The cytotoxicity of advanced glycation end products was attenuated by UCMSCs in human vaginal wall fibroblasts by inhibition of an inflammatory response and activation of PI3K/AKT/PTEN

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SUMMARY Pelvic organ prolapse (POP) occurs when the pelvic organs (bladder, bowel or uterus) herniate into the vagina, causing incontinence, voiding, and bowel and sexual dysfunction, negatively impacting upon a woman's quality of life. Intermediate intermolecular cross-links and advanced glycation cross-links increase in prolapsed tissue. Stem cells are able to participate in tissue repair due to their ability to differentiate into multiple lineages, and thus into various types of connective tissue cells, so they therefore hold great promise for treating pelvic floor dysfunction. The current study found that advanced glycation end products (AGEs) inhibited the viability and proliferation of human vaginal wall fibroblasts (VWFs), were cytotoxic to VWFs, and also induced the apoptosis of VWFs. In contrast, umbilical cord-derived mesenchymal stem cells (UCMSCs) secreted anti-inflammation cytokines to protect against the cytotoxic effects of fibroblasts induced by AGEs and attenuated the cytotoxic effect of AGE on fibroblasts by activation of the PI3K/Akt-PTEN pathway. This study demonstrated that UCMSCs inhibited the cytotoxic effect of AGE in cells from patients with POP by inducing an anti-inflammatory reaction and activating the PI3K/AKT/PTEN signaling pathway. The current results provide important insights into use of stem cells to treat POP.

Keywords pelvic organ prolapse; advanced glycation end products; umbilical cord-derived mesenchymal stem cells; cytokines; PI3K-AKT

1. Introduction

Pelvic floor dysfunction (PFD) is the term for a group of clinical conditions, including stress urinary incontinence (SUI), pelvic organ prolapse (POP), overactive bladder syndrome, and fecal incontinence (1,2). In the general population, POP is an exceedingly common condition for mature women, with an estimated 41% presenting to their primary gynecologist with prolapse (3). PFD is primarily caused by aging and parity, and there are bimodal peaks of POP in these women at the ages of 46 and 71 (4). Treatments for this condition are still conservative and symptom-based. Women with symptoms who failed to respond to or who chose not to receive conservative treatment are candidates for surgery. Traditionally, surgeries include anterior, posterior, or total repair of the vagina, with concomitant hysterectomy, but the rate of recurrence can be as high as 20-30 % (5,6). Synthetic and biomaterial meshes have recently been used during surgery to provide improved long-term outcomes; however, about one-third of meshes cause scarring, erosion, and pain (7). Alternative methods are therefore needed to promote the repair and regeneration of damaged tissues.

In the supportive system of the pelvic floor, fibrous connective tissues surrounding the pelvic organs form fascia and ligaments to provide mechanical strength to support the vagina and its adjacent organs. Due to their specific anatomical location, these tissues are subjected to constant mechanical tensile loading from abdominal pressure and gravity (8). The fascia and ligaments of the pelvic floor mainly consist of dense connective tissues containing fibroblasts and extracellular matrix (ECM) secreted by fibroblasts.

Advanced glycation end products (AGEs), the products of nonenzymatic glycation and oxidation of proteins and lipids, accumulate in diverse biological settings including: diabetes, inflammation, renal failure, and aging (9). In a study examining the actual role of AGEs in the pathological physiology of POP, Jackson et al. found that both intermediate intermolecular cross-links and advanced glycation cross-links increased in
prolapsed tissue (10,11). AGEs can affect the metabolism of collagen through the receptor for AGEs (RAGE) but not directly through changes in expression or structure. AGEs activate the p-p38 MAPK and NF-κB-p-p65 pathways, thereby regulating collagen metabolism, although other pathways may also be involved (12). Taken together, these findings provide an enhanced understanding of the mechanism through which AGEs contribute to collagen metabolism in pelvic tissue of POP and the pathophysiology of POP.

Stem cells are able to participate in tissue repair due to their ability to differentiate into multiple lineages, and thus into various types of connective tissue cells, so they therefore hold great promise for treating PFD (13). Bone marrow-derived mesenchymal stem cells (BMSCs) are one of the most well-characterized stem cell sources, have great differentiation capability, and secrete bioactive factors that facilitate tissue repair (14,15). In animal models of SUI, periurethral injection of BMSCs restored the damaged external urethral sphincter and significantly alleviated SUI symptoms (16). Tissue engineering (TE) approaches have been used in different areas of medicine to improve long-term outcomes of surgical interventions (17). BMSCs are believed to regulate the repair process at sites of injured tissue by interacting with essential endogenous cells involved in the healing process: fibroblasts, endothelial cells, and epithelial cells (18,19). Umbilical cord-derived mesenchymal stem cells (UCMSCs) are isolated from the human umbilical cord and have better cell content and greater ability to proliferate than BMSCs. UCMSCs have lower immunogenicity than BMSCs, are easy to obtain, and cause no ethical controversy, so they have attracted increasing attention from researchers (20).

