Hematological inflammatory biomarkers affecting the success rate of in vitro fertilization among cases of unexplained infertility
Short running title: Hematological biomarkers and IVF outcome.

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The authors guarantee that he:

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Abstract

Objective: This study aimed to evaluate the possible relation between the outcome of in-vitro fertilization (IVF) in non-obese females with unexplained infertility, and inflammatory indices obtained from complete blood count (CBC), including white blood cell and platelet counts (WCC and PC), neutrophil-to-lymphocyte-ratio (NLR) and platelet-to-lymphocyte-ratio (PLR).

Methodology: Forty lean cases (BMI <25 kg/m^2) undergoing IVF for unexplained infertility were involved. This study evaluated the effects of CBC inflammatory markers, measured at the outset of ovarian stimulation protocol, on IVF outcomes.

Results: The mean values of CBC parameters were normal, except for 30% leukocytosis and 4% thrombocytosis. Despite similar embryological findings, the women who eventually got pregnant had significantly lower WCC, neutrophil counts (NC), platelet counts (PC) and NLR (all $p<0.001$). On the other hand, thrombocytosis, lymphocyte counts and PLR were similar between women with positive and negative IVF outcomes. A significant correlation was found between platelet count and the number of oocytes and embryos, as well as the number of day-3 and grade I embryos.

The WCC, NC, PC as well as the NLR all had a significant diagnostic performance in predicting clinical pregnancy. The NLR showed the highest AUC (0.89), with a cutoff of ≤2.4, a sensitivity of 0.78 and a specificity of 0.9. On logistic regression analysis, the NC and PC were the most significantly affecting clinical pregnancy rate (p=0.011 and 0.049, odds ratio =0.25 and 0.95, respectively).

Conclusion: Poorer IVF clinical outcomes may be expected if unexplained infertility is associated with elevated WCC, NC, PC or NLR

Keywords: In-Vitro Fertilization, Complete Blood Count, Leukocytosis, Thrombocytosis.

Introduction

Accounting for almost 20% of infertile couples, unexplained infertility is defined whenever the usual cornerstones of fertility, such as ovulation, sperm function and deposition as well as tubal patency and endometrial receptivity, are all apparently normal [1,2].

The previously recorded higher failure rates of IVF among unexplained couples may imply that there may be other factors, such as a hostile inflammatory milieu, immune problems or functional abnormalities in the gametes and the endometrium, having a crucial role [3-9].

Embryonic implantation is a complex molecular and cellular process relying on various factors, most of which have not been clarified completely until now. Implantation needs coordination between various cellular events, such as the trophoblastic evolution and timing of some molecular action processes, which play a crucial role in embryonic apposition, penetration and invasion to the endometrial lining. In spite of good quality embryo transfer, failure of implantation is a relatively common issue that has been attributed to various factors, including a low-grade chronic inflammatory status [10-14]. The later may be defined as raised inflammatory biomarkers, including complete blood count (CBC) indices, such as total leukocytic count (TLC), neutrophil count (NC), and neutrophil-to-lymphocyte ratio (NLR) [5-9]. Furthermore, NLR is a recognised marker of systemic inflammation [15], PLR was recently introduced as a marker for inflammation and thrombosis [16]. The effect of raised inflammatory biomarkers on the outcome of IVF/ICSI remains a poorly studied topic [17,18].

This study will examine the theory whether IVF success rates may be correlated with the level of such inflammatory markers.

Methodology

This was a retrospective study conducted in the IVF unit of Ain Shams University Maternity Hospital on 40 cases with unexplained infertility undergoing long protocol of stimulation for IVF. All results and research data were obtained from medical records between January and August 2018. Inclusion criteria involved cases of unexplained infertility having ≥2 previous IVF failures, with adequate ovarian reserve (FSH >12 mIU/ml, AMH <1 ng/ml, or AFC <5) and no apparent causes of infertility (e.g. male factor, tubal factor) and with BMI ≤25 kg/m^2.

This study also excluded women with endocrine disturbances, chronic renal, hepatic, hypertensive or diabetic women. It excluded those with pelvic inflammatory disease or other chronic inflammatory conditions, hematologic diseases and splenectomy.

All cases had been evaluated by full clinical history and full infertility workup, including Day 3 FSH, LH, estradiol, TSH and prolactin, as well as antral follicle count (AFC). All cases had a CBC test and all indices of interest, particularly CBC inflammatory parameters, were recorded for comparative analysis as regards IVF outcomes.

