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Are the cytokines TNF alpha and IL 1Beta early predictors of embryo implantation? Cross sectional study

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ABSTRACT

The cross-talk between endometrium and embryo is not accessible to the researcher for obvious ethical reasons that let understand why implantation remains the black box of reproduction. We aimed to detect of the concentrations of IL-1β and TNF-α in endometrial secretion at the time of oocyte retrieval for early prediction of implantation. One hundred twenty women participated in the study during ICSI cycles. All women participating in the study included the following criteria; age; 22–36 years, BMI; less than 35 kg/m^2, a husband with oligo- or oligoasthenospermia. All women received controlled ovarian hyperstimulation and immediately after ovum pickup, an intrauterine flushing was done. Embryo transfer was done at the blastocyst stage five days after ovum pick up. Serum pregnancy tests were done for all women. The clinical pregnancy was defined as the appearance of the gestational sac and positive embryo cardiac activity was confirmed by TVS. The ongoing pregnancy was detected by abdominal ultrasound at 12 weeks. The participants were divided into two groups: the pregnant group and the non-pregnant group. Thirty-two and half percent of women got pregnant. There were non-significant differences between the two groups regarding the demographic, clinical and laboratory data except for the duration of infertility and concentrations of TNF-α and IL-1β. The concentrations of TNF-α and IL-1β were significantly higher in the pregnant group than the non-pregnant group.Therefore,The use of TNF-α and IL-1β to predict implantation in IVF is promising especially before embryo transfer.

Clinical trial.gov registration NCT02854514

1. Introduction

Ovarian hyperstimulation protocols and laboratory culture conditions in IVF/ICSI ET cycles has been improved greatly after the first successful pregnancy. The etiology of implantation failure mainly includes three categories; decreased endometrial receptivity, embryonic defects, and non-synchronized dialogue between maternal and embryonic tissues (Guzeloglu-Kayisli et al., 2009; Liang et al., 2015).

The human endometrium appears to be receptive to implantation for a limited period of time during the mid-luteal phase of the normal ovulatory cycle. Implantation is primarily determined by sex steroids which regulate the expression of locally acting growth factors, transcription factors, cytokines and chemokines (Boomsma et al., 2009). Cytokines which have pro-inflammatory effects (type 1) include interferon-γ (INF-γ), interleukin (IL-17, IL-1β) and tumor necrosis factor (TNF)-α. Those with anti-inflammatory effects (type 2) include IL-10, IL-4 and IL-1ra (Monastero and Pentyala, 2017). The physiological balance between type 1 and type 2 is critical for implantation (Liang et al., 2015).

Identifications of such cytokines may provide more information about implantation in both normal and ART cycles. Furthermore, the identification of these molecules might be useful in determining the best time for embryo transfer (Rahiminejad et al., 2015).

Routinely, less than 5% of oocytes were collected in IVF cycles and only 20–25% of embryos were transferred lead to a birth. Implantation processes remain the black box of fertility (Cartwright et al., 2010).

It is known that TNF-α increases the production of collagenases (Johnson et al., 1999) whereas IL-I induces the production of

Abbreviations: OHSS, ovarian hyperstimulation syndrome; E2, estradiol; GnRH-a, gonadotropin-releasing hormone agonist; LH, luteinizing hormone; IVF, in-vitro fertilization; ICSI, intra-cytoplasmic sperm injection; TVS, trans vaginal ultrasound; hCG, human chorionic gonadotropin; IL, interleukin; ET, embryo transfer; ART, assisted reproductive technology; %, percent; Kg/m^2, kilogram per square meter Kg/m^2; HMG, human menopausal gonadotropin; mIU, milli international unit; mg, milligram; C, centigrade; mm, millimeter; NO, number; MMP, matrix metalloproteinase; SD, standard deviation; pg, picogram; INF-γ, interferon gamma; M11, metaphase 2; OR, odd ratio; CI, confidence interval

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prostaglandin, nitric oxide, vascular endothelial growth factor and adhesion molecules (Dinarello, 2003). Karagouni et al. (1998) reported that gonadotropins administered during controlled ovarian stimulation induce both local and systemic production of IL-I. There is also evidence indicating that IL-1β stimulates progesterone production by luteal cells (Barak et al., 1992; Miceli et al., 2003). Cytokines have a paracrine and autocrine actions that do not require high concentration.

