

# Follicular fluid dehydroepiandrosterone sulfate is a credible marker of oocyte maturity and pregnancy outcome in conventional *in vitro* fertilization cycles

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## ABSTRACT

**AIM:** To investigate if the level of dehydroepiandrosterone sulfate (DHEA-s) in follicular fluid (FF) influences the competence of oocytes to fertilize, develop to the blastocyst stage, and produce a viable pregnancy in conventional *in vitro* fertilization (IVF) cycles. **SETTINGS AND DESIGN:** Prospective study of age-matched, nonpolycystic ovary syndrome (PCOS) women undergoing antagonist stimulation protocol involving conventional insemination and day 5 blastocyst transfer. **MATERIALS AND METHODS:** FF levels of DHEA-s and E2 were measured by a radio-immuno-assay method using diagnostic kits. Fertilization rate, embryo development to the blastocyst stage and live birth rate were main outcome measures. Cycles were divided into pregnant/nonpregnant groups and also into low/medium/high FF DHEA-s groups. Statistical analysis was done by GraphPad Prism V software. **RESULTS:** FF DHEA-s levels were significantly higher in pregnant ( $n = 111$ ) compared to nonpregnant ( $n = 381$ ) group ( $1599 \pm 77.45$  vs.  $1372 \pm 40.47$  ng/ml;  $P = 0.01$ ). High ( $n = 134$ ) FF DHEA-s group had significantly higher percentage of metaphase II (MII) oocytes ( $91.5$  vs.  $85.54$  vs.  $79.44\%$ ,  $P < 0.0001$ ), fertilization rate ( $78.86$  vs.  $74.16$  vs.  $71.26\%$ ,  $P < 0.0001$ ), cleavage rate ( $83.67$  vs.  $69.1$  vs.  $66.17\%$ ,  $P = 0.0002$ ), blastocyst formation rate ( $37.15$  vs.  $33.01$  vs.  $26.95\%$ ,  $P < 0.0001$ ), and live birth rate ( $29.85$  vs.  $22.22$  vs.  $14.78\%$ ,  $P = 0.017$ ) compared to medium ( $n = 243$ ) and low ( $n = 115$ ) FF DHEA-s groups, respectively despite comparable number of oocytes retrieved and number of blastocysts transferred. FF DHEA-s levels correlated significantly with the attainment of MII oocytes (Pearson  $r = 0.41$ ) and fertilization rates (Pearson  $r = 0.35$ ). **CONCLUSION:** FF DHEA-s level influences the oocyte maturation process and is predictive of fertilization, embryo development to the blastocyst stage and live birth rates in non-PCOS women undergoing conventional IVF cycles.

**KEY WORDS:** Blastocyst, dehydroepiandrosterone sulfate, fertilization, *in vitro* fertilization, pregnancy, oocyte

## INTRODUCTION

*In vitro* fertilization (IVF) involves retrieval of oocytes by super-ovulation which is achieved by exogenously supplementing follicle stimulating hormone (FSH) and stimulating the ovaries to produce multiple follicles. Injection human chorionic gonadotropin (hCG) is administered to attain final oocyte maturation. Several pathways have been postulated for the gonadotropin-induced meiotic maturation;<sup>[1-4]</sup> all of which ultimately function to enhance synthesis of stimulatory factor (s) for induction of nuclear and cytoplasmic maturation of oocytes.<sup>[5]</sup>

Involvement of steroids such as estradiol and testosterone in the regulation of meiotic maturation in conjunction with gonadotropins, although contemplated, still remains unclear.<sup>[6]</sup> An earlier study had

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cited that oocyte priming with estrogen is necessary to develop  $\text{Ca}^{2+}$  oscillations during maturation.<sup>[7]</sup> Based on the corollary, that oocytes are particularly sensitive to steroids while still at the germinal vesicle stage; it was speculated that any imbalance in the estrogen-to-androgen ratios *in vivo* may cause an abnormal  $\text{Ca}^{2+}$  response thus affecting fertilization.<sup>[7]</sup>

Dehydroepiandrosterone (DHEA), the most abundant circulating steroid hormone in humans,<sup>[8]</sup> may be presumed to play an immensely significant role in this context. Since DHEA is converted to testosterone in ovarian connective tissue (theca/stroma) and is subsequently processed by the granulosa cells to form estradiol, it assumes the prohormone status of a predominant endogenous precursor and a metabolic intermediate in ovarian follicular steroidogenesis.<sup>[9,10]</sup> DHEA may thus be considered a key molecule at the crossroads, maintaining a critical balance between androgen and estrogen production on one hand and affecting oocyte maturation on the other. However, earlier studies with serum/follicular fluid (FF) DHEA levels in nonpolycystic ovary syndrome (PCOS) women have provided contradictory results.<sup>[11,12]</sup>

