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THE EFFECT OF DRAG FORCE AND FLOW RATE ON MESENCHYMAL STEM CELLS IN PACKED-BED PERFUSION BIOREACTOR

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ABSTRACT

Packed-bed bioreactors provide larger surface area to volume ratio compared to the static culture on flasks. Therefore, these systems offer ideal production environment for large-scale culture of mesenchymal stem cells (MSCs), but the effect of fluid dynamics on the cell-behavior of MSCs is not fully elucidated. In this study, packed-bed perfusion reactor loaded with different size of polymethyl methacrylate carriers was used to apply different rates of shear stress and drug forces at constant flow rate. The cell viability, cell-expansion, apoptosis and protein secretion levels were analyzed for both unmodified and Vascular Endothelial Growth Factor-positive ($VEGF^+$) MSCs. The superficial stress was estimated to between 0.21-0.25 N/m^2. The results showed that the shear stress reduced the VEGF secretion, and Caspase-3 was activated at high drag force, which cause the reduction of the cell numbers in the bioreactor. The reduction of cytoskeletal actin structures seemed to play the central role in this adverse effect of the non-planar shear stress. The expression reduction of VEGF might also have critical impacts on the tissue engineering applications, in which the formation of vascular construct is essential.

Keywords: Bioreactor, Mesenchymal stem cells, Packed bed column, Protein secretion, Vascular endothelial growth factor

1. INTRODUCTION

The large selection of bioreactor designs is available for the production of biochemicals. Due to the fragile nature of adult stem cells under dynamic flow conditions, alternative systems have been developed to the continuous stirred or air-lift reactors, like as the perfusion bioreactors [1]. These types of bioreactors enable the continuous flow of medium through the system and eliminate the transport limitations of both flow and mass. Perfusion bioreactors are the system of choice in bone tissue engineering in vitro mostly because of their mechanical stimulus to cell expansion and to differentiate stem cells to particular tissue cells [2, 3]. Fluid shear forces in these systems cause mechanical stimulation of the culture and has been shown to enhance the expression of the osteoblastic phenotype in MSCs [4]. However, the shear stress within the packed bed bioreactor has been also reported to affect adversely the stemness of stem cells [5, 6].

MSCs are the cells of choice in many applications of regenerative medicine [7]. In addition to their characteristics of in vitro expansion and differentiation into other mesenchymal cells, they also involve in the regulation of immune system. To supply the cell requirement in regenerative medicine, packed bed bioreactor designs have been proposed for the expansion of MSCs [5], and a tubular perfusion bioreactor system was used to increase the glycosaminoglycan production [8]. Their multi-characteristics could be attributed to their secretion of a number of soluble bioactive molecules, like cytokines and growth factors [9].

In most of the studies analyzing the effect of the shear-stress, the selected design of the culture system provides planar flow to the surface [10, 11]. These parallel-plate flow chambers enable researchers calculate the shear-stress rate easily, but neglect the other flow parameters in packed-bed column and in other 3D-scaffolds, like the drag force effect [6]. Similarly, most of the blood vessels have curled and twisted structures in the tissues.

Cell adhesion plays an important role in the cell behaviour, and affected by the hydrodynamic forces created by fluidic flows. Drag force is another concept of the fluid hydrodynamic forces beside the shear-

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stress. In fact, drag force is the primary coupling force between the fluid and particulate phases, and therefore, it is quite important in a fluidized packed bed column [12]. In a larger set-up, the effect of drug force might be neglectable. However, the developement of microfluidic systems also caused the needs for detailed understanding for biomechanical mechanism including the issue of drug force [13].

Thus many other factors should be considered for the measurement like the drag force effect. In this study, it was aimed to analyze the effect of shear stress and drag force on the VEGF secretion by non-encapsulated MSCs. For this purpose, both rat bone marrow derived MSCs (rBM-MSCs) and VEGF gene transferred rBM-MSCs were used to culture on collagen coated polymethyl methacrylate (PMMA) beads, packed in a column. The amount of VEGF secretion and the cell viabilities were compared.

2. MATERIALS AND METHODS

2.1. Cell Culture

The study protocol was approved by the local ethical committee under protocol no. [KOU-HADYEK 6/5-2011]. rBM-MSCs used in this study was isolated and characterized in our previous study [14]. Briefly, MSCs were isolated from the bone marrow aspirates by gradient centrifugation (Ficoll-histopaque gradient, 1.077 g/mL). The isolated cells were cultured in the culture media; L-DMEM (Thermo, Gibco, Paisley, UK) supplemented with 10% FBS and 1% antibiotics (Thermo, Gibco) at 37°C in a humid atmosphere containing 5% CO_2.

