Multi-factorial Analysis of the Follicular Fluid Milieu to explore the discrepant effect of follicular fluid endometrial flushing on outcome of Assisted Reproduction Trial

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Abstract

Objectives: To evaluate the effect of endometrial flushing (EF) with aspirated follicular fluid (FF) on outcome of assisted reproduction procedure and to explore any possible relationship between FF cytokine milieu and such outcome.

Study setting : Randomised controlled trial
Patients & Methods: Eighty infertile women were randomly categorized into: Group EF (n=40) had EF after oocyte retrieval and Control group (n=40) did not have EF. All women were subjected to the standard down-regulation regimen followed by controlled ovarian hyper stimulation . Oocytes were retrieved 34–36 h after hCG administration and aspirated FF was collected and centrifuged at 600 rpm for 10 min and 5-ml sample of supernatant was obtained for ELISA estimation of tumor necrosis factor-α (TNF-α), granulocyte colony-stimulating factor (G-CSF), leptin and anti-Mullerian Hormone (AMH) levels in both groups. The remaining amount was used for EF in EF group and was discarded in control group. Pregnancy was diagnosed by measurement of β-HCG level and confirmed by transvaginal sonography as clinical pregnancy.

Results: Embryologic data and estimated levels of studied parameters showed non-significant difference between both groups, despite being in favor of EF group. Mean FF levels of AMH and G-CSF were significantly higher and levels of TNF-α were significantly lower in women got clinical pregnancy (n=24) compared to those had failed trial (n=56). Regression analysis defined high FF levels of AMH as positive and TNF-α as negative significant predictors for clinical pregnancy. ROC curve analysis defined low FF levels of TNF-α as a significant sensitive and high AMH as significant specific predictors for clinical pregnancy.

Conclusion: Cytokine milieu of FF may affect outcome of IVF/ICSI procedures in contradictory manner and could explain the discrepant outcome of endometrial FF flushing. The obtained results may behave the way for the use of artificial flushing fluids containing anti-TNF-α, AMH or G-CSF for promotion of implantation and increasing pregnancy rates.

Keywords: Follicular fluid, endometrial flushing, ICSI outcome, TNF-α, AMH, leptin, G-CSF

Introduction

In-vitro fertilization (IVF), a popular assisted reproduction technique, is a widely accepted procedure for the treatment of infertility. Unfortunately, the success rate of this technique, measured as the average pregnancy rate per cycle, is only 30–40% with an overall live-birth rate of 54.4% [1, 2].
Follicular fluid may be regarded as a biological 'window' reflecting metabolic and hormonal processes occurring in the microenvironment of the maturing oocyte before ovulation and also as a predictor of outcome parameters such as fertilization, embryo cleavage and pregnancy rates in IVF [3].

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The peri-ovulatory follicular fluid (FF) provides microenvironment for the oocyte and contains likely key immunological factors for the regulation of its growth and development. There is growing evidence that interactions between the immune and endocrine systems can influence ovarian function. Consequently, the composition of regulatory factors in the follicular fluids is indirectly linked to fertilization and early embryonic development [4, 5].

Matrix metalloproteinases 9 level in FF could be a good predictor for successful IVF outcome [6]. Elevation of FF levels of epidermal growth factor-like growth factors, such as amphiregulin may result in increasing the number of oocyte retrieval and embryo generation, consequently increased cumulative pregnancy rate and so might be a good indicator to predict the number of oocytes and embryos [7].

Oxidative stress markers in FF were reported to significantly affect the outcome of IVF as malondialdehyde was found to be significantly different in pregnant and non-pregnant women and had a good sensitivity profile in predicting pregnancy, so it may be considered a marker for predicting IVF success [8].

Follicular fluid and surrounding tissue contain various lymphocyte subsets (resident and infiltrating) which synthesize numerous cytokines, such as interleukin (IL)-1 and tumor necrosis factor-α (TNF-α) are important modulators of mammalian ovarian function. In IVF patients high levels of intrafollicular IL-1beta were associated with good fertilization rates [9]. On the other side, IL-18 and IL-18 binding protein are detectable in follicular fluid but do not determine IVF outcome in women with "tubal factor" and so IL-18 and IL-18 binding protein are not promising prognostic markers for IVF success in this subgroup of patients [10].

