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A Novel Protocol for the Establishment of in *Vitro* Intrauterine Adhesions Model

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ABSTRACT

Background and Aim: Intrauterine adhesions (IUAs), also known as Asherman's syndrome, is a prevalent gynecological condition that presents with various clinical manifestations, including atypical menstrual patterns, pelvic discomfort, reduced endometrial thickness, repeated pregnancy loss, and potential infertility. Despite advancements in therapy, the successful pregnancy rate remains low, especially in severe cases, posing significant therapeutic challenges and poor prognoses. Therefore, extensive research is essential to understand the underlying mechanisms of IUAs and develop effective therapeutic interventions. The objective was to examine the co-culture endometrial stem cells (EnSCs) of the human uterus and intraperitoneal macrophages as an *in vitro* model for IUAs. The aim was to establish a physiologically relevant model that replicates the complex cellular interactions and microenvironment observed in the endometrium during IUAs development.

Methods: This study introduces a novel method of incubating co - culture of stem cells and intraperitoneal macrophages with hydrogen peroxide (H_2O_2) to mimic impaired endometrial stromal cells, considering the regenerative properties of macrophages, and prominent leukocytes in the endometrium. We investigate the influence of macrophages on the response of stem cells to H_2O_2 by examining the amount of cell migration after scratching and the cell viability by MTT test and evaluating the amount of secreted NO in the presence of H_2O_2 and without it. Statistical analysis utilized Student's *t-test* or *one-way ANOVA*, with GraphPad Prism version 6 software, considering results with $p< 0.05$ as statistically significant.

Results: The study involved culturing and preparing EnSCs in a 24-well plate, while peritoneal macrophages were isolated through lavage in mice. Four experimental groups were established: EnSCs (group A), EnSCs treated with H_2O_2 (group B), EnSCs co - cultured with macrophages (group C), and EnSCs co - cultured with macrophages treated with H_2O_2 (group D). Cell viability was assessed using the MTT test, measuring the reduction of tetrazolium salt to formazan crystals. Nitric oxide (NO) production was measured using the Griess reaction, and cell migration was evaluated through a scratch wound healing assay.

Conclusion: The findings indicated that H_2O_2 treatment had a negative impact on EnSCs viability, but co-culturing with macrophages provided a protective effect. Moreover, H_2O_2 treatment led to increase NO levels, suggesting macrophage activation against oxidative stress. Interestingly, H_2O_2 treatment promoted cell migration in both EnSCs alone and the co - culture, while macrophages inhibited migration. These results underscore the significance of the interaction between EnSCs and macrophages in understanding IUAs and developing effective treatments.

INTRODUCTION

Intrauterine adhesions (IUAs), also known as Asherman's syndrome, is a prevalent gynecological condition characterized by various clinical presentations, including atypical menstrual patterns, pelvic discomfort, reduced endometrial thickness, repeated pregnancy loss, and potential

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infertility (1). The primary cause of IUAs is damage to the basal layer of the endometrium, which can occur as a result of uterine cavity surgery, induced abortion, or infections (2, 3). The incidence of IUAs has been observed to increase in recent years, likely due to the rising frequency of cesarean section and uterine cavity operations (4). In a study conducted on a population of infertile women, the incidence of IUAs was found to be 4.6% (5). The primary pathological characteristic of this condition is the presence of endometrial fibrosis, which is characterized by abnormal accumulation of collagen, scarring / adhesions in the endometrium, proliferation of endometrial stromal cells, elevated fibroblast levels, and increased secretion of extracellular matrix (ECM) (6). At present, hysteroscopy adhesiolysis is considered the preferred treatment for Asherman syndrome (7). Despite breakthroughs in therapy modalities, the successful pregnancy rate remains low, and severe IUAs provide a significant therapeutic challenge with a poor prognosis. Serious injury to the basal layer may result in the loss of the majority of endometrial cells, and the endometrium may finally fail to recover (8). Therefore, it is imperative to conduct thorough research on the mechanisms underlying IUAs and devise efficacious therapeutic interventions. The implementation of a suitable *in vitro* is necessary for a research inquiry and a suitable alternative for animal models. In 1978, Prianishnikov suggested that there are stem cells located in the deepest basal layer of the human endometrium which have the ability to differentiate into different types of endometrial cells (9). Since then, subsequent studies have explored the involvement of endometrial stem cells (EnSCs) as a potential means to develop *in vitro* models of IUAs following treatment with hydrogen peroxide (H_2O_2) (10). Exposure to H_2O_2 is a widely employed technique in cellular models to induce oxidative damage or stress. The Fenton reaction involving the reaction between H_2O_2 and Fe^{2+} ions generates the highly reactive hydroxyl radical (OH), which is considered to be the principal mechanism underlying oxidative damage (11).

The incubation of stem cells with H_2O_2 is a method that might serve as a means to replicate some of the characteristics of impaired endometrial stromal cells (12), as macrophages are prominent leukocytes in endometrium f(13) and have regenerative features (14). The objective of this study was to examine the co-culture of EnSCs and intraperitoneal macrophages as a novel *in vitro* model for IUAs, aiming to establish a physiologically relevant model that replicates the complex cellular interactions and microenvironment observed in the endometrium during IUAs development and investigate the influence of macrophages on the response of stem cells to H_2O_2.

