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Monocyte chemotactic protein-1 concentrations and expression of women with endometriosis undergoing IVF cycles

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ABSTRACT

To research the local action of endometriosis on follicular inflammatory microenvironment of patients undergoing *in vitro* fertilization (IVF) cycles. A prospective case control study was organized at Kamal AL-Samarai IVF center (Baghdad/Iraq), January 2021 - May 2021. The study included (80) infertile women undergoing IVF cycles were split to two groups: Patients with endometriosis group (n = 40) and Patients without endometriosis group (n = 40). Follicular fluid (FF) and blood samples were gathered at the period of oocyte pick-up. Levels of MCP-1 mRNA were investigated with the reverse transcription polymerase chain reaction (RT-PCR) technique. Concentrations of cytokines (monocyte chemo attractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), and interleukin-10 (IL-10)) were investigated in FF by sandwich enzyme-linked immunosorbent assay (ELISA). The mRNA expression of MCP-1 was significantly (P = 0.00) higher in patients with endometriosis group (1.95 ± 0.69) compared with patients without endometriosis group (0.40 ± 0.15). The studied cytokines concentrations in FF of women with endometriosis group were significantly (P = 0.00) higher than those without endometriosis group: MCP-1 (548.07 ± 199.43 pg/ml *vs*. 45.98 ± 18.72 pg/ml), TNF-α (912.15 ± 316.12 pg/ml *vs*. 261.89 ± 90.69 pg/ml), IL-10 (258.94 ± 68.82 pg/ml *vs*. 52.12 ± 14.46 pg/ml). The immune milieu of FF of patients with endometriosis is modified, which could explain the effect of endometriosis on infertility. Furthermore, elevated MCP-1 expression in endometriosis patients suggesting systemic inflammatory changes.

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1. Introduction

Endometriosis is gynecologic condition recognized by existence of endometrial tissue out uterus [16]. The disease influences women of childbearing age causing infertility and abdominal ache [7].Table 1.

During endometriosis, systemic and local immunity is changed. The aggregation of T lymphocytes and activated macrophages which mediate the inflammatory reactions related to endometriosis that could influence endometrial and ovarian function [43]. Macrophages secrete cytokines. The Cytokines are peptides involved in cell proliferation, differentiation, and inflammation (Arango Duque *et al*., 2014). An alteration of cytokines has been demonstrated in peritoneal fluids, ectopic lesions, endometrial tissue and blood in patients with endometriosis [28,23,33,21]. Whereas, other studies have focused on cytokine profile in the follicular fluid (FF) in women with endometriosis [26,41].

Follicular fluid (FF) frames granulosa cell and oocyte combination and is interceding agent in correspondence between the cells in follicle [2]. Changes in molecular components of FF caused by endometriosis may influence fertility. Inflammatory factors in the follicular environment may affect the combination among infertil-

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Table 1
The designed Primers.

Primer	Sequence (5′→3′ direction)
MCP 1(primer for Gene Expression)	
Forward	**5'-AGAATCACCAGCAGCAAGTGTCC-3'**
Reverse	**5'-TCCTGAACCCACTTCTGCTTGG-3'**
TEGT(Testis Enhanced Gene Transcript)	
Forward	**5'-TGCTGGATTTGCATTCCTTACA-3'**
Reverse	**5'-ACGGCGCCTGGCATAGA-3'**

ity and endometriosis [20]. In women with endometriosis, higher FF concentrations of cytokines and macrophages have been demonstrated [44]. Changed balance of the immune agents could be contribute in alterations on oocyte maturation, folliculogenesis, and ovulation [29]. Certain cytokines, such as tumor necrosis factor-α (TNF-α) [13] and monocyte chemo attractant protein-1 (MCP-1) [5] found to stimulate leakage of the monocytes and macrophages from blood to peritoneal cavity. The MCP-1 is a monocyte and macrophage chemoattractant protein consists of 76 amino acids produced by monocytes and lymphocytes [37].