Preliminary studies demonstrated that G-CSF stimulated neutrophilic granulocyte proliferation and differentiation, acted on macrophages of decidual cells, and finally affected the implantation. What [11.12] is more, known and reported immune effects of G-CSF are recruitment of dendritic cells, promoting Th-2 cytokine secretion, activating T regulatory cells, and also stimulation of various proangiogenic effects [12,13]. The current prospective study aimed to evaluate the effect of endometrial flushing with aspirated follicular fluid (FF) on the outcome of women undergoing assisted reproduction and to explore any possible relationship between cytokine milieu of aspirated FF and such outcome.

Patients & Methods

The present study was conducted at Assisted Reproduction Unit of Agial center, Nasr city, Cairo, Egypt. The study protocol was approved by the Local Ethical Committee and was conducted since Jan 2013 till March 2014. Eighty women signed written fully informed consent for both the approach and the procedure were included in the study. Inclusion criteria included age younger than 37 years, having a regular and proven ovulatory menstrual cycle with a length of 26–35 days and body mass index (BMI) of <35 kg/m^2, and serum FSH and estradiol were within normal range. Indications for IVF were tubal pathology, unexplained infertility, and male factor.

Patients were randomly, using sealed envelopes, categorized into two groups. Group EF included 40 women subjected to FF endometrial flushing after oocyte retrieval and Control group included 40 women would not have FF endometrial flushing.

Controlled ovarian stimulation

The protocol for controlled ovarian hyperstimulation preceded by the standard down-regulation regimen described by **Chang et al.**, [14]. Pituitary down-regulation was evaluated by a determination of serum estradiol (E_2), LH concentration and transvaginal sonography of the ovaries. Serum E_2 and LH was assayed using a commercially available competitive immunoassay with the Immulite Analyzer (DPC Coat-a Count; Diagnostic Products Corp., USA) at Unit laboratory. All patients received triptorelin acetate (Decapeptyl; Ferring, Germany) 0.1 mg injected subcutaneously once daily, beginning on day 21 of the previous cycle until the 1st day of the next cycle. If the serum E_2 level was <35 pg/ml, LH <10 mIU/ml and no follicles >10 mm in diameter were noted on TVS, Decapeptyl was decreased to half a dose and continued until and including the day of hCG administration. If the pituitary was not suppressed, Decapeptyl was continued at the same dose and the serum E_2 LH level was rechecked daily until suppression was achieved.

Patients received hMG (Menogon; Ferring Pharamceutical Co, Germany) in a dose of 225 IU/ day after pituitary suppression. Gonadotrophin was administered daily for 6 days, after which the dose was individualized according to ovarian follicular growth. Patients were monitored every other day starting on day 6 of stimulation with TVS and serum E_2. Intramuscular hCG (Pregnyl; Organon, Holland) 10,000 IU was administered when at least 5 follicles were ≥18 mm in diameter and with adequate serum E_2 levels. Progesterone was measured only on the day of hCG administration. Patients were divided into low,

moderate and high responders, according to the total dose of hMG used up to the day of hCG injection [15].

Oocytes were retrieved 34–36 h after hCG administration and aspirated FF was collected in a sterile container and was centrifuged at 600 rpm for 10 min at room temperature and a 5-ml sample of the supernatant was obtained for laboratory workup, while the remaining amount of supernatant was used to flush the endometrium through an applied uterine catheter in FF group and was discarded in the other group.

Oocyte preparation

For ICSI, the oocyte–corona–cumulus complexes were assessed shortly after retrieval. The complexes were denuded by placing them in a medium with 80 IU/ml of hyaluronidase for 5 sec. The cumulus and corona cells were removed mechanically by a set of pipettes with consecutive inner diameters of 220, 200, 180 and 160 μm. According to nuclear maturation grading, the oocytes were classified into categories, metaphase II or non-metaphase II that included oocytes at the metaphase I and germinal vesicle stages. The denuded oocytes were cultured in an M2 culture medium for 3–8 h, and then were examined for the presence of the first polar body. After confirmation of the first polar body, ICSI was performed on the heated stage of an inverted microscope according to **Tsai et al.** [16]. All embryos were scored on the day of embryo transfer for developmental stage and morphology, using the described criteria by **Steer et al.** [17] and good quality embryos were transferred. A good-quality embryo was defined embryo in G1 and G2 grade, having four blastomeres on day 2 or ≥8 blastomeres on day 3, less than 20% fragmentation, and no multinuclear blastomeres [17].

