

Effect of PLGA Nanoparticle-Mediated Delivery of miRNA 503 on The Apoptosis of Ovarian Endometriosis Cells

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Received: 11/July/2022, Accepted: 29/August/2022

Abstract

Objective: One of the challenges in gene therapy is the transfer of the gene to the target cell. MicroRNAs (miRNAs) regulate gene expression after transcription by binding directly to the messenger and play a vital role in cell behaviors and the pathogenesis of some diseases. This study was aimed at developing poly (lactic-co-glycolic acid) (PLGA)-based nanoparticles (NPs) for gene delivery to endometriotic cyst stromal cells (ECSCs).

Materials and Methods: In this experimental study, endometriosis cells were isolated from women with severe endometriosis (DIE) and digested by the enzymatic method (40 µg/ml DNAase I and 300 µg/ml collagenase type 3). PLGA-based NPs were synthesized and characterized. The size of sole PLGA NPs and PLGA/miRNA were 60 ± 4 nm and 70 ± 5.1 nm respectively. Poly lactic-co-glycolic-based NPs were used as vector carriers for miRNA 503 transfection in endometriosis cells. The cells were divided into the five groups of control and four doses (25, 50, 75, and 100 µm) of miRNA 503/PLGA at 12, 24, 48, and 72 hours. Viability and apoptosis were evaluated by the MTT assay and Annexin Kits. Data were analyzed by one-way analysis of variance.

Results: The results show that the size of PLGA/miRNA complex with dynamic light scattering (DLS) was 70 ± 5.1 nm and zeta potential values of the PLGA/PEI/miRNA complexes were 27.9 mV. Based on the MTT assay results, the optimal dose of miRNA 503/PLGA was 75 µm, at which the viability of ECSCs was 52.6% ± 1.2 (P≤0.001), and the optimal time was 48 hours. The apoptotic rates of ECSCs treated with PLGA/miRNA503 (34.75 ± 4.9%) were significantly higher than those of ECSCs treated with PLGA alone (3.35 ± 2.58%, P≤0.01).

Conclusion: Cell death increased with increasing the concentration of miRNA; thus, it can be suggested as a treatment for endometriosis.

Keywords: Apoptosis, miRNA 503, Nanoparticle, Ovarian Endometriosis

Cell Journal (Yakhteh), Vol 24, No 11, November 2022, Pages: 697-704

Citation: Chaichian Sh, Mehdizadeh Kashi A, Tehermanesh K, Pirhajati Mahabadi V, Minaeian S, Eslahi N. Effect of PLGA nanoparticle-mediated delivery of miRNA 503 on the apoptosis of ovarian endometriosis cells. Cell J. 2022; 24(11): 697-704. doi: 10.22074/CELLJ.2022.557554.1069.
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Introduction

Endometriosis is an estrogen-dependent gynecological disease in which endometrial tissue is found in unusual places outside the uterus, ranging from minor lesions on other healthy pelvic organs to large endometriotic ovarian cysts. This condition changes the ovaries and causes the viscera to stick to each other, and this adhesion is the main cause of pain and infertility in these people (1). Ding et al. (2) show that the cause of estrogen sensitivity in endometriosis tissues is related to the activity of neurons. Inflammation affects the growth of the oocyte, and the unfavorable environment created by the activity of macrophages reduces the quality of the oocyte, resulting in infertility (3). There is also an endocannabinoid system in the human ovary that is effective in the neuro protection of cells and has an anti-inflammatory function through modulating cell survival and proliferation and inducing apoptosis in normal cells.

In one study a relationship between this system and

ovarian pathologies was shown (4). In endometriosis, receptors of this system are inhibited and increased cell inflammation, disease progression, and pain (5). Endometriosis is classified as a tumor disease by the World Health Organization (WHO) because it has a tumor-like structure and behaves like cancer in terms of invading other tissues. In fact, endometriosis, like cancer cells, attacks tissues, induces angiogenesis, increases the production of estrogen, impairs immune function, and causes inflammation (6). At present, the best method for the diagnosis of endometriosis and isolation of invasive tissues is laparoscopic examination with the histological confirmation of glands in removed lesions (7). Therefore, people's fear of surgery and assuming dysmenorrhea as normal delay the timely diagnosis of the disease (8).