MATERIALS and METHODS

Table 1. Materials used in this study along with their manufacturing company

Material Name	Manufacturer	Country
EnSCs	IBRC	Iran
DMEM/F12 Culture Medium	Gibco	USA
Fetal Bovine Serum (FBS)	Gibco	USA
Falcon Tube (15 and 50 ml)	SPL Life Science	South Korea
Flask (25 and 75 cm2)	SPL Life Science	South Korea
Petri Dish	SPL Life Science	South Korea
Sample Cap	Anacell	Iran
ELISA 96-well Plate	SPL Life Science	South Korea
Microtube (0.5 and 1.5 ml)	Anacell	Iran
Cryo Vial 2 ml	SPL Life Science	South Korea
Ethanol (Alcohol)	Kimia Alcohol Zanjan	Iran
Penicillin/Streptomycin	Biosera	France
Trypsin-EDTA	Gibco	USA
MTT	Anacell	Iran
NO Measurement Kit	Sib Zist Fan	Iran

Table 2. The devices used in this study along with their manufacturing company

Device Name	Manufacturer	Country
CO_2 Incubator	Memmert	Germany
Class II Laminar Flow Hood	Jal Tajhiz	Iran
Centrifuge	Eppendorf	Germany
Inverted Microscope	OPTIKA	Italy
Optical Microscope	Nikon	Japan
Image J2(FIJI)	NIH	USA

Culture and preparation of EnSCs

Endometrial stem cells (EnSCs) of the human uterus were obtained from the Center of Genetic and Biological Resources of Iran. To initiate the cell culture, EnSCs cells were counted, and approximately 5×10^4 cells were placed at the bottom of each 24-well plate in a complete cell culture medium. Subsequently, the cells were given 24 hours to attach to the plate's surface. For the passage, the cells were first washed with PBS buffer. Then, trypsin was added to a T75 flask (2 ml) and a T25 flask (1 ml), followed by incubation at 37°C for 1 minute. This process helped detach the cells from the bottom of the flask. The detached cells were separated by pipetting, ensuring that EnSCs were in a single - cell state, without any cell aggregations. Microscopic observation confirmed the presence of single cells. To deactivate trypsin, the culture medium containing FBS was added, and pipetting was performed several times. The cells obtained from one flask were then transferred to several other flasks to promote their growth and expansion.

Macrophage isolation and culture

Peritoneal lavage was performed twice to obtain macrophages. In each instance, 5 ml of ice-cold RPMI medium without FBS was injected into the peritoneal cavity of mice with a 27 g needle. The needle was inserted slowly into the peritoneum, taking care to avoid puncturing any organs. Following the injection, a gentle massage of the peritoneum was performed to dislodge any cells attached to the PBS solution. A 25 - gauge needle, bevel up, attached to a 5 ml syringe was inserted into the peritoneum to collect the fluid, with the needle tip moved gently to prevent clogging by fat tissue or other organs. The maximum amount of fluid was collected, and the collected cell suspension was deposited into tubes kept on ice after removing the needle from the syringe (15).

Macrophage and EnSCs co - culture

The lavage fluid was collected near a flame and centrifuged at 1500 RPM for 5 minutes. The supernatant was discarded, and 1 ml of DMEM/F12 medium containing 10% FBS was added to the cells. The cells were then pipetted and counted using a Toma slide, and 5×10^5 cells were seeded in the wells of a 24-well plate. After allowing the cells to adhere to the bottom of the wells for two hours, the wells were washed and incubated with fresh complete medium for 24 hours. Next, 25×10^3 EnSCs were counted and added to each well, creating a ratio of 1 macrophage to 20 EnSCs.

Model establishment

The following experimental groups were established to examine the effects of different treatments on the EnSCs and their co - culture with intraperitoneal macrophages. Group A comprised the EnSCs without any additional treatment, serving as the control group. Group B involved the EnSCs treated with 1µM H_2O_2. For the co - culture experiments, Group C consisted of the EnSCs and intraperitoneal macrophages co - cultured together without any H_2O_2 treatment. Lastly, Group D represented the co-culture of EnSCs and intraperitoneal macrophages, but this group was subjected to the treatment with 1µM H_2O_2. The allocation of samples into these distinct groups allowed for the evaluation and comparison of the effects of H_2O_2 treatment on EnSCs alone and in the presence of macrophages in an *in vitro* model of IUAs. The treatment groups (B and D) were exposed to H_2O_2 for 2.5 hours and then the cell mediums were changed. All the groups were incubated for an additional 24 hours. As a final step, the cell medium was replaced, and the cells were subjected to a scratch using a crystal tip. This scratch created a gap in the confluent monolayer of cells, replicating a wound-like condition (16).

