

## Relevance of serum ascorbic acid status in ovulation and pregnancy outcome of non-PCOS women undergoing intrauterine insemination cycles

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### Abstract

Ovulation is an important process for successful conception in intra-uterine insemination (IUI) cycles. The ovulatory process is initiated by an E2 induced LH surge causes a series of dramatic physiological and biochemical alterations in the ovary leading to follicle rupture and oocyte release. Ascorbic acid (AA) has implicated in the process of ovulation and folliculogenesis. Also its altered levels have been correlated to luteal-steroidogenesis. Hence, the study attempts to carry out an inter-phasic comparison of various hormones and AA between the early follicular, periovulatory and luteal-phases in non-PCOS women during IUI-cycle so as to obtain a cogent view about their implications in ovulatory process and subsequent pregnancy-outcome. AA level is found to be at its nadir in the ovulatory phase while LH level is at its zenith. The utilization of AA in the ovulatory phase is seen to hold the key for future course of events i.e. synthesis of 17-OHP and E2, leading to pregnancy. A significant decrease was observed in the serum-AA levels from follicular to ovulatory phase within the pregnant group but not in the non-pregnant ones. Thus, serum ascorbic-acid seems to have a prominent bearing for ovulation and pregnancy outcome in non-PCOS women undergoing IUI-cycles.

**Keywords:** Ascorbic acid, Ovulation, IUI, Pregnancy

### 1. Introduction

In intra uterine insemination (IUI) cycles, ovulation assumes utmost importance for successful conception. The ovulatory process which is initiated by an E2 induced LH surge causes a series of dramatic physiological and biochemical alterations in the ovary leading to follicle rupture and oocyte release (1, 2, 3, 4, 5, 6, 7). Several earlier workers have recognized the importance of estradiol (E2), luteinizing Hormone (LH), progesterone (P) and 17- $\alpha$  hydroxyprogesterone (17-OHP) in the ovulatory process of women undergoing IUI and IVF cycles (2, 8, 9, 10, 11, 12, 13, 14, 15, 16).

Apart from the above stated hormones, ascorbic acid (AA) has also been implicated in the process of

ovulation and folliculogenesis (17). Alterations in the levels of this molecule have been correlated to luteal steroidogenesis (18). In fact, a LH induced regulation of ascorbic acid throughout the ovarian cycle was evidenced by some workers earlier (18, 19). Besides, AA is reported to enhance the effect of clomiphene citrate on ovulation induction of anovulatory women (20). Although, Henmi et al (14) found an enhancement in IUI pregnancy rates upon AA supplementation in women with luteal phase defects, Griesinger et al (21) failed to recognize any significant alteration in pregnancy outcome upon AA uptake of subjects during IVF cycles. Thus, there remains a contradiction regarding the role of exogenous AA on pregnancy outcome.

Despite various implications of AA in reproductive processes, surprisingly none of the earlier IUI studies have attempted to correlate circulating levels of AA with ovulatory process and

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pregnancy outcome. Hence, the present study attempts to carry out an inter-phasic comparison of various hormones and AA between the early follicular, periovulatory and luteal phases in non-PCOS women during IUI cycle so as to obtain a cogent view about their implications in ovulatory process.

## 2. Materials and Methods

### 2.1. Study design

The study included 216 cycles of Intra-Uterine-Insemination (IUI) in non-PCOS women (mean age  $28.25 \pm 4.16$  years, BMI  $23.84 \pm 3.84$ , Waist/Hip ratio  $0.88 \pm 0.07$ ). 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 ovaries visualized in transvaginal ultrasound sonography.
- 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 from last 6 months.
- 3) Spontaneous onset of puberty and sexual development.
- 4) No current or past diseases affecting ovaries or gonadotropins or sex steroid secretion, clearance, or excretion.

#### *Exclusion Criteria*

In order to obtain a homogenous group of women for the study, the following criteria were excluded:

- 1) Women older than 40 years of age.
- 2) Women suffering from bilateral tubal block and Women with polycystic ovary syndrome defined as according to the Rotterdam consensus 2004 (22).
- 3) Women with serum hyperprolactinemia (Serum PRL  $> 21$  ng/ml) and hyper or hypo thyroid (Serum TSH levels  $< 0.3$  or  $> 5.2$   $\mu$ U/ml).
- 4) Women with associated severe male factor infertility.

### 2.2. IUI treatment cycle protocol

#### 2.2.1. Ovulation Induction

Non-PCOS women were selected for the study on the basis of their baseline scan and baseline hormone analysis on day 2 of natural menstrual cycle. The selected women inducted for IUI were subjected to ovulation study by TVS. After confirming the

absence of any cyst; stimulation was started with Letrozole 2.5 mg HS from D3 to D7 added HMG (75 IU) from D8 onwards till at least 1-3 follicle/s attained a size of 17 to 18 mm. TVS monitoring was done daily from D8 onwards, till lead follicles reaches 18 mm diameter, after which injection hCG (5000 IU FertiGyn Sun Pharma India) was administered. Rupture of follicle observed on ultrasound confirmed ovulation.