Today, methods such as gene therapy have been used in the diagnosis and treatment of diseases. MicroRNAs (miRNAs) regulate post-transcriptional gene expression playing a crucial role in proliferation, differentiation, and

apoptosis, which are key to the diagnosis of endometriosis (9). miRNAs are involved in cell survival, proliferation, angiogenesis, and apoptosis, therefore, they are effective in the pathogenesis of endometriosis (10). In endometriosis, programmed cell death, which is called apoptosis, is reduced. Thus, endometriotic tissues develop in the abdominal and pelvic cavities. Accordingly, there is an inverse relationship between the severity of endometriosis and the rate of apoptosis (11). Viral and non-viral vectors are used for miRNAs transmission. Non-viral gene delivery vectors, such as inorganic nanoparticles (NPs) and liposomes, have been used in recent years (12).

One of the best carriers is poly (lactic-co-glycolic acid) (PLGA), which has been widely used in drug delivery because it escapes from the endo-lysosome system and keeps its contents longer. Therefore, it is also suitable for gene transfer (13). Various miRNAs have been observed in endometrial and atopic lesions (14). One of the miRNAs inhibited in endometriosis is miR-503, which is involved in endometriotic cyst stromal cells (ECSCs) cell apoptosis. miR-503 inhibits cell cycle in the G0/G1 phase and prevents cell proliferation (15). Since to date, there is no study that have been measured the effects of this nanoparticle on apoptosis of endometriosis cells, in this study we performed the effect of PLGA-micro RNA delivery on the apoptosis of stromal cells of ovarian endometrium cysts *in vitro*.

Materials and Methods

Sampling and culture of human endometriosis tissue

In this experimental study, ovarian endometrioma cyst walls were removed from women 30 to 40 years at Hazrat-e Rasool Hospital during laparoscopic surgery. After washing, the tissue fragments were enzymatically digested with collagenase and DNAase and incubated at 37°C with 5% CO₂ for 90 minutes. Then, the tissue pieces were passed through filters (45 µm) (16). Cells cultured at 37°C in a 5% CO₂ atmosphere in DMEM/F12 medium were supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), gentamycin (40 µg/mL), and 5% fetal bovine serum for three weeks, and the culture medium was changed every 2-3 days when they reached 80% confluency. The research was approved by the Research Ethics Committee of Iran University of Medical Sciences (IR.IUMS.REC.1397.1176).

Immunocytochemical analysis of cultured endometriosis cells

In order to confirm the cyst wall endometrial tissue-isolated cells, immunocytochemistry was performed for CD10 markers (17). After 3 weeks, endometriosis cells were detached from the flask using trypsin/EDTA. The cells were smeared and incubated with 4% paraformaldehyde at room temperature for 10 minutes. Triton X-100 was added to the cells to make the cell membrane permeable to antibodies, then antibodies were added to the cells at appropriate concentrations in a 1:200 ratio overnight at 4°C. Also, the cells were washed

thoroughly and stained with Fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ig antibodies 1:500 for 1 hour at room temperature in the dark. After a further wash, they were mounted in glycerol. CD markers were analyzed by fluorescence microscopy.

Preparing microRNAs

miRNAs 503, including primer (sequence [5' → 3']: TAGCAGCGGGAACAGTTCTGCAG) (Fluorescent marker), were purchased from Pishgam Co. (St Kargar, Tehran, Iran), and PLGA (Resomer RG502H) with a 50:50 mole ratio of glycolic acid to lactic acid and a molecular weight of 12,000 g/mol, polyvinyl alcohol (PVA, 89 mol% hydrolyzed), Span 80, and Tween 80 were purchased from Sigma (St Louis, MO, USA).