During IVF program, FF TNF-α levels was higher in endometriosis patients group [14]. An old study [19] reported that intrafollicular TNF-α concentrations were same in both unexplained infertility and endometriosis whom undergoing ICSI..

Recently, MCP-1 in both peritoneal fluid and FF were showed elevated concentrations in patients with endometriosis [18,8]. Various studies have demonstrated higher interleukin 10 (IL-10) concentrations in blood and FF of patients with endometriosis than control subjects [25,35].

The current research directed to examine the local impact of endometriosis on follicular inflammatory milieu. Hence, we studied some cytokines (TNFα, IL-10, and MCP1) regarding their local follicular levels in IVF cycles of women with and without endometriosis. Furthermore, the MCP1 origin was studied by mRNA analysis of patients with and without endometriosis.

2. Patients, materials, and methods

2.1. Study participants

A prospective case control study organized at Kamal AL-Samarai IVF center (Baghdad/Iraq), January 2021 - May 2021. The medical principle committee in Al-Nahrian University agreed the current research. All participants signed a documentary approval for research.

The research enrolled (80) infertile patients that underwent IVF cycles were split into following groups:

Patients with endometriosis group (n = 40; age = 32.20 ± 4.87 years). The endometriosis diagnosis by laparoscopy and other pathological diagnosis as reported by the American Fertility Society Classification [3].

Patients without endometriosis group (n = 40; age = 32.01 ± 4. 99 years). They were diagnosed with tubal infertility and normal laparoscopic findings.

The exclusion criteria: women with premature ovarian failure, cancer, polycystic ovarian syndrome, and infectious diseases.

2.2. Stimulation protocol

All patients had controlled ovarian stimulation by a standard long protocol, which begun on the day 21 of the mid-luteal (former menstrual cycle) with daily administration of subcutaneous GnRH-a injection (triptorelin) and continue to the day of HCG administration. When pituitary down-regulation was done (E2 level less than 50 pg/ml, menstruation take place, and endometrial thickness by ultrasound examination was ≤ 2–3 mm (Hugues *et al*., 2012). Ovarian stimulation begun with recombinant human follicle stimulating hormone (rhFSH) ampoules in a dose of 150 IU. The follicle growth and doses of Gonal-F® were monitored by *trans*-vaginal ultrasound (at cycle day 5) as well as by serum E2 levels (at day 6–8 of the Gonal-F® injection and until the day of HCG administration) (Dale *et al*., 2020). When two or three follicles reached 17–18 mm, ovulation induction was done by the recombinant hCG administration (rhCG 6500 IU, Ovitrelle®; Merck, Italy) subcutaneously [42].

2.3. Oocyte collection and FF and blood sampling

Oocytes were collected by needle. Uncontaminated samples of FF were separated (3000xg, 10 min) at lab temperature. The FF supernatant was conveyed to tubes and saved (-20 °C) till estimated. Venous blood was gathered at oocyte pickup day.

3. MCP-1 mRNA expression

3.1. Blood samples preservation with TRIzol reagent:

From each blood sample, 0.25 ml EDTA treated blood was placed to 0.25 ml RNase-free water in a separated tube to dilute and the levels of contaminating materials were reduced. From this mixture, 0.25 ml was aspirated and placed to 0.75 ml of TRIzol® LS Reagent in another tube. The ratio was (3 TRIzol: 1 RNase - water diluted blood). The samples were left at –23 C° overnight (Trizol LS Reagent, 2012).

3.2. Total RNA extraction with TRIzol

Total RNA extracted using the TRIzol® LS Reagent dependent on the protocol supplied by the manufacturer (Trizol LS Reagent, 2012), as follows:

3.3. Phase separation

The homogenized blood incubated at lab temperature for 5 min to accomplish nucleoprotein complex separation. Chloroform and TRIzol LS Reagent were placed in a tube. The tube was rattled robustly by hand, incubated at lab temperature, and separated by centrifugation. The mixture split up to upper aqueous phase (colorless) and lower phenol–chloroform phase (red). The RNA remained in aqueous phase which conveyed into microcentrifuge tube to be subjected to RNA isolation procedure.

