Mitochondrial transfer restores the proliferation of injured endometrial stromal cell

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Research Article

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Abstract

Background: The uterine endometrium is a tissue that needs sufficient energy to support its active physiological function, the damage of which is responsible for intrauterine adhesion. In this study, we investigated the evidence that mitochondrial transfer is involved in endometrial repair and explored the effects and possible mechanism of mitochondrial transfer.

Methods: The study was approved by the regional ethics committee of Ningbo Women and Children's Hospital (Date: 12/3/2021/No: EC2021-M004). Endometrial stromal cells were pretreated with hypoxia and cocultured with human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) in vitro. Confocal imaging was used to assess mitochondrial transfer between the cells. The proliferation and ATP production of endometrial stromal cells were detected to evaluate the physiological function of the cells.

Results: We found that coculture of hUCB-MSCs and endometrial stromal cells partially restored the proliferation and ATP production of hypoxic cells. Mitochondrial transfer was observed from hUCB-MSCs to hypoxic endometrial stromal cells. This process can be blocked by hUCB-MSCs pretreated with carbenoxolone disodium (CBX), which can block TNT connection formation.

Conclusions: The mitochondria of stem cells could be transferred completely into hypoxic endometrial stromal cells, partially restoring the proliferation of injured cells in vitro. This process is proactive.

Trial registration: The study was approved by the regional ethics committee of the Ningbo women and Children's Hospital (Date: 12/3/2021/No: EC2021-M004).

Background

The damage to endometrial stromal cells caused by various factors can affect the proliferation of the endometrium. When the repair mechanism of endometrial injury is decompensated, the intrauterine muscle wall and/or cervical canal may adhere to each other, and intrauterine adhesion is formed. Intrauterine adhesion is one of the major factors that may affect women's menstruation and reproductive function during their child-bearing period [1]. Hysteroscopic uterine adhesion separation combined with an artificial cycle after surgery is a classic method for treating intrauterine adhesion at present [2]. However, because the proliferation ability of the endometrium is impaired, the incidence of adhesion rerudescence after surgical treatment is nearly 20%-62% [3–4]. Therefore, there is great clinical value to determine the factors that may affect endometrial proliferation to adjust the treatment plan and improve the prognosis of patients with intrauterine adhesions.

Generally, the uterine endometrium is a tissue with active oxygen metabolism changes that needs a sufficient energy supply to support its active physiological function [5]. Mitochondria are the energy factories of cells, and 95% of the energy produced by cells comes from oxidative phosphorylation in mitochondria [6]. At the same time, mitochondria are also the main organelles of oxygen consumption

and are the most sensitive organelles to hypoxia [7]. Studies have confirmed that damaged endometrial cells of patients with intrauterine adhesions show a series of cell ischemia and hypoxia, such as lysosome disappearance and mitochondrial swelling, which may be caused by mechanical injury or inflammation [8–9].

Mitochondria are ancient and special organelles that can actively undergo intercellular transfer. Research on ischemic and hypoxic cardiomyocytes shows that bone marrow mesenchymal stem cells (BMSCs) can transfer their complete mitochondria with normal functions to hypoxic cardiomyocytes and rebuild their aerobic respiration capacity after coculture of these two kinds of cells. This transfer process is called mitochondrial transfer [10]. Studies by Islam et al. also found that when BMSCs were perfused into the airway of mice with acute lung injury, mitochondria of BMSCs were found in the lung epithelial cells of mice. The transferred mitochondria increased the ATP content of local alveolar cells and the secretion of surface-active substances of type II alveolar cells, which can repair the function of injured cells [11]. Other subsequent studies confirmed that mitochondrial transfer is a universal phenomenon in stem cells, vascular smooth muscle cells [12] and various malignant tumor cells [13]. Therefore, it is reasonable that mitochondrial transfer is a biological phenomenon of cells under stress. In fact, the intrauterine operation or the occurrence of intrauterine infection also puts endometrial cells into a stress state of ischemia and hypoxia, which may damage the mitochondrial function of endometrial cells.

