Original article

PLASMA AND FOLLICULAR FLUID LEVELS OF HIGH SENSITIVITY C REACTIVE PROTEIN AS PREDICTORS OF IMPLANTATION OUTCOME IN IN-VITRO FERTILIZATION TREATMENT. A PROSPECTIVE COHORT STUDY IN ABUJA, NIGERIA

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Abstract:

Context: *In-vitro* fertilization (IVF) treatment has achieved the highest pregnancy rates for infertile couples than any other fertility treatment option. However success rates from IVF procedures are still low at 11-46%. Implantation failure is the commonest cause of IVF failure. The role of inflammatory mediators in determining implantation outcome and use of follicular fluid as markers of oocyte quality are currently being investigated into.

Aims: To investigate the relationship between plasma and follicular fluid levels of high sensitivity C - reactive protein (hs-CRP), and implantation outcome.

Settings and Design: A prospective cohort study consisting of 150 women undergoing IVF treatment at the National Hospital, Abuja.

Methods and Material: Plasma levels of hs-CRP were measured at down regulation. Follicular fluid (FF) levels of hs-CRP were also measured during oocyte retrieval and results compared with plasma levels of β-hCG measured on the 14th day post embryo transfer.

Statistical analysis was done using the Statistical Package for Social Sciences (Chicago, Illinois, USA) version 20.

Results: Median plasma hs-CRP level was 6.00mg/L (IQR=6.81) in the study population, with values of 7.48mg/L (IQR = 7.50) and 3.62mg/L (IQR = 7.17) in subjects with negative and positive implantation outcome respectively.

The *p–value* =0.259, an insignificant difference. Median FF hs-CRP was 3.69mg/L (IQR = 5.18) in the study population, 4.72mg/L (IQR = 5.18) and 2.25mg/L (IQR = 5.01) in subjects with a negative implantation outcome, and positive implantation outcome respectively. This difference was statistically significant with a *p-value* of 0.035.

Conclusions: Higher FF level of hs-CRP 72mg/L (IQR = 5.18) was found in subjects with a negative implantation outcome and Lower FF level of hs-CRP 2.25mg/L in subjects with a negative implantation outcome, *p- value* of 0.035 and statistically significant.

Key-words: Inflammatory mediators, IVF Outcome predictors, Follicular Fluid, high sensitivity, C reactive protein, Implantation outcome.

Introduction:

The world has witnessed a marked increase in patients presenting to infertility clinics in the past three decades [1]. In Nigeria, the prevalence of infertility is about 15% with approximately 12 million couples facing infertility related problems. [2, 3] Societal pressure is great on an infertile couple with several misconceptions as to the cause and treatment of infertility.[4,5] A local study recently documented the overwhelming importance of childbearing and the suffering caused by infertility in African societies, leading to a wide range of psycho social consequences, loss of marital stability, loss of social status and isolation, problems with gender identity, loss of continuity of family lines and general emotional distress.[6]

The World Health Organization (WHO) and International Committee for Monitoring Assisted Reproductive Technology (ICMART), defined infertility clinically, as a disease of the reproductive system characterized by a failure to achieve a successful pregnancy after twelve months or more of regular unprotected sexual intercourse.[7]

In-vitro fertilization (IVF) is the recommended treatment of choice for unresolved infertility, and it offers the highest pregnancy rate irrespective of the cause of infertility.[8] The first

successful IVF procedure was documented in 1978, this treatment option has recently become widely available in Nigeria, being offered in Private and Government owned health facilities.[9] Pregnancy rates from IVF procedures have however remained low ranging from 11-46% worlwide.[10] A recent study in Abuja, showed a clinical pregnancy rate of 9.4 - 69.4 % in women aged 26 - 43 years that had IVF treatment.[11] Irrespective of the IVF treatment protocol, the commonest cause of failure in IVF is implantation failure.[10] Implantation is a precisely synchronized series of molecular interactions that result in the attachment of a blastocyst to the uterine epithelium.[10] [10] For implantation to occur, a competent semi-allogenic blastocyst breaches a receptive maternal endometrium and invades the underlying stroma.[12]