Inadequate uterine receptivity is responsible for approximately two thirds of implantation failures, whereas the embryo itself is responsible for one third of these failures (Simon et al., 1998; Ledee-Bataille et al., 2002). The aim of this study was to estimate the concentration of IL-1β and TNF-α in endometrial secretion at the time of oocyte retrieval for early prediction of implantation.

2. Patients and methods

2.1. Patient data

This cross-sectional study was conducted at Dar El Teb Hospital, Dokki, Giza, Egypt, from the period from August 2015 till April 2017. The study was conducted after approval of the Local Ethical Committee and after obtaining consents from all participants in the study. One hundred twenty women participated in the study with the following criteria; age; 22–36 years, BMI; less than 35 kg/m^2, Husband with oligo- or oligoasthenospermia. Exclusion criteria included 1-Gross uterine and tubal pathology, 2-Development of OHSS, 3-Poor responders, 4-Failure of oocyte fertilization or failure of the embryos to reach the blastocyst stage and 5-Refusal to participate in the study at any step of the ICSI cycle.

2.2. Protocol of induction of superovulation

All women underwent long agonist protocol for controlled ovarian hyperstimulation described by Chang et al. (1993). Pituitary suppression was achieved by GnRH agonist starting in the previous mid-luteal phase (decapeptyl R 0.1 mg, Triptorelin-Acetate, Ferring GmbH, Wittland 11, D-24109, and Kiel, Germany). The pituitary downregulation was confirmed by serum LH less than 5 m IU/ml and serum E2 less than 50 pg /ml. The HMG ampoules were started after the confirmation of pituitary downregulation by 225 IU/day (Merional 75 IU, IBSA Institute Biochimique SA, Switzerland). During the follow up of stimulation, the doses were adjusted according to the patient's response. All women were followed up by TVS until at least three dominant follicles were reached in every woman. When the dominant follicles reached 16–20 mm, HCG 10,000 IU (Choriomon 5000 IU, IBSA Institute Biochimique SA, Switzerland) was administered. Ovum pick up was done after 35 h following HCG administration. Immediately after retrieval, intrauterine flushing was done by 5 ml saline. It is the first time to investigate the endometrial cytokines at the time of oocyte pick up. Previous studies tried to detect IL-1beta before cycle day 23 like (Boomsma et al., 2009; Rahiminejad et al., 2015). The luteal phase was supported by progesterone 300 mg per day (progest, micronized progesterone 100 mg, Technopharma, Egypt, for pharco pharmaceuticals, Amriya- Alexandria). Five days following ovum pick up, the embryos were transferred at the blastocyst stage. Twelve days later to embryo transfer, serum pregnancy test was done and if positive, the clinical pregnancy was confirmed by TVS for detection of gestational sacs and embryo cardiac activity. Antenatal care of the pregnancy was done elsewhere because our unit is specialized for IVF only.

2.3. Primary outcome

1 The clinical pregnancy was detected by TVS at 6 weeks gestation (gestational sacs and embryo cardiac activity).
2 The ongoing pregnancy was detected by abdominal ultrasound at 12 weeks.
3 Detection of chemical pregnancy that was defined as a positive pregnancy test but the pregnancy did not appear on ultrasound examination.

2.4. Intrauterine flushing

The cervix was exposed by insertion of bivalve speculum into the vagina. The outer sheath of the embryo transfer catheter was introduced into the uterine cavity smoothly. Five ml saline was installed by syringe into the uterine cavity. The intrauterine fluids were aspirated quickly without contamination by the vaginal fluid. Centrifugation was performed at 1000 *g* for 10 min. The supernatant fluids were stored at −80 °C. The volume of fluid retrieved from the uterine cavity varied as it did in studies for pp14 and LIF assays (Li et al., 1993; Mackenna et al., 1993; Ledee-Bataille et al., 2002). Therefore, like those authors, we considered only the cytokine concentration, but not the amount of cytokine harvested.

