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Original Article

Adipocytes exposed to high glucose potentiate angiogenesis and endothelial dysfunction

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Abstract: Background: Endothelial dysfunction accounts for ultrafiltration failure and high morbidity of cardiovascular disease in peritoneal dialysis patients. However, mechanisms of endothelial dysfunction have not been well understood by now. It shows an expanded visceral adipose tissue with increased adipocytokines in dialysis patients. Here, we investigated whether adipocytes exposed to high glucose could promote angiogenesis and the release of cytokines related to dysfunction of endothelial cells. Methods: 3T3-L1 cells were differentiated into adipocytes. Adipocytes were exposed to medium with 139 mmol/L glucose or isotonic mannitol, and VEGF levels in supernatant were measured by ELISA. Tube formation and migration of bEnd.3 endothelial cells were investigated with or not the addition of anti-VEGF antibody. VEGF, ICAM-1 and ET-1 levels of endothelial cells were assessed by Western Blot. Results: Compared with control group, significant up-regulation of VEGF level was observed in supernatant of adipocytes treated with high glucose ($P<0.05$). There were no difference in VEGF levels when endothelial cells were cultured with different adipocyte supernatants ($P>0.05$). Compared with endothelial cells cultured with high glucose, those cells cultured with supernatant of adipocytes pre-treated with high glucose formed more tubular structures accompanied by stronger migration ability ($P<0.05$), and anti-VEGF antibody partially blocked these effects ($P<0.05$). We also observed significantly up-regulation of ICAM-1 and ET-1 in endothelial cells cultured with the supernatant of adipocyte treated with high glucose ($P<0.05$). Conclusion: Adipocytes exposed to high glucose enhanced VEGF release and potentiated tube formation and migration of endothelial cells, and participated in the regulation of ICAM-1 and ET-1 expression of endothelial cells. It indicated that adipocytes play an important role in endothelial cell dysfunction in high glucose condition simulating the condition of peritoneal dialysis.

Keywords: Adipocytes, angiogenesis, endothelial cells, glucose, peritoneal dialysis

Introduction

As one of the major replacement therapies for end stage renal disease (ESRD), peritoneal dialysis (PD) shows great advantages [1-4]. Sometimes its utilization is partially diminished owing to ultrafiltration failure (UFF) and poor solute clearance [5]. Meanwhile, PD patients also suffer from several complications, and cardiovascular disease (CVD) related to endothelial dysfunction ranks the leading cause of death in long-term PD patients [6].

Peritoneal membrane is the medium for change of metabolic wastes and liquid between internal and external environments. As one of the major components of peritoneum, endothelium charges for the exchange of small solutes [7]. However, endothelial dysfunction is common in PD patients. For one thing, angiogenesis is observed in the peritoneum of PD patients [8], leading to the increase of effective solute transport area [9] and eventually accelerating the process of UFF. For another, endothelial dysfunction is also considered as one of the initial steps of cardiovascular events in PD patients. Once damaged, increasing intercellular adhesion molecule-1 (ICAM-1) [10], vascular cell adhesion molecule-1 (VCAM-1), endothelin-1 (ET-1), and decreasing nitric oxide synthase (NOS) of endothelial cells (ECs) [11, 12] may trigger and exacerbate the process of CVD.

Conventional peritoneal dialysis fluid (PDF), a medium characterized by high glucose, glucose degradation products (GDPs), advanced glyca-

tion end products (AGEs) and low pH, makes use of the osmotic gradient generated by glucose to bring wastes away [13]. It has been verified that non-physiological components in PDF like glucose can do harm to ECs directly [14], or through the impact on other peritoneal cells [15]. However, mechanisms of endothelial dysfunction or interaction among cells are still not well understood.

Meanwhile, studies have proved that patients undergoing PD show obvious increase of visceral adipose tissue despite their low lean body mass [16]. As a powerful endocrine organ, adipose tissue secretes different kinds of adipocytokines including Leptin, Tumor Necrosis Factor-α (TNF-α) and Vascular Endothelial Growth Factor (VEGF) [17]. And through the function of secretion, adipocytes alter the peritoneal physiology [18]. However, whether adipocytes could interact with ECs in peritoneum and got involved in peritoneal angiogenesis and endothelial dysfunction during PD have not been well understood until now.

