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Effect of volume flows on the viability of MSCs during injection through a cannula

Abstract: Introduction: Clinical trials currently evaluate the use of mesenchymal stem cells (MSCs) for the treatment of non-union bone fractures. The stem cells are injected directly into the non-union area of a bone via a cannula. During this injection process, pressure and shear forces affect the MSCs which could influence the viability of the cells. One parameter that influences the level of the shear forces is the volume flow. The aim of this study is to show whether the injection process with two different volume flows influences the cell viability. Methods: MSCs were isolated from bone tissue, harvested during arthroplasty. Afterwards, they were diluted to a concentration of 1 million cells/mL and 1 mL of this suspension was injected through a cannula with 200 mm length and 2 mm diameter (14 G) with volume flows of 38 and 100 mL/min. The evaluation was performed by detecting living, apoptotic, and dead cells using flow cytometry. The statistical analysis was performed with a Kruskal-Wallis-test to identify significant differences and with a TOST procedure for significant equivalence. The significance level was set to 5 % and the equivalence margin to 20 %. Results: The cell population of healthy cells was in the control group

85.88±2.98 %. After an injection with 38 mL/min the population of healthy cells was 86.04±2.53 % and with 100 mL/min 85.48±1.64 %. The statistical analysis revealed no significant difference between these groups ($p = 0.99$), but a significant equivalence between the control group and the two volume flows (38 mL/min: $p = 0.002$, 100 mL/min: $p = 0.001$). In addition the results show no increase of apoptotic and dead cells in the population after injection. Conclusion: The results indicate that the injection process through the cannula with these volume flows has no effect on the viability of the MSCs.

Keywords: mesenchymal stem cells, cell viability, non-union, volume flows, shear forces

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1 Introduction

The research to use mesenchymal stem cells (MSCs) in clinical applications is constantly proceeding. One application field is the usage of MSCs in bone regeneration due to their high differentiation potential in the osteogenic cascade [1]. Clinical studies are presently investigating the influence of stem cells on the healing process of non-unions [2]. The gold standard therapeutic procedure is the surgical stabilization of the bone fracture and the stimulation of the bone healing process by using autologous bone grafting. Instead of bone grafting, MSC products are injected directly into the fracture gaps via cannula to stimulate bone growth in clinical trials [2]. In contrast to bone grafting, the injection process is minimally invasive. To achieve an optimal therapeutic result, a high viability of the injected MSCs in the fracture gap is required [3]. However, various flow regimes can influence the viability by damaging and destroying the cells [4]; thus leading to a negative influence in the therapeutic effect of the cells [5], [6]. The main influencing factor affecting the viability of the cells during the passage of the cannula are shear stresses which depend on the length and the cross-sectional area of the cannula as well as the viscosity of the fluid [7]. Moreover, the level of the shear forces are affected through the volume flow of the fluid, because the shear forces increase proportionally to the volume flow. The influence of this shear stress has already been investigated in some studies on adult stem cells [8], [6], [5]. However, due to their plastic adherence and their larger size of 12 – 15 μm compared to mononuclear cells (MNCs) with 5 – 6 μm , these results are not comparable for

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MSCs. Thus, Walker et al. examined the influence of catheters with different diameters and volume flows also on the viability of MSCs. The used volume flows and catheters in that study are useful for assessing the viability of MSCs after intravascular administration as this is the main used transplantation route in most clinical studies [5]. The utilized catheters are not comparable to those for the injection into non-unions of bone. These cannulas are considerably longer as they not only have to penetrate the skin but also the fat layer, the fascia and muscle groups, e.g. in the area of the femur. Since these parameters (volume flow, length of the cannula) influence the viability, the aim of this study was to investigate the effect of the injection process through a cannula with for the treatment of non-unions realistic volume flows on MSC viability.

2 Materials and Methods

The used MSCs were isolated from human bone material harvested during routine orthopedic surgery at the University Hospital Schleswig-Holstein, Department for orthopaedic and trauma surgery in Lübeck. The bone material was collected from five patients older than 18 years and after signing consent. Thereby, the collected materials were considered by the operating surgeons as “waste material” and the harvesting process thus without extra risk for the patient. The study was approved by the Ethics committee of the University of Lübeck (ID: 16-307).