2.2. Co-transfection of rBM-MSCs with VEGF and GFP Genes

VEGF gene was obtained kindly from Dr. Duran Ustek, Department of Genetics, Institute of Experimental Medicine, Istanbul University [15]. The gene was ligated to the downstream of CMV promoter of pGFP vector (Clontech, Palo Alto, CA) deleting the GFP gene on the plasmid. The constructed plasmid was named as pVEGF. After isolation with EndoFree plasmid isolation maxi kit (Qiagen, Hilden, Germany), the plasmid mix of pVEGF/pGFP (10:1) were transfected by electroporation (Neon Transfection System, Invitrogen, Carlsbad, CA) with respect to the instructions provided by manufacturer. The transfection parameters for rBM-MSCs were 990V/40ms/2 pulse. After 48 h of culture in DMEM-medium with 10% FBS, the transformed cells were selected with respect to their resistance against 400 µg/mL G418 (Roche, Mannheim, Germany) under standard culture conditions.

2.3. Collagen Coating of Beads

PMMA beads, in shape of polygonal spheres, were supplied commercially from market in three different sizes; ∅2.5 mm, ∅3 mm and ∅4 mm (∅, stands for diameter of the bead). The surface of beads was first washed 2 times with distilled water, 3 times with 70% ethanol, and 2 times with PBS (Thermo, Gibco), consecutively. The beads were dried under laminar flow cabin, and coated with 5 µg/cm^2 of Rat Tail Collagen (Type I) (BD Biosciences, San Jose, CA) coating solution for 1 h at room temperature under constant rotation at 100 rpm. The beads were washed with PBS once, air dried and stored at 4ºC until use. The coating efficiency was estimated by BCA protein assay kit (Sigma-Aldrich, St Louis, MO). The effect of flow on collagen coating was analyzed by incubating the coated beads without cell for 3 days in the perfusion system, and the remaining collagen was measured.

2.4. Cell Seeding on Beads and Cell Counting

The collagen coated beads were embedded in standard culture medium in 6-well culture plate (BD Bioscience), and 10^6 cells/well were seeded following with gently mixing a few times. The cells were cultured for 48 h to allow attachment. The attached cells on the surface of beads were checked by staining with hematoxylin, followed with the examination under light microscope. The number of the cells attached on the surface of the beads was estimated by WST-1 Assay (Roche, Mannheim, Germany). After the

removal of culture medium, 100 µL of 10% WST-1 (in serum-free medium) was added to the cells. The plates were pre-read immediately on a plate reader at 450 nm to obtain a background count. After incubation at 37°C for 3 h, the color intensity of plates was determined for any changes. The number of cells was estimated by using standard chart prepared for this cell line with known number of cells.

2.5. Flow Perfusion Bioreactor Setup

The system included a media reservoir, multi-channel peristaltic pump, and a packed bed column with heating jacket. The column (culture chamber), reservoir and pump are connected to each other in series. The media was kept warm at 37°C on heating plate while the media was aerated (gas composition of 5% CO_2 and 95% air) at the rate of 70 mL/min (0.1 MPa, 20°C). The flow is controlled by peristaltic pump (Ismatec, ISM834, Wertheim-Mondfeld, Germany) at constant flow rate of 1.633 mm/s. The 2 mL columns were filled with beads as tight as possible. During operation the column was kept at constant temperature at 37°C by heating jacket (MI-IBC, Tokai hit, Shizuoka, Japan).

2.6. Estimation of Shear Stress Profiles

The Reynold's number (Re) (1) for each experimental set-up was estimated from the relation given for packed bed columns [16]:

$$Re = \frac{4}{6}\left(\frac{1}{1-\varepsilon}\right)\left(\frac{\mathrm{D}(\varepsilon\nu)\rho}{\mu}\right) \quad (1)$$

, where ε is void fraction in column; D is diameter, ν is velocity, ρ is density and µ is viscosity. The viscosity of fluid (µ) was taken to be 10^{-3} Pa.s [6].The system was operated under constant flow rate at 1.633 mm/s. In the system, beads with three different sizes were employed so that every column of each set-up was operated with different contact (wetted) area. Flow variations in the direction transverse to the flow were assumed to be negligible. The friction factor (f) was calculated according to the Ergun's equation (2) in the column:

$$f = \frac{150}{Re} + 1.75 \quad (2)$$

The shear stress (τ)was estimated by following equation (3):

$$\tau = f\left(\frac{D}{L\rho v^2}\frac{\varepsilon^3}{1-\varepsilon}\right)^{-1} \quad (3)$$

, where L is length of column. The Schmidt Number (S_c) was estimated by following equation (4) given by Geankoplis [16]:

$$Sc = \frac{\mu}{\rho\, Dm} \quad (4)$$

, where Dm stands for molecular diffusivity. The molecular diffusivity was taken to be 7.8-8.6 ×10-4 m2/s [17].

2.7. Quantification of VEGF by ELISA

Secretion of VEGF (Invitrogen, Life Technologies, Carlsbad, CA) was determined in culture media by ELISA according to the manufacturer's recommendations. The minimum detectable dose was 5 pg/mL for VEGF. All these experiments were repeated at least thrice. Absorbance differences were measured by UV-visible spectrophotometer (VersaMax microplate reader, Molecular Devices, Sunnyvale, CA, USA).