Here, we imitated the high glucose situation of PDF and observed the role of adipocytes differentiated from an immortalized mouse preadipocyte cell line 3T3-L1 on an isogenous microvascular endothelial cell line bEnd.3 in an *vitro* study, aiming to find a new way to protect the integrity of peritoneum and prevent endothelial dysfunction in PD patients.

Materials and methods

Differentiation of preadipocyte

3T3-L1 cells (American Type Culture Collection, USA) were cultured in dulbecco's modified eagle medium (DMEM) (Gibco, USA) with 10% fetal bovine serum (FBS, USA) in a humidified incubator at 37°C under 5% CO_2 and 95% air atmosphere. The medium was replaced every 2 days, and cells were allowed to differentiate under standard conditions as described before [19]. In brief, the cells were treated with adipocyte differentiation medium containing 1 μM Dexamethasone (Sigma, USA), 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX) (Sigma, USA) and 10 μg/mL Insulin (Sigma, USA) 2 days after confluence (Day 2). Three days later, this medium was discarded and replaced with regular DMEM supplemented with 10 μg/mL insulin (Day 5). Cells were ready for assay and experiment at Day 12.

Identification of adipocytes

We used Oil Red O (ORO) staining to identify the differentiated adipocytes. For the preparation of ORO working solution, 0.7 g ORO powder (Aladdin, China) was dissolved in 200 mL 100% Isopropanol and stirred on the shaker overnight. After mixing the ORO stock solution and ddH_2O at a 3:2 ratio and staying at room temperature for 20 min, we filtered the mixture through 0.22 μm membrane filter (Millipore Biotech, USA) and ORO working solution was ready. For ORO staining assay [19], the plates were incubated with fresh Paraformaldehyde in dark for 1 h after the medium was discarded. The wells were then washed with 60% Isopropanol, and fresh ORO working solution was added when the plates dried completely. Pictures were taken after removing all ORO and washing 3 times with ddH_2O (10×10).

Culture of bEnd.3 and treatment of adipocytes

bEnd.3 (American Type Culture Collection, USA), microvascular endothelial cells which exhibit the characteristics of ECs, were cultured in DMEM with 10% FBS, as described [20]. Mature adipocytes (Day 12) were used in our study. Briefly, after been starved with serum free medium for 12 h, adipocytes were cultured with DMEM containing high D-glucose (Sigma, USA) (139 mmol/L, HG) which shared the same concentration with that of 2.5% PDF. Meanwhile, isotonic mannitol (Sigma, USA) (HM) was set as a control of osmotic pressure, and regular DMEM (25 mmol/L glucose) was set as a blank control (Control group). Supernatant of different groups (Con-adipocyte, HG-adipocyte and HM-adipocyte) was harvested at 0 h, 24 h, 48 h and spun down at 3000 rpm for 10 min at 4°C. All supernatant was immediately frozen at -80°C until measurement or used as conditioned-medium.

Enzyme-linked immunosorbent assay (ELISA)

VEGF levels of mature adipocytes in regular, HG or HM condition at 0 h, 24 h and 48 h were determined by ELISA kits (R&D Systems, USA) according to the manufacturer's instructions.

Tube formation assay

bEnd.3 cells could spontaneously form capillary tubes in Matrigel matrix [21]. This property