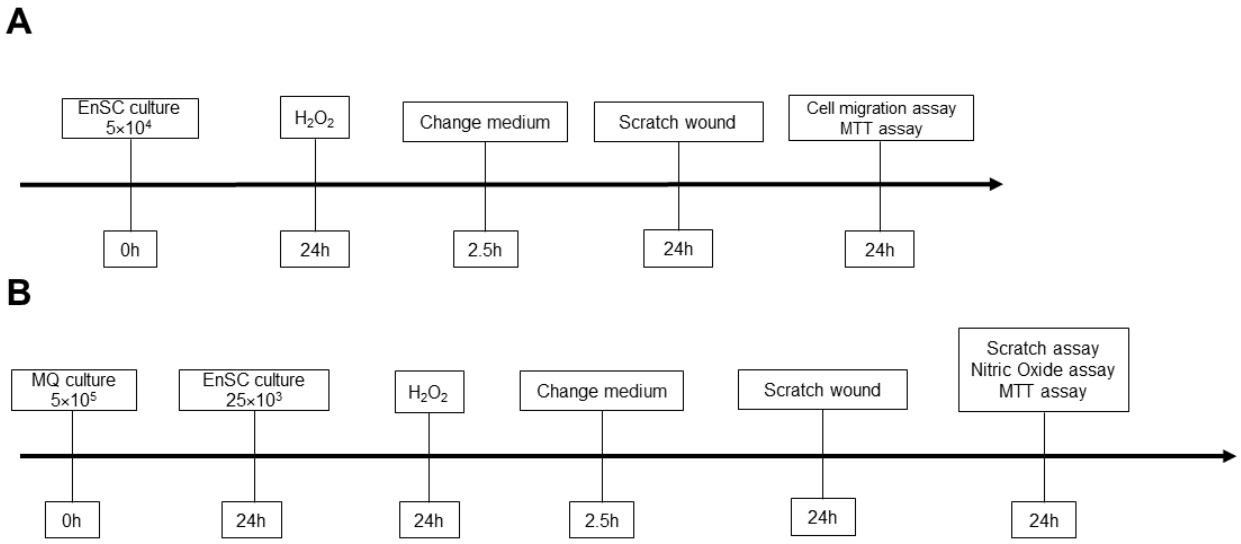

FIGURE 1. This schematic timeline shows the procedures of experiments performed in this study. A: EnSCs were treated with H_2O_2. B: The co-cultures of EnSCs with macrophages were treated with H_2O_2

Scratch Wound Healing Assay

The scratch assay is a commonly used method for evaluating cell migration, especially in adherent cell lines such as fibroblasts, endothelial cells, and epithelial cells. This assay is preferred due to its cost-effectiveness and simplicity, making it accessible for researchers to perform (19, 20).

The following steps were performed for the scratch test using cells in a 24-well plate:

1. 5×10^4 EnSCs were placed at the bottom of each 24-well plate in a complete cell culture medium.
2. 5×10^5 macrophages were seeded in the wells of a 24-well plate.
3. In co - culture, 25×10^3 EnSCs were counted and added to each well, creating a ratio of 1 macrophage to 20 EnSCs.

* In the co - culture groups, macrophages must be cultured first, and then stem cells must be cultured so that they can stick to the bottom of the plate.

* It should be noted that the number of cells in the co - culture group should be limited so that they adhere well to the bottom of the plate and do not overlap.

4. All the groups were incubated for an additional 24 hours.
5. After 24 hours, H_2O_2 was added to each well in B and D groups to achieve a final concentration of 1 μM. This substance creates conditions similar to damage caused by intrauterine surgery in the body.
6. The cells were then incubated for 2.5 hours.
7. After 24 hours, the culture medium of all the plates was emptied and washed once with PBS.
8. After washing, fresh DMEM - F12 culture medium (1 ml) was added to all the plates.

* Since H_2O_2 is lethal for the cells, it should not stay in the vicinity of the cell for more than 2.5 hours and it should be removed quickly.

9. The bottom of the wells in all groups was scratched using a crystal tip.

* The path of scratching and the movement of the crystal tip should be vertical from the top to the bottom of the plate.

10. After scraping, the plates were gently placed in a 37°C incubator without shaking.
11. Images of the scratch site were taken at 0, 6, 12, and 24 hours.

* All tests were performed in triplicate.

12. The images were analyzed using Image J2 (FIJI) software in the following way.

Image J2 (FIJI) software

ImageJ was created by Wayne Rasband at NIH and provides easy installation on arbitrary platforms and a simple user interface. It can calculate the area and pixel value statistics of user-defined selections and intensity - threshold objects. Also, it can measure distances and angles and create density histograms and line profile plots. It is a free, open-source image processing program that can display, edit, analyze, process, save, and print various image types. ImageJ allows multiple images to be displayed on the screen at one time. The active window has its title bar highlighted. All operations will be performed on the active image. ImageJ is written in Java, which allows it to run on Linux, Mac OS X, and Windows, in both 32-bit and 64-bit modes. As good as ImageJ is, we strongly encourage users to use FIJI instead. FIJI is a free, enhanced version of ImageJ2 that includes many pre-installed macros, including the Bio-Formats plugin (1). It is a great tool to process images and performs analysis. It is used in many scientific peer-reviewed publications, with over 1000 articles in diverse fields such as life sciences, astronomy, and physics (2).

Steps to work with software to analyze scratch test results:

1. First the Image J2 software was downloaded and opened (Figure 3-1).
2. The images taken of the scratches created at different times were dragged and dropped into the Image J2 software (Figure 3-2).
3. After entering the images into the software, the wound healing size tool option is clicked (Figure 3-3).
4. On the opened page, we clicked OK and did not change the default settings of the software (Figure 3-4).
5. Image J2 software identifies the scratched area and separates it from the border with a different color (Figure 3-5).
6. Finally, the software analyzed selected images Taken from 10 objects using a microscope and specified the percentage of the scratched area on a new page (Figure 3-6).

