Studying the effect of ovulation stimulation by using clomiphene citrate on serum level of tumor necrosis factor alpha and interleukin-1ß in sub-fertile women in Holy Kerbala Province

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Objectives The overall goal of this study was to figure out the effect of ovulation induction on the levels of TNF-α and IL-1ß, and to investigate the possible interaction between the cytokines and hormones in respect to anovulation.
Methods The study comprised of 53 women; 33 sub-fertile and 20 fertile. Their ages ranged from 17–39 years. The anovulation in the sub-fertile group were diagnosed based on the standard criteria (clinical and laboratory investigations). All patients were scheduled for the induction of ovulation by treatment with clomiphene citrate. The blood samples have been drawn on the 2nd day of the menstrual cycle. The hormones and cytokines were measured. 2–4 weeks after commencing the treatment with clomiphene citrate (clomiphene, a non-steroidal compound, structurally similar to oestrogen, blocks oestrogenic hypothalamic receptors, resulting in blinding of the hypothalamus–pituitary axis to endogenous circulating oestrogen), the second sample of blood was drawn again to re-measure the levels of the same cytokines measured before the treatment. By SPSS software for windows, version 20, IBM, US, 2010, data of all participants have been entered, descriptive data analysis, paired sample T test, one way ANOVA test and person's correlations have been used as appropriate.
Results In this study, the levels of TNF-α and IL-1ß before treatment were lower than those in after treatment; however, these differences were significant only in case of TNF-α ($P = 0.041$). These results indicate that clomiphene citrate has a specific effect on TNF-α expression and to a lesser extent on other cytokines.
Conclusion The results of this study may implicate a direct role of TNF-α in the fertility and, thus, could raise questions about the possibility of using immune modulation in the treatment of sub-fertility.
Keywords ovulation stimulation, clomiphene citrate, tumour necrosis factor alpha, interleukin-1ß, fertility, anovulation

Introduction

The ovulation is the development and release of an ovum from the ovaries. It is the most fertile period of the menstrual cycle. By about 14 days into the reproductive cycle, an oocyte reaches maturity and is released as an ovum.[1] The gonadotropins FSH and LH are produced by the anterior pituitary gonadotrophic cells and are responsible for the ovarian follicular stimulation.[2] The FSH targets the receptor expressed only on granulosa cells and induces the maturation of ovarian follicle.[3] The LH plays a key role in the initiation of the ovulatory process of preovulatory follicles by activating multiple cellular signaling pathways.[4] The most common cause of female infertility is ovulatory disorder characterised by anovulation or by infrequent and/or irregular ovulation.[5] Anovulatory dysfunction is a common problem and is responsible for about 40% of female infertility.[6]

Sometimes women does not ovulate (release an egg from an ovary each month) or ovulate irregularly, this will interfere with the ability to become pregnant. The ovulation induction is the term for the use of medical therapy to treat women who do not ovulate by themselves.[7] The first-line medication for the ovulation induction is clomiphene citrate. It is a nonsteroidal compound, structurally similar to oestrogen and it blocks oestrogenic hypothalamic receptors, resulting in blinding of the hypothalamus–pituitary axis to endogenous circulating oestrogen. This in turn triggers the release of FSH from the anterior pituitary following alterations in GnRH pulsatility.[6]

The gonadotropins and gonadal steroids play the central role in ovarian folliculogenesis. However, the variable fate of follicles within the same ovary suggests the existence of additional intraovarian modulatory systems.[8] The cytokines assist granulosa cell growth during the follicular development and thus, they maintain the normal ovarian function. In addition, the immune mediators may play role in ovulation. In this context, it has been shown that an influx of immune cells takes place during the LH peak and this influx is associated with the release of several cytokines.[9] IL-1β augmented the FSH-stimulated accumulation of 20-dihydroprogesterone. There are studies in the rat ovary indicate that the rat ovarian theca-interstitial cell is a site of IL-1β gene expression, the preovulatory acquisition of which is gonadotropin dependent.[10] TNF-α engages in the differentiation of a variety of cell types. In the ovary, the TNF-α was found capable of attenuating the differentiation of cultured granulosa cells from immature rats.[11] The detection of TNF-α activity in some luteal tissue on day 5, and the scarcity of macrophages at this stage raise the possibility that cells other than macrophages may also produce TNF in the corpus luteum.[12] The TNF-α stimulates progesterone synthesis in differentiated ovaries, while in undifferentiated ovarian cells TNF-α inhibits steroidogenesis.[13] The IL-1 and TNF-α suppress 17β-estradiol (E_2) and progesterone release from granulosa and luteal cells *in vitro*. The TNF-α affects negatively folliculogenesis and ovarian maturation.[14] The principal aim of this study is to investigate the possible side effects of ovulation stimulation by studying its effects on the immune system as well as the certain biochemical systems. It is worthy to mention that understanding the side effects of ovulation stimulation may help better intervention with those effects by therapeutic or preventive measures.