2.5. Enzyme-linked immunosorbent assay (ELISA) of cytokines

The protocol for estimation of enzyme immune assay was the same as the manufacturer's protocol. Quantities ELISA kits for detecting IL 1Beta and TNF-alpha were obtained from R&D system (DIA source Immuno Assays S, A. Rue du Bosquet, 2, B-1348 Louvain-la-Neuve, Belgium). The limits for the detection of IL-1β and TNF-α were 0.35 Pg/ml and 0.7 Pg/ml, respectively.

2.6. Statistical analysis

The sample size was calculated using Open Epi (version 3, open source calculator- SSProor) according to the number of women coming to Dar El Teb hospital for IVF and fulfilling inclusion criteria in 6 months expected to be 185 women and percent of success of IVF in a previous study 32.4%(3). So at power of study 80% and CI 95%, the size was estimated to be 120 women. Obtained data were presented as mean ± SD, ranges, numbers, and ratios. Results were analyzed using independent *t*-test within-group variability in normally distributed data, Wilcoxon; ranking test for unrelated data, and Mann -Whitney-test for variability between groups if not normally distributed. Receiver operating characteristic (ROC) curve analysis was used to identify optimal cut-off values of different markers with maximum sensitivity and specificity of ICSI outcome. The validity of markers was measured by the area under the ROC curve. Statistical analysis was conducted using the SPSS program (Windows statistical package for social science) Version 18. A. P value < 0.05 was considered statistically significant.

3. Results

From 134 women consented to participate in the study, 14 women were excluded from the work; 2 women developed OHSS, 2 women were poor responders,3 women showed failed fertilization, 4 women failed to reach the blastocyst stage and the remaining 3 women refused to participate at the time of ovum pick up. Fig. 1

Only one hundred twenty women completed the study with a mean age 26.8 years, mean BMI 27.7 kg/m^2 and a mean duration of infertility 5.5 years. After stratification of the age of women every five years, it was found that the significant proportion of pregnant women > 26 years to pregnant women ≤26 was 69.2% and 30.8%, respectively,-while significant the proportion of non pregnant women > 26 years to nonpregnant women ≤26 was 44.4% and 55.6%, respectively.The percentage of women≤ 26 years was 47.5%.All women were primary infertile and had normal basal FSH and LH (6–8, 5–7mIU/ml, respectively). Table 1

The mean duration of induction was 13.1 days consuming mean number of HMG ampoules 34.8 while the mean number of follicles larger than 16 mm at the time of HCG administration was 11.9 follicles

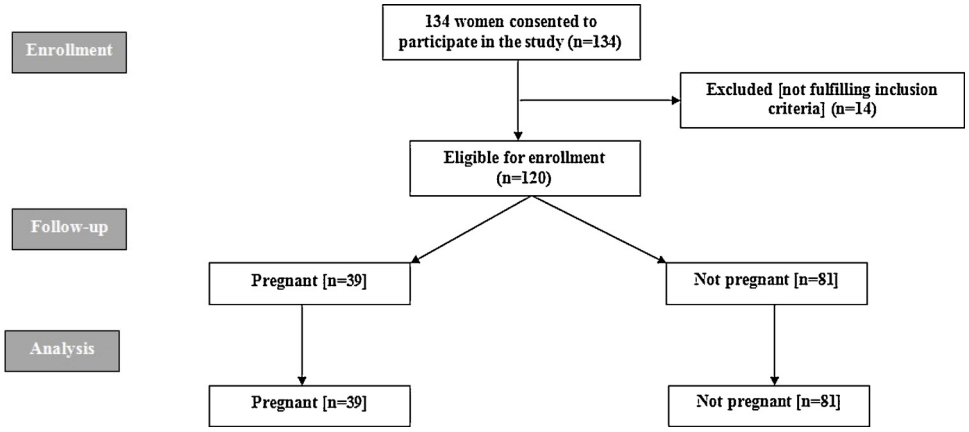

Fig. 1. Consort flow sheet.

