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Original Article

Progestin Repairs the Mitochondria Membrane Potential by Preventing Membrane Hyperpolarization in Mitochondria Transferred Endometrial Stromal Cells

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Abstract

Background: The regenerative capacity of the endometrium is attributed to stem/progenitor cells. Despite their remarkable regenerative capacity, in some cases, impairments in regeneration can be observed. Endometrial mitochondria transplantation into the uterine cavity improves the uterine environment in Asherman's syndrome. Mitochondria transfer shows therapeutic advantages by supporting tissue metabolism and viability. However, the disruptive environment of the endometrium could affect mitochondrial health adversely. Increasing mitochondrial activity with progestin protects against apoptosis. The aim was to investigate the effect of progestin supplementation during exogenous mitochondrial transplantation. The study was designed as an in vitro cell culture of endometrial stromal cells. Mitochondria were isolated from the same cell line, representing autologous mitochondria transplantation.

Method: The transplantation of mitochondria was detected by fluorescence labeling of mitochondria. Viability was assessed by CCK8, and apoptosis was detected by AnnexinV/PI staining. Gene expression analysis was performed for Ki67, p38, and Erk1/2.

Results: Mitochondria were successfully transferred into endometrial stromal cells. The viability was not significantly altered due to the exogenous mitochondria, despite the increase in the reactive oxygen level. The addition of progestin is also well tolerated. The combined application of both mitochondria and progestin further supports the viability of cells without inducing the level of reactive oxygen species. Apoptotic levels were decreased in the presence of progestin even in the co-transfection group of mitochondria and progestin. The mitochondrial cell membrane was evaluated with JC-1 showing that the disrupted membrane potential was recovered by progestin, improving the damaged membrane potential of the mitochondria.

Conclusion: The damaged membrane potential improved in the presence of progestin, helping to improve the overall output of the mitochondrial transplantation.

Keywords: Dienogest, Stromal Cells, Membrane Potentials, Mitochondria, Progestin

INTRODUCTION

Endometrial tissue experiences cyclic changes during the menstrual cycle. Following the shedding during menstruation, the upper layer of the endometrium undergoes physiological alterations in response to estrogen and progesterone. Re-epithelialization of the endometrium is achieved by the formation of new luminal epithelial cells derived either from the activity of residual basalis glands or by mesenchymal-to-epithelial transition in the basalis. During this period, stromal/stem cells play significant roles in tissue regeneration (1). In menstrual/regeneration disorders, the recovery of endometrial tissue becomes impaired, as in Ascherman's syndrome or endometriosis, which leads to infertility. In these cases, the critically damaged endometrium could be repaired by intrauterine injected stem cells, which support the regeneration of epithelium and stroma by promoting cell proliferation (2,3). Several tissue sources of mesenchymal stem cells have been proposed in endometrial tissue repair of severe injury to the basal

layer of the endometrium (4-6). However, the ability of these stem cells to repair the damaged endometrium has some limitations (7). Drug therapy could support stem cell activities in several ways (8,9). As an alternative to drug or cell-based therapies, exosomes derived from cells have been shown to substantially improve tissue injury (10-12).

Mitochondria transfer between cells could support a diverse set of physiological processes by maintaining mitochondrial homeostasis (13). This cell-to-cell contact-mediated natural process was found to be common especially in some diseases, such as cardiovascular diseases and obesity (14,15). Mitochondria transfer occurs naturally, but this process can be induced by the activities of cancer cells (16,17). Contact-independent mitochondria delivery or transplantation into damaged tissue to maintain the tissue homeostasis is also proposed as an alternative for pharmaceutical applications. Mitochondria transplantation shows therapeutic advantages in human infertility and embryo quality (18). Hwang et al. indicate that mitochondria derived from endometrial organoids could play a crucial role in facilitating uterine repair, which in turn enhances fertility by restoring the disrupted metabolic conditions of the endometrium in Asherman's syndrome (19). There are several ways to transplant exogenous mitochondria into cells to prompt tissue regeneration. However, it is challenging to improve the transplantation medium to enhance the treatment efficiency in the delivery of mitochondria.