Luteal phase support (LPS) was started the day after ovum pick up by the vaginal administration of progesterone (Prontogest 200 mg suppositories. Nile Company, Pharmaceuticals, Egypt) thrice daily for 16 days and was continued for up to12 weeks if pregnancy occurred. Pregnancy was diagnosed by measurement of β-HCG level and was confirmed by later transvaginal sonography (TVU) as clinical pregnancy.

Laboratory investigations

Collected FF samples were stored –80°C until ELISA assayed for estimation of:

1. Tumor necrosis factor-α (TNF-α) measured with an ELISA kit from Pelikine™ Inc., Concord, USA [18].
2. Granulocyte Colony-stimulating Factor (G-CSF) levels using Quantikine G-CSF kit (R&D, Wiesbaden, Germany) [19].
3. Leptin levels using an ELISA kit from RayBio^{R} Inc., Parkway, Norcross, GA USA [20].
4. Anti-Mullerian Hormone (AMH) using an ELISA kit from MyBioSource, Inc. San Diego, California, USA [21].

Statistical analysis

Sample Power was calculated according to Kraemer & Thiemann [22] using their proposed figure showed that the sample size for 60% power would require an N of 31/group and 80% power would require an N of 51/group. This hypothesis was documented by Murphy & Myors [23], so sample size was chosen to be 40 patients to provide a power in range of 60-80% Obtained data were presented as mean±SD, ranges, numbers and ratios. Results were analyzed using paired t-test for within group variability and Wilcoxon; ranked test for unrelated data (Z-test) and Chi-square test (X^2 test) paired t-test for variability between groups. Statistical analysis was conducted using the SPSS (Version 15, 2006) for Windows statistical package. P value <0.05 was considered statistically significant.

Results

The study included 80 infertile women with mean age of 29.3±3.6; range: 24-36 years and mean duration of infertility of 5.8±2; range: 3-10 years. Twelve women were overweight with body mass index (BMI) of <30 kg/m^2 and 68 women were obese with BMI in range of 30-35 kg/m^2. Fifty-nine women were primary infertile and 21 women were secondary infertile. Thirty women were infertile secondary to male factor, 30 women had tubal infertility and 20 women had unexplained infertility. There was non-significant (p>0.05) difference between studied groups as regards enrollment demographic and clinical data and baseline laboratory data as shown in table 1.

All embryologic data showed non-significant difference between the studied groups. However, the reported implantation rate and clinical pregnancy rate were non-significantly higher in EF group compared to control group, (Table 2).

Estimated levels of FF studied parameters showed non-significant (p>0.05) difference between both studied groups, irrespective of decrease or increase of these levels in aspirated follicular fluid as shown in table 3.

Mean FF levels of AMH were significantly (p=0.014) higher, but leptin levels were non-significantly higher in women had got clinical pregnancy (n=24) compared to those had failed trial (n=56). On contrary, mean FF levels of TNF-α were significantly (p=0.049) lower in women had got clinical pregnancy compared to those had failed trial (Fig. 1). However, mean FF levels of G-CSF were significantly (p=0.001) higher in women

had got clinical pregnancy compared to those had failed trial (Table 4, Fig. 2).

Clinical pregnancy rate showed positive significant correlation with FF levels of AMH (r=0.345, p=0.002) and G-CSF (r=0.249, p=0.026), while showed positive non-significant (r=0.184, p>0.05) correlation with FF leptin levels. On contrary, clinical pregnancy rate showed negative significant (r=-0.326, p=0.003) correlation with FF levels of TNF-α.