The process is not completely understood but has been shown to be associated with elevations in endometrial cytokines, prostaglandins, and leukocytes.[13] A potent inflammatory response of T helper (Th1) and production of inflammatory cytokines such as interleukin (IL) 6, IL8, is also necessary for the initiation and maintenance of implantation and pregnancy.[14] Maintenance of a crucial albeit delicate balance between Th1 and Th2 activities is essential for a successful implantation.[15] An abnormal increase in the Th1/Th2 ratio could result in implantation disorders and failure. [15]

C-reactive protein (CRP) is synthesized by the liver and serves as an early marker of inflammation or infection it belongs to the family of pentraxins and exists in different isoforms, the most prominent of which are the native pentameric CRP (pCRP) and monomeric CRP (mCRP).[12, 16] The pCRP possesses both pro-inflammatory and anti-inflammatory properties while mCRP exerts potent pro-inflammatory actions and may amplify the inflammatory response [16]

Maternal age, obesity and smoking are factors significantly associated with increasing levels of CRP, and are also known to negatively influence implantation.[17] High sensitivity C reactive protein (hs-CRP) assays are basically

nephelometric measurements that allow for detection of smaller quantities of CRP in specimen than conventional CRP assays.

Recent studies have support that controlled ovarian hyperstimulation (COH) can increase plasma CRP concentration [18]

]. Noteworthy is the finding of higher pregnancy rates in women with elevated CRP levels on the transfer day compared with women who had decreased CRP levels [19]

pregnancy occurred in 68.4% of women who had an elevated CRP level on the day of ET compared with OPU day; therefore, serum levels of CRP on the day of ET has been suggested as a biomarker for success in IVF/ICSI patients [19]. Chun-Xia et al.

have also shown that serum levels of CRP on the day of ET were significantly higher in women who became pregnant compared with non-pregnant women.[20] Furthermore, higher serum levels of CRP on the day of gonadotropin initiation, day of hCG administration, and day of OPU have been reported in non-pregnant women than those who became pregnant [20 as been seen that pregnant women following frozen embryo replacement (FER) had higher CRP levels compared with women who did not become pregnant. [21]

Therefore, the higher systemic inflammation in the in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) treatment can increase the chance of implantation and pregnancy However, the results regarding the association of CRP with ART outcomes are controversial [21] However, the results regarding the association of CRP with ART outcomes remain controversial, Seckin and colleagues found no significant difference in serum levels of high-sensitivity C-reactive protein (hs-CRP) between pregnant and non-pregnant patients.[22]

A similar study indicated that CRP levels were not always different between pregnant and non-pregnant women in ART cycles. Other studies associated high CRP levels with IVF failure[23 - 25] Embryologists have recently proposed the use of Follicular Fluid (FF) for the purpose of assessing oocyte competence. Follicular fluid is easily accessible as it is aspirated with the

oocyte during OCR. It has been postulated that some biochemical characteristics of the FF surrounding the oocyte may play a critical role in determining oocyte quality and the subsequent potential to achieve fertilization and embryo development.[26] A successful embryo implantation has been shown to be dependent on oocyte competence and endometrial receptivity.[12] Oocyte quality is currently, routinely assessed only visually by morphological criteria under a conventional microscope. This practice is able to predict poor oocyte quality but lacks the potential to positively predict good quality oocyte.[12] Another disadvantage is that it lacks objectivity as it is based on purely qualitative criteria.[12] It also limits the potential to optimize the choice of oocyte prior to fertilization and encourages oocyte overproduction. Understanding the role of CRP in determining oocyte quality and hence implantation outcome could improve the implantation rate (the commonest cause of IVF failure) in women undergoing IVF treatment.