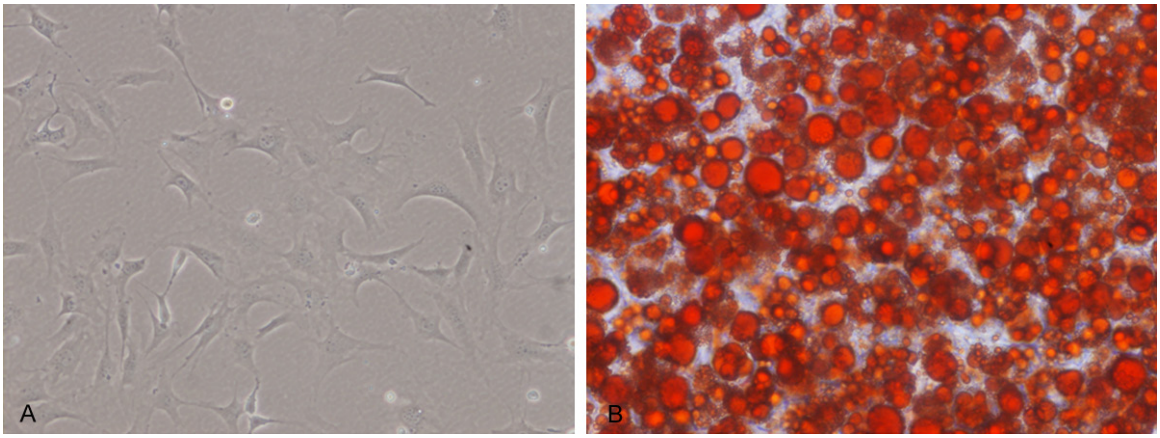

Figure 1. Morphology of 3T3-L1 and adipocytes. A. The microscopic morphology of 3T3-L1 (10×10). B. Oil Red O staining of mature adipocytes (10×10).

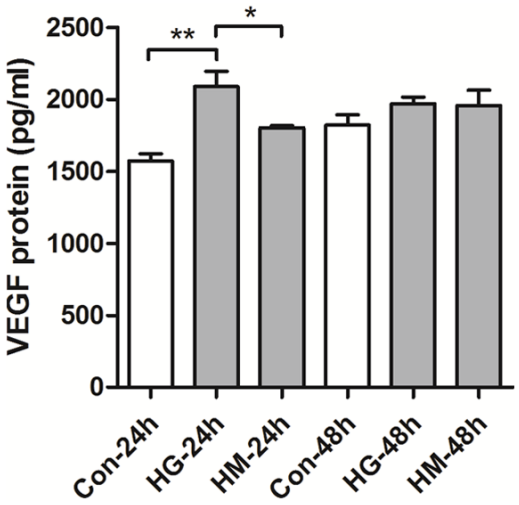

Figure 2. VEGF levels increased in adipocyte supernatant. Con-24h: adipocytes cultured in regular DMEM for 24 h; HG-24h: adipocytes cultured in 139 mmol/L glucose for 24 h; HM-24h: adipocytes cultured in 139 mmol/L mannitol for 24 h; Con-48h: adipocytes cultured in regular DMEM for 48 h; HG-48h: adipocytes cultured in 139 mmol/L glucose for 48 h; HM-48h: adipocytes cultured in 139 mmol/L mannitol for 48 h. **: P<0.01; *: P<0.05.

was used to assess the effect of adipocytes stimulated by glucose on bEnd.3 cells as a model for angiogenesis. Matrigel (Becton Dickinson, USA) was thawed for 24 h at 4°C and diluted into 10 mg/mL with sterile PBS using pre-cooled pipet tips. 50 μL diluted Matrigel was added into pre-cooled 96-well cell culture plates and left at 37°C for 1 h to promote gelling. bEnd.3 cells were digested and 100 μL cells (1.5×10^4 cells/mL) suspended with regular DMEM, HG or HM DMEM were cultured on Matrigel. All test samples were performed in triplicate. After 12 h, images were captured and tube formation was quantified by counting the number of tubular structures formed per mm^2. A three branch point event was scored as one tube.

The effects of adipocyte supernatant preincubated with or not anti-VEGF antibody (Neutralizing goat anti-mouse VEGF polyclonal antibody, Proteintech, China) (10 ng/mL) on tube formation of bEnd.3 were also observed.

Cell migration assay

The migration of bEnd.3 cells was evaluated by polycarbonate membrane of transwell chambers (8 μm pore size, 24-well plate, Corning Costar, USA). 100 μL bEnd.3 cells (1×10^5 cells/mL) were cultured in the upper chamber with serum-free DMEM, and 600 μL media in accordance with that in tube formation assay were added into the lower wells. Cells were cultured for 12 h at 37°C under 5% CO_2 and 95% air atmosphere. Cells affixed to the inside of the membrane were scraped with cotton swabs, and the chambers were fixed with 100% Methylalcohol for 5 min. Number of the migrated cells was measured by counting the cells stained with 0.5% crystal violet solution (Beyotime, China) at 10×20 magnification (high power, HP). The role of anti-VEGF antibody (10 ng/mL) was also observed as above.