2.8. Immunostaining

Samples were fixed in ice-cold methanol for 10 min and treated with 0.025% Triton X-100 (Merck, Darmstadt, Germany) for permeabilization. Cells were incubated with 1.5% blocking serum solution (Santa Cruz Biotechnology, Heidelberg, Germany) for 30 min at 37 ºC, and incubated overnight at 4ºC with the primary antibodies after washing with PBS. Samples were incubated with appropriate secondary antibodies for 25 min and covered with mounting medium containing DAPI (Santa Cruz Biotechnology). The mounted cells were immediately examined under fluorescence microscope (Leica DMI 4000B, Wetzlar, Germany). The antibodies for VEGF, alpha smooth muscle actin (ASMA) and active Caspase3 were supplied from Santa Cruz Biotechnology.

2.9. Statistics

All experiments were performed in triplicates. Data are reported as means ± standard deviation. All statistical analyses were performed using SPSS 10.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed using one-way ANOVA or paired t-test. Differences between the experimental and control groups were regarded as statistically significant when $P<0.05$.

3. RESULTS

3.1. Ectopic Expression of VEGF

The VEGF synthesis in cells was determined by immunohistochemical stainings (Figure 1). The measurement showed the VEGF secretion of 52.3 pg/mL ±5.7, whereas the untransformed rBM-MSCs could secrete higher amount of VEGF, 578.4 pg/mL ±5.3, in static culture.

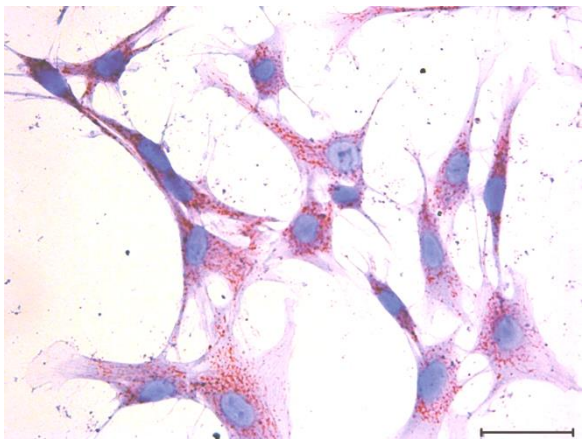


Figure 1. Immunohistochemical staining of secreted VEGF by rBM-MSCs transfected with pVEGF. Scale bar, 50 µm.

3.2. Effect of Flow on Collagen Coating and Attached Cells

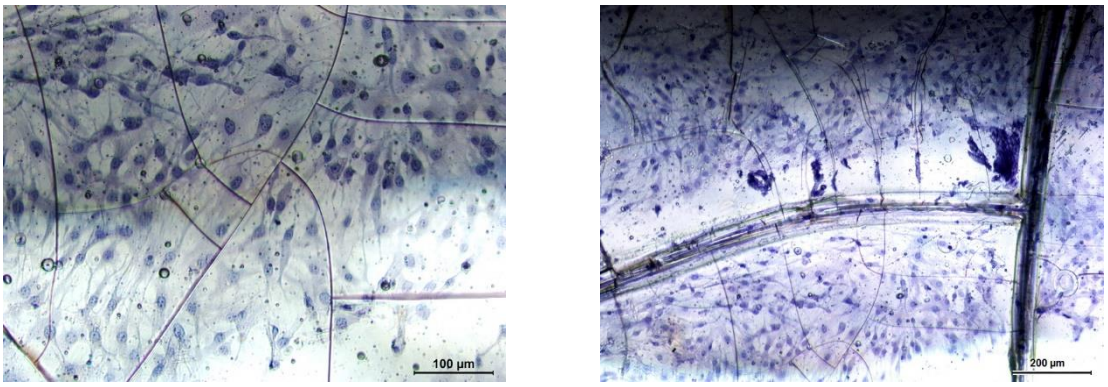
The culture conditions and setting were optimized for perfusion bioreactor. The flow rate was adjusted for the system by keeping the Re below 10.0 to sustain the laminar flow in packed bed column (Table 1). After 3 days of operation, the cellular adhesion and viability were assessed (Figure 2). Three types of beads in identical composition but in different sizes were used to assess the different shear stress rates only in columns. The total surface areas of columns with small to large beads were 5.89 cm^2, 5.65 cm^2 and 6.53 cm^2 (except the inner wall), respectively. The flow rate of the system was adjusted to 1.633 mm/s to obtain laminar flows in three columns (10.0>Re). The shear stress on the beads was estimated between 0.20-0.25 N/m^2 (2.0-2.5 dyn/cm^2).