2.1 MSC isolation and cultivation

The isolation of the MSCs was executed in two steps, first by isolating MNCs using density gradient centrifugation with Lymphoprep™ (StemCell Technologies, Canada) and second by separation on the basis of the plastic adherence properties of MSCs. Therefore, the isolated cells were firstly cultivated with Minimum Essential Medium (MEM) (Gibco® by Life Technologies, USA) supplemented with 10 % Fetal Bovine Serum (FBS) (Gibco® by Life Technologies, USA) and 1 % Penicillin-Streptomycin (P/S) (Gibco® by Life Technologies, USA). After adhesion of the MSCs to the bottom of the culture flask, 1 % GlutaMAX™ Supplement (Gibco® by Life Technologies, USA), 1 % Sodium Pyruvate (Gibco® by Life Technologies, USA) and 1 % MEM Non-Essential Amino Acids Solution (Gibco® by Life Technologies, USA) were accessorially added to MEM and the medium change was conducted by replacing the medium completely. The cultivation was operated under standard cultivation conditions with a CO₂ concentration of 5 % and a temperature of 37 °C. In case of a 80 % confluency of the cells,

they were harvested with Trypsin-EDTA (0.05%) (Gibco® by Life Technologies, USA) and used for the injection simulation experiments.

2.2 Procedure of the simulated injection process

For the experimental execution of the simulated injection process the cells were dissolved in Dulbecco's phosphate-buffered saline (PBS) (Thermo Fisher Scientific, USA) at a concentration of 1,000,000 cells/mL and obtained with a standard 1 mL syringe. For the simulation process a cannula with a length of 200 mm and a diameter of 2 mm (14 G) from the company unimed® (Lausanne, Switzerland) was used. These medical products correspond to the currently used cannulas in clinical trials to inject MSCs into non-unions. To avoid a contamination of the MSCs during the simulation, the cannula was placed in a 12 mL sterile bottle with a septum silicon cap. This ensures a closed system during the procedure. To guarantee the passaging of the complete cell suspension through the cannula, it was rinsed with sterile PBS before and after the transition of the MSCs through the cannula. The cells for the trials were injected with two different volume flows (38 and 100 mL/min). The cells of the control group were treated exactly the same as the cells for the trials, except for the injection procedure.

2.3 Viability measurement

To measure the viability of the MSCs after the simulated injection process, an “apoptotic/necrotic/healthy cells detection kit” (PromoCell GmbH, Heidelberg, Germany) was applied. After the simulation procedure the cell suspension was centrifuged and the cell pellet resuspended with a 1:5 diluted Binding Buffer to a cell concentration of 1,000,000 cells/mL. The detection of apoptotic, necrotic, and healthy cells was performed by staining. Therefore 4 µL Fluorescein (FITC) – Annexin ($\lambda_{\text{abs}}/\lambda_{\text{em}} = 492 \text{ nm}/514 \text{ nm}$) and 4 µL Ethidium Homodimer III ($\lambda_{\text{abs}}/\lambda_{\text{em}} = 528 \text{ nm}/617 \text{ nm}$) were added to a 100 µL cell suspension and incubated for 15 min at room temperature. To determine the viability by flow cytometry, 200 µL buffer was conducted to each sample. Data were obtained with a BD LSRII Bench Top Analyser (BD Bioscience, USA) in three technical repeats.

2.4 Statistics

Due to the small sample size an underlying normal distribution of the data cannot be assumed, therefore a Kruskal-Wallis-test was performed with a significance level of $\alpha = 0.05$. In addition to the determination of significant differences, also equivalence tests were performed. Therefore a robust TOST for equivalence was used with an equivalence range of 20 % and a significance level of 5 % [9].

3 Results

3.1 Test-setup to simulate the injection process

The test-setup to simulate the injection process consists of several components (see Figure 1). One main element was a linear actuator (a) which moves a slide by means of a threaded rod (b). The linear actuator ensures a constant volume flow through the cannula (c) during the simulated injection process. The plunger of the syringe was shifted by the movement of the carriage. To enable this action, the threaded rod ran in a threaded nut inserted in the carriage, which allows the carriage to move forwards and backwards depending on the direction of the rotation. The linear actuator was controlled by a stepper motor, which can adjust the volume flow in the range of 10 – 100 mL/min. Another component of the test-setup is the syringe holder (d), which was designed to use different syringe sizes (1, 5 and 10 mL).

Figure 2: Construction of the test-setup to investigate the influence of the occurring shear stress on the viability of the cells after passing the cannula. The test-setup consisted of a linear actuator (a), which moves a slide by means of a threaded rod (b), the cannula to be tested (c) and the syringe holder (d).