Western blot analysis

Western blot analysis was conducted using whole cell lysates of ECs. In brief, ECs were cul-

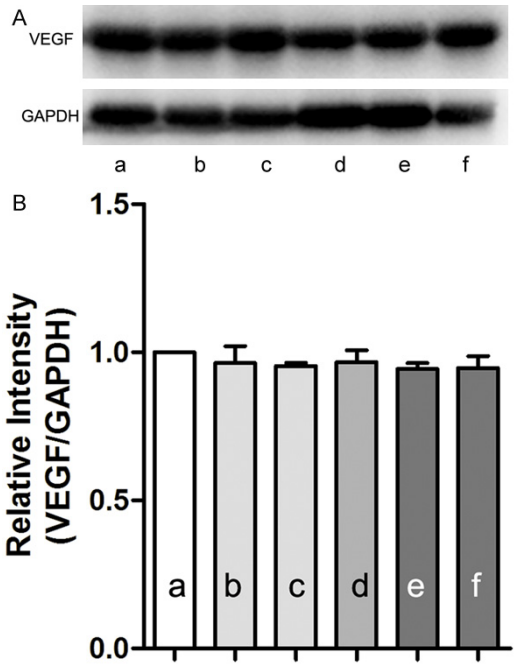

Figure 3. VEGF protein level of endothelial cells. A. VEGF protein level of endothelial cells cultured in different kinds of media for 12 h. B. The quantitative data of VEGF levels in endothelial cells. a. Endothelial cells cultured in regular DMEM; b. Endothelial cells cultured in 139 mmol/L glucose; c. Endothelial cells cultured in 139 mmol/L mannitol; d. Endothelial cells cultured in supernatant of regular DMEM treated adipocytes; e. Endothelial cells cultured in supernatant of high glucose treated adipocytes; f. Endothelial cells cultured in high mannitol treated adipocytes.

tured with or not the supernatant of adipocytes for 24 h. After been washed 3 times with 1×PBS, the cells were solubilized by the direct addition of cell lysis buffer for Western and IP (Beyotime, China) and cell debrises were removed by centrifugation at 4°C. The protein concentration was determined using Enhanced BCA Protein Assay Kit (Beyotime, China), and equal amounts of proteins were separated by 8%~10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) (Beyotime, China) and transferred onto nitrocellulose filter membranes (Millipore Biotech, USA). Nonspecific bindings were blocked with 5% bovine serum albumin (BSA, Beyotime, China) for 2 h and the membranes were incubated overnight at 4°C with primary antibodies of VEGF (Rabbit anti-mouse IgG polyclonal antibody, ab46154, Abcam, USA), ICAM-1 (Rabbit anti-mouse IgG polyclonal antibody, 10831-1-AP, Proteintech, China), ET-1 (Rabbit anti-mouse IgG polyclonal antibody, ab117757, Abcam, USA) and GAPDH (Rabbit anti-mouse IgG monoclonal antibody, ab181602, Abcam, USA). After been washed 3 times (5 min each), the membranes were incubated for 1 h with HRP-conjugated anti-Rabbit antibody (Proteintech, China) and detected with enhanced chemiluminescence reagents (Millipore Biotech, USA). Gray value of each band was detected with Photoshop software and the destined protein expression levels were expressed as a relative value to that of GAPDH.

Statistics

All experiments were completed in triplicate. Normal distribution data were expressed as means ± standard deviation, t-test was used to compare between groups, and abnormal distribution data were analyzed by non-parametric test, using SPSS 17.0. $P<0.05$ was considered statistically significant. Graphs were generated using GraphPad Prism 5.0 (GraphPad Software Inc, California).

Results

Culture and identification of adipocytes

Preadipocytes 3T3-L1 (**Figure 1A**) were successfully differentiated into adipocytes. ORO staining showed that 90% of preadipocytes were differentiated into mature adipocytes after 12 days of differentiation (**Figure 1B**).