Table 1. The laboratory data of the studied group revealed a mean number of M11 oocytes 9.01 and a mean number of cleaved embryos 6.3, while the mean number of transferred blastocyst was 2.6. Table 1.

After analysis of the stored intrauterine fluids, it was found that the mean concentrations of TNF-α and IL-1β were positively correlated with pregnancy. The pregnancy was significantly associated with higher concentrations of TNF-α and IL-1β collected by intrauterine flushing immediately after ovum pick up (9.6 ± 8.7, 202.01 ± 185.1. respectively) Table 1.

Comparing both groups regarding the demographic data, the duration of infertility of less than 5 years was significantly associated with pregnancy ($p < 0.001$) while the other demographic data did not differ significantly. Table 1

Assessment of serum pregnancy tests detected chemical pregnancy that was confirmed by TVS at 6 weeks gestation in only 32.5% of women (clinical pregnancy rate = 32.5%). Only one woman aborted at 10 weeks gestation (ongoing pregnancy rate = 31.7%) Table 2.

The ROC curve was used to assess the predictability of implantation by cytokines.

The areas under the curve for TNF-α and IL-1β were (0.8 & 0.7 at a cut off ≥4.8 & ≥37.9, respectively) indicating that the TNF-α was more predictive to pregnancy than IL-1β Fig. 2, Table 3.

Logistic regression analysis for the predictors of pregnancy excluded the duration of infertility of less than 5 years and transferred blastocyst number. The TNF-α > 4.8, IL-1β ≥37.9 and age > 26 years remained to be the significant predictors to pregnancy with a confidence interval (1.04–4.7, 2.05–7.8, 1.3-5.6. respectively), and OR (2.3, 3.8, 2.01. respectively) Table 4.

Table 1
Shows demographic, clinical and laboratory data and their relations to pregnancy.

Variables	Total (n = 120)		Non-pregnant (n = 81)		Pregnant (n = 39)		χ^2	p
	No	%	No	%	No	%		
Age group:								
22 – 26 y	57	47.5	45	55.6	12	30.8	6.67	
27 – 31 y	45	37.5	25	30.9	20	51.3		0.04*
32 – 36 y	18	15	11	13.5	7	17.9		

Variables	Mean ± SD	Mean ± SD	Mean ± SD	t	p
Age (years)	26.78 ± 4.3 (22-36)	27.31 ± 4.42 (22-36)	25.65 ± 3.86 (22-36)	1.64	0.11 NS
BMI (kg/m^2)	27.7 ± 2.04 (24.22-31.25)	27.42 ± 2.15 (24.22-31.25)	28.27 ± 1.7 (27.05-31.25)	1.77	0.08 NS
Duration of infertility (year)	5.46 ± 2.12 (3- 10)	6.07 ± 2.06 (3–10)	4.19 ± 1.63 (3–7)	4.08	< 0.001**
No. of HMG amp	34.78 ± 2.29 (32–40)	35.04 ± 2.61 (32–40)	34.23 ± 1.31 (33-36)	1.49	0.14 NS
Duration of induction : (day)	13.11 ± 1.71 (11-17)	13.44 ± 1.71 (11-17)	12.83 ± 1.5 (11-15)	1.90	0.06 NS
No. of follicles ≥ 16 mm	11.9 ± 3.67 (6–18)	11.98 ± 2.84 (6–15)	12.81 ± 4.44 (7–18)	1.24	0.22 NS
TNF-α (pg/ml)	6.72 ± 7.11	5.82 ± 5.36	9.55 ± 8.7	MW 2.98	0.003**
Median (range)	5.4 (0 – 25)	4.1 (0–18.5)	5.7 (3.1– 25)		
Interleukin 1β(pg/ml)	183.64 ± 264.94	173.48 ± 301.48	202.01 ± 185.1	MW 2.95	0.010*
Median (range)	39.7 (9–935)	36(9–935)	39.7(32.5-448)		
No. of Metaphase II oocytes:	9.01 ± 1.60 (7– 12)	8.68 ± 1.3 (7–11)	9.12 ± 1.61 (8–12)	1.60	0.11 NS
No. of fertilized oocytes	7.36 ± 1.82 (5– 11)	8.41 ± 1.12 (5–10)	8.35 ± 1.32 (6–11)	0.26	0.80 NS
No. of cleaved embryos:	6.27 ± 1.68 (3-9)	6.83 ± 1.31 (3-8)	7.04 ± 0.72 (4-9)	0.35	0.39 NS
No. of blastocyst	4.59 ± 1.85 (2-7)	4.94 ± 1.24 (2-6)	5.27 ± 0.43 (3-7)	1.61	0.11 NS
No. of transferred blastocyst:	2.58 ± 0.50 (2–3)	2.57 ± 0.50 (2-3)	2.73 ± 0.34 (2-3)	1.81	0.07 NS

