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Use of anti-interleukin 6 receptor monoclonal antibody for the treatment of Endometriosis

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IL-6 dysregulation is implicated in numerous clinical symptoms of endometriosis. Recent anti-IL-6 and anti-TNF therapies have resulted in clinical improvements in endometriosis patients' signs and symptoms as well as their quality of life. This study has shown the efficacy of tocilizumab drug as an anti-IL-6 receptor monoclonal antibody for endometriosis therapy in an experimental rat model. Il-6 expression is consistent with endometriosis in humans. As a result, this model might be useful in researching the pathogenesis and therapy of endometriosis. 18 female Sprague Dawley rats were employed as an experimental model for endometriosis. Following the induction surgery of endometriosis, the surviving rats were separated into two groups: the treatment group, which obtained tocilizumab, and the control group, which received saline. The volumes and histopathological properties of the implants were evaluated before and after the induction surgery and the usage of the TCZ medication. IL6 expression was evaluated via qRT-PCR in all lesions. Immunohistochemistry was also used to detect tumor necrosis factor alpha (TNF-α) intensity in ectopic& eutopic endometrium. There was a significant difference in post-treatment spherical volume between the TCZ and control groups. Implant volumes of TCZ group were lower than those of the control group respectively (70.74± 51.15 VS 113.58± 50.69; P= 0.04^{b}). Comparing eutopic with ectopic lesions, we demonstrated that IL-6 gene expression inside the control group was higher than in the TCZ group. According to histopathological evaluation, the epithelium of eutopic endometrium in both groups was observed to be more preserved than the ectopic implant. Interleukin 6 blockers, which have been utilized to treat related auto-immune disorders, might open up a recent avenue for the use of an immune-modulatory biologic medication for endometriosis therapy.

Keywords: Infertility. Endometriosis. Interleukin-6.Tumor Necrosis Factor-α.

INTRODUCTION

Infertility has been known as a failure to institute a typical pregnancy after one year of regular, unprotected sexual intercourse or due to debilitation of a person's ability to reproduce alone or with his /her partner (Zegers-Hochschild et al. 2017). About 18% of general society is prone to infertility (Hanson et al. 2017). However, fewer studies have focused on the pathophysiology of the infertility. Infertility is not a distant condition and can affect various systems in the body. Despite the fact that both genders were equally likely to be involved in the individual's infertility, women have historically suffered from the scar of infertility (Turner et al. 2020). Disorders in female reproductive system are linked to many abnormalities. These anomalies can cause major symptoms such as discomfort, frequent urination, and irregular menstruation, as well as reproductive issues such as miscarriage and infertility. The most prevalent disorders that can lead to infertility in women are premature ovarian failure (POF), polycystic ovary syndrome (PCOS), endometriosis, Asherman syndrome, and preeclampsia (Zhao et al. 2019).

Endometriosis is one of the most prevalent gynecological disorders; It is diagnosed in 21-40% of infertile women. Clinically, pain and infertility are considered to be the most common symptoms of endometriosis. These symptoms may be accompanied by dyspareunia, dysuria, dyschezia, and aperiodic urinary symptoms (Parasar et al. 2017). It is characterized by the growth of extra-uterine tissue leading to infertility in nearly half of these cases. Several causes have been suggested, including the placement of the endometrium near the fallopian tubes during menstruation and immune dysfunction (Lorzadeh and Kazemirad, 2020). Immune system plays a major role in the development of the endometriosis, especially by inhibiting lymphocytes, natural killer cells, cytotoxic T cells, exacerbated macrophages, and inflammatory responses (Lorzadeh and Kazemirad, 2018). In addition, any disruptions occur in the secretion of cytokines, chemokines, and hormones may exacerbate the disease (Lin et al. 2018).