3.2 Viability of MSC after simulated injection procedure

The previously performed experiments to determine the realistic volume flows occurring during the injection of a stem cell product into a non-union revealed a maximum volume flow of 37.65 mL/min (data not shown). Therefore the simulated injection process was carried out with a volume flow

of 38 mL/min. In addition, the MSCs were also injected through the cannula with a volume flow rate of 100 mL/min to investigate whether any possible high-volume flow would lead to an impairment of viability.

The viability measurement indicated that the cell population of the control group consisted of 85.88 ± 2.98 % alive, 1.1 ± 0.21 % apoptotic and 13.02 ± 2.94 % dead cells. The results showed that the viability of MSCs remained the same after injection with the two different volume flows (38 and 100 mL/min). The population of living cells was 86.04 ± 2.53 % at a flow of 38 mL/min and 85.48 ± 1.64 % at 100 mL/min. Simultaneously, the number of dead cells with 13.02 ± 2.94 % (0 mL/min), 12.85 ± 2.6 % (38 mL/min), and 13.4 ± 1.84 % (100 mL/min) as well as the apoptotic cell fraction with 1.1 ± 0.21 % (0 mL/min), 1.11 ± 0.38 % (38 mL/min), and 1.13 ± 0.47 % (100 mL/min) did not raise with increasing volume flow (see Figure 2). There was no significant difference between the live ($p = 0.99$), apoptotic ($p = 0.88$) and dead cell population ($p = 0.93$) for different volume flows, but there is a significant equivalence between the control group and the two different volume flows in the living cell population (38 mL/min: $p = 0.002$, 100 mL/min: $p = 0.001$).

Figure 1: The different cell fractions of MSC after injection with different volume flows (0 mL/min, 38 mL/min and 100 mL/min). There was no effect of the volume flow on the live cell, dead cell and apoptotic cell populations, but there was a significant equivalence between the control group and the two different volume flows in the living cell population. Statistic significant equivalence $p \leq 0.05$ (#); $p \leq 0.01$ (##); $p \leq 0.001$ (###).

4 Discussion

During the injection process via a cannula, shear forces act on the cells, which can influence the viability [7]. In a laminar flow field cells deform due to various influences, like viscosity and the strength of the shear forces. If the deformations of the cells become severe, the cell membrane will rupture and cell death will occur [4]. The maximum shear stress without

damage varies from cell type to cell type [4]. For these reasons the aim of the study was to investigate if the injection via a cannula affects the cell viability. The results show no significant decrease of the cell viability after the injection via cannula but a significant equivalence between the control group and the two volume flows and also no significant increase of the population of death and apoptotic cells with increasing volume flow. This indicates that the evaluated volume flows have no influence on the cell viability during the injection process.

Preliminary experiments resulted in a maximum volume flow of 37.65 mL/min, which is why one of the injection tests was performed with a volume flow of 38 mL/min. As the shearing forces increase proportionally with the volume flow, an additional volume flow two and a half times higher than the maximum measured volume flow were used for the injection tests. With a maximum volume flow rate of 100 mL/min the aim was to investigate whether different volume flow rates during the injection process into a human non-union generate shear forces, which could lead to cell death. The results with different volume flows showed no significant difference between the volume flows in any of the three cell fractions (live, dead, apoptotic), but a significant equivalence between the control group and the two volume flows in the living cell population. This result assured that the prevailed volume flows during an injection into a human non-union do not negatively affect the cell viability. Furthermore, the fraction of viable cells observed with the used volume flows lays substantially above the 70% threshold requested by the FDA. Therefore, the evaluated flows allow an even higher percentage of living cells to reach the human non-union by cannula injection than requested and thereby can support the bone fracture healing process.

This is in accordance with the results of the working group of Walker et al., who also could not detect any changes in cell viability at volume flows of 1 – 8 mL/min [5]. Thus, with these results it was demonstrated, that even at higher volume flows MSCs can be injected without affecting the cell viability. In addition, the results show no influence on the cell viability via the used stainless steel cannula with a diameter of 2 mm (14 G) and a length of 200 mm. Other studies were also not able to determine influences on the cell viability by using cannulas with smaller diameters of 19 to 27 G. Nevertheless, the cannulas had shorter lengths of 5 to 15 cm [10].

In summary, the presented results clearly demonstrate the possibility of injecting MSC via cannula into a human non-union without losing viability.

5 Conclusions

The aim of this study was to investigate whether a high volume flow influences the viability of the MSCs. The results show that the cell population of living cells, dead cells and apoptotic cells are not affected by the tested volume flows, which indicates that the injection of MSCs into a non-union via cannula is feasible in terms of shear stress with higher volume flows.

Author Statement

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