#### 2.2.2. IUI Procedure

Washed and concentrated sperm sample was inseminated in the intrauterine cavity using 'Gynetics' IUI catheter between 36-48 hours of the hCG injection. Progesterone supplement in the form of soft gelatin capsule (200 mg three times in a day after each 8 hour interval NeoGest VHB Life Sciences Limited) for luteal phase support was given up to day-28 of the cycle. On day-28 of the withdrawal bleeding, serum  $\beta$ -hCG level  $>50$  m IU/ml was considered as a positive indicator of pregnancy.

### 2.3. Clinical pregnancy

Appearance of a fetal sac with functional heart on ultrasound in the sixth or seventh week of gestation was considered as clinical pregnancy.

### 2.4. Hormonal Estimations

Fasting serum sample obtained on different representative days of IUI cycles was estimated for E2 (DSL-4400), P (DSL3900), 17-OHP (DSL-10-6800) and LH (DSL4600) using radioimmunoassay (RIA) and immunoradiometric assay (IRMA) or 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, 0.12 ng/ml, 0.0346 ng/ml, 0.12 mIU/ml respectively. Ascorbic acid concentration in serum was determined by spectrophotometer at 700 nm using acid phosphotungstate (23).

#### *Definition of Study Groups*

Cycles were classified into Pregnant and Non-Pregnant groups depending on pregnancy outcome.

### 2.5. Statistical Analysis

The Data was statistically analyzed for study 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  $\pm$  SD unless otherwise specified.

In all cases a p-value <0.05 was considered statistically significant. Statistical significance between the three phases was determined by one-way analysis of variance. P < 0.05 was considered to be statistically significant.

### 3. Results

Intercomparison on the contents of AA and certain hormones between pregnant and non-pregnant non-PCOS subjects at varied phases of menstrual cycle in IUI cycle i.e. early follicular phase (day-2), periovulatory (day of hCG) and luteal phase (day-21) have been presented here.

It may be seen that there exists almost no alterations in the contents of serum ascorbic acid, E2, LH, P and 17-OHP between the two study groups at Day-2 (Table 1).

Table 1. Baseline (day-2) serum levels of ascorbic acid and hormones in pregnant and non pregnant groups

Parameter	Pregnant (44)	Non-Pregnant (172)	P value
Ascorbic Acid (µg/ml)	5.48 ± 1.57	5.76 ± 1.51	ns
Estradiol (pg/ml)	43.06 ± 18.95	48.18 ± 21.63	ns
LH (mIU/ml)	4.25 ± 1.88	4.47 ± 1.83	ns
Progesterone (ng/ml)	1.06 ± 0.18	1.12 ± 0.28	ns
17- OHP (ng/ml)	1.01 ± 0.17	1.04 ± 0.18	ns

All values are mean ± SD. Values in parenthesis indicate number of subjects in each group. P value obtained using Student's 't' test. p>0.05 represents non significant (ns).

Table 2 represents intercomparison of serum ascorbic acid and various hormones between the two study groups at day of hCG (periovulatory phase). Although significant reduction in the levels of ascorbic acid and progesterone is expressed in the pregnant subjects as compared to non pregnant ones (AA: 4.64 ± 1.43 vs. 5.58 ± 1.51 p = 0.0017 and P: 1.72 ± 1.06 vs. 2.62 ± 0.87 p = 0.0103 respectively), insignificant variations are observed in the levels of E2, LH and 17-OHP between the two groups under study. Comparison of Day-21 levels of serum ascorbic acid and hormones between pregnant and non-pregnant groups is depicted in Table 3.

Although, the level of AA is found to be significantly lesser in pregnant group (5.71 ± 2.50 vs. 6.94 ± 2.45 p = 0.0213). The pregnant group exhibits significant enhancement in the levels of both E2 (316.4 ± 113.2 vs. 213.1 ± 75.63 p = 0.001) and 17- α hydroxyprogesterone (5.32 ± 1.23 vs. 3.15 ± 1.24 p = <0.0001) as compared to non-pregnant group. However, the level of progesterone remains comparable between the groups.

