



Evidence Supporting the Diethylstilbestrol Induction of Endometriosis Via Immune-Inflammatory Pathway in Rat Model

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Abstract: Endometriosis is a benign reproductive disorder distinguished by chronic inflammation and anomalous progress of stromal cells and glands outside the uterus. Though the etiology of endometriosis is still a matter of debate, the immune-inflammatory mediators are thought to play a major role. Macrophage infiltration and proinflammatory intermediaries in the peritoneal environment peritoneal milieu impact ovarian, uterine function and pelvic morphology, resulting in endometriosis symptoms and signs. The endometriosis model system was developed using diethylstilbestrol (DES) which is examined to develop the disease via the immune-inflammatory pathway. The immune-inflammatory markers (NFkB, MAPK, TNF- α , IL-6 and STAT-3) were investigated in this study. The results demonstrated that the DES induced endometriosis animal model had a higher level of CA-125, a biomarker of endometriosis. Endometriosis-induced groups had increased levels of NFkB, MAPK, TNF- α , IL-6 and STAT-3 which clearly depicts the immune-inflammatory role in the induction. Finally, the results conclude that DES induced endometriosis via the immune-inflammatory pathway.

Keywords: CA-125, DES, endometriosis, inflammatory pathway.

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1. INTRODUCTION

Endometriosis is an estrogen-reliant benign gynecological disorder which is described by the proliferation of endometrial glandular structures and stromal cells outside the uterine cavity.¹ Endometriosis is known to cause deleterious effects in the reproductive aged women including the quality of life and affects 5-10% of them.² Endometriosis is clinically subdivided in to peritoneal superficial lesions, deep infiltration endometriosis and endometriomas and scored on their severity as per American Fertility Society Classification -minimal, mild, moderate and severe.³ It is diagnosed by clinician with several factors which includes severe pelvic pain, heavy menstrual bleeding, dysmenorrhea, infertility, dyspareunia and pelvic inflammatory disease or irritable bowel syndrome.⁴ The pro-inflammatory mediators and the endometrial cells invasion causes severe pelvic pain. Endometriosis also reduces the ability of implantation, increasing the chance of pregnancy loss. Furthermore, endometriosis symptoms reduce women's life quality by interfering with their productivity, social life, and emotional health. Due to the lack of knowledge in the pathological mechanisms of endometriosis, the diagnosis and treatment of endometriosis remains a challenge.⁵ There are several theories on the pathophysiology of endometriosis and the widely acknowledged of which is retrograde menstruation theory. Endometriosis pathophysiology is influenced by many factors, including hormonal imbalance, estrogen reliance, progesterone resistance, angiogenesis, inflammation, oxidative stress, and immunological signals, all of which may aid in the formation and spread of the lesion.⁶ Although various mechanisms contribute to the etiopathogenesis of endometriosis, it is thought that endometrial lesion development is caused by disruptions in immune surveillance. Indeed, patients' immune responses to endometriotic lesions have been dysregulated, with elevated inflammatory cytokines and over active macrophages and neutrophils in the peritoneal cavity.⁷ The existence of endometrial lesions promotes stress and the gradual progress of inflammation, while macrophages trigger growth and angiogenesis, inflammatory cytokines, which may be responsible for the endometriosis development, maintenance, and impairment of reproductive functions.⁸ The intricate proinflammatory environment swarming endometriotic lesions promotes their proliferation and vascularization. Inflammatory agents excite nerve fibers in the endometrium and endometriotic lesions.⁹ During the time of immune-inflammatory reaction, immune cells get triggered and produce high amounts of growth factors, cytokines and angiogenic factors. Out of which, cytokines including Interleukin, VEGF, TNF- α etc., has been studied extensively in relation of the pathogenesis of endometriosis and considered as the diagnostic markers.¹⁰ Interleukin performs an essential part in the inflammatory and immune response. Activated macrophages produce a pro-inflammatory cytokine - Tumor necrosis factor α . TNF- α triggers other important pro-inflammatory cytokines, such as IL-6, IL-1 etc., Pro-inflammatory cytokines are required for the endometrial growth, proliferation, and the shedding during the normal reproductive physiology.¹¹ Besides the human based *in vitro* systems, extensive studies on immune-inflammatory responses in endometriosis needs a model organism to study effectively. The development of such a model sounds difficult which should exhibit main characteristics of endometriosis. Researchers develop endometriosis in the animal model by surgical method which might be difficult. In a previous study an alternative induction