The current study investigated the anti-inflammatory role of UCMSCs and signaling pathways to inhibit the cytotoxic effect of AGEs in POP. Those findings were analyzed to determine if UCMSCs could serve as a potential treatment that reduces cell damage.

2. Materials and Methods

2.1. Culture of human UCMSCs and human vaginal wall fibroblasts (VWFs)

Human UCMSCs were purchased from the Shanghai Branch of Chinese Academy of Science and cultured in Gibco Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in a 5% CO₂ atmosphere.

Human fibroblasts derived from the vaginal wall were obtained from patients suffering from POP or other diseases who underwent a hysterectomy at the Obstetrics and Gynecology Hospital of Fudan University. Ethical approval was obtained from the Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University. Briefly, fresh vaginal wall tissue specimens from the surgical margin of the free wombs were rinsed 3 times with phosphate-buffered saline (PBS) (containing 1% penicillin, streptomycin, amphotericin B) at 4°C for 5 min and digested at 37°C for 30 min in PBS containing 2% collagenase. After separation, the cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (containing 10% fetal bovine serum, 1% penicillin, streptomycin, and amphotericin B) at 37.5°C in a 5% CO₂ atmosphere, with replacement of the culture medium every 2-3 days. VWFs were identified using anti-vimentin antibody staining and subsequently stored in liquid nitrogen for further study.

2.2. Co-culture of human UCMSCs with VWFs

Human UCMSCs were co-cultured with VWFs by seeding UCMSCs (5 × 10⁴ cells/dish) and fibroblasts (1 × 10⁵ cells/dish) onto DMEM/F-12 culture medium.

2.3. Cell treatment and chemicals

VWFs or co-cultured cells were treated with AGEs at various concentrations (0, 25, 50, 100, and 200 μg/mL) in DMEM/F-12 containing 10% FBS for 2 days and then used for subsequent experiments. Untreated cells served as the control group (con). The PI3K inhibitor LY294002 and the Akt inhibitor GSL 690693 were purchased from Selleckchem (Houston, TX, USA).

2.4. Annexin V/PI double-staining

The fibroblasts from each treatment group were harvested and washed with PBS twice before being labeled with Annexin V/PI double-staining (KeyGen Biotech, China) in the dark, as described previously. All samples were analyzed with flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) using the analytical software Cell Quest (BD, USA).

2.5. Real-time cell analyzer (RTCA) system

The xCELLigence RTCA DP System (ACEA Biosciences, San Diego, California, USA) allows label-free and real-time monitoring of cellular processes, such as cell proliferation, cytotoxicity, adhesion, viability, invasion, and migration, using electronic cell sensor array technology.

In brief, 50 μL of cell culture medium at room temperature was added to each well of E-plate 16 plates for analysis with the xCELLigence RTCA DP System. The E-plate 16 was then connected to the cell culture incubator and electrical contacts were checked. Background impedance was measured for 24 hours. UCMSCs were resuspended in cell culture medium and
adjusted to 5,000 cells/well. The cell suspension (100 μL) was added to wells containing 50 μL of medium on the E-plate 16 in order to determine the optimum cell concentration. After incubation at room temperature for 30 minutes, the E-plate 16 was placed in the cell culture incubator. Cell adhesion, growth, and proliferation were monitored every hour for a period of up to 24 hours via the incorporated sensor electrode arrays of the E-plate 16. After 24 hours, different concentrations of AGEs were added to 200 μL of cell culture medium, and live cells were monitored every 15 minutes for a period of up to 96 hours. Electrical impedance was measured with the RTCA-integrated software of the xCELLigence system as a dimensionless parameter termed CI.

2.7. Cytokine analysis

A Bio-Rad Bio-plex 200 suspension array system was used to measure the cytokine levels in cell culture medium. This bead-based Luminex technology allows for analysis of multiple proteins in a single sample. The experimental protocol allows for simultaneous reporting of standards, controls, blanks, and cytokines of interest in duplicate. The human cytokine 15-plex kit included the following cytokines: IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, IFN-γ, sCD40L, and TNF-α. Bio-Rad Bio-plex Data Pro software was used for data analysis to identify the extreme values, data distribution, and to select the range.