In this study, we investigated the evidence that mitochondrial transfer is involved in endometrial repair and explored the possible mechanism of mitochondrial transcellular transfer, which can provide a new starting point for promoting the repair of injured endometrium in the future.

Methods

Endometrial stromal cell isolation and maintenance in culture

Endometrial samples were obtained during the proliferative stage of the menstrual cycle from women (n = 8) aged 30–36 years who underwent hysterectomy for uterine fibroids. The study was approved by the regional ethics committee of the Ningbo Women and Children's Hospital (Date: 12/3/2021/No: EC2021-M004). Written informed consent was obtained from all participating women. All donors had normal menstrual cycles (25–35 days) and were proven to be fertile at least once. Donors were examined for the absence of hormonal diseases and any other uterine pathologies (e.g., polycystic ovary syndrome and/or previous infertility records, endometriosis, fibroids), sexually transmitted diseases and human immune deficiency disease. None of the women had used hormonal contraception or an intrauterine device for a minimum of 3 months prior to biopsy. The subject's proliferative stage (cycle day [CD] 5–10) was calculated based on their previous time of menstruation.

The endometrial samples were washed and finely minced in Minimum Essential Medium α (MEM α; Thermo Scientific, USA) with 5% pooled human platelet lysate (Thermo Scientific), 100 IU/ml penicillin, 0.1 mg/ml streptomycin (Thermo Scientific), and 20 µl/ml heparin 1000 IE/ml (Sigma Aldrich, Germany) before digestion with dispase II (0.75 U/ml; Sigma Aldrich) in complete media with agitation for 45 min at 37°C/5% CO_2. The endometrial stromal cells were isolated and characterized according to previously published methods[14].

Hypoxia treatment

Endometrial stromal cells were seeded in 24-well culture dishes, and fresh medium was used to keep the cells healthy by providing fresh nutrients before hypoxia treatment. The culture dishes were incubated in a modular incubator chamber (Thermo Scientific) containing humidified hypoxic air (1% O_2, 5% CO_2, 94% N_2) for 2 hours at 37°C. Stromal cells cultured under normoxic conditions (20% O_2, 5% CO_2 and 75% N_2) were used as controls. Hypoxia-treated cells were collected at the indicated time points and prepared for follow-up analysis.

Human umbilical cord blood-derived mesenchymal stem cell (hUCB-MSC) isolation and in vitro coculture system

Human umbilical cord blood was collected from umbilical veins at the time of neonatal delivery with informed consent from the mother. hUCB-MSCs were isolated and characterized according to previously published methods[15].

Hypoxic stromal cells and hUCB-MSCs were seeded at a density of $1 \times 10^4/cm^2$ in a 1:1 ratio and supplemented with Dulbecco's modified Eagle's medium (DMEM) with 10% FBS.

Inhibition of gap junction intercellular communication

hUCB-MSCs were collected and seeded in 24-well plates and incubated with the gap junction blocker carbenoxolone disodium (CBX, 50 mM in DMEM, Sigma Aldrich) under the same conditions of activator treatment for 6 hours. Control cells were incubated with DMEM alone. The pretreated hUCB-MSCs were collected and prepared for follow-up analysis.

Mitochondrial transfer

For assessment of mitochondrial transfer, MSC mitochondria and human endometrial stromal cells were prestained with MitoTracker Deep Red and Green, respectively (Thermo Fisher Scientific). Hypoxic cells and hUCB-MSCs were cocultured at a 1:1 ratio for 24 hours, 48 hours or 72 hours. The cells were fixed with 4% paraformaldehyde and washed. Mitochondrial transfer was visualized using the EVOSFL Auto Imaging System (Life Technologies, USA), and red mitochondria were observed in green endometrial cells.

We imaged cells by confocal imaging (LSM 510 META, Zeiss). The optical thicknesses were 2 and 0.6 µm for confocal imaging. For fluorescence recovery after photobleaching, we photobleached the cytosolic

fluorescence of calcein in selected mBMSCs using high-power laser excitation and then imaged fluorescence recovery at 1 frame/min. In all runs, we obtained optical sections across the cell's vertical diameter to confirm that the photobleaching was complete throughout the cell. We analyzed the images using commercial software (MetaMorph 6.3, Universal Imaging). Three imaged fields were obtained from every well in each group.

