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Research Article

# Impact of Mitochondrial Oxidative Phosphorylation Dysfunction in the Patient with Endometriosis

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## ABSTRACT

**Background:** Endometriosis is a chronic inflammatory condition where the precise cause of endometriosis is still not unclear. Mitochondrial dysfunction has been reported in severe forms of endometriosis. However, the role of mitochondrial dynamics, mitophagy, and mitochondrial-related complexes in endometriosis and its relation with Epithelial-Mesenchymal Transition (EMT) is not clear.

**Objective:** Therefore, this study aims to elucidate the role of mitochondrial dysfunction and its relation with EMT in the pathogenesis of endometriosis.

**Material and methods:** At present, cell viability, immunophenotypic enrichment using flow cytometry, and gene expression by real-time quantitative PCR were carried out.

**Results:** A significant decrease in the expression of OXPHOS genes, DRP1, Pink-1, Parkin, and E-cadherin in endometrial biopsies collected from women with severe endometriosis compared to biopsies collected both from healthy women and women with mild endometriosis was noted. Enhanced expression of mesenchymal stem cell markers (CD73, CD90, and CD105), N-cadherin, hypoxia-inducible factor-1α, TWIST, SNAIL, and SLUG were severe endometrial forms compared to mild form and controls.

**Conclusion:** Our observations revealed the presence of EMT in severe forms of endometriosis, accompanied by the expression of MSC markers and strongly imply the role of EMT in the pathogenesis of endometriosis. Additional research endeavours are necessary to validate and build upon these discoveries, with the ultimate goal of enhancing care and outcomes for individuals afflicted by endometriosis.

**Keywords:** Endometriosis; Mitochondrial oxidative phosphorylation dysfunction; Epithelial to mesenchymal cell transitioning

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## INTRODUCTION

Endometriosis is a chronic inflammatory condition that relies on estrogen, characterized by the presence of tissue resembling the endometrial tissue outside the uterus [1]. This tissue comprises stromal fibroblasts, epithelial cells, immune cells, nerves, and vascular/perivascular cells. These components react to hormonal fluctuations during the menstrual cycle, causing inflammation, scarring, and pain. While the precise cause of endometriosis remains unclear, it likely involves a complex interplay of genetic, hormonal, immune, and environmental factors [2]. The condition impacts fertility through various mechanisms, including pelvic anatomy distortion, adhesions, fallopian tube scarring, pelvic inflammation, immune system dysregulation, hormonal changes affecting egg quality, impaired embryo implantation, and alterations in egg quality [3]. The delayed diagnosis often leads to unexplained infertility, exacerbating stress levels for affected individuals [4]. Female infertility has been linked to increased risks of cancer and chronic diseases, which are the primary contributors to morbidity and mortality [5]. The diagnosis of endometriosis typically relies on laparoscopic visualization, considered the gold standard, although non-surgical methods such as transvaginal ultrasound and MRI can detect ovarian and deep endometriosis [6, 7]. Furthermore, the use of contraceptive pills and pain relievers may contribute to further delays in diagnosis [8]. Endometriosis has been associated with various comorbidities such as asthma, cardiovascular disease, and certain cancers, highlighting the need for improved diagnostic standards [9].

The potential involvement of EMT in endometriosis is supported by observations of increased invasiveness and survival of endometrial cells in ectopic locations. Several studies have reported alterations in EMT-related markers in endometriotic lesions, including decreased E-cadherin expression and increased N-cadherin and vimentin expression. Furthermore, the role of hypoxia in endometriosis has gained attention. Hypoxia-Inducible Factor-1α (HIF-1α) is a key transcription factor activated under low oxygen conditions and has been implicated in various cellular processes, including angiogenesis and cell survival.

Recent research has emphasized the role of mitochondrial dysfunction in the pathophysiology of endometriosis, with alterations in Oxidative Phosphorylation (OXPHOS), mitochondrial dynamics, and mitophagy contributing to cellular dysfunction [10]. OXPHOS, the process by which mitochondria generate ATP, is disrupted in endometriosis, leading to cellular energy deficits and oxidative stress [11]. Pathogenic mutations in mitochondrial DNA (mtDNA) affecting genes encoding subunits of the mitochondrial respiratory complexes could potentially promote tumorigenesis by disrupting the mitochondrial respiratory chain function and increasing Reactive Oxygen Species (ROS) production. Dysfunctional mitochondria exhibit impaired respiratory chain complexes, which can be quantified by assessing the expression of OXPHOS genes in endometrial tissues [12]. Moreover, abnormal mitochondrial dynamics and impaired mitophagy exacerbate cellular damage and promote disease progression [13]. However, the role of OXPHOS genes in endometriosis in Indian patients is not clear.

Epithelial-Mesenchymal Transition (EMT) refers to the process whereby epithelial cells transform into a mesenchymal phenotype [14]. This transformation involves the loss of epithelial markers, including E-cadherin, β-catenin, occludin, claudin, plakophilin, cytokeratin, and desmoplakins, and the acquisition of mesenchymal markers like N-cadherin, vimentin, and fibronectin. Various studies have indicated that dysregulation of specific transcription factors, oncogenes, tumor suppressors, miRNAs, and growth factor signaling pathways can instigate EMT. This phenomenon is recognized as a significant contributor to the unfavorable prognosis observed in gynecological cancers. The EMT process can be a major contributing factor in infertility-related morbidity [15]. Molecular analysis of EMT-related genes can elucidate the molecular pathways driving disease progression. However, the role of mitochondrial dynamics, mitophagy, and mitochondrial-related complexes in endometriosis and its relation with EMT is not clear. In vitro studies using endometrial tissue biopsies and isolated cells from women with mild and severe endometriosis can provide insights into EMT, a process implicated in the invasive nature of endometriotic lesions [16]. Therefore, this study aims to elucidate the role of mitochondrial dysfunction and EMT in the pathogenesis of endometriosis. By investigating the molecular mechanisms underlying these processes, we hope to identify potential therapeutic targets for mitigating disease progression from mild to severe forms. This multidimensional approach may pave the way for personalized treatment strategies and improve clinical outcomes for patients with endometriosis.