Interleukin 6 (IL6) is an inflammatory cytokine that has

a crucial role in inflammation and tumor development. It is a major agent in IL6 cytokine family that is responsible for a variety of biological, metabolic and immune tasks (Hunter and Jones, 2015). It is a pleiotropic cytokine secreted by macrophages that stimulates endometrial cell proliferation and angiogenesis in endometriosis (Symons et al. 2018). It is elevated in peritoneal fluid (PF)and serum of women with endometriosis (Kang et al. 2014; Kashanianet al. 2015). Studies indicated that IL-6 disorders may participate to the pathology of endometriosis. Elevated IL6 levels have been reported to suppress natural killer cell (NK) action in endometriosis patients by regulating the expression of the tyrosine phosphates-2 (SHP2) (Kang et al. 2014).

In patients with such inflammatory disorders, the overregulation of IL-6 and its soluble receptor (IL-6R) results in a combination of IL-6 and soluble IL-6R that activates gp130 and creates inflammatory impulses. To heal these patients, we established an anti-IL-6R antibody to block the IL-6 signaling resulting from interactions of IL-6 with its surface receptor as well as the neutralization of its soluble ligands (Nishimoto et al. 2000).

Tocilizumab (TCZ) is a humanized recombinant monoclonal antibody which inhibits the downstream classic signaling and trans-signaling cascades involving the Janus-activated kinase-signal transducer and activator of transcription (JAK-STAT) pathway by targeting both membrane & soluble IL-6R (Tanaka et al. 2014). This action may prevent IL-6 from binding to both the IL-6R and the signal transducer glycoprotein 130 complex. TCZ also reduces serum macrophage migration inhibitory factor levels (Richez et al. 2012; Kasama et al. 2014), as well as T helper 17 (Th17) cell levels, while raising regulatory T cell (Samson et al. 2012; Pesce et al. 2013).

Surgical and hormonal therapies for endometriosis are currently available; however, the high risk of disease recurrence and the negative effects of these treatments limit their usage over time (Greene et al. 2016). The purpose of this research was to investigate more about the humanized monoclonal anti-IL6 receptor antibody (tocilizumab) as a biological treatment for endometriosis.

MATERIALS AND METHODS

Animal Model Preparation

In this experiment 18 Female Sprague-Dawley rats of cycling reproductive age from Mansoura Experimental Research Center (MERC) animal house, weighing 180 to 250 grams were employed. This study took into account animal care recommendations and laboratory animal care principles.

Experimental Induction of Endometriosis

Endometriosis was induced as previously described (Lebovic et al. 2004). Rats were administrated intramuscularly (IM) with 50 mg / kg mix of ketamine and xyline before being opened through 5-cm vertical abdominal incision. The left horn remain to represented normal endometrium while right horn had been incised longitudinally, divided into two sections, and fixed in 37°C phosphate buffered saline (PBS). These sections had then been sutured to the right side of the peritoneal cavity with 5-0 Vicryl (Polyglactin 910, Ethicon, NJ). All rats were injected with 5 mg / kg estradiol benzoate IM biweekly for a month.

A month later, the second surgery was performed to reveal endometrial implants. Photos were taken for these implants with a digital camera. Implant volume was determined using prolate ellipsoid equation = a x b x c x $\pi/6$mm3 where a, b &c signify width, length, and height, respectively. Tissues were then removed from these endometrial implants and quickly converted to paraffin blocks for staining with hematoxylin and eosin stain (HE). To establish the existence of endometriosis, the tissue was observed via a light microscope.

Because the implants were not viable, one rat was discarded from the trial. Also two rats died due to surgical complications. The remaining rats (15) were divided into two groups: TCZ-group (10 rats) and control group (5 rats). Rats in the TCZ-group obtained 8 mg/kg tocilizumab (Actemra; Roche, Switzerland) intraperitoneal biweekly till 4 weeks, whereas rats in control group received saline (0.9 %) at a comparable dose and frequency.

One month later, final surgery was performed. All rats were anesthetized, photos were taken and implants volumes were also calculated after theusage of the medication. The eutopic and ectopic endometrial tissues were removed from each left and right uterine horns, then all rats were sacrificed.