PLGA NPs containing miRNAs were synthesized by using the water-in-oil-in-water solvent vaporization technique consisting of an organic phase and two aqueous phases in one medium (18). To stabilize the dispersed phase, a stabilizer is needed, the most commonly used of which is polyvinyl alcohol. This substance in the external aqueous phase creates a thin layer around the particles. In the organic phase, the active substance was used at the Span 80 level, and in the aqueous external phase, Tween 80 level. All the solutions were prepared in DEPC-treated water, and RNase-free media were used at all stages. To prepare the desired nano capsule, the internal aqueous phase was first obtained by creating a polyplex derived from polyethylene imine) PEI 25 (KDa and miRNA with a 3: 1 mass ratio. Polyethylene imine is capable of compressing high molecular chain genetic content that can produce NPs of appropriate size entering the cell as endocytosis. First, 0.1% solution of PEI was prepared in DEPC-treated water. A miRNA solution was also prepared using DEPC-treated water at a concentration of 100 pmol/µl. It was then combined in 80 µl of PEI solution containing 90 µg of PEI and 40 µl of miRNA containing 30 µg of miRNA, and the volume was reached to 0.5 ml by phosphate-buffered saline (PBS, Merck, USA) and incubated in a thermocycler at 37°C for 30 minutes.

To prepare the organic phase in solvent evaporation, 10 mg of PLGA was dissolved in 2 ml of organic solvent ethyl acetate. To form an initial emulsion of 0.5 ml of internal aqueous phase, 0.5 ml of Span 80 solution at a concentration of 5 mg/ml was added to the organic phase using Vertex and an ultrasonic bath. The initial water emulsion was created in the oil.

This water emulsion was then added dropwise to an oil containing 5 ml of 8% PVA and 10 mg of Tween 80 as an outer phase for 3 minutes using a probed ultrasonic device. The power of 50 watts was subjected to sonication to form a secondary emulsion. In the final step, the final dual emulsion solvent diffusion was added to 4 ml of 0.5% PVA and subjected to magnetic stirring for 4 hours until the ethyl acetate solvent was used to diffuse the external aqueous phase containing solid polymer particles. After forming the nanoparticles, they were separated twice and purified by centrifugation at 12,000 rpm for 30 minutes and also

suspended in distilled water to remove unloaded miRNA and additional surfactants in the external aqueous phase from the surface of NPs (the supernatant was investigated by dynamic light scattering to determine the separation efficiency). The nano capsules were finally dried for 24 hours and stored in a refrigerator at a temperature of 4°C for 24 hours. All of the above steps were performed with 0.5 ml distilled water without drug as internal aqueous phase (19). Poly lactic-co-glycolic (Resomer RG502H) with a 50:50 mole ratio of glycolic acid to lactic acid and a molecular weight of 12,000 g/mol, PVA (89 mol% hydrolyzed), Span 80 and Tween 80 were purchased from Sigma (St Louis, MO, USA).

Nanoparticle characterization

Some of the dried powder of PLGA/PEI/miRNA was dispersed in 1 ml of saline phosphate buffer at pH=7.4 using an ultrasonic bath, and the zeta potential was measured using a zeta meter device. The size and morphology of the PLGA NPs and PLGA modified with PEI /miRNA complexes onto a copper sheath, carbon coated, were characterized via transmission electron microscopy (TEM). Finally, the cellular uptake of NPs was examined by TEM. For the TEM technique, ECSCs were washed with PBS, then 2.5% glutaraldehyde was used as a primary fixation for 2 hours. The cells were rinsed 2-3 times with PBS, and free glutaraldehyde was removed. Then, 1% osmium tetroxide was used as a secondary fixation for 1.5 hours. The cells were dehydrated in acetone (50%, 70%, 90%, 100%), infiltrated by resin, and finally, embedded in pure resin (Epon 812, TAAB, UK). Then, 50 nm sections were stained with uranyl acetate and lead citrate on copper grade and then imaged with TEM (LEO 906, Zeiss).

Cytotoxicity assay

In this study, ECSCs were divided into the five groups of control and experimental groups, with cells distributed in a 96 well plate at a cell density of 20×10^3 cells per well in the different concentrations of PLGA/miRNA (25, 50, 75, and 100 μ M) and different incubation periods (12, 24, 48, and 72 hours). We performed the MTT assay to determine the toxicity of PLGA/miRNA. To evaluate the survival rate, the cells were centrifuged and washed with PBS and incubated with 100 μ l of MTT solution (MTT tetrazolium salt 5 mg/ ml) for 3-4 hours. Finally, the cells were centrifuged, and the supernatant was removed. Next, 100 μ l of dimethyl sulfoxide (DMSO, Merck, USA) was added to the wells, and the plates were shaken for 10 minutes in a microplate shaker before observation with the ELISA reader at 570 nm. The cells were then treated with the optimum dose obtained, and survival rates compared with and without nanoparticles.