All cases underwent down regulation using long agonist protocol. All cases have had ovarian stimulation using human menopausal gonadotropins (hMG). Human chorionic gonadotropins (hCG, 10,000 iu) were used to trigger ovulation. Transvaginal oocyte retrieval under sonographic guidance was conducted for all women. All cases underwent intracytoplasmic sperm injection (ICSI) as per the routine protocol in our unit. Recorded embryologic data were obtained for determining oocyte and embryonic features. The embryologic data were correlated to CBC inflammatory markers. The recorded oocyte features included the total number of retrieved oocytes, metaphase II (MII),

metaphase I, germinal vesicles, oocyte anomalies, degeneration and empty zona pellucida. The embryonic features included the total number of embryos, grades 1 to 3 embryos, day 3 embryos with 7-8 cells, day 5 embryos with blastocyst features, transferred embryos. Oocyte die-off ratios (DOR) was calculated from the ratio between MII's and day-3 embryos. The embryo DOR (EDOR) was calculated from the ratio between pronucleate to day-3 embryos [19]. Pregnancy was defined as beta HCG result >25 iu on day 15 post egg collection. Clinical pregnancy was defined as cardiac pulsations detected at 6 weeks of pregnancy. Implantation was defined as visualization of an intrauterine gestational sac by transvaginal ultrasonography. Biochemical pregnancy was defined as one ending before detection of a gestational sac on ultrasonography. Livebirths were defined as living births at a gestational age ≥ 26 weeks. The implantation rate per embryos transferred was defined as the number of gestational sacs detected by ultrasonography divided by the number of embryos transferred. The implantation, biochemical pregnancy, clinical pregnancy and take-home baby rates per embryo transfer were calculated to compensate for different numbers of embryos transferred.

Statistical methods

The collected data were coded, tabulated, and statistically analyzed using IBM SPSS statistics (Statistical Package for Social Sciences) software version 18.0, IBM Corp, Chicago, USA, 2009. The collected variables were compared between the IVF successful and failed cycles. Descriptive statistics was conducted for quantitative research data as minimum and maximum of the range as well as mean ± standard deviation (SD) for quantitative normally distributed research data, while it was done for qualitative research data as number and percentage. Data assumed to be normal for their size, independent t-test in cases of two independent groups. In qualitative data, inferential analyses for independent variables were done using Chi square test for differences between proportions and Fisher's exact test for variables with small expected numbers. Correlations were done using Pearson correlation. Receiver operating characteristic curve (ROC) curve was used to evaluate the performance of different tests differentiating between certain groups. The level of significance was taken at p value <0.05.

Results

The forty patients included were young (<35 years old), normoweight cases of unexplained infertility with adequate ovarian reserve (normal gonodotropin levels and antral follicle counts). They had a mean duration of infertility of 3.5±1.1 years and a mean of 2.9±1 previous IVF failures. Their mean WCC and PC were normal, but twelve women (30%) had leukocytosis while 12.9% (n=4) had thrombocytosis (table 1).

The included patients had a mean of 9.6±2.2 eggs retrieved, with an MII rate of 68.3% and a fertilization rate of 67.7 %. They had a mean of 4.5±1.8 embryos. They had a mean Biochemical pregnancy rate was 5% (n=2), their clinical pregnancy rate was 22.5% (n=9) and the take home baby rate was 20% (n=8). Two women developed twin pregnancies (5%) while one miscarried (2.5%). Out of the 92 embryos which were transferred, the biochemical pregnancy rate per embryo transferred was 12% (n=11), the implantation rate per embryo transferred was 12% (n=11), the clinical pregnancy rate per embryo transferred was 9.8% (n=9) and the take home baby rate per embryo transferred was 8.7% (n=8). Their mean oocyte DOR was 1.8±0.4, while their EDOR was 1.2±0.2 (Table 2).

Despite similar embryological findings (Table 2), the women who eventually got pregnant had significantly lower WCC, NC and platelet counts (PC) and NLR ($p<0.001$) than those who failed to conceive. The former had fewer cases with absolsute leukocytosis ($p=0.04$) (Table 1). On the other hand, thrombocytosis, lymphocyte counts (LC) and PLR were similar between both groups (Table 1). A significant correlation was found between platelet count and the number of oocytes and embryos, as well as the number of day-3 and grade I embryos. (Table 3). The WCC, NC and PC as well as the NLR all had a significant diagnostic performance in predicting clinical pregnancy (Table 4) The NLR showed the highest AUC (0.89), with a cutoff of ≤2.4, a sensitivity of 0.78 and a specificity of 0.9. (Table 4)

On Logistic regression analysis (Table 5), the NC and PC were the ones most significantly affecting the clinical pregnancy rate ($p=0.011$ and 0.049, odds ratio =0.25 and 0.95, respectively).