of endometriosis using Diethylstilbestrol was attempted. Diethylstilbestrol is an endocrine disrupting chemical known to be synthetic estrogen. In the early 1940s DES was believed to reduce pregnancy losses. But in the 1970s, a case study showed the risk of cervical and vaginal carcinoma for the patient prescribed with DES and was banned in early 1980s. Several patients exhibited various reproductive anomalies especially infertility, cysts in uterus, miscarriage.¹² Our previous study demonstrated the successful development of endometriosis by diethylstilbestrol (DES) instead of the traditional surgical induction in the animal model.¹³ Further in this study we aimed to prove endometriosis induction of diethylstilbestrol in animal model ensues via immune-inflammatory pathway.

2. METHODS

2.1 Animal model

The female Wistar albino rats of 10- 12 weeks (N =10; Control – 5 & Endo - 5), with the weights of 150 - 200 g were used in this study. The animals were maintained at $21 \pm 2^\circ\text{C}$, a humidity of 60%, and in a 12-hour light and 12-hour dark cycle. The experiment was conducted under the approval of regulatory body at Bharathidasan University, Tiruchirappalli controlled by CPCSEA, India (BDU/IAEC/P24/2018 dt. 07.08.2018).

2.2 Endometriosis induction

Endometriosis was induced in the rat model which was described in previous study.¹³ Briefly, the animals showing regular estrus cycles were chosen for the study and grouped into Control (N=3) and Endo (N=3). Endo group animals received DES (Diethylstilbestrol, Sigma Aldrich, USA) for 20 days at a concentration of 1mg/kg/bw via subcutaneous route of injection. The animals were sacrificed after the experimental period, and the samples were collected and stored for further process.

2.3 Measurement of serum level of CA-125

The serum samples required for the experiment were collected and level CA-125 was assayed using commercially available Enzyme Linked Immunosorbent Assay kit (AFG Scientific, USA) as per the guided instructions from the manufacturer. Briefly, the sample and standards were added to the 96 well plate and incubated for 30 mins at 37°C . After incubation, the plates were washed with a washing buffer five times. HRP conjugated enzyme ($50\mu\text{L}$) was added and incubated for 30 mins at 37°C . After washing, the chromogen solution ($50\mu\text{L}$) was added and incubated for 15 min at 37°C . A stop solution was added ($50\mu\text{L}$) when the color changes were observed, and optical density (OD) was measured at 450 nm Microplate Spectrophotometer (Thermo Fisher Scientific, USA).¹⁴

2.4 Histological assessment

After the experimental period, the dissected uterine samples were fixed using 10% formalin and dehydrated using different concentrations of xylene and ethanol. Uterine samples were embedded in paraffin wax and tissue sections with the thickness of $5\mu\text{m}$ were taken using a rotary microtome (Leica, USA) and mounted on a glass slide and dried. The sections were then deparaffinized, rehydrated and stained using

Hematoxylin and eosin (Himedia, USA) and pathologically identified using light microscope (Magnus, India).¹⁵

2.5 Real Time PCR expression analysis

The Trizol reagent (Invitrogen, USA) was used to isolate total RNA from the uterine horn. The mRNA concentration was measured using the Microplate Spectrophotometer (Thermo Fisher Scientific, USA) at 260/280 nm. The RNA isolated was reverse transcribed into cDNA using Super Script III First Strand Synthesis System (Invitrogen, USA) and the relative expression of NFKB, MAPK, TNF- α , IL-6 and STAT-3 was studied using quantitative PCR (Roche, USA) with β -actin as the internal control.¹⁶

2.6 Statistical analysis

The GraphPad Prism software version 7.0 was used to analyze the data acquired. The values obtained were represented as Mean \pm Standard Deviation with student t-test to measure the statistical significance.

3. RESULTS

3.1 Higher levels of CA-125 confirms the endometriosis induction

The experimental animals were screened throughout the period. The estrus cyclicity was continuously monitored in both the control and endo group animals to confirm the regularity and alterations in the estrus cycle and the body weight was also monitored. The endo group displayed alterations in the estrus cycle showing irregular pattern of the cycle and the body weight which is evidenced in the previous study¹³. Further to confirm the induction, the experimental animals were analyzed for the levels of CA-125 which is an effective biomarker in the diagnosis of endometriosis. The serum levels of CA-125 in the endometriosis induced group was significantly increased (Control: 236 ± 2 vs Endo: 452 ± 6) when compared with the control which supports the successful induction of endometriosis in the model (Fig 1).