Apoptosis evaluation

After determining the effective dose of microRNA and PLGA in cell viability, the cells were cultured with a dose of NPs and microRNA/NPs with the lowest viability for 48 hours. Then, the apoptosis rate of cells was assessed using Annexin V-FITC Apoptosis Detection Kit. That way,

500 μ l of the binding buffer was added to the cell plate. Afterward, 5 μ l of Annexin V-FITC and 5 μ l of PI at room temperature were added to the cells and incubated in foil for 10 minutes. Finally, flow cytometry was performed, and the rate of apoptosis in cells was evaluated (20).

Transplantation of endometriosis cells to mice

In this experiment, 12 mice NMRI (n=6 in each groups) (6-8-weeks-old) with 25 ± 1 g weight were divided into two groups. In the first group, cells that had only PLGA added to their culture medium were injected, and in the second group, cells treated with PLGA/miRNA were injected. The animals were kept in university laboratory's animal house. To weaken the immune system of the mice, they were treated with a single dose of 7.5 Gy γ -irradiation for 6 min (21). After 72 hours, cells were transplanted to the back of the thigh of the mice. The mice were anesthetized with the intra peritoneal injection of a mixture of 100 mg/kg ketamine hydrochloride 10% (Rotexmedica, Germany) and 10 mg/kg xylazine 2% (Alfasan, Holland). Then, 20 μ L of suspension, including 2×10^6 cells at the fourth passage, was injected subcutaneously into the back of the right front limb in each group. All the mice were observed for 3 weeks.

Statistical analysis

Data were analyzed using One-way analysis of variance (ANOVA) to compare different groups. The analysis was performed by using of SPSS, version 16 (Chicago, IL, USA). Results were expressed as mean \pm SEM, and a $P < 0.05$ was considered significant.

Results

Sampling and culture of human endometriosis tissue

The stromal cells were harvested from endometriosis cell. One week after digestion and culture, size and morphology of them were similar to fibroblast cells. At the end of third week cell confluency was 2×10^5 cells/ml. An aspect on the phase contrast microscopy of the third passage of the culture derived from an ovarian endometrioma is presented in Figure 1A, B. The cultured cells were confirmed to be positive for CD10 antigen (Fig.1C).

Characterization of nanoparticles/miRNA complexes

The particle size and surface morphology of the NPs were examined by TEM, DLS, and Zeta potential (Fig.2A-C). PLGA NPs with a size below 100 nm are effective in gene transfer (22). In this study, the mean diameter of the sole PLGA NPs was 60 ± 4 nm, whereas the size of PLGA/miRNA complex with DLS was increased to 70 ± 5.1 nm. The surface charge index of NPs is determined by Zeta potential. For the endocytosis of particles into the cell, a more positive particle load leads to a stronger bond to the cell membrane surface and easier penetration to the cell. In this study, zeta potential values of the PLGA/PEI/miRNA complexes were 27.9 mV. The cellular uptake of NPs is shown by TEM (Fig.2D).