The isolated RNA from the samples was reversely transcribed to cDNA using TransStart GreenqPCR Super MixKit(Trans Gen Bio-Tech,China). The procedure carried out in (20 μl) reaction volume and total RNA volume was (20 μl). The RT-qPCR was accomplished by the GoTaq Green Master Mix System Kit (Promega,USA) and cDNA as a template. Forward and reverse oligonucleotide primers of target *MCP-1* gene were designed and showed in table(1). The forward and reverse primers of the housekeeping gene TEGT were also given [27],as illustrate in table (1)

The reaction mix set to (10 μl) final volume and included (5 μl) GoTaq_ qPCR Master Mix (1X), for 2-Step RT-qPCR , 0.5 μl of each primer (10 mM), (2 μl) cDNA, and (2 μl) nuclease-free water. The mix transferred to real time thermocycler (MIC-4 Real-time PCR System, Australia). The expression was used as relative fold change ($2^{-\Delta\Delta Ct}$). Hence, data expressed as the fold change in expression of target gene which normalized to housekeeping gene and depending on calibrator, which is control subjects target gene [32].

3.4. Measurement of MCP-1, TNF-α, and IL-10 concentrations in FF

Human MCP-1 levels were measured in FF by human MCP-1 (Monocyte Chemotactic Protein 1) ELISA Kit, using sandwich ELISA method according to the manufacture's protocol from (MyBiosource incorporation, USA). Human TNF-α levels were measured in FF by human TNF alpha ELISA Kit, using sandwich ELISA method according to the manufacture's protocol from (abcam incorporation, UK). Human IL-10 levels were measured in FF by human IL-10 ELISA Kit (Interleukin-10) a, using sandwich ELISA method according to the manufacture's protocol from (abcam incorporation, UK).

3.5. Statistical analysis

Result analysis was carried out using SPSS version 26 (SPSS Inc. Chicago, IL, United States). Shapiro–Wilk normality test was utilized to set if the studied variables followed Gaussian distribution. Data were appeared as mean ± standard deviation (SD). Student's *t*-test was utilized to test variation among the studied groups. Statistical significant *p* value was less than 0.05 (Forthofer *et al.*, 2014).

4. Results

4.1. The mRNA analysis of MCP-1 in blood samples

The mRNA expression analysis of MCP-1 was found to be more highly expressed in patients with endometriosis group (1.95 ± 0.69) compared with patients without endometriosis group (0.40 ± 0.15). This result was statistically significant (P = 0.00) (Fig. 1).

4.2. Concentrations of cytokines (MCP-1, TNF-α, and IL-10) in FF:

The concentrations of MCP-1 in FF from patients with endometriosis group (548.07 ± 199.43 pg/ml) elevated significantly (P = 0.00) in comparison to women without endometriosis group (45.98 ± 18.72 pg/ml) (Fig. 2).

Similarly, concentrations of TNF-α in FF in patients with endometriosis group (912.15 ± 316.12 pg/ml) elevated significantly (P = 0.00) in comparison to women without endometriosis group (261.89 ± 90.69 pg/ml) (Fig. 3).