Data are presented as mean ± SD, median and ranges are in parenthesis; *: Significant ($p < 0.05$); **: Highly Significant ($p < 0.01$).

Table 2
Shows outcome among the studied group.

Variables	(n = 120)	
	No.	%
Pregnancy test		
-ve	81	66.7
+ve	39	32.3
Clinical pregnancy rate	39	32.5
Ongoing pregnancy	38	31.7
No. of gestational sac		
1	10	25.6
2	20	51.3
3	9	23.1

We think that the cause of lower implantation and pregnancy rates was due to the influence of severe male factor (severe oligoasthenozoospermia or increased sperm DNA fragmentation) and its impact on genetic and morphological state of the embryo, therefore, the detrimental paternal effect on blastocyst competence. The age of women ≤26 years may be an addional factor for the lower implantation and prgnancy rates.

4. Discussion

The present study proposed that the period of implantation window is an immunological and inflammatory response and before the arrival of the blastocyst, the endometrial stromal cells secret pro-inflammatory cytokines like TNF-α and IL-1β to initiate inflammatory action (Hinduja et al., 2018) that may reach its optimal efficacy at the implantation window. Based on this idea, early detection of initiators (like TNF-α and IL-1β) of immunological and inflammatory response, can predict implantation.

During most days of the menstrual cycle, the endometrium is hostile towards the embryos, so major physiologic endeavor is needed to reverse this paradoxical condition. During this reverse period, the endometrium has to acquire an accurate morphological and functional state (decidualization) to reach the period of implantation window (Asimakopoulos et al., 2010).

Achache and Revel (2006) stated that IL-1β is the key mediator of this immunological and inflammatory response and the secretion of IL-1β is induced by TNF-α (Chimote et al., 2010).

For many years, the prevailing concept (derived from animal studies) for establishing pregnancy required a shift toward anti inflammatory cytokines (Wegmann et al., 1993; Arck et al., 1997) and the immunological regulation of tolerance of conceptus required a simple predominance of anti-inflammatory state (Chaouat et al., 2004). In agreement with this concept, several studies reported that the recurrent pregnancy loss was due to dysregulation in the balance of TH 1 and TH 2 immunity to trophoblast, and excess local TNF-α prevents implantation and triggers immunological pregnancy loss (Hill, 1997; Reid et al., 2001; Chaouat et al., 2007; El- Far et al., 2007). Also, prolonged exposure of pro-inflammatory cytokines to pregnancy is detrimental (Chimote et al., 2010). The previous studies evaluated the pregnancy as a single event of anti-inflammatory TH 2 predominance when, in reality, it has three distinct immunological phases. The first immunological phase requires a strong inflammatory response. The second immunological phase is associated with an anti-inflammatory state. The third phase is inflammatory (Mor et al., 2011).