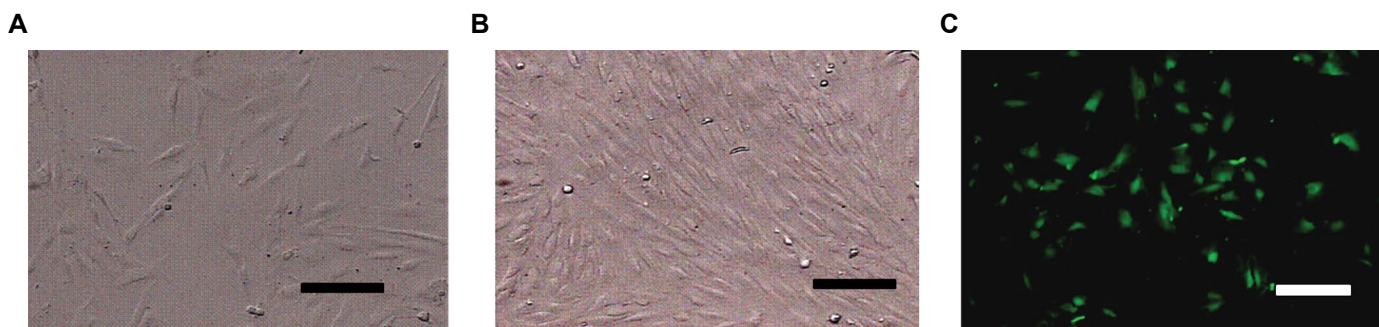


Fig.1: The structure of endometriosis cells in the culture medium. Size and morphology of them were similar to fibroblast cells. **A.** Endometriosis cells one week after planting, **B.** The end of the third week of culture (scale bar: 50 μ m). **C.** Immunocytochemistry of endometriosis cells with CD10 marker (scale bar: 30 μ m).

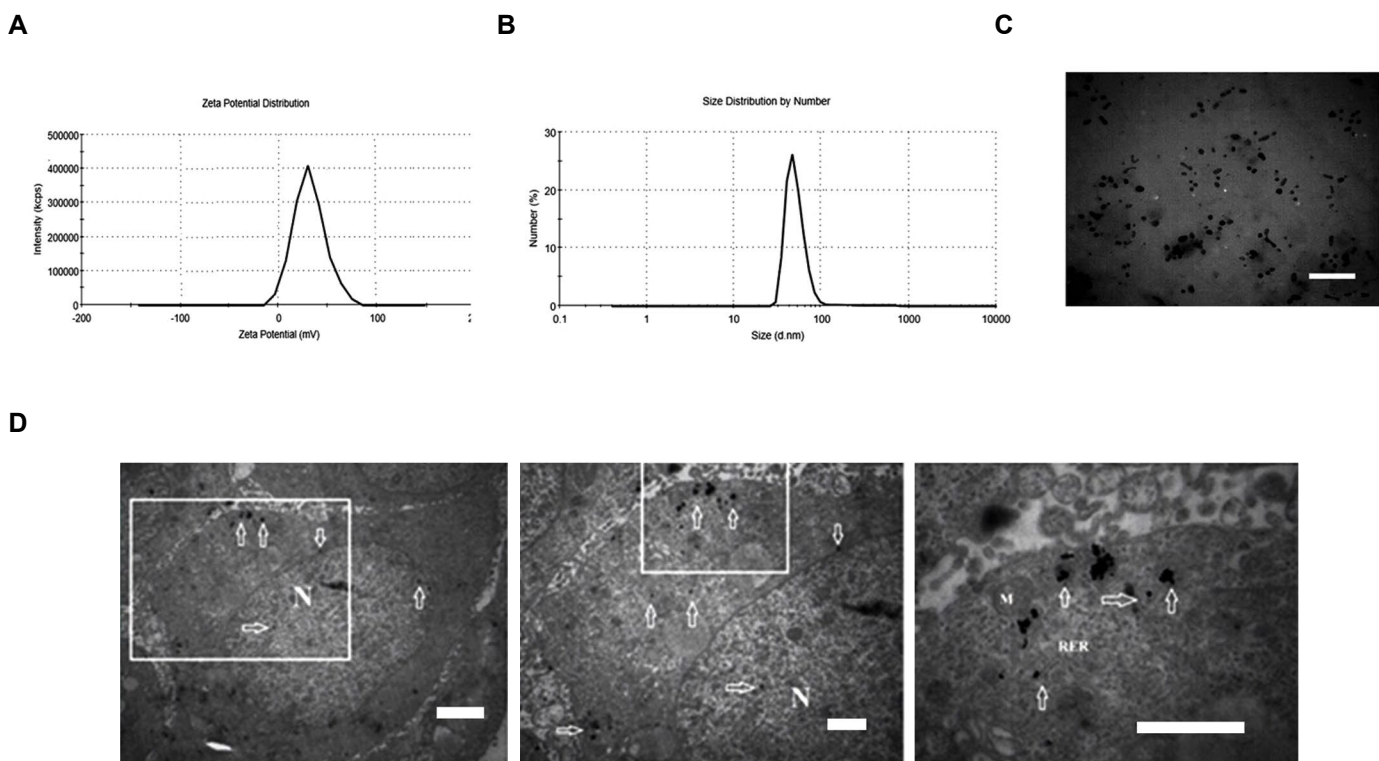


Fig.2: Nanoparticle evaluation tests. **A.** Zeta potential, **B.** The particle size based on the DLS test, **C.** Electron microscope image of PLGA showed spherical surface in all nanoparticles (scale bar: 500 nm), and **D.** As shown, the nanoparticles have accumulated in the nucleus and cytoplasm (scale bar: 1 μ m). DLS; Dynamic light scattering and PLGA; Poly lactic-coglycolic acid.