DHEA is reversibly catalyzed by sulfotransferase (SULT2A1) to its sulfate ester DHEA sulfate (DHEA-s). Most DHEA in blood is found as DHEA-s with levels that are about 300 times higher than those of free DHEA. Whereas DHEA levels naturally reach their peak in the early morning hours, DHEA-s levels show no diurnal variation. Thus from a practical point of view, measurement of DHEA-s seems more preferable than DHEA since its level remains stable. DHEA-s is also the most abundant steroid in follicular fluid of preovulatory follicles being present at micromolar levels.<sup>[13]</sup> Although a few *in vitro* studies indicate the utilization of DHEA-s by granulosa cells via sulfatase for androgen and estrogen production,<sup>[14,15]</sup> no study has as yet been able to obtain a correlation of FF DHEA-s levels with fertilization and pregnancy outcome.

Hence, we attempted to investigate if levels of FF DHEA-s correlate with the retrieval of mature oocytes possessing the competence to fertilize, effect early embryonic development, and produce a viable pregnancy in non-PCOS women undergoing IVF cycles.

## MATERIALS AND METHODS

A prospective cohort study of women undergoing IVF cycles ( $n = 521$ ) was carried out at our academically affiliated private infertility clinic from January 2011 to April 2014. Informed consent was obtained from all individual participants included in this study. The study protocol was approved by our own Hospital Ethical Committee. No

approval from any other ethical committee was required since the procedures did not involve animals or any invasive techniques in humans. All estimations were carried out in FF, which is easily obtained during ovum pick-up (OPU), a basic step of IVF treatment; or in serum samples obtained from blood collected by simple venipuncture.

DHEA-s levels were estimated in pooled FF by a radio-immuno-assay (RIA) method using diagnostic kits obtained from Beckman Coulter (Immunotech IM0729, Analytical/functional sensitivity 2.64  $\mu\text{g}/\text{dL}$ , the intra-assay and interassay coefficient of variations  $\leq 4.93\%$  and  $\leq 9.32\%$ , respectively). Correspondingly, FF E2 levels were also measured using Beckman Coulter Immunotech RIA kit (A21854, Analytical/functional sensitivity 9.58/13.11  $\text{pg}/\text{ml}$ , the intra-assay and interassay coefficient of variations 14.4% and 14.5%, respectively). For assay purposes, only the original follicular aspirate was collected (even if oocyte was retrieved in the flush) from all follicles  $>16$  mm in size and from which oocyte had been retrieved. For each patient per cycle, FF so obtained from all follicles was mixed in equal volume and centrifuged at 3000 g for 15 min to remove blood contaminant/cellular elements and the clear supernatant was used for further assay and analysis.

All women were endocrinologically normal, non-PCOS and age-matched (age range 25–35 years) normal responders (based on their baseline serum AMH levels and/or antral follicle count). Cycles, where intra-cytoplasmic sperm injection had to be performed due to severe male factor, were excluded so as to avoid any bias in fertilization outcome resulting from compromised sperm quality. Although FF DHEA-s levels were measured in total fertilization failure (TFF) cycles ( $n = 29$ ), these cycles were obviously not included while comparing pregnancy rates. Therefore, clinical pregnancy and live birth rates were analyzed in  $n = 492$  cycles.

All cycles involved standard stimulation protocol with recombinant FSH (Gonal F, Merck-Serono, India) from day 2 and daily administration of antagonist from day 6 until the day of the trigger with injection hCG. Transvaginal ultrasound-guided oocyte retrieval under patient sedation was done between 34 and 36 h after hCG administration. Conventional insemination was done after oocyte incubation, and morphological assessment of oocyte quality was based on the appearance of COC complex. 16–18 h postinsemination, the metaphase II (MII) status of oocytes was ascertained and fertilization rate assessed. On day 1 post-OPU, the presence of one extruded polar body indicated MII status of oocyte whereas the presence of two polar bodies and two pronuclei confirmed fertilization.