## MATERIALS AND METHODOLOGY

### Study approval

The study protocols were approved by the Institutional Ethics Committee of MHRT Hospital & Research Centre, Hyderabad. All the samples were collected after getting the signed informed consent from each subject enrolled in the study.

### Selection of subjects

All subjects were clinically diagnosed and confirmed for the presence of endometriosis lesions by laparoscopy and histo-pathological analysis. Further, the grading of these endometriosis lesions was performed as per the guidelines of the American Society of Reproductive Medicine Revised System. A total of 240 participants (including 120 healthy control women and 120 women with endometriosis) were enrolled in the Department of Obstetrics and Gynecology, MHRT Hospital & Research Centre, Hyderabad, India as per the inclusion and exclusion criteria. Out of 120 women with endometriosis, 60 had minimal/mild (I/II) forms and 60 women had moderate/severe (III/IV) forms of endometriosis (as depicted in Tables 1 and 2). A total of 120 healthy women from the general population were enrolled as controls. The control group comprised women who had undergone sterilization for family planning and tubectomy, exhibiting a

complete absence of any pathology. Selection of the participants was carried out as per the following inclusion and exclusion criteria:

## Inclusion criteria

- Women clinically diagnosed with endometriosis.
- Reproductive age women between 18 years-55 years.
- Women capable of giving an Informed Consent Form (ICF).

## Exclusion criteria

- Female patients less than 18 years old and above 55 years.
- Women with pelvic inflammatory disease, and adhesions due to previous surgeries or infections.
- Women were unable to give an Informed Consent Form (ICF) and were unable to provide tissue for the study due to physical or psychological reasons.

**Table 1:** Subjects with surgically confirmed endometriosis.

| No. of subjects | No. of patients undergoing laparoscopy | No. of the patients confirmed diagnosis of Endometriosis |
|---|---|---|
| 240 | 180 | 120<br>Stage I-II (Minimal to mild): 60<br>Stage III-IV (Moderate to severe): 60 |

**Table 2:** Demographic factors and clinical outcome measures in individuals with surgically confirmed endometriosis.

| Baseline Parameter | Surgical Endometriosis (n=120) | | Controls (n=120) |
|---|---|---|---|
| | Minimal to mild (60) | Moderate to severe (60) | |
| Age (mean SEM) | 26±0.8 | 33 ± 0.4 | 35.3 ± 0.7 |
| BMI (median, [IQR]) | 23.2 ± 0.7 | 23.5 ± 0.4 | 35.3 ± 0.4 |
| FSH (mean SEM) | 6.13 ± 0.5 | 7.13 ± 0.4 | 7.02 ± 0.2 |
| **Marital Status** | | | |
| Single | 4 | 6 | 12 |
| Married | 54 | 51 | 94 |
| Divorced | 2 | 3 | 4 |
| **Parity, n (%)** | | | |
| 0 | 49 | 51 | NA |
| 1 | 11 | 9 | NA |
| Duration of infertility in years | 7 ± 1.8 | 9 ± 2.5 | 5 ± 1.1 |
| Primary infertility | 42 | 48 | NA |
| Secondary infertility | 18 | 12 | NA |
| Number of subjects | 60 | 60 | 120 |

## Isolation of single cells from each biopsy sample

Isolating individual cells from endometrial biopsy samples from endometriosis is a crucial step for understanding the cellular components and mechanisms involved. This process enables to study of the characteristics of each cell independently, providing valuable insights into the complexities of endometriosis at a cellular level.

## Methodology

Human endometrial tissue was obtained from consenting subjects with a diagnosis of endometriosis under a protocol approved by the Institutional Review Board (IRB). Primary cell cultures were prepared from the tissue collected from the endometrium of endometriosis patients (n=7). The finely minced tissue was incubated in Hanks balanced salt solution containing 4-(2-Hydroxyethyl)-1-Piperazine Ethane Sulfonic Acid (HEPES; 25 mm), 1% penicillin/streptomycin, collagenase (1 mg/mL, 15 U/mg) and DNase (0.1 mg/mL, 1500 U/mg) for 45 minutes at 37°C with agitation. During and at the end of the incubation, the tissue was pipetted gently to disperse the cells every 15 minutes. Endometrial cells were pelleted, washed, and suspended in Ham's Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (DMEM/F12) (1:1) containing 10% Fetal Bovine Serum (FBS), 1% penicillin/ streptomycin and 1% amphotericin B. A mixture of endometrial cells (epithelial and stromal) was passed through a 40 mm sieve, which allowed stromal cells to pass through while epithelial cells were retained on the sieve (Millipore, Billerica, Massachusetts). Backwashing with the

same isolation media was used to collect the epithelial glands retained in the strainer.

## Cell viability testing and counting

The viability of isolated cells from endometriosis tissue biopsy was assessed by Trypan Blue Exclusion Assay and Fluorescein Di-Acetate Assay. Cell viability testing, counting, and enumeration were carried out using a hemocytometer.