Discussion

This study recruited relatively young cases with adequate ovarian reserve in an attempt to minimize the effects of embryo number and quality. Furthermore, only cases with a BMI of <25 kg/m2 were enrolled to eliminate any alleged effect of obesity on the development of an inflammatory process [20-22]. It showed a statistically significant difference between women who had a successful IVF cycle versus a failed one in terms of most of their CBC inflammatory variables. Namely, the number of retrieved oocytes as well as the number and quality of the resulting embryos were all significantly correlated to the platelet count. The WCC, NC, PC and the NLR all were predicting clinical pregnancy.

This study disagrees with that of Cakiroglu et al [18], who found that MPV is correlated to the clinical pregnancy rate among PCOS women while the women's age was the only factor affecting success rate among women with unexplained infertility. This may be due to the difference in the study design since the latter did not really examine the differences in CBC between successful and failed IVF cycles, they rather compared obese to normal weight women. On the other hand, TOLA et al found no correlation between the clinical pregnancy rate and any of the CBC inflammatory markers [17]. This may be due to the fact that their study included women of an older age group (up to 40 years old) which may be more affected by problems related to embryo quality or endometrial receptivity [17]. Furthermore TOLA et al did not discuss the details of their differences between the successful and failed cycles in terms of embryo quality [17].

Increasing research evidence implies that infertility is correlated with a chronic low-grade inflammatory process at cellular and molecular levels. In harmony with the current research results, prior studies have revealed higher levels of IFN-γ, TNF-α, IL-2, IL-6 and IL-21, and reduced TGF-β among cases with unexplained infertility during the luteal phase of the menstrual cycle [4,8,23-27].

Previous studies have shown lower IVF success rates among cases of unexplained infertility in comparison to other infertile women [9,11,20]. There was no statistical correlation between the CBC inflammation markers and FSH levels [17]. Few research studies investigated the role of CBC indices on ICSI clinical outcomes [17]. It is hypothesized that implantation failure in unexplained infertility may result from reduced endometrial receptivity and abnormal immune responses. It has been suggested that increased platelet counts, and reduced lymphocytic counts are suggestive of chronic low-grade inflammation [21,23]. Already NLR, PLR and MPV (mean platelet volume) are being suggested as markers of chronic inflammation which may be significantly higher among women with PCOS [28,29].

The chief restrictions in the current study are the small cohort size, mainly related to infertile women in a retrospective design, and using data from a single IVF center. Although this may potentially undermine the value of the observed differences, yet since the clinical, embryological and endocrine parameters were similar in the pregnant and non-pregnant groups; this suggests that the results of the current study are most probably significant. Yet still, the current findings merit more attention and call upon further larger and prospective studies in this field.

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Table (1): Demographic characteristics and laboratory findings of the studied cases; and comparison of clinical pregnancy according to demographic characteristics and laboratory findings

Findings (Total N=40)	Mean±SD	Range	Positive (N=9)	Negative (N=31)	p value
Age (years)	28.5±2.5	25–34	28.3±2.3	28.5±2.6	^0.88
BMI (kg/m^2)	23.1±0.9	21.1–24.5	23.1±0.7	23±0.9	^0.86
Infertility duration (years)	3.5±1.1	2–6	3.4±0.9	3.5±1.1	^0.86
Previous IVF cycles	2.9±1	2-6	4.1±1.2	3.8±1	^0.39
FSH (mIU/L)	7.1±1.5	3.9–12	7.1±1	7.1±1.6	^0.95
LH (mIU/L)	5.1±1.1	3.4–7.2	4.9±0.9	5.1±1.1	^0.59
Prolactin (ng/mL)	13.1±2.4	9.5–19.5	13.4±2.7	13.0±2.4	^0.71
Estradiol (pg/mL)	54.6±7.7	34–67.5	51.8±8.9	55.4±7.3	^0.22
TSH (mIU/L)	2.2±1.3	0.5–5.4	1.9±1.5	2.3±1.3	^0.46
Antral follicle count (AFC)	7.9±1.8	5–11	7.4±1.6	8±1.9	^0.41
White cell count (WCC) (x10^3/mL)	8.5±1.7	5.1–13.9	7.7±1.5	10.1±1.4	^<0.001*
Neutrophil count (NC) (x10^3/mL)	6.1±1.3	3.2–9.7	4.6±1.3	6.5±1	^<0.001*
Lymphocyte count (LC) (x10^3/mL)	1.6±0.6	0.9–4.2	2.3±1.2	2.2±0.6	^0.74
Platelet count (PC) (x10^3/mL)	364.1±49.7	166.0–452.0	316.7±29.7	377.9±45.9	^<0.001*
Neutrophil-to-lymphocyte ratio (NLR)	4.1±1.5	1.2–7.9	2.1±0.5	3.1±0.7	^0.001*
Platelet-to-lymphocyte ratio (PLR)	178.4±59.5	58.5–337.1	159.5±57.4	183.9±59.9	^0.28
	Number	**Percentage (%)**	**Number and Percentage (%)**		**p value**
Leukocytosis (>11.0x10^3/mL)	12	30	0 (0%)	12 (38.7%)	#0.04*
Thrombocytosis (>450x10^3/mL)	4	12.9	0 (0%)	4 (12.9%)	#0.56