The basic IVF procedure involves the following steps; down regulation therapy this involves administration of Gonadotrophin releasing hormone (GnRH) analogue to inhibit its endogenous production from the hypothalamus. Ovarian stimulation, Oocyte Retrieval (OCR) and fertilization, embryo culture, and Embryo Transfer (ET).[9]

Aim;

To investigate the relationship between plasma and follicular fluid hs – CRP levels with the outcome of implantation in women undergoing *in-vitro* fertilization.

Objectives;

1. To determine the plasma levels of hs-CRP at down regulation in women undergoing IVF.
2. To determine the follicular fluid level of hs-CRP in women undergoing IVF at OCR
3. To determine the plasma level of β-hCG on the 14th day post embryo transfer in women undergoing IVF.

4. To determine the association between plasma levels of hs-CRP before at down regulation with plasma levels of β-hCG on the 14th day post ET.
5. To determine the relationship between follicular fluid levels of hs-CRP at OCR with plasma levels of β-hCG on the 14th day post ET

Subjects and Methods:

This was a prospective cohort study carried out at the IVF unit and Chemical Pathology department of National Hospital, Abuja (NHA). The study population was composed of 150 women aged between 18 and 35 years with history of infertility (primary or secondary), undergoing IVF treatment at NHA. Women with obesity, cardiovascular, renal and liver diseases, women with significant history of smoking, significant alcohol consumption and those on immunosuppressive therapy were excluded from the study. Participants were given full information about the study and written consent was obtained before enrolment. Ethical clearance was sought for and obtained from the Health ethics research committee of NHA; NHA/EC/218/2013 on the 20th of June 2013. Patient confidentiality was maintained by omitting identifiable data and data protection was ensured during the study. There was no conflict of interest. Sample size was determined using the Fisher's formula and approximated to 150 participants.

We recruited study participants using the purposive sampling method; consenting women who met the eligibility criteria, presenting at the IVF unit for treatment were enrolled in the study until the sample size was met. Three millilitres of venous blood was withdrawn from a peripheral vein under sterile conditions into lithium heparin specimen containers from study participants on the day of commencement of down regulation therapy (prior to administration of GnRH agonists). Plasma was subsequently obtained by spinning the drawn blood sample in a centrifuge at 5,000 rpm for five minutes and then frozen in a cryo tube at -20^0 C for subsequent hs-CRP analysis within 3 months of storage. Similarly, we obtained plasma samples

from study participants on day 14, post embryo transfer and analysed for β human chorionic gonadotropin. Follicular fluid was collected at OCR and analysed for CRP as follows; Oocyte retrieval, an aseptic procedure was performed under transvaginal ultrasound guidance approximately 36 hours after a successful ovulation induction. The mature follicles were aspirated into heated sterile falcon tubes maintained at a temp of 37^{o}C. Suitable oocyte(s) were identified, aspirated and inseminated with the prepared semen at a stable temperature of 37^{o}c and pH of 7.2-7.3.[9. 27] The residual follicular fluid (from which the oocyte) was then collected and centrifuged at 5000 rpm for 5 minutes. The supernatant was stored in a cryotube at -20^{o}c in a freezer and subsequently analysed for hs-CRP within three months of storage.

Plasma and FF samples for hs CRP were both analysed on the Roche Cobas c311® a fully automated random access analyser for clinical chemistry and homogenous immunology assay (HIA). The test principle is based on immunoturbidimetry while the test procedure is based on latex particles coated with antibody specific to human CRP aggregating in the presence of CRP from the sample to form immune complexes, the immune complexes lead to an increase in light scattering which was proportional to the concentration of CRP in the serum. The light scattering was measured by reading turbidity (absorbance) at 570 nm. The CRP concentration was determined from a calibration curve developed from CRP standards of known concentration.[28]