## Trypan blue exclusion assay

Cell viability can be assessed by microscopic observation using dyes that help in distinguishing between viable and non-viable cells. Trypan blue is the most common dye used for the assessment of cell viability. Live cells containing intact membranes will exclude the trypan blue, whereas dead cells with disrupted membranes intake the dye and appear in dark blue under a microscope. In our study, we have used 0.2% trypan blue for checking the viability percentage and cell number. The calculations for determining the percentage of cell viability and viable cell concentrations were determined using the following formulas:

$$\text{Percentage cell viability} = (\text{No. of viable cells}/\text{No. of total cells}) \times 100$$

$$\text{Dilution factor (DF)} = \text{Total volume}/\text{Volume of cells}$$

## Cells concentration

- Viable cells conc. (cells/mL) = Average number of viable cells/square × DF × $10^4$
- Dead cells conc. (cells/mL) = Average number of dead cells/square × DF × $10^4$
- Total cells conc. (cells/mL) = Average number of total cells/square × DF × $10^4$

## Fluorescein Di-Acetate (FDA) assay

FDA is a hydrophobic non-fluorescent dye that can penetrate the cell membrane. Intracellular esterases present in the intact cell membranes hydrolyze the diacetate present in the FDA to produce fluorescence. These fluorescent molecules accumulate in metabolically active cells with intact membranes and fluorescence green that can be used as a marker for cell viability. Cells with inactive metabolism and without an intact cell membrane fail to accumulate fluorescent molecules and don't fluoresce in green.

## Short-term culture assay

- **In vitro enrichment of isolated single cells:** The in vitro enrichment of endometriosis cells is crucial for isolating specific cell populations, conducting functional studies, and performing molecular analyses. This protocol aims to obtain pure cell populations, enabling investigations into cellular behavior, and molecular studies. Enriched cells can be used for various downstream applications like ex vivo cell polarization, Real-Time Reverse Transcription PCR (qRT-PCR), flow cytometry, in vitro expansion, etc.
- **Morphological assessment of enriched cells:** Morphological characterization of Cultured MSCs was performed using phase contrast confocal microscopy (Carl Zeiss, Germany) at days 0, 1, 3, and 5. Cultures were monitored under constant microscopic observation to detect the changes in morphology and confluency.

## Trypsinization

The process of trypsinization uses trypsin (proteolytic enzyme) that helps in detaching the adherent cells from the culture vessels by disturbing the cell adhesion proteins, and cell-cell and cell-matrix interactions. Trypsinization is frequently performed to pass cells into a new culture vessel.

## Immunophenotypic of enriched cells using flow cytometry

Immunophenotyping is carried out through the flow cytometry technique. This technique was discovered in the 1950s by Wallace Coulter. Its principle is based on the direction and amount of light reflected during the passage of single cells in suspension in front of a laser beam. Activation of fluorescent-labeled antibodies or dyes tagged to the cells helps in detecting, sorting, and counting cells. This technique allows quantitative analysis of a single-cell population present in a heterogeneous population of cells in a shorter period. This technique is widely used in basic as well as clinical research.

## Molecular and gene expression studies

Isolation of Mitochondria from Tissue Biopsy: Mitochondria were isolated from tissue biopsies using a mitochondrial isolation buffer as described by (Mashayekhi, Vida et al., 2014). Endometriosis tissue biopsy was taken in a 1.5 mL microcentrifuge tube with saline and centrifuged at 3000 rpm for 5 min for wash and removing RBCs and debris. The supernatant was discarded and the tissue was placed on a slide. The tissue was mashed using a sterile scalpel and blade. The mashed tissue was transferred into a 1.5 mL microcentrifuge tube. 1 mL of mitochondrial isolation buffer was added to the mashed tissue and vortexed. The sample was incubated with mitochondrial isolation buffer at 40°C for 15 min followed by centrifugation at 2000 g for 10 min at 40°C. The supernatant was collected in a fresh microcentrifuge tube and centrifuged at 10,000 g for 10 min at 40°C. The supernatant was discarded and the pellet containing isolated mitochondria was used for the isolation of RNA.

## Ribonucleic Acid (RNA) isolation

Extraction of RNA from the cells was performed using the Guanidinium Thiocyanate (GITC) method. Isolated cell suspension was taken in a 1.5 mL microcentrifuge tube and centrifuged at 3000 rpm for 5 min. The supernatant was discarded and 400 µL of the solution–D was added to the pellet Mixture thoroughly white color precipitate was observed with 250 µL of water-saturated phenol, 250 µL of Chloroform: Isoamyl alcohol and 30 µL of Sodium acetate was added.

Mixed thoroughly and incubated for 30 min at 40°C. The sample was mixed and centrifuged at 10,000 rpm for 10 minutes at 40°C.

The supernatant was collected in a fresh microcentrifuge tube and an equal volume of Chloroform: Isoamyl alcohol (24:1) was added. Centrifuged at 10,000 rpm for 10 min at 40°C. The supernatant was collected in a fresh microcentrifuge tube and an equal volume of Isopropanol was added. White thread-like structure was observed which was supposed to be RNA. If RNA was not observed the solution was incubated overnight at -20°C. Centrifuged the solution at 10,000 rpm for 10 minutes at 40°C.The supernatant was decanted and the pellet was washed with 70% ethanol. The supernatant was drained and the RNA pellet. About 30 µL–50 µL of Diethyl Pyrocarbonate (DEPC)-treated water was added to dissolve the RNA pellet. RNA was stored at -200°C.

## Complementary DNA (cDNA) preparation and PCR

PCR reagents were taken except enzyme at RT before 5 minutes of starting the reaction. Labeled the PCR tubes for required reactions mentioned in table 3. First PCR buffer and then other reagents were added to make a master mix. Aliquot the mix equally throughout the tubes and add the distinct sample separately in all the tubes. Give a short spin to all tubes to make sure no bubbles are tacked into the tubes. Set the PCR tubes in a thermocycler and RUN the program. After finishing the program, switch off the thermocycler, remove the tubes, and check the amplified products on the agarose gel. The PCR reaction mixture and amplification conditions are as follows in table 4.