Data are presented as mean ±SD or number and percentage
^Independent t-test, #Chi-square test, *statistically significant.

Table (2): Embryological findings of the studied cases; and comparison of clinical pregnancy according to embryological findings.

	Findings	Mean±SD	Range	Positive (n=9)	Negative (n =31)	p value
Oocytes	**Total**	9.6±2.2	6–14	10.6±2.5	9.4±2.1	^0.15
	MII	6.6±1.7	4–9	7.2±1.9	6.4±1.7	^0.21
	MI	1.6±0.7	1–3	2.1±1.4	1.5±0.7	^0.22
	Germinal vesicles	1.2±0.9	0–3	0.8±0.8	1.2±1	^0.29
	Anomaly	0.2±0.4	0–2	0.1±0.3	0.1±0.3	^0.9
	Degenerated	0.1±0.3	0–1	0.3±0.5	0.1±0.4	^0.23
	Empty zona pellucida	0.1±0.3	0–1	0±0	0.1±0.3	^0.08
		Number	**Percentage (%)**	**Number and Percentage (%)**		**p value**
	MII Rate (MII/total)	263/385	68.3%	65/95 (68.4%)	198/290 (68.3%)	#0.98
	Fertilization Rate (Fertilized Oocytes/MII)	178/263	67.7%	65/95 (68.4%)	198/290 (68.3%)	#0.98
		Mean±SD	**Range**			
Embryos	**Total**	4.5±1.8	2–8	5.1±2	4.3±1.7	^0.21
	Grade I	4±1.8	1–8	4.9±2.2	3.7±1.6	^0.08
	Grade II	0.3±0.6	0–2	0.1±0.3	0.4±0.7	^0.24
	Grade III	0.1±0.3	0–1	0.1±0.3	0.1±0.3	^0.89
	Day-3	3.9±1.6	2–8	4.9±2.1	3.6±1.4	^0.14
	Day-5	3.1±1.8	1–7	4.1±2.4	2.8±1.6	^0.17
	Oocyte die-off ratio (DOR; MII/Day-3 embryos)	1.8±0.4	1–3	1.7±0.7	1.9±0.4	^0.19
	Embryo die-off ratios (EDOR; Day-3/Day-5 embryos)	1.2±0.2	1–2	1.1±0.2	1.2±0.2	^0.23
	Embryos Transferred	2.3±0.9	1–3	2.6±0.5	2.2±0.9	^0.19

Data are presented as mean ±SD or number and percentage.
^Independent t-test, #Chi-square test.

Table (3): Correlations of CBC with the different patients's characteristics.