RT-PCR for IL6

RNA Isolation

Tissues were collected and promptly in liquid-frozen nitrogen for RNA isolation. The expression of IL-6 had been evaluated in eutopic and endometriotic lesions. The TRIzol (Invitrogen, USA) has been used to extract RNA from the samples according to manufacturer guidelines. Nano-Drop 2000c (Thermo Scientific, Germany) was utilized to assess the RNA's purity and integrity. The ratio of optical density at wavelengths 260and 280(260/280) was used to determine the quality of RNA. A ratio of 1.8 to 2 indicates a good quality of RNA.

cDNA Synthesize

RNA was reverse transcribed using a Complementary DNA (cDNA).The IL-6 cDNA amplification process began through10-minute denaturation at 95°C, followed by 30 seconds of denaturation at 94°C, 60 seconds of annealing at 56°C, and 60 seconds of extension at 72°C. After normalization with the reference gene (GAPDH), transcripts were measured.

GAPDH cDNA amplification began with a 5-minute denaturation at 95°C, followed by cycles of 60 seconds denaturation at 95°C, 60 seconds annealing at 75°C, and 60 seconds extension at 72°C. IL-6: sense 5'-ACC CTT CAG GAA CAG CTA TGA-3', antisense: TCT CAA CAA CAT CAG TCC CAA GA-3'& GAPDH: sense: 5'-AGA CAG CCG CAT CTT CTT GT-3', antisense: 5'-TTC CCA TTC TCA GCC TTG AC3'. Threshold cycle (CT) and fold change in expression of each gene was calculated by using the following equation $2^{-\Delta\Delta Ct}$.

Histologic Examination

Endometrial tissues were obtained and fixed in 4% par formaldehyde at 5°C for a week. After that, the tissues were rinsed in sterile water for a few hours. The tissue was dehydrated in ethanol solutions, then combined with equal parts ethanol and xylene. After 15 minutes of incubation, the tissue was combined with an equivalent amount of xylene for another 15 minutes. These techniques were continued until the tissue was clear. The tissue was then fixed in paraffin for five-minutes at room temperature, sectioned at a thickness of 5m, and stained for 3 minutes at room temperature with H&E. A light microscope had been used to capture images (Olympus CX31, USA).

Immunohistochemistry

Antibodies against TNF-α (Thermo scientific, USA) were used for immunohistochemical staining. The techniques were undertaken in conjunction with manufacturer's instructions. Lesions were washed then incubated for 15 mins in citrate buffer at room temperature till 20 minutes. After being processed with 3 percent H2O2, the portions were rinsed in PBS in order to inactivate endogenous peroxidase. After washing, The portions were probed with HRP conjugated for one hr. DAB substrate was used to visualize immunolabeling, portions were rinsed and dehydrated before being covered. Bright field microscope was used to examine the sections (Olympus CX31)

Statistical Evaluation

SPSS for Windows version 25.0 was used to analyze the data. GraphPad Prism was used for the statistical analysis (GraphPad Software 9). Where possible, numerical variables were provided as mean + standard deviation. Mann– Whitney U test had been performed to assess the volume changes and the expression level of IL-6. the data for immunohistochemistry for IL6 and TNF-α was subjected to a Student's t-test, A P value of less than.05 was considered significant.

RESULTS

The relative expression level for IL-6 gene was determined using the $2^{-\Delta\Delta Ct}$ equation, with GAPDH as a reference gene.

In the control group, IL-6 expression in the ectopic lesions (2.38 ± 0.39) were be higher than the eutopic (preserved left horn) (1.04 ± 0.36) with p value 0.008^b. In the treated group, cytokine IL-6 expression in the eutopic lesions (0.3± 0.25) was higher than its expression in the ectopic lesions (0.12 ± 0.26) with p value 0.002^b ($p < .05$).

In eutopic lesions, IL-6 expression in control group had been found to be higher than TCZ group respectively (1.04 ± 0.36 VS 0.3 ± 0.25; $P = 0.001^b$). In ectopic lesions, IL-6 expression levels of the control group was found to be higher than the TCZ group respectively (2.38± 0.39 VS 0.12± 0.26; $P = 0.001^b$).