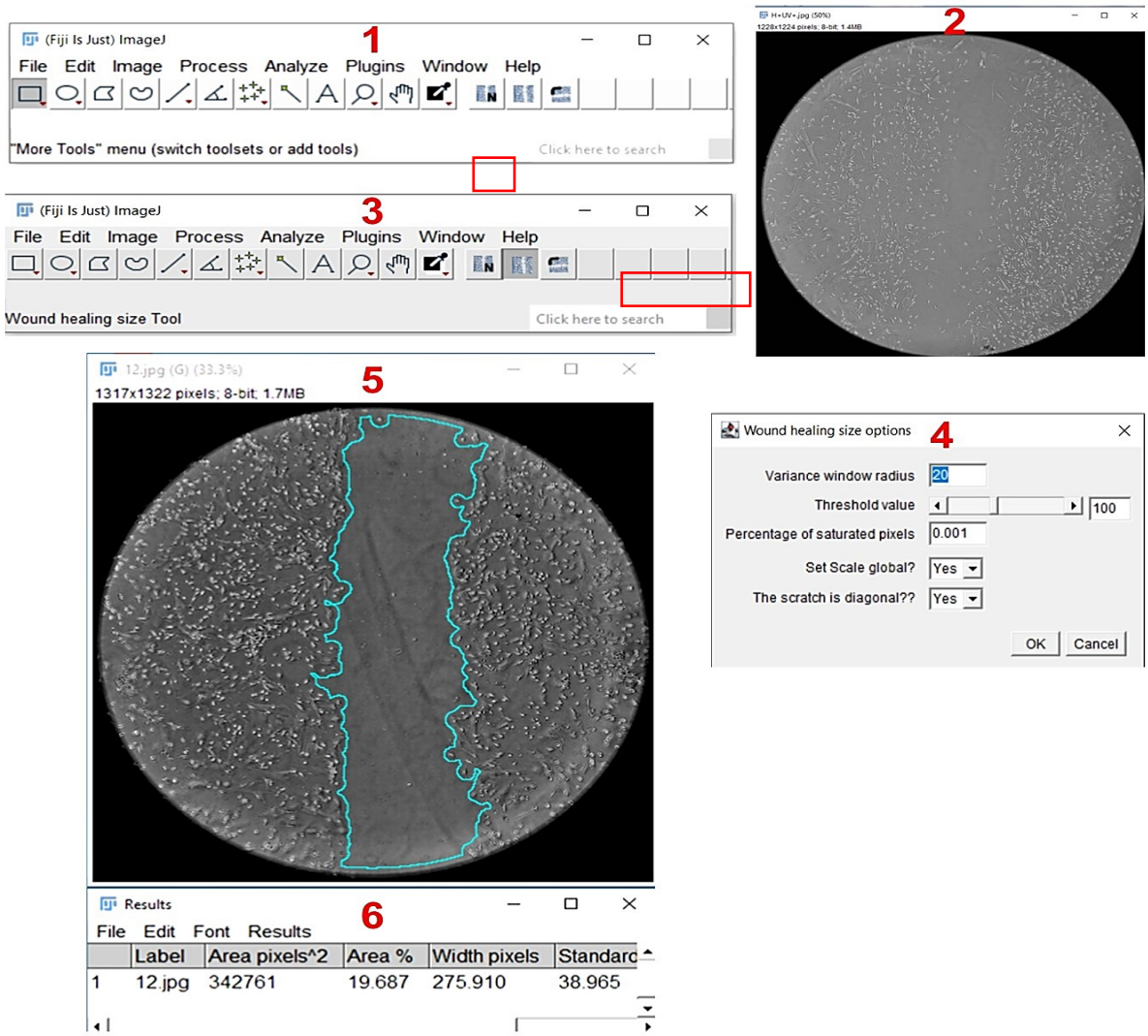

FIGURE 2. Steps to work with Image J2 software. The steps are indicated by numbers 1 to 6. This software is version 64.

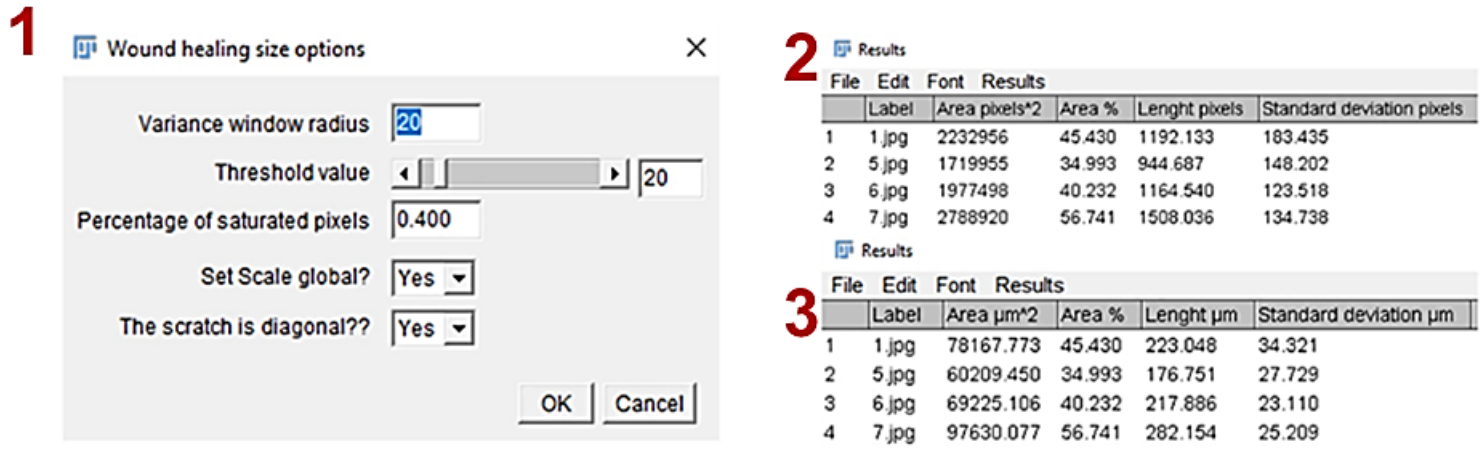

FIGURE 3-1. Wound healing size measuring tool. 3-2. Interface window to adjust parameters. 3-3. Snapshots of the results in table format in pixels show the area of the wound, wound coverage of the total area, and the average of the width and its standard deviation.