Mitochondria transplantation is still an experimental technique and information on mitochondria transplantation is quite limited, especially in endometriosis. To manage endometriosis symptoms, progestin treatment is a common and effective way. However, the effect of progestin on the transplanted mitochondria has not been revealed yet. In this study, it aim was to investigate the effect of progestin supplementation in the medium on exogenous mitochondria during transplantation. As the endometrial stromal cells play a central role in regeneration, the effect of mitochondria transplantation was analyzed in the presence of the progestin in vitro. The mitochondria derived from these cells were delivered into the cultured endometrial stromal cells.

METHODS

Isolation of Endometrium Stromal/Stem Cells (E-SCs)

E-SCs were isolated by an enzymatic method as previously described by Rencber et al. (20). Tissues were obtained from seven women without endometriosis (age, 42.0±1.29 years) who had operated for benign gynecological conditions. The female volunteers had regular menstrual cycles and had not received exogenous hormone treatment in the three months prior to surgery. The obtained E-SCs were cultured in DMEM/F12 medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C, under 5% CO2. Later, these cells were characterized by a flow cytometer with positive (CD44, CD73, CD90 and CD105) and negative markers (CD34 and CD45) for mesenchymal stem cells.

For the cell culture with progestin, Dienogest (Visanne, Bayer Weimer, Weimer, Germany) was used. It was dissolved in 20% DMSO in PBS at pH7.4. In drug supplementation assays, the final concentration of dienogest was adjusted to 10 nM to the level of EC50 dose to activate the progesterone receptor (9).

Mitochondria Isolation and Transplantation in Vitro

Mitochondria were isolated from E-SCs using Standard Hydrogen Electrode (SHE) buffer [0.25 M sucrose, 20 mM HEPES (pH 7.4), 2 mM EGTA, 10 mM KCl, 1.5 mM MgCl2 and 0.1% BSA]. Cells were suspended in SHE buffer at 4 °C, and cell membranes were disrupted by an injector using a 26G needle. The suspension was centrifugated at 250g for 5 min at 4 °C, the supernatant was transferred into another tube, and the mitochondria were recovered by centrifugation at 1500xg for 15 min at 4 °C. The pellet was dissolved in the SHE buffer. The Bicinchoninic acid method (SMART BCA kit, Intron) was used to determine the protein content in the mitochondria according to the manufacturer's instructions. Transfer of mitochondria is performed by adding mitochondria directly into the medium.

TMRM Staining and Detection of Exogenous Mitochondria in E-SCs

Before the isolation, mitochondria were stained first with Tetramethyl rhodamine methyl ester (TMRM), and then isolated from the cells. The aim of staining was to verify the transfer of exogenous mitochondria. During cell culture, cells were stained with TMRM dye at a final concentration of 10 µM for 20 min under standard culture conditions. The mitochondria were isolated and transferred as described. The mitochondria delivered by cells were fixed with PFA cells were visualized.

Cell Viability by CCK8

The viability of cells was determined by CCK8 assay (Elabscience Biotechnology, Wuhan,China). The CCK8 solution was diluted to 10% in RPMI1640 media without serum, according to manufacturer's instructions. Cells were incubated for 24 hours. Absorbance at 450nm was assessed using a microplate reader (VersaMax plus, Molecular Devices). The viable cell numbers were expressed as a percentage of absorbance observed in control cells to 100%.

Detection of Apoptosis and Level of Reactive Oxygen Species (ROS)

Apoptosis analysis was performed by Annexin V-FITC/PI Apoptosis kit (Elabscience), according to the kit instructions. Briefly, cells were washed with PBS, and

centrifuged at 250g, and cells were suspended in 500 µL of Annexin V Binding Buffer (5 µL of Annexin-V FITC and PI 5 µL) were added to each group. The cells were gently mixed and incubated at room temperature for 30 min. After incubation, the samples were analyzed using a FACS Calibur (BD Biosciences). The data obtained was later analyzed using the Cell Quest program (BD Biosciences).

To determine cellular ROS levels, 2′,7′-dichlorofluoresceindiacetate (DC-FDA) staining was performed. Briefly, the cells were washed twice with PBS and suspended in serum-free RPMI-1640 medium. DC-FDA dissolved in serum-free medium was added at a final concentration of 10 µM and incubated for 30 min at 37 °C, 5% CO2. At the end of incubation, the cells were washed again with PBS and analyzed by flow cytometry.

