The status of ascorbic acid in follicular fluid of non-PCOS women during IVF-ET cycles is an indicator for aromatization and pregnancy outcome

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Abstract

Follicular fluid (FF) serves as a natural 'culture medium' for the maturation of both granulosa cells and oocytes. Ascorbic Acid (AA) known to maintain reproductive integrity and reported to either accumulate in the follicular fluid or other ovarian cells. A relatively higher bioavailability of AA within the graafian follicle as compared to serum suggests importance of AA in the graafian follicle biology. As a pre-eminent water soluble antioxidant, this molecule has also been reported to provide protection of oocyte and granulosa cells against cellular injuries but earlier studies were limited with exogenous supplementation. The present study evaluated the significance of endogenous FF-ascorbic acid in aromatization and pregnancy outcome in non-PCOS women undergoing IVF-ET treatment cycles. The positive relationship of FF-AA with clinical pregnancy rate observed in this study clearly denotes the importance of maintaining the antioxidant status within the microenvironment in order to achieve pregnancy. The present study has attempted to establish for the first time a successful correlationship between endogenous FF-AA levels with pregnancy outcome as against the exogenous supplementation theory and also aided to determine the critical requirement of AA within the follicular milieu for achieving the objective.

Keywords: Ascorbic acid, DHEA-S, E2, Aromatization, Pregnancy

1. Introduction

Follicular fluid aspirated at the time of ovum pickup (OPU) in an IVF cycle serves as a natural 'culture medium' for the maturation of both granulosa cells and oocytes (1, 2, 3). Ascorbic Acid (AA) is known to maintain reproductive integrity (4, 5, 6, 7) and is reported to either accumulate in the follicular fluid (8, 9) or other ovarian cells i.e. granulosa, theca and luteal cells (10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20). A relatively higher bioavailability of AA within the Graafian follicle as compared to serum (21) suggests importance of AA in the Graafian follicle biology. As a pre-eminent water soluble antioxidant (22, 23, 24). This molecule

has also been reported to provide protection of oocyte and granulosa cells against cellular injuries (21).
Human studies have indicated beneficial effects of AA not only on ovulation through induction of clomiphene citrate resistant anovulatory women (25, 26) but also on enhancement in pregnancy rate of non-IVF infertility treatment cycles (27). Though a higher pregnancy rates in women undergoing IVF-ET cycles have been correlated to raise in the concentrations of FF-AA, the difference in pregnancy rates between the control and experimental groups was not found to be statistically significant (28). It is strange that all these studies attempted to evaluate the impact of exogenous AA supplementation on ovarian function and reproductive outcome without attempting to associate endogenous follicular fluid-AA as a determinant for pregnancy outcome in IVF-ET cycles.

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The present study therefore seeks to evaluate the significance of endogenous FF-ascorbic acid in affecting aromatization and pregnancy outcome in non-PCOS women undergoing IVF-ET treatment cycles.

2. Materials and Methods
2.1. Subjects
The study included 105 in-vitro fertilization embryo transfer (IVF-ET) cycles in non-PCOS women (mean age 32.23 ± 3.69 years, BMI 23.55 ± 2.847 (kg/m^2), Waist/Hip ratio 0.887 ± 0.057, duration of infertility 8.28 ± 4.34 years).

Informed consent was obtained from each patient for participation before commencing the study. The study protocol was approved by the local Hospital Ethical Committee and was in conformity with the provisions of Declaration of Helsinki (as revised in Edinburgh 2000).

Inclusion Criteria
1) Both side morphologically normal and adequately visualized ovaries under transvaginal ultrasound scans.
2) Menstrual cycle length range between 25 and 35 days i.e. regularly menstruating women with normal nutritional habits and not receiving any kind of medication for the last 6 months.
3) No current or past diseases affecting ovaries or gonadotropins or sex steroid secretion, clearance, or excretion
4) Repeated 3 failed IUI cycles.

Exclusion Criteria
1) Pre-menopausal women older than 42 years of age.
2) Women with polycystic ovary syndrome as defined according to the Rotterdam consensus 2004 (29).
3) Women suffering from renal or gastrointestinal diseases, history of pelvic surgery, endocrine disorders.
4) Severe male factor abnormality patients who were considered for ICSI.