Cell viability

The viability indices of cells for the control and 25, 50, 75, and 100 μ M PLGA/miRNA concentrations were 97.3%, 91.3%, 85.6%, 81.4%, and 70.2% in 12 hours, 98%, 88.4%, 80.4%, 78.8%, and 68.1% in 24 hours, and 97.3%, 70.3%, 64.2%, 52.6%, and 47.9% in 48 hours, and 98%, 66.8%, 60.3%, 49.9%, and 44.6% in 72 hours, respectively. The survival rate of stromal cells at the concentrations of 25, 50, 75, and 100 μ M PLGA/miRNA decreased compared to the control group at 12, 24, 48, and 72 hours in a time- and dose- dependent manner.

The results showed that with increasing incubation time

from 24 hours to 48 hours, the survival rate decreased, and with increasing time to 72 hours, cell survival decreased, but no significant difference was observed in this regard between 24 hours and 72 hours. Therefore, the incubation time was 48 hours. Also, in comparison with the survival rate in different doses, the survival rate decreased with increasing concentration, but as the figures shows, no significant difference was observed between 75 μ m and 100 μ m concentrations; therefore, 75 μ m doses were selected ($P \leq 0.001$, Fig.3). The cells were then incubated at a dose of 75 μ m of PLGA for 48 hours, and the survival rate was assessed. The cell viability rates in the control and treated groups were 98.4% and 89.9%, respectively,

which showed a significant difference in the survival of cells treated with PLGA/miRNA (Fig.4).

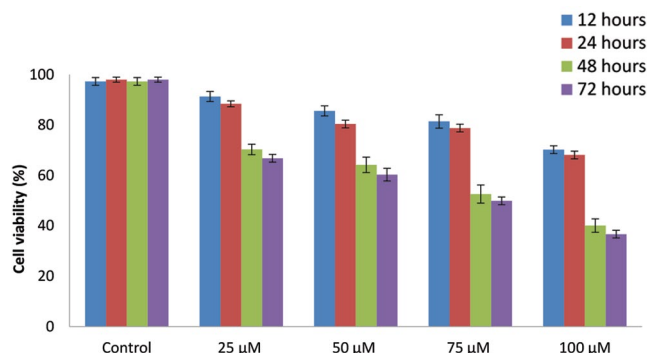


Fig.3: Based on MTT test, treatment with 75 μm miR-PLGA for 48 hours was selected; significant differences between groups were observed ($P \leq 0.001$). MTT; (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and PLGA; Poly lactic-co-glycolic acid.

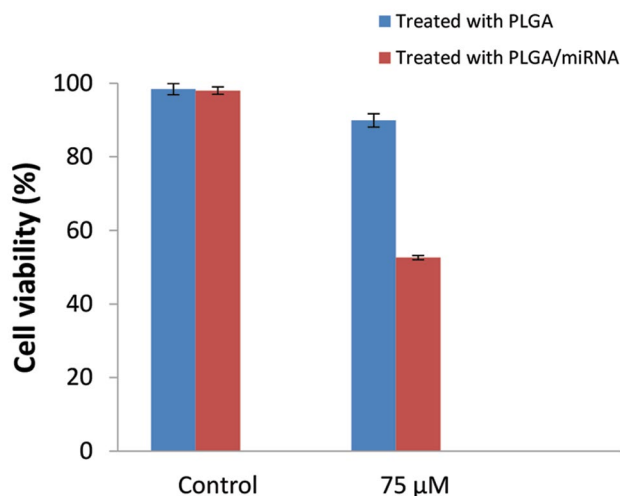


Fig.4: Based on MTT test, significant differences in survival rate with and without miRNA were observed ($P \leq 0.001$). MTT; (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and PLGA; Poly lactic-co-glycolic acid.