Regression analysis for FF levels of leptin, G-CSF, AMH and TNF-α, as independent variables, as predictors for clinical pregnancy as dependent variable defined high FF levels of AMH as positive significant predictor and high FF levels of TNF-α as negative significant predictor for clinical pregnancy (Table 5). ROC curve analysis defined low FF levels of TNF-α as a significant sensitive and high AMH as significant specific predictors for clinical pregnancy (Table 6, Fig. 3).

Discussion

The current study found endometrial flushing using aspirated follicular fluid resulted in non-significant increase of implantation rate and clinical pregnancy rate compared to women did not have endometrial flushing. This finding was not surprising and supported that previously reported in literature wherein **Wongtra-Ngan et al.** [24] searched the Menstrual Disorders and Subfertility Group Specialized Register of controlled trials and found no evidence to suggest an association between follicular aspiration and flushing and ongoing clinical pregnancy per woman and no evidence of significant differences in increased oocyte yield per woman, but also reported no evidence of a difference in adverse events reported between follicular aspiration and flushing and aspiration only. **Levy et al.** [25] conducted systematic review and meta-analysis of 518 patients who participated in 6 randomized trials and results of this meta-analysis indicated no significant differences in the oocyte yield (oocytes retrieved divided by follicles aspirated), total oocytes retrieved, fertilization or pregnancy rates when comparing the non-flushing and flushing groups.

Also, **Kara et al.** [26] detected no difference in the retrieved oocyte number, however, the clinical pregnancy rate was higher and cycle cancellation rate was lower and number of metaphase I, germinal vesicle numbers were higher in follicular flushing group than control group but, this difference was not statistically significant. Recently, **Hashish et al.** [27] stated that in nature, the fallopian tube picks-up the FF with the oocyte during ovulation. As this fluid contains factors responsible for endometrial growth and improve implantation. That's why flushing the endometrial cavity with FF after ovum pick-up might improve endometrial receptivity and implantation rates; the limiting step in ICSI.

They found clinical pregnancy and implantation rates were higher in women had endometrial flushing using follicular fluid group compared with the control group who did not had endometrial flushing, however, the difference was not statistically significant.

However, studies that found no significant difference in the number of the retrieved oocyte or in its frequency per woman spotted light on an illogic hypothesis as logically there will not be any relation between endometrial flushing and these numbers as the follicular fluid itself is a yield of women preparation and flushing after retrieval mostly will not affect the number of the retrieved oocytes which had already retrieved. Thus, the current study proposed another hypothesis, that the cytokines, irrespective of being hormones, growth factors or pro-or anti-inflammatory cytokines, may modulate the endometrial receptivity and so affected the implantation and pregnancy rates.
Mean levels of estimated parameters showed non-significant difference between follicular fluids aspirated from women of both groups; a finding reflecting equality of response to preparation protocol applied in the current study. However, there were some reported interesting findings; firstly, irrespective of endometrial flushing, mean FF levels of AMH and G-CSF were significantly higher with significantly lower TNF-α level in women had successful implantation and got clinical pregnancy compared to those failed to have, but FF level of leptin showed non-significant difference despite being higher in those got pregnant. Secondly, there was positive significant correlation between FF levels of AMH and G-CSF and negative significant correlation with FF levels of TNF-α and clinical pregnancy rate. Regression and ROC curve analyses defined elevated FF level of G-CSF and TNF-α as a significant positive and negative predictive for clinical pregnancy, respectively.

Multiple recent works supported these findings of the current study, where **Lédée et al.** [28] found combined follicular G-CSF and IL-15 quantification appears as an efficient and noninvasive method to define oocyte competence for subsequent successful conception in modified natural IVF/ICSI cycles. Thereafter, **Lédée et al.** [29] documented that follicular G-CSF was highly predictive of subsequent implantation and monitoring FF G-CSF for the selection of embryos with a better potential for pregnancy might improve the effectiveness of IVF by reducing the time and cost required for obtaining a pregnancy.

Kim et al. [30] found FF AMH levels correlated positively with the matched embryo score on day 3 after fertilization, normal fertilization rate was significantly lower in women had low than in those of intermediate group as regards the levels of FF AMH and concluded that the FF AMH level could be a predictor of the ensuing oocyte and embryo quality. Moreover, **Chang et al.** [31] found FF leptin concentration and leptin/adiponectin ratio is not significantly related to oocyte maturity and corresponding embryo development.