**Table 3:** PCR reaction mixture.

| Components | Volume |
|---|---|
| 10X PCR buffer | 2.50 L |
| DNTPs | 0.50 L |
| Primer (F) | 0.50 L |
| Primer (R) | 0.50 L |
| Taq polymerase | 0.50 L |
| $dH_2O$ | 18.75 L |
| DNA | 1.0 L |
| Total reaction volume | 25 L |

**Table 4:** PCR cycling conditions.

| S.NO | PCR Steps | Time Period | Temperature in °C |
|---|---|---|---|
| 1 | Predenaturation | 5 min | 95°C |
| 2 | Denaturation | 40 sec | 95°C |
| 3 | Annealing | 30 sec | 55°C |
| 4 | Elongation | 40 sec | 72°C |
| 5 | Final elongation | 5 min | 72°C |

Steps 2-4 were repeated for 40 cycles.

## Real-time quantitative PCR (RTq-PCR)

RTq-PCR allows specific, reproducible, and sensitive quantification of nucleic acids. RTq-PCR readout is given in the form of "Cycle Threshold" (CT) essential to attain a particular point of fluorescence. RTq-PCR principle depends on the utilization of fluorescent dye which helps in quantification of the of nucleic acids amount in a given sample. Data generated during the logarithmic phase in a given reaction yields quantitative data regarding the initial quantity of an amplified target. Over the course of the reaction, the change in the fluorescence will be measured by a Real-time PCR machine combined with a thermal cycler having fluorescent dye scanning capability. An amplification plot can be generated by plotting the obtained fluorescence *vs.* cycle number over the duration of the complete PCR reaction. SYBR Green I is the most frequently used DNA-binding fluorescent dye. It binds to all ds-DNA (Double-Stranded DNA) and is detected by measuring increasing fluorescence till the end of the cycle.

cDNA from all the variables was quantified by nanodrop reading, and 5 ng of cDNA from each sample was used for quantitative PCR. The reaction mixture was prepared for all the primers as follows in table 5. Reaction conditions were adjusted as per the Melting Temperature (TM) of each primer following the first step of initial denaturation for 5 min at 94°C, second step is a denaturation step for 30 sec at 94°C, third step is an annealing step for 40 sec at 52°C -56°C and fourth step is an extension step for 40 sec at 72°C. Steps 2-4 were repeated for 40 cycles. Final denaturation was kept at 72°C for 5 min followed by melting curve analysis for 15 min. Melt curve analysis was carried out to separate the amplified products and primer dimers. GAPDH primer was used as an endogenous gene primer for normalizing test samples. For every sample values were collected and used for statistical analysis to calculate fold difference. All reactions were carried out in triplicates to overcome the technical error and gel electrophoresis was done.

**Table 5:** Reaction mixture for RTq-PCR.

| Components | Volume |
|---|---|
| 2X SYBR Green Mix | 10 µL |
| Forward primer (10 pmol/µl) | 0.5 µL |
| Reverse primer (10 pmol/µl) | 0.5 µL |
| cDNA template | 2.0 µL |
| $dH_2O$ | 7.0 µL |
| Total | 20 L |

## STATISTICAL ANALYSIS

The entire data obtained was expressed in mean ± standard deviation. One-way ANOVA and two-way ANOVA was used for statistical analysis of variance by using GraphPad Prism software (Version 5). Spearman test was used for correlation analysis. p values with $< 0.05$ were considered statistically significant. A chi-square test was performed to assess the statistical association between categorical variables representing histopathology grades, specifically distinguishing between mild and severe forms and various clinical parameters of endometriosis. Step One Version 2.2 software was used to calculate the RT–qPCR efficiency. RT-qPCR was carried out in triplicates for all genes. Target gene expression was compared to control gene expression. Calculation of relative fold change was done by normalizing target gene expression against endogenous gene expression (GAPDH) according to the Livak method (Livak and Schmittgen 2001) and Pfaffl's method (Pfaffl, 2001) of relative quantification was used for further validation of fold difference values for every transcript. $\geq 0.99$ regression value (R2) was considered to be significant with 100% PCR efficiency. R programming (R Version 3.1.2) run on the UNIX platform was used for the graphical representation and statistical computing of fold difference values for different mRNA transcripts analyzed through RT-qPCR. p-value of $\leq 0.05$ was considered to be statistically significant.

## RESULTS

### Invitro culture of cells isolated from different forms of endometrial tissue biopsies

Single cells isolated from the endometrial tissue biopsy samples were tested for viability using fluorescent di acetate. The cells were then cultured and allowed to proliferate under appropriate conditions to study the changes in EMT at the cellular and molecular levels. The adherent cells started changing their morphology from spherical to spindle-shaped (Figure 1).

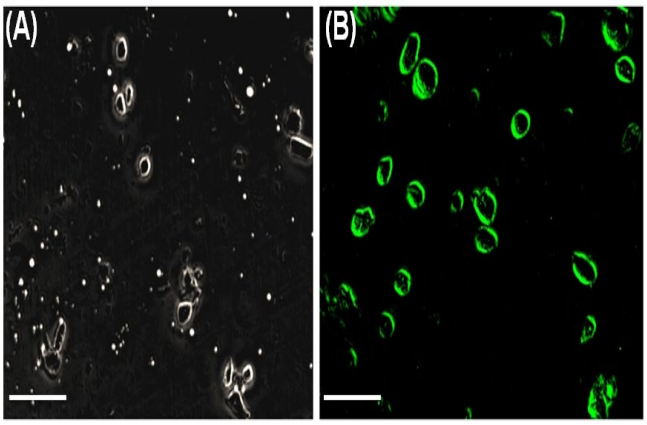


**Figure 1:** The integrity of the membrane: "A" represents unstained cells and "B" represents FDA-stained cells. Original magnification: 40X, Scale bar: 20 µm.

### Morphological identification of EMT progression in different forms of endometrial tissue biopsies

The morphological characterization was done by microscopic examination. As the culture progressed from day 0 to day 5, we noted that there was a gradual change in the morphology of epithelial cells into mesenchymal cells. We noticed that there was an increase in the population of mesenchymal cells in severe forms of endometrial cultures on day 5. Our study results demonstrated an enhanced EMT progression in severe forms of endometriosis when compared to mild forms of endometriosis (Figure 2).