Development of embryo was monitored until the formation of blastocysts on day 5. Blastocyst evaluation was done as per Gardner's classification.<sup>[16]</sup> Embryo transfer (ET) involved transcervical transfer of not more than 2 top/good quality day 5 blastocysts using Cooks ET catheter. Post transfer, the luteal phase was supported by the administration of micronized progesterone 100 mg daily (Susten, Sun Pharma, India) until day 14 of ET. Serum  $\beta$ -hCG concentration >50 mIU/mL recorded on day 14 of ET was considered as a positive indicator of pregnancy. Clinical pregnancy was confirmed by the presence of gestational sac with positive cardiac activity.

Inter-comparison of various parameters were performed between pregnant ( $n=111$ ) and nonpregnant groups ( $n=381$ ) excluding the TFF cycles. The patients were then classified into low (<760 ng/ml), medium (760–1850 ng/ml), and high (>1850 ng/ml) FF DHEA-s groups on the basis of 25<sup>th</sup> and 75<sup>th</sup> percentiles of FF DHEA-s concentration. Fertilization rate, embryo development to blastocyst stage and live-birth rate were the main outcome measures. Statistical analysis was done using GraphPad Prism V software (Infact, Graph Pad Software is the company). Chi-square test, Student's *t*-test, one-way ANOVA, Pearson correlation and odds ratio (OR) were calculated as applicable to obtain statistical significance. Values are expressed as  $\pm$  standard error of the mean.  $P < 0.05$  was considered statistically significant.

## RESULTS

All patients included in the study were age matched and did not differ significantly w.r.t age either in pregnant versus nonpregnant ( $30.21 \pm 0.27$  vs.  $30.80 \pm 0.17$  years;  $P = 0.09$ ) or in low, medium and high groups ( $31.44 \pm 0.28$  vs.  $31.0 \pm 0.2$  vs.  $30.73 \pm 0.3$ ,  $P = 0.10$ ). Similarly, all women were non-PCOS with insignificant differences in their baseline serum AMH levels ( $2.83 \pm 0.24$  vs.  $2.61 \pm 0.19$  ng/ml;  $P = 0.12$ ). Overall live birth rate achieved was 22.56% (111/492). Inter-comparison of the basic and embryological parameters between pregnant and nonpregnant groups is depicted in Table 1. Significantly higher levels of FF DHEA-s ( $1599 \pm 77.45$  vs.  $1372 \pm 40.47$  ng/ml;  $P = 0.01$ ) and FF E2 ( $244,500 \pm 54,090$  vs.  $105,500 \pm 11,490$  pg/ml;  $P = 0.016$ ) were observed in pregnant as compared to non pregnant group. Interestingly, cycles

involving TFF evinced significantly lower levels of FF DHEA-s as compared to remaining cycles in which fertilization occurred ( $686.7 \pm 52.76$  vs.  $1423 \pm 36.10$  ng/ml;  $P < 0.0001$ ). These findings prompted us to further classify the cycles depending upon 25<sup>th</sup> and 75<sup>th</sup> percentile value into low, medium, and high FF DHEA-s groups. We analyzed the data within these three groups after including as well as excluding the TFF cycles. On including the TFF cycles, although low ( $n = 131$ ), medium ( $n = 256$ ), and high ( $n = 134$ ) groups were similar in terms of age ( $31.44 \pm 0.28$  vs.  $31 \pm 0.2$  vs.  $30.73 \pm 0.30$ ,  $P = 0.10$ ), day of hCG E2 levels ( $1880 \pm 63.45$  vs.  $2160 \pm 72.38$  vs.  $2320 \pm 77.58$ ,  $P = 0.32$ ) and number of eggs retrieved (1112 vs. 2550 vs. 1424,  $P = 0.55$ ); they displayed highly significant differences with respect to number/percent of MII oocytes (799 [71.85%] vs. 2115 [82.94%] vs. 1303 [91.50%],  $P = 0.0003$ ), number of oocytes fertilized/percent (714 [64.21%] vs. 1831 [71.80%] vs. 1123 [78.86%],  $P < 0.0001$ ), number of embryos cleaved/percent (663 [59.62%] vs. 1706 [66.90%] vs. 1049 [73.67%],  $P = 0.0039$ ), and number of blastocysts formed/percent (270 [24.28%] vs. 815 [31.96%] vs. 529 [37.15%],  $P < 0.0001$ ), respectively. Number of TFF cycles were significantly higher in the low group compared to medium and high FF DHEA-s groups (16 [12.21%] vs. 13 [5.08%] vs. 0 [0%]  $P = 0.0002$ ).