Gaafar et al. [32] reported that on the day of oocyte retrieval, G-CSF was positively correlated with the number of fertilized oocytes, while TNF-α detection was associated with reduced number of fertilized oocytes, only G-CSF showed significant positive effect to the pregnancy outcome and concluded that the functions of cytokines in reproduction are likely to be complex, and cytokine evaluation may offer insight to the understanding of the mechanisms leading to success or failure of assisted reproduction.

The obtained results and review of literature allowed concluding that cytokine milieu of follicular fluid may affect outcome of IVF/ICSI procedures in contradictory manner and could explain the discrepant outcome of endometrial follicular fluid flushing. However, the obtained results may behave the way for the use of artificial flushing fluids containing anti-TNF-α, AMH or G-CSF for promotion of implantation and increasing pregnancy rates.

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Table (1): Patients' enrolment data

			Control	EF group	p value
Age (years)			29.9±3.7	28.7±3.4	=0.147
Weight (kg)			87.9±5.4	88.2±5.8	=0.851
Height (cm)			165.7±3.3	166.1±3.8	=0.443
BMI data	Number	≤30	7 (17.5%)	5 (12.5%)	=0.492
		>30	33 (82.5%)	35 (87.5%)	
	Mean (kg/m^2)	≤30	29.4±0.3	29.1±0.5	=0.782
		>30	32.6±1.6	32.3±1.4	=0.843
	Total (kg/m^2)		32±1.9	31.9±1.7	=0.837
Duration of infertility	≤5 years		15 (37.5%)	13 (32.5%)	=0.286
	>5 years		25 (62.4%)	27 (67.5%)	
	Mean (years)		5.4±2.2	6.1±1.8	=0.099
Type of infertility	Primary		28 (70%)	31 (77.5%)	=0.115
	Secondary		12 (30%)	9 (22.5%)	
Cause of infertility	Male factor		16 (40%)	14 (35%)	=0.103
	Tubal factor		13 (32.5%)	17 (42.5%)	
	Unexplained		11 (27.5%)	9 (22.5%)	
Baseline hormonal data	Serum E2 (pmol/L)		30.2±6	31.4±6.4	=0.065
	Serum FSH (IU/L)		7.3±0.8	7.23±0.8	=0.535

Data are presented as mean±SD & numbers; percentages are in parenthesis; BMI: body mass index; p>0.05: non-significant; EF: endometrial flushing

Table (2): Embryological characteristics and cycle outcome in studied groups

		Control	EF group	p value
Number of oocytes retrieved		10.2±2.7	10.5±2.5	=0.370
Number of metaphase II oocytes retrieved		8.7±2.5	9.2±2.1	=0.271
Fertilization rate (%)		87.3±6.3	89.5±5.2	=0.076
Number of available embryos	G1	4.1±1.4	3.8±1.6	=0.228
	G2	4.3±1.2	4±1.8	=0.057
	G3	0.7±0.5	0.8±0.6	=0.301
Number of embryos transfered	G1	1.6±0.5	1.8±0.6	=0.050
	G2	1.4±0.5	1.5±0.5	=0.241
Implantation rate (%)		9.8±6.6	10.5±3.4	=0.079
Clinical pregnancy rate (%)		10 (25%)	14 (35%)	=0.092

Data are presented as mean±SD & numbers; percentages are in parenthesis; p>0.05: non-significant; EF: endometrial flushing

Table (3): Mean (±SD) level of parameters estimated in aspirated FF of both groups

	Control	EF group	p value
Leptin (ng/ml)	57.5±10	60.5±7.7	=0.145
TNF-α (ng/ml)	5.9±2.3	6.2±1.8	=0.541
AMH (ng/ml)	4.1±2.5	4.4±2.8	=0.761
G-CSF (pg/ml)	22.8±6.9	21.5±7.2	=0.282

Data are presented as mean±SD; TNF-α: tumor necrosis factor-α; AMH: Anti-Mullerian hormone; G-CSF: Granulocyte Colony-stimulating factor; EF: endometrial flushing

