subgroups according to (WHO, 2012) plasma folate, there was significant association between plasma folate level and positive pregnancy outcome ($P < 0.05$), when comparing the pregnancy rate of deficient group, lower normal group and high group with that of normal group. The highest pregnancy rate was observed in women with normal plasma folate and it equals 56.7 %, while the pregnancy rate of deficient folate group was 11.1 %, of lower normal folate group was 10.0 % and of high folate group was 12.2 %. In addition, it was found that normal plasma folate was correlated significantly with higher fertilization rate. Therefore, it can be suggested that normal plasma folate of 6-20 ng/ml resulted in highest fertilization rate and thereby highest biochemical pregnancy rate.

Experimental studies have shown that FA is essential in successful implantation by providing favorable receptive environment to receive the implantation-competent blastocyst for a successful pregnancy (Chandracharya et al. 2018). So it increases the chances of successful implantation by providing much more favorable decidua environment (Chandracharya et al. 2018).

In the current study, it was shown that normal plasma folate concentration was significantly correlated to pregnancy outcome and fertilization rate. The finding of significant correlation between pregnancy outcome and follicular fluid folate is in accordance with a previous study in which follicular fluid concentration of folate was correlated to biochemical pregnancy (Boxmeer et al. 2009); however, in the contrary to results of Boxmeer et al. the current study not revealed significant correlation of follicular fluid folate concentration to oocyte or embryo quality. On the other hand, the association between fertilization rate and serum and follicular fluid folate in this study was in line with the study carried out by other authors O'Neill, 1998, since an association between folate and fertilization rates has been observed in *in vitro* models. Researches on mouse pre-implantation embryos have demonstrated that intrinsic folates are necessary for development of embryo because of their participation in thymidine generation (O'Neill, 1998; Kwong et al. 2010). Furthermore, substantial quantities of reduced folates are needed to accumulate in the oocyte at time of gametogenesis because accumulation of thymidine does not happen in cells. These events will lead to the exponential rise in DNA formation which happens at time of early embryonic development. This experimental information supports the suggestion that embryo survival is increase with response to folate (Matte et al., 1984, Habibzadeh et al. 1986; Tremblay et al. 1989) and is in accordance with present results on implantation.

It has been shown that the concentration of gelatinase MMP-2(matrix metalloproteinases) in women follicular fluid can serve as a predictor of oocytes maturation, in addition, the expression of MMP-2 plays a significant role in association with higher fertilization rate(Laflamme and Wolfner, 2013), folic acid found to be the major epigenetic regulator of MMP-2(Sundrani et al. 2011) but further research work is still needed to reach a consensus about such role.

Studies on the contribution of diet in fertility are limited; however, they suggest that a number of nutrients, especially folate, can upgrade fertility (Chavarro et al. 2008, Gaskins et al. 2012; Steegers-Theunissen et al. 2013). Folate is required for the DNA synthesis, RNA transfer, methionine and cysteine. For that reason, the need for folate is greatly increased at time of rapid cell growth, including the peri-conceptional period, (Ebisch et al. 2007; Forges et al. 2007). Folate serves as a methyl group donor in the pathway of homocysteine in addition for many other protein synthesis pathways. It has been shown that high follicular fluid content of homocysteine is associated with

bad fertility outcome and this may be related to poor oocyte quality (Ocal et al. 2012).

The inadequacy of a number of folate-dependent proteins is hypthesized to participate significantly in the peri-conception period (Bliek et al. 2004). Folate insufficiency may also result in mutations due to the introduction of uracil instead of thymine into the DNA (Blount et al. 1997; Koury et al. 1997). Chromosome instability and double-strand breaks may follow the inability to repair because of failure of removal of misincorporated uracil with subsequent apoptosis (Blount et al. 1997; Koury et al. 1997). Formation and repair of DNA in addition to DNA methylation are critical in gametogenesis ,fertilization, and pregnancy (Jaroudi and SenGupta, 2007; Kiefer, 2007).

In an American study, it was found that higher folate intake was associated with higher rates of implantation, clinical pregnancy, and live birth in 232 women undergoing assisted reproductive technology (Gaskins et al. 2014). However, the later study mentioned nothing about the association between follicular fluid folate concentration and fertility outcomes during IFV/ICSI cycles.

It has been inferred that folate may improve fertility outcome in couples undergoing ART according to some European studies (Szymanski et al. 2003, Boxmeer et al. 2009). For instance it has been shown that pregnancy outcome is 3 times greater in women with adequate follicular fluid folate levels in the Netherlands (Boxmeer et al. 2009). And I t has been inferred that a rise in the chance of pregnancy by a factor of 3.3 (95% CI: 1.09, 9.71) can follow a rise in follicular fluid folate by 2 folds (Boxmeer et al. 2009).

In a polish study, the oocyte quality and maturation rate was significantly higher in women receiving folate supplementation than in women receiving no folate supplementation (Szymanski et al. 2003). Nevertheless, results have not been totally controversial across researches, as a study among British women undergoing ART described no such association between the chance of a successful pregnancy and pre-pregnancy folate and (Haggarty et al. 2006).

Indeed, these findings reflect the need to encourage folic acid campaigns in order to improve fertility outcome. However, in the current study, serum folate within normal range (6-20 ng/ml) was the best predictor of highest pregnancy outcome and high plasma folate had bad effect on pregnancy rate. This findings suggest limitation of folate administration to infertile women to the degree that assure adequate serum folate in order to avoid excess serum folate that may have negative drawback on pregnancy outcome in women undergoing IVF/ICSI cycles. Actually, this hypothesis was based on the current finding that high serum folate (> 20 ng/ml), was associated with low pregnancy outcome in comparison with normal serum folate in a highly significant manner. It has been shown that high intake of folic acid supplement may lead to increase in the plasma level of un metabolized folic acid and this can cause toxic effects on cellular levels including oocyte development and maturation (Obeid and Herrmann, 2012; Xie et al. 2019).

Therefore, it should be recommended that plasma folate should be monitored when prescribing folate to infertile women and that it should be discontinued once its level reaches the maximum normal serum range. The current study finding that an optimal level of plasma folate and not the higher possible concentration in line with general observation that the majority of the women had normal to high folate concentrations; however, the optimal folate concentration in monofollicular fluid to raise the probability of pregnancy is till now not clear (Boxmeer et al. 2009). Indeed, the current observation that follicular fluid concentration is directly correlated to plasma folate is in line with previous observations (Steegers-Theunissen et al., 1993; Boxmeer et al. 2009). This correlation has an advantage of predicting how follicular fluid changes with changes in plasma folate; therefore, an idea about follicular folate can be made when plasma folate is estimated despite the difference in the exact folate concentration between plasma folate and follicular fluid folate.

One possible limitation of the current study was the inability to correlate the daily intake of folate to both plasma and follicular fluid folate in women enrolled in the current study in order to make an idea about how much changes will happen in folate concentration in response to distinct change to oral folate intake. In a prospective cohort of women undergoing infertility treatment in the United States it was found that “pre-treatment supplemental folic acid above 800µg/day was related to a higher probability of live birth among women undergoing assisted reproductive technology on the background of a fortified food supply” (Gaskins et al. 2014).

CONCLUSION

In conclusion, both high and low plasma folate are associated with low pregnancy rate; therefore, it should be recommended that plasma folate should be monitored when prescribing folate to infertile women and that it should be in the optimal effective level and discontinued once its level reaches the maximum normal plasma range.

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