VEGF levels increased in supernatant of adipocytes

VEGF level of adipocytes was 1572.5±83.9 pg/mL in control group with regular DMEM (Con-24h). VEGF level increased in supernatant of adipocytes exposed to high glucose after 24 h (HG-24h, 2088.4±107.2 pg/mL; $P<0.01$), while not in a time-dependent manner (**Figure 2**). Mannitol showed similar function (HM-24h, 1803.2±25.9 pg/mL), but was not as significant as high glucose ($P<0.05$). Furthermore, VEGF levels of ECs cultured in conditional supernatant of adipocytes were also measured, however, we found no significant difference among groups (**Figure 3**), ensuring that the functional VEGF in our system was produced by adipocytes.

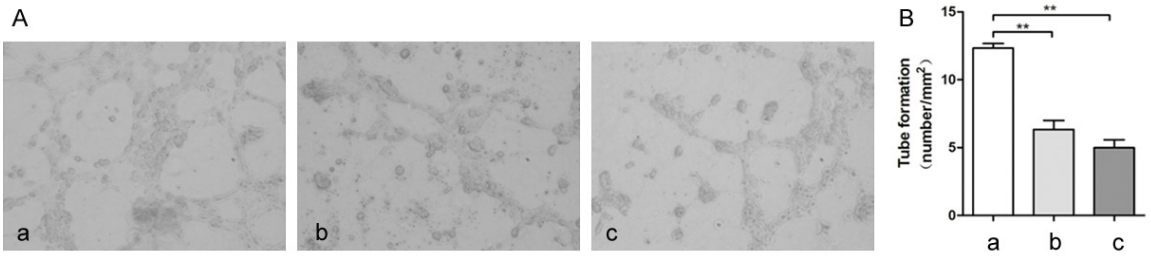

Figure 4. High glucose inhibited tube formation of endothelial cells. A. Morphology of tubes in 12 h (10×20). B. The quantitative data of tube number per mm^2. a. Endothelial cells cultured in regular DMEM; b. Endothelial cells cultured in 139 mmol/L glucose; c. Endothelial cells cultured in 139 mmol/L mannitol. **: P<0.01.

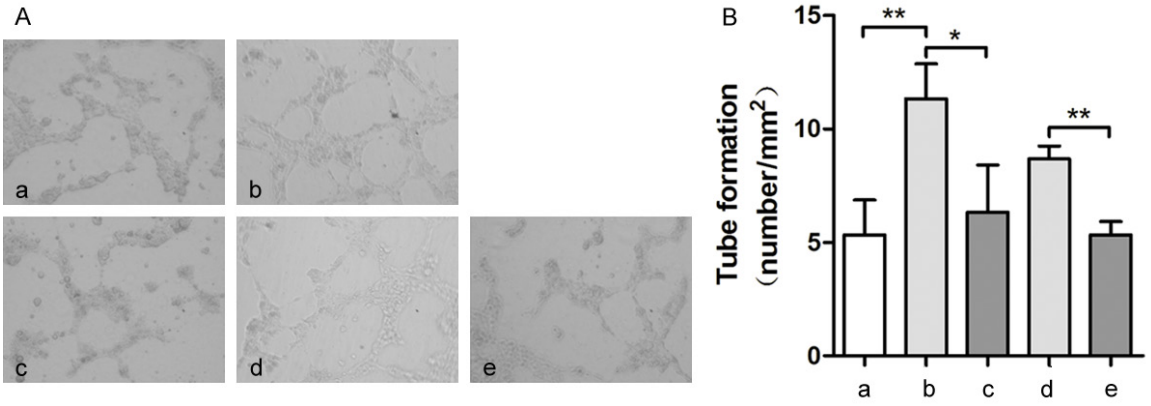

Figure 5. Increased tube formation of endothelial cells in conditional supernatant of adipocytes was inhibited by anti-VEGF antibody. A. Morphology of tubes in 12 h (10×20). B. The quantitative data of tube number per mm^2. a. Endothelial cells cultured in supernatant of regular DMEM treated adipocytes; b. Endothelial cells cultured in supernatant of 139 mmol/L glucose treated adipocytes; c. Endothelial cells cultured in the same condition with 'b' yet preincubated with anti-VEGF antibody (10 ng/mL); d. Endothelial cells cultured in supernatant of 139 mmol/L mannitol treated adipocytes; e. Endothelial cells cultured in the same condition with 'd' yet preincubated with anti-VEGF antibody (10 ng/mL). **: P<0.01; *: P<0.05.