Findings	WCC		NC		LC		PC		NLR		PLR	
	r	p	r	p	r	p	r	p	r	p	r	p
Woman's age	0.28	0.09	0.18	0.26	0.46	0.1	-0.13	0.44	-0.26	0.11	-0.36	0.12
Woman's BMI	0.001	0.99	0.01	0.93	-0.03	0.88	0.07	0.68	0.05	0.78	0.04	0.79
Infertility duration	-0.15	0.36	-0.21	0.2	-0.2	0.21	0.06	0.7	-0.07	0.68	0.11	0.49
Previous IVF	-0.1	0.54	-0.04	0.81	0.11	0.49	-0.004	0.98	0.02	0.92	-0.11	0.49
FSH	0.06	0.73	0.09	0.6	0.16	0.34	-0.06	0.73	-0.05	0.74	-0.19	0.24
LH	0.17	0.29	0.18	0.28	0.21	0.19	0.09	0.59	-0.04	0.83	-0.05	0.74
Prolactin	-0.05	0.77	-0.03	0.88	0.16	0.32	-0.22	0.17	-0.12	0.45	-0.39	0.11
Estradiol (pg/mL)	0.31	0.06	0.27	0.09	0.29	0.07	0.12	0.46	-0.12	0.46	0.2	0.22
TSH (mIU/L)	-0.08	0.63	-0.02	0.93	-0.2	0.21	-0.21	0.2	0.24	0.14	-0.25	0.11
Antral follicle count (AFC)	0.18	0.27	0.22	0.18	0.07	0.69	-0.03	0.86	0.03	0.84	-0.15	0.37
Total oocytes	0.07	0.69	0.01	0.95	0.04	0.81	-0.36	0.02*	-0.14	0.39	-0.11	0.49
MII	-0.19	0.23	-0.18	0.28	0.02	0.89	-0.26	0.1	-0.09	0.59	-0.06	0.72
MI	-0.17	0.3	-0.14	0.38	0.02	0.91	-0.38	0.02*	-0.07	0.69	-0.09	0.6
Germinal vesicles	-0.27	0.09	-0.31	0.06	-0.16	0.31	0.06	0.71	-0.14	0.4	-0.12	0.47
Anomaly	0.21	0.21	0.24	0.14	0.2	0.21	-0.18	0.26	0.03	0.85	-0.13	0.41
Degenerated	-0.001	0.99	-0.03	0.85	0.05	0.75	-0.05	0.75	-0.13	0.43	0.1	0.55
Empty zona pellucida	-0.22	0.17	-0.21	0.21	-0.01	0.94	0.09	0.6	-0.03	0.88	0.17	0.31
MII Rate	0.07	0.68	0.08	0.63	-0.12	0.46	0.07	0.69	0.26	0.11	0.08	0.61
Fertilization Rate	-0.02	0.89	0.004	0.98	-0.02	0.88	-0.38	0.02*	0.04	0.82	-0.18	0.28
Total Embryo	-0.16	0.32	-0.16	0.34	0.07	0.67	-0.35	0.03*	-0.13	0.44	-0.13	0.44
Grade I	-0.21	0.19	-0.19	0.24	0.08	0.63	-0.45	0.01*	-0.13	0.42	-0.14	0.4
Grade II	-0.26	0.11	-0.22	0.18	0.07	0.66	0.11	0.51	-0.13	0.42	-0.01	0.98
Grade III	0.04	0.8	0.03	0.84	-0.02	0.92	0.27	0.1	0.01	0.97	0.06	0.73
Day-3	0.15	0.35	0.09	0.58	0.07	0.65	-0.35	0.03*	-0.02	0.9	-0.09	0.58
Day-5	-0.24	0.15	-0.2	0.21	0.07	0.65	-0.28	0.08	-0.11	0.51	-0.05	0.77
DOR	-0.29	0.07	-0.27	0.1	0.03	0.86	0.27	0.09	-0.12	0.46	0.14	0.37
EDOR	0.14	0.38	0.12	0.47	-0.07	0.67	-0.08	0.61	0.07	0.68	-0.11	0.51

r: Pearson correlation, p: p-value, *: statistically significant.

Table (4): Diagnostic performance of CBC in predicting clinical pregnancy

CBC	AUC	SE	p-value	95% CI	Cut off	Sensitivity	Specificity
White cell count	0.88	0.071	0.001*	0.71–1	≤8.2	0.78	0.9
Neutrophil count	0.87	0.07	0.001*	0.71–1	≤5.5	0.78	0.87
Lymphocyte count	0.57	0.12	0.56	0.34–0.79	≤1.9	0.56	0.65
Platelet count	0.86	0.06	0.001*	0.74–0.98	≤345	0.89	0.74
Neutrophil-to-lymphocyte ratio	0.89	0.06	<0.001*	0.74–1	≤2.4	0.78	0.9
Platelet-to-lymphocyte ratio	0.58	0.11	0.51	0.36–0.78	≤209	0.89	0.36

AUC: Area under curve, SE: Standard error, CI: Confidence interval
*: statistically significant

Table (5): Logistic regression for factors affecting clinical pregnancy rate

Factor	β	SE	p-value	OR (95% CI)
Neutrophil count	-1.39	0.55	0.011*	0.25 (0.09–0.73)
Platelet count	-0.05	0.03	0.049*	0.95 (0.9–1)
Constant	24.19	10.02	0.016*	--

β: Regression coefficient, SE: Standard error, CI: Confidence interval, OR: Odds ratio.
*: statitistically significant