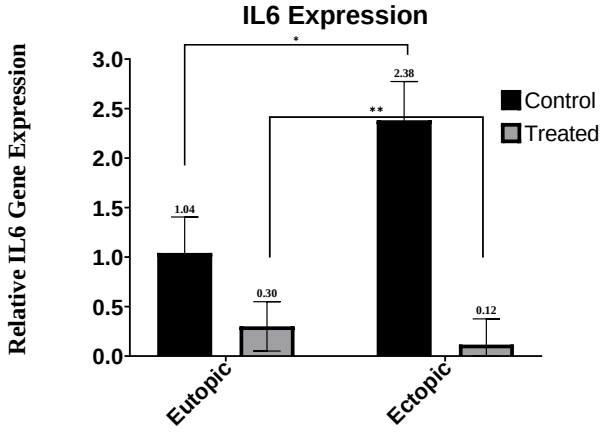

Figure 1: The relative fold change in interleukin (IL)6 expression normalized against the expression of reference gene GAPDH in eutopic&ectopic endometrial tissues when treated with TCZ compared to control group.

The data is available as a mean± standard deviation. *P < 0.05 and **P < 0.01, respectively, were regarded as statistical differences.

Morphologic and Histologic Evaluation

Pre- and post-treatment implants volumes inside TCZ and control groups were shown in (Table 1). TCZ and control displayed similar pretreatment spherical volumes $P= 0.7^b$. After TCZ therapy, implant volumes of TCZ group had been significantly lower than control group (70.74± 51.15 VS 113.58± 50.69) respectively $P= 0.04^b$. For TCZ group, the difference between pre-treatment (110.1± 82.4)& post-treatment (70.74± 51.15) was considerably significant P= 0.007.

Table 1: Pre-& post-treatment of endometrial implants volumes in treated and control groups.

Group	Pre-treatment	Post-treatment	P
Control (n=5)	109.32 ± 47.09	113.58 ± 50.69	0.088
TCZ (n=10)	110.1± 82.4	70.74± 51.15	0.007
P	0.7^b	0.04^b	

Values presented as mean ± SD. N represent to number of rats in each group.

Histopathological examination of the endometrial

implant has made it possible to make an experimental diagnosis of endometriosis. Morphologically, endometrial cysts had been found to be strongly vascularized similar to human peritoneal endometriosis (Plate 1).

Histopathological inspection of the ectopic lesions in the control group stained with H&E revealed hemorrhage, congested blood vessels, inflammatory cell infiltration with excessive collagenous stroma, while the TCZ group demonstrated a decrease in inflammatory cell infiltration, blood vessel congestion, but there was a little interstitial hemorrhage (Plate 2). In addition to that, the epithelium of the cystic implants in control group was observed to be more preserved (normal).

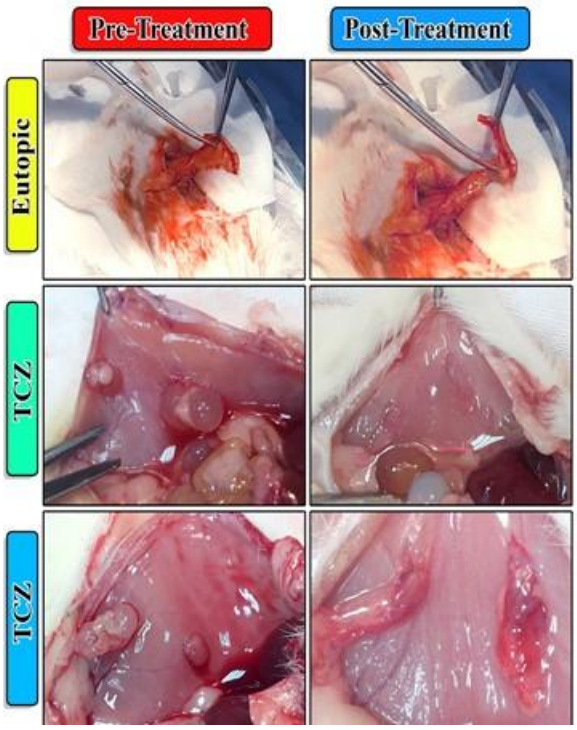

Plate 1. Morphological appearances of endometriosis foci in a rat model. Pre- and post-treatment of the eutopic endometrium (left horn) remained intact in both control and TCZ groups. Endometriotic vesicles before TCZ treatment appeared to be cystic and highly vascularized however, after TCZ treatment the cystic became tiny and in some cases, it completely disappeared.