Increased tube formation of endothelial cells in conditional supernatant of adipocytes was inhibited by anti-VEGF antibody

Under normal culture condition, bEnd.3 spontaneously formed tubular structures on Matrigel (12.3±0.6/mm^2) (**Figure 4**). Compared with ECs cultured with regular DMEM containing 25 mmol/L glucose, ECs cultured with 139 mmol/L glucose or isotonic mannitol formed much less tubular structures (6.3±1.2/mm^2 and 5.0±1.0/mm^2; P<0.01), which was consistent with previous studies [22].

bEnd.3 cells cultured with the supernatant of adipocytes treated with 139 mmol/L glucose or isotonic mannitol formed more tubular structures on Matrigel compared with those cultured with the supernatant of adipocytes treated with regular DMEM (11.3±1.5/mm^2 or 8.7±0.6/mm^2 *vs.* 5.3±1.5/mm^2; P<0.001). The addition of anti-VEGF antibody to the supernatant partially reduced the number of tubular structures (6.3±2.1 and 5.3±0.6; P<0.01) (**Figure 5**).

Increased migration of endothelial cells in conditional supernatant of adipocytes was inhibited by anti-VEGF antibody

To further establish the angiogenic properties of adipocyte supernatant, we performed the ECs migration assay. ECs treated with high glucose showed significant migration compared with the control group (6.8±1.7/HP *vs.* 2.3±1.2/HP; P<0.05) (**Figure 6**), however, HG-adipocyte supernatant showed a more significant effect compared with high glucose directly on ECs (46.3±7.1/HP *vs.* 6.8±1.7/HP; P<0.05) (**Figure 6B**). Similar phenomena could also been seen in HM-adipocyte group (29.0±4.7/HP *vs.* 5.7±

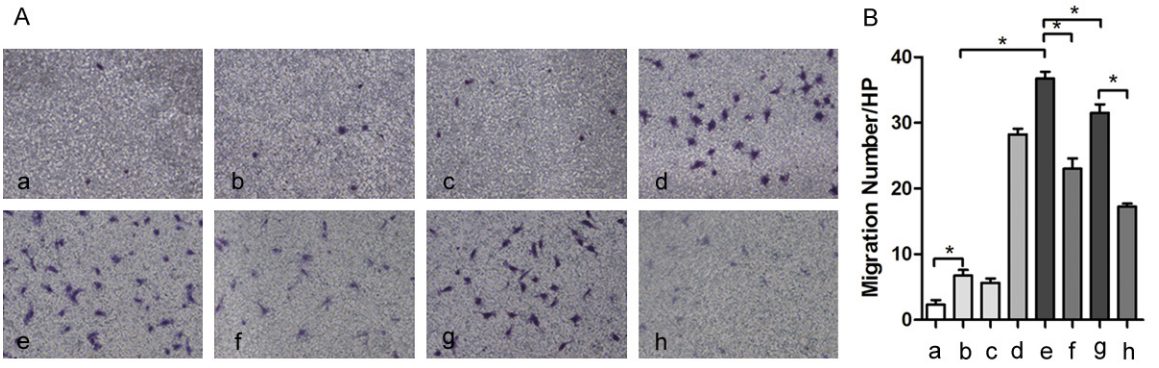

Figure 6. Increased migration of endothelial cells in supernatant of conditioned adipocytes was inhibited by anti-VEGF antibody. A. Endothelial cell migration in 12 h per HP (10×20). B. The quantitative data of migration number per HP (10×20). a. Endothelial cells cultured in regular DMEM; b. Endothelial cells cultured in 139 mmol/L glucose; c. Endothelial cells cultured in 139 mmol/L mannitol. d. Endothelial cells cultured in supernatant of regular DMEM treated adipocytes; e. Endothelial cells cultured in supernatant of 139 mmol/L glucose treated adipocytes; f. Endothelial cells cultured in the same condition with 'e' yet preincubated with anti-VEGF antibody (10 ng/mL); g. Endothelial cells cultured in supernatant of 139 mmol/L mannitol treated adipocytes; h. Endothelial cells cultured in the same condition with 'f' yet preincubated with anti-VEGF antibody (10 ng/mL). *: P<0.05.