The proliferation of endometrial stromal cells

Cell proliferation was evaluated by the MTT method. To inhibit the proliferation of cells, mesenchymal stem cells were treated with mitomycin C (10 μg/mL) in DMEM with 10% FBS (200 μL) at 37°C for 90 minutes. Hypoxic endometrial stromal cells were seeded into 96-well plates at a density of 5,000 cells/well and cocultured with mitomycin-C-pretreated mesenchymal stem cells at a 1:1 ratio for 24, 48 and 72 hours. The cells were then incubated with 20 μL of MTT solution (5 mg/mL in PBS) at 37°C for 4 hours, and the formed formazan crystals were dissolved in 150 μL of dimethyl sulfoxide at room temperature for 10 minutes. The optical density at 492 nm was measured using a GF-M3000 microplate reader (Caihong Co., China), and the cell viability was determined to be the absorbance ratio of treated and untreated cells.

Measurement of ATP production

Measurement of ATP production was performed on 10,000 cells per group using the ATPlite luminescent detection assay (Perkin Elmer, USA) according to the manufacturer's instructions. Measurements were expressed as relative luciferase units (RLU) and calculated as fold of RLU, as measured in the control group.

Statistical Analysis

Analysis was performed using SPSS 17.0 software. Experiments for each donor were performed at least in triplicate; the average of three technical replicates was taken as a single data point for each donor, and the data points were pooled together for statistical analysis. Pooled data were presented as the mean with SD. For parametric data, Student's t test or one-way analysis of variance with post hoc analysis using Bonferroni's selected comparisons was performed. The statistical significance level was set at $P < 0.05$.

Results

Coculture of hUCB-MSCs and endometrial stromal cells partially restored the proliferation and ATP production of hypoxic cells.

When endometrial stromal cells were oxygen deprived, the proliferation of hypoxic cells was significantly decreased compared with that of normal endometrial stromal cells ($p < 0.001$). There was no difference in proliferation between normal endometrial stromal cells cocultured with or without hUCB-MSCs ($p = 0.853$). After coculture of hypoxic endometrial stromal cells with hUCB-MSCs, the proliferation of hypoxic cells was significantly increased compared with that of hypoxic endometrial stromal cells alone ($p <$

0.001) after 72 hours, while there was no difference in the first 48 hours. However, there was still a decrease in proliferation compared with that of normal cells even after 72 hours ($p = 0.013$). (Fig. 1a).

ATP production in hypoxic cells was significantly decreased compared with that in normal endometrial stromal cells ($p < 0.001$). There was no difference in ATP production between normal endometrial stromal cells cocultured with or without hUCB-MSCs ($p = 0.756$). After coculture of hypoxic endometrial stromal cells with hUCB-MSCs, ATP production in hypoxic cells was significantly increased compared with that of hypoxic endometrial stromal cells alone at 48 h ($p = 0.003$) and 72 h ($p < 0.001$). However, there was still a decrease in ATP production compared with that of normal cells cocultured with hUCB-MSCs even after 72 hours ($p = 0.037$). (Fig. 1b).

Mitochondrial transfer was observed from hUCB-MSCs to hypoxic endometrial stromal cells.

Mitochondrial transfer was evident in that hUCB-MSCs lost red fluorescence, while endometrial stromal cells internalized the fluorescence. When we imaged the endometrial stromal cells at different optical sections, the internalization of hUCB-MSC mitochondria was evident in the merger of the DsRed and calcein colors, which resulted in intermediate pseudocolors. Trypan blue, a cell-impermeable fluorescence quencher that decreases extracellular fluorescence, failed to decrease internalized DsRed fluorescence, affirming internalization. We found that the mitochondria of hUCB-MSCs were transferred into hypoxic endometrial stromal cells (Fig. 2).