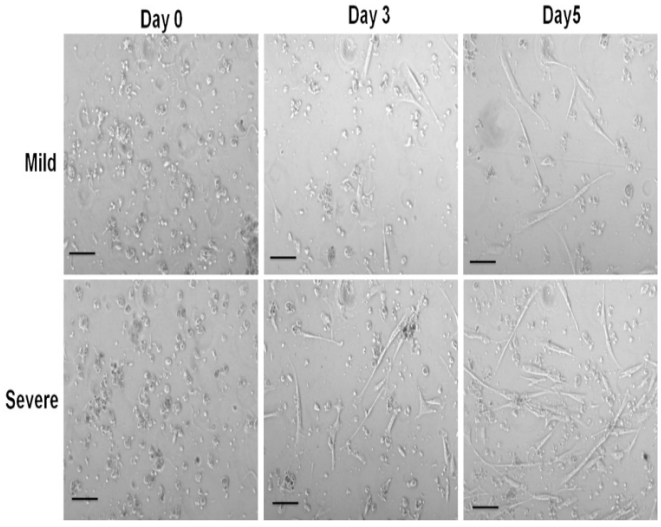


**Figure 2:** Morphology of cells isolated from mild and severe forms of endometrial tissue biopsies. Microscope magnification: 10X, Scale bar: 20 µm.

## Quantifying the expression levels of OXPHOS, mitochondrial dynamics fission, and mitophagy genes in endometrial biopsies

At present, we examined the expression of genes that have a vital function in mitochondrial functioning and homeostasis. Further, we studied the expression of the genes involved in different complexes of OXPHOS in mild and severe forms of endometriosis in comparison with controls. A significant decrease in the expression of OXPHOS genes in endometrial biopsies collected from women with severe endometriosis compared to biopsies collected both from healthy women (controls) and women with mild endometriosis (***p<0.0001) was noted (Figure 3). However, a non-significant association in the relative gene expression of all these genes was noted between control and mild forms. These decreased expressions in the OXPHOS genes in severe form endometriosis might ultimately result in decreased ATP synthesis.

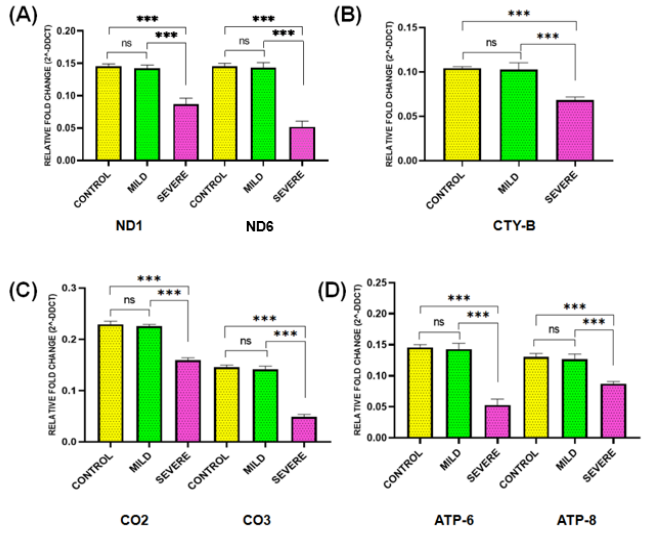


**Figure 3:** Relative expression of OXPHOS genes in endometrial biopsies collected from healthy women (controls), women with mild endometriosis, and women with severe endometriosis: A) Complex-1 (ND1 & ND6); B) Complex-3 (CYT-B); C) Complex-4 ($CO_2$ & $CO_3$); D) Complex-5 (ATP-6 & ATP-8).

Note: (***p<0.0001) represents significance; ns= non-significant.

## Relative quantification of mitochondrial dynamics fission gene

We also examined the relative gene expression of DRP1, a master regulator of mitochondrial fission in mild and severe forms of endometriosis in comparison with controls. A significant downregulated in the expression of the DRP1 gene in endometrial biopsies collected from women with severe forms of endometriosis compared to biopsies collected both from healthy women (controls) and women with mild endometriosis (***p<0.0001) was noted (Figure 4). The results from the DRP1 gene expression analysis indicate that there is a failure in mitochondrial homeostasis.

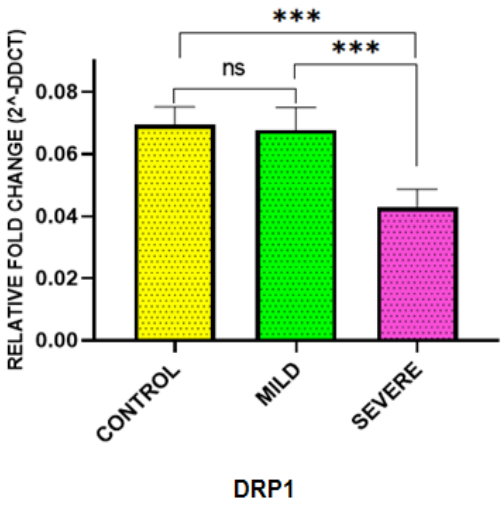


**Figure 4:** The relative quantification of DRP1 gene expression in endometrial biopsies collected from healthy women (controls), women with mild endometriosis, and women with severe endometriosis.

Note: (***p<0.0001) represents significance; ns= non-significant.

## Relative quantification of mitophagy genes

We also assessed the relative gene expression of Pink 1 and Parkin in endometrial tissues might help in understanding the disease pathogenesis. Results from our data demonstrated a significant down-regulation of these two mitophagy genes in the endometrial biopsies collected from women with severe forms of endometriosis compared to biopsies collected both from healthy women (controls) and women with mild endometriosis (***p<0.0001; Figure 5). However, a non-significant association in the relative gene expression of all these genes was noted between control and mild forms.