Table 3. Troubleshooting table

Steps	Problems	Solution
1	After adding H_2O_2, the cells died	Check H_2O_2 final concentration, which should be1µM. Check the incubation time should not be more than 2.5 hours
2	After adding stem cells, the cells do not attach	The number of added macrophages should not be high
3	The edge of the scratch is not smooth because the unscratched part of the cell monolayer is detached	Increase the speed of scraping. Wash the cell monolayer with medium once times after scraping
4	Cells are still round, not attached or spread well after 24 h	Check the shape and appearance of the cells, as well as the color and pH of the medium, and if possible, replace the cells
5	The cells in the co - culture are placed on top of each other and cannot adhere well	The number of cells added to each plate should be reduced
6	After scratching, the accumulation of dead cells	After scratching, It should be washed with PBS
7	The ImageJ2 software cannot detect the scratched area and separate it from other areas	Contrast adjustment
8	Cells are damaged or dead	Continuously lighting up the same field for image acquisition mayproduce heat enough to damage cells. The intensity of light should be Reduced

Cell viability assessment

The MTT (3- (4, 5- dimethylthiazol - 2 - yl) - 2, 5 - diphenyltetrazolium bromide) test was employed to assess cell viability and determine the potential cytotoxicity of H_2O_2 on cells. This colorimetric method relies on the reduction and conversion of yellow tetrazolium crystals by the succinate dehydrogenase enzyme, leading to the formation of insoluble purple crystals. Initially, a soluble yellow MTT solution, which contains tetrazolium salt, penetrates the cell membrane. In viable cells, the mitochondrial reductase enzyme reduces the tetrazolium salt to formazan, a purple-colored substance that is insoluble in water. This enables the measurement of cell proliferation and survival rates, providing an indication of cell viability. The purple formazan crystals are then dissolved in either dimethyl sulfoxide (DMSO) or acid isopropanol, and the optical absorption intensity is measured at 540 or 570 nm using a spectrophotometer or plate ELISA reader. The level of light absorption corresponds to the concentration of formazan accumulated within and on the cell surface, with higher concentrations resulting in a more intense purple color and greater absorption (17).

NO production measurement test:

Nitric Oxide (NO) is a gaseous molecule produced by various cells in the body, including immune myeloid cells like macrophages. It serves important biological functions such as signaling and acting as an effector molecule or metabolic regulator. In response to inflammatory signals, macrophages increase cytokine production and generate NO, which plays a vital role in pathogen killing and immune responses (17). NO is produced through the intracellular oxidation of L-arginine by nitric oxide synthetases. Nitric oxide is highly unstable and undergoes oxidation, converting into nitrate and nitrite. The amount of nitrite in cell culture supernatant or serum can be determined using the Griess reaction, a colorimetric method. In this reaction, nitrite reacts with sulfanilic acid in the first stage, producing a diazonium ion. In the second step, this ion couples with N- (1-naphthyl) ethylenediamine, resulting in a mixture of pink-colored azo derivatives. NO was measured using the Cib Biotech kit (cat: 3201-200).

Statistical analysis

The data was presented as mean values accompanied by standard deviations. Statistical analysis was performed using ordinary *one-way ANOVA*. GraphPad Prism version 6 software (GraphPad Software, La Jolla, USA) was utilized

for calculating significant differences. Results were considered statistically significant when $p < 0.05$. The all tests were performed in triplicate.

RESULTS

Comparison of migration rate at 4 different time points

The scratch test was conducted to assess cell migration capacity in both EnSCs and co-cultured EnSCs - macrophage cells. The cells were subjected to imaging at 0, 6, 12, and 24 hours after scratch initiation, and the resulting images were analyzed using Image J2 software.

In the EnSCs groups, no significant differences in cell migration were observed at 0 and 6 hours among the groups, ($P > 0.05$). However, at 12 and 24 hours, a significant difference was found between the group not treated with H_2O_2 (group A) and the group treated with H_2O_2 (group B) ($P < 0.05$), indicating enhanced cell migration in the H_2O_2 - treated group.

Similarly, in the co - culture groups, no significant differences in cell migration were observed at 0 and 6 hours among the groups ($P > 0.05$). However, at 12 and 24 hours, a significant difference was noted between the H_2O_2 - untreated co - culture group (group C) and the H_2O_2 -treated co-culture group (group D) ($P < 0.05$), indicating promoted cell migration in the H_2O_2 - treated co-culture group as well.

Interestingly, at the time points of 12 and 24 hours, cell migration in all the co - culture groups (both H_2O_2 - untreated and treated) was significantly lower compared to the groups with EnSCs alone, even in the absence of H_2O_2 treatment. This suggests that the presence of macrophages in the co - culture system may have an inhibitory effect on cell migration, regardless of H_2O_2 treatment.

These findings indicate that H_2O_2 treatment significantly accelerates cell migration in both EnSCs alone and their co - culture with macrophages. Moreover, the co - culture of EnSCs and macrophages itself appears to have an inhibitory effect on cell migration compared to EnSCs alone, regardless of H_2O_2 treatment.