Table 2. Serum ascorbic acid and hormone levels at day of hCG administration in pregnant and non-pregnant groups

Parameter	Pregnant (44)	Non-Pregnant (172)	P value
Ascorbic Acid (µg/ml)	4.64 ± 1.43	5.58 ± 1.51	**
Progesterone (ng/ml)	1.72 ± 1.06	2.62 ± 0.87	*
Estradiol (pg/ml)	419.3 ± 197.2	402.3 ± 180.1	ns
LH (mIU/ml)	15.08 ± 12.68	14.68 ± 13.14	ns
17- OHP (ng/ml)	2.00 ± 0.65	2.29 ± 0.99	ns

All values are mean ± SD. Values in parenthesis indicate number of subjects in each group. P value obtained using Student's 't' test. P < 0.05= significant (\*), P < 0.0001= Extremely significant (\*\*\*) and p>0.05= non significant (ns).

Table 3. Serum ascorbic acid and hormone levels at day-21 in pregnant and non-pregnant groups

Parameter	Pregnant (44)	Non-Pregnant (172)	P value
Ascorbic Acid (µg/ml)	5.71 ± 2.50	6.94 ± 2.45	*
Estradiol (pg/ml)	316.4 ± 113.2	213.1 ± 75.63	**
Progesterone (ng/ml)	44.52 ± 17.07	37.11 ± 14.60	ns
17-OHP (ng/ml)	5.32 ± 1.23	3.15 ± 1.24	***

All values are mean ± SD. Values in parenthesis indicate number of subjects in each group. P value obtained using Student's 't' test. P < 0.05 = significant (\*), P < 0.001= Highly significant (\*\*), p< 0.0001 = Extremely significant (\*\*\*) and p>0.05 represents non significant (ns).

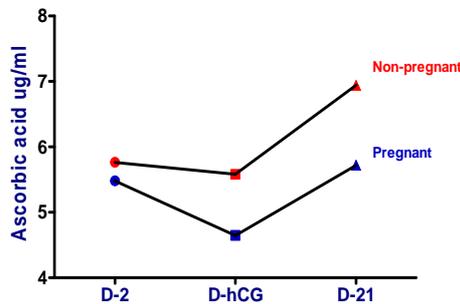


Figure 1. Intercomparison of serum ascorbic acid between pregnant and non-pregnant groups at different phases of menstrual cycle in IUI treatment cycles.

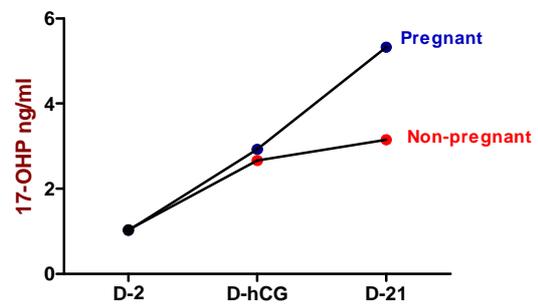


Figure 4. Intercomparison of serum 17- $\alpha$  Hydroxyprogesterone between pregnant and non-pregnant groups at different phases of menstrual cycle in IUI treatment cycles.

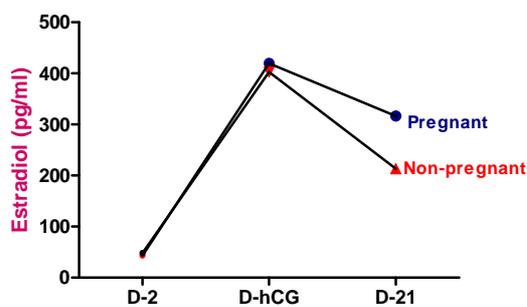


Figure 2. Intercomparison of serum estradiol between pregnant and non-pregnant groups at different phases of menstrual cycle in IUI treatment cycles.

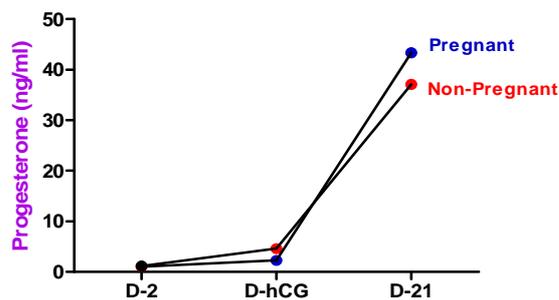


Figure 3. Intercomparison of serum progesterone between pregnant and non-pregnant groups at different phases of menstrual cycle in IUI treatment cycles.

#### 4. Discussion

This is the first ever study that has attempted to correlate pregnancy outcome in non-PCOS women with serum AA at varied phases of menstrual cycle in IUI cycles. A general elevation in day-2 serum AA contents observed in both pregnant and non-pregnant groups denotes that elevated AA through its antioxidant behaviour probably aids folliculogenesis and imparts a protective role against follicular apoptosis as envisaged by certain workers earlier (24, 25).