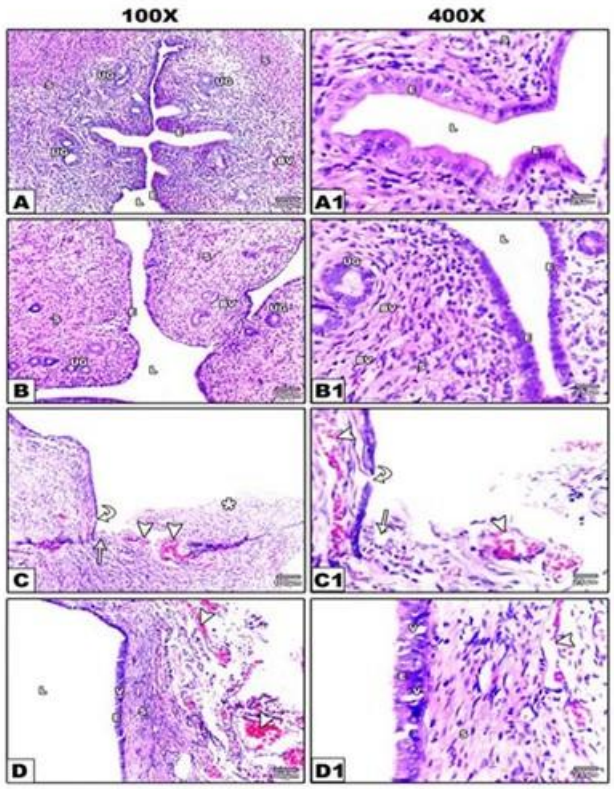

Plate 2. Photomicrograph of histopathological appearance of eutopic& ectopic endometrial lesions in control and TCZ groups. where A, B, C &D with bar= 100µm A1, B1, C1&D1 with bar= 25µm. H&E staining. Curved arrow represented damaged epithelial layer, arrow represented unique epithelium& arrow head represented hemorrhage.
Abbreviation: Stroma, S; Uterine Gland, UG; Lumen, L; Vessels, V; Glandular Epithelium, E; Vacuole, V**.**

TNF-α Immunodistribution

TNF-α immunoreactivity was found in the cytoplasm of endothelial cells, glandular epithelial cells, and diffusely in stromal cells in both eutopic and ectopic endometrial tissues (Plate 3). Endometriosis immunoreactions to TNF-α was stronger than eutopic endometrium, as predicted.

The intensity of TNF-α immunostaining in the ectopic lesions of the control implants were considerably higher than the TCZ implant. When we evaluated eutopic endometrial tissue for TNF-α staining in both control & TCZ groups, we detected that TNF-α immunoreactivity intensity was similar in both group.

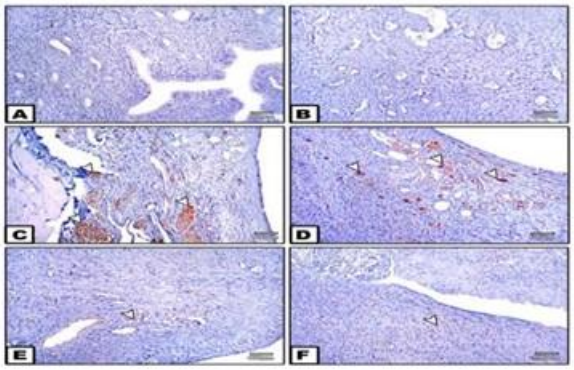

Plate 3: Representative photomicrographs of TNF-α immunostaining in the experimental rat model. Immunostaining in endometrial eutopic lesions exhibited week TNF- α immunoreaction in (A) TCZ eutopic lesions and (B) control eutopic. (C) and (D) control ectopic implant demonstrated strong TNF-α immunoreaction. (E) and (F) TCZ group ectopic implant demonstrated mild TNF-α immunoreaction.