We analysed plasma β hCG using the Roche Cobas e411®, a fully automated immunoassay analyser based on Electrochemiluminescence.[29] The Cobas e411® β-HCG employs the use of a competitive assay, sandwich principle.[29] The test procedure involves incubating 10 µL of sample, biotinylated monoclonal hCG-specific antibodies, a monoclonal hCG-specific antibody labelled with a ruthenium complex for eighteen minutes to form a sandwich complex. In a second incubation, streptavidin-coated

microparticles were added; the complex was bound to the solid phase via interaction of biotin and streptavidin.[29] The reaction mixture was aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with ProCell.[29] Application of voltage to the electrode induced chemiluminescent emission which was measured by a photomultiplier.[29]

Quality assurance measures included; pre analytical measures such as patient counselling on nature and timing of samples, prompt storage of plasma and follicular fluid samples at -20^{o}C for less than three months and ensuring that all samples were thawed only once. Analytical measures involved carrying out precision and accuracy studies before each run with using commercially prepared control solutions.

Results were analysed and data obtained followed a skewed distribution, attempts at log transformation of skewed distribution did not produce a symmetrical distribution hence use of statistical tools was limited to; the median used as a measure of central tendency and the interquartile range used to describe dispersion. A non-parametric test (Mann-Whitney U test) was the statistical test of choice. *P*-value of < 0.05 was considered indicative of statistical significance. All data generated were processed and analyzed using the Statistical Package for Social Sciences version 20 (SPSS, Chicago, Illinois, USA) statistical software.

Results

The median baseline plasma level of hs-CRP in the study subjects was 6.00 mg/L(IQR 6.81). T his was higher than the reported reference value ≤ 5.0mg/L. The median FF hs-CRP level in this study was 3.69 mg/L (IQR= 5.18 mg/L), this was lower than the median plasma hs-CRP level 6.00 mg/L at down regulation therapy in study subjects.

Table 1; Baseline plasma and follicular fluid levels of high sensitivity C reactive protein in study subjects.

Analytes	Median	IQR	Manufacturer's Reference Values
Plasma hs C-reactive protein levels (mg/L)	**6.00**	**6.81**	**≤ 5.0**
Follicular fluid hs C reactive protein (mg/L)	**3.69**	**5.18**	**≤ 5.0**

Implantation marker plasma β-hCG, was predictive of positive implantation outcome in 55 (36.67%) of the study subjects, and predictive of a negative implantation outcome in 95 (63.33%) of the study population, using < 200 IU/mL as a cutoff of successful implantation at 14 days post ET.[30] Implantation rate was found to be 37% in this study.

Table 2; Plasma β-human chorionic gonadotrophin (β-hCG) levels on the fourteenth day post embryo transfer

Analyte		Frequency	Percentage
Plasma β-HCG	**Negative** (<200 IU/mL)	**95**	**63.3%**
	Positive (≥200 IU/mL)	**55**	**36.7%**

A higher median baseline plasma hs-CRP of 7.48 mg/L and IQR 7.50 mg/L was found in subjects with a negative implantation outcome while subjects with a positive implantation outcome had a lower baseline plasma hs-CRP median of 3.62 mg/L (IQR=7.17), the difference however, was not of statistical significance (*p-value* = 0.25). The median FF hs-CRP was 4.72mg/L, (IQR=5.18) in subjects with a negative implantation outcome, and determined as 2.25mg/L, (IQR=5.01) in subjects with a positive implantation outcome. This difference was found to be of statistical significance with a *p-value* of 0.035. Higher FF hs-CRP levels were found to be associated with negative implantation outcome in this study. Plasma to FF hs-CRP ratio was similar in study subjects (1.58:1.00 in subjects with negative implantation outcome and 1.60:1.00 in subjects with positive implantation outcome).

Table 3: Comparison of plasma and follicular fluid levels of hc-CRP with plasma β-human chorionic gonadotrophin levels on the fourteenth day post embryotransfer.