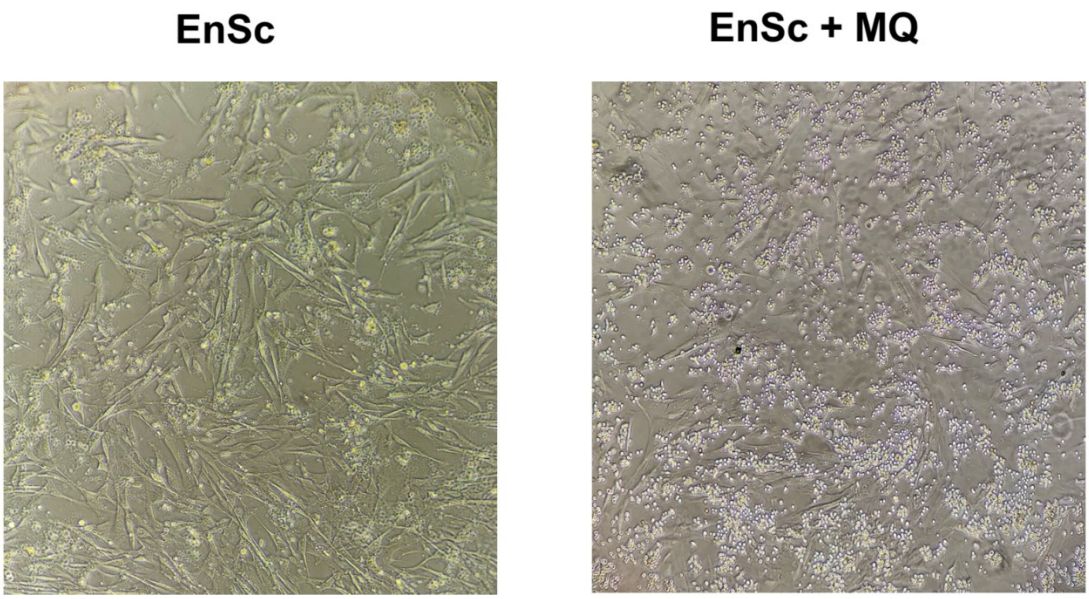

FIGURE 4. EnSCs and co - culture with macrophages before scratch.

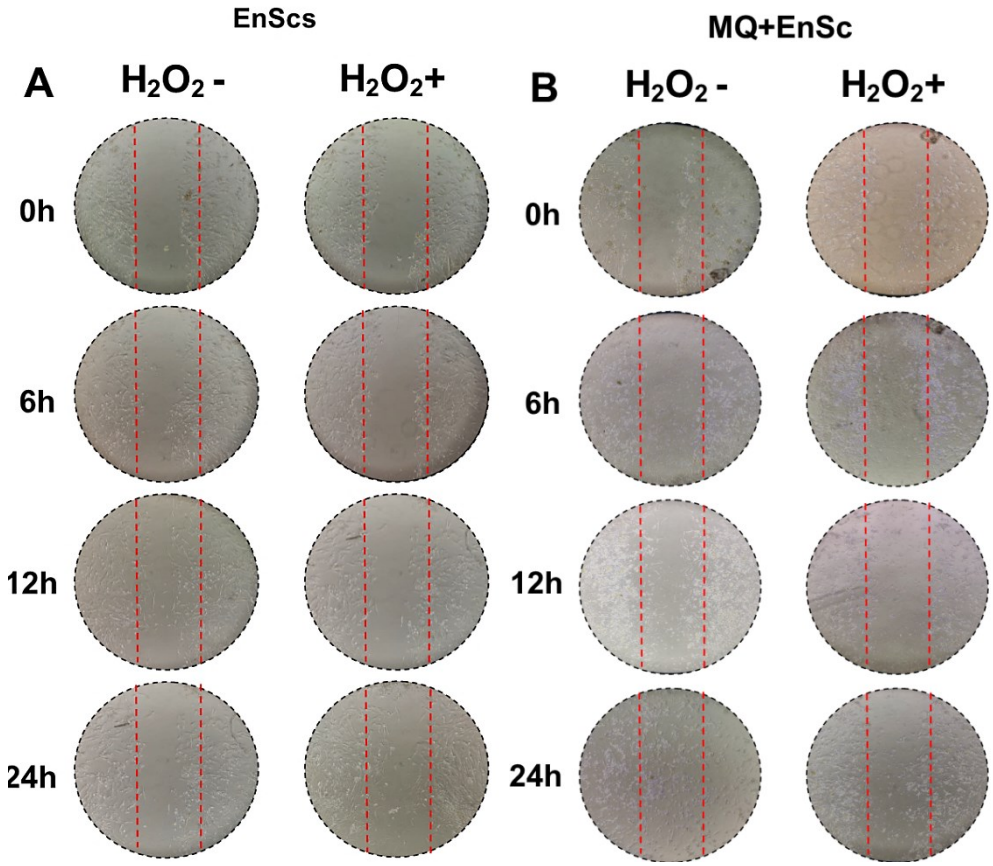

FIGURE 5. The scratch test of EnSCs and co - culture groups treated with H_2O_2 and the group not treated with H_2O_2 at 4 different times after scratching. The magnification in all the above images is 100x. Image analysis was done with ImageJ software.