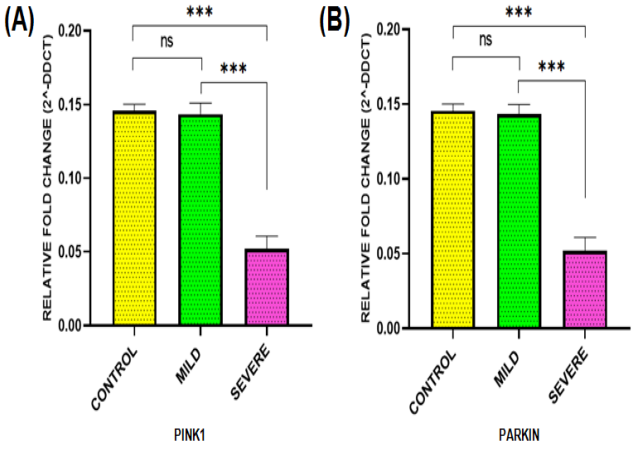


**Figure 5:** Relative expression of mitophagy genes in endometrial biopsies collected from healthy women (controls), women with mild endometriosis, and women with severe endometriosis: A) Pink1; B) Parkin.

Note: (***p<0.0001) represents significance; ns= non-significant.

## Increased relative gene expression of Hypoxia-Inducible Factor-1α (HIF1α) in severe forms of endometriosis

We further assessed the expression of HIF-1α in different forms of endometrial biopsies. Our findings demonstrated a significant elevation in the mRNA expression of the HIF1α gene in the endometrial biopsies collected from women with severe forms of endometriosis compared to biopsies collected both from healthy women (controls) and women with mild endometriosis (***p<0.0001; Figure 6).

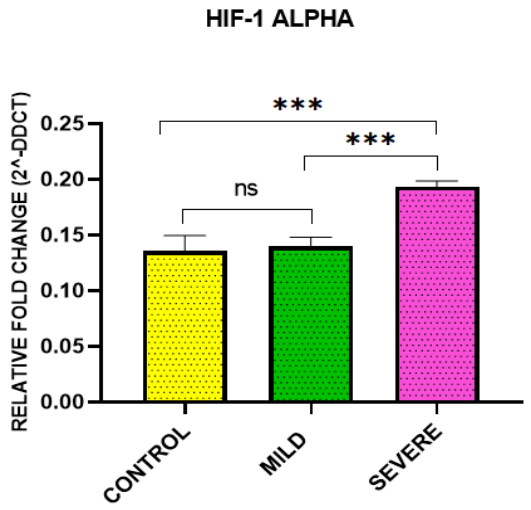


**Figure 6:** Relative expression of HIF1α gene in endometrial biopsies collected from healthy women (controls), women with mild endometriosis, and women with severe endometriosis.

Note: (***p<0.0001) represents significance; ns= non-significant.

## Immunophenotypic characterization using flow cytometry to demonstrate EMT progression using mesenchymal positive markers

Immunophenotypic analysis of cultured cells isolated from mild and severe forms of endometrial tissue biopsies was performed using flow cytometry at day 5. Severe endometrial forms showed an enhanced expression for mesenchymal positive markers CD73 (96.9%), CD90 (97.5%), and CD105 (97.1%). Whereas mild endometrial forms showed depleted expression mesenchymal positive markers CD73 (53.2%), CD90 (50.4%) and CD105 (64.5%). Details of immunephenotypic characterization of mesenchymal stem cells on the 5th day of in-vitro culture were given in figure 7.

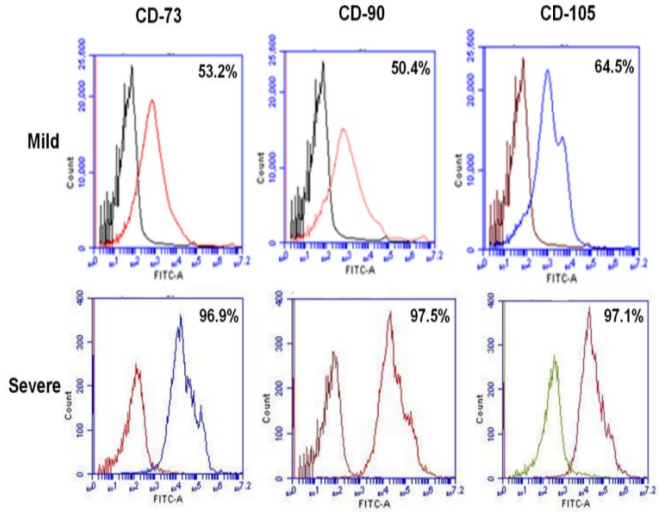


**Figure 7:** Immunophenotypic characterization of mesenchymal stem cells isolated from endometrial tissue biopsies collected from women with mild endometriosis, and women with severe endometriosis on the 5th day of in-vitro culture.

## Up-regulation in the expression of mesenchymal markers indicates an enhanced EMT in severe forms of endometriosis

Further validation of in-vitro culture data was done using real-time quantitative polymerase chain reaction (RT-qPCR) expression analysis for mesenchymal positive gene transcripts CD73, CD90, and CD105 at day 5 in both mild and severe forms (Figure 8). The results showed a significantly higher expression of all these gene transcripts in severe forms of endometriosis compared to mild and healthy controls (***p<0.0001). Further, a slight increase in the expression of all these gene transcripts was noted in mild forms of endometriosis compared to healthy controls (***p<0.01).