Table 1. Effect of flow on collagen coating and cell number

Bead Size ∅ (mm)	N_{Re}	Drag Force (N)	Collagen Coating (mg/cm^2)	Attached Cells (# cells/cm^2)	
				Normal	VEGF+
2.5	3.10	4.86 x 10^{-6}	1.14±0.05	15812±4070	11068±781
3.0	3.80	5.89 x 10^{-6}	1.19±0.03	9698±2387	4511±37
4.0	5.57	11.13 x 10^{-6}	0.29±0.03	4301±892	4801±7

The collagen coating was significantly decreased under the effect of flow (Table 1). With respect to the results, the reduction of collagen coating could not be correlated with the rate of the shear-stress. Rather shear stress, the drag force could have an effect on the diminution of coating. The unitless constant Schmidt Number (S_c) was calculated for the process to be S_c<1.0 (1.16-1.28 × 10-3), which indicated that molecular diffusion is significant as momentum transport in all columns.

The same experiment was repeated for the beads with cells to determine the number of attached cells after process. The flow caused wash-off the attached cells from the surface of the beads at different degrees. The number of the cells decreased with the decreasing amount of collagen coated on surface (Table 1). As the cells loosely attached on PMMA surface, the elimination of the protein coating from surface could result in reduction in the attached cell numbers.



(**a**) (**b**)

Figure 2. Attached cells on the surface of the collagen coated PMMA beads (∅2.5mm). The cells were stained with hematoxylin dye before (a) and after (b) culture in perfusion system for 3 days. (Original magnifications: A,X200; B, X100).

3.3. VEGF Secretion under Shear Stress

In the static culture (no shear stress), VEGF secretion was detected to be approximately 52.3 pg/mL, and 578.4 pg/mL in unmodified and VEGF transfected cell lines respectively. The ectopic expression of gene could trigger the control mechanism for VEGF expression that might cause the shut-down of its wild-type expression. The amount of secreted VEGF by the attached cells on beads was measured, and the data were normalized with the total cell number in each column (Table 2). The effect of shear stress was significant on the VEGF secretion in unmodified rBM-MSCs. Compared to the level in static culture, VEGF secretion was decrease to 2.8-4.8%. At higher shear stress, the reduction of expression was higher (Table 2). This reduction was not observed in genetically modified cell line at the moderate stress level (0.2085 N/m^2). However, the fall in the cytokine level was observed also at the higher shear stress (0.2470 N/m^2). VEGF could not be detected in the process with the column packed with 4 mm beads. The low number of the cells attached on beads might result in the secretion of inadequate level of VEGF.

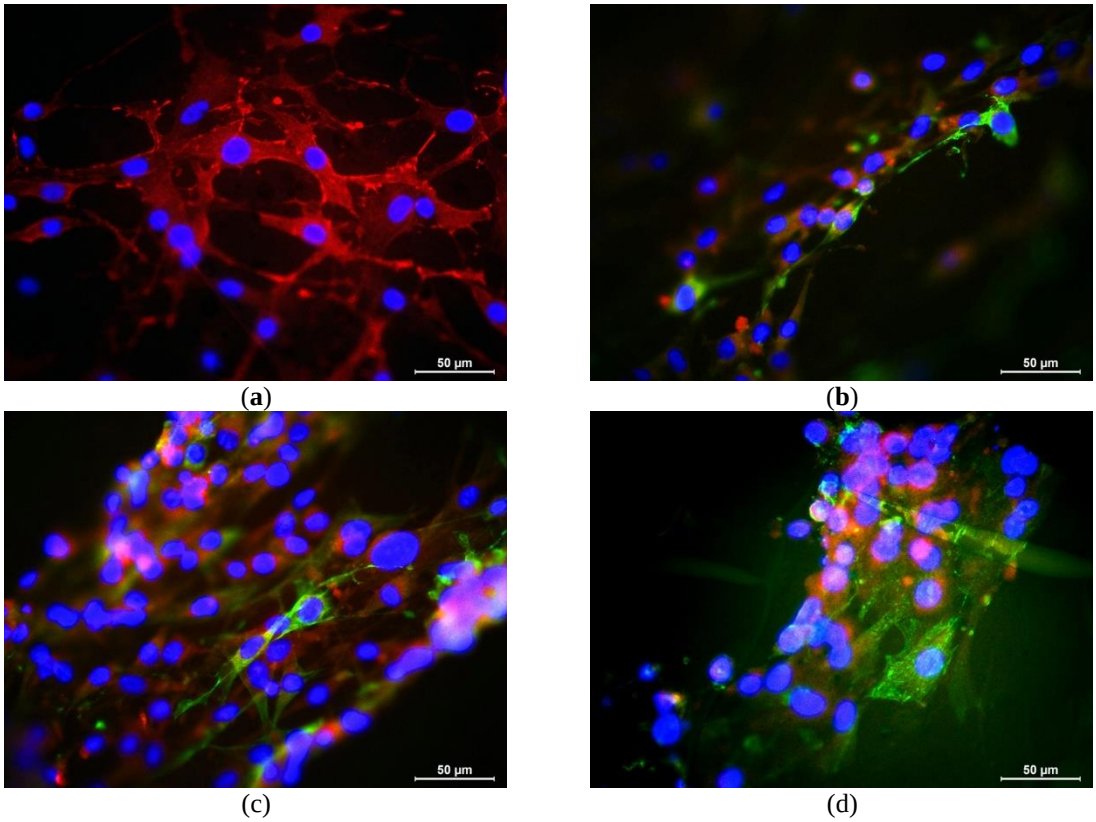
Table 2. Alteration of VEGF secretion in response to shear stress by normal and VEGF gene transfected stem cell lines