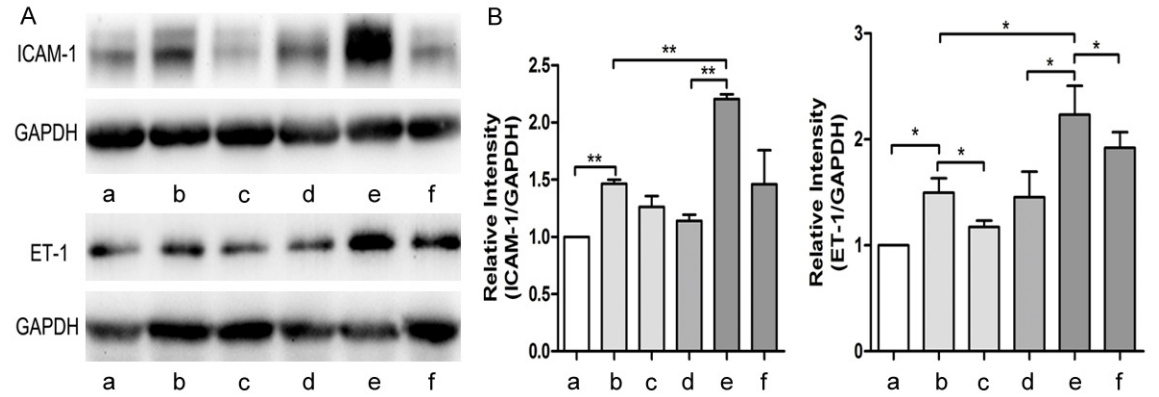

Figure 7. Increasing ICAM-1 and ET-1 expression in endothelial cells in conditional supernatant of adipocytes. A. ICAM-1 protein levels of endothelial cells cultured in adipocytes supernatant for 24 h. B. The quantitative data of ICAM-1 and ET-1 levels in endothelial cells. a. Endothelial cells cultured in regular DMEM; b. Endothelial cells cultured in 139 mmol/L glucose; c. Endothelial cells cultured in 139 mmol/L mannitol. d. Endothelial cells cultured in supernatant of regular DMEM treated adipocytes; e. Endothelial cells cultured in supernatant of 139 mmol/L glucose treated adipocytes; f. Endothelial cells cultured in supernatant of 139 mmol/L mannitol treated adipocytes. **: P<0.01; *: P<0.05.

1.2/HP; P<0.05), however, the effect was less significant than the supernatant of adipocytes treated with high glucose (P<0.05). The addition of anti-VEGF antibody to HG-adipocyte and HM-adipocyte supernatant partially inhibited the migration (23.0±3.2/HP and 17.3±0.9/HP; P<0.05).

ICAM-1 and ET-1 expression increased in endothelial cells cultured in conditional supernatant of adipocytes

We further explored the trends of ICAM-1 and ET-1, the representative proteins of endothelial dysfunction, in our experiment. As been shown in **Figure 7**, HG-adipocyte supernatant significantly promoted the expression of ICAM-1 protein in ECs compared with high glucose directly acted on the cells (2.2±0.4 *vs.* 1.5±0.4; P< 0.01). Similar phenomena could be seen in the expression of ET-1 (2.2±0.3 *vs.* 1.2±0.1; P<0.01).

Discussion

In this *in vitro* study, we found significant up-regulation of VEGF in conditional supernatant of adipocytes. VEGF produced by adipocytes in

high glucose condition promoted tube formation and migration of ECs. Furthermore, we also found significant up-regulation of ICAM-1 and ET-1 of ECs cultured in conditional supernatant of high glucose treated adipocytes, demonstrated the role that adipocytes played in angiogenesis and endothelial dysfunction in PD patients.