ANALYTE	B-HCG LEVEL (IQR)		*P*-VALUE
	NEGATIVE (< 200IU/L)	POSITIVE (≥200IU/L)	
Plasma hs c-reactive protein levels (mg/L)	**7.48 (7.50)**	**3.62 (7.17)**	**0.259**
Follicular fluid hs c-reactive protein levels (mg/L)	**4.72 (5.18)**	**2.25 (5.01)**	**0.035**
Plasma : follicular fluid (hs-Crp)	**1.58 : 1.00**	**1.60 : 1.00**	

Discussion:

From the results of this study a baseline median plasma hs-CRP level of 6.00mg/L was observed in the study subjects, this was higher than the manufacturer's reported reference value of ≤ 5.00mg/L. The reference range studies for hs-CRP were done in non-African populations. This could be attributed to environmental factors (such as increased prevalence of communicable diseases) or genetic differences in the genotype and haplotype of the CRP gene locus in the study subjects.[31, 32]

Positive implantation defined by plasma β-hCG level (≥ 200 IU/L) on the 14th day post ET was recorded in 36.67% (≈37%) of study participants. This was comparable to results from a recent study in Abuja, which showed a clinical pregnancy rate of 9.4 - 69.4 % in women that had IVF treatment.[11] Worldwide pregnancy rates from IVF range from 11-46% worldwide.[10]

The difference between baseline plasma levels of hs-CRP in subjects with positive implantation outcome and those with negative implantation outcome on the 14th day post embryo transfer was found to be insignificant in this study. This finding was expected as factors known to affect hs-CRP levels were excluded for in the study population (exclusion criteria). Similar studies done to determine serum and FF levels of CRP in patients undergoing controlled ovarian hyperstimulation for IVF-ET and their correlation to controlled ovarian hyperstimulation, also concluded that there was no significant correlation between serum CRP levels and IVF outcome.[22 -25]

Conversely a prospective study carried out to investigate the levels of CRP in women treated by IVF/ET showed that CRP levels increase significantly during the first week following OCR and successful outcome is associated with an increment in CRP.[33] In another study investigating maternal level of CRP at 4 weeks gestation, serum level of CRP was measured on the 14th day of post ET in 135 women undergoing IVF pregnant women had significantly higher CRP levels than those who were not pregnant.[34]

In our study, we found a significant difference between median FF hs-CRP; 4.72mg/L, (IQR=5.18) and 2.25mg/L, (IQR=5.01) in subjects with a negative implantation and those with a positive implantation outcome respectively (p = 0.035). Higher FF hs-CRP levels were observed in subjects with negative implantation outcome. As follicular fluid is composed mainly of plasma ultra-filtrate and secretions of the granulosa and theca cells this difference could be attributed to the differential activity in granulosa and theca cells of women with positive and negative implantation outcome, and is in agreement with the study that associated negative implantation outcome with increased inflammatory response.[35] This contradicts the findings from a previous study that did not find significant difference in FF CRP levels and IVF outcome in women undergoing IVF.[23]

Conclusion: It can be concluded from the findings in this study that:-

1. The baseline plasma level of hs-CRP was higher 6.00 mg/L(IQR 6.81). than the caucasian based reference ranges of ≤ 5.0mg/L.
2. The median (IQR) FF levels of hs-CRP was determined as 3.69 (5.18) mg/L during OCR (this has not been determined in previous similar studies).
3. The plasma β-hCG measured on day fourteen post ET was found to be predictive of successful implantation in 55 out of the 150 study subjects representing an implantation rate of 37%

4. Baseline plasma hs-CRP was not significantly different between study subjects that had a positive implantation outcome and those with a negative implantation outcome on the 14th day post ET.
5. Follicular fluid level of hs-CRP was significantly higher in study subjects with a negative implantation outcome than in subjects with positive implantation outcome.

Recommendation;

It is our recommendation that collaborative multi-center research be undertaken in the numerous IVF treatment centres in Nigeria with the aim of Investigating the relationship between follicular fluid level of hs-CRP and implantation outcome in IVF cycles.

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