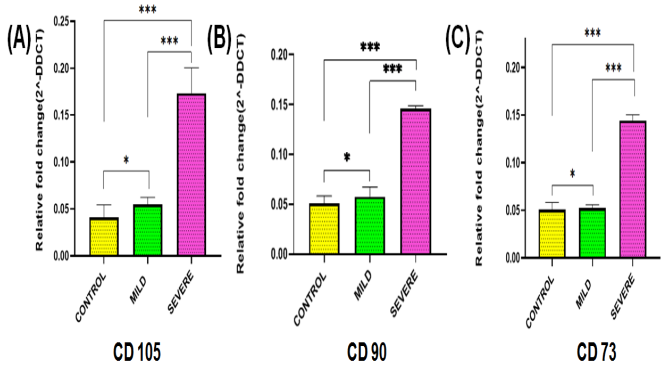


**Figure 8:** Real-time quantitative polymerase chain reaction (RT-qPCR) for mesenchymal stem cell-specific genes (CD73, CD90, and CD105) on the 5th day of the in-vitro culture of mesenchymal stem cells isolated from endometrial tissue biopsies of controls, women with mild endometriosis, and women with severe endometriosis. "A" represents transcript levels of CD105, "B" represents transcript levels of CD90; "C" represents transcript levels of CD73.

Note: (***p<0.0001 and ***p<0.01) represents significance.

### Diminished E-cadherin expression and increased N-cadherin expression promote the progression of EMT

Using Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR), the expression levels of E-cadherin and N-cadherin were assessed in both mild and severe forms of endometriosis. A significant decrease in the E-cadherin expression in severe forms of endometriosis compared to the mild and control, after enrichment (***p<0.0001) was noted. Further, a significant increase in the expression of N-cadherin was noted in the severe forms of endometrial cultured cells when compared to mild and control cultured cells after enrichment (***p<0.0001). However, a non-significant association in the expression of both E-cadherin and N-cadherin was noted between mild and control cultured cells (Figure 9). Our findings demonstrated that severe endometrial forms had an increased EMT progression.

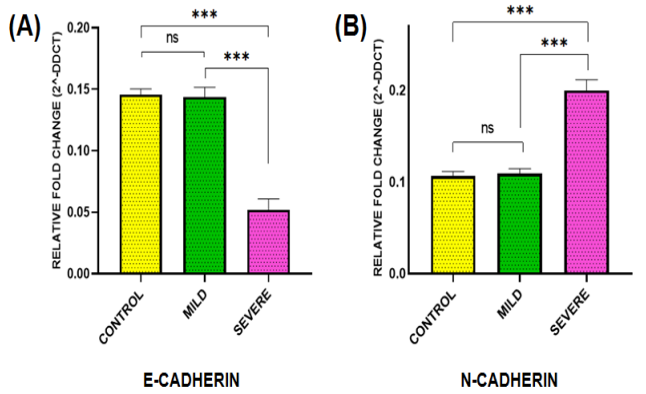


**Figure 9:** Identification of EMT progression through the gene expression analysis of E-cadherin (**A**) and N-cadherin (**B**) in endometrial tissue biopsies of controls, women with mild endometriosis, and women with severe endometriosis.

Note: (***$p$<0.0001) represents significance.

### Increased expression of TWIST, SNAIL, and SLUG occur concordantly in severe endometrial forms

TWIST, SNAIL, and SLUNG are the transcription factors inducing EMT by repressing the transcription of E-cadherin. The relative gene expression of all these three transcription factors was found to have a significantly increased expression in severe forms of endometrial cultured cells when compared to mild and control cultured cells after enrichment (***p<0.0001). However, a non-significant association in the expression of all these genes was noted between mild and control cultured cells (Figure 10). Further, our data at the cellular and molecular level supports that an increased TWIST, SNAIL, and SLUG expression and decreased E-cadherin expression play a crucial role in EMT progression.

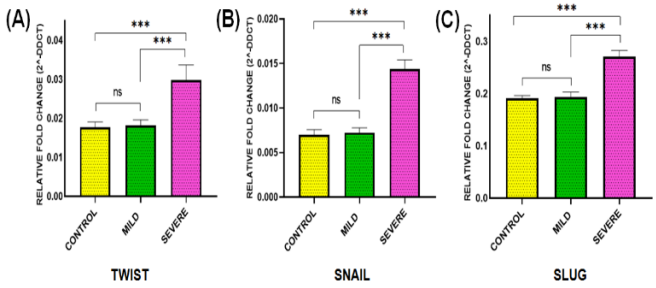


**Figure 10:** Bar graph representing relative expression levels of TWIST (A), SNAIL (B), and SLUG (C) in endometrial tissue biopsies of controls, women with mild endometriosis, and women with severe endometriosis.

Note: (***p<0.0001) represents significance.

The data in this study provides evidence of the severity of endometriosis and its effect on enhanced EMT progression, marked by the characterized changes in cell morphology, increased mesenchymal marker expression, and alterations in cadherin expression. Additionally, the research indicates that mitochondrial dysfunction might play a role in severe endometriosis pathogenesis, as shown by the downregulation of OXPHOS, mitochondrial fission, and mitophagy genes. The increased expression of HIF-1α further supports the hypothesis that hypoxic conditions may contribute to disease progression. These findings could have important implications for understanding the mechanisms underlying the pathogenesis of endometriosis and for developing novel therapeutic approaches.

## DISCUSSION

Epithelial-Mesenchymal Transitions (EMTs) involve the transformation of epithelial cells into mesenchymal ones, a phenomenon observed in various biological contexts. These transitions are typically categorized into three types: the initial type is prevalent in embryonic development, the second type is linked with the regeneration of adult tissues, and the third type is characteristic of cancer advancement. The process of EMT occurring during tumor progression is profoundly dysregulated. EMT facilitates the progression of solid tumors, enhancing their malignancy by promoting increased invasiveness and metastatic potential [17]. EMT stands as a crucial cellular process, marked by the gradual departure from the epithelial phenotype and the successive acquisition of a Mesenchymal Stem Cell (MSC) phenotype. As per our study, immunophenotypic characterization of MSCs positive markers (CD73, CD90, and CD105) performed in mild and severe forms of endometriosis in vitro cultures at day 5 revealed an increased percentage of cells expressing MSCs positive markers. Similarly, real-time PCR data for mesenchymal positive gene transcripts of CD105, CD90, and CD73 showed a significant upregulation in severe forms and mild forms of endometrial cultures when compared with control endometrial cultures.