Table (4): Mean (±SD) levels of parameters estimated in aspirated FF of studied women categorized according to outcome defined as diagnosis of clinical pregnancy

	Succeeded	Failed	P value
AMH (ng/ml)	4.81±2.65	2.94±2.16	=0.014
Leptin (ng/ml)	61.5±8.6	57.9±9	=0.280
G-CSF (pg/ml)	25.8±7.2	20.5±6.5	=0.001
TNF-α (ng/ml)	5.72±2.1	6.82±1.7	=0.049

Data are presented as mean±SD; TNF-α: tumor necrosis factor-α; AMH: Anti-Mullerian hormone; G-CSF: Granulocyte Colony-stimulating factor

Table (5): Regression analysis of parameters estimated in FF as predictors for clinical pregnancy

		Standardized coefficient (β)	t	P
Model 1	AMH (ng/ml)	0.356	3.641	0.0007
	TNF-α (ng/ml)	-0.340	-3.472	0.001
	Leptin (ng/ml)	0.246	2.506	0.014
Model 2	AMH (ng/ml)	0.336	3.337	0.001
	TNF-α (ng/ml)	-0.317	-3.143	0.002
Model 3	AMH (ng/ml)	0.345	3.243	0.002

AMH: Anti-Mullerian hormone; TNF-α: tumor necrosis factor-α; G-CSF: Granulocyte Colony-stimulating factor

Table (6): ROC curve analysis of parameters estimated in FF as predictors for clinical pregnancy

	AUC	SE	p	Levels of 95% CI
AMH (ng/ml)	0.725	0.060	0.002	0.606 to 0.843
G-CSF (pg/ml)	0.654	0.064	0.029	0.529 to 0.779
TNF-α (ng/ml)	0.260	0.064	0.001	0.135 to 0.385
Leptin (ng/ml)	0.613	0.068	0.122	0.480 to 0.745

AMH: Anti-Mullerian hormone; G-CSF: Granulocyte Colony-stimulating factor; TNF-α: tumor necrosis factor-α

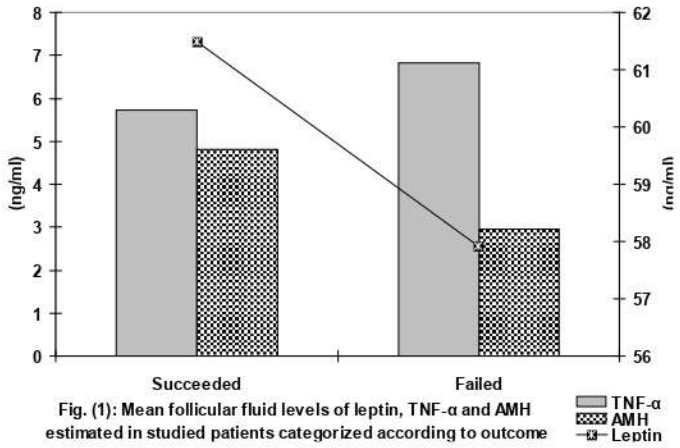

Fig. (1): Mean follicular fluid levels of leptin, TNF-α and AMH estimated in studied patients categorized according to outcome

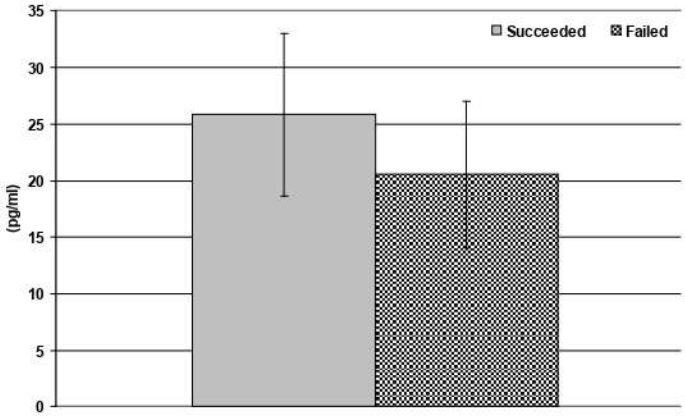

Fig. (2): Mean FF level of G-CSF estimated in studied women categorized according to outcome (clinical pregnancy)