Counting the number of sites with mitochondrial transfer according to DsRed fluorescence showed that the number of mitochondria in hypoxic endometrial cells cocultured with hUCB-MSCs was significantly higher than that in normal endometrial cells cocultured with hUCB-MSCs (Fig. 2d).

hUCB-MSCs transferred mitochondria into hypoxic endometrial stromal cells through tunneling nanotube connections (TNTs)

TNTs are a kind of gap junction intercellular communication that can be blocked by CBX. The hUCB-MSCs were incubated with CBX for 6 hours to block TNT formation. Then, the two kinds of cells were cocultured for 72 hours.

Few mitochondria of hUCB-MSCs pretreated with CBX were transferred into hypoxic endometrial stromal cells ($p < 0.001$) (Fig. 3). The proliferation of hypoxic endometrial stromal cells was inhibited when they were cocultured with hUCB-MSCs pretreated with CBX compared with those without CBX ($p = 0.017$). Oxygen-deprived endometrial stromal cells were mixed with hUCB-MSCs pretreated with CBX, and it was found that fewer ATP were produced than that in the coculture group without CBX ($p = 0.008$). There was no significant difference in proliferation ($p = 0.415$) or ATP production ($p = 0.617$) between the coculture group with CBX and the group of hypoxic cells alone (Fig. 4).

Discussion

The uterine endometrium changes periodically from proliferating to shedding every month, and a sufficient energy supply is necessary to maintain its active physiological state. Abundant blood support from the uterus also shows that the endometrium is a tissue with very active oxygen metabolism [16]. When the endometrium is affected by mechanical damage or inflammation, it is in a state of ischemia and hypoxia [17]. Mitochondria are the most important organelles for providing energy to maintain normal cell function [18]. Under hypoxic conditions, the mitochondrial electron transport chain is disordered because there are not enough oxygen molecules to act as the final acceptor of electrons, and a large amount of reactive oxygen species (ROS) is produced through the electron transport chain. Excessive ROS destroys the balance between ROS and antioxidant levels and finally leads to disorder of endometrial cell proliferation [19, 20]. Our study also confirmed that when endometrial stromal cells are in a state of hypoxia, ATP production in mitochondria is significantly reduced and the proliferation of hypoxic cells is impaired. Lysosomal hydrolase release and lysosomal dysfunction are found in endometrial stromal cells, which may lead to mitochondrial dysfunction [21]. Our previous studies on mitochondrial DNA also found that the polymorphism of genes that transcribe and translate proteins related to oxidative phosphorylation in mitochondrial DNA can affect the repair and proliferation of the endometrium, which indirectly confirmed that the change of mitochondrial function is related to the repair and proliferation of the endometrium [22].

Mitochondria are organelles capable of active transcellular transfer. When BMSCs were injected into the airways of mice with acute lung injury, mitochondria of BMSCs were found in the lung epithelial cells of the mice, confirming the presence of mitochondrial transfer. Moreover, mitochondria that transferred into local alveolar cells increased the ATP of local and adjacent alveolar cells and the secretion of surface-active substances of type II alveolar cells, showing an obvious repair effect on injured cells [10, 23]. We found in our study that when hUCB-MSCs and endometrial stromal cells pretreated with hypoxia were cocultured in vitro, the red fluorescently labeled mitochondria of hUCB-MSCs were transferred into endometrial stromal cells, ATP production was increased, and cell proliferation was partially restored. When mitochondrial transfer is inhibited, ATP production and cell proliferation are also inhibited. These findings indicate that there is mitochondrial transfer between MSCs and hypoxic endometrial stromal cells, and mitochondrial transfer is a way of functional recovery of hypoxic endometrial stromal cells. In addition to alveolar cells and endometrial cells, the phenomenon of mitochondrial transfer also exists in nerve cells [24], vascular smooth muscle cells [25], cardiomyocytes [26] and various malignant tumor cells [27], suggesting that the transcellular transfer of mitochondria is a biological phenomenon of cells under stress. Studies have even found that human mitochondria can be found in the alveolar epithelial cells of mice after infusing human bone marrow mesenchymal stem cells into acute lung injury model mice, indicating that mitochondrial transfer is not limited by cell types and populations [28]. Supplementing stem cells and increasing mitochondrial transfer between cells may be a way to improve the prognosis of endometrial injury.

Similar to other cell-to-cell signaling pathways, mitochondrial transcellular transfer also takes place through connections between cells [29]. Tunneling nanotubes (TNTs) are a type of long membrane structure 100–800 nm in width and 100 μm in length. Based on F-actin as their framework, TNTs are wrapped by a phospholipid bilayer extending from the cell membrane, connecting the cytoplasm of two cells. They exist extensively in a variety of physiological and pathological cells, such as kidney cells, astrocytes, and myocardial cells[30]. CBX can block cells from forming tunneling nanotubes (TNTs) to connect with other cells. In this study, CBX prevented hUCB-MSCs from forming TNT connections with endothelial stromal cells. Intercellular mitochondrial transfer was also significantly affected, ATP production in hypoxic endometrial stromal cells was reduced, and cell proliferation was inhibited. This indirectly indicates that hUCB-MSCs can form TNT connections with hypoxic endometrial stromal cells. This linkage is a major mode of mitochondrial transfer between hUCB-MSCs and hypoxic endometrial stromal cells. In addition to endometrial stromal cells, TNT connections are the main mode of intercellular communication in the mitochondrial transfer between BMSCs and damaged alveolar epithelial cells or hypoxically injured cardiomyocytes [31, 32]. Furthermore, in our study, we blocked the formation of TNT junctions in hUCB-MSCs rather than hypoxic endometrial stromal cells. Mitochondrial transfer was significantly affected, indicating that stem cells form intercellular connections with injured endometrial stromal cells and actively achieve mitochondrial transfer rather than passively.

Conclusions

Endometrial stromal cells were cocultured with hUCB-MSCs after hypoxia treatment in vitro, and the mitochondria of stem cells could be transferred into hypoxic cells, partially restoring the proliferation of injured endometrial stromal cells. Mitochondrial transfer between cells may be realized by TNT connections, which are actively formed from hUCB-MSCs to endometrial stromal cells. Therefore, exogenous hUCB-MSCs act as a "Trojan horse", which can provide fresh mitochondria to damaged endometrial stromal cells, thus improving the repair of injured endometrial cells and restoring their biological function.

However, the research on the mechanism of mitochondrial transfer in this study only provides some indirect evidence, and it requires further observation of cell connections under electron microscopy to obtain direct evidence of the formation of TNT connections between cells and mitochondrial transfer through TNT connections. In addition, this study was completed under in vitro cell culture conditions. In vivo, the function of hUCB-MSCs may also be affected by the physiological environment, including rejection of immune cells and interference of other cells. Therefore, the practical application of mitochondrial transfer in clinical treatment needs further verification by more in vivo experiments.

Abbreviations

hUCB-MSCs
human umbilical cord blood-derived mesenchymal stem cells
MEMα

Minimum Essential Mediumα
DMEM
Dulbecco's modified eagle medium
CBX
carbenoxolone disodium
RLU
relative luciferase units
TNTs
Tunneling Nanotube connections

Declarations

Ethics approval and consent to participate: The study was approved by the regional ethics committee of the Ningbo women and Children's Hospital (Date: 12/3/2021/No: EC2021-M004).

Consent for publication: Not applicable.

Availability of data and materials: Not applicable.

Competing interests: The authors have no competing interests.

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Authors' contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Liang Chen, Jue Zhu, Yichen Chen, Miaohhua Zhu and Qiming Wang. The first draft of the manuscript was written by Jing Zhang and Jue Zhu, all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

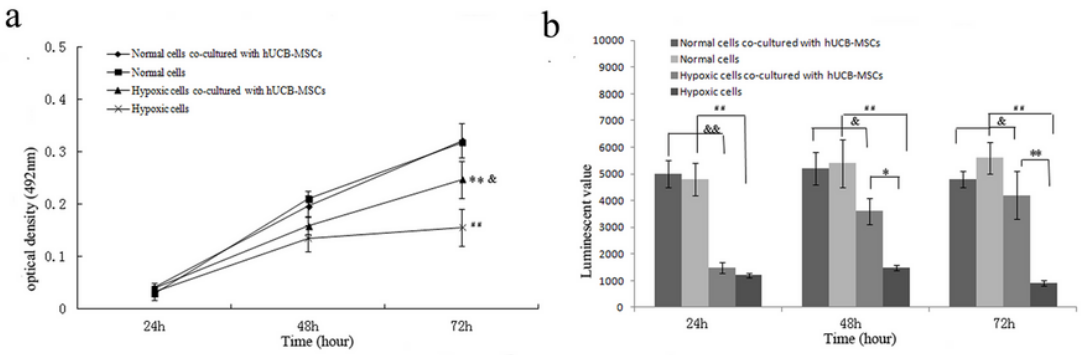

Figure 1

Proliferation and ATP production of hypoxic endometrial stromal cells cocultured with hUCB-MSCs

(a): The proliferation of hypoxic endometrial stromal cells. **: hypoxic cells cocultured with hUCB-MSCs vs. hypoxic cells alone, p<0.001; ##: hypoxic cells vs. normal cells, p<0.001; &: hypoxic cells cocultured with hUCB-MSCs vs. normal cells cocultured with hUCB-MSCs, p<0.05; (b): ATP production of hypoxic endometrial stromal cells. **: hypoxic cells cocultured with hUCB-MSCs vs. hypoxic cells alone, p<0.001; *: p<0.05; ##: hypoxic cells vs. normal cells, p<0.001; &&: hypoxic cells cocultured with hUCB-MSCs vs. normal cells cocultured with hUCB-MSCs, p<0.001, &: p<0.05.

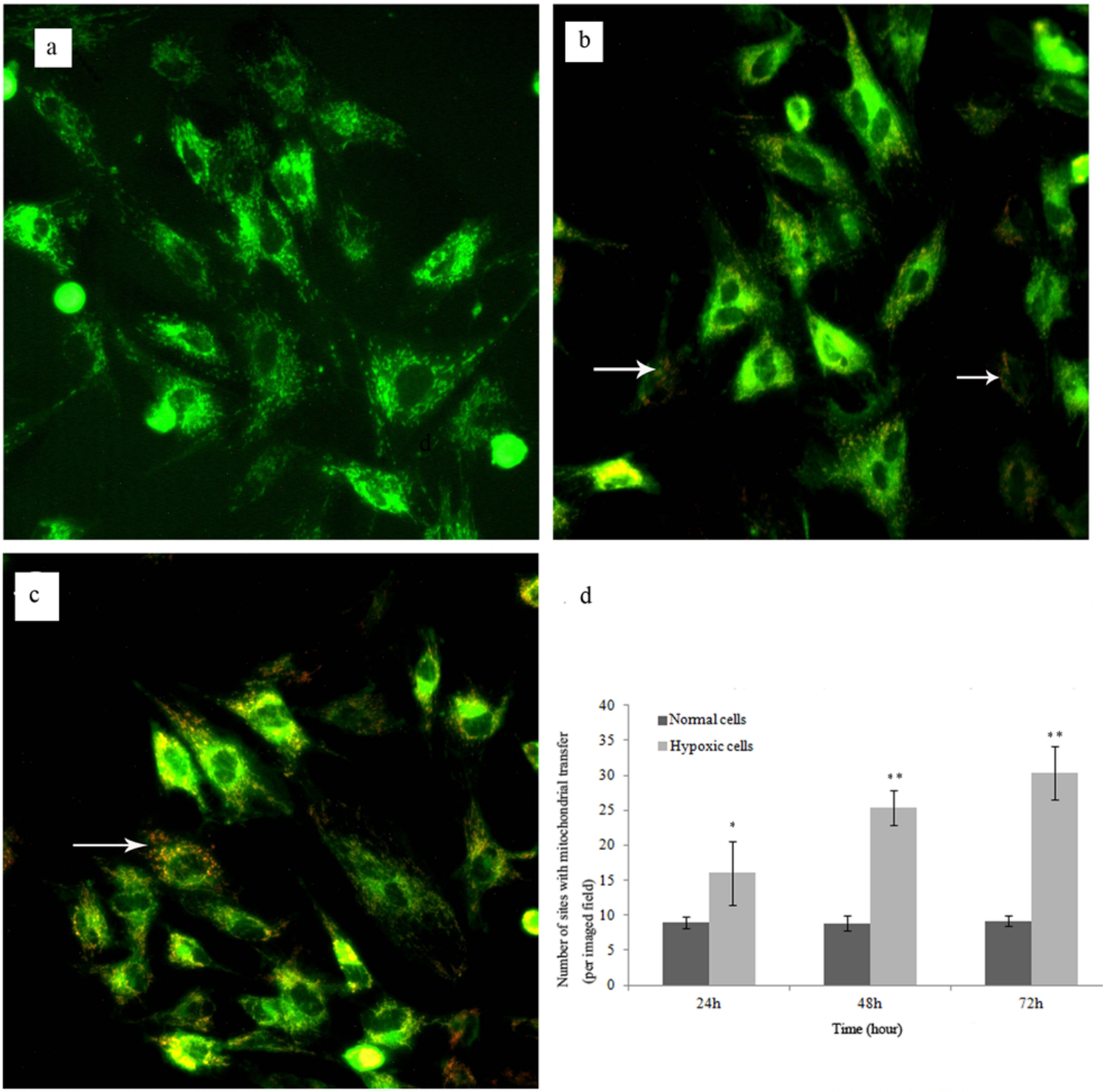

Figure 2

Mitochondrial transfer from hUCB-MSCs to hypoxic endometrial stromal cells (×400) (a):

Hypoxic endometrial stromal cells; (b): Endometrial stromal cells cocultured with hUCB-MSCs at 0 h, the arrow represents the mitochondria in hUCB-MSCs; (c): Mitochondria of hUCB-MSCs expressing red fluorescence entering the endometrial stromal cells, the arrow represents the transplanted mitochondria; (d): Number of sites with mitochondrial transfer for cells cocultured with hUCB-MSCs (per imaged field),

hypoxic endometrial stromal cells cocultured with hUCB-MSCs vs. normal endometrial stromal cells cocultured with hUCB-MSCs, *: p<0.05; **: p<0.001.

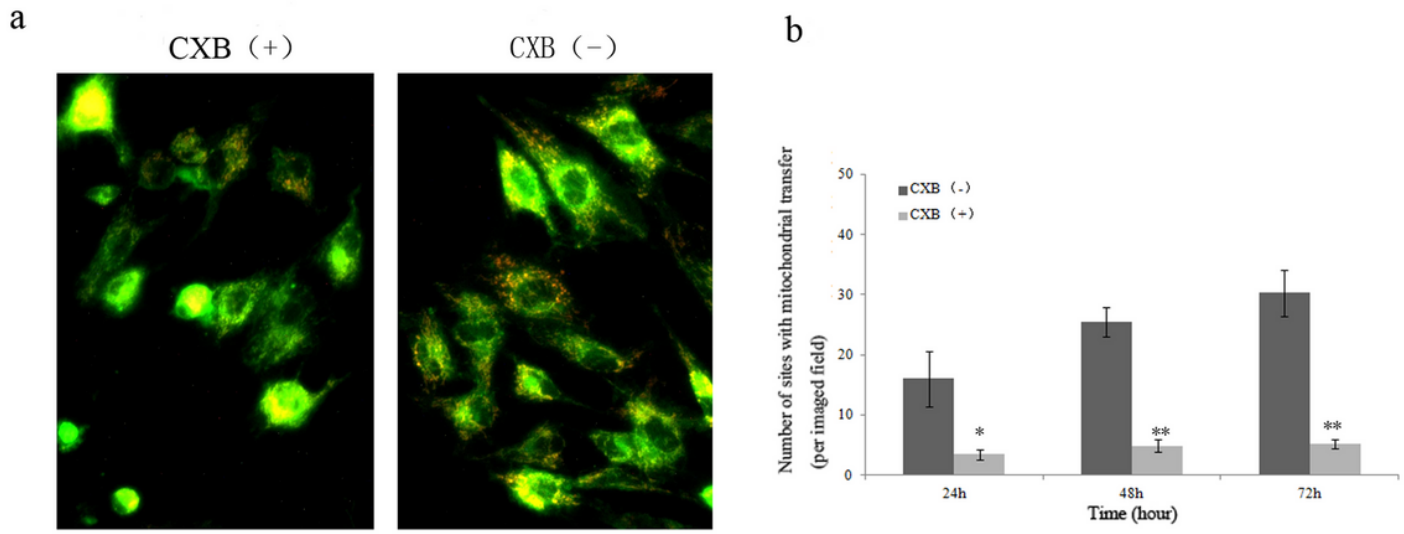

Figure 3

Mitochondrial transfer from hUCB-MSC-pretreated CXB to hypoxic endometrial stromal cells (×400)

(a): Images of mitochondrial transfer; (b) Number of sites with mitochondrial transfer for cells cocultured with hUCB-MSCs pretreated with or without CXB, hypoxic cells cocultured with hUCB-MSCs+CXB vs. hypoxic cells cocultured with hUCB-MSCs, *: p<0.05; **: p<0.001.

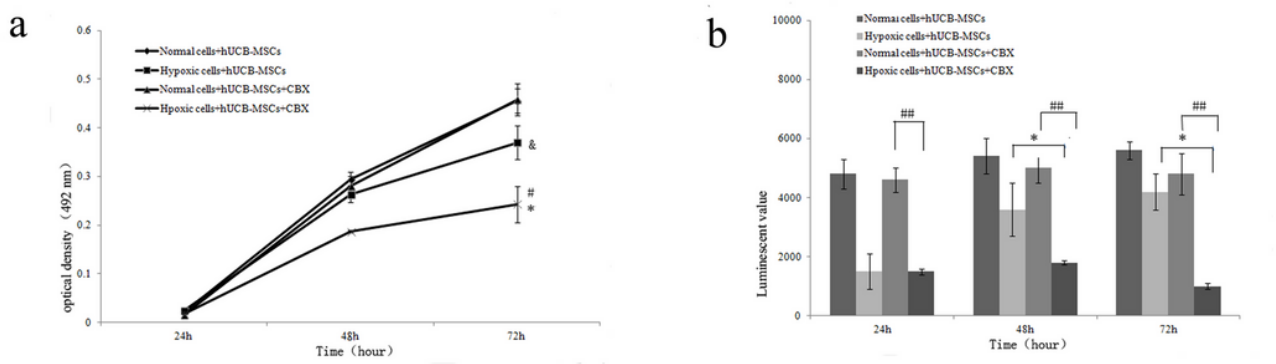

Figure 4

Proliferation and ATP production of hypoxic endometrial stromal cells cocultured with hUCB-MSCs pretreated with CXB

(a): The proliferation of hypoxic endometrial stromal cells. *: hypoxic cells cocultured with hUCB-MSCs+CXB vs. hypoxic cells cocultured with hUCB-MSCs, p<0.05; #: hypoxic cells cocultured with hUCB-MSCs+CXB vs. normal cells cocultured with hUCB-MSCs+CXB, p<0.05; &: hypoxic cells cocultured with hUCB-MSCs vs. normal cells cocultured with hUCB-MSCs p<0.05; (b) ATP production of hypoxic

endometrial stromal cells. *: hypoxic cells cocultured with hUCB-MSCs+CXB vs. hypoxic cells cocultured with hUCB-MSCs, $p<0.05$; ##: hypoxic cells cocultured with hUCB-MSCs+CXB vs. normal cells cocultured with hUCB-MSCs+CXB, $p<0.001$.