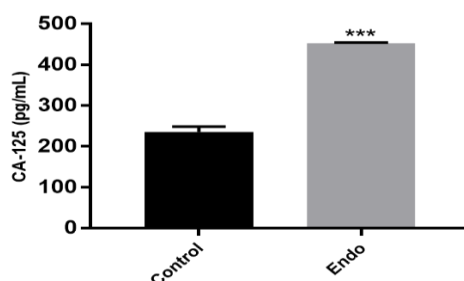


Fig 1: Endo group showed higher levels of CA-125 compared with control. The data is represented as mean \pm SEM, n = 3, *p < 0.001**

3.2 Histopathology of uterine horn confirms the endometriosis induction

The histopathological examination of uterine horn revealed the induction of endometriosis. The sections were observed in the microscope. The control uterine horn showed the normal pathological structure of endometrium containing

glands, stroma and epithelium. Whereas the endo group animal exhibited abnormal pathology such as degenerated endometrium, increased glandular structures with variations in shape. The number of macrophages has also increased in the endo group which altogether confirms the induction of endometriosis (Fig 2).

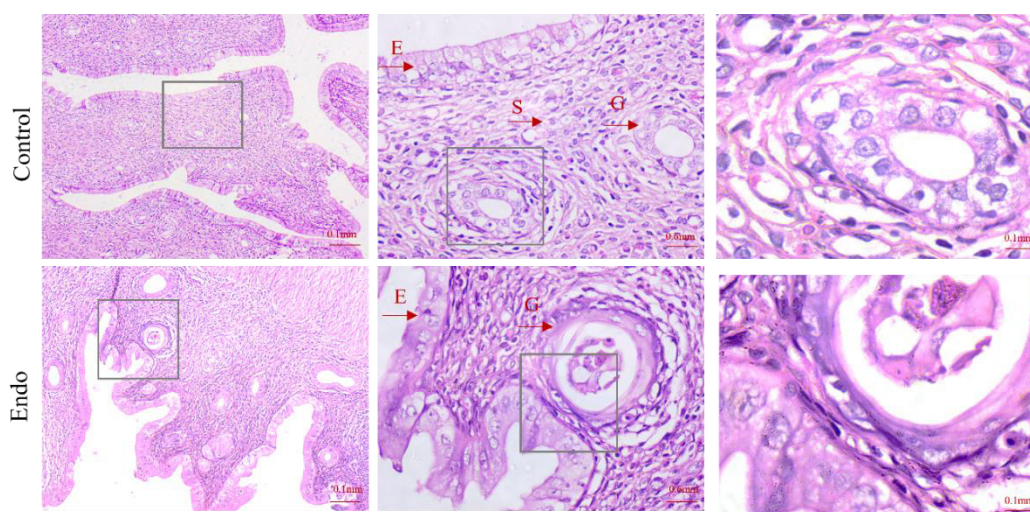


Fig 2: The histopathological images of the control and Endo uterine horn stained with H&E showed the restructured epithelium, degenerated glandular cells in the endo group. S – Stromal cells, E- Epithelium, G- Glandular cells

3.3 Diethylstilbestrol alters the inflammatory markers expression

Extensive evidence to support the efficiency of DES in inducing human mimicked endometriosis in a rodent model is needed. As endometriosis is the severe inflammatory disorder, the immune-inflammatory markers were found to be abnormally expressed. Hence, the study focused on the expression profiling of key immune-inflammatory markers (NFKB, MAPK, TNF- α , IL-6 and STAT-3) in the

endometriosis induced group compared with the control group. NFKB, a transcription factor found to be increased in the endo group compared with the control. The level of MAPK in the endo group was significantly increased when compared to the control. The expression of inflammatory cytokine TNF- α was also upregulated in the endo group. Compared to the control group, endometriosis induced animals displayed higher expression of IL-6 and STAT3. (Fig 3).