Depletion of ovarian AA in rats, guinea pigs and cows at periovulatory stage is well recognized (26, 27, 28, 29, 30, 31, 32, 33). Although LH mediated follicular depletion of AA at preovulatory phase is recorded in humans (19, 26, 34), the exact explanation for such depletion is still forthcoming. LH induced superoxide radicals in ovary (35) may cause decrease in AA. Indeed, the peak value of LH concurrent to decrease in serum AA on day of hCG of non-PCOS subjects presented here clearly lends support to the contention that probably LH have direct control on AA depletion at the time of ovulation.

The observation of a decline in serum AA recorded in both the study groups at periovulatory phase indicates an excessive ovarian consumption of AA in order to encounter reactive oxygen species induced by LH surge and thus aid follicular maturation. Interestingly however the decline pattern of AA from follicular to ovulatory phase was found to exist predominantly among the pregnant group as compared to the non-pregnant counterpart. This denotes that pregnancy outcome depends on the dynamic status of AA and conceivably excess

utilization of AA at periovulatory phase is an indicator for successful conception.

Observation of a proportional raise in serum AA during the transition from periovulatory to luteal phase irrespective of pregnancy outcome suggests that further flux in AA occurs. However, on comparison of AA contents at luteal phase between the pregnant and non-pregnant groups revealed that the contents were found to be relatively lower in the pregnant group as compared to non-pregnant group indicating an increased utilization of AA at luteal phase as a prerequisite for pregnancy.

The observed elevation in serum E2 level during the time of both follicular maturation and ovulation (d-hCG) lends support to the earlier contention that peak E2 level prior to ovulation causes a positive feedback for LH surge (1, 2). The observation of significant decline in serum E2 level concurrently with raise in AA levels between the ovulatory and luteal phase in all the women shows that there exists an inverse relationship between the two important components at these phases. However, on comparison of their contents between the study-groups revealed that in the pregnant group as compared to non-pregnant group, E2 levels were significantly higher and AA levels significantly lower at mid luteal phase. While, raised AA may facilitate E2 production and favor pregnancy, levels over and above its threshold value might prove futile.

The profile of serum progesterone from periovulatory to mid luteal phase in women has been shown to register a gradual hike (36). The present work indicates that in all non-PCOS women irrespective of pregnancy outcome replicated a similar pattern of progesterone alteration most probably due to the use of intravaginal progesterone support as a precautionary measure against luteal phase insufficiency. It thus becomes apparent that progesterone is probably a non starter as far as pregnancy outcome in IUI cycles is concerned. However, its biochemical conversion to its hydroxylated product (17-OHP) may alternatively be considered as a possible determinant for the prediction of pregnancy outcome. The present evidence of a relative elevation in the levels of 17-OHP at luteal phase in pregnant group as compared to non-pregnant group certainly suggests its importance in defining fertility and is in consonance with earlier observations where probable role of 17-OHP in the progression of pregnancy was proposed (14, 37, 38, 39, 40).

The present study also demonstrates the existence of a predominantly higher utilization of AA in pregnant group which corresponded to elevation in

17-OHP at luteal phase. Thus vindicates that AA possesses a vital role in pregnancy-outcome probably through its ability to hydroxylate progesterone (41, 42).

Although, Sanyal and Datta (43) found direct decrease in aromatase activity at high ascorbic acid concentrations, Henmi et al (14) obtained enhancement in E2 as a response to AA supplementation. This conflicting account on the effect of AA on aromatase activity provokes future examination, especially to establish its optimum level for both steroidogenesis and establishment of pregnancy.

This is the first ever study that attempts to obtain a coherent view regarding relationship of serum AA with the entire factors involved in sequential events of menstruation cycle namely from folliculogenesis to luteinization and establishment of pregnancy. Whereas AA level is found to be at its nadir in the ovulatory phase, LH level is at its zenith in this phase. The utilization of AA in the ovulatory phase is seen to hold the key for future course of events i.e. synthesis of 17-OHP and E2, leading to pregnancy. Thus, serum ascorbic acid seems to have a prominent bearing for ovulation and pregnancy outcome in non-PCOS women undergoing IUI cycles.

### Conclusion

The study for the first time presents the relevance of serum AA at early follicular, periovulatory and luteal phase of menstrual cycle in IUI cycles in non-PCOS women. A significant decrease was observed in the serum AA levels from follicular to ovulatory phase within the pregnant group but not in the non-pregnant ones. The decreased status of serum AA at periovulatory phase possibly indicates an excess utilization of AA for successful conception. In luteal phase, increased serum levels of AA were recorded in these subjects implicating its role in the facilitation of hydroxylation, aromatization and luteal steroidogenesis necessary for effective conception. This is further exemplified by the finding of higher utilization of AA in pregnant women as compared to non-pregnant ones at periovulatory phase that holds the key for luteal phase events and subsequent pregnancy outcome in IUI treatment cycles.

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