Bead Size ∅ (mm)	Shear Stress (N/m^2)	VEGF Secretion (pg/mL)	
		Normal	**VEGF+**
2.5	0.2470	16.33±2.41	26.16±4.99
3.0	0.2085	27.71±4.88	59.90±6.85
4.0	0.2215	n.d. [1]	n.d. [1]

[1] *n.d., not detectable*

3.4. Viability of Cells and Apoptosis

The cell numbers attached on the surface of the beads were estimated by WST-1, which detected the metabolically active cells (Table 1). The detachment of cells from the bead surface was more significant in the culture with modified cell line. Independently of the cell line examined, the cell numbers on support were observed to decrease over the process. The lower number of cells could be attributed to increasing drag force, which functioned in the removal of collagen coating and the detachment of cells from surface. To observe whether the reduction was due to apoptosis, the cells were immunostained with active Caspase3 and ASMA (Figure 3). Before culture in flow, the cells showed no sign of apoptosis (Figure 3a). However, the culture in perfusion bioreactor caused the increase in the active Caspase3 levels in all columns. The expression of active Caspase3 was observed highest in the cells exerted with high drag force in 4 mm bead column (Figure 3d). In other two columns, a slight, but insignificant level of active Caspase3 elevation was detected. The shear stresses at these levels appeared to be not effective on apoptosis (Table 1; Figure 3 b,c).



(**a**) (**b**)

(c) (d)

Figure 3. Effect of shear stress on cell apoptosis. The cells on beads were stained with Caspase3 (green) and ASMA (red) antibodies before perfusion culture (a), and after 3 days culture on ∅2.5 mm (b), ∅3.0 mm (c) and ∅4.0 mm (d) beads. The nuclei were stained with DAPI (Blue). Scale bar, 50 µm.

4. DISCUSSION

The expression of both endogenous and recombinant VEGF in rBM-MSCs was reduced under the effect of shear stress in perfusion bioreactor system. The stress was not applied on a planar system, like in most studies [10, 11], but uniform spherical support (collagen coated PMMA) was used to mimic the flow characteristics in 3-dimensional (3D) scaffolds in this study. A variety of bioreactor types has been using in tissue engineering, and they were proven to be applicable to 3D culture systems, which improves the tissue formation capacities of stem cells [18, 19]. MSCs are sensitive to external physical effects, and their differentiation into osteoblasts was improved by shear stress under appropriate perfusion conditions [20]. The mechanical stimulus by shear stress was noticed to be critical also for MSCs in endothelial differentiation [21, 22]. Although in those studies, the effect of the shear-stress was on favor to the cell expansion and differentiation, it affected unfavorably the cell cultures in this study. The cell behavior of a number of progenitor and stem cells was analyzed under different shear stress levels including embryonic stem cells (0–1 N/m^2), endothelial progenitor cells (0.01–0.25 N/m^2), mesenchymal stem cells (0.5–1.5 N/m^2) [23]. Furthermore, the study by Kim et al. (2017) also revealed that adult stem cells respond distinctively to small differences in shear stress at the level of 0.0019 N/m^2, 0.0022 N/m^2 and 0.0024 N/m^2. The actin structures change in response to altered shear stress rate, and the generated signals cause to decrease the proliferation rate as the shear stress increases. The intensivity toward the shear stress response could be as low as 0.002 N/m^2 [23]. The cell number reduction in this study might not only be explained by the effect of shear stress, but other forces, like drag force, might play an important role.

As bioreactors are becoming increasingly important in developing constructs for tissue engineering and in vitro physiological model systems, the hydrodynamic characteristics of perfusion bioreactor configurations and its influence both on the cell microenvironment and in modulating morphogenesis should be defined cautiously [19, 24]. The main difference was the utilized packed bed column which developed significant level of drag force. A closed-loop perfusion bioreactor setup for bone tissue engineering was designed by inserting the polyuretan circular inserts into the chamber [25]. This system provides a large surface for cells to attach and to expand, while allowing large scale stem cell culture. Although, the effect of shear stress has not been investigated in that study, the beneficial effect of the design toward osteogenic differentiation has been clearly revealed. In another study, a shear stress of 0.015 N/m^2 has been also reported to up-regulate the osteogenic pathways in human bone marrow MSCs [5, 6]