In recent findings, EMT has been elucidated in the context of endometriosis. Numerous EMT-specific pathways, including Twist, Snail, Slug, Zinc finger E-box-binding homeobox 1/2

(ZEB1/2), as well as E/N-cadherin, keratins, and claudins, have been implicated in this process [18]. In our study, we studied the impact of mitochondrial dysfunction and the effect of the EMT markers by using in vitro cultures of endometrial cells and tissues. Our investigation has centered on the relative quantification of various genes, aiming to aid in the identification of mitochondrial functionality. Numerous proteins and signaling pathways play pivotal roles in EMT. Notably, the loss of E-cadherin, a transmembrane protein crucial for interconnecting epithelial cells at adherens junctions, is regarded as a foundational event in EMT. Several Transcription Factors (TFs), including Snail1, Slug, and Twist either directly or indirectly repress E-cadherin [19]. These TFs often exhibit a hierarchical regulation pattern, with Snail1 and Slug typically being induced first, subsequently activating the aforementioned factors [20]. Interestingly, they also regulate the expression of claudins and desmosomes, thus facilitating EMT. For instance, Slug and Snail can repress claudin-1 messenger RNA (mRNA) and protein expression in vitro, and Slug can initiate desmosomal disruption, which represents the initial and essential phase of EMT [21]. Currently, relative expression levels of TWIST, SNAIL, and SLUG (transcription factors inducing EMT) showed a significant increase in gene expression in severe forms of endometrial cell cultures. TWIST, SNAIL, and SLUNG are the transcription factors inducing EMT by repressing the transcription of E-cadherin [16]. An essential aspect in the progression of EMT is the cadherin switch coupled with the decrease in the expression of the epithelial E-cadherins and an increase in the expression of mesenchymal N-cadherins [22]. Similarly, our study finds that the gene expression analysis of E-cadherin and N-cadherin depicts a significant decrease in the E-cadherin expression in severe forms of endometrial cultured cells and a significant increase in the N-cadherin expression in severe forms of endometrial cultured cells.

Hypoxia-Inducible Factors (HIF) are the major transcriptional factors that regulate hypoxia in several pathological and biological processes [23]. Our findings demonstrated a significant elevation in the mRNA expression of the HIF1α gene in the endometrial biopsies collected from women with severe forms of endometriosis compared to biopsies collected both from healthy women and women with mild endometriosis. It was reported that increased HIF1α enhances leptin expression and promotes the proliferation of endometrial cells [24].

The core of mitochondrial energy metabolism is the series of reactions involved in the OXPHOS that generates ATP. Several studies reported that bioenergetics failure resulting from mitochondrial dysfunction is the basis for many [25-27]. To date, there has been limited investigation into the expression of genes associated with OXPHOS complexes, mitochondrial dynamics, and mitophagy in endometrial tissue biopsies. At present, our findings suggest relative gene expression of OXPHOS genes was significantly decreased in the severe endometrial biopsies when compared to control and mild endometrial biopsies. Mitochondrial dynamics, involving the process of fission and fusion orchestrate the functioning and morphology of mitochondria. At the molecular level, the expression of DRP1 involved in the process of fission in mild and severe forms of endometriosis has not yet been studied. Currently, relative gene expression of DRP1 expression was found to be significantly decreased in the severe endometrial biopsies when compared to control and mild endometrial biopsies. According to our study, the relative quantification of the mitophagy genes *i.e.* PINK1 and Parkin were found to be significantly downregulated in severe forms of the disease compared to mild forms and controls. It is reported that mitophagy plays a crucial role in the selective removal of dysfunctional mitochondria through the pathways mediated by PINK1 and Parkin [28, 29]. Failure in the process of mitophagy results in improper mitochondrial functioning and cellular homeostasis leading to several diseases [30].

## CONCLUSION

This study provides strong evidence that severe endometriosis is characterized by enhanced EMT progression and mitochondrial dysfunction. It provides the association of the severity of endometriosis is closely linked to cellular and molecular changes that promote a more invasive and adaptable cellular phenotype. The observed mitochondrial abnormalities may contribute to the pathogenesis of the disease, possibly through altered energy metabolism and cellular stress responses. Mitochondrial dysfunction has been reported in severe forms of endometriosis. Mitochondrial energy production and metabolism in endometriosis tissues was observed to be diminished. However, the precise underlying cause of this phenomenon remains elusive. Furthermore, our observations revealed the presence of EMT in severe forms of endometriosis, accompanied by the expression of MSC markers. These findings strongly imply that EMT plays a significant role in the pathogenesis of endometriosis. Comprehending the roles of EMTs holds promise for developing more efficacious management strategies for this debilitating condition. This study highlights potential targets for therapeutic interventions, such as modulating EMT processes or improving mitochondrial function. Future research endeavors are necessary to validate these findings in larger cohorts and explore the functional consequences of these cellular and molecular changes in endometriosis pathogenesis.

## LIMITATIONS AND FUTURE DIRECTIONS

The sample size has to be increased and future research has to be conducted. This study involves in-vitro cell culture which may not replicate the complex in-vivo conditions.

For future directions, a large cohort should be done with a more diverse patient population to increase generalizability and to explore potential therapeutic strategies targeting EMT, mitochondrial function, or hypoxia-related pathways in endometriosis.

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## AUTHORS' CONTRIBUTIONS

RR and AAK contributed equally to this work; RR and AAK designed the study; RR and AAK conducted the laboratory work; SSA, WT, VAA, and VB wrote the manuscript. SSA performed the statistical analysis. All authors reviewed and approved the final manuscript.

## CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

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