2.8. Cell Counting Kit-8 (CCK-8) Assay

Following the protocol for the CCK8 assay (Do-jindo Laboratories, Kumamoto, Japan), cell growth by transfected cells in 96-well plates was assessed at 48 hours. A spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the absorbance at 450 nm.

2.9. Statistical analyses

Data were analyzed using the software GraphPad Prism (Version 7). Experimental results were expressed as the mean ± standard error of the mean (SEM). Each value is the mean of the data from an assay performed in triplicate. Data were subjected to analysis of variance (ANOVA), and the Tukey test was used to separate the means. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. AGEs inhibited the viability and proliferation of VWFs from patients with PFD

To analyze the effect of AGEs on PFD, human fibroblasts were obtained from the vaginal wall of patients with POP. These fibroblasts were isolated, cultured, and treated with different concentrations of AGEs. Changes in biological function including cell viability, cell apoptosis, and cell proliferation were detected. A CCK-8 assay was used to detect cellular dehydrogenase activity to evaluate the viability of fibroblasts. AGEs were found to significantly decrease the viability of fibroblasts in a dose-dependent manner in all treated groups compared to the control group (Figure 1A). Annexin V/PI double-staining was performed to examine cell apoptosis. AGEs significantly induced the apoptosis of fibroblasts in a dose-dependent manner compared to control cells (Figure 1B). The xCELLigence RTCA DP System was used to monitor cell proliferation in real time. AGEs markedly inhibited the growth of treated cells in a time- and concentration-dependent manner (Figure 1C).

Figure 1. Effect of different concentrations of AGEs on cell viability, apoptosis, and proliferation of vaginal wall fibroblasts from patients with pelvic floor dysfunction. (A) AGEs decreased the viability of fibroblasts in a dose-dependent manner in all treated groups compared to the control group. (B) AGEs induced the apoptosis of fibroblasts in a dose-dependent manner compared to control cells. (C) AGEs inhibited the growth of treated cells in a time- and concentration-dependent manner. *$p < 0.05$. All results are expressed as the mean ± SEM, and data are representative of at least three experiments.

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3.2. UCMSCs protect fibroblasts against cytotoxic effects induced by AGEs

To determine whether UCMSCs protected against the cytotoxic effects of AGEs, fibroblasts were co-cultured with human UCMSCs and then treated with different concentrations of AGEs. Directly cultured fibroblasts treated with different concentrations of AGEs served as the corresponding control group. UCMSCs significantly increased the viability of fibroblasts until treatment with AGEs at a concentration of 100 μg/mL compared to the control (Figure 2A). Compared to the control group, cells co-cultured with UCMSCs exhibited a significant reduction in the rate of apoptosis starting at the concentration of 50 μg/mL when treated with AGEs (Figure 2B), and co-culturing attenuated the inhibitory effect of AGEs on cell proliferation (Figure 2C).

3.3. UCMSCs secrete anti-inflammation cytokines to attenuate the cytotoxic effect of AGEs

To explore whether UCMSCs secreted cell factors that affect the cytotoxic effect of AGEs, cytokines from the supernatant of UCMSCs co-cultured with fibroblasts that were treated with AGEs at a concentration of 100 μg/mL were analyzed. The anti-inflammatory cytokines IL-4, IL-6, and IL-10 increased in UCMSCs while the pro-inflammatory cytokines IL-1β, IFN-γ, and TNF-α decreased in UCMSCs compared to fibroblasts cultured alone (Figure 3).

3.4. UCMSCs attenuate the cytotoxic effect of AGE on fibroblasts by activating the PI3K/Akt/PTEN pathway

Compared to the control, co-culturing with UCMSCs significantly enhanced the expression of PI3K and Akt mRNA in fibroblasts that were treated with 100 μg/mL of AGEs. The signaling pathway for that effect was analyzed. PEN modulates apoptosis, and the expression of PTEN mRNA decreased in co-cultured cells (Figure 4A).

Fibroblasts were pre-treated with the PI3K inhibitor LY294002 and the Akt inhibitor GSK 690693. An enhanced cytotoxic effect of AGEs at a concentration of 100 μg/mL was noted in UCMSCs co-cultured with fibroblasts; the rate of apoptosis increased compared to that in untreated cells (Figure 4B). Treatment with these inhibitors had an additive inhibitory effect on proliferation (Figure 4C) and cell viability (Figure 4D).