As been mentioned above, PDF is characterized by high concentration of glucose which significantly exceeds the normal value of internal environment. Many studies have reported the damage of high glucose on ECs, including endothelial mitochondriopathy [23], increased oxidative stress levels [24] and DNA strand breaks [25]. And as for angiogenesis, apart from buffer in PDF and sterilization methods (heat or filter) [26], glucose exerts its effect mainly through three different ways, means: (i) glucose itself by promoting the secretion of proangiogenic factors from other cell lines such as macrophages, mast cells and mesothelial cells (MCs), (ii) two pathways of glucose degradation, means the formation of GDPs [27] and AGEs [22], and (iii) enhance angiogenesis by other factors like buffer [28]. Here, we observed that in a short time (within 12 h), high glucose inhibited tube formation of ECs, demonstrating that in our experiment, angiogenesis induced by high glucose is not due to the increase of vessel branches. Microvessels formed in the pathologic neovascularization increase the peritoneal permeability, and the increased effective solute transport area further accelerates the course of UFF [9]. Furthermore, Boulanger et al. have verified that 'mesothelial RAGE activation by AGEs enhances VEGF release and potentiates capillary tube formation' [22], proving that apart from glucose itself, cross-talk among different kinds of peritoneal cells in high glucose condition may be another promoter for angiogenesis.

Peritoneum is composed of MCs, basement membrane and submesothelial layer, and the latter contains collagen, blood vessels, adipose tissue and so on [29]. For PD patients, denudation of the mesothelial monolayer [30], together with passive diffusion of solutes in PDF through the peritoneal barrier, and adipocytes protrude from the omental surface actively [29], create opportunities for the direct contact between PDF and adipocytes [13].

PD patients show an increase of body fat [31, 32], especially for visceral fat tissue [16], which may be partially due to the absorption of glucose in PDF [33]. Visceral fat tissues exist extensively in abdominal cavity, including omentum and mesentery, and these lying in the submesothelial layer of parietal peritoneum. As an endocrine organ, adipose tissue secretes different kinds of adipocytokines and exerts important metabolic and proinflammatory effects [34]. Studies have shown that visceral but not subcutaneous fat tissue is the origin of many cytokines such as TNF-α and interleukin [35, 36]. Previous studies about adipocytes in PD were mainly focused on inflammation. They found that adipose tissue in obese produced more inflammatory mediators compared with the lean controls [37, 38]. Similarly, adipocytes exert important proinflammatory effects on peritoneal membrane for PD patients [39, 40]. However, the effect of adipocyte on ECs dysfunction in PD is seldom reported. Here in our study, we demonstrated that VEGF level increased in adipocytes exposed to high glucose, but not EC itself, which was in accordance with previous reports [22]. And conditional supernatant of adipocytes promoted tube formation and migration of ECs compared with glucose directly-treated groups, indicating the role of adipocykines on peritoneal angiogenesis.

Endothelial dysfunction is also associated with CVD events. Increased expression of adhesion molecules, such as ICAM-1, by ECs is one of the earliest indications during the initiation of atherosclerosis [41]. These molecules are involved in the recruitment and attachment of leukocytes to the endothelium [42]. And ET-1, a kind of short regulatory peptide, is recognized among the most potent vasoactive regulators [43]. Apart from the consistent results that high glucose promoted the expression of these cytokines [44, 45], we found that conditional supernatant of adipocytes exerted a more significant effect, which demonstrated that adipocytes exposed to high glucose could lead to endothelial dysfunction by secreting cytokines, then trigger or aggravate the process of CVD.

One of the limitations of our experiment is that it contains only an *in vitro* study, and it would be interest to expand this study to an *in vivo* study and clinic research. Also, we only observed one kind of cytokines (VEGF) of adipocytes in this study which is closely related to angiogenesis and endothelial dysfunction, other adipocytokines may also be explored in further experiments.

Taken as a whole, by exploring the effects of adipocytes exposed to high glucose on ECs, we supposed that expanding adipocytes during PD may play an important role in peritoneal angiogenesis and endothelial dysfunction in PD patients, which may give us a new way to protect the integrity of peritoneum in PD patients.

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Disclosure of conflict of interest

None.

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