On the contrary to the fore mentioned concept, some investigators tried to examine the relation between different cytokines and uterine implantation either locally (Von Wolff et al., 2000; Boomsma et al., 2009; Rahiminejad et al., 2015) or systemically (Rehman et al., 2018).The results obtained from local studies provided evidence that IL-1β and TNF-α mRNA expressions increased in human endometrium by the end of the proliferative phase and early luteal phase (Von Wolff et al., 2000) but IL-1β mRNA expression was lower at the time of implantation window while TNF-α mRNA expression remained elevated. This finding (provided at implantation window) was supported by the local detection of the concentrations of both cytokines (using multiplex immunoassay) just prior to embryo transfer (Boomsma et al., 2009). Six years later, another study was conducted at the same time point and found that the clinical pregnancy rate had a significant inverse relation with the local concentrations of TNF-α similar to earlier animal studies and no significant relation with the local concentrations of IL-1 β (Rahiminejad et al., 2015) but they detected the concentrations of endometrial cytokines by ELISA and attributed the discrepancy to some

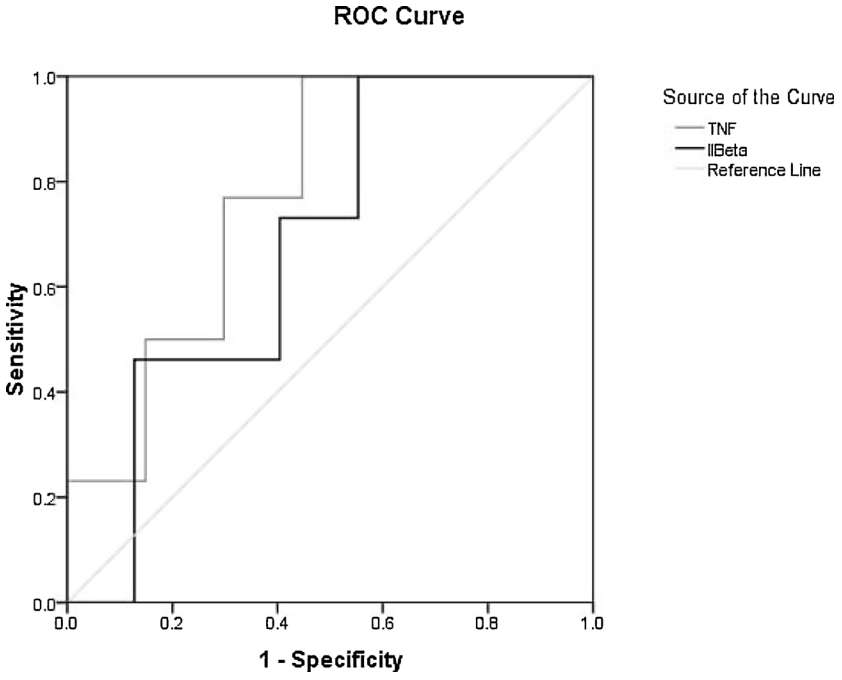

Fig. 2. Shows the predictive values of TNF-α and IL 1β.

Table 3
Shows validity of TNF-α & IL-1β in prediction of success ICSI.

Variable	Cutoff	AUC	CI	Sens.	Spec.	+PV	-PV	Accuracy	p-value
TNF-α	≤4.75	0.78	0.67-0.88	76.9	70.4	55.6	86.4	72.5	** < 0.001
IL-1β	≤37.85	0.68	0.56-0.8	73.1	72.3	55.9	83	72.5	*0.01

AUC: Area under curve; CI: Confidence interval; +PV: Positive predictive value; -PV: Negative predictive value; Sens.: Sensitivity; Spec.: Specificity; *: Significant ($p < 0.05$); **: Highly Significant ($p < 0.01$).

Table 4
Shows Logistic regression analysis for significant predictors of ICSI success among the studied groups.