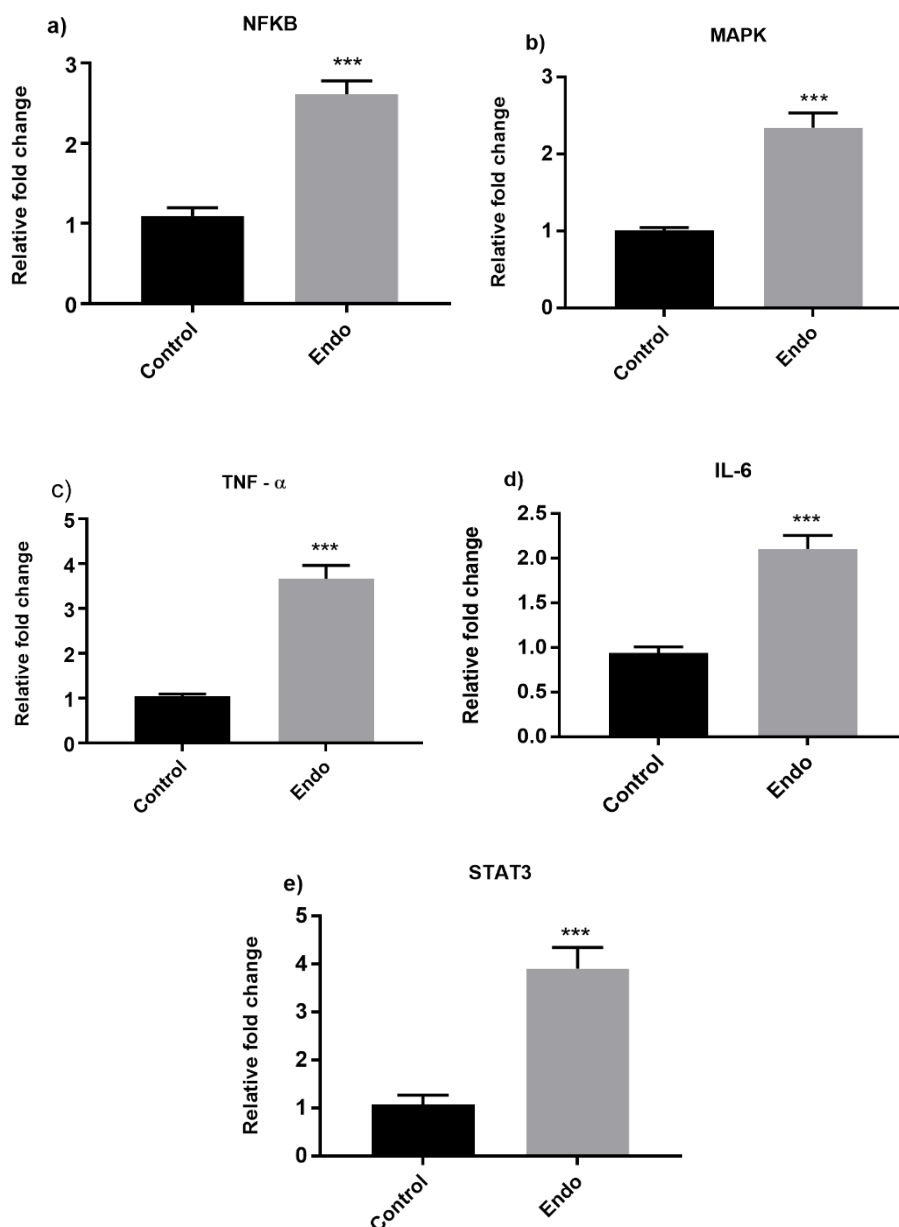


Fig 3: The mRNA expressions of NFKB (a), MAPK (b), TNF- α (c), IL-6 (d) and STAT-3 (e) were upregulated in the DES group compared with control. The data is represented as mean \pm SEM, n = 3, *p < 0.001**