Mitochondria Membrane Potential

The mitochondria membrane potential was assessed by incubating cells in JC-1. The cells were incubated in the RPM 1640 culture medium supplemented with 0.5 mL tetrachloro-tetraethylbenzimidazol carbocyanine iodide 1 (JC-1) staining working solution and cultured for 20 min at 37°C. Cells were washed with PBS twice by centrifugating at 250g for 5 min. The membrane potential was detected by a flow cytometer. The cells were incubated in the RPM 1640 culture medium supplemented with 0.5 mL tetrachloro-tetraethylbenzimidazol carbocyanine iodide 1 (JC-1) staining working solution and cultured for 20 min at 37°C. Cells were washed with PBS twice by centrifugating at 250g for 5 min. The membrane potential was detected by a flow cytometer.

Gene Expression Analysis

After 24 hours following the transfer of mitochondria, total RNA was isolated by Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA), and cDNA was synthesized by the cDNA synthesis kit (Thermo Scientific, Rockford, IL, USA), according to manufacturer's instructions. The amplification of target genes (Ki67, p38 and Erk1/2) was performed by Power SYBR-Green Master Mix (Thermo, Applied Biosystems Life Technologies, Carlsbad, CA, USA) with gene-specific primers in LightCycler 480-II (Roche) according to manufacturer's recommendations. Cp values were calculated by LightCycler 480 Software (release 1.5). Beta-actin was used as a housekeeping gene in normalization. ΔΔCp values were calculated with respect to control.

STATISTICAL ANALYSIS

The experimental groups were analyzed in triplicates (n=3). Statistical evaluation of the data obtained from the analyzes was performed using a parametric one-way ANOVA test for multiple group analyzes in the Minitap 14 program. Data acquired from the analyzes was analyzed using the student's paired t-test. Results were considered statistically significant at $p < .05$.

RESULTS

To assess the effect of progestin, E-SCs were culture in RPMI 1640 medium with progestin first for 24 hours. The mitochondria were transferred after then at the amount of 5 µg, equivalent to the protein concentration. The amount of mitochondria is almost equal to the mitochondria mass derived from the E-SCs at the number of 1x106 cells. As control, cells without treatment with progestin or mitochondria were used. The transfer efficiency was evaluated by flow cytometer, and it revealed that 90.17% ±1.22 of cells were positive after 24 hours of incubation, indicating that they were transplanted with exogenous mitochondria. However, the level of mitochondria penetrated inside the cells was not homogenous within the cell population (**Figure 1**). After 48 hours of incubation, the labeled mitochondria were still present in their cytoplasm with a signaling ratio of 84.61% ±5.24.

As the highest level of mitochondria was detected in the cells after 24 hours of transplantation, cells were analyzed at that time. The cell viabilities were not adversely affected by the transplanted mitochondria (**Figure 2A**). The progestin even affected viability. Remarkably, viability was improved in the cell

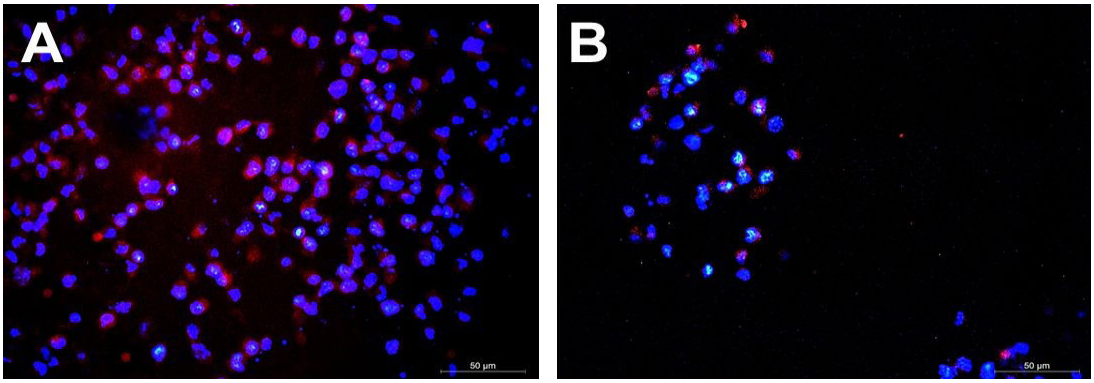

Figure 1. E-SCs after being transplanted with exogenous mitochondria derived from E-SCs, were labeled with TMRM dye and transplanted by adding the mitochondria to the culture medium. The cells were cultured and after 24 hours (A) and 48 hours (B), and mitochondria were visualized in red fluorescence. Cell nuclei was stained with DAPI, showing blue fluorescence. Scale bar,