Shear stress is the friction force exerted on the cells and exists, where there is fluid flow. As the endothelial cells localized at the inner surface of the blood vessels, they constantly exposed to shear stress caused by blood flow at variable rates, and this physical effect was shown to be more effective in migration of endothelial cells than chemotaxis [26,27]. This stimulus was responded by loosening the cell-cell contact, activation of several signaling pathways and remodeling cytoskeleton structures in cells [28]. However, the expression of death-associated protein kinase (DAPK) was also reported to be regulated by the mechanical stimulus affecting cytokine-activated apoptosis [29]. In a microfluidic culture system, the effect of shear stress on human annulus fibrosus cells was evaluated [30]. As stated, a shear stress of 0.1 N/m^2 caused an anabolic effect on collagen type-1 and -3, while the catabolic effects on the aggrecan were reported. On the other hand, 1.0 N/m^2 had an improved anabolic effect on collagen type-1, but it has a catabolic effect on collagen type-3 and aggrecan. Although the applied force was lower than the physiological condition of tissue, the genes, which should involve in the tissue regeneration, were acted differently. That study supported the idea that shear stress plays a significant role in the niche of cell. In this study, the flow within the column packed with ∅4mm beads created moderate shear-stress, but significantly high level of drag force, which both induced the apoptosis in the cell (Figure 3d) and tear-off the collagen coating from the surface decreasing the cell adhesiveness. In both cases, the cell number reduction was observed affecting the lower VEGF production, although the shear-rate was not at highest level.

Another question about the stress effect might be the stem cells committed to differentiate under the effect of the shear-stress. VEGF is the main cytokine regulating angiogenesis in both wound healing and tumor development [31, 32]. In the study by Beckermann et al. (2008), the migration of MSCs to blood vessels lead to increase the secretion of VEGF and other growth factors, but very few or no differentiation was observed [33]. VEGF secretion was further enhanced by hypoxia in this model. In our reactor system, lower levels of shear-stress and flow rate were exerted to the cells, and cell differentiation did also not observed. The laminar shear stress was found to induce the Tie2/PI3K/Akt/eNOS and PI3k/Akt-SIRT1-Ac-H3 pathways and leads to improve the endothelial cell markers in early endothelial progenitor cells [34-36]. Even a shear stress at 0.05 N/m2 is sufficient to phosphorylate beta-catenin, translocation into nucleus and inhibit the Wnt signaling, which and favor paracrine cardiovascular support of MSCs [37]. However, the effect of drag force was still not analyzed because of the system design.

Here it was showed that shear stress significantly influenced the VEGF secretion both in normal and genetically engineered MSCs. As the gene expression is not regulated endogenously, the only effector determines the VEGF secretion rate was the protein transport cascade in the cytoplasma. The VEGF expression in unmodified rBM-MSCs was under the control of only its promoter. However, there were two kind of expression of VEGF: the one, which was under the control of its promoter as like in the unmodified MSCs, and the other one, which was expressed constitutively in the cell under the control of CMV promoter. Interestingly, the secretion was reduced at high shear stress level. The reduction in secretion might regulate at the post-transcriptional period. This is particularly important in the neo-organogenesis applications, where adequate vascularization in 3D tissue constructs is desired for long-term functioning after implantation [38]. Shear stress also augmented the expression of VEGF receptors [39] and VEGF [40] itself, but the expression of TGF-β1 was reduced and activating TGF-β1/SMAD pathway under certain shear stress [40,41]. In the study by Cui et al. (2012), endothelial progenitor cells were exposed to relatively high shear stresses (0.2-2.0 N/m2), and it was demonstrated that the higher the shear-stress in a flow chamber, the higher was the expression of endothelial markers (CD31 and vWF) [42]. However, the VEGF expression was decreased at higher shear stress rates in this study. The VEGF might show a different expression profile than CD31 or vWF, but the drag force effect could also play an important role in the expression profile on behalf of the shear stress in a 3D-culture system.

The hypoxic condition in culture was shown to affect the half-life of the VEGF mRNA in cells [43]. This might partially clarify the VEGF level difference between the static culture and the bioreactor. The difference in the secretion by the MSCs on the beads might be still explained by the effect of the fluid shear stress on cells. The alteration in VEGF secretion could be explained by the effect of drag force on ion channels, which might be 'pushed' open due to the drag force. However, the single effect of drag force is not sufficient, and in addition to drag force, shear stress induced production of NO and Erk1/2 inhibition might also alter the ion channel function directly or indirectly [44].

5. CONCLUSION

The use of 3D bioreactors instead the static planar systems are essential to mimic the neo-organogenesis. The characterization of factors affecting the cell behavior in 3D culture systems is crucial in tissue engineering. In this study, VEGF secretion was observed to be reduced by rBM-MSCs under the effect of continuous shear stress in bioreactor, but the drag force might also be an important factor in the stem cell culture in packed bed bioreactors. The study results indicated that the VEGF secretion was effected by mechanical stimulus, which should be considered as one of the process parameters of the bioreactor operations in tissue engineering, considering both the shear-stress and the drag force exerted by the flow.

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REFERENCES

[1] Zhang ZY, Teoh SH, Teo EY, Khoon Chong MS, Shin CW, Tien FT, Choolani MA, Chan JK. A comparison of bioreactors for culture of fetal mesenchymal stem cells for bone tissue engineering. Biomaterials 2010; 31: 8684-8695.