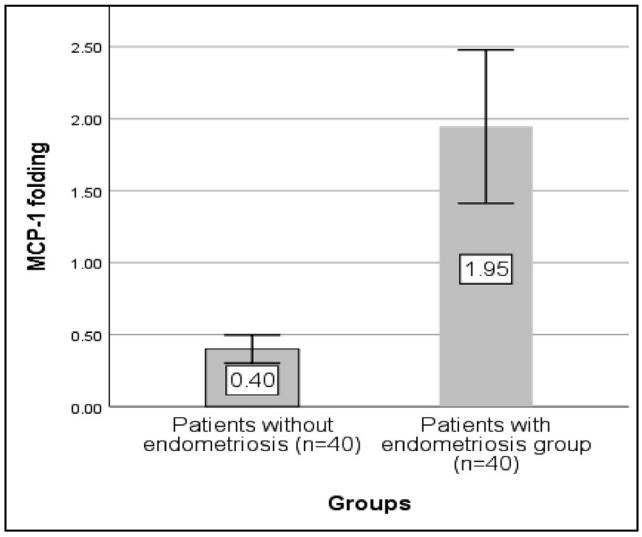

Fig. 1. Mean value ± SD of MCP-1 mRNA expression in blood of patients with and without endometriosis undergoing IVF cycles.

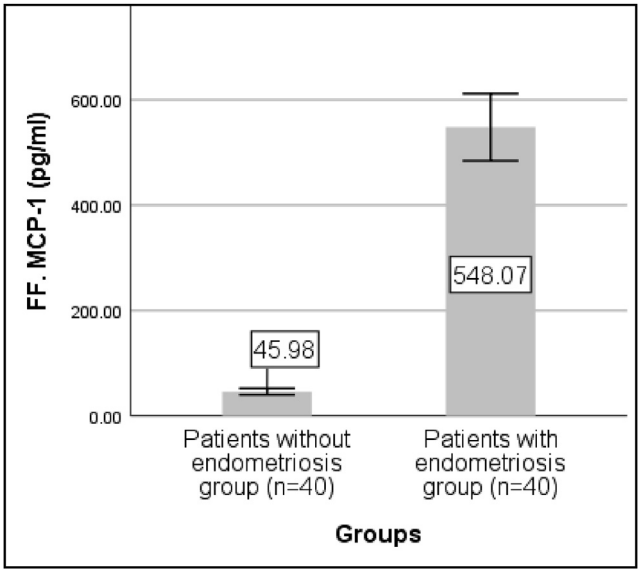

Fig. 2. Mean value ± SD of MCP-1 levels in follicular fluid of patients with and without endometriosis undergoing IVF cycles.

Concentrations of FF IL-10 elevated significantly (P = 0.00) in patients with endometriosis group (258.94 ± 68.82 pg/ml) compared with women without endometriosis group (52.12 ± 14.46 p g/ml) (Fig. 4).

5. Discussion

Different immune effectors play important roles during ovarian cycle; e.g. ovulation. Immune effectors in peripheral blood such as cytokines, are inducted to endometrium and ovary at luteal phase to plan for ovulation. The cells in follicles also secrete cytokines, which affect oocytes development. The immune-ovarian cell interactions are important combination of ovulation [39]. Abnormal immune responses in endometrium and ovary cause reproductive failures such as poor oocyte quality [38]. Accordingly, it is essential to know immune response throughout ovarian cycle. The cells in

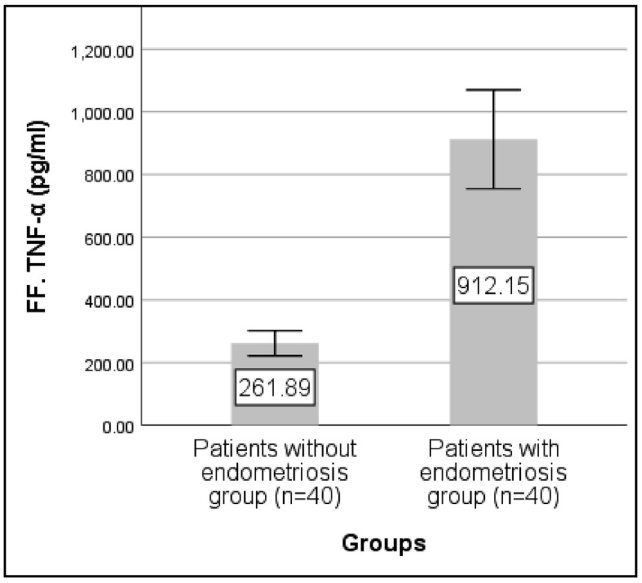

Fig. 3. Mean value ± SD of TNF-α levels in follicular fluid of patients with and without endometriosis undergoing IVF cycles.