2.2. IVF-ET treatment cycle protocol
2.2.1. Down-regulation protocol
Pituitary down-regulation/desensitization was initiated with gonadotropin-releasing hormone (GnRH) agonist (500 µg/day Lupride, Sun Pharma, India) started in the mid-luteal phase (D-21) of the previous menstrual cycle or 7 days prior to the earliest expected date of next menstrual cycle.

After the onset of menstrual bleeding, down regulation was confirmed by measurement of serum E2 and FSH levels <20 pg/ml and <1.0 mIU/ml respectively and at the same time absence of ovarian cyst was confirmed by transvaginal ultrasound examination. Controlled ovarian hyperstimulation (COH) was started either on the day of onset of menstruation or 1-2 days at the most after the onset of menses.

2.2.2. Controlled Ovarian Stimulation (COH) Protocol
After confirmation of down regulation with GnRH agonist a standard long protocol was used for the controlled ovarian hyperstimulation (COH). Controlled ovarian hyperstimulation was started with daily administration of recombinant FSH (rFSH) 225 IU/day (Foligraph, Bharat Serum Pvt. Ltd. India,). The gonadotropin (rFSH) dosage was decided according to follicular growth. Ovarian response was monitored by transvaginal ultrasonography (TVS) and monitoring was done daily from D8 onwards till final oocyte maturation. Triggering of ovulation was induced with 5,000 IU of hCG (Fertigyn, Bharat Serum Pvt. Ltd. India) when at least 1-3 lead follicles reached a mean diameter of 18 mm and 2-3 other follicles reached mean diameter of 16 mm.

2.3. FF collection, oocyte pickup and insemination
Approximately 34-36 h after hCG administration, transvaginal ultrasound-guided oocyte retrieval was done. Follicles were aspirated under patient sedation. Oocytes were placed in the insemination medium (Sydney IVF Fertilization Medium; Cook Women's Health, Spencer, IN). After 4-5 h of oocyte retrieval, insemination was done with approximately 1 million/sperms per oocyte.

Also FF was maintained at steady temperature conditions (37° C). Equal volume of FF from each follicle was mixed together to obtain pooled FF and centrifuged at 3000 *g* for 15 min at 4° C to eliminate cellular elements. The clear supernatant was used for estimations. Ascorbic acid estimation was performed immediately and remaining supernatant was frozen at -80° C and kept for analysis of estradiol and dehydroepiandrosterone sulphate (DHEA-S).

2.4. Assessment of fertilization and cleavage
Fertilization was assessed 16 to 18 hours after insemination by the presence of two pronuclei and two polar bodies and the cleavage done by Veeck's gradation system (30).

2.5. Ascorbic Acid Estimation
Ascorbic acid concentration in FF was determined by spectrophotometer at 700 nm using acid phosphotungstate (31).

2.6. Hormone estimation

Evaluation of FF-E2 and DHEA-S of FF was performed by using radioimmunoassay (RIA) diagnostic kits obtained from Diagnostic Systems Laboratories, Texas (USA). Protocols were followed as per manufacturer's instructions provided in the kits. Theoretical sensitivity or lowest detection limits were 4.7 pg/ml and 0.06 ηg/ml respectively.

2.7. Embryo Transfer

After embryo evaluation on day-3, embryo transfer was done andduring the luteal phase, vaginal progesterone pessaries (200 mg NeoGest VHB Life Sciences Limited) were administered starting from day-ET until day-14 of embryo transfer. On day-14 of embryo transfer, serum β-hCG >50 m IU /ml was considered as a positive indicator of pregnancy.

2.8. Clinical pregnancy

Clinical pregnancy was assessed by evaluation of number of gestational sac observed at ultrasound scans at around 7 weeks of amenorrhea.

2.9. Definition of Study Groups

1) Cycles were classified into pregnant and non-pregnant groups depending on pregnancy outcome.
 2) Cycles were randomly sorted into two different groups according to FF Ascorbic acid concentrations. Cutoffs for defining low and high AA concentrations corresponded to the 50th centile/median value of each measurement.
Thus the FF Ascorbic acid groups were divided into:
1) High FF Ascorbic acid (> 12.00 µg /ml) group.
2) Low FF Ascorbic acid (≤ 12.00µg /ml) group.