Variables	B	S.E.	Wald	P	OR	95% CI	
						Lower	Upper
Age > 26 years	1.02	0.59	1.23	0.04*	2.01	1.26	5.62
Duration of infertility (< 5 years)	−0.04	0.06	0.65	0.48	1.23	0.97	1.97
High TNF -α > 4.75	1.04	0.62	1.94	0.03*	2.34	1.07	4.93
High interleukin 1β > 37.85	1.18	0.68	3.13	0.002**	3.95	2.12	8.20
No of transferred embryo =3	0.16	0.21	0.98	0.14	1.27	0.84	2.16

+++S.E.: standard error; OR: Odd Ratio; CI: Confidence interval; *: Significant ($p < 0.05$); **: Highly Significant ($p < 0.01$).

possible reasons like differences in technical procedure and volume of the sample. Recently, in a prospective cohort study on women undergoing IVF-ET, the serum levels of pro inflammatory cytokines (TNF-α) detected at the time of implantation had direct correlation with pregnancy rate (Rehman et al., 2018).

Our studies showed significantly higher concentrations of the pro inflammatory cytokines IL-1β and TNF-α (using ELISA because of its feasibility) in endometrial secretion early at the time of ovum pick up and were correlated with pregnancy.

In line with our findings, it was shown that IL-1β promotes the human in vitro decidualization and stimulates the secretion of chemokines and other factors, required for implantation, from uterine natural killer cells (Prutsch et al., 2012) and the local macrophages secret LIF and IL-1β to increase the fucosylated structures to allow trophoectoderm attachment prior to attachment of the embryo to the endometrial surface (Jasper et al., 2011; Nakamura et al., 2012; Robertson and Moldenhauer, 2014). Also, IL-1β and TNF-α regulate trophoblastic MMP-2, MMP-3, and MMP-9 which they allow the trophoblast to invade the endometrium (Meisser et al., 1999; Staun-Ram et al., 2004; Cohen et al., 2006; Prutsch et al., 2012). The TNF-α was associated with an inflammatory process related to implantation, placentation, and pregnancy outcome (Alijotas-Reig et al., 2017). High IL-1beta concentration in day 3 culture-conditioned medium was positively correlated with pregnancy after IVF treatment indicating a possible role of embryonic IL-1 beta in the implantation process (Sequeira et al., 2015). In support to our results, some studies found that the clinical pregnancy rate was increased due to slight endometrial damage in women with repeated IVF failure by modifying endometrial secreted inflammatory cytokines (El-Toukhy et al., 2012; Granot et al., 2012).

Cytokines in follicular fluid (FF) are important for reproduction as they modulate oocyte maturation and ovulation which influence subsequent fertilization, development of early embryo and potential for implantation (Gaafar et al., 2014).

The most limiting and difficult issue to evaluate is the dialogue at the materno-fetal interface during the process of implantation. Embryo can cross-talk with the endometrium through different factors. It actively participates to its own implantation and influences endometrial gene expression (Kashiwagi et al., 2007).

Although there are several studies on cytokines in recurrent implantation failures, there was a considerable variation in the findings from several studies that may be due to the different population studied, different timing of the sample collection and whether the cytokines were measured in whole tissues or specific cell population. (Laird et al., 2006).

This non invasive method of aspiration of uterine secretions enabled the researchers of this study to predict the endometrial receptivity early giving the chance for other researches on a large scale of cytokines. We think that the results will be of great benefit for women undergoing IVF either to freeze or transfer embryos.

Limitation of the study: The study was limited by the cost of the cytokines as we studied only two cytokines and the expensive cost of the analysis of the samples. Another limitation was the cooperation of the patients to participate in the study.

5. Conclusion

The use of TNF-α and IL-1β to predict implantation in IVF is a promising especially before embryo transfer.

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Availability of data and materials

The datasets used and/or analyzed during the current study were available from the corresponding author on reasonable request.

Authors' contributions

KMS: Analysis, Manuscript Drafting, Acquisition of data, Critical Discussion, Management and Follow up of cases; MKA: Study Design, Manuscript Drafting, Acquisition and interpretation of data, Management and Follow up of cases; RA: participated in the design of the study, performed the statistical analysis, and revised Manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study was approved by the Local Ethical Committee of Benha University Hospital and written informed consent was obtained from each participant before the study.

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Declaration of Competing Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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