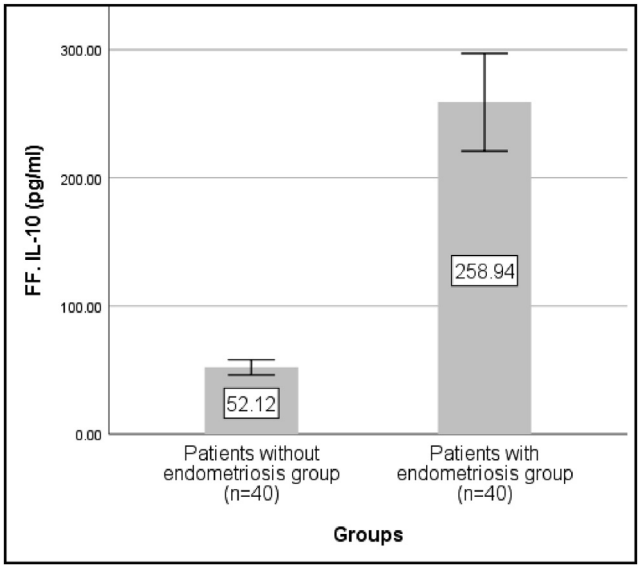

Fig. 4. Mean value ± SD of IL-10 levels in follicular fluid of patients with and without endometriosis undergoing IVF cycles.

follicles secrete cytokines, which influence development of oocytes. The immune-ovarian cells are important components of ovulation [39].

The FF provides a milieu that affects oocyte maturation and it has an important function in ovulation that affects fertilization and embryo development (Driancourt *et al.*, 1998).

In different infertility etiologies and following IVF treatment, intrafollicular cytokine were influenced oocyte, fertilization, and embryo quality [30]. Elevated FF cytokines may impair folliculogenesis and adversely affects oocyte quality in endometriosis conditions [24]

Current study revealed elevation in mRNA expression and concentrations of MCP-1 in human FF in endometriosis patients group, in comparison to without endometriosis patients group. An old research by Boucher *et al.* investigated the influence of ovarian hormones on MCP-1 production. The study found that higher MCP-1 mRNA expression and levels of patients with endometriosis in reply to interleukin-18. These results may explain elevated MCP-1 in serum, peritoneal fluid and eutopic endometrium (Boucher *et al.*, 2000).

Another study studied cytokines levels in FF and mRNA analysis of granulosa cells, in 153 individuals underwent IVF with different infertility etiologies (polycystic ovary syndrome PCOS, tubal factor, male factor, endometriosis, unexplained infertility, and other reasons). One of the studied cytokines was MCP-1, non-significant difference was found in MCP-1 mRNA of cumulus granulosa cells compared to mural granulosa cells. Whereas, MCP-1 in FF of endometriosis group were lowered than tubal factor group. The study found that different infertility etiologies are joined by different intrafollicular cytokines and some of the studied cytokines influence oocyte fertilization and embryo quality following IVF treatment [30]. The variation between the current study and Sarapik *et al.* study regarding FF MCP-1 levels between the studied group may be due to the difference in ovarian stimulation protocol, which in the current study is GnRH agonist long protocol whereas in Sarapik *et al.* study was GnRH antagonist.

Yih *et al.* study hypothesized that MCP-1 expression is early situation in endometriosis development and therefor participate to macrophages gathering in peritoneal fluid that start inflammatory reaction and lead to infertility by producing different cytokines. Furthermore, MCP-1 secreted by peritoneal macrophages into pelvic cavity [40].