4. Discussion

The current study investigated whether UCMSCs could attenuate the cytotoxic effect of AGE-induced cell apoptosis in POP and the possible molecular mechanisms allowing UCMSCs to resist AGE-induced cytotoxicity.

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in POP. The current study found that AGEs inhibit the proliferation of VWFs and have dose-dependent cytotoxic effects on those cells. AGEs also induce the apoptosis of VWFs. In contrast, UCMSCs protect fibroblasts against the cytotoxic effects of AGEs by secreting anti-inflammatory cytokines to improve cell proliferation and cell viability and decrease the rate of apoptosis. Moreover, the PI3K/AKT/PTEN pathway is involved in UCMSCs inhibiting the cytotoxic effect of AGE-mediated cell apoptosis.

Human fibroblasts have the advantages of being easily harvested, cultured, and expanded in vitro, which make them an ideal cell source for regenerative medicine. VWFs play an important role in the pathophysiology of POP, which controls the integrity of collagen, and thereby impacts the mechanical properties of the pelvic floor (21,22). Primary culture of VWFs is commonly used to evaluate connective tissue in POP (23). Previous studies have described the impacts of AGEs on fibroblast proliferation. One study reported that AGEs promote the proliferation of fibroblasts, but another reported that AGEs induced the apoptosis of or inhibited the proliferation of fibroblasts (24,25). In the current study, the proliferation of fibroblasts from patients with POP was significantly inhibited by increasing concentrations of AGEs, suggesting that fibroblasts are more likely to be inhibited in POP. These results explain the smaller number of fibroblasts in the pelvic floor of patients with POP.

MSCs have been extensively used as cell-based therapies predominantly for their anti-inflammatory and immunomodulatory non-stem cell properties (26). They have also potential for tissue engineering purposes for regenerating new tissues or promoting the activity of endogenous stem cells (27). MSC populations have the capacity for self-renewal, a high proliferative potential, and differentiate into a variety of mesodermal and other lineages. Recent advances in cellular identification using more specific markers have shown that MSCs can be extracted from most tissues including bone marrow, the umbilical cord, the placenta, adipose tissue, and the endometrium, although not all of these sources have demonstrated clonogenicity for their MSC populations (28,29).

Typically, MSCs actively respond to stress or injury in a similar manner to the way cells of the innate immune system respond to pathogen exposure. When supplied systemically, exogenous MSCs home in on sites of injury in response to inflammation (30,31). There, MSCs operate in a paracrine manner secreting large amounts of diverse proteins, growth factors, cytokines, and chemokines that promote a variety of actions including neo-angiogenesis, tissue regeneration and remodeling, immune cell activation, suppression of inflammation, and cellular recruitment (32).

The potential of MSCs to serve as a cell-based therapy has recently been explored in numerous clinical applications. The ability to direct BMSCs to differentiate into other cell types and lineages has shown that these cells maintain a phenotype lacking tissue-specific characteristics until they are exposed to signals in damaged tissues (33,34). MSCs obtained from dental pulp have been used to repair related tissues such as the periodontal ligament, dental papilla, and dental follicle (35). The ability of adipose tissue and bone marrow MSCs to act as precursor cells has also

Figure 4. UCMSCs attenuate the cytotoxic effect of AGE on fibroblasts by activating the PI3K/Akt/PTEN pathway. (A) The levels of expression of PI3K, Akt, and PTEN mRNA in POP and co-cultured UCMSCs treated with different concentrations of AGEs. (B) The PI3K inhibitor LY294002 and the Akt inhibitor GSK 690693 increased the rate of apoptosis in co-cultured UCMSCs treated with 100 μg/mL of AGEs. (C) The PI3K inhibitor LY294002 and the Akt inhibitor GSK 690693 inhibited proliferation in co-cultured UCMSCs treated with 100 μg/mL of AGEs. (D) The PI3K inhibitor LY294002 and the Akt inhibitor GSK 690693 inhibited cell viability in co-cultured UCMSCs treated with 100 μg/mL of AGEs. *p < 0.05. All results are expressed as the mean ± SEM, and data are representative of at least three experiments.