4. DISCUSSION

Endometriosis is always considered to be an inflammatory disease.¹⁷ Endometriosis associated inflammatory response is reliant on increased activated macrophages and cytokines released in peritoneal fluid. Endometriosis will proliferate and be maintained by a local inflammatory milieu via proliferation of, endometrial-peritoneal adhesion, angiogenesis, and invasion. Inflammatory status in endometriosis produces two major symptoms of endometriosis - infertility and pelvic

pain.¹⁸ The local pelvic inflammatory process alters the immune related cells which is thought to be a cause of endometriosis.¹⁹ According to the recent research, women with endometriosis exhibited increased of activated macrophages, which secrete growth factors, cytokines and oxidative markers, resulting in the growth and proliferation of endometriosis.^{20,21} Although the detailed role of immune-inflammatory process in the proliferation and progression of the endometrial lesions remain unknown, recent researches proposes that mast cells (MCs) play an important part in the

immune-inflammatory mechanisms in endometriosis. In fact, endometriotic lesions contain many degranulated MCs.²² Hence, we attempted to prove the inflammatory mediated endometriosis induction of DES in the rodent model. Biomarkers are used to diagnose endometriosis which is essential in addition to the hormone profiling to validate the disease. CA-125, a frequent blood biomarker for endometriosis, has received a lot of attention. A meta-analysis published found that the patients with endometriosis have higher levels of CA-125, particularly in the most severe stages.²³ In this study, serum CA-125 level was increased in the serum of DES group. Also, pathological identification revealed degenerated glandular cells, epithelium in the endo group confirms the endometriosis induction. The pro inflammatory transcription factor NFkB is involved in both inflammation and physiological immunity. Peritoneal oxidative stress has been shown to impair cellular function by boosting proinflammatory gene expression via NFkB activation regulation.^{24,25} Endometriosis has been shown to stimulate the MAPK signaling pathway, which is important in the inflammatory process and the development of endometriosis. Women with endometriosis have increased uterine smooth muscle cell proliferation, which is accompanied by MAPK activation.²⁶ ROS mediated activation of MAPK pathway leads to the aggressive proliferation of ectopic endometrial cells.²⁷ Thus, the developed rat model of endometriosis exhibits the alteration in the NFkB and MAPK which can promote the proliferation of endometriosis. Cytokines are responsible for inflammatory responses and tissue neovascularization. TNF- α and interleukin-6 (IL-6) have previously been investigated in the pathophysiology of endometriosis.²⁸ The estrogenic microenvironment activates peritoneal macrophages, causing them to secrete pro-inflammatory cytokines TNF- α and IL-1. The presence of IL-6 and TNF- α in the peritoneal fluid of individuals with aberrant immune cell activity demonstrates the function of cytokines in pathogenesis.²⁹ In endometriosis, IL-6 is one of the most significant pro-inflammatory cytokines and angiogenic agents. It is highly expressed by peritoneal macrophages and endometriotic lesions, and considerably higher IL-6 levels have been recorded in peritoneal fluid and endometriotic lesions of endometriotic patients compared to non-endometriotic controls.³⁰ As supporting the above, the DES group showed increased expression of TNF- α and IL-6 compared with the control. Recent studies showed that the levels of IL-6 shown to be upregulated in the stromal cells isolated from endometriosis biopsies compared with control. IL-6 mostly signals via the STAT family of transcription factors, notably STAT3.³¹ The STAT pathway is used by several inflammatory cytokines to communicate their

message. This signaling pathway is critical for immune cell signaling.³² JAKs phosphorylate STAT proteins upon cytokine binding, which then translocate to the nucleus and affect transcription.³³ As STAT3 is known to regulate these cellular processes, this suggests that ectopic lesions may also exhibit STAT3 over activity, which may influence lesion genesis and survival.^{34,35} In the current study, STAT3 level was found to be upregulated upon activation of IL-6 in the DES group. Although our previous study demonstrated the induction of endometriosis, our findings now revealed that DES induces endometriosis by triggering the immune-inflammatory pathway. The inflammatory pathway triggered helps in proliferation and growth of the endometriosis which is supported by the data represented in this study.

5. CONCLUSION

Our study demonstrated that DES induces endometriosis by activating the inflammatory markers. Immune-inflammatory factors believed to play an important role in the pathophysiology of endometriosis. The elevated level of CA-125 confirmed the effective induction of endometriosis. The altered expression of NFkB, MAPK, TNF- α , IL-6 and STAT-3 associated with proliferation and sustained growth of endometriosis compared with control, further supports the study.

6. AUTHOR CONTRIBUTIONS STATEMENT

KB and SPK designed the study. SPK is involved in the data collection and analysis process. The initial draft of the manuscript was prepared by SPK and KB involved in revision of the article provided constructive criticism and recommendations and the final manuscript approved.

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8. CONFLICT OF INTEREST

Conflict of interest declared none.

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