Apoptosis evaluation in ECSCs after treatment with PLGA/miRNA503

Apoptosis was measured using the annexin V-FITC apoptosis detection kit. The rate of apoptosis in control group was 0.98 ± 0.1 (Fig.5A). The result showed that total apoptosis in ECSCs treated with PLGA miRNA503 ($35.66 \pm 4.6\%$) were significantly higher than those of cells treated with PLGA ($3.76 \pm 1.19\%$, $P \leq 0.01$, Fig.5B, C).

The gross morphology of endometriosis lesion

The macroscopic observation of endometriosis lesions

in the two models are presented in Figure 6. In the first group, cells were treated with PLGA alone, and in the second group, they were treated with PLGA/miRNA. As shown in the figure, in the second group, the rate of cell apoptosis was higher, and tumor size was smaller. These lesions had a cystic morphology and were distinguished from the surrounding tissues.

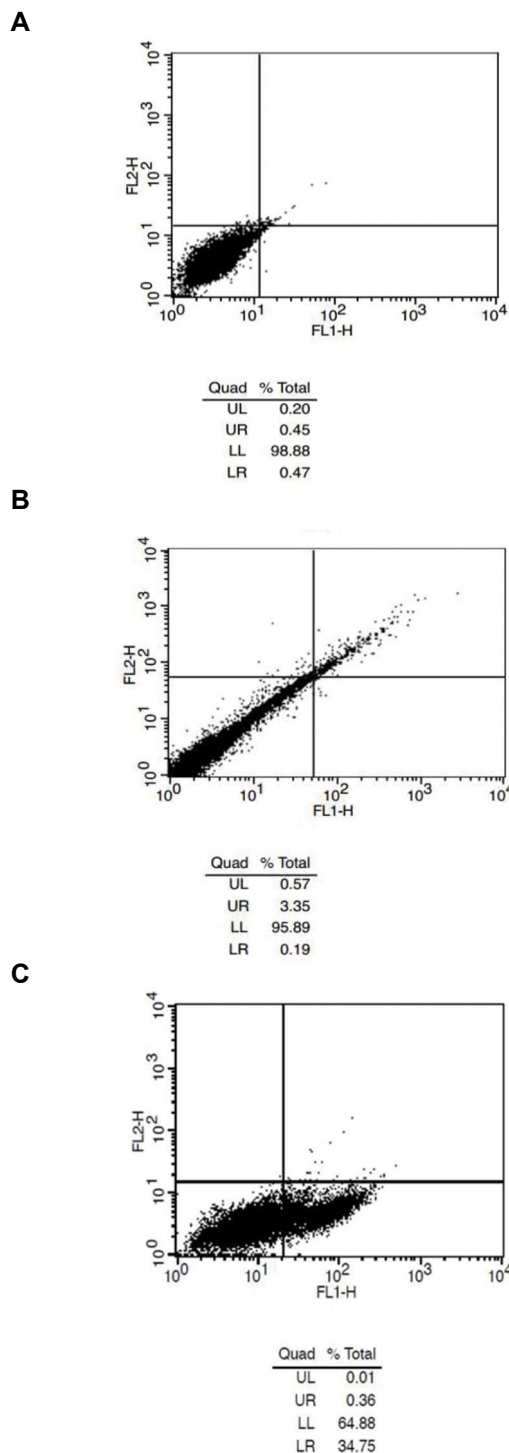


Fig.5: Based on the annexin assay. **A.** Flow cytometry of cells in the control group, **B.** PLGA-treated group, and **C.** PLGA/miRNA 503-treated group, result show that the apoptotic rates of the ECSCs treated with PLGA/miRNA 503 were significantly higher than those of cells treated with PLGA ($P \leq 0.01$).

A**B**

Fig.6: In both groups, cells was injected subcutaneously into the back of the right front limb. After 3 weeks, the endometriosis lesions in two models were shown. **A.** PLGA-treated cell injection and **B.** PLGA/miRNA503-treated cell injection.

Discussion

Endometriosis is a benign disease of the female reproductive system, which is associated with increased angiogenesis and defects in cellular apoptosis (23) that behaves like a cancer in terms of aggressiveness (24). In one study, plant compounds with anti-inflammatory properties have been used to upturn the apoptotic effect of drugs in the treatment of cancer, and it has been observed that cell proliferation and angiogenesis were inhibited (25). Due to increase in the percentage of women with endometriosis and its common complications, including chronic pelvic pain and infertility, a non-surgical diagnosis is absolutely desirable (26). As the standard diagnostic modality for endometriosis is still laparoscopy, which carries many risks for the patient (27), a number of studies have been performed in this regard. For example, Samartzis et al. (28) used Doxycycline to inhibit the progression of endometriotic stromal cells *in vitro*.