After excluding the TFF cycles, there was a highly significant rise in embryo development rates from low ( $n = 115$ ) to medium ( $n = 243$ ) to high ( $n = 134$ ) FF DHEA-s groups [Table 2]. FF DHEA-s levels correlated significantly with percentage of MII oocytes (Pearson  $r = 0.41$  [95% confidence interval (CI): 0.34–0.48,  $P < 0.0001$ ]) and fertilization rates (Pearson  $r = 0.35$  [95% CI: 0.27–0.42,  $P < 0.0001$ ]). OR and relative risk (RR) of oocytes remaining unfertilized is presented in Figure 1. Clinical pregnancy and live birth rates were significantly higher in the high group as compared to both medium and low groups [Figure 2].

## DISCUSSION

Our study for the first time evaluates FF DHEA-s levels and emphasizes its significance in the process of oocyte maturation and its competence to fertilize. A Higher fraction of MII oocytes and subsequently higher fertilization and

**Table 1: Inter-comparison of various parameters between pregnant and nonpregnant groups**

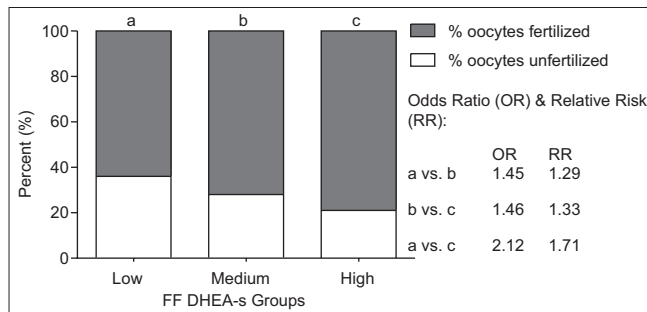
Parameter	Pregnant ( $n=111$ )	Nonpregnant ( $n=381$ )	<i>P</i>
Day of hCG E2 (pg/ml)	2380 $\pm$ 88.92	2000 $\pm$ 57.68	0.31
Number of eggs retrieved	1211	3684	0.24
Number of eggs fertilized (%)	988/1211 (81.58)	2680/3684 (72.75)	0.02
Number of cleaved embryos (%)	935/1211 (77.21)	2483/3684 (67.4)	0.007
Number of blastocysts formed (%)	479/1211 (39.55)	1135/3684 (30.81)	<0.0001
Mean number of d5 blastocysts transferred	1.91 $\pm$ 0.07	1.82 $\pm$ 0.05	0.11

hCG= Human chorionic gonadotropin

**Table 2: Inter-comparison of data between low, medium and high FF DHEA-s groups**

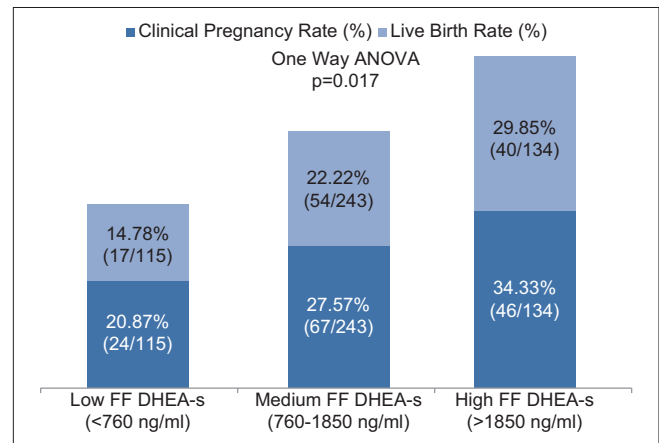
Parameter	Low FF DHEA-s (<760 ng/ml) (n=115)	Medium FF DHEA-s (760-1850 ng/ml) (n=243)	High FF DHEA-s (>1850 ng/ml) (n=134)	One-way ANOVA (Kruskal-Wallis test) <i>P</i>
Age (years)	31.36±0.29	30.93±0.21	30.73±0.30	0.18
day of hCG E2 (pg/ml)	1950±66.31	2100±75.43	2320±77.58	0.29
FF E2 (pg/ml)	80193±6819	102777±6012	186713±20709	0.01
Number of eggs retrieved	1002	2469	1424	0.64
Number of MII oocytes (%)	796 (79.44)	2112 (85.54)	1303 (91.5)	<0.0001
Number of eggs fertilized (%)	714/1002 (71.26)	1831/2469 (74.16)	1123/1424 (78.86)	<0.0001
Number of embryos cleaved (%)	663/1002 (66.17)	1706/2469 (69.1)	1049/1424 (73.67)	0.0002
Number of blastocysts formed (%)	270/1002 (26.95)	815/2469 (33.01)	529/1424 (37.15)	<0.0001
Mean number of d5 blastocysts transferred	1.82±0.04	1.85±0.07	1.90±0.05	0.23

hCG= Human chorionic gonadotropin, FF= Follicular fluid, MII= Metaphase II, DHEA-s= Dehydroepiandrosterone sulfate