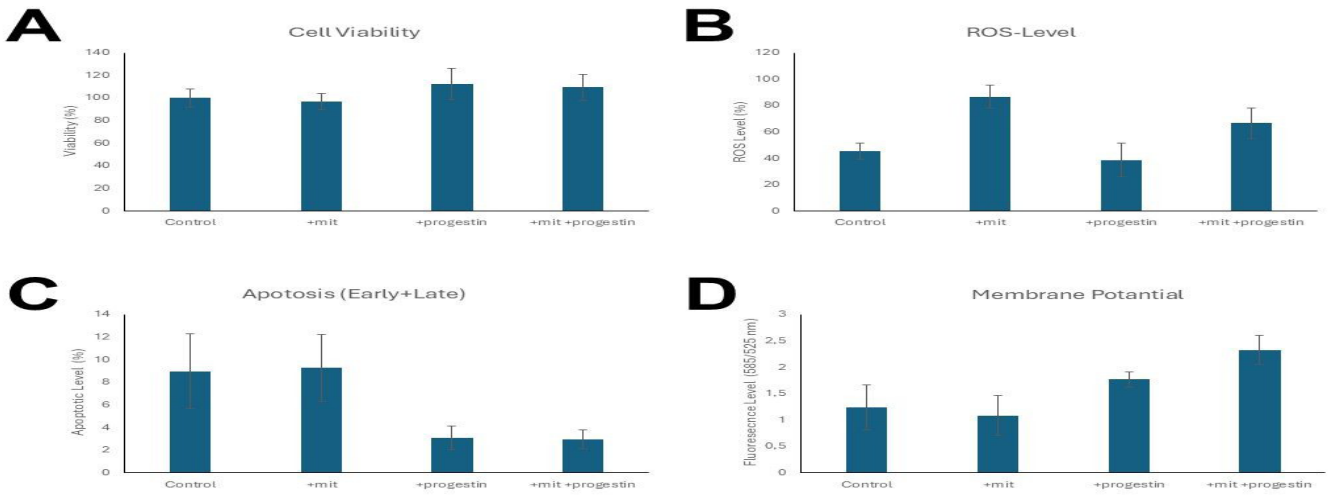

Figure 2. After 24 hours of transplantation, cells were analyzed for cell viability by CCK8, reactive oxygen species by DC-FDA staining, apoptosis by AnnexinV/PI staining, and mitochondrial membrane potential by JC-1 staining. The control group (without transplantation and progestin) was compared with sole mitochondria-transplanted group (+mit), sole progestin-treated group (+progestin), and the group (+mit +progestin) co-treated with mitochondria and progestin.

treated with both mitochondria and progestin. On the other hand, the ROS levels were found highest in the mitochondrial transplanted groups, indicating that the mitochondria might be damaged during isolation and the transplantation. The progestin negatively affected the production of the ROS level and decreased even below the level of the control group. This effect of progestin improved the mitochondria transplantation group in which the ROS level decreased in the co-treated cells (**Figure 2B**).

The apoptotic level of cells increased significantly after mitochondria transfer (**Figure 2C**). This increment was in parallel with the ROS level. Later, the increased apoptotic level was attenuated in the presence of the progestin, and this supportive effect of the progestin continued in the co-treated groups with mitochondria and progestin. This was also observed in the results of the mitochondrial membrane potential.

The attenuated level of ROS and apoptotic level due to the progestin could be linked with the activity of the mitochondria. The analyzes with JC-1 showed that the disruption in the mitochondrial membrane potential was improved due to the progestin, which improved the overall cell viability. The cell membrane potential level was found highest in the groups with progestin treated ones (**Figure 2D**).