Because of this negative effect of MCP-1, the effect of some drugs has been studied to reduce MCP-1 expression in endometriosis. Recently, Kolahdouz-Mohammadi *et al.* revealed that Resveratrol treatment reduce MCP1 expression. They recommended to obtain more results about resveratrol effectiveness in endometriosis treatment (Kolahdouz-Mohammadi *et al.*, 2021).

Similarly, Statins lowered the expression of MCP-1 in endometriotic cell and endometriotic implants of nude mouse model. Statins apply anti-inflammatory impact in the cultured endometriotic cells and give an expected therapy of endometriosis [9].

MCP-1 levels were studied in systemic (peripheral blood) and local (peritoneal fluid, FF, endometriotic lesions). Akoum *et al.* have shown that patients with endometriosis had increased leukocyte chemotaxis in their peritoneal fluid in endometriosis early stages (stages I and II) and suggested that MCP-1 may function in the inflammatory reaction [1]. These information are in agreement with other researches, which revealed higher MCP-1 concentrations in peritoneal fluid in endometriosis different stages [40,18].

Similarly, other researchers demonstrated higher FF MCP-1 in severe endometriosis cases. The study showed the oocyte microenvironment change combined with high FF concentrations of MCP-1. Therefore, they subdivided endometriosis patients group with a possible worse prognosis. Hence, they demonstrated that FF MCP-1 levels could be potential biomarker for endometriosis [8]. Indeed, data from Dahm-Kähler *et al.* indicated elevation MCP-1 concentrations in FF obtained from patients undergoing IVF procedure. At ovulation, MCP-1 is prompt by IL-1 and expressed in theca cells [10]. Similar to current research, Yland *et al.* found that levels of FF MCP-1 were higher from endometrioma affected ovaries. They proposed that endometriosis is associated with a local proinflammatory milieu, which has negative impact on oocyte development. They suggested presence of other agents to further explain the association between endometriosis and infertility [41].

Present study showed an elevation in FF TNF-α and IL-10 concentrations in endometriosis group. TNF-α and IL-10 detected in peritoneal fluid in endometriosis group complicated with infertility. Concentrations of IL-10 and TNF-α in peritoneal fluids were elevated in endometriosis conditions [34]. Another study observed significant increase in levels of TNF-α and IL-10 in serum and FF in endometriosis subjects subjected IVF program in comparison to control subjects [31], which would be in agreement with the present study results. Moreover, higher concentrations of FF TNF-α and the rest studied cytokines indicated the cytokines could affect quality of oocyte in women with endometriosis by negatively affecting the follicle milieu [36]. At ovulation, FF is a supporter of peritoneal fluid in endometriosis conditions and it would be coherent to contain higher levels of peritoneal cytokines [6].

6. Conclusion

The existence of elevated concentrations of FF MCP-1, TNF-α and IL-10 in endometriosis patients perhaps be consideration of local persistent inflammation (suggesting either local production or recruitment). Hence, the immune microenvironment in FF is changed. These changes may participate in the disease mechanism that affect adversely oocyte development and the subsequent poor IVF outcome in patients with endometriosis. Furthermore, elevated MCP-1 expression in blood of patients with endometriosis suggesting systemic inflammatory changes.

Future researches on mRNA local expression of MCP-1 of ovarian cells e.g., granulosa cells, as well as, investigating additional

factors in follicles local environment of patients with endometriosis, and their association with infertility and IVF outcomes.

7. Recommendation

Endometriosis group must be treated by anti-inflammatory drugs, to reduce higher MCP-1 mRNA expression and higher cytokines levels.

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CRediT authorship contribution statement

Zina F.H. Al-Obaidi: Writing – original draft, Software. **Farah Thamer Samawi:** Validation. **Rusul Hashem:** Visualization. **:** . **Bushra J. Al-Musawi:** Investigation. **Saad S. Al-Dujaily:** Conceptualization. **Hala Baher:** Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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