**Figure 1:** Odds ratio and relative risk in low, medium, high follicular fluid dehydroepiandrosterone sulfate groups

embryo developmental rates in the high FF DHEA-s group over the other two groups obtained in our study justifies our contention. Although sulfonated steroids have been predominantly regarded to function as inactive storage reservoirs for steroid hormones,<sup>[17-20]</sup> it may be speculated that the sulfate group of DHEA-s plays a role in this maturation process. Indeed, in a recent study Neunzig and Bernhardt, 2014,<sup>[21]</sup> noted that DHEA-s stringently regulates the first step of steroidogenesis and surmised that the negatively charged sulfate group of DHEA-s upregulates the catalytic activity of enzyme CYP11A1 by 75% which binds with higher efficiency to substrate cholesterol to display a 26% increase in pregnenolone formation in presence of DHEA-s but not in presence of DHEA.<sup>[21]</sup> Earlier, Liu and Dillon, 2002<sup>[22]</sup> had identified a putative membrane receptor for DHEA/DHEA-s suggesting that they may possess some physiological role. These studies and our own results indicate that the contribution of DHEA-s in the process may be more substantial than DHEA. Therefore, our initial contention that DHEA-s may bear a more significant role than just being a reservoir for DHEA molecule holds substance. This may probably also be the reason why previous studies obtained contradictory results with DHEA.

Our results demonstrate a concomitant raised FF E2 level along with higher FF DHEA-s levels in the pregnant and

**Figure 2:** Clinical pregnancy and live birth rates in low, medium, and high follicular fluid dehydroepiandrosterone sulfate groups

high groups as against lower levels observed in the non pregnant and medium/low FF DHEA-s groups. This result corroborates the relevant role of E2 in the fertilization process and in stipulating endometrial receptivity.<sup>[23-26]</sup> At the same time, it may be noted that our results attribute a more significant role for DHEA-s than E2 in this process. Our findings display a significantly higher fertilization rate and subsequent enhancement in embryo development rates up to the blastocyst stage in the high FF DHEA-s group. Most importantly, our study denotes that higher fertilization rates correlate more significantly with FF DHEA-s (Pearson  $r = 0.35$ ,  $P < 0.0001$ ) than with FF E2 (Pearson  $r = 0.12$ ,  $P = 0.03$ ). These results emphasize that DHEA-s may act as the primary regulator of estrogen formation. Indeed, there are reports and pathways indicating direct utilization of DHEA-s as a precursor for estrogen production.<sup>[15,27]</sup> Estrogens in turn, enhance the oscillation frequency of calcium (Ca) spikes.<sup>[28]</sup> It has earlier been documented that an abnormal  $Ca^{2+}$  response during fertilization consequently impairs developmental potential of the resulting embryo.<sup>[7]</sup> DHEA-s, owing to its conversion to estriol seems to aid in the generation of  $Ca^{2+}$  wave necessary for oocyte maturation.<sup>[7]</sup> This study,

therefore, marks DHEA-s as a primary entity in the overall process of oocyte maturation.

The exact mechanism by which DHEA-s initiates this process still needs to be elucidated. Nevertheless, a strong correlation of FF DHEA-s levels with the attainment of MII stage oocytes as well as the significantly lower odds/RR of fertilization failure [Figure 1] with high FF DHEA-s levels obtained in our study validate its involvement in this process. Finally, significantly higher embryo development rates presently observed not just at cleavage stage, but also at blastocyst stage leading to significantly higher live birth rates in the high FF DHEA-s group establishes FF DHEA-s as a robust marker for oocyte competence.

## CONCLUSION

FF DHEA-s level influences the oocyte maturation process and is predictive of fertilization, embryo development to the blastocyst stage and live birth rates in non-PCOS women undergoing conventional IVF cycles.

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## Conflicts of interest

There are no conflicts of interest.

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