The cellular response was evaluated also with the gene expression profile for Ki67, Erk1/2 and p38. Ki67 is a marker for cell proliferation. It was observed that either mitochondria transplantation or progestin treatment had an inductive effect on the proliferation (**Figure 3**). This could also explain the high level of cell viability even in the presence of an increased level of apoptosis and ROS levels. However, the treatment of both progestin and mitochondria did not show a combined improving effect so that the level of mitochondrial activity could be covered by the progestin effect in the cells. Although the reason was not clear, the expressions of Erk1/2 and

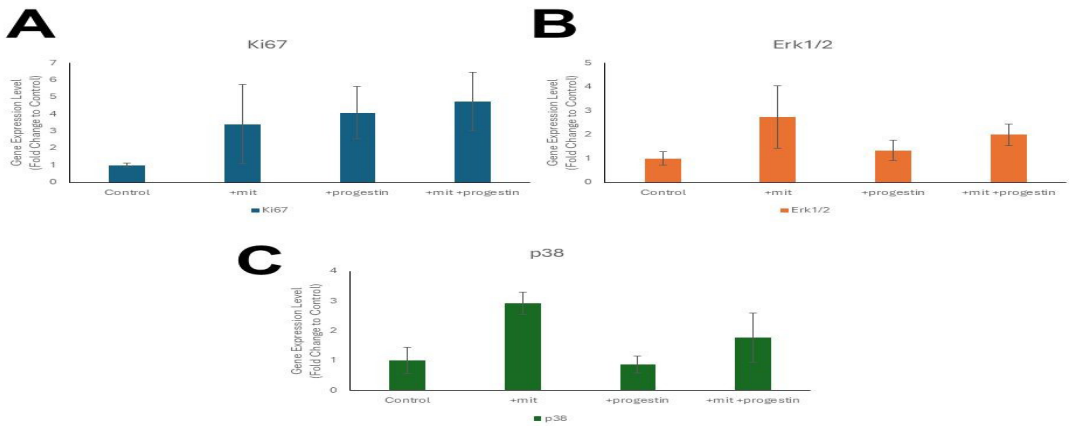

Figure 3. Gene expression analysis of mitochondria-transplanted and progestin-treated E-SCs for Ki67 (A), Erk1/2 (B), and p38 (C) after 24 hours of incubation following transplantation and/or progestin treatment. The control group (without transplantation and progestin) was compared with the sole mitochondria-transplanted group (+mit), the sole progestin-treated group (+progestin), and the group (+mit +progestin) co-treated with mitochondria and progestin. Gene expression levels were calculated as fold change relative to control cells.

p38, markers for the MAPK signaling pathway, were suppressed by the progestin. This suppression might improve the adverse effect of the exogenous mitochondria in the cells regulating the apoptotic levels by decreasing the apoptosis. Alternatively, the MAPK pathway might also induce mitophagy in the cells, which could explain the decreased level of transplanted mitochondria in cells after 48 hours compared to the cells after 24 hours.

DISCUSSION

Dienogest is a selective progestin that combines the pharmacological properties of 19-norprogestin and progesterone derivatives. Therefore, it has a strong progestational effect on the endometrium and a systemic anti-androgenic effect. There is a randomized placebo-controlled study showing that dienogest at a dose of 2 mg/day reduces pelvic pain and dysmenorrhea (21-23). In addition, case series have been published showing its effectiveness in both reducing lesion size and reducing pain in deep endometriosis and extrapelvic endometriosis (24). Progestins inoks prevent implantation of regurgitated endometrium, inhibit angiogenesis and the expression of matrix metalloproteinases, and reduce inflammation of endometriotic implants and the subsequent immune response (25,26). Dienogest (DNG) is a therapeutic drug used in the treatment of endometriosis. There is limited data regarding its mechanism of action on endometrial cells. Changes induced by DNG treatment in human endometrial stromal cells (E-SCs) were investigated using in vivo and in vitro models (27).

E-SCs are the cells at the center of endometrial regeneration. Any improvement in the viability of these cells would positively support the regenerative process of the tissues. So far, it is unknown how the cells are affected after mitochondrial transplantation in the presence of progestin. Progestin stimulates mitochondrial respiration by inducing biochemical and molecular parameters (28). The progesterone-induced increase in mitochondrial activity is not a precursor to apoptosis, but rather is protective (29). It was shown that the progestin positively affected the transplantation output, improving viability while decreasing the ROS and apoptotic level. Here, the mitochondrial membrane potential was focused on, as progestin improved the overall mitochondrial membrane potential. Depending on progestin-stimulated ATP production may contribute to prevention of cell apoptosis cooperatively with growth factor-stimulated signaling (30). The direct relation of progestin / progesterone has not been revealed, but the indirect relation was shown in studies in other cell lines (22). The energy status of the cell is key in the decision about cell proliferation. The higher the level of ATP present in the cells, the more it would improve the cellular metabolism in many aspects. This increased level of ATP could also support the antioxidant protective mechanism inside the cells, which provides defense against the increased ROS levels in the cells. Therefore, the induced ROS levels after the mitochondria transplantation did not undesirably affect viability and proliferation. Therefore, the progestin in the media improved the transplantation outcome. This challenges the dogma that exogenous mitochondria affect the cells toxically after transplantation due to increased ROS levels (16). The level of ROS still in cells is still a concern in viability. Its negative effect might be shed by the progestin.