NPs through structural mitochondrial damage are effective in causing apoptosis and cell necrosis (29). Nanomaterials in the treatment of endometriosis are

accumulation in endometriotic tissues. Chaudhury et al. (30) used cerium oxide NPs in an endometriosis-induced mouse model and observed the inhibition of angiogenesis. In another study, plant nanocomposites were used to induce apoptosis and necrosis of endometriotic stromal cells (31). NPs can be synthesized from various natural or synthetic lipids, proteins, metals, and polymers, one of the synthetic polymers used in numerous biomedical applications is PLGA (32). Poly lactic-co-glycolic NPs as drug delivery systems in antibiotic therapy, chemotherapy, and anti-inflammatory drugs have proven their potential (33). Shabani et al. (34) investigated the anticancer activity of cisplatin conjugated with PLGA NPs for elimination of mouse malignant cells from normal cell and observed that apoptosis rate of tumor cell was higher than free drug. Singh et al. (35) used the combination of doxycycline and PLGA NPs for the treatment of endometriosis and observed that angiogenesis was inhibited. Also used from letrozole and curcumin loaded-PLGA NPs for endometriosis in a mouse model. Guo et al. (36) injected endometriosis cells subcutaneously, and then the animal model was treated with two different types of NPs of different sizes (10 nm vs. 40 nm) by intravenous injection; they observed a regression of endometriosis. Li et al. (37) evaluated iron oxide NPs (15 nm) modified with hyaluronic acid in a rat model of endometriosis and reported that these NPs could accumulate in CD44 expressing tumors.

One biomarker that can be used in research is miRNAs, which raise as potent regulators of gene expression in proliferation, cell survival, and angiogenesis in some disease such as endometriosis (38). Shams et al. (19, 39) have shown that miRNAs are tumor suppressors, they used two effective miRNAs (143 and 206) to induce apoptosis in cancer cells. Hirakawa et al. (15) found that one miRNA in stromal cell of ovarian endometriosis, which inhibits cell proliferation and induction of apoptosis, is miRNA 503 that was epigenetically inhibited. Thus, we developed a PLGA-based nanoparticle polyplex with miRNA expression to induce apoptosis in endometriosis cells. Our results demonstrated that the cytotoxicity PLGA/miRNA 503 increased in ECSCs in comparison to PLGA. After the incubation of ECSCs with PLGA/miRNA 503, apoptosis evaluation was performed by using an annexin V-FITC apoptosis detection kit. The results showed that the apoptotic rates of PLGA miRNA 503 were significantly higher than those of PLGA, which is consistent with other studies (40). After the incubation of cells with PLGA miRNA and imaging by TEM, the presence of NPs in the nucleus and cytoplasm of cells was confirmed, which is in line with studies that showed that NPs with a size of 10-150 nm and surface charge of +30 – -20 mV could accumulate in endometriotic tissues (36).

Conclusion

We reported the synthesis and characterization of PLGA/miRNA and its *in vitro* effects on the viability of ECSCs. In addition, our results demonstrated that

miRNA 503 reduced cell proliferation and progressed apoptotic rate in endometriotic cells. The obtained results support the use of the optimal dose of PLGA/miRNA as an effective approach for preventing the progression of endometriosis.

Acknowledgments

This study was funded by a grant from Iran University of Medical Sciences (IUMS). We would like to give great thanks to the Nanotechnology Department of Iran University of Medical Sciences. The authors declare that there is no conflict of interest in this study.

Authors' Contributions

N.E., Sh.Ch., A.M.K., K.T., V.P.-M., S.M.; Contributed to conception and design, data and statistical analysis, and interpretation of data. N.E.; Contributed to experimental work and wrote the manuscript, V.P.M.; Performed transmission electronic microscopy process. Sh.Ch.; Was responsible for overall supervision. All authors performed editing and approving the final version of this manuscript for submission, also approved the final draft.

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