Materials and Methods

The study comprised of 53 women; 33 sub-fertile and 20 fertile (used as the control group for cytokine profile analyses). Their ages ranged from 17 to 39 years. The community of our study was the female partner of sub-fertile couple from Karbala city attending the Karbala Fertility Unit (KFU) which is found in the Karbala Maternity Hospital. The samples of our study were female, selected from the local community who were complaining of primary or secondary sub-fertility. The anovulation has been diagnosed based on the patient's history, hormonal level (day 2) and pelvic U/S in the in the unit of X-ray and Sonar in Karbala Maternity Hospital. Whereas, the control group (fertile women) has been collected from the local community. The sample collection was during the period from April 2015 to August 2015. The laboratory study has been carried out in the research laboratories of biochemistry department and the microbiology department, College of Medicine, University of Karbala. The patients and control women have been informed about the study and its aims and their agreement have been taken. The patients have been selected by the gynaecologist after taking history and investigations. The patients were not on any form of ovulation induction strategy by either pharmacological or nonpharmacological way. The blood samples have been drawn on day 2 of the cycle. The patients have been examined by the gynaecologist to ensure that there is no any overt inflammation. In addition, C-reactive protein has been measured in each patient and any patient showed high titers has been excluded from the study. The hormones and cytokines were measured by using standard sandwich enzyme-linked immunosorbent assay technology. After obtaining the blood samples, the patients have been given the ovulation induction drugs (clomiphene citrate) for 3 months. After that, another sample of blood has been drawn again to re-measure the levels of the same cytokines measured before the treatment. By using the statistical package for social sciences, SPSS software for windows, version 20, IBM, US, 2010, the data of all the participants have been entered, the descriptive data analysis, paired sample T test, one way anova test and person's correlations have been used as appropriate.

Results

A total of 33 patients were suffering from anovulation and 20 fertile subjects were enrolled. The mean age of the patients was 25.09 years old. As shown in Table 1, a significant positive correlation was found between age and BMI (R = 0.364, P = 0.038). A highly significant positive correlation was found between BMI and BMI categories (R = 0.854, P = 0.00). Significantly, there were no correlations between age and BMI categories, FSH, LH and oestrogen (R = 0.304, P = 0.085); (R = 0.351, P = 0.092); (R = 0.28, P = 0.185); (R = 0.277, P = 0.266); respectively.

In Table 2, the mean age of secondary type of infertility was higher in comparison with the primary type of infertility (27.1 vs. 24.4) and this difference was statistically significant (P = 0.014). In addition, the mean BMI of the secondary type of infertility was higher than in the primary type of infertility (31.7 vs. 25.7) and this difference was statistically highly significant (P = 0.00). However, no significant differences were found between both the types of infertility in respect to sex hormones.

Table 1. **Correlation matrix among the patient's characteristics**

		BMI	BMI categories	FSH	LH	Oestrogen
Age in years	Pearson correlation	0.364*	0.304	0.351	0.280	0.277
	Sig. (2-tailed)	0.038	0.085	0.092	0.185	0.266
BMI	Pearson correlation		0.854**	0.215	0.241	0.173
	Sig. (2-tailed)		0.000	0.314	0.257	0.493
BMI categories	Pearson correlation			0.088	0.159	−0.373
	Sig. (2-tailed)			0.682	0.457	0.128
FSH	Pearson correlation				0.273	−0.413
	Sig. (2-tailed)				0.196	0.089
LH	Pearson correlation					0.179
	Sig. (2-tailed)					0.478

Table 2. **Characteristics of patients according to the type of sub-fertility**

	Type of sub-fertility				*P*-value
	Primary n = 15		Secondary n = 18		
	Mean (±SD)	Std. error	Mean (±SD)	Std. error	
Age (years)	24.4 (±5.4)	1.8	27.1 (±4.3)	1.4	0.014
BMI	25.7 (±2.1)	0.72	31.7 (±3.2)	1	0.00
FSH (mIU/ml)	5.59 (±1.97)	0.65	5.99 (±1.4)	0.48	0.812
LH (mIU/ml)	6.8 (±3.8)	1.2	10 (±6.7)	2.2	0.655
Oestrogen (pg/ml)	99.3 (±211.2)	70.4	34.9 (±22.4)	7.4	0.377

Table 3. **Cytokine levels before and after treatment with clomiphene citrate for sub-fertile subject**