The expression of Ki67, Erk1/2, and p38 genes constitutes a critical determinant of cellular viability and functionality (31). Ki67, a recognized marker of cellular proliferation, signifies the maintenance of functional integrity through the process of cellular division, a metabolic event necessitating substantial ATP. Under conditions of healthy mitochondrial transfer, these organelles effectively supply the requisite energetic substrates to support this process. Conversely, in instances of mitochondrial damage or dysfunction, characterized by disruption of the mitochondrial membrane potential, a reduction in ATP synthesis and an elevation in reactive oxygen species (ROS) generation are observed (32). This physiological stressor induces the transcriptional upregulation of Erk1/2 and p38, both members of the mitogen-activated protein kinase (MAPK) superfamily, which are integral to cellular stress response pathways (33). Notably, p38 has been implicated in the modulation of cellular proliferation under conditions of physiological stress. The addition of the progestin in the culture was known to support cell viability and proliferation. It was shown that progesterone can induce TERT expression in cells transiently within 12 hours after exposure. This induction was reported to mediate by MAPK signaling pathway (34,35). It was shown even to increase the telomerase activity by inducing TERT gene expression, which is expressed during the cell cycle process at the S-phase, where genomic DNA was duplicated. Addition of progestin positively affect the cell viability directly, but the mitochondria membrane potential was shown to improve in the presence of the progestin. Progesterone could have a wide range of beneficial effects on mitochondrial function. Interestingly, progesterone could protect neurons from injury by increasing the mitochondrial membrane potential and ATP production in neurons (36). In another study, progesterone was shown to improve mitochondrial function in sperm by increasing the mitochondrial membrane potential and ATP production (37). Progestin, like progesterone, could improve mitochondrial membrane potential and ATP production, while attenuating ROS production. In our study, it was shown that the progestin could also improve the cell viability and support the mitochondrial function in E-SCs.

Limitations of the Study

This study has several limitations. It was conducted entirely in vitro, which limits the ability to predict in vivo outcomes. The use of endometrial stromal cells from healthy individuals may not fully represent the cellular environment in disease states such as endometriosis or Asherman's syndrome. Additionally, the short observation period did not allow for evaluation of long-term effects or stability of the transplanted mitochondria. The molecular mechanisms underlying the effects of progestin on mitochondrial function were not fully explored. Further in vivo studies are necessary to confirm these findings and assess their clinical relevance.

CONCLUSION

Progestin could be used as an effective agent to control cell viability after mitochondria transplantation. In the treatment of endometriosis, progestin is commonly used. Therefore, tissue regeneration might be improved by mitochondria transplantation in addition to progestin treatment. Here, it was shown that the progestin did not disrupt the cellular event. Conversely, it supported the proliferation by decreasing the apoptotic levels. Mitochondria transplantation could possess some negative sides, as the process might, induce the ROS level in the tissue, even undesirably contributing to the degenerative nature of the endometrium. Progestin might be used to support mitochondria transplantation success in other cells or tissues. These findings also need to be confirmed through in vivo studies. In conclusion, cotreatment of mitochondria transfer and progestin has a significant effect on cell viability.

DECLARATIONS

Ethics committee approval: This study was carried out in accordance with the ethical principles of the Declaration of Helsinki. Patient data and tissue samples were used under ethical guidelines. Approval for the study protocol was obtained from the Ethics Committee of Kocaeli University (approval date: 12 March 2020; approval number: KU-GOKAEK 2020/55).
Informed consent form: Since the study was performed using previously collected tissue samples and conducted in vitro, and patient data were anonymized before analysis, an informed consent form was not required.
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The author has read and approved the final version of the manuscript.
Conflict of interest: The author declares that no conflicts of interest.
AI: Artificial intelligence tools were used to assist in language editing and improving the clarity of the manuscript. However, all scientific content, data analysis, and interpretations were carried out by the author, who bear full responsibility for the content and conclusions of this work.

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