2.10. Statistical Analysis:

The data was statistically analyzed by using the Graph Pad Prism Version-5.0 statistical software package. Student's 't' test was used to evaluate difference between means and statistical significance. All values are expressed as Mean ± SD unless otherwise specified. Receiver Operating Characteristics Curve was obtained to establish cutoff threshold values. Correlation was performed by using Pearson correlation test. In all cases a P value <0.05 was considered statistically significant.

3. Results

All non-PCOS women undergoing IVF-ET treatment cycles were classified into pregnant and non-pregnant groups according to their pregnancy outcome. It was observed that FF ascorbic acid (FF-AA) in the pregnant group (n=36) as compared to non-pregnant ones (n=69) was significantly higher (16.76 ± 7.87 vs. 9.93 ± 4.69 P = <0.0001).

FF ascorbic acid values were further classified into high and low groups on the basis of median values to emphasize their effect on pregnancy outcome. There were significant rise in follicular fluid DHEA-S (1142 ± 899.3 vs. 732.9 ± 361.7 P=0.0256) and E2 (346276 ± 263555 vs. 160607 ± 136911 P= 0.0003) in high FF-AA group as compared to low FF-AA group (Figure 1).
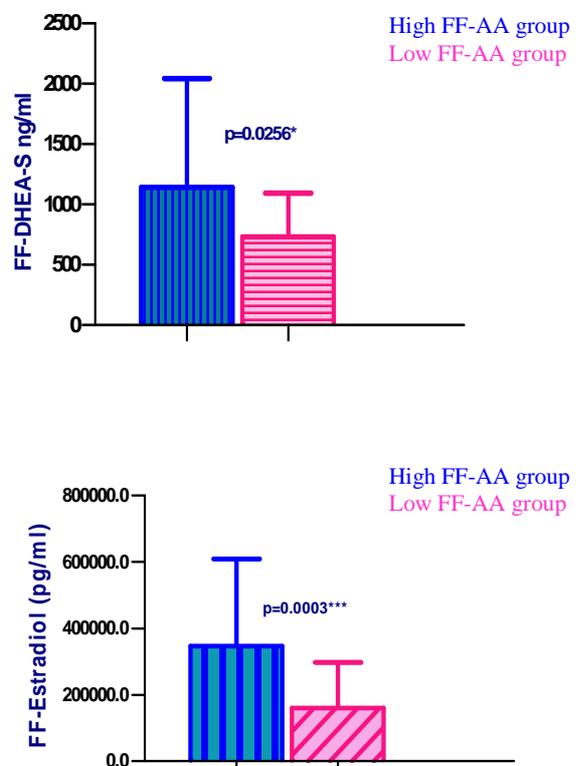

Figure 1. FF-DHEA-S (a) and FF-E2 (b) contents in High vs. Low FF-AA groups.

Table 1 represents comparison of embryology parameters in High vs. Low FF Ascorbic Acid Groups. Although, no significant variations in the number of retrieved oocytes and embryo transfer between the two study groups could be perceived, the high FF-AA group were found to possess significantly higher percentage of in fertilization rate (86.40 ± 19.28 vs. 75.66 ± 21.33 P=0.0206) and cleavage rate (79.22 ±22.87 vs. 66.56 ± 23.39 P=

0.0126) as compared to low FF–AA group. Furthermore, high FF-AA group recorded a higher clinical pregnancy rate (38.60 % vs. 19.57 %) as compared to low FF-AA group.

Table 1. Embryology parameters in High vs. Low FF Ascorbic Acid Groups.

Parameter	High FF-AA (>12.00μg /ml) (n=58)	Low FF-AA (≤12.00μg /ml) (n=47)	P value
Number of retrieved eggs	448	320	
Fertilization Rate (%)	86.40 ± 19.28	75.66 ± 21.33	*
Cleavage Rate (%)	79.22 ± 22.87	66.56 ± 23.39	*
Number of transferred embryo	103	79	ns
Clinical Pregnancy Rate (%)	38.60	19.57	*

All values are mean ± SD. P < 0.05 =significant (*), P < 0.001= Highly significant (**), P < 0.0001 = Extremely significant (***) and P>0.05 represents non significant (ns).