Cytokines conc. in pg/ml	Before treatment mean (±SD) $n = 20$	After treatment mean (±SD) $n = 20$	*P*-value
TNF-α	58.47 (±26.49)	74.88 (±23.39)	0.041
IL-1ß	10.32 (±7.86)	12.24 (±5.37)	0.266

Table 4. **Correlation of cytokine level between sub-fertile and fertile subject**

Cytokines conc. in pg/ml	Patients mean (±SD) $n = 33$	Control mean (±SD) $n = 17$	*P*-value
TNF-α	44.9 (±26.7)	78.6 (±18.2)	0.00
IL-1ß	11.2 (±6.2)	13.2 (±10.4)	0.396

As shown in Table 3, the paired sample T test has been used to compare the means of the cytokine levels between before and after treatment with clomiphene citrate. The levels of cytokines before treatment were lower than those in after treatment; however, these differences were significant only in case of TNF-α ($P = 0.041$).

Table 4 shows that the mean concentration of TNF-α in the sub-fertile group (44.9) was <1 fold lower than its mean concentration (78.6) in the fertile group. The mean concentration of IL-1ß in sub-fertile (11.2) also was less than its mean concentration (13.2) in fertile group but they were convergent.

Discussion

The immune cells are associated with the regulation of every level of the hypothalamus–pituitary–ovarian axis. The cytokines assist granulosa cell growth during the follicular development and thus they maintain the normal ovarian function. It has been shown that on the influx of immune cells takes place during the LH peak and this influx of immune cells is associated with the release of several cytokines. In addition, the rupture of the ovarian follicles is believed to be an immune-mediated reaction with IL-1, the TNF-alpha are the cytokines playing the major role in this process.[9] The crosstalk between the endocrine and immune systems regulates a large number of biological processes that affect target tissues, and this crosstalk involves gene expression, cytokine and/or lymphokine release and hormone action.[15] The mean age of the patients was 25.09 years old. The age of the female is the single most important determinant with a gradual decline in fertility especially after the age of 35 years.[16,17]

A significant positive correlation was found between the age and BMI ($P = 0.038$). This correlation may be caused by the natural behaviour of patients who tend to engage in more secondary life style with increasing age. The increasing secondary life style may be caused by several reasons such as the social influence, lack of outdoor activities, or accompanied by other changes:- life events and the kind of responsibilities in addition to the pathological changes. The various environmental and social factors relating to diet and physical activity have been identified that could contribute to obesity.[18]

The levels of cytokines before treatment were lower than those in after treatment, however these differences were significant only in case of TNF-α ($P = 0.041$). These results indicate that the clomiphene citrate affects specifically on TNF-α expression and to a lesser extent on other cytokines. The clomiphene citrate treatment blocks the oestrogenic hypothalamic receptors, resulting in blinding of the hypothalamus–pituitary axis to endogenous circulating oestrogen. This in turn triggers the release of FSH from the anterior pituitary following alterations in GnRH pulsatility.[6] And because the oestrogens inhibit IL-1 and TNF-α production,[19–22] we could assume that the clomiphene citrate treatment has the same role on these cytokines. There is evidence that cytokines are involved in both the inhibition and stimulation of follicular responsiveness to gonadotrophins.[23] TNF-α activates neutrophils, induces IL-1 gene expression, enhances the expression of class I major histocompatibility complex (MHC) antigens and adhesion molecules on the endothelial cells, and is involved in bone marrow resorption and the production of prostaglandin and collagenase from human synovial cells and fibroblasts.[24]

The mean concentration of TNF-α in the sub-fertile group was <1 fold lower than its mean concentration in the fertile group. The mean concentration of IL-1ß in sub-fertile also was less than its mean concentration in fertile group but they were convergent. Abnormally, a low concentrations of sex hormones are associated with a higher serum levels of TNF-alpha and which could suggest a suppressive effect of estradiol and progesterone on pro-inflammatory cytokine secretion.[25] The *in vitro* studies by Chao et al. have shown that estradiol can lower the tumour necrosis factor production.[26]

Conclusion

The very interesting results were emerged when comparisons, were made between the sub-fertile and fertile groups in respect to the cytokine levels. The levels of TNF-α and IL-1ß were significantly lower in the sub-fertile group compared to the fertile subjects indicating a possible role in the aetiology of sub-fertility status. They were low in the sub-fertile groups, and their low concentrations might possibly associate with the anovulation status. Generally, the results of the current study may implicate a direct role of certain cytokines (TNF-α and IL-1ß) in the fertility and, thus, could raise questions about the possibility of using immune modulation in the treatment of sub-fertility. ■

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