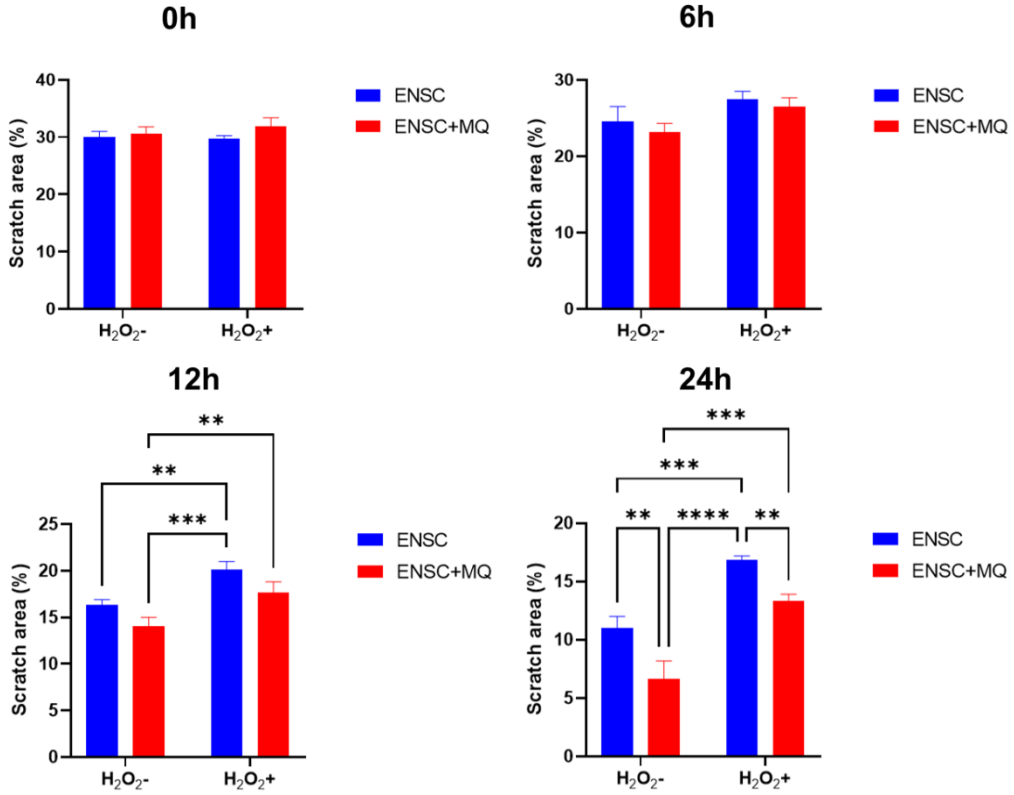

FIGURE 6. Comparison of migration rate in 4 different time points between two groups. Data are shown as mean ± standard deviation. A statistical difference of less than 0.05 is considered significant. The sign * indicates a significant difference between groups (* for P < 0.05, ** for P < 0.01).

Comparison of the cell viability in different groups

Cell viability was evaluated using the MTT test. Both groups A and C demonstrated high cell viability, implying that EnSCs maintained their viability. Comparing group B to group A, the cell viability of EnSCs in group B was found to be significantly lower ($P < 0.05$), indicating that H_2O_2 treatment had an adverse effect on cell viability. Similarly, group D exhibited significantly lower cell viability ($P < 0.05$) compared to group C. Moreover, comparing H_2O_2 - treated groups, group D demonstrated significantly higher cell viability.

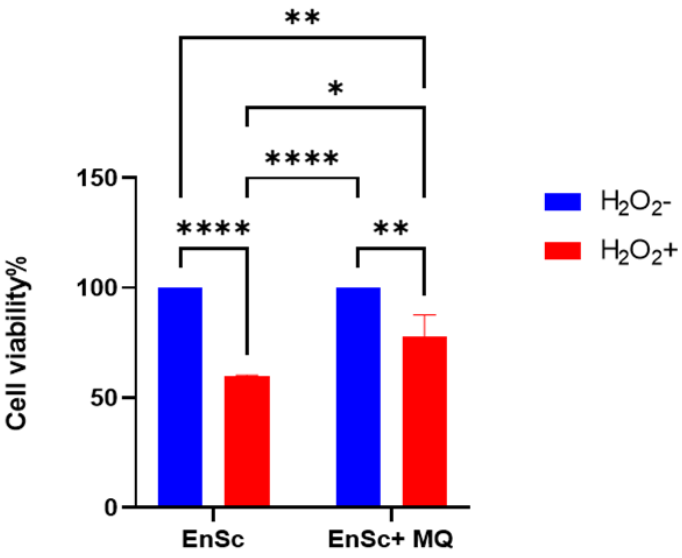

FIGURE 7. Comparison of the cell viability in EnSCs with co - culture group treated with H_2O_2 and not treated (A). Comparison of the cell viability in EnSCs groups with co - culture group treated with H_2O_2 (B) Data are shown as mean ± standard deviation. A statistical difference of less than 0.05 is considered significant. The sign * indicates a significant difference between groups (* for $P < 0.05$, ** for $P < 0.01$, * for $P < 0.001$, and **** for $P < 0.0001$).**

Comparison of the nitric oxide production in the co-culture groups

To assess the impact of H_2O_2 treatment on macrophage and EnSCs co - cultures, the cell supernatant of two groups (group C and D) was collected, with 200 microliters sampled from each. The levels of NO in the supernatant were measured, and triplicate samples were taken for each group to obtain average values.

The findings revealed a significant difference between the group treated with H_2O_2 and the group not treated with H_2O_2 ($P < 0.05$). This suggests that H_2O_2 treatment has an impact on NO levels in the co-cultures of macrophages and EnSCs.

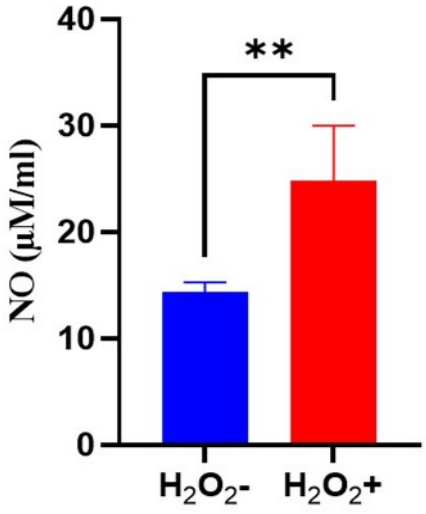

FIGURE 8. Comparison of the nitric oxide production in EnSCs co - culture group treated with H_2O_2 and not treated. Data are shown as mean ± standard deviation. A statistical difference of less than 0.05 is considered significant. The sign * indicates a significant difference between groups (for $P < 0.01$).**