DISCUSSION

Endometriosis is a condition that affects child bearing female and is distinguished with symptoms ranging from pelvic pain to infertility. Despite being a benign gynecological disease, endometriosis remains to be a debilitating disease for females, due to the symptoms that develope. Complex interactions between genetic profile, hormonal activity, menstrual cycle, inflammatory status, and immunological factors determine the appearance of the endometriosis phenotype (Filip et al. 2020).

Many anti-endometriosis medicines or prospective treatments, such as hormonal and immunomodulatory agents, impact the amount and profile of cytokines in endometriosis. Therefore, treatment of endometriosis targets both inflammatory and anti-inflammatory cytokines, innate macrophages and NK cells when compared to suppressing only inflammatory cytokines. May be more effective for the treatment of endometriosis (Zhao et al. 2019).

In this study, endometriosis experimental rat model was used to explain tocilizumab (TCZ) as an effective medicine for endometriosis. According to our current results treatment with TCZ suppressed IL-6 gene expressions in endometrial tissues, shown histological differences in epithelial layer, decreased implant volumes, and also reduce immunoreactive intensity of TNF-α.

IL-6-encoding mRNA transcripts were detected in ectopic &eutopic lesions using quantitative RT-PCR in order to investigate gene expression. Endometriotic lesions had excessive proportion of IL-6 mRNA transcripts than eutopic endometrium in control group (Fig. 1). After the usage of TCZ drug, expression level of IL-6 in the TCZ group in ectopic endometrium was lower than those in the eutopic endometrium (0.12 ± 0.26 VS 0.30 ± 0.25; $P=0.002^{b}$). which mean that in control group the endometriotic implants shown higher levels of IL6 expression than in normal eutopic lesions due to inflammation, while after TCZ usage the expression of IL-6 reduced by blocking IL-6 signals.

Previous research has shown that anti-IL-6 therapy can help cure endometriosis in the Wistar rat model (Taskin et al. 2016). They used a similar method to induce endometriosis, our findings are consistent with their. They revealed that TCZ drug decreased implant sizes and significantly debilitated the epithelial layer in ectopic endometriotic lesions compared with the eutopic one. They also found IL-6 efficacy appeared to be largely dependent on VEGF inhibition, despite the fact that they considered TCZ as an IL-6 inhibitor, since immunohistochemistry investigations in their work indicated that TCZ group reduced strength of VEGF immunohistochemistry, whereas interleukin 6 reactivity had been comparable across control and TCZ-group, in a contrast El-Zayadi et al. (2020) discovered that the immune-histochemical stain strength of ectopic epithelium had been strong across all control animals, but weak in 9 of 14 tested animals with no statistically significant.

Also our recent study shown that TNF-α immunoreactivity intensity in the ectopic lesions of the control group was considerably moderate while it was mild in ectopic implant after usage of TCZ drug showing the impact of the anti-IL-6 in the expression of TNF-α.

Previous researches have indicated the use of tumor necrosis factor alpha inhibitors as recent approved treatment for endometriosis. According to D'Hoogheet al. (2001), anti-tumor necrosis factor drugs were viable non-hormonal medications for endometriosis. In the course of their research, endometriosis had been averted in baboons whom pelvic cavity had been injected with menstrual aspirate cured with recombinant TNF receptor-1. Endometriotic lesions were identified in control animals, but no lesions were found in the tested animals whose menstrual aspirate had been depleted of TNF. In a separate trial, Barrier et al. (2004) provided etanercept to baboons with spontaneous endometriosis 3 times a week via subcutaneous injection. Etanercept is one of the medications used to inhibit TNF-α effects on endometriotic tissues. They discovered a statistically significant decrease in the treatment group's red lesion, indicating that etanercept lowers quantity of the developing endometriosis inside baboons.