[2] Bancroft GN, Sikavitsas VI, Mikos AG. Design of a flow perfusion bioreactor system for bone tissue-engineering applications. Tissue Eng 2003; 9: 549-554.

[3] Kavlock KD, Goldstein AS. Effect of pulse frequency on the osteogenic differentiation of mesenchymal stem cells in a pulsatile perfusion bioreactor. J Biomech Eng 2011; 133: 091005.

[4] Sikavitsas VI, Bancroft GN, Holtorf HL, Jansen JA, Mikos AG. Mineralized matrix deposition by marrow stromal osteoblasts in 3D perfusion culture increases with increasing fluid shear forces. Proc Natl Acad Sci USA 2003; 100: 14683–14688.

[5] Osiecki MJ, McElwain SDL, Lott WB. Modelling mesenchymal stromal cell growth in a packed bed bioreactor with a gas permeable wall. PLoS One 2018; 13: e0202079.

[6] Zhao F, Chella R, Ma T. Effects of shear stress on 3-D human mesenchymal stem cell construct development in a perfusion bioreactor system: Experiments and hydrodynamic modeling. Biotechnol Bioeng 2007; 96: 584-595.

[7] Le Blanc K, Davies LC. MSCs-cells with many sides. Cytotherapy 2018; 20: 273-278.

[8] Guo T, Yu L, Lim CG, Goodley AS, Xiao X, Placone JK, Ferlin KM, Nguyen BN, Hsieh AH, Fisher JP. Effect of Dynamic Culture and Periodic Compression on Human Mesenchymal Stem Cell Proliferation and Chondrogenesis. Ann Biomed Eng 2016; 44: 2103-2113.

[9] Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol 2007; 213: 341–347.

[10] Knippenberg M, Helder MN, Doulabi BZ, Semeins CM, Wuisman PI, Klein-Nulend J. Adipose tissue-derived mesenchymal stem cells acquire bone cell-like responsiveness to fluid shear stress on osteogenic stimulation. Tissue Eng 2005; 11: 1780-1788.

[11] Huang Y, Jia X, Bai K, Gong X, Fan Y. Effect of fluid shear stress on cardiomyogenic differentiation of rat bone marrow mesenchymal stem cells. Arch Med Res 2010; 41: 497-505.

[12] Ghadirian E., Arastoopour H. CFD simulation of a fluidized bed using the EMMS approach for the gas-solid drag force. Powder Technology 2016; 288: 35-44.

[13] Wiklund K, Zhang H, Stangner T, Singh B, Bullitt E, Andersson M. A drag force interpolation model for capsule-shaped cells in fluid flows near a surface. Microbiology. 2018;164(4):483-494.

[14] Turac G, Duruksu G, Karaoz E. The Effect of Recombinant Tyrosine Hydroxylase Expression on the Neurogenic Differentiation Potency of Mesenchymal Stem Cells. Neurospine 2018; 15: 42-53.

[15] Adas G, Percem A, Adas M, Kemik O, Arikan S, Ustek D, Cakiris A, Abaci N, Kemik AS, Kamali G, et al. VEGF-A and FGF gene therapy accelerate healing of ischemic colonic anastomoses (experimental study). Int J Surg 2011; 9: 467-471.

[16] Geankoplis CJ. Transport Processes and Unit Operations. 3rd ed. Englewood Cliffs, NJ, USA: Prentice Hall Press, 1993.

[17] Bueno EM, Ruberti JW. Optimizing Collagen Transport Through Track-Etched Nanopores.J Memb Sci 2008; 321: 250-263.

[18] Matsuura T. Bioreactors for 3-dimensional high-density culture of human cells. Hum Cell 2006; 19: 11-16.

[19] Mitra D, Whitehead J, Yasui OW, Leach JK. Bioreactor culture duration of engineered constructs influences bone formation by mesenchymal stem cells. Biomaterials 2017; 146: 29-39.

[20] Li YJ, Batra NN, You LD, Meier SC, Coe IA, Yellowley CE, Jacobs CR. Oscillatory fluid flow affects human marrow stromal cell proliferation and differentiation. J Orthop Res 2004; 22: 1283–1289.

[21] Hosseinkhani H, Inatsugu Y, Hiraoka Y, Inoue S, Tabata Y. Perfusion culture enhances osteogenic differentiation of rat mesenchymal stem cells in collagen sponge reinforced with poly(glycolic acid) fiber. Tissue Eng 2005; 11: 1476–1488.

[22] Yourek G, McCormick SM, Mao JJ, Reilly GC. Shear stress induces osteogenic differentiation of human mesenchymal stem cells. Regen Med 2010; 5: 713-724.

[23] Kim SH, Ahn K, Park JY. Responses of human adipose-derived stem cells to interstitial level of extremely low shear flows regarding differentiation, morphology, and proliferation. Lab Chip 2017; 17(12): 2115-2124.

[24] Dong JD, Gu YQ, Li CM, Wang CR, Feng ZG, Qiu RX, Chen B, Li JX, Zhang SW, Wang ZG, et al. Response of mesenchymal stem cells to shear stress in tissue-engineered vascular grafts. Acta Pharmacol Sin 2009; 30: 530-536.