DISCUSSION

IUAs represent a significant health concern that can lead to female infertility, irregular menstruation, and recurrent miscarriages (22). At present, there is a lack of effective treatment strategies for treating this condition (23, 24). Research findings have indicated that a notable factor contributing to the development of IUAs is the reduction in stem cells within the endometrium basal layer (25, 26). Stem cells are essential for tissue homeostasis, repair, and regeneration, as they possess the unique ability to self - renew and differentiate. In the context of IUAs, a study revealed that endometrial stem cells isolated from patients with IUAs exhibit diminished angiogenic activity, impaired capacity for clone formation, and reduced proliferation compared to stem cells obtained from healthy females (27). Endometrial stem cells (EnSCs) are versatile cells with multipotent capabilities, believed to originate within the endometrium and bone marrow (BM). Their remarkable plasticity makes them a valuable source for regenerative medicine, particularly as an autologous option. ESCs play a crucial role in reproductive processes, impacting both successful outcomes and instances of failure. Consequently, they hold potential as targets for therapeutic interventions in reproductive disorders, including recurrent implantation failure, thin endometrium, Asherman's syndrome, and recurrent pregnancy loss. The unique properties of ESCs offer promising avenues for advancing treatment strategies in these areas (28). These findings highlight the potential mechanisms underlying the

development of IUAs and suggest that the compromised functionality of endometrial stem cells may contribute to the pathogenesis of this condition. Therefore, in order to develop therapies for IUAs, it is necessary to enhance our understanding of adult stem cells and their regulatory mechanisms in the human endometrium (29).

Macrophages are a significant type of leukocytes found in the endometrium (13), and they play a crucial role in the regulation of tissue repair, regeneration, and fibrosis in various tissues (30). Macrophages in the uterus, specifically, are a diverse and adaptable subset of immune cells that respond to signals from the surrounding microenvironment (20). In addition to their well - established functions in reducing inflammation and fighting against infections, these macrophages actively contribute to the successful establishment and maintenance of pregnancy (31).

By co - culturing EnSCs and macrophages, we aimed to establish an *in vitro* model that can replicate the complex cellular interactions and microenvironment observed in the endometrium during IUAs development. This co - culture model allows for the investigation of the crosstalk between EnSCs and macrophages, providing insights into the underlying mechanisms involved in adhesion formation and potential therapeutic interventions.

H_2O_2 has been utilized in several studies as a means to imitate damaged endometrial cells (10, 12). In the study conducted by Li et al. (10), endometrial stem cells of the human uterus were treated with H_2O_2. The findings revealed a significant decrease in cell viability of hEnSCs following exposure to H_2O_2. Consistent with this study, our results indicated that H_2O_2 treatment negatively affected the cell viability of EnSCs, and the co - culture of EnSCs with macrophages had a potential protective effect. This effect is probably related to macrophages roles in enhancing repair and regeneration (30). The combination of H_2O_2 treatment and macrophage co - culture (group D) showed improved cell viability compared to EnSCs treated with H_2O_2 alone (group B). These findings highlight the importance of considering the interaction between EnSCs and macrophages in understanding the cellular response and potential therapeutic strategies for intrauterine adhesions.

Also, NO production has been recognized as a protective mechanism of macrophages against H_2O_2 - induced damage (32). In this study, the findings revealed a significant difference ($P < 0.05$) in NO levels between the group treated with H2O2 and the group not treated with H_2O_2. This suggests that H2O2 treatment has an impact on NO levels in the co - cultures of macrophages and EnSCs. The increase in NO levels in response to H_2O_2 treatment suggests that macrophages may have activated their defense mechanisms and up regulated NO production to counteract the damaging effects of H_2O_2. NO is known to possess antioxidant and anti-inflammatory properties, and its elevated production can serve as a protective response against oxidative stress.

Previous studies have provided evidence supporting the effect of H_2O_2 on accelerating cell migration (33). For example, in Li et al's study, H_2O_2 treatment led to a decrease in adherent endometrial stem cells. These findings suggest that H_2O_2 can influence cell migration processes. In line with these studies, the current findings demonstrate that both EnSCs alone and the co - culture of EnSCs and macrophages exhibit enhanced cell migration when exposed to H_2O_2. Significant differences in cell migration were observed at 12 and 24 hours compared to the respective untreated groups. Interestingly, at the 12 and 24-hour time points, cell migration in all co-culture groups (both H_2O_2 - untreated and treated) was significantly lower compared to the groups with EnSCs alone, even without H_2O_2 treatment. This suggests that the presence of macrophages in the co - culture system may have an inhibitory effect on cell migration, regardless of H_2O_2 treatment.

The findings indicate that H_2O_2 treatment significantly accelerated cell migration in both EnSCs alone and their co - culture with macrophages. Furthermore, the co - culture of EnSCs and macrophages itself appeared to have an inhibitory and protective effect on cell migration compared to EnSCs alone, regardless of H_2O_2 treatment. These results highlight the complex interaction between EnSCs and macrophages and the protective effects on macrophages.

This established model provides a platform for testing various treatments, such as immunomodulators, scar healing agents, or anti - fibrotic compounds. The effectiveness of these treatments can be assessed by evaluating cell migration, macrophage polarization, and tissue damage through the analysis of gene expression, protein expression, and cellular functions.

CONCLUSION

In conclusion, the co - culture of EnSCs and macrophages established an *in vitro* model that replicates some of the cellular interactions and microenvironment seen in the endometrium during IUAs development. This model seems to provide valuable insights into the mechanisms involved in adhesion formation and potential therapeutic interventions. H_2O_2 treatment negatively affected EnSCs viability, but the co - culture with macrophages exhibited a protective effect. Additionally, H_2O_2 treatment caused an increase in NO levels, suggesting macrophage activation to counteract oxidative stress. H_2O_2 treatment accelerated cell migration in both EnSCs alone and the co - culture, while macrophages inhibited migration. These findings emphasize the

importance of the EnSCs - macrophage interaction for understanding IUAs and developing effective treatments. This study was reviewed at Shahid Beheshti University of Medical Sciences in Tehran and registered with the code of ethics IR.SBMU.AEC.1402.003.

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CONFLICT OF INTEREST

All authors declare no conflicts of interest.

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