Also as stated in Zulfikaroglu et al. (2011) study, by induction of endometriosis using mature female Wistar-Albino rats, Compared to the control group, etanercept

dramatically reduced the extent of endometriosis as well as lower PF and serum of several cytokines in ectopic lesions. Furthermore, histopathological evaluation shown stroma and endometrial glands in endometrial implants and the control cystic implant epithelia were shown to be more persistent than tested group. In contrast to their results, no improvement in endometriosis was seen during laparoscopy after years of continuous usage in a patient with stage four who underwent successful *in vitro* fertilization (IVF). Such instance demonstrated that the use of TNF-inhibitors prior IVF improves the chances of success in severe endometriosis despite having no impact on infertile, demonstrating that immune anomalies in the pelvis weren't the problem (Shakiba and Falcone, 2006).

Immune deregulation and systemic inflammation play critical roles in the progression of endometriosis in the peritoneal microenvironment (Ahn et al. 2015) due to increased levels of pro-inflammatory cytokines and chemokinesthat have been detected in the PF of endometriotic patients compared to non-endometriotic participants, these secretions suggested, they had a role in the onset and evolution of endometriosis by stimulating endometrial angiogenesis, adhering, invasion, and proliferate (Borrelli et al. 2014 ; De Andrade et al. 2017; Jiang et al. 2019).

Under various inflammatory, environmental, and genetic effectors, interleukin 6 contribute to genesis of endometriosis via inhibiting ectopic implants apoptosis in the peritoneum with TNF (Dyson and Bulun, 2012). It can also promote endometriotic implant migration, which can lead to the development of extra-pelvic endometriosis (Woo et al. 2017).

E2 has been discovered to greatly enhance endometrial cancer growth and invasion (Zhang et al. 2018; Yaguchi and Onish, 2018). Furthermore, evidence showed that blocking IL-6 antibodies inhibited the proliferating and invasive capacity of endometrial tumour cells stimulated by E2, indicating that E2 may stimulate tumor development via the IL-6 signaling pathway. The Food and Drug Administration (FDA) has approved inhibitors of the IL-6 pathway, including IL-6, IL-6 receptor, and janus kinase (JAK), for treatment of a variety of malignancies, and additional novel inhibitors of the IL-6/JAK/Stat3 signaling pathway are in clinical and/or preclinical trials (Jones et al. 2011; Huynh et al. 2017;Johnson et al. 2018).

Furthermore, it could be shown that the inhibition of IL6 not only significantly suppresses the proliferating and invasive capacity of E2-induced endometrial cancer cells, but also reduces the increase in the expression of pStat3. During malignant transformation, Stat3 is constitutively activate by phosphorylation in response to IL6 stimulation and then becomes homo- or heterodimers, which migrate from the cytoplasm into the cell nucleus and act as specific transcription activators for a number of downstream genes (Johnson et al. 2018). It was discovered that E2 may stimulate IL6 / Stat3 phosphorylation while attenuating IL6-Ab enhancement, implying that E2 enhanced IL6 production, which subsequently triggered Stat3 phosphorylation, while the IL6-neutralizing antibodies prevented Stat3 activation (woo et al. 2017).

Anti-IL6 inhibition has been shown in animal experiments to be beneficial in the treatment of endometriosis. It has also been reported that blocking IL-6 signaling using an anti-IL6R antibody can limit the proliferation of ectopic tissues in animal models, resulting in an improvement in disease activity due to a reduction in inflammation.

CONCLUSION

Tocilizumab suppression IL6 gene expression and vesicle volume of endometrial implants. This is obviously confirmed in our study by the histological and immunohistochemical data demonstrating morphologic degenerative alterations with TCZ therapy. If proven in people, this therapy might pave the way for effective non-hormonal therapy for endometriosis. More experimental and clinical research is needed before this therapy may be used in clinical practice.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEDGEMENT

It has been a delight to work with such a kind and encouraging team. We attempted to provide a new overview that may be useful in the future in endometriosis therapy as one of the most prevalent diseases that affect women, as well as to highlight certain crucial topics without drowning the reader in superfluous detail.

AUTHOR CONTRIBUTIONS

NMF designed and performleed the experiments and also wrote the manuscript. AME, AAE, and YMT performed animal treatments, RT-PCR, tissue collection, and data analysis. AME and AAS suggested the work. designed experiments and reviewed the manuscript. All authors read and approved the final version.

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