The results of FF ascorbic acid Receiver Operator Characteristics (ROC) curve have been presented in Table 2. The ROC_{AUC} was found to be 75.8 % with a threshold (cut off) value > 11.55μg/ml of FF-AA for pregnancy. A strong correlationship was found between FF-AA and clinical pregnancy rate (Pearson r (95% CI) = 0.48, r2= 0.23, P= < 0.0001) (Table 3).

Table 2. Receiver Operating Characteristic (ROC) curve analysis of FF Ascorbic acid.

ROC Analysis	FF-ascorbic Acid (μg/ml)
Area	0.758
95% confidence interval	0.6603 to 0.8558
Threshold value for pregnancy (μg /ml)	> 11.55
Sensitivity%	70.59
Specificity%	62.50
Likelihood ratio	1.88
P value	0.0001***

Extremely significant (***)=P<0.0001.

Table 3. Correlation of FF–AA with Clinical Pregnancy rate.

Pearson r (95% CI)	0.48 (0.31 to 0.62)
r2	0.23
P value	< 0.0001***

Value in parenthesis represents 95% confidence interval (CI).
P <0.05 (*)= significant.
Pearson correlation was used to draw correlation with pregnancy outcome.
r2= correlation coefficient.

4. Discussion

Exogenous AA supplementation has earlier been reported to result in enhanced serum levels of E2 as well as higher pregnancy rates in both non-IVF (27) and IVF-ET treatment cycles (28), thus denoting the essentiality of AA in pregnancy probably through enhanced E2 production. It was Luck et al (8) who proposed the likely role of AA in hormone production and high levels of follicular fluid estradiol, associated with ovarian response, oocyte protection, and higher fertilization as well as pregnancy rates (32). Infact, DHEA-S, the most abundant steroid present in follicular fluid of preovulatory follicles (33) which was suggested to act as a precursor for estradiol synthesis by granulosa cells (34) was found to be relatively elevated in high FF-AA group as compared to low FF-AA group. Thus the present observation of elevated levels of E2 and DHEA-S in high FF-AA group as compared to low FF-AA group indicates the possible role of endogenous FF-AA in the promotion of FF-E2 probably through enhanced aromatization.

Improvement in embryo development rate probably through antioxidant role of AA from its supplementation within the culture media was reported by several studies earlier (35, 36, 37, 38, 39, 40). The demonstration of higher and significant percentage of fertilization, cleavage and clinical pregnancy rate in high FF-AA group as compared to low FF-AA group observed here may be due to a protective role afforded by AA against cellular injuries to oocyte and granulosa cells. This view is backed by similar propositions made by few studies earlier (8, 9, 21) though these studies were performed by exogenous supplementation of AA. The positive correlationship of FF-AA with clinical pregnancy rate as observed in this study clearly denotes the importance of maintaining the antioxidant status within the microenvironment in order to achieve pregnancy.

It is noteworthy that the present study has not only attempted to establish for the first time a successful correlationship between endogenous FF-AA levels with pregnancy outcome as against the exogenous supplementation theory, but also aided to determine the critical requirement of AA within the follicular milieu for achieving the objective. Thus, the threshold value of FF-AA pregnancy which was found to be >11.55 µg/ml could serve as the possible determinant for successful conception.

Conclusion

The present study for the first time has attempted to show the relevance of FF-AA for pregnancy outcome in IVF-ET cycles of non-PCOS women. It was found that higher levels of FF-AA recorded in pregnant group compared to non-pregnant group corresponded to better quality oocyte as well as higher fertilization, cleavage and pregnancy rates. Simultaneously, elevation of DHEA-S the androgen precursor corresponding to higher AA and E2 obtained in follicular fluid suggests that endogenous AA probably plays an important role in facilitating aromatization process of DHEA-S into E2.

The threshold value of FF-AA for pregnancy outcome was found to be >12.38 µg/ml, which may serve as an important determinant for successful conception for non-PCOS women undergoing IVF-ET treatment cycles.

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