[25] Bhaskar B, Owen R, Bahmaee H, Rao PS, Reilly GC. Design and Assessment of a Dynamic Perfusion Bioreactor for Large Bone Tissue Engineering Scaffolds. Appl Biochem Biotechnol 2018; 185(2): 555-563.

[26] Porter B, Zauel R, Stockman H, Guldberg R, Fyhrie D. 3-D computational modeling of media flow through scaffolds in a perfusion bioreactor. J Biomech 2005; 38: 543–549.

[27] Lamalice L, Le Boeuf F, Huot J. Endothelial cell migration during angiogenesis. Circ Res 2007; 100: 782-794.

[28] Li S, Huang NF, Hsu S. Mechanotransduction in endothelial cell migration. J Cell Biochem 2005; 96: 1110–1126.

[29] Rennier K, Ji JY. Shear stress regulates expression of death-associated protein kinase in suppressing TNFα-induced endothelial apoptosis. J Cell Physiol 2012; 227: 2398-2411.

[30] Chou PH, Wang ST, Yen MH, Liu CL, Chang MC, Lee OK. Fluid-induced, shear stress-regulated extracellular matrix and matrix metalloproteinase genes expression on human annulus fibrosus cells. Stem Cell Res Ther 2016; 7: 34.

[31] Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, Isner JM. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 1999; 18: 3964-3972.

[32] Bao S, Wu Q, Sathornsumetee S, Hao Y, Li Z, Hjelmeland AB, Shi Q, McLendon RE, Bigner DD, Rich JN. Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. Cancer Res 2006; 66: 7843-7848.

[33] Beckermann BM, Kallifatidis G, Groth A, Frommhold D, Apel A, Mattern J, Salnikov AV, Moldenhauer G, Wagner W, Diehlmann A, et al. VEGF expression by mesenchymal stem cells contributes to angiogenesis in pancreatic carcinoma. Br J Cancer 2008; 99: 622-631.

[34] Cheng BB, Yan ZQ, Yao QP, Shen BR, Wang JY, Gao LZ, Li YQ, Yuan HT, Qi YX, Jiang ZL. Association of SIRT1 expression with shear stress induced endothelial progenitor cell differentiation. J Cell Biochem 2012; 113(12): 3663-3671.

[35] Yang Z, Xia WH, Zhang YY, Xu SY, Liu X, Zhang XY, Yu BB, Qiu YX, Tao J. Shear stress-induced activation of Tie2-dependent signaling pathway enhances reendothelialization capacity of early endothelial progenitor cells. J Mol Cell Cardiol 2012; 52(5): 1155-1163.

[36] Ohtani-Kaneko R, Sato K, Tsutiya A, Nakagawa Y, Hashizume K, Tazawa H. Characterisation of human induced pluripotent stem cell-derived endothelial cells under shear stress using an easy-to-use microfluidic cell culture system. Biomed Microdevices 2017; 19(4): 91.

[37] Chen WT, Hsu WT, Yen MH, Changou CA, Han CL, Chen YJ, Cheng JY, Chang TH, Lee OK, Ho JH. Alteration of mesenchymal stem cells polarity by laminar shear stimulation promoting β-catenin nuclear localization. Biomaterials 2019; 190-191: 1-10.

[38] Laschke MW, Harder Y, Amon M, Martin I, Farhadi J, Ring A, Torio-Padron N, Schramm R, Rücker M, Junker D, et al. Angiogenesis in tissue engineering: breathing life into constructed tissue substitutes. Tissue Eng 2006; 12: 2093-2104.

[39] Yamamoto K., Takahashi T, Asahara T, Ohura N, Sokabe T, Kamiya A, Ando J. Proliferation, differentiation, and tube formation by endothelial progenitor cells in response to shear stress. J Appl Physiol 2003; 95: 2081-2088.

[40] Wang H, Riha GM, Yan S, Li M, Chai H, Yang H, Yao Q, Chen C. Shear stress induces endothelial differentiation from a murine embryonic mesenchymal progenitor cell line. Arterioscler Thromb Vasc Biol 2005; 25: 1817-1823.

[41] Park JS, Huang NF, Kurpinski KT, Patel S, Hsu S, Li S. Mechanobiology of mesenchymal stem cells and their use in cardiovascular repair. Front Biosci 2007; 12: 5098-5116.

[42] Cui X, Zhang X, Guan X, Li H, Li X, Lu H, Cheng M. Shear stress augments the endothelial cell differentiation marker expression in late EPCs by upregulating integrins. Biochem Biophys Res Commun 2012; 425(2): 419-425.

[43] Levy AP, Levy NS, Goldberg MA. Post-transcriptional regulation of vascular endothelial growth factor by hypoxia. J Biol Chem 1996; 271: 2746-2753.

[44] Johnson BD, Mather KJ, Wallace JP. Mechanotransduction of shear in the endothelium: basic studies and clinical implications. Vasc Med 2011; 16(5): 365-377.