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been exploited by directing their differentiation toward the chondrogenic lineage in order to produce cartilage-
synthesizing chondrocytes (36). Although MSCs show
promise as cell-based therapies, greater understanding of
their mechanism of action and their potential is needed.
Early use of MSCs has not always met expectations,
often leading to inconsistent results. This may be due
to lesser refined methods of isolating and cultivating
MSCs resulting in the administration of fibroblasts
and myofibroblasts rather than undifferentiated MSCs.
Production of significant numbers of MSCs posed a
challenge until recently since the regenerative potential
of MSC declined during culture expansion, which is
required due to the small numbers of perivascular MSC
present within tissues (37).

POP is a common hidden disease burden for large
numbers of women. Compounding this burden is the
inadequacies of current surgeries with or without
mesh. Recent advances in cellular phenotyping and
gene profiling suggest endometrial MSCs as a possible
complement to mesh-based POP treatment (38). The
capacity of eMSCs to regenerate tissue is exemplified
during a woman’s reproductive life, where they
regenerate at least one centimeter of endometrial lining
each menstrual cycle for over 400 menstrual cycles.
Seeding eMSCs onto polyamide/gelatin composite
mesh and implanting them into the vaginal wall allow
favorable modulation of the innate immune response
and accelerate organized tissue repair. The first attempt
at combining eMSCs and mesh to treat a fascial defect
was successful in rodent models. This is encouraging,
suggesting that further development of this approach
using an ovine model is warranted (39-41).

In a recent in vivo study on PFD, transplantation
of BMSCs resulted in new tissue growth and collagen
deposition in a wound healing model. In the context
of PFD, an appropriate amount of elastic fibers in the
connective tissue is extremely crucial to functionally
restoring pelvic floor support. Simple deposition of
collagen would cause formation of dense connective
tissues and eventually scar tissues (42,43).

UCMSCs have shown great potential in regenerative
medicine for their extensive sources, potential to
differentiate into multiple lineages, low immunogenicity,
and self-renewal ability (44). The safety and therapeutic
potential of human UCMSCs have been increasingly
studied in the context of regenerative medicine and
immune modulation. The immunosuppressive and anti-
inflammatory properties of cultured/expanded UCMSCs
have led these cells to be tested for their therapeutic
potential in preclinical animal models since the mid-
2000s, and their differentiation characteristics and
responses to external environment have been extensively
documented in in vitro single and co-culture setups
(45-47). They have multiple advantages such as easy
isolation and harvesting, no posing of ethical concerns,
no tumor susceptibility, and low immunogenicity (48).

As a result, UCMSCs hold significant promise for tissue
engineering and regenerative medicine applications (49).
To date, UCMSCs have been widely used in multiple
studies to treat conditions such as acute lung injury,
insulin-resistant diabetes, Alzheimer's disease (AD),
acute myocardial infarction, graft-versus-host diseases
(GVHD), aplastic anemia, arthritis, liver disease, spinal
cord injury, systemic lupus erythematosus, and stroke
(50-52).

Previous studies demonstrated that phosphatase
and tensin homolog (PTEN) can negatively regulate the
PI3K/AKT pathway, which in turn influences the nuclear
factor kappa-light- chain-enhancer of activated B cells
(NF-κB) signalling to modulate cell survival, migration,
and proliferation. AGES adjust the metabolism of target
proteins through RAGE and activate an array of signal
transduction cascades, such as MAPK, ROS, p38, NO,
and NF-κB (53,54). The current authors hypothesized
that the PI3K/Akt/PTEN pathway may be involved in
governing the observed effects of UCMSCs to resist the
cytotoxic effect of AGES in POP. PI3K is a lipid kinase
that induces cell cycle progression, cell survival, and cell
migration, and many pieces of evidence have indicated
that PI3K/AKT signaling is constitutively activated in
many tumors with PTEN dysfunction.

The current findings further substantiate the
contention that UCMSCs play a beneficial anti-
inflammatory role by inhibiting the cytotoxic effect of
AGES. PI3K-AKT signaling, which is closely related to
cell proliferation, is firmly considered to be involved in
inflammatory action as well (55). In the current study,
the anti-inflammatory conditions created by cells co-cultured
with UCMSCs inhibited the expression of PTEN to
reverse the apoptosis and improve cell proliferation in
POP.

In conclusion, the anti-inflammatory role of
UCMSCs may help to reverse the cytotoxic effect of
AGES in patients with POP and activate the PI3K/AKT/
PTEN signaling pathway to increase proliferation and
decrease apoptosis. These roles might provide important
